

Crystallization of soluble proteins in vapor diffusion for x-ray crystallography

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The preparation of protein single crystals represents one of the major obstacles in obtaining the detailed 3D structure of a biological macromolecule. The complete automation of the crystallization procedures requires large investments in terms of money and labor, which are available only to large dedicated infrastructures and is mostly suited for genomic-scale projects. On the other hand, many research projects from departmental laboratories are devoted to the study of few specific proteins. Here, we try to provide a series of protocols for the crystallization of soluble proteins, especially the difficult ones, tailored for small-scale research groups. An estimate of the time needed to complete each of the steps described can be found at the end of each section.

INTRODUCTION

The awareness that extensive structural knowledge is an indispensable tool in understanding, on a chemical basis, the molecular mechanism of protein function, has led to an enormous expansion of the field of protein crystallography over the past three decades, as indicated by the exponential growth of protein structures from x-ray diffraction data deposited in the Protein Data Bank (PDB) (<http://www.wwpdb.org/stats.html>)¹. The successful determination of the crystal structure of a biological macromolecule requires the preparation of crystals suitable for x-ray analysis, usually single crystals—and to a lesser extent and with bigger difficulty twinned crystals—which are characterized by a high degree of homogeneity, long-range order² and suitable size³, larger than 10 μm^3 , with the optimum being somewhere between 10⁻⁴ and 10⁻³ mm³.

The process of obtaining crystals suitable for diffraction experiments from a protein or other biological material is cumbersome and not trivial. Despite the considerable effort devoted to advancing the theory and practice of macromolecular crystallization, our understanding of the phenomenon is still limited and different approaches can be used to reach the goal. Our aim is neither to repeat what has been already very well done in dedicated series of monographs⁴ and books⁵⁻⁷, nor to review the enormous amount of literature available on the subject. Here, we outline our approach to the crystallization of the particular protein of interest which might be called 'target oriented crystallization' and to summarize our experience as a small-scale crystallography laboratory dedicated to structure–function studies of specific proteins, in a series of procedures to be followed in order to maximize the chance of crystallizing a soluble protein, especially the 'difficult' ones. Please note that the crystallization of membrane proteins is outside the scope of this protocol, and the reader should refer to specialized literature⁸⁻¹³ (and references therein).

Despite substantial progress over the years, crystallization still represents the major rate-limiting step in structure determination, due to the large number of variables that must be systematically altered for optimal crystal formation (these variables are collectively known as 'crystallization space'). Typical variables that influence crystal formation include the concentration and nature of the

protein (e.g., full-length protein versus truncations, chemical modifications and mutations), the methods for setting up crystallization trials (e.g., vapor diffusion, dialysis, free interface diffusion, batch under oil), and physical and chemical parameters (e.g., temperature, electric/magnetic field, pH, type of buffer, additives and precipitants). Due to the many parameters capable of influencing the appearance and growth of a protein crystal, the process of protein crystallization is not linear, but requires constant re-evaluation of the feedback one gets from many of the steps involved in the procedure in an iterative manner. Careful observation of the outcome of the different experiments is therefore key to the successful implementation of the protocol.

A generalized experimental protocol to obtain diffracting crystals for every well-folded protein cannot be devised, since every protein has its own distinctive chemical properties. What can be done is to detail a procedure that affords the highest probability of success and to consider each crystallization trial exactly for what it is: a scientific experiment. The careful observation of the outcome of each experiment will provide some information about the system of interest that can be used to improve conditions in successive rounds of trials that may ultimately lead to success. Given this specification, the term 'protocol' will be still used here, although in the meaning just illustrated.

The crystallization strategy described here relies heavily on the chemical knowledge of the protein, since we believe that learning as much as possible of the chemistry of our protein will maximize the chance of success. We have several positive cases, which will be addressed here. For this reason, we think that a successful crystallization laboratory should have at hand a bunch of different strategies, in terms of protein characterization, crystallization setup and chemical modification of the protein under study, which have been already described in several publications, review papers and book chapters^{3-7,14-23}, but mostly should use 'experiment-driven thinking'.

The reader is directed in particular to consult four sources of alternative descriptions of the techniques reported in this protocol⁴⁻⁷. In the following method, we have tried to coordinate five individual and sequential protocols or 'blocks' for the crystallization of soluble proteins and to present a general timing for

the procedure whenever possible. Successful soluble protein crystallizations could be obtained, if the information presented here is taken with open mind: the conditions that work with one protein are not necessarily optimal for a different protein. However, the ability to explore correlations among the five blocks in which this protocol is subdivided may help to overcome the bottleneck of protein crystallization.

The protocol detailed in the following section represents (or is close to) 'the optimum'. We are aware that whether or not a researcher will be able to go through all the steps described herein depends on the amount of protein and on the equipment available. Our suggestion is to characterize the protein as much as possible beforehand. For example, in our experience, in the more difficult cases, it is extremely useful to perform nuclear magnetic resonance (NMR) measurements to ascertain the presence of mobile (or even unfolded) parts or domains of the protein in order to plan mutagenesis experiments²⁴ (see ANTICIPATED RESULTS). Mutagenesis is a formidable tool to achieve protein crystallization^{25,26}. However, even largely unstructured proteins can sometimes be crystallized, if the correct deductions are drawn from the observation and analysis of the protein behavior (see ANTICIPATED RESULTS).

As mentioned earlier, the present protocol can be subdivided into the series of sequential procedural 'blocks' described as follows:

Preliminary sample preparation and characterization

The most important substance that will be used in the crystallization trials is the protein, which has to be handled properly following rigorous general laboratory precautions and a few specific precepts and caveats:

- (i) It is essential to avoid combining different purification batches for crystallization trials. Purification conditions and procedures are never identical, and each protein batch should be screened individually.
- (ii) The foundation for a successful crystallization experiment is laid at the purification stage. The purity, homogeneity and monodispersity of the protein sample should be evaluated prior to crystallization. Proteins should be as pure, homogeneous and monodisperse as possible. It should be free from structural and charge heterogeneity. Heterogeneity of a protein solution can impair crystal growth, and the crystallization of an impure macromolecule is often a doomed undertaking. Specifically, not only do the molecules to be crystallized have to be pure in terms of contaminating agents, but also in terms of sequence integrity and conformation. Lack of 'total purity' in the protein sample may lead (a) to the complete failure to form crystals, (b) to the production of small crystals unsuitable for x-ray structural analysis or (c) to the formation of crystals which do not scatter x-rays well, because incorporation of impurities in the crystal causes lattice strain and reduces the crystalline order causing decreased quality of the diffraction pattern in terms of both resolution and intensity^{27–31}.
- (iii) Lyophilization is a practice to be avoided as it has been shown to induce conformational changes and even denaturation of a protein^{32,33}. Although some proteins crystallize after lyophilization in general, if possible, this practice should be altogether shunned.

