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Small-Molecule High-Throughput Screening Utilizing *Xenopus* Egg Extract

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Abstract

Screens for small-molecule modulators of biological pathways typically utilize cultured cell lines, purified proteins, or, recently, model organisms (e.g., zebrafish, *Drosophila*, *C. elegans*). Herein, we describe a method for using *Xenopus laevis* egg extract, a biologically active and highly tractable cell-free system that recapitulates a legion of complex chemical reactions found in intact cells. Specifically, we focus on the use of a luciferase-based fusion system to identify small-molecule modulators that affect protein turnover.

Keywords

Xenopus egg extract; *Xenopus laevis*; Cell-free; Small molecules; High-throughput screening; Protein turnover; Protein degradation

1 Introduction

Traditionally, small-molecule screening to identify potential therapeutic leads and/or biological tools have been performed using in vitro (purified components) or in vivo (cultured cells/whole organism) approaches. Each approach has its own strengths and weaknesses. The use of purified proteins simplifies the process considerably because one is sampling only molecules that directly bind and alter the activity of the protein being targeted. The major weakness of this approach, however, is that the biological consequences of inhibition/activation by the small molecule at the organismal level are less clear. Screening for phenotypic changes using cultured cells or whole organisms is obviously more biologically relevant, although manipulations are more complex. Lack of effects may be due to failure of compounds to pass through the plasma membrane, expulsion via efflux pumps, or cell death. Additionally, target identification remains a major hurdle.

The *Xenopus laevis* egg extract system overcomes some of the limitations of using purified proteins or cells/organisms for small-molecule screening by providing a cell-free, yet robust, biologically active system that can be readily manipulated [1]. Because *Xenopus* egg extract lacks intact plasma membranes, small molecules are allowed unfettered access to putative targets. In addition, *Xenopus* egg extract contains all of the eukaryotic cellular machinery and complex signaling pathways required for the early development of an organism. Finally, large amounts of homogenous *Xenopus* egg extract can be prepared at one time, an important consideration for large-scale screens and reproducibility [1–3]

Xenopus egg extract is a homogenous mixture of cellular components including cytoplasmic proteins, cellular organelles, amino acids, and nucleotides at near physiological levels [4].

This system has been used to answer numerous biological questions regarding the cell cycle, cytoskeletal dynamics, signal transduction, apoptosis, nuclear assembly, nucleocytoplasmic transport, ubiquitin metabolism, and protein turnover [5–32]. While the versatility of the *Xenopus* egg extract system in reconstituting a large number of complex biological reactions is a major strength for small-molecule screening, different methodologies for extract preparation must be used to optimize the system for a particular pathway or biological event. Thus, the preparation methodology of *Xenopus* egg extract is a major consideration in performing a high-throughput screen to ensure that one has the best chance of identifying useful small molecules. Additional methods for *Xenopus* egg extract preparation have been described elsewhere [6, 10, 13, 25, 32–36].

Xenopus egg extract is a particularly robust system for studying protein turnover that lacks the potentially confounding influence of gene transcription. The method of *Xenopus* egg extract preparation described within this chapter is optimized for analyzing protein turnover of β -catenin, the key effector protein of the Wnt signaling pathway; also, we found that it supports the degradation of another Wnt component, Axin, as well as other signaling pathway proteins that are known to rapidly turn over [5, 37, 38]. The usefulness of *Xenopus* egg extract for studying key aspects of cytoplasmic Wnt pathway regulation is supported by multiple studies that identify important regulatory proteins/steps that contribute to β -catenin degradation [2, 3, 5, 38–44]. Significantly, the preparation of *Xenopus* egg extract described herein was successfully used to screen and identify small molecules that stimulate β -catenin turnover and inhibit Wnt signaling [2, 3].

In this chapter we provide a detailed method for using *Xenopus* egg extract preparations that are optimized for examining protein turnover. We take advantage of firefly luciferase (Luciferase), a protein normally stable in *Xenopus* egg extract that, when fused to proteins of interest, provides a simple and rapid readout of protein turnover. We describe herein how these Luciferase fusion proteins can be used to perform high-throughput (HTS) biochemical screens in *Xenopus* egg extract to identify biologically active small-molecule compounds.

