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Preparation of Protein Crystals for X-Ray Structural Study

Soichi Takeda

Summary

The knowledge of accurate molecular structures obtained by X-ray protein crystallography is now inevitable for rational drug design and for understanding the molecular basis underlying genetic disorders found in patients. However, preparing protein crystals suitable for structural analysis is currently the bottleneck in structure determination by this method. The intent of this chapter is to present current methods of preparing protein crystals for structural studies for a wide range of biologists who have access to macromolecules but do not know how to handle them for crystallization. The chapter includes the pretreatment of a protein prior to the crystallization experiment, initial screens, and optimization of the crystallization conditions for further X-ray study. Finally, handling considerations that are important for a protein intended for crystallization experiments are discussed.

Key Words: X-ray crystallography; structural biology; crystallization; protein structure; rational drug design; synchrotron.

1. Introduction

The importance of solving protein structures continues to grow in fields ranging from basic biochemistry and biophysics to pharmaceutical development, medical science, and, of course, cardiovascular research. In order to obtain high-resolution, three-dimensional structural knowledge of proteins by X-ray crystallography, crystals diffracting at high resolution are needed. This chapter focuses on the essential principles and procedures involved in crystallization so as to provide a general understanding of what is entailed in this key step of solving X-ray protein structures. It is not our aim to convert biologists into X-ray crystallographers; complete explanation of the physical basis of the techniques and methods currently practiced is beyond the scope of this chapter.

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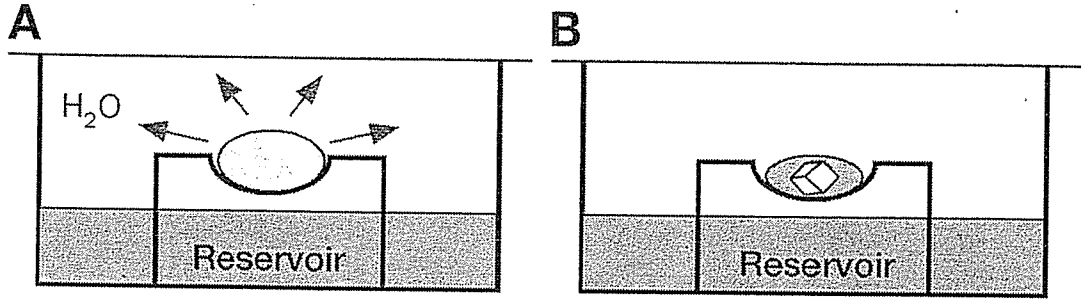

Fig. 1. Principle of the vapor diffusion (sitting drop) method for crystallization. The concentration of the reservoir is initially twice that in the droplet (**A**), and the two become equal to that of the reservoir that has a volume several orders of magnitude larger than the droplet. The drop decreases volume during the process so that the concentration of all components in the drop, including the protein, rise significantly. If the variables are right, this results in protein crystallization (**B**).

This chapter includes neither protein expression and purification protocols nor methods associated with X-ray diffraction data collection and structural analysis; rather, it simply focuses on the basis of crystallization experiments. If one needs further information as well as more detailed protocols on crystallization, please see the textbooks listed in the references *(1–7)*.

Although there are a variety of methods for setting up protein samples for crystallization, the most widely used method is crystallization by vapor diffusion. In this method, vapor diffusion occurs in a closed system because of a difference in concentration between a small droplet of protein, typically 0.3–10 μL, and a larger body of liquid, typically 0.1–1 mL (*see* **Fig. 1**). The protein drop is made by diluting protein twofold with liquid from the larger body, usually referred as the reservoir solution; therefore, the protein drop starts at half the concentration of the contents in the reservoir solution. This concentration gradient drives vapor diffusion, resulting in the gradual concentration of protein and, if the variables are right, protein crystallization. Reservoir solutions can contain a wide range of chemical variables, including buffers for pH control, salts (NaCl, ammonium sulfate, and so on), precipitating agents (polyethylene glycol, organic solvents), reducing agents (dithiothreitol and so on), and detergents.