- (iv) Proteins displaying a tendency to form aggregates in dilute solution do not usually crystallize.
- (v) For the initial crystallization screening, the protein sample should be at least 90–95% pure on a Coomassie stained SDS–polyacrylamide gel electrophoresis (SDS–PAGE). A 1 p.p.m. contaminant in a typical 10–20 mg ml⁻¹ protein solution amounts to approximately 10⁹–10¹⁰ molecules per milliliter. Such contaminations might either interfere with the nucleation process and with the growth of nuclei by poisoning the developing crystal. For the above reason, high purity (at least 95–99%) is required for optimization of crystallization conditions. Even highly purified macromolecules intended for crystal growth, may, for a great variety of reasons, be chronically heterogeneous due to modifications, denaturation or the binding of ligands. Even when the protein is only 90% pure, crystallization trials should still be conducted, as the crystallization process may be considered as a purification process in itself. Moreover, heterogeneous masses have frequently been known to act as nucleation centers, allowing crystals to grow around them³⁴.

Protein solubility screens

Probing the solubility of the proteins at different concentrations, in different buffers conditions, with different additives/stabilizers, and with different precipitating agents (see PROCEDURE) affords the data necessary to draw the supersolubility curve of the protein. The supersolubility curve is a curve in the phase diagram of a protein solution (i.e., protein concentration versus precipitant concentration at constant temperature) which divides the conditions where phase separation (liquid, amorphous solid and crystal nuclei) occurs from those where the solution remains above the saturation point of the protein for an indefinite length of time without phase separation. In other words, the supersolubility curve identifies that region of the phase diagram above saturation, where kinetic phenomena dominate the process of phase separation^{7,35}. Once the supersolubility curve has been determined, the experiment can be designed to reach the metastable zone, namely that part of the phase diagram contained between the solubility and the supersolubility curves, where thermodynamic parameters govern the growth of nuclei into crystals. The transition from the supersaturation zone to the metastable zone can be obtained by dilution experiments, by transferring the solution or by controlling evaporation^{14,35–40}.

Fast screening of crystallization conditions

In crystallization trials, the researcher should alter the solubility of the protein in search for the favorable intermolecular interactions that lead to crystal formation. All the screens should carefully be reviewed, and one should try and rationalize the outcome of every condition tested keeping in mind that even negative results give precious information. Variables that alter the protein solubility should be actively sought after. The most important parameters that affect protein solubility are protein concentration, precipitant type and concentration, pH, and temperature, which is an often neglected variable despite its proven impact^{5,6,41}. In any event, other variables should never be ignored either, and, most important of all, one should always try to learn as much as possible on the chemical behavior of the protein under investigation.

Optimization of crystallization conditions

Once the appearance microcrystals, polycrystalline aggregates, thin plates, etc., of protein has been observed in the screenings just described, the crystallization conditions should be optimized using the sitting drop approach detailed in refs. 5–7. Even in the unusual fortunate instance when crystals suitable for x-ray diffraction analysis are obtained from the initial screening, crystallization optimization should still be carried out. One should in fact pursue other conditions, which may offer crystals of different morphology that could in turn prove valuable during a crystallographic structure determination.

If the crystals obtained at the screening stage are not suitable for x-ray diffraction analysis or diffract only at low resolution, one should proceed with the optimization of the crystallization conditions. Although it is always preferable to pursue the largest, better-looking crystals, there are plenty of reports where small, ‘ugly’ crystals have eventually produced the best data. It is worth mentioning that in protein crystal growth, the formation of crystals is often hard to reproduce even following scrupulously the original conditions. Optimization experiments should therefore also be viewed and used as a way to improve reproducibility.

Optimization of crystallization conditions—crystal nuclei transfer techniques

One of the paradoxes in crystallization is that the optimal solution conditions for nucleation of the crystals are not the ideal ones to support their subsequent growth³⁸ (and references therein). Nucleation only occurs in a supersaturated solution, while optimum growth occurs in the metastable region (between solubility and supersolubility curves—see the phase diagrams reported in ref. 7, chapter 9). The ideal experiment therefore must uncouple nucleation from growth to satisfy the different requirements of the two events. A method to transfer the crystal nuclei to metastable conditions involves diluting the drops^{42,43} after incubating them for some hours at spontaneous nucleation conditions or transferring nucleating drops over reservoirs at different concentrations for equilibration at metastable conditions^{36–38}. Among the techniques for the separation of nucleation and growth, seeding is the most popular way of moving the system in ‘phase space’ avoiding the ‘hazards’ of the nucleation zone^{6,44–47}. These so-called ‘seeding’ experiments fall in four general categories: (i) macroseeding (or seed transfer), (ii) microseeding, (iii) streak and (iv) cross-seeding and epitaxial.

The choice among the preceding four seeding techniques depends on the results of the crystallization screens. Microseeding techniques can always be used for crystal optimization. If only microcrystals (less than 5 μm in size), microcrystalline precipitate, dendritic crystals or spherulites have been obtained, then microseeding is the only possible technique. If crystals of appreciable size in one or two dimensions (rods or plates; 10–50 μm), but very thin in the other dimensions, have been obtained, then either macroseeding or streak seeding can be used to grow 3D crystals. When sizable crystals that present imperfections like multiple crystals, twinned crystals and the like have been obtained, then streak seeding can be used.

If all crystallization trials have been unsuccessful and failed to provide any crystal, the cross-seeding or epitaxial seeding can be attempted. It is possible that the discovery of new materials having nucleating properties will make epitaxial seeding the method of choice in the future⁴⁸.

The strategy of macroseeding consists in slightly melting the surface of a very small crystal (very thin plates or rods) before transferring it to a new equilibrated drop in the metastable zone containing fresh protein, which will optimistically continue to crystallize on the washed surface of the small crystal. The success of the macroseeding experiment depends largely upon how well one determines the lowest precipitant concentration where the crystal will not melt completely.

Microseeding provides another way to control nucleation as it consists of introducing crystal nuclei in the equilibrated metastable protein solution, where the seeds might grow.

In streak seeding, an animal whisker (usually cat or rabbit) or another tool is utilized as a seeding wand. The wand is used to touch or stroke the surface of the parent crystal to dislodge and trap the crystallization nuclei. The whisker is then drawn through the new equilibrated drop, depositing the seed in a streak line.

Seeds can also consist of heterogeneous material that can be any solid substance having nucleating properties. When a molecule not only nucleates on another but also shares some of the structural features of the substrate, the growth is said to be epitaxial. Different mineral samples (i.e., mica and apophyllite) may be used as heteroepitaxial nucleant. Recent studies demonstrated that porous materials (i.e., porous silicon), containing pore sizes that are comparable with the size of the protein molecules, display nucleation-inducing properties^{48–51}.

A further technique uses crystals of a similar protein (e.g., mutant protein, same protein from a different species) to promote nucleation. In this case, we speak of cross-seeding. Vice-versa, when working with mutants or variants of a protein already crystallized, cross-seeding, with crystals of the native protein into drops containing the mutant or variant can be attempted.