2 Materials

2.1 *Xenopus* Egg Extract Preparation

1. 100 U/mL pregnant mare serum gonadotropin (PMSG): Stock is prepared fresh before injections by dilution of 1,000 units (U) of PMSG in 10 mL of purified deionized water.
2. Storage water: 40 L of 20 mM sodium chloride. Weigh out 46.72 g of sodium chloride into 40 L of deionized water.
3. 20 $\times$ stock Marc's Modified Ringers (MMR): 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 40 mM potassium chloride, 2 M sodium chloride, 20 mM magnesium chloride, and 40 mM calcium chloride, pH 7.4. Weigh out 35.7 g of HEPES, 4.5 g of potassium chloride, 175.2 g of sodium chloride, 2.9 g of magnesium chloride, and 6.7 g of calcium chloride. Mix these into a total volume of 1.25 L purified deionized water. Once all is dissolved, adjust the pH of

the solution to 7.4 with NaOH and fill to a final volume of 1.5 L with deionized water.

4. 750 U/mL human chorionic gonadotropin (HCG): HCG is prepared fresh before injections by dilution of 10,000 U of HCG in 13.3 mL of purified deionized water.
5. 2 % (w/v) cysteine solution: 8 g of cysteine is diluted into 400 mL of deionized water, and pH is adjusted to 7.7 with NaOH.
6. Leupeptin, pepstatin, aprotinin (LPA): 10 mg/mL leupeptin, 10 mg/mL pepstatin, and 10 mg/mL aprotinin. Dissolve 10 mg of leupeptin, 10 mg of pepstatin, and 10 mg of aprotinin in 1 mL of dimethyl sulfoxide (DMSO).
7. 10 mg/mL cytochalasin D: Dilute 10 mg of cytochalasin D into 1 mL of DMSO.
8. 10 mg/mL cycloheximide: Dilute 10 mg of cycloheximide into 1 mL of purified deionized water.

2.2 Luciferase-Fused Proteins

1. Several in vitro-transcription/translation kits are commercially available. We typically use a rabbit reticulocyte system in which the cDNA of interest is subcloned into the pCS2+ plasmid with transcription driven by the SP6 promoter.

2.3 Active Extract

1. 20× energy reaction (ER) mix: 20 mM adenosine triphosphate, 150 mM creatine phosphate, 20 mM magnesium chloride, and 600 µg/mL creatine phosphokinase. Weigh out 10.1 mg of adenosine triphosphate, 31.7 mg of creatine phosphate, 1.7 mg of magnesium chloride, and 600 µg creatine phosphokinase. Mix these into a total volume of 1 mL of purified deionized water. Divide ER mix into 50 µL aliquots and store at −80 °C until needed.

2.4 Z-Factor Scoring and Screening

1. White 96-well plate (*see Note 1*).
2. Small-molecule library of choice.
3. Luciferin reagent/commercially available kit to assess luciferase activity.

3 Methods

3.1 Preparation of *Xenopus* Egg Extract for Screening of Protein Turnover

As described above, this purification method is optimized for analyzing β -catenin protein turnover. The method described is for preparing extract from ten frogs. For larger or smaller preparations, the amount of buffer should be adjusted accordingly. Typically, each frog yields ~1 mL of extract with a protein concentration of ~50 mg/mL.

1. To induce frog egg production, female frogs are primed with 100 U of PMSG injected subcutaneously into the dorsal lymph sac using a 3 mL tuberculin syringe and 27 G needle.

