

A Sparse Matrix Approach to Crystallizing Ribozymes and RNA Motifs

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1. Introduction

With the discovery of RNA catalysts and the role of RNA in many essential biological processes, our need to learn the fundamentals of RNA structure has become acute. X-ray crystallography is the only means available to determine the three-dimensional structure of many biologically interesting RNAs owing to their size. To begin an RNA crystallography project, one needs to synthesize milligram quantities of homogeneous RNA, as described in Chapters 38 and 39.

This chapter covers an efficient way to obtain leads to crystallization conditions for an RNA macromolecule. Initial crystals can then be optimized for diffraction studies in subsequent experiments. Owing to the large number of variables one could screen in trying to obtain crystals, initial crystallization screens have been developed that are biased toward conditions that have worked for other macromolecules (1-5). The method presented below is based on a sparse matrix screen for RNA (3) using vapor diffusion to bring the RNA to supersaturation (6). See Chapter 41 for a related approach.

First, the specialized materials that need to be prepared to set up crystallization experiments are described. The second section covers the preparation of solutions. Third, the setup and storage of crystallization trays are described. Finally, the analysis of the crystallization attempts is briefly discussed.

2. Materials

2.1. Trays and Cover Slips

1. Linbro tissue-culture trays (24-well, ICN-Flow).
2. 22 mm Glass circular cover slips.

Table 1
Solutions for Annealing

Tube no.	Solution set II, pH, and salt ^a	RNA + set II to anneal, total volume (μL)
2.1	5.5, MgCl ₂	4.4
2.2	5.5, MgSO ₄	4.4
2.3	6.0, MgCl ₂	39.6
2.4	6.0, MgSO ₄	4.4
2.5	6.5, MgCl ₂	35.2
2.6	6.5, Mg(OAc) ₂	8.8
2.7	7.0, MgCl ₂	44.0
2.8	7.5, MgCl ₂	44.0
2.9	8.0, MgCl ₂	8.8

^a100 mM buffer, 10–20 mM Mg salt.

3.2. Preparation of Solutions

We have found that using DEPC-treated water and disposable pipets and tubes minimizes RNA degradation owing to contaminating RNases. For crystallization experiments, all solutions should be filtered through 0.2 μm filters. In addition, the divalent salts should be of high quality to reduce degradation problems from contaminating transition metals, such as zinc and manganese (see Note 4).

1. To each liter of distilled water, add 200 μL of DEPC and autoclave.
2. Filter and store the RNA sample in approx 10 mM buffer (pH <7.0) at 4°C. The RNA should be about 10–15 mg/mL; 1 OD at 260 nm = 40 μg/mL (see Note 5).
3. Make the following well solutions with DEPC-treated water and filter (all % values are v/v): 1, 2, and 3 M ammonium sulfate, 7 and 15% isopropanol, 8 and 10% *t*-butyl alcohol, 5, 15, 25, and 30% 1,4-dioxane, 5, 10, 15, and 25% MPD, 20% ethanol, 4% PEG 8000, 10% 1,6-hexanediol, and 2.5 mM spermine. Fifty milliliters of each are sufficient for several sparse matrix setups. The spermine solution should be made fresh or stored at –20°C between uses. Store PEG 8000 at 4°C to avoid bacterial growth.
4. Make solution set II (2X solutions) shown in Table 1 with filtered stock solutions. One milliliter of each is more than sufficient. Use 100 mM buffer and 10–20 mM Mg²⁺.
5. Make solution set III shown in Table 2 with filtered stock solutions. One milliliter of each is more than sufficient. Since many of these solutions contain spermine or spermidine, they should be stored at –20°C between uses. The amounts listed in Table 2 are for 5X stocks.

3.3. Crystallization Setup, Storage, and Observations

1. Prepare the nine annealing mixes in 0.7 mL microcentrifuge tubes as described in Table 1.

3. 2% Dimethyldichlorosilane in toluene (this solution should be used in the hood and disposed of properly; dimethyldichlorosilane should be stored desiccated at 4°C).
4. 95% Ethanol.
5. Forceps, flat blade (i.e., Millipore MF filter forceps).
6. Pressurized and filtered air (0.2 μm filter).
7. 10 mL Syringes.
8. Vaseline.
9. Modeling clay.