The seed(s) must be placed into a new experiment equilibrated at the metastable zone. In practice, since determining the phase diagram is expensive in terms of the researcher’s time and protein consumption, most seeding experiments are done without knowing the protein phase diagram. To move to the metastable zone of supersaturation, the concentration of at least one of the components (precipitant or protein) must be lowered. Different results can be obtained depending on which of the concentrations is lowered: (i) protein and/or (ii) precipitant.

The relationship between protein solubility and precipitant concentration is a function of the type of precipitant (i.e., polymeric precipitant or salts), particularly, protein solubility decreases proportionally as the polymer concentration increases. The relationship between salt concentration and protein solubility is on the other hand more complex. The nature of the salt affects the solubility independently of the ionic strength⁴⁵. Thus, lowering the salt concentration may have less predictable consequences than lowering the protein concentration.

MATERIALS

REAGENTS

- SDS gel preparation kit (Sigma-Aldrich, cat. no. 08091) or, as an alternative, see REAGENT SETUP
- Isoelectric focusing (IEF) gel (see REAGENT SETUP)
- Buffer solutions (see Table 1)
- Sodium azide **! CAUTION** Sodium azide is a poison. It is harmful by inhalation, ingestion or by skin contact. Ingestion or inhalation may be fatal, and the material is readily absorbed through skin. Sodium azide is also a mutagen.
- Thymol (optional) **! CAUTION** Thymol is harmful if swallowed, inhaled or absorbed through the skin. All mercury compounds are very toxic. Care should be taken not to come in contact with them by inhalation, swallowing or through the skin. There is danger of cumulative effects.
- Thimerosal [ethyl (2-mercaptobenzoato-(2-)-O,S) sodium mercurate] **! CAUTION** Thimerosal in particular is an experimental neoplastigen and teratogen. It is harmful by inhalation and ingestion and may cause reproductive damage.
- Organomercuric compound: 4-(hydroxy-mercury) benzoic acid (Sigma Aldrich, cat. no. 25010)
- Preformulated buffers, precipitants (salts, polymers, volatile and nonvolatile organic solvents), detergents, surfactants, additives, reductants and ligands are available at the retailer's web sites listed under the EQUIPMENT and as analytical grade and biochemical grade products from Sigma-Aldrich
- PCT kit (Hampton Research)
- EasyXtal pre-screen assay (Qiagen-Nextal Biotechnologies, Canada)
- JBS solubility kit (Jena Biosciences, Germany)
- ADA [N-(2-acetamido)iminodiacetic acid], MES [2-(N-morpholino)ethanesulfonic acid] or cacodylate (optional) **! CAUTION** Many of the reagents used in crystallization experiments are toxic. Please follow the safety sheets pertinent to each precipitant. **▲ CRITICAL** The quality and purity of all products are the most important requirements for successful crystal formation.

EQUIPMENT

- Pharmacia Electrophoresis Power Supply – EPS3005 (Pharmacia)
- Ampholine PAGplate (Amersham Pharmacia Biotech, Uppsala, Sweden)
- Multiphor II apparatus (Pharmacia)
- Electrospray mass spectrometer Finnigan LTQ equipped with an ion spray source (Thermo Electron Co. Schaumburg, IL) **▲ CRITICAL** Availability only required via dedicated facilities and not in the laboratory itself.
- Static and dynamic light scattering (LS) (Scitech Instruments or Wyatt Technology Corporation) **▲ CRITICAL** Availability only required via dedicated facilities and not in the laboratory itself.
- JASCO J-815 CD spectrometer (JASCO) **▲ CRITICAL** Availability only required via dedicated facilities and not in the laboratory itself.
- Dialysis membrane
- 22 µm pore size membranes
- NMR Bruker 400–800 MHz spectrometers (Bruker) **▲ CRITICAL** Availability only required via dedicated facilities and not in the laboratory itself.
- X-ray diffractometer equipped with a sealed tube or rotating anode x-ray source, focusing optics and 2D detector (charge coupled device or image plate). Access to a synchrotron x-ray source is desirable **▲ CRITICAL** Availability only required via dedicated facilities and not in the laboratory itself.

PROCEDURE

Preliminary sample preparation and characterization ● TIMING 30 h–5 d

- 1| Prepare approximately 1 ml solution of the protein with concentration approximately 10 mg ml⁻¹ in the buffer used for protein preparation and purification.
- 2| Use approximately 1 µl solution prepared in Step 1 to carry out an overloaded SDS-PAGE according to the Laemmli's method⁵² in order to check the protein purity (the protocol described in ref. 7, p. 26 can be followed). In case of proteins obtained from natural sources, also a N-terminal sequence or even an overall protein sequence analysis is recommended. **▲ CRITICAL STEP** Avoid combining different purification batches for crystallization trials. Purification conditions and procedures are never identical: each batch should be screened individually.
- 3| Prepare a sample of the protein that is free of nonvolatile salts by buffer exchange over a dialysis membrane with either no buffer at all or with volatile buffers like ammonium acetate (keep the buffer concentration below 10 mM) and determine the

TABLE 1 | Suitable buffers for protein solubility screenings.

Potassium/sodium phosphate, pH 5.0
Potassium/sodium phosphate, pH 6.0
Potassium/sodium phosphate, pH 7.0
Sodium citrate, pH 5.5
Bis-Tris propane, pH 6.5
HEPES, pH 7.5
Sodium acetate, pH 4.5
Sodium citrate, pH 4.7
Sodium acetate, pH 5.0
Sodium citrate, pH 5.5
Cacodylic acid, pH 6.5
Ammonium acetate, pH 7.3
Imidazole, pH 8.0
Bicine, pH 8.5
Bicine, pH 9.0
MES, pH 5.8
MES, pH 6.2
MES, pH 6.5
HEPES, pH 7.0
HEPES, pH 8.0
TRIS, pH 7.5
TRIS, pH 8.0
TRIS, pH 8.5

- Stereomicroscopes Nikon SMZ 800 (Nikon) and MOTIC SMZ 143 (Motic)
- Bench top centrifuge
- Centricon centrifugal filter (Centricon)

REAGENT SETUP

IEF Analytical IEF of crude cell extracts is performed in precast 5% polyacrylamide gels (Amersham Biosciences) containing ampholytes (pH range, 3.5–9.5) with Multiphor II apparatus.