As the variability of conditions required for crystallizing proteins is too large for exhaustive searches, more practical approaches for initial searches are employed. This is termed "sparse matrix" screening. The sparse matrix design was first introduced in 1991 by Jancarik and Kim *(8)* (*see* **Table 1** and **Note 1**). Various ready-made kits, some are the extensions of the original one and the others are based on different strategies, are currently commercially available and are used effectively for initial crystallization screens of large numbers of proteins.

Table 1
Fifty Solutions for the Original Sparse Matrix Screen *(7)*

No.	Salt	Buffer	Precipitant
1	0.02 *M* Ca chloride	0.1 *M* Na acetate pH 4.6	30% MPD
2	None	None	0.4 *M* K/Na Tartrate
3	None	None	0.4 *M* ammonium phosphate
4	None	0.1 *M* Tris-HCl pH 8.5	2.0 *M* ammonium sulfate
5	0.2 *M* Na citrate	0.1 *M* HEPES/NaOH pH 7.5	30% MPD
6	0.2 *M* Mg chloride	0.1 *M* Tris/HCl pH 8.5	30% PEG4000
7	None	0.1 *M* Na cacodylate pH 6.5	1.4 *M* Na acetate
8	0.2 *M* Na citrate	0.1 *M* Na cacodylate pH 6.5	30% iso-propanol
9	0.2 *M* ammonium acetate	0.1 *M* Na citrate pH 5.6	30% PEG 4000
10	0.2 *M* ammonium acetate	0.1 *M* Na acetate pH 4.6	30% PEG 4000
11	None	0.1 *M* Na citrate pH 5.6	1.0 *M* ammonium phosphate
12	0.2 *M* Mg chloride	0.1 *M* HEPES-Na pH 7.5	30% iso-propanol
13	0.2 *M* Na citrate	0.1 *M* Tris-HCl pH 8.5	30% PEG 400
14	0.2 *M* Ca chloride	0.1 *M* HEPES/NaOH pH 7.5	28% PEG 400
15	0.2 *M* ammonium sulfate	0.1 *M* Na cacodylate pH 6.5	30% PEG 8000
16	None	0.1 *M* HEPES/NaOH pH 7.5	1.5 *M* Li sulfate
17	0.2 *M* Li sulfate	0.1 *M* Tris/HCl pH 8.5	30% PEG 4000
18	0.2 *M* Mg acetate	0.1 *M* Na cacodylate pH 6.5	20% PEG 8000
19	0.2 *M* ammonium acetate	0.1 *M* Tris-HCl pH 8.5	30% iso-propanol
20	0.2 *M* ammonium sulfate	0.1 *M* Na acetate pH 4.6	25% PEG 4000
21	0.2 *M* Mg acetate	0.1 *M* Na cacodylate pH 6.5	30% MPD
22	0.2 *M* Na acetate	0.1 *M* Tris-HCl pH 8.5	30% PEG 4000
23	0.2 *M* Mg chloride	0.1 *M* HEPES/NaOH pH 7.5	30% PEG 400
24	0.2 *M* Ca chloride	0.1 *M* Na acetate pH 4.6	20% iso-propanol
25	None	0.1 *M* imidazole pH 6.5	1.0 *M* Na acetate
26	0.2 *M* ammonium acetate	0.1 *M* Na citrate pH 5.6	30% MPD
27	0.2 *M* Na citrate	0.1 *M* HEPES/NaOH pH 7.5	20% iso-propanol
28	0.2 *M* Na acetate	0.1 *M* Na cacodylate pH 6.5	30% PEG 8000
29	None	0.1 *M* HEPES/NaOH 7.5	0.8 *M* K/Na tartrate

(Continued)

Table 1 *(Continued)*
Fifty Solutions for the Original Sparse Matrix Screen *(7)*