2.2. Solutions

1. Diethylpyrocarbonate (DEPC), which is toxic, should be stored at 4°C.
2. Sterile distilled water, DEPC-treated (see Section 3.2.).
3. 1 M Stocks of the following buffers and salts in DEPC-treated distilled water: potassium succinate, pH, 5.5, potassium cacodylate, pH, 6.0 (toxic), potassium PIPES, pH, 6.5, potassium MOPS, pH, 7.0, potassium HEPES, pH, 7.5, Tris-HCl, pH, 8.0, MgCl₂, Mg(OAc)₂, MgSO₄, CaCl₂, SrCl₂, BaCl₂, NiCl₂, CuSO₄, CrCl₃, CoCl₂, spermine-HCl, spermidine-HCl. Spermine and spermidine stocks should be stored at –20°C.
4. Precipitating agents: ammonium sulfate, polyethylene glycol 8000 (PEG 8000), 2-methyl-2,4-pentanediol (MPD), 1,6-hexanediol, *t*-butyl alcohol, ethanol, isopropanol, 1,4-dioxane (possible carcinogen).
5. 0.2 μm Syringe filters.
6. RNA solution at ≥10.0 mg/mL (see Section 3.2. for details).

2.3. Crystallization Setup, Storage, and Observations

1. Dissecting microscope with crosspolarizers (×60 magnification is sufficient).
2. Styrofoam boxes capable of holding Linbro trays.

3. Methods

3.1. Trays and Cover Slips

1. Drop the cover slips individually into a 2% dichlorodimethylsilane solution with shaking for 15 min. This should be done in the hood. Pour off the liquid into a waste container in the hood (see Note 1).
2. Rinse the cover slips thoroughly with 95% ethanol to remove the toluene, dichlorodimethylsilane, and HCl generated during the silanization process.
3. Blow dry the cover slips individually with the filtered pressurized air, and store in a container free of dust particles (see Note 2).
4. Melt vaseline, and pull into 10 mL syringes by aspiration (see Note 3).
5. Blow out each well with the pressurized air to remove plastic shavings.
6. After the Vaseline cools and solidifies, place a bead of Vaseline on the lip of each well of two Linbro trays. Leave a very small gap to allow air to escape before the cover slip is sealed in place (see Section 3.3.).
7. Place small balls of modeling clay in the inside corners of the Linbro tray lid to keep the lid suspended above grease.

Table 2
Solution Set III (Additives)

Tube no.	Polyamine, mM	Mg salt ^a , mM	Other divalent, 10 mM
3.1	Spermine (spm), 2.5	80	Ca
3.2	spm, 2.5	80	
3.3	spm, 2.5	55	Ba
3.4	spm, 2.5	80	
3.5	spm, 2.5	80	
3.6	spm, 2.5	5	
3.7	spm, 2.5	30	Co
3.8	spm, 2.5	80, MgSO ₄	
3.9	spm, 2.5	30	
3.10	spm, 5.0	5	
3.11	spm, 5.0	80	Co
3.12	spm, 5.0	55	
3.13	spm, 5.0	80	Co
3.14	spm, 5.0	80	
3.15	spm, 5.0	55	
3.16	spm, 5.0	5	
3.17	spm, 5.0	5	
3.18	spm, 5.0	55	Ca
3.19	spm, 7.5	30	
3.20	spm, 7.5	80	Co
3.21	spm, 7.5	5	Ca
3.22	spm, 7.5	5	Co
3.23	spm, 7.5	30	Co
3.24	spm, 7.5	30	
3.25	spm, 7.5	30, Mg(OAc) ₂	
3.26	spm, 7.5	5	Sr ^b
3.27	spm, 7.5	30	Cr
3.28	Spermidine (spd), 5.0	55	Ni
3.29	spd, 5.0	80, MgSO ₄	
3.30	spd, 5.0	30	CuSO ₄
3.31	spd, 5.0	5	
3.32	spd, 5.0	380	
3.33		30	Ca
3.34		30	
3.35		30, Mg(OAc) ₂	Ca
3.36		30	Ba
3.37		5	
3.38		80	Sr
3.39		55	Ni
3.40		5	
3.41		55	CuSO ₄
3.42			
3.43		55	
3.44		55	Co

^aMgCl₂, unless otherwise indicated. Divalent metals are chloride salts, unless otherwise indicated. Chromium is trivalent.

^bSrSO₄ crystallizes often at 4°C.