SDS-PAGE analysis The individual components needed are: acrylamide/bisacrylamide 30% solution 29:1 (Sigma-Aldrich, cat. no. A3574); SDS 10 and 20% solutions (Sigma-Aldrich, cat. nos. 71736 and 05030); Ammonium persulfate (Sigma-Aldrich, cat. no. A3678); N,N,N',N'-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T9281); Tris-HCl buffer (Sigma-Aldrich, cat. no. T3253); dithiothreitol (DTT) or 2-mercaptoethanol (BME) (Sigma-Aldrich, cat. nos. 43817 and M6250); Glycerol (Sigma-Aldrich, cat. no. G5516); Gly (Sigma-Aldrich, cat. no. 241261); and Coomassie brilliant blue R (Sigma-Aldrich, cat. no. B8647)

EQUIPMENT SETUP

Crystallization trials Full lists (including a wide choice) of all equipment needed for vapor diffusion experiments (sitting/hanging drop setup), batch/microbatch experiments, free interface diffusion and gel-based crystallization are available from Axygen Biosciences, Emerald Biostructure, Hampton Research, Jena Bioscience, Molecular Dimensions and Qiagen-Nextal Biotechnology.



molecular weight of the protein by mass spectroscopy (MS). Please note that MS is the ideal analytical method as a routine check for sample impurities of all kinds.

- 4| Determine isoelectric homogeneity of the protein sample by performing an IEF gel⁵³ (the protocol described in ref. 7, p. 28 can be followed).
- 5| In case of enzymes, check the activity, if you have an assay. An absent or too low specific activity might depend on several factors (lost enzyme, misfolding and inhibition by impurities). In this case, there is no point in pursuing crystallization. The enzyme preparation and purification steps should be reconsidered.
- 6| Detect aggregate formation in dilute solution by LS^{54–60} or by NMR/NMR dispersion^{61,62}. In case of aggregate content of over 15–20% of the protein present in solution (monodispersity lower than 85–80%), reconsider protein purification and buffer composition⁶³.
- 7| Check the circular dichroism (CD) spectrum of your protein to see if it has secondary structure. Use the stock protein solution and operate according to refs. 63 and 64.
- 8| Record 2D ¹⁵N/¹H NMR protein spectra to ensure that the protein domains are folded at submillimolar concentrations⁶⁴.

Buffer screening ● TIMING 24 h

9| Prepare concentrated buffer solutions (0.5 M) of all different buffers listed in **Table 1**. The buffer solutions should be sterilized by filtration through 0.22 μm pore size membranes and the glassware should be autoclaved. Add 0.1–1.0 mM sodium azide to keep buffer solutions free from microbial presence.

10| Dispense your protein (at 1–2 mg ml⁻¹) in sitting drops (1 μl) (for sitting drop setup see ref. 7, pp. 86–88) over 1 ml well solutions containing series of diluted buffers (100 mM) at various pH, with additives/stabilizers according the purification protocol⁶⁵.

▲ **CRITICAL STEP** If you plan to use metal ions (besides group 1 metal ions) as additives, avoid using phosphate and carbonate buffers to prevent crystallization of insoluble inorganic salts.

- 11| Visually identify those drops that are clear.
- 12| Choose one set of conditions out of those identified as suitable (clear solutions) in Step 10, which guarantees that the protein is stable and soluble (i.e., useful to crystallization).

Samples storage ● TIMING 2 h

13| Centrifuge protein solutions in a bench top device (2,000g, 5 min, 4 °C) and decant the supernatant protein solution.

14| Filter the protein solution through 0.22 μm pore size membranes into a sterile vessel. Use filters with the smallest possible dead volume to minimize protein sample loss.

▲ **CRITICAL STEP** The protein may adhere to the filter membrane. To avoid sample loss, test a small aliquot of the solution that remains on the filter for the presence of protein (SDS and/or activity).

? TROUBLESHOOTING

15| Concentrate the protein solution to 10 mg ml⁻¹ through a Centricon centrifugal filter of appropriate molecular weight cutoff (centrifuge 5,000–14,000g, 4 °C; the time and speed depend on the protein and on the filter used). Nonspecific aggregation of the protein may occur at this stage. In any case, it is advisable to repeat a LS measurement (if available) to evaluate the monodispersity of the protein in the concentrated solution. In any case, check the protein concentration by UV-visible spectroscopy and/or by activity test (if available).

? TROUBLESHOOTING

16| Use 0.1–1.0 mM sodium azide to keep protein solutions free from microbial presence. Please note that sodium azide is a strong Lewis base and an inhibitor for metallo-proteins and enzymes as it may act as a ligand for the metal center. Similarly, azide can also interfere with heavy atom derivatization. As an alternative to azide, one may use thymol, Thimerosal to a comparable concentration of 50–100 μM or implement rigorously sterile laboratory procedures and materials. In any event, it is worthwhile mentioning that also organomercury compounds are able to form covalent bonds with protein, especially if free Cys are available for binding. On the other hand, we find organomercury compounds very helpful in preventing protein precipitation by inhibiting the formation of intermolecular disulfide bonds. A classical example of the usefulness of mercury compounds for obtaining high-quality crystals is given by the crystallization of human carbonic anhydrase⁶⁶. A more recent example of the use of organomercury compounds is provided by the successful crystallization of both bacterial and mammalian CutA proteins⁶⁷ (see the ANTICIPATED RESULTS for the discussion).

PROTOCOL

17| Aliquot the protein sample into multiple small Eppendorf tubes: transfer 100 μl aliquots of protein solution for the initial screenings (Steps 25–52) and 20 μl aliquots of protein solution for optimization (Steps 53–98).

18| Label protein samples clearly with the sample identification, batch identification and date of storage. Labels should include (i) name of protein; (ii) sample identification: batch, storage temperature and date; (iii) sample buffer composition, additives, ligands, etc.; and (iv) sample concentration.

19| Freeze the protein samples aliquots in the freezer and store them at $-20\text{ }^{\circ}\text{C}$.

▲ CRITICAL STEP Repeated freezing and thawing of the protein sample should be avoided, because it may alter the protein. Make aliquots small enough so that the entire aliquot can be consumed in the experiment after thawing.

■ PAUSE POINT Frozen samples of most protein solutions can be safely stored at $-20\text{ }^{\circ}\text{C}$ for at least 3 months. In special cases, storage at $-80\text{ }^{\circ}\text{C}$ is required. A convenient way of storing proteins at very low temperatures is to use the same vessels where frozen crystals are stored under liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). Check with the molecular biologist(s) who prepared the protein or compare the protein to a similar protein in the literature for the best storage temperature.

Protein sample handling

20| Unfreeze the protein sample and allow the sample to equilibrate to the temperature at which the crystallization experiments will be set up.

▲ CRITICAL STEP When thawing a sample, do not shake or vortex the protein in order to avoid foaming.

? TROUBLESHOOTING

21| Once the protein aliquot is unfrozen, protect the sample from microbes by storing the thawed protein solution at $4\text{ }^{\circ}\text{C}$, but use it within 1 d.

22| Set up the trials as soon as possible not leaving the protein sample for extended time at room temperature ($20\text{--}22\text{ }^{\circ}\text{C}$).

▲ CRITICAL STEP Over time, proteins may break down and the mixture may become heterogeneous.