No.	Salt	Buffer	Precipitant
30	0.2 *M* ammonium sulfate	None	30% PEG 8000
31	0.2 *M* ammonium sulfate	None	30% PEG 4000
32	None	None	2.0 *M* ammonium sulfate
33	None	None	4.0 *M* Na formate
34	None	0.1 *M* Na acetate pH 4.6	2.0 *M* Na formate
35	None	0.1 *M* HEPES/NaOH 7.5	0.8 *M* Na/K phosphate
36	None	0.1 *M* Tris-HCl pH 8.5	8% PEG 8000
37	None	0.1 *M* Na acetate pH 4.6	8% PEG 4000
38	None	0.1 *M* HEPES/NaOH pH 7.5	1.4 *M* Na citrate
39	2.0 *M* ammonium sulfate	0.1 *M* HEPES/NaOH pH 7.5	2% PEG 400
40	None	0.1 *M* Na citrate pH 5.6	20% iso-propanol, 20% PEG 4000
41	None	0.1 *M* HEPES/NaOH pH 7.5	10% iso-propanol, 20% PEG 4000
42	0.05 *M* K phosphate	None	20% PEG 8000
43	None	None	30% PEG 1500
44	None	None	0.2 *M* Mg formate
45	0.2 *M* Zn acetate	0.1 *M* Na cacodylate pH 6.5	18% PEG 8000
46	0.2 *M* Ca acetate	0.1 *M* Na cacodylate pH 6.5	18% PEG 8000
47	None	0.1 *M* Na acetate pH 4.6	2.0 *M* ammonium sulfate
48	None	0.1 *M* Tris-HCl pH 8.5	2.0 *M* ammonium phosphate
49	1.0 *M* Li sulfate	None	2% PEG 8000
50	0.5 *M* Li sulfate	None	15% PEG 8000

PEG, polyethylene glycol; MPD: 2-methyl-2,4-pentanediol.

Because good-quality crystals are not usually obtained in the first screen, once crystals (most often microcrystals) have been observed in trial experiments, crystallization conditions must be improved for crystal size and quality. At this stage, we usually perform a "grid screen." For each condition, two variables (such as pH and polyethylene glycol [PEG] concentration) are altered in a two-dimensional (x,y) grid. After fine-tuning of all the parameters,

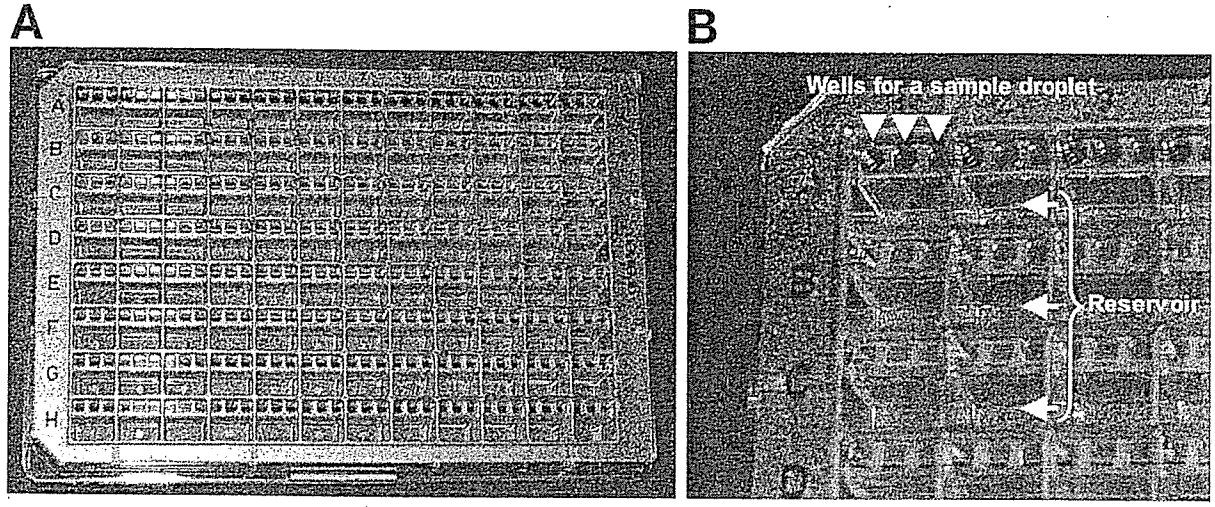

Fig. 2. Greiner CrystalQuick 96-well sitting drop crystallization plate. The plate has 8 vertical wells and 12 horizontal wells. Within each well is a rectangular reservoir with 0.1 mL of fill volume. Adjacent to the reservoir is a ledge containing three drop-support wells for each sample.

crystals suitable for X-ray study are obtained. If the crystals have appropriate size and are really made of protein, not salt, then this is the time to start characterizing them by X-ray diffraction with the help of a protein crystallographer.