Sparse Matrix Approach

- Heat the annealing mixes for 10 min at 65°C in a heating block. Keep the tubes covered with aluminum foil to minimize evaporation of the solutions and condensation on the lids of the tubes.
- Allow the tubes to cool slowly to room temperature. Centrifuge the tubes briefly after they have cooled to collect any condensation that may have formed. See Note 4 for additional annealing protocols.
- Prepare 44 0.7 mL microcentrifuge tubes with the appropriate amount of the annealing mix and solution from set III as described in Table 3.
- Add 1 mL of well solution to each of the wells of the Linbro tray as described in Table 3. The Linbro trays have numbered columns 1-6 and lettered rows A-D. For example, condition 22 is well D4 in the first tray, and condition 36 is well B6 in the second tray.
- On a cover slip, mix the RNA mix for condition 1 with well solution in ratios of 2:1, 2:2, and 1:2 (in μL). Flip the cover slip gently, and seal over well A1. Repeat for the other 43 conditions (see Note 6).
- Using the dissection microscope, make notes on the drops immediately after finishing the trays (see Note 7).
- Replace the lid of the tray, and store the trays in a styrofoam box or a constant-temperature incubator to buffer against temperature changes (see Note 8).
- Repeat steps 1-8 for screening at additional temperatures; i.e., 4 or 30°C. Use styrofoam boxes to move trays between temperatures (see Note 9).
- Trays should be analyzed under the dissection microscope after about a week to see which drops contain precipitate, which drops are clear, and with luck, which drops have crystals. These observations will be useful in designing a second round of screening.
- Store the trays and make observations over the next 3-4 mo, increasing the time between observations (see Note 10).

4. Notes

- Some companies sell presilanized cover slips, but these are more expensive. Other silanizing reagents have not worked as well in our hands. The cover slips must be well-silanized because the organic precipitants used in this screen lower the surface tension of the drops, which causes them to spread out. MPD seems to be the worst in this regard. If the cover slips are not well silanized, it is (nearly) impossible to set up 3 drops/cover slip (see Note 6 and Section 3.3.).
- Some sources of pressurized air can contain tiny droplets of oil in the delivered air, which can be removed by an in-line filter.
- Vacuum grease can be used for room-temperature or 30°C trays, but is very difficult to work with at 4°C if one needs to remove a cover slip to recover drop contents.
- Though we generally start by using 60-65°C for annealing, other temperatures may be more useful. Several annealing temperatures and cooling speeds could be tested by running the annealed RNA on native polyacrylamide gels or using dynamic light scattering (see Chapter 39). A different screen recently published

Table 3
Tray Setup

Tube no., tray no., and well ID	Well solution	Drop solution, 4.4 μ L, annealed + 1.1 μ L additives
1 (1.A1)	15% Isopropanol	2.3 + 3.1
2 (1.A2)	4% PEG 8000	2.3 + 3.2
3 (1.A3)	7% Isopropanol	2.5 + 3.3
4 (1.A4)	3 M Ammonium sulfate	2.1 + 3.4
5 (1.A5)	2 M Ammonium sulfate	2.7 + 3.5
6 (1.A6)	10% MPD	2.5 + 3.6
7 (1.B1)	2 M Ammonium sulfate	2.7 + 3.7
8 (1.B2)	25% MPD	2.4 + 3.8
9 (1.B3)	15% 1,4-Dioxane	2.5 + 3.9
10 (1.B4)	25% 1,4-Dioxane	2.7 + 3.10
11 (1.B5)	10% 1,6-Hexanediol	2.8 + 3.11
12 (1.B6)	1 M Ammonium sulfate	2.8 + 3.12
13 (1.C1)	4% PEG 8000	2.8 + 3.13
14 (1.C2)	2 M Ammonium sulfate	2.8 + 3.14
15 (1.C3)	20% Ethanol	2.5 + 3.15
16 (1.C4)	7% Isopropanol	2.5 + 3.16
17 (1.C5)	5% 1,4-Dioxane	2.8 + 3.17
18 (1.C6)	8% <i>t</i> -Butanol	2.3 + 3.18
19 (1.D1)	5% 1,4-Dioxane	2.9 + 3.19
20 (1.D2)	15% MPD	2.5 + 3.20
21 (1.D3)	15% Isopropanol	2.3 + 3.21
22 (1.D4)	25% MPD	2.5 + 3.22
23 (1.D5)	5% MPD	2.5 + 3.23
24 (1.D6)	15% MPD	2.8 + 3.24
25 (2.A1)	3 M Ammonium sulfate	2.6 + 3.25
26 (2.A2)	5% MPD	2.7 + 3.26
27 (2.A3)	8% <i>t</i> -Butanol	2.8 + 3.27
28 (2.A4)	25% 1,4-Dioxane	2.8 + 3.28
29 (2.A5)	5% MPD	2.2 + 3.29
30 (2.A6)	30% 1,4-Dioxane	2.7 + 3.30
31 (2.B1)	10% <i>t</i> -Butanol	2.7 + 3.31
32 (2.B2)	2.5 mM Spermine	2.8 + 3.32
33 (3.B3)	15% MPD	2.7 + 3.33
34 (2.B4)	3 M Ammonium sulfate	2.3 + 3.34
35 (2.B5)	25% 1,4-Dioxane	2.6 + 3.35
36 (2.B6)	30% 1,4-Dioxane	2.7 + 3.36
37 (2.C1)	7% Isopropanol	2.3 + 3.37
38 (2.C2)	15% 1,4-Dioxane	2.3 + 3.38
39 (2.C3)	1 M Ammonium sulfate	2.8 + 3.39
40 (2.C4)	20% Ethanol	2.9 + 3.40
41 (2.C5)	15% Isopropanol	2.3 + 3.41
42 (2.C6)	15% 1,4-Dioxane	2.7 + 3.43
43 (2.D1)	20% Ethanol	2.3 + 3.43
44 (2.D2)	1 M Ammonium sulfate	2.7 + 3.44