23| The protein solution is better handled by using a gel-loading pipette tip (available at: [http://catalog2.corning.com/Lifesciences/en-US/Shopping/Product.aspx?categoryname=Pipet+Tips\(Lifesciences\)%7cPipet+Tips%2c+Gel+Loading\(Lifesciences\)](http://catalog2.corning.com/Lifesciences/en-US/Shopping/Product.aspx?categoryname=Pipet+Tips(Lifesciences)%7cPipet+Tips%2c+Gel+Loading(Lifesciences)); <http://www.biocompare.com/matrix/3943/Gel-Loading-Pipette-Tips.html>; <http://www.lightlabsusa.com/home.php?cat=263>; http://www.vwrs.com/catalog/product/index.cgi?object_id=0011814&class_id=5002473) to dispense small drops with better control and accuracy of drop placement in the subsequent steps of the procedure.

Protein solubility screens—selection of the appropriate protein concentration for crystallization ● TIMING 4 h (A) or 2–4 d (B)

24| Perform protein solubility tests using one of the approaches detailed in the following options A, B or C. The selection of appropriate protein concentration for crystallization can minimize or prevent situations where a screen results in an over abundance, either of precipitate or clear drops and can improve probability of crystallization and crystal size.

(A) Use of commercially available kits

- (i) The PCT kit (Hampton Research)⁶⁵, with only four unique, preformulated precipitant solutions, provides information on protein solubility by using a small quantity of sample (i.e., 5–10 μl of 10–20 mg ml^{-1} of protein solution).
- (ii) The EasyXtal pre-screen assay (Qiagen-Nextal Biotechnologies, Canada) is arranged in three mini-grids (six conditions each). The initial protein concentration should be at least 20 mg ml^{-1} and at least 24 μl of protein solution is required.
- (iii) The JBS solubility kit (Jena Biosciences, Germany) is based on the principle reported in option B⁶⁵, and it comprises two individual kits for successive use: 'A—buffer kit' and 'B—additives kit'. A minimum of 38 μl of protein solution at the concentration of at least 3 mg ml^{-1} is required to perform the two screens.
- (iv) Perform the screenings using one of the commercially available kits above by following the procedure described in the respective user guides.
- (v) By analyzing the results of the assay, the concentration of protein to be used in initial crystallization screenings can be easily determined.

Note: Allows to save protein and/or to save time, while optimizing protein concentration.

(B) Optimum solubility buffers and additive screens^{65,68}

- (i) Prepare your own buffers solutions and evaluate the protein solubility using a procedure similar to that reported^{65,68}. For this purpose, utilize protein sample concentrations in the range of 3–50 mg ml^{-1} (as high as possible, depending on solubility of the purified protein), and use buffers from **Table 1** (modified from ref. 68).
- (ii) Use the hanging drop set up (see ref. 7, pp. 84–85) with a 24-well tissue culture plate and siliconized glass cover slips. In each well, pipette 1 ml of 100-mM buffer solution (reservoir buffer).
- (iii) Pipette 2 μl aliquots of protein solution in starting buffer onto the glass cover slips.

- (iv) To each drop of protein solution, add 2 μ l of 100 mM reservoir buffer, and mix the solutions by gently drawing and expelling the solution in the pipette tip.
- (v) Invert the glass slips and seal them onto the wells using petroleum jelly.
- (vi) Allow the plate to rest undisturbed at room temperature so that vapor diffusion may take place.
- (vii) Measure the amount of precipitate in the drops by placing the tray against a black background, illuminating it from the side, and visually examining each drop under a microscope. Under these lighting conditions, precipitate appears as a white spot against the black background and is scored based on the fraction of the drop covered by precipitate (scale of 0–4, with 0 for no precipitate and 4 for precipitate completely covering the drop).
- (viii) Score the drops at 12–24 h intervals for several days till the drops reach their final equilibrium concentrations.
- (ix) Perform a stabilizer screen using the common stabilizers/additives from **Table 2** and the buffer conditions in which the protein is most soluble in according to the buffer screen just carried out. The additives listed in **Table 2** help to find the optimal solubility conditions for the protein⁶⁸.

TABLE 2 | Suitable additives/stabilizers for protein solubility screenings and concentration ranges to be used.

Salts (NaCl, KCl)	25, 50, 100 mM
Reductants (DTT, BME)	1–10 mM
Glycerol	5–10% (vol/vol)
Mild detergents (i.e., octylglucoside, dodecyl maltoside)	6–10 mM
If available and if needed: inhibitors, ligands or cofactors (the concentration to be used depends on the binding affinity)	

Note: Option B is more time or sample consuming than option A. The protein concentration has to be as high as possible. Details on the procedure are reported in refs. 65 and 68. One might also consider repeating the screen at different temperatures (i.e., room temperature and 4 °C). Proteins crystallize at the temperature at which they are most soluble⁶⁹. The ease of use of this screen and the capability of testing many conditions makes this a very efficient means of searching for the best solvent condition for a particular protein that initially shows high levels of aggregation. Very often, proteins do not crystallize, because the purified protein sample is not conformationally homogenous. This optimum solubility screen allows to obtain the most homogeneous and monodisperse proteins conditions for proteins that usually aggregate and cannot be concentrated prior to setting up crystallization screens. Recently, the efficiency of a similar optimum solubility screen on improving crystal growth, quality and size has been reported^{69,70}.

(C) Alternative techniques

- (i) Solubility measurements may also be done by alternative techniques such as the scintillation method and Michelson laser interferometry^{71,72}. These methods have, however, the drawback of requiring specialized equipment and training.

Fast screening of crystallization conditions ● TIMING 2 h for manual preparation of 96 different conditions

25 | Centrifuge the protein sample at 2,000g for 10–15 min at 4 °C immediately prior to setting up the crystallization screen and use the supernatant for the following steps of the protocol. This ensures the removal of aggregates and amorphous material.

26 | (Optional) When free sulfidryl residues (Cys) are present in the protein, oxidation may occur. In this case, in order to avoid degradation of the protein, prepare solutions of mild reducing agents and add them to the protein solutions so that their final concentration in the crystallization drop is approximately 1–10 mM. Common reducing agents that can be used for this purpose are DL-DTT (greater than 99.0% purity; see MATERIALS) and BME (greater than 99.0%; see MATERIALS).

27 | In a crystallization experiment, solutions of these reducing agents remain active for 2–3 d and 3–7 d, respectively⁵. Alternatively, organomercury compounds may be used (see Step 16). Organomercury compounds protect the free sulfidryl residues from reacting with them by establishing Hg–S covalent bonds.