2. Materials

2.1. Preparation of Proteins for Crystallization

1. Tris-buffer(TB): 10 m*M* Tris-HCl, pH 7.5 (*see* **Note 2**). Prepared by diluting 100X stock solution (1 *M* Tris-HCl, pH 7.5) with deionized water.
2. Dialysis tubes: Spectra/Por 7 or its equivalent, whose membrane has been treated to minimize the content of heavy metals that can affect crystallization of the protein. Rinse with TB prior to use to remove sodium azide that has been added as a preservative when shipped.
3. Centrifugal filter device: Amicon Ultra-4 (Millipore). There are five devices with different nominal molecular weight limits (NMWLs; 5000, 10,000, 30,000, 50,000, and 100,000). For higher recovery, use a NMWL device with a cutoff range a bit smaller than the molecular weight (MW) of the protein of interest. Prerinse the devices with TB prior to use.

2.2. Initial Screening of Crystallization Conditions

1. Crystal screen: Crystal Screen (Hampton Research) or Crystal Screen Basic (Sigma-Aldrich) containing 50 solutions (*see* **Table 1** and **Note 1**) described in the original sparse matrix by Jancarik and Kim *(8)*.
2. Crystallization plate: Greiner CrystalQuick 96-well sitting drop crystallization plate (Hampton Research; *see* **Fig. 2** and **Note 3**).

3. Sealing tape: a roll of 4-in. wide Crystal Clear Sealing Tape (Hampton Research) or its equivalents. Tapes or films used for sealing crystallization plate should be optically transparent for viewing with a microscope.

3. Methods

Crystallization experiments start with "crystal quality" protein (*see* **Note 4**). Prior to a crystallization experiment, the protein sample should be concentrated in a low-ionic-strength buffer. The crystallization of the protein can be divided into the following two stages: (1) initial screening to obtain any kind of crystals or promising precipitates and (2) optimization to improve the crystals for X-ray diffraction data collections. Ninety-six-well crystallization plates (*see* **Fig. 2** and **Note 2**), which have been developed for sitting drop vapor diffusion to facilitate high-throughput crystallization, are used for both for screens and optimizations. Commercially available "sparse matrix" screens are currently the best choice for initial trials (*see* **Table 1** and **Note 1**). The crystallization experiments are examined daily by observation of the protein droplets using a stereo microscope. The conditions that produce microcrystals should be optimized by "grid screening" to grow high-quality (free of cracks and defects) single crystals of appreciable size (0.05–0.1 mm at least for the dimensions of a face) suitable for X-ray study.

3.1. Preparation of the Proteins for Crystallization

1. Prepare several milligrams of the protein of interest with appropriate purity (*see* **Note 5**).
2. If the protein is lyophilized, dissolve the protein in approx 1 mL of TB (*see* **Note 6**). If the protein is hard to dissolve, then incrementally increase the salt (50, 100, 150, and 200 m*M* NaCl) until it is fully in solution.
3. Dialyze the protein against 1 L of TB (*see* **Note 7**). If the protein is already in solution, start from this step. Change the dialysis solution twice at 6-h intervals. If the sample precipitates, add NaCl in the dialysis solution in order to prevent precipitation, and repeat the step. The salt concentration of the protein sample should be as low as possible. Ideally, it is best to prepare samples in a lower buffer concentration without salt.
4. Remove undisclosed particles by centrifugation (20,000*g* for 10 min).
5. Concentrate the protein solution with a centrifugal filter device Amicon Ultra-4. In general, 10 mg/mL is a good starting protein concentration for initial crystallization trials (*see* **Note 8**). Add the sample to the Amicon Ultra filter unit and spin at a maximum 4000*g* for approx 10–60 min in a swinging-bucket rotor.
6. Recover the concentrated protein by inserting a pipet into the bottom of the filter unit.
7. Store the protein at 4°C until it is used in the crystallization experiment. For longer storage, –70°C is better; however, freezing and rethawing of the sample should be avoided. Therefore, aliquot the protein into several tubes (100–200 µL each) and quick-freeze each one in a liquid nitrogen bath before placing at –70°C.