performs the annealing in the presence of polyamine and buffer with little or no other salts present (5). If the RNA is degrading during the annealing step, addition of 0.1 mM EDTA may reduce the problem.

- The concentration of RNA for storage needs to be high enough for the dilutions involved in setting up the drops, but not so high as to form aggregates. We have observed the formation of gels if the RNA is stored at high concentrations. These gels will often dissolve if the RNA is warmed to room temperature or a little warmer.
- To support the cover slip while setting up drops, one can use the lid of the Linbro tray with one part of the cover slip slightly off the edge. We often use a 1.5 mL microcentrifuge tube firmly secured in a tube rack. This has the advantage that the edge of the tube lid is approximately the boundary within which all three drops must be set up. We usually pipet the solutions onto the cover slip in the shape of an L (an asymmetric shape) so that the drops can be distinguished later. We usually do not pipet up and down to mix the well and drop solutions. Layering one on the other works fine for mixing such small volumes.
- To avoid heartache later, it is important to observe the drops right after setting them up to look for foreign particles that may have fallen into the drops (talc, pieces of plastic from the pipet tips, and so forth). We always wear gloves when setting up RNA crystallization experiments, making sure to wear talc-free gloves or wash off any talc before handling the solutions. One should also write down whether precipitate formed immediately or not. This will help in the design of future experiments.
- Since this screen uses several volatile precipitants (including MPD), many of the drops will actually grow in size over time. This is probably owing to the fact that there is no salt component in the well solutions for the volatile compounds. Thus, the drop size tends to grow to decrease the ionic strength of the drop. One possible means of avoiding this problem is to take 1 mL aliquots of the well solutions to use in addition to the RNA solution on the cover slip, and add 150 mM NaCl to the solutions that actually go in the well. This approach also helps the 2.5 mM spermine condition, which, in the absence of additional ionic strength in the well, will not drive the drops to concentrate as in a standard vapor diffusion experiment.
- We have had good results for crystallization at room temperature and 30°C, whereas some of the tRNAs (including tRNA^{Phe}) yielded crystals at 4–8°C (7). Precipitates are generally along a continuum between granular (disincent particles) and flocculent. Granular precipitates in general are better than flocculent precipitates in terms of how close the conditions are to ones in which crystals will grow (2). In addition, one should also cross the polarizers to see if putative crystals or precipitates are birefringent, i.e., glow when the background is dark. Birefringence occurs because crystals rotate plane-polarized light (6). One should be aware of the fact that birefringence is harder to interpret when the polarized light passes through plastic. One should remove the lid of the Linbro tray before making observations. Then only the plastic at the bottom of the well will interfere with birefringence, though not too severely.

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Crystallographic Analyses of Chemically Synthesized Modified Hammerhead RNA Sequences as a General Approach Toward Understanding Ribozyme Structure and Function

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1. Introduction

Solid-support chemical synthesis of RNA (1), though costly, has the advantage of allowing the incorporation without restriction of any desired nucleotide sequence, including sequences which contain special modified nucleotides. For example, the crystal structure of an all-RNA hammerhead ribozyme containing a modified 2'-O-methylcytosine at the active site to prevent cleavage has recently been solved (2,3). Incorporation of the 2'-methoxyl moiety specifically at the cleavage site of the ribozyme can only be accomplished by using the chemical synthesis approach. Many "unnatural" hammerhead RNA sequence modifications, including modified purine and pyrimidine bases, 2'-fluoro- and 2'-amino-modified riboses, and phosphorothioates (among many other examples), have been synthesized by a variety of research groups for probing the hammerhead RNA reaction mechanism and structure. Modified sequences, including ones containing unnatural bases, can be synthesized and crystallized in the same conditions as those used to produce the original crystals for a variety of applications. For example, a hammerhead RNA substrate strand containing a photolabile moiety protecting the active site in a manner analogous to the 2'-O-methylcytosine at the active site, has recently been crystallized in the same conditions and space group as the originally solved crystal form. This will allow the structure of the modified hammerhead RNA to be solved with a single data set based on the (publicly distributed) coordinates of the original structure. The ultimate purpose of this particular modified hammerhead RNA is for time-resolved crys-