28 | Start fast screening of crystallization conditions by using sitting drop techniques (as described in ref. 7, pp. 82 and 88–89), 96-well plates and sealing film. Use commercially available sparse matrix or grid screen solution kits^{73–75} (plates and kits available at: Emerald Biostructures Inc.; Hampton Research; Molecular Dimensions; Qiagen-Nextal Biotechnology, Canada; Jena Bioscience, Germany; Axygen Biosciences) like the ones listed next, following the order: (i) sparse matrix, (ii) matrix screen PEG/ions, (iii) grid screen ammonium sulfate, (iv) grid screen PEGs, (v) grid screen PEG/LiCl, (vi) grid screen alcohols, (vii) grid screen salts. Shake crystallization solutions (i.e., sparse matrix or grid screen kit solutions) thoroughly before use. Stored solutions present heterogeneous concentration that may hamper the experiment reproducibility. Dispense 100 μ l of crystallization solution in the reservoir and drops consisting of 1–2 μ l of protein solution + 1–2 μ l of reservoir solution (equal amounts). Pipette the crystallization solution into the protein solution and let the two diffuse together without mixing. Once taken from the refrigerator, the screening solutions should be kept approximately 30 min at room temperature to allow equilibration from the 4 °C storage temperature. Do not open the solution vials before equilibration to avoid moisture condensation. Start crystallization trials with kits (i–iv) and set them up according to each kit user guide. Use protein solution at the best concentration as determined from Step 24.

PROTOCOL

▲ **CRITICAL STEP** In crystallization trials, avoid vibrations because of their negative influence on this process.

? TROUBLESHOOTING

29| Perform screens at two different temperatures: 4 and 20 °C (controlled room temperature).

30| (Optional) When the amount of available protein sample is not sufficient to perform duplicate screens, set up screens at room temperature first. After 2/4 weeks move the plates to 4 °C and monitor changes in solubility.

31| Observe the drops using a light microscope with magnification between $\times 20$ and $\times 100$.

▲ **CRITICAL STEP** Do not use polarized light because of interference from the plastic tray with the polarized beam. Check the entire drop by focusing; try dark field illumination to check for gelatinous precipitate. At this stage, the color of the precipitate is important.

32| If some or all drops are clear, do not examine or disturb them in any way. The observation of each trial should be performed according to the following schedule 3 d, 1 week, 10 d, 2 weeks, 18 d, 3 weeks, 4 weeks.

■ **PAUSE POINT** Store the crystallization trials up to 1 year, observe the crystallization drops once a month.

33| To classify the appearance of the drops, record the results of the experiment using the numbering scheme⁷⁵ detailed in **Figure 1**.

34| If kits (i–iv) do not provide any useful initial conditions promoting crystallization, use kits (v–vii).

35| After each observation, score the results of the screen being careful to assign an order of magnitude to the description of what is observed in the drop. Use the 1–10 scale⁷⁵, ranging from precipitates to crystals, as described in Step 33. This will assist the optimization step and will help to distinguish subtle differences between precipitates, which can be helpful during optimization trials.

? TROUBLESHOOTING

36| A reasonable precipitation is achieved when one-third to one-half of the drops contains some kind of precipitate. Once a precipitate is seen in a drop, pay attention to that drop and look for crystal appearing in the precipitate cloud. Be sure to scan the entire drop searching for crystals at the top, the bottom and in between. It is possible that crystals will grow from the precipitate after some time^{76,77} (see Step 53).

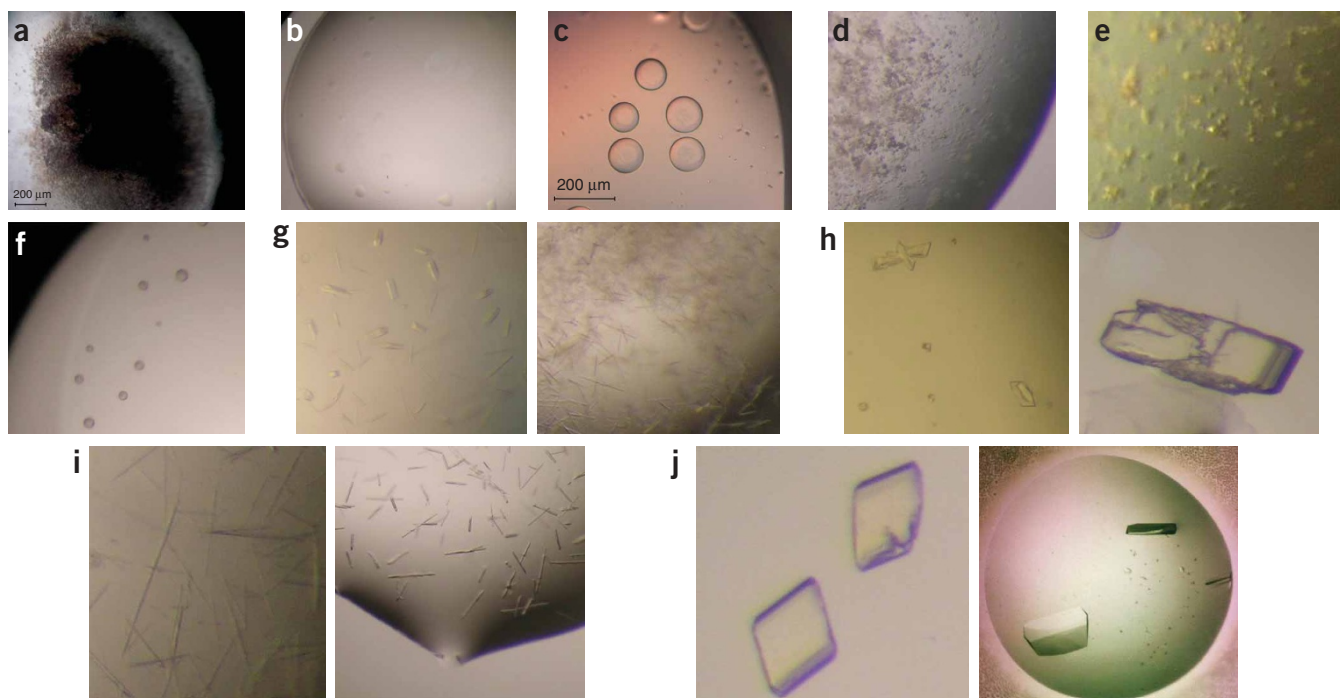


Figure 1 | Classification scheme for the results of a crystallization screen taken from ref. 75. (a) Heavy brown or flocculent precipitate (score of 1), (b) clear drop (score of 2), (c) phase separation or light precipitate (score of 3), (d) granular precipitate (score of 4), (e) microcrystalline precipitate (score of 5), (f) spherulites (score of 6), (g) micro-crystals (score of 7), (h) multiple crystals (score of 8), (i) small crystals (needles, thin plates; score of 9), (j) well shaped single crystals (score of 10).

37| One of the most difficult aspects for beginners is to recognize promising precipitates and distinguish them from precipitates that are not worth pursuing. Except for drops where the protein is denatured (i.e., the precipitate does not redissolve upon dilution), regard precipitate as a positive drop result. This is because precipitate occurs in a state of supersaturation. Crystals can only grow from supersaturated solutions; hence they can grow from a solution that contains precipitates. Reversible precipitates might be microcrystalline or amorphous while irreversible precipitates are formed by denatured macromolecules. Amorphous precipitate does not show birefringence, while microcrystalline precipitate does. Please note that a useful guidance to evaluate the results of the crystallization screenings is provided by the Terese Bergfors website (<http://xray.bmc.uu.se/~terese/crystallization/library.html>) where the reader can find a pictorial representation of all possible outcome of a crystal screen and suggestions about the actions to be undertaken in each different case.