8. Do not mix different purification batches in crystallization trials. Because it is not uncommon for one batch of protein to crystallize whereas the next will not, it is vital to keep a history of each sample and to track each batch separately.

3.2. Setting Up Protein Drops for Initial Crystallization Screening

1. These instructions assume the use of a Greiner CrystalQuick 96-well sitting drop crystallization plate (**Fig. 2**). Depressions in the plate may be sprayed with pressurized air or some inert gas to blow away dust just before dispensing reservoir solutions. Pipet 0.1 mL of each crystallizing solution of the screen in each of the 96 reservoirs.
2. Pipet 0.5 µL of the protein solution onto the drop-support well of A1 (*see* **Notes 9** and **10**). To carry out crystallization experiments on such a submicroliter scale, it is recommended to use Pipetman P2 (Gilson) or its equivalent.
3. Pipet 0.5 µL of reagent from the reservoir onto the drop-support well of A1 and mix with the protein solution.
4. Repeat **steps 2** and **3** for the remaining reservoirs.
5. Seal the plate with a strip of clear sealing tape.
6. Leave the plate in an incubator, and maintain a fairly constant temperature. Crystallization trials should be performed at a minimum of two temperatures, usually 20 and 4°C, because most protein crystals have been obtained at these temperatures. If possible, try another temperature between 10 and 15°C.
7. The crystallization plates are examined using a stereo microscope (1) immediately after setup, (2) once a day for the first week, and (3) once a week for several weeks (*see* **Note 11**).
8. In wells in which the protein precipitated immediately after making the drop, repeat the setup with modification. Halve the reservoir solution concentration with deionized water and repeat **steps 2–5**. It may slow crystal nucleation.
9. If enough protein sample is left, repeat **steps 1–8** with other screening kits, e.g., Crystal Screen II, PEG/ION Screen, INDEX, SALTRx (Hampton Research), Extension Kit, Low Ionic Kit, PEG Grid Screening Kit (Sigma-Aldrich), Wizard Screens (Emerald BioStructures), and so on. Different screens consist of solutions designed based on different strategies, thus they may compensate for gaps in the original sparse matrix screen *(8)*.

3.3. Observation of the Drops by a Stereo Microscope

1. Scan the drops at about ×20–40 magnification, and when something suspicious appears, increase the magnification to ×80 or ×100 for a better view (*see* **Notes 12** and **13**). Scan the entire depth of the drop, because crystals will form at different levels.
2. Crystals occur in a great variety of shapes such as needles, blades, walnuts, plates, and various geometric shapes and in various sizes (hardly observable 10 µ to 1 mm). An example is shown in **Fig. 3**.
3. Crystals are often distinguished from amorphous substances by their flat faces with sharp edges and by their anisotropy. Anisotropy of the crystals is examined by putting them between a crossed polarizer attached to a stereo microscope. They

