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Preparation of Homogeneous Ribozyme RNA for Crystallization

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1. Introduction

The development of in vitro transcription methods has facilitated research in RNA structural biology in recent years. There is now increasing interest in determining atomic resolution structures of RNA by X-ray crystallography. The first step in this process is the production of a homogeneous sample of the RNA molecule of interest for crystallization trials.

Although in vitro transcription routinely generates milligram quantities of a specific RNA sequence, the RNA must be purified away from other reaction components prior to use. In addition, bacteriophage polymerases, such as T7 and SP6, usually add an extra nucleotide onto the 3'-end of about half of the transcripts, resulting in a heterogeneous population of RNA *(1)*. Since the ends of the RNA can affect the folding of the molecule and may be involved in crystal contacts, it is important to remove the heterogeneous 3'-ends prior to crystallization.

A method is presented here for generating homogeneous RNA transcripts and purifying them for crystallization trials. My colleagues and I have used this method to produce RNAs ranging in size from 70–160 nucleotides, and several of these samples have subsequently been crystallized.

2. Materials

2.1. Transcription, Processing, and Precipitation of the RNA Sample

1. One milligram of a plasmid construct consisting of the exact sequence of interest flanked by a T7 RNA polymerase promoter sequence upstream and a self-cleaving hepatitis δ ribozyme sequence *(2)* downstream. The plasmid should be linearized with a restriction enzyme that cuts immediately after the hepatitis δ ribozyme sequence (*see* Note 1).

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2. Sterile 40 mL polypropylene centrifuge tubes.
3. 1 *M* Stock solutions of Tris-HCl, pH 8.1, $MgCl_2$, dithiothreitol (DTT), and spermidine: all solutions should be prepared with sterile diethylpyrocarbonate (DEPC)-treated water.
4. 0.1 *M* Stocks of ATP, CTP, GTP, and UTP, pH adjusted to 8.0 with NaOH.
5. 1% Triton X-100.
6. Sterile DEPC-treated water (*see* Note 2).
7. 37°C Water bath.
8. 65°C Water bath.
9. 0.5 *M* EDTA.
10. 3 *M* NaCl.
11. Ice-cold 100% ethanol.
12. 2X Formamide loading buffer: 90% (v:v) deionized formamide, 1X TBE, 0.4% (w:v) each of xylene cyanol, and bromophenol blue.

2.2. Gel Purification, Concentration, and Analysis of RNA

1. 40% Acrylamide stock: dissolve 76 g of acrylamide and 4 g of bis-acrylamide in water to give 200 mL of solution.
2. 10% Ammonium persulfate (APS) solution (w:v) in water.
3. TEMED.
4. 10X TBE: 890 m*M* Tris-borate, pH 8.3, 20 m*M* EDTA.
5. 3 mm Thick Teflon spacers and comb for gel 24 × 29 cm.
6. Razor blades.
7. Plastic wrap.
8. Sterile disposable 50 mL centrifuge tubes (e.g., Fisher).
9. Handheld short-wave UV lamp.
10. Fluorescent thin-layer chromatography (TLC) plate for UV shadowing: silica gel 60 or cellulose impregnated with GF254 or equivalent fluor (e.g., #801063, Machery-Nagel, Germany).
11. 10 mL Syringes.
12. 0.45 μm Filtering apparatus.
13. Amicon ultrafiltration apparatus and Argon source.
14. YM3, 10- or 30-mol-wt cutoff membranes from Amicon, as appropriate.
15. 0.2 μm Spin filters (e.g., Amicon).

3. Methods

3.1. Transcription, Processing, and Precipitation of the RNA Sample

In this procedure, RNA transcripts consisting of the sequence of interest followed by a self-cleaving hepatitis δ ribozyme are produced at 37°C, followed by incubation at 65°C to facilitate δ ribozyme cleavage. The processed transcripts are then precipitated to concentrate the RNA prior to polyacrylamide gel purification.