▲ CRITICAL STEP Although one may not obtain good-quality crystals from the initial screening, the presence of oils, gels, phase separation, spherulites, microcrystals, needles or plates in some of the crystallization experiments should be carefully noted as these are still good starting points for the subsequent optimization step.

38| Determine and classify which precipitants always cause the appearance of a precipitate, regardless of precipitant concentration and which only cause precipitation at higher precipitant concentrations but not at lower concentration (or vice versa). Initially one might select precipitants that fall in the latter category as precipitant candidates for secondary screens.

39| Determine the pH dependence of the solubility of your protein.

40| Determine how the presence of metal ions, cations and anions affects the protein solubility.

41| (Optional) Design a grid or a sparse matrix screen about precipitant variables from observations described in Steps 38–40. For example, one might note that all drops are clear in the presence of PEG 6000, below 10% wt/vol concentration, but that heavy precipitate is observed at 30% wt/vol PEG 6000 and above. One might design a screen to evaluate PEG 6000 concentrations between 10 and 30% (wt/vol) versus an appropriate pH range. If reviewing the effects of pH on solubility shows that drops are clear at pH values below 7, while a heavy precipitate is observed above pH 7, then it seems reasonable to evaluate a pH range between 3 and 7 (refs. 5,65) (see ANTICIPATED RESULTS for an example of a grid).

42| Always take into consideration the effect that salts, polymers, organic solvents and additives might have on the pH of the buffer selected for crystallization experiment. To be safe, check the pH of an experimental solution after all components have been added, mixed and the solution has been equilibrated at the temperature where the buffer was titrated and the experiment is to be performed.

43| (Optional) If the protein is known to bind small molecules or cofactors and inhibitors, then try screening crystallization conditions by including the known ligands. Cofactors, inhibitors and other compounds that bind or interact with the protein may alter the protein conformation and solubility. This may impose a greater degree of structural homogeneity and increase the likelihood of crystallization⁷⁸.

▲ CRITICAL STEP When working with chemically unstable ligands (e.g., ADP, ATP, NAD and NADH), be sure to use freshly prepared working solutions or properly stored stocks (see the material property data sheet of each of these compounds about the storing instructions). These agents are sensitive to degradation over time and this can deeply influence the outcome of a crystallization experiment. Also, consider the acid–base properties of these ligands since the additive can influence the final pH of the drop and, vice versa, the binding properties of the ligand are influenced by its protonation state. It might be necessary to adjust the pH of the ligand solution before the addition to the drop. The binding of metal ion cofactors also depend on pH: at pH below 5, metal ions might be released from the protein and this might alter the protein conformation even before the denaturation point is reached. On the other hand, at pH above 8, the hydroxide anion might compete with the protein for binding the metal ion and insoluble metal hydroxides might form.

44| Detergents are often used as additives where hydrophobic interactions might lead to nonspecific aggregation and preclude crystallization. The presence of protein nonspecific aggregation can be detected by dynamic LS or simply deduced by observing amorphous precipitate formation, but not crystal growth. In this case, it is worth trying using detergents even though there are literature reports indicating the general usefulness of detergents in improving crystallization⁷⁹. When screening detergents as additives, be sure to evaluate small amphiphiles such as benzamidine, ethanol, dioxane, 1,6-hexanediol, ethylene glycol and butyl ether for their ability to influence micelle stability^{80,81}. When you increase the length of the detergent chain, you should decrease the amount of precipitant, because the chain length changes the micelle size.

45| Always take into consideration the amino acid sequence, protein isoelectric point (calculated from sequence) and overall charge of the protein at the working pH⁸². Use in millimolar concentrations charged, short chained, water soluble molecules, termed ‘electrostatic agents’ in the field of protein crystal growth, such as spermine, spermidine, hexadecanoic acid, 1,7-diaminoheptane and ions to neutralize free charges.

PROTOCOL

46| If all attempts to alter protein solubility and obtain crystals by manipulation of the environment fail, manipulate the protein according to the following optional Steps 47–51, and repeat all the crystallization experiments.

47| (Optional) If dealing with glycosylated proteins: remove all or part of the glycosylation by following reported procedures^{83–86}.

48| (Optional) Utilize site directed mutagenesis to replace relevant amino acids and alter protein solubility⁸⁷. This is one of the most powerful tools available^{25,26}, and it might represent the key factor to achieve crystallization in the most difficult cases²⁴. However, be aware that the mutated protein is by definition different from the ‘wild-type’ and the influence of the introduced mutation(s) on the protein chemical behavior should be evaluated thoroughly (see ANTICIPATED RESULT for an example).

49| (Optional) Isolate, clone or purify the protein from an alternate source^{88,89}.

50| (Optional) Generate a fusion protein to alter solubility⁸⁹.

51| (Optional) Truncate N- or/and C-termini or entire domains if you have evidence of conformational disorder²⁴. The same precautions described in Step 48 apply.

52| If all drops in the screen are either clear or contain denatured protein, go back to repurify the protein and repeat Steps 1–51.

Optimization of crystallization conditions—chemical and physical variables ● TIMING 24–48 h

53| When the appearance of protein microcrystals, polycrystalline aggregates, thin plates, etc., has been observed, i.e., for those conditions that have resulted in scores 7–10 (Step 33), carry out trials for optimization of crystallization conditions using sitting drop methodology. However, it is known that small crystals may form initially, but after some time, they slowly disappear and are replaced by few that grow larger at the expense of the small ones. The smaller crystals act as ‘nutrients’ for the bigger crystals. This is a spontaneous process, the so-called Ostwald ripening^{76,77}, that occurs, because larger crystals are energetically favored with respect to the smaller ones. If the protein is available in sufficient quantity, we suggest to run the optimization described below in parallel with seeding experiments described in Steps 72–98. Use variable volume of precipitant and protein solutions as reported below in Steps 64–66.

▲ **CRITICAL STEP** Even if you are fortunate enough to obtain crystals suitable for x-ray diffraction analysis from an initial screen (Steps 25–46), do not stop your crystallization efforts. Pursue other conditions that may offer crystals of different morphology, which could be valuable during a crystallographic structure determination project. If the crystals are not suitable for x-ray diffraction analysis or diffract only at low resolution, proceed with the optimization of the crystallization condition. It is always preferable to optimize the largest, better-looking crystals. However, there are plenty of reports where small, ugly crystals have eventually produced the best data.