2. Primed frogs are stored in 4 L of 20 mM NaCl at 18 °C for 5–10 days (*see Note 2*).
3. Prepare a 0.5× MMR solution to be used in the next step. This is performed by diluting a 20× MMR stock to make 40 L of a 0.5× MMR solution. Set up ten 4 L buckets (*see Note 3*). These buckets should be prepared and kept in a 16 °C incubator overnight prior to injecting frogs with HCG.
4. After 5–10-day incubation, inject the dorsal lymph sac of each primed frog using a 3 mL tuberculin syringe and 27 G needle with 750 U HCG. Each injected frog should be placed in a bucket containing 4 L of 0.5× MMR at 16 °C.
5. Allow the frogs to lay eggs for 15–16 h at 16 °C.
6. A day prior to injections, dilute 20× MMR to 4 L of a 1× MMR solution and 50 mL of a 0.1× MMR solution. The morning of the egg extract prep, prepare fresh 400 mL 2 % cysteine solution, pH 7. These solutions should be stored at 16 °C (*see Note 4*).
7. After the 15–16-h egg laying period, gently squeeze the abdomen and lower back of each frog to expel additional eggs, and place the frogs into a separate container of deionized water.
8. Remove the majority of the MMR from each bucket, leaving the eggs in the smallest volume possible. Make sure, however, that the eggs remain covered in MMR.
9. Remove poor-quality eggs with a plastic transfer pipet as these will decrease the quality of the overall extract. If greater than 10 % of the eggs appear poor in quality, the entire batch should be thrown away (*see Note 5*).
10. Combine cleared, high-quality eggs and remaining MMR in a 500 mL glass beaker.
11. Again, pour out the majority of the MMR keeping the eggs submerged.
12. Estimate volume of egg bed, and wash eggs by carefully adding twice the volume of 1× MMR along the inside of the beaker. Gently swirl the eggs and pour off debris and the majority of the MMR. Repeat this twice and continue to remove any poor-quality eggs.
13. To de-jelly the eggs, pour 100 mL of 2 % cysteine along the inside of the beaker. Swirl the beaker gently to mix and allow the eggs to settle at 16 °C for 5 min. Pour off the majority of the cysteine, keeping the eggs submerged. Repeat until the eggs appear tightly packed (*see Note 6*).
14. Wash off the cysteine by adding 1× MMR along the inside of the beaker, gently swirl, and again, pour off most of the solution. Repeat until the 1× MMR solution is no longer cloudy. Poor-quality eggs should be continually removed during this process.
15. Gently rinse the eggs with 30 mL of 0.1× MMR, and pour off the majority of solution.

16. Add LPA (10 $\mu\text{g/mL}$ final) and cytochalasin D (20 $\mu\text{g/mL}$ final) to the remaining 20 mL of 0.1 $\times$ MMR.
17. Add the 0.1 $\times$ MMR solution containing LPA and cytochalasin D to the washed eggs, swirl gently, and incubate at 16 $^{\circ}\text{C}$ for 5 min.
18. Transfer the eggs into prechilled 50 mL centrifuge tubes using a 25 mL pipet (*see Note 7*). After the eggs have settled, the excess buffer should be removed. The eggs should remain covered with buffer.
19. Pack the eggs by centrifugation at $400 \times g$ for 60 s at 4 $^{\circ}\text{C}$ using a fixed-angle rotor.
20. Remove any excess buffer from the packed eggs.
21. Crush the eggs by spinning tubes at $15,000 \times g$ for 5 min at 4 $^{\circ}\text{C}$.
22. The egg extract will now be separated into three layers. The bottom and darkest layer contains yolk, pigmented granules, etc.; the middle layer contains cytoplasmic fraction (the desired material), and the top layer contains lipid-enriched material. In order to collect the cytoplasmic layer, the lipid layer must first be disrupted, which can be accomplished by piercing the lipid layer with a P1000 pipet tip so as to create a hole.
23. Using a new P1000 pipet tip, collect the cytoplasmic layer (straw-colored middle layer) through the hole in the lipid layer and transfer the collected cytoplasm to a new centrifuge tube on ice (*see Note 8*).
24. Spin the collected cytoplasmic layer at $15,000 \times g$ for 10 min at 4 $^{\circ}\text{C}$ (*see Note 9*).
25. Again, collect the cytoplasmic layer into a new prechilled centrifuge tube and add LPA, cycloheximide, and cytochalasin D to final concentrations of 10 $\mu\text{g/mL}$ each.
26. Dispense the extract into 100–1,000 μL aliquots and snap-freeze in liquid nitrogen for storage (*see Notes 10 and 11*).

3.2 Preparation of Recombinant In Vitro-Transcribed/Translated Luciferase-Fusion Proteins

It is important to be able to readily produce sufficient amounts of recombinant Luciferase-fused protein(s) in order to perform high-throughput screening. We have found that recombinant protein production by in vitro-transcription/translation (IVT), bacterial expression, or the *Sf9*/baculovirus systems all work well. Protein production by IVT is the quickest and easiest of the three, although the limited protein yield can be an issue. A much greater protein yield can be obtained using the bacterial or *Sf9*/baculovirus systems, but these are much more labor intensive.