1. Set up a 10 mL transcription reaction in a 40 mL sterile centrifuge tube. Final concentrations of reagents should be as follows: 30 m*M* Tris-HCl, pH 8.1, 25 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* DTT, 0.01% Triton X-100, 4 m*M* each of ATP, CTP, GTP, and UTP, 0.25–0.5 mg of linearized plasmid template (*see* Note 3), and 0.5 mg T7 RNA polymerase (*see* Note 4).
2. Incubate the transcription reaction at 37°C for 3 h (*see* Note 5).
3. Remove and save 10 μL for analysis.
4. Incubate the transcription reaction at 65°C for 1 h.
5. Remove 10 μL for analysis.
6. Check for complete processing of the δ ribozyme sequence by adding 10 μL of 2X formamide loading buffer to each reserved sample and loading samples onto a 10% denaturing polyacrylamide gel containing 7 *M* urea.
7. When the bromophenol blue dye reaches the bottom of the gel, stop the electrophoresis; stain gel with ethidium bromide solution (10 μL of 10 mg/mL stock solution in 300 mL of water) to visualize the RNA bands. If δ ribozyme processing is complete, proceed to next step. If processing is incomplete, see Note 6.
8. Add 0.5 mL of 0.5 *M* EDTA solution to the transcription reaction, and mix well to dissolve any magnesium pyrophosphate precipitate.
9. Add 1 mL of 3 *M* NaCl to the transcription reaction, mix, and add 22 mL of 100% ethanol. Mix and freeze at –80°C (or on dry ice) for at least 1 h or overnight.

3.2. Gel Purification, Concentration, and Analysis of RNA

In this procedure, an RNA sample from a transcription reaction is separated from nucleotides, aborted transcripts, salts, and polymerase by preparative denaturing polyacrylamide gel electrophoresis. The RNA is eluted from the gel, concentrated by ultrafiltration, and analyzed for purity by analytical gel electrophoresis.

1. Resuspend the RNA sample (after precipitation) in 1 mL of sterile DEPC-treated water. Add 1 mL of 2X formamide loading buffer, and heat the sample in a 65°C water bath to dissolve the pellet completely.
2. Prepare 200 mL of polyacrylamide gel solution of the appropriate percentage for the RNA sample (*see* Table 1). The gel solution should contain 1X TBE buffer and 7 *M* urea.
3. Prepare gel plates. Wash plates thoroughly with detergent, rinse copiously with distilled water, rinse with ethanol, and dry with absorbent tissue. Wash Teflon spacers the same way, and then assemble the gel plates with 3 mm spacers between the plates on either side. A Teflon rectangle 24 × 3 cm is used to form the well for the sample. Clip together the plates with binder clamps, and tape the bottom and sides of the assembled gel plates with 4 cm wide tape.
4. Pour 30 mL of the gel solution into a beaker, add 0.4 mL of 10% APS solution and 0.05 mL of TEMED, mix well, and pour into the gel plate assembly. Allow to polymerize completely (about 20 min, depending on the gel percentage).

Table 1
Separation Range of Different Acrylamide Gel Percentages[a]

Acrylamide conc. (%)	Effective size range for separation (bp)	XC (bp)	BPB (bp)
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12	40–200	70	20
15	25–150	60	15

[a]This table can be used as an approximate guide for choosing the percentage of polyacrylamide to use, where XC is xylene cyanol FF marker dye, BPB is bromophenol blue marker dye, and sizes are for double-stranded DNA (from ref. *3*).

5. Add 1 mL of 10% APS and 0.15 mL of TEMED to the remaining gel solution, mix well, and pour into the gel plate assembly. Insert the comb 1-1.5 cm deep and allow to polymerize completely (about 1 h).
6. Remove the tape from the plates, clip plate assembly to the gel apparatus, add 1X TBE buffer to cover top and bottom of the gel, remove comb, and rinse well with buffer.
7. Apply the sample to the well in a thin, even stream. Run the gel at 25 W (*see* Note 7).
8. When dye migration indicates the gel is ready, turn off the power, remove the plate assembly, and pry the plates open carefully. The gel will remain stuck to one plate. Cover the gel with plastic wrap, flip over, and pull off the other glass plate. Cover the exposed side of the gel with plastic wrap, place the gel on top of a fluorescent TLC plate, and visualize the RNA bands with handheld UV lamp.
9. Excise band(s) of interest with a clean razor blade, slice into 4 cm segments, and place in the barrel(s) of sterile 10 mL syringe(s). Squeeze band(s) through the syringe(s) into a sterile 50 mL plastic centrifuge tube(s) (*see* Note 8). Place tube(s) on dry ice or at –80°C for 15 min, and then in a 37°C water bath for 15 min. Add 25 mL of sterile DEPC-treated water to tube contents, agitate overnight at 4°C.
10. Filter each eluted RNA sample through a 0.45 µm filter to remove crushed acrylamide. Wash filtrate twice with 50 mL of sterile DEPC-treated water. Place eluate in Amicon ultrafiltration apparatus, using appropriate mol-wt cutoff membrane for sample. Apply Argon pressure to filter urea, salts, and acrylamide away from the RNA sample. When the sample volume in the Amicon apparatus becomes <5 mL, add 50 mL of DEPC-treated water and reconcentrate. Repeat once.
11. Remove concentrated RNA sample, and take OD at 260 nm to determine concentration. Analyze 1 and 20 µg amounts on an analytical polyacrylamide gel, and visualize by ethidium bromide staining to check for purity of the sample. Filter through 0.2 µm spin filter, and store at 4°C. *See* Chapter 39 for further analysis of RNA prior to crystallization and Chapter 40 for crystallization techniques.