54| Explore as many optimization opportunities for crystallization as possible. Optimize every hit from a screen. When you reach an optimization dead end for a particular hit, review the screens again and optimize crystal leads from other hits. Optimize different hits simultaneously if protein availability permits. Please note that in protein crystal growth, crystals are often seen, but cannot be reproduced later under the same conditions. Use optimization experiments also to improve reproducibility.

? TROUBLESHOOTING

55| Note the presence and relative concentration of every component in the solution that appears to affect positively protein crystallization. In particular, concentrate on the effect of the following: (i) polymer (PEGs, PEG monomethyl ethers, ethylene imine polymers, JEFFAMINES); (ii) salt; (iii) ions; (iv) organic solvents; (v) pH; (vi) protein concentration; (vii) temperature; (viii) buffer; (ix) crystallization method (setup) ((a) crystallization by vapor diffusion; (b) batch crystallization⁹⁰; (c) crystallization under oil (microbatch)^{16,40,91}; (d) crystallization by dialysis^{7,92}; (e) crystallization in gels^{93–95}); (x) protein purity, homogeneity and monodispersity.

56| After the main variables have been noted (see Step 55), ‘play around’ with them. Alter the precipitant concentration, the pH and the protein concentration. Design a screen by bracketing the precipitant conditions and pH values above and below those that produced the crystals. For example, if crystals were obtained in 25% wt/vol PEG 6000, pH 7.5, a subsequent screen might be designed to vary PEG between 15 and 35% wt/vol versus pH 6.5–8.5. To save protein, larger increments have to be tried initially, with more fine increments as the results dictate. When using PEG as precipitant, evaluate the effects of changing the molecular weight of the PEG. PEG sizes from $M_r = 400$ to 20,000 have successfully provided protein crystals, but the experience indicates the range 2,000–8,000 as the most successful.

▲ **CRITICAL STEP** In some cases, varying the pH by 0.1 pH units is enough to stop crystallization. Always check the pH of the final solution containing protein and precipitant.

▲ **CRITICAL STEP** Take care of PEG solutions. PEGs are inherently unstable and often produce aldehydes and peroxides as by-products. The presence of these compounds becomes more likely as the PEG solution ages, and their formation is promoted by UV light. Store PEG solutions at low temperature (4 °C), in the dark, and possibly even under nitrogen, to avoid oxidation processes initiated by atmospheric oxygen. Mould growth in PEG solutions can be prevented by adding 0.1 mM sodium azide.

57| (Optional) Switch from PEG 400 to 2-methyl-2,4-pentanediol (MPD), or vice versa, if you experience limited success with either of these compounds.

58| Evaluate the effect of different and/or related salts. For example, when ammonium sulfate appears to be effective but does not provides high-quality crystals, evaluate lithium sulfate or other sulfates.

59| Vary salt concentration (increase and decrease) in the presence of a fixed amount of PEG.

60| Try different buffers, e.g., at pH 6.0 try ADA [*N*-(2-acetamido)iminodiacetic acid], MES [2-(*N*-morpholino)ethanesulfonic acid]] or cacodylate.

61| Go back to the initial crystallization screening plates. Review the plates, looking for conditions where neither precipitate nor crystals were/are observed. Review the results to identify a common precipitant/ingredient in several or all of these protein samples. For example, you might find that all drops with isopropanol remained clear.

62| Add the 'common ingredient' from Step 61 to the current crystallization conditions and determine whether its presence improves the crystal size and/or quality. If, for instance, you observe a difference in the crystal in the presence of isopropanol, consider evaluating other additives in the class of alcohols such as ethanol, methanol, butanol and the like. Please note, however, that alcohols and dioxane have the effect of poisoning the crystals and of slowing down nucleation. Furthermore, alcohols like methanol and isopropanol are volatile and may render problematic the crystal mounting for diffraction data collection because of rapid solvent evaporation and subsequent crystal damage. In this case, use heavier alcohols like isobutanol or 2-butanol^{96–99}.

▲ **CRITICAL STEP** Carefully mix additives with the precipitating solution (i.e., reservoir) to avoid errors in the final concentration. It is possible that additives, like glycerol and detergents, will create concentration gradients in the drop that could make reproducing the experiment difficult.

63| Evaluate the effect of ligands, inhibitors, cofactors and all the suggestions reported in **Table 3**.

64| Increase the drop volume, that is, going from 4 to 8 µl. This has a twofold effect: an increase in the amount of protein available for crystallization and an increase in the equilibration time of the drop with well solution as a consequence of the lower reservoir/drop volume ratio. It may produce larger crystals.

65| Increase protein concentration⁶: crystals hardly ever form unless the protein concentration largely exceeds its solubility¹⁴. Higher nucleation might be observed.

66| Perform tests using different reservoir volumes. A smaller volume of the reservoir can alter the kinetics of equilibration between the reservoir and the drop^{100,101}. A lower reservoir/drop volume ratio will in fact slow down equilibration rates. As a general rule, larger crystals with fewer imperfections are obtained if the growth rate is minimized. If your trials produce only microcrystals, reduction of their growth rate can be achieved by the more gradual equilibration, with the reservoir brought about by a decrease in reservoir/drop volume ratio.

67| Use agarose or silica gel media for crystal growth. (ref. 7, pp. 127–142) This approach reduces the equilibration rate and provides substantial flexibility and control^{93,102–104}.

68| If no crystals and only precipitate are the results of the initial screens, bring precipitant/protein concentration ratio to values just below supersaturation, and vary pH and temperature to change the protein solubility.

69| If optimization experiments lead to the formation of 'multiple crystals' try obtaining single crystals by (i) exploring new additives as done in the screening experiments, (ii) attempting gel crystallization¹⁰⁵, (iii) shifting the phase equilibrium toward the aqueous phase (e.g., increasing ionic strength) to destabilize the crystalline form and prompt the separation the coprecipitated crystals, (iv) changing the pH of crystallization solutions, (v) changing the crystallization temperature,

TABLE 3 | Useful crystallization additives with relative advantages and disadvantages.

Additive	Advantage	Disadvantage	Conditions
Glycerol ^{110–112}	By increasing viscosity, it reduces turbulent motions in the solution and promotes the growth of fewer crystals of larger size. Cryoprotectant	In high concentrations, greater than 25%, may inhibit crystal growth	1–25% vol/vol of the protein solution
Alcohols, acetone and dioxane	Reduce dielectric constant of the medium ^{96–99} . The modification of the dielectric constant induces changes of the various interactions (intramolecular solute–solute, solvent–solute and so on). It has been verified experimentally that for a given concentration of electrolyte, the reduction of ionic strength decreases macromolecular solubility under salting-in condition and facilitates the protein crystallization	Alcohols and dioxane poison the crystals and inhibit nucleation. Volatile alcohols may cause problems in transferring the crystals from the solution to the goniometer head for data collection	2–25% vol/vol of the protein solution
Divalent cations such as Mg ²⁺ , Ca ²⁺ , Zn ²⁺ and Cd ²⁺ (avoid redox active