1. Produce IVT protein(s) using commercially available kits (*see Note 12*).
2. Luminescence activity at this point should be assessed by measuring a small sample (typically 1 μL of IVT protein). This can be performed by mixing 1 μL of protein and 25 μL of luciferin reagent in a 96-well white plate. The IVT protein is then aliquoted and stored at -80°C until used. The size of protein aliquots is determined based on considerations in Subheading 3.3 and **Note 13**.

3.3 Assessing Z-Factor Score

Assessment of a screen's Z-factor is important to ensure its usefulness and/or probability of finding small molecules. Thus, at this point it is important to optimize the screen in order to obtain the most effective Z-factor score and ensure the best chance of reliably identifying biologically active small molecules. The method described is for a 96-well plate. With appropriate scaling, however, this protocol can be modified for a 384-well format or alternative well formats.

1. Place a white 96-well plate on ice to cool. This step is important to inhibit the degradation reaction until setup is complete.
2. Quickly thaw frozen aliquots of *Xenopus* egg extract and 20× ER mix by rubbing the tubes between one's hands or gently swirling in a 30 °C water bath until only a small amount of frozen extract remains. Place the extract on ice. Add the ER mix (1× final) to the extract and mix by brief vortex pulses to generate the reaction mix. Place the reaction mix on ice.
3. Quickly thaw frozen recombinant Luciferase-fusion protein by rubbing the tubes between one's hands until only a small amount of frozen protein remains. Place the protein on ice.
4. Add the appropriate amount of luciferase-fusion protein to the reaction mix such that the relative luminescence units (RLU) will be approximately 10,000 RLU/μL (*see Note 13*).
5. Dispense 10 μL of the reaction mix plus Luciferase-fusion protein into each of the 96 wells on ice.
6. For Z-factor scoring, load vehicle and control in alternating wells, mimicking a checkerboard design. We typically use DMSO (vehicle, negative control) and MG132 (proteasome inhibitor, positive control) when screening proteins that are degraded in a proteasome-dependent manner. Compounds are added at ~500 μM (0.5 μL of each compound from 10 mM stocks) to respective wells. If using DMSO as vehicle, add an equal volume of the positive control (*see Note 14*).
7. Mix the plate by lightly shaking either by hand or vortexing at low speed, being careful not to eject liquid from the wells.
8. Incubate the plate at room temperature for a predetermined optimal period of time (*see Note 15*).
9. Stop the reaction by addition of 75 μL of luciferin reagent to each well.
10. Mix the plate by lightly shaking either by hand or vortexing at low speed, again being careful not to eject liquid from the wells.
11. Measure luciferase activity using a luminometer.
12. The Z-factor can be assessed by calculation as previously described [45]. A Z-factor score of 1 is ideal, a score between 1 and 0.5 indicates that the assay is

excellent, a score between 0.5 and 0.0 indicates that the assay is weak, and a score 0 indicates that the assay is error prone and is, therefore, not reliable.

3.4 Screening for Small Molecules

1. After optimizing the screen for an effective Z-factor score, a small-molecule pilot screen can be performed under similar conditions. Perform the pilot screen with identical conditions used to achieve an optimized Z-factor score in Subheading 3.3. Load the same volume of compounds as was used for addition of controls when determining the Z-factor (*see* **Notes 14** and **16**). It is important to load both negative and positive controls (typically in triplicate) in order to assess the effectiveness of the screen. For proteasome-mediated degradation screens, we use DMSO and MG132 as negative and positive controls, respectively.
2. We have found that it is important to run a Luciferase-only control screen in order to identify compounds that directly inhibit/enhance Luciferase activity [46]. The enzymatic activity of the Luciferase protein requires ATP. Thus, it is possible that some compounds may alter the activity of the Luciferase protein by altering ATP levels.
3. Upon completion of the HTS screen, assess whether the screen ran optimally by comparing values of the negative and positive controls, which should reflect values that were observed when assessing the Z-factor for the screen.
4. Effective small molecules are those that increase or decrease the luminescence by >3 standard deviations. Screens should be repeated at least three times. Small molecules that repeatedly cause greater than a threefold change in standard deviation are likely to represent “true hits.”