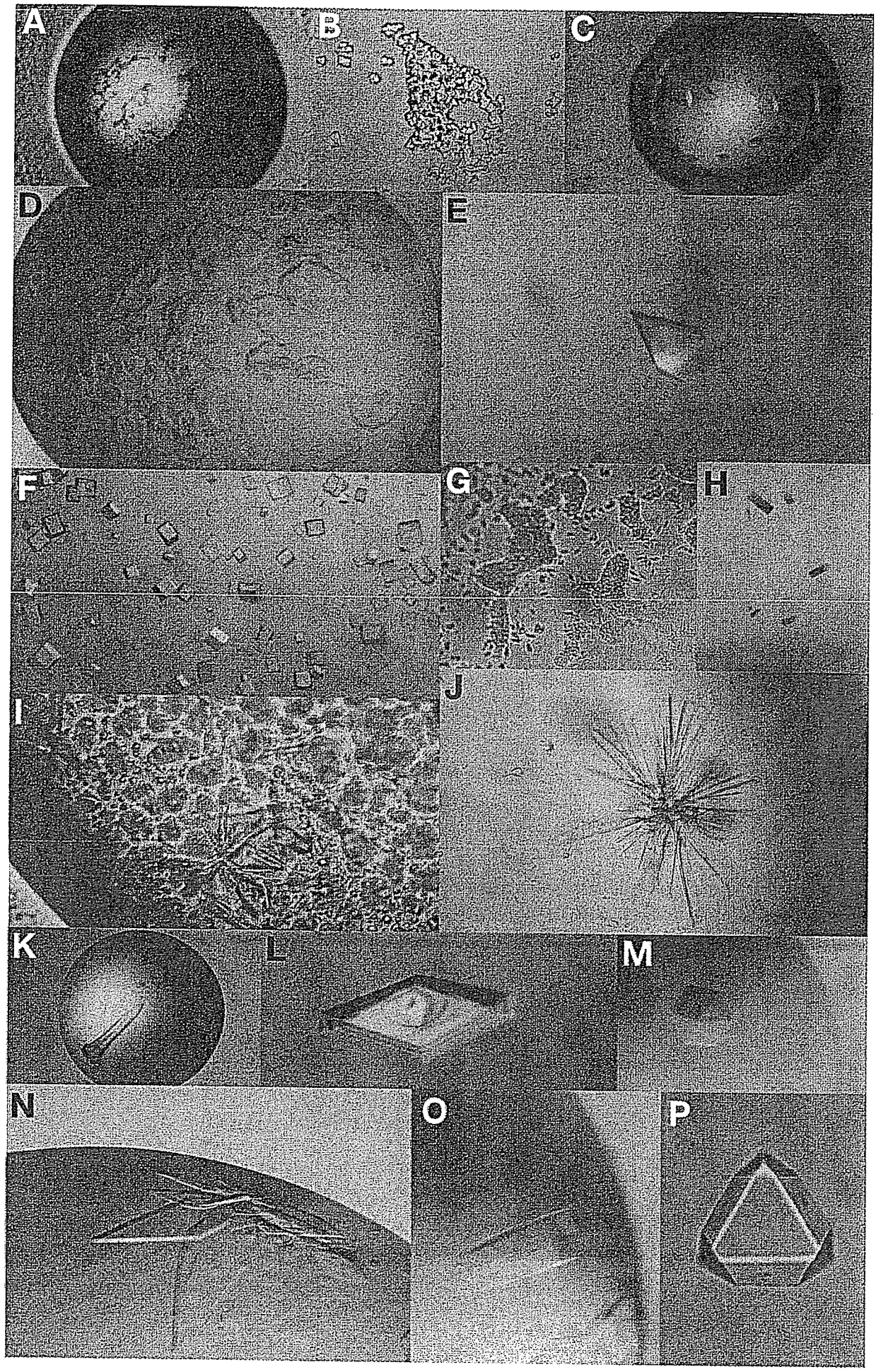

Fig. 3.

sometimes display beautiful colors, because the crystals are birefringent (*see* **Fig. 3C,E,H,L**).

4. Carefully observe everything to be familiar with the differences between microcrystals, amorphous aggregates, and sweater fuzz. If something suspicious is found, make new drops under the same conditions and check its reproducibility. True crystals should be reproduced if the protein is not degraded.
5. When either crystals or micro crystals are found, it is important to verify that the crystals are protein and not salt. IZIT (Hampton Research) is an example of a protein-binding dye. If the crystals obtained are suspected to be salts, a grain of the dye can be added to the drop. Salt crystals will not absorb the dye (*see* **Note 14**).
6. Another method of verifying protein crystals is to take a needle and try to crush the crystals. Salt crystals are so hard to break that one can often hear them snap. Protein crystals are much more fragile and easier to smash.

3.4. Optimization of the Crystallization Conditions

1. Once the crystals (most often microcrystals or suspicious crystals) are obtained in trial experiments, crystallization conditions must be improved for crystal size and quality. pH and precipitant concentration are two of the most important determinants of protein solubility; therefore, they will be the first parameters optimized by a grid screen.
2. Prepare reservoir solutions for the grid screen with variations in pH (in steps of 0.5 pH units initially, then 0.1 pH units finally) vs precipitant concentrations (decreasing in steps of 5%, initially), beginning around the pH value and the precipitant concentration that was found in the initial screen (*see* **Note 15**). Refine those parameters until the optimal crystallization conditions are found.
3. Protein concentration, temperature, and droplet size should also be optimized.
4. Add inhibitors, substrate, or co-factors to the protein drop using the optimized well conditions. Repeat the initial screen with the additive compound if an effect is found (*see* **Note 16**).

Fig. 3. *(Opposite page)* Photo micrographs of the results of crystallization. Crystals or microcrystals of various morphologies obtained from various proteins, including troponin complexes *(9,10)*, in initial crystallization screens are shown in **A**, **B**, **D**, and **F–J**. Transparent spherulite clusters made of tiny crystals were observed (**B**) in a close up view of **A**, suggesting that the well condition was a good starting point for optimization. After optimization of the conditions, single crystals with sharp edges were obtained (**C**) and successfully used for X-ray study. Crystals grown too quickly without edges (**D**) were improved by lowering both the protein and the precipitant concentrations (**E**). Single separated crystals (**K–M**, **O**, and **P**) or single crystals in the cluster of plates (**N**) were obtained after refining the crystallization conditions and were used for diffraction studies. Some crystals, not all, show birefringence (**C**, **E**, **H**, and **L**). Sometimes crystals eventually appear in the oil phase of a drop of salt-polyethylene glycol (PEG)-protein (**I**), because the protein may preferentially partition into the PEG-rich oily phase, thereby becoming locally concentrated enough to nucleate.

5. Repeat **steps 2–4** for other conditions that were not optimized before.
6. Once crystals with the appropriate size are obtained, start to characterize them by X-ray diffraction. For structural studies, it is not morphology but the diffraction quality of the crystals that is important.

4. Notes

1. Although sparse matrix screens are a biased sampling of crystallization parameters, selected from known or published successful crystallization conditions, they are an efficient method to screen a large number of parameters with a limited amount of protein. In our experiences, the first crystals from nearly half of the proteins tested in the initial screens were obtained using such sparse matrix-based ready-made kits. For example, the first crystals of the complexes of troponin, TnC/TnI(1-47) dimer *(9)* and TnT/TnC/TnI trimer *(10)*, were obtained from the solutions no. 38 and no. 17 of Crystal Screen (Hampton Research), respectively.
2. The solution in which the protein is finally dissolved should be as simple as possible (*see* **Note 7**), and 10 m*M* HEPES/NaOH, pH 7.0 is also a common example. It is better not to use phosphate buffers for proteins intended for crystallization even during the preparation procedure, because they often give rise to salt crystals. For proteins susceptible to oxidation, one often needs to add an excess amount of dithiothreitol (~20 m*M*) both in the drop and in the reservoir in order to keep the sample homogeneous (*see* **Note 10**).
3. 96-well plates are much more compact and use less material than 24-well plates, which have been conventionally used. In our experience, there are no obvious differences between the two systems in the results from the initial screens.
4. The protein should be at least 90–95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) *(11)* and staining with Coomassie Brilliant Blue. Always consider further purification of the protein (1) if the initial screen does not produce any promising results or (2) to improve crystal quality when optimizing. It is our belief that poor purity is the most common cause of unsuccessful crystallization, and the purity requirements of macromolecules must be higher than in other fields of molecular biology. On the other hand, it is not always true that proteins with high purity are easily crystallized. In our case, before truncating the fragile portion of the troponin complex *(10)*, full-length molecules that are extremely pure (more than 99% on SDS-PAGE) would never crystallize. In such cases, try screens with different constructs of varying lengths, different isoforms, or the protein from different sources. Small changes in the physical properties of the proteins can substantially affect its ability to crystallize. Isolated structural domains of proteins are very often easily crystallized with high quality, and limited proteolysis is one of the best methods to figure out which structural domains are suitable for crystallization experiments *(12)*.
5. The initial gels prior to the crystallization experiments are important for documentation of the homogeneity of the protein, possible batch variations, and for verification of protein stability. As proteins degrade with time, comparison of the gels from failed crystallization drops with the original ones will establish if the protein

has deteriorated. Aging results from the action of contaminants either already present or introduced into the samples or from modifications generated by oxidants. In some cases, changes in crystal formation are owing to the presence of fungi or mold that multiply in the stored protein solutions. Store solutions in airtight bottles to prevent contamination by airborne micro-organisms and wear gloves during manipulations; fingers are always contaminated by proteases and bacteria that may degrade the proteins.

6. Handle the protein solution gently. When mixing a lyophilized sample into solution or thawing a sample, one should take care not to make foam, because foam can be a sign of denaturation.
7. Dialysis will remove nonvolatile buffers and other chemicals that may have been present either before lyophilization or in the solution of the final purification step. Sometimes trace amounts of impurities can affect the ability of proteins to crystallize. The buffer concentration of the sample solution (10 m*M*) is 10 times lower than the buffer concentration in the reservoir solutions of the screens, which usually contain 100 m*M* of buffer. Hence, the pH of the crystallization drops becomes closer to that of the reservoirs, which allows the screens to be more effective.
8. For obtaining the protein concentration, spectrophotometry is an accurate and easy method. Theoretical molar absorption coefficients ε of polypeptides can be calculated from the content of tryptophan and tryrosine using:

$$\varepsilon_{280\text{nm}} = 5500n_x + 1490n_y,$$

where 5500 and 1490 are the good estimates of the molecular absorption coefficients for tryptophan and tyrosine residues in proteins at 280 nm ***(13)***, and n_x and n_y are the numbers of those residues, respectively. Thus, protein concentration is obtained from:

$$c\ (\text{mg/mL}) = A_{280\text{nm}} \times \varepsilon_{280\text{nm}}/M_r\ (M_r\text{; molecular weight of the protein})$$

9. Before a crystallization experiment, solid particles such as dust, denatured proteins, and solid contaminants from purification columns should be removed. This can be achieved simply by centrifugation (20,000*g* for 5 min) immediately prior to setting up the crystallization trials.
10. It is better to make drops as small as possible. Saving the sample allows screening of more conditions. In addition, there is a great advantage in that a smaller drop reaches equilibrium faster, resulting in faster crystallization. One of our proteins crystallizes within a day from a drop consisting of 0.3 μL of protein and 0.3 μL of reservoir solution, however is never crystallizes from the larger drops. After solving structure, it turned out that the protein was oxidized to form a disulfide bond between two of three neighboring cysteine residues within a few days, resulting in sample heterogeneity that prevented crystallization (*see* **Note 2**).
11. When the storage space is large enough for the plates, the experiment may be continued for as long as 1 yr. Because the crystallization plates are made of polystyrene that allows for some evaporation overtime, this long-term storage can eventually result in crystallization.

12. The stereomicroscope should have an observation platform large enough to support the crystallization plate when looking at all the drops. It is better to have transmitted light with a separate light source connected with an optical fiber in order to prevent heating the base, because crystals can be dissolved easily by temperature variation.
13. It is highly recommended to use a microscope with a higher magnification lens (~×100) because it is sometimes very difficult to distinguish microcrystals and amorphous precipitates using microscopes with lower magnification (~×50). We use the Leica MZ-16 stereo microscope for viewing drops. If a stereo microscope with a high magnification lens is not available, a standard microscope with a ×10 objective lens and ×10 eyepieces for a better view may be used.
14. In protein crystals, the molecules are loosely packed with large solvent-filled channels that normally occupy 40–60% of the crystal volume. This is an advantage when reacting the protein with small reagent molecules such as dyes or heavy metal compounds to be used for phasing, because they can diffuse through these channels and reach reactive sites in the all the protein molecules in the crystal.
15. It is important to find the threshold at which the protein starts to precipitate, because nucleation of crystals usually occurs close to this point, and crystals grow under the supersaturated condition.
16. Inhibitors, substrates, or co-factors (coenzymes) induce some conformational changes in proteins upon binding, and this might result in a more compact and stable state. The apoprotein and protein–ligand complex may be sufficiently different in solubility and physical behavior in many cases. Thus, this may provide a second or third chance at growing crystals unless one does not have any crystals from the apoprotein. In addition, such a complex is inherently more interesting than the apoprotein alone when the structure is eventually determined.

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