4. Notes

1. Although other self-cleaving RNA motifs (the hammerhead and hairpin ribozymes) have been used for homogeneous RNA preparation *(4,5)*, the hepatitis δ ribozyme was chosen because it has almost no sequence requirements upstream of the cleavage site *(6)*. Virtually any RNA sequence of interest can be used in this system, with the exception of sequences with a G immediately preceding the cleavage site.
2. To prepare DEPC-treated water, add 1 mL of DEPC to 5 L of distilled water, shake well, let sit at room temperature for 30 min, and autoclave. This procedure destroys any contaminating proteins and microorganisms that might interfere with RNA preparation and storage.
3. We routinely optimize transcription conditions for each new plasmid template by setting up 20 µL reactions in which different concentrations of magnesium (20–40 m*M*), DTT (10–40 m*M*), NTPs (2–5 m*M*), and template concentration (200–800 n*M*) are tested. Following incubation for 1–2 h, each reaction is quenched by the addition of an equal volume of 2X formamide loading buffer, and 5–10 µL of the resulting sample are analyzed on a denaturing polyacrylamide gel and visualized by ethidium bromide staining.
4. T7 RNA polymerase is prepared in our laboratory and stored in 50% glycerol at –20°C at a concentration of 1 mg/mL.
5. Sometimes the progress of the transcription reaction can be monitored by the presence of magnesium pyrophosphate, which forms a flocculent white precipitate as transcription proceeds. The lack of precipitate should not be interpreted as a sign of poor transcription, however, since pyrophosphate precipitation can be affected by the concentration of salts and DTT in the reaction.
6. In our hands, the efficiency of δ ribozyme processing varies depending on the context of the cleavage site. In general, cleavage is enhanced in the presence of higher magnesium ion concentrations (>20 m*M*) and at temperatures above 37°C. Inefficient processing can be improved by adding additional magnesium ion to the reaction, heating at 65–70°C, and in some cases, by adding 5–10% formamide or 1–2 *M* urea to the reaction. RNA sequences that are highly structured (i.e., base-paired) near the cleavage site have been problematic; it may be necessary to change the sequence around the cleavage site or include an "antisense" DNA strand in the reaction to compete with the interfering structure.
7. It is important to avoid overheating of the gel plates during electrophoresis, since this may result in aberrant migration of the RNA as well as plate cracking.
8. For gels >12% in acrylamide, crush with a glass rod or by dicing bands finely with a razor blade.

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Establishing Suitability of RNA Preparations for Crystallization

Determination of Polydispersity

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1. Introduction

Successful crystallization of ribozymes and ribozyme domains depends on covalent homogeneity of the sample, conformational homogeneity of the preparation, and an efficient and broad sampling of solution conditions where crystals might nucleate and grow. Chapter 38 presents methods to prepare multimilligram quantities of pure RNA, and Chapter 40 describes strategies for determining crystallization conditions. This chapter presents two methods to determine the conformational homogeneity (polydispersity) of RNA preparations. These methods have been successfully used to determine which of several related constructs of a given target molecule are likely to crystallize, enabling the experimenter to focus on the most promising candidate, and constitute an important step in a successful strategy for crystallization.

The first method is polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. The equipment needed for its implementation is readily available in laboratories engaged in RNA research, and the technique is familiar to many experimenters. Native PAGE, however, is limited to evaluation of polydispersity under low-ionic-strength conditions and is relatively time-consuming. Its principal drawback is that it involves separation, not examination of the sample directly in solution. Size-exclusion or gel-filtration chromatography, a technique not considered in this chapter, suffers from the same limitation. The second method presented is examination of RNA samples by dynamic light scattering (DLS) *(1)*. This nondestructive technique allows determination of the conformational homogeneity of RNA in solutions containing a wide

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