4 Notes

1. Plates with round- or flat-bottom wells work equally well.
2. It takes at least 5 days in order for priming to take full effect, and priming should last for 10 days. After 10 days the effect of priming is diminished, and a decreased amount of eggs are obtained.
3. The use of large-sized buckets containing multiple frogs increases the risk that a given frog might lay poor-quality eggs; in that case, a significant amount of time and effort will be required to separate poor-quality from high-quality eggs. This additional time increases the likelihood that high-quality eggs will lyse or otherwise degenerate. Thus, it is not worth the risk to use fewer tanks.
4. At this point, it is important to maintain the temperature at 16 °C throughout the remainder of the extract preparation. It is also important to work as rapidly as possible. As noted above, the longer the amount of time needed to process the eggs, the greater the likelihood of spontaneous egg lysis.
5. High-quality eggs will have a high dark-to-light contrast between the darkly pigmented animal hemisphere and the lightly colored vegetal hemisphere. Poor-quality eggs will appear stringy (immature eggs) or white and puffy (lysed eggs).

6. Eggs will become more compact as the jelly coat is removed, which will float above the eggs. Three cysteine treatments are usually required for full de-jellying to occur. Once the eggs have been de-jellied, they will become very fragile and prone to lyse, so it is important to swirl gently and ensure that eggs are not exposed to the air.
7. When pipetting, to prevent eggs from being exposed to air, first draw up some buffer before suctioning up the eggs.
8. For preparation of high-quality extract for β -catenin degradation, it is important to minimize the amount of lipid or pigmented layers transferred.
9. This clearing step may be repeated if the cytoplasmic layer still contains a significant amount of pigment or lipid material, which may result in proteolysis of β -catenin by non-Wnt pathways. Excessive spins, however, decrease the robustness of the extract to support β -catenin degradation mediated by Wnt components.
10. Do not freeze extract if you wish to maintain the translational capacity of the egg extract. Once frozen, *Xenopus* egg extract loses significant capacity to translate exogenously added mRNA. For more information, see [5, 47].
11. *Xenopus* egg extract, once prepared, is stable for long-term storage in liquid nitrogen. Extract can alternatively be stored at -80°C ; however, it should be used within 2 months.
12. To confirm that a protein of interest is produced using an IVT reaction, immunoblot analysis can be performed. Alternatively, proteins can be radiolabeled with [^{35}S]methionine and analyzed by SDS-PAGE/autoradiography.
13. We found that a readout of 10,000 RLU/ μL provides a robust initial signal for monitoring changes in β -catenin protein turnover. It should be noted that the more dilute the extract, the less efficient the degradation reaction becomes. Thus, to maintain robustness of the degradation reaction, it is important to minimize the volume of reagents added to the extract. We found that diluting the volume of the extract more than 35 % significantly lowered the capacity of the extract to degrade β -catenin.
14. Because *Xenopus* egg extract is highly concentrated ($\sim 50\text{ mg/mL}$), we find that small molecules need to be added in the μM range to be effective. Also, for small molecules dissolved in DMSO, it is important to add as little volume as possible. We found that adding more than 10 % DMSO will significantly inhibit the degradation reaction.
15. Different proteins will require different reaction times depending on the half-life of the protein. This is a key optimization step that should be properly assessed during Z-factor determination. The key is to identify the time in which the protein of interest has degraded by roughly half of its initial concentration. Working near this threshold will allow one to more readily identify small molecules that either inhibit or enhance degradation of the protein of interest. However, the bigger the difference/change between the positive and negative controls, the easier it is to

achieve a robust Z-factor. Additionally, for longer incubation times it may be necessary to incubate the plate in a humidity chamber to prevent evaporation due to the small volume. This can be accomplished by placing damp paper towels within the bottom of a plastic box that can be closed completely. The plate can then be set on the paper towels with the box lid closed during the incubation.

16. Arraying the small molecules themselves in a 96-well format significantly simplifies the transfer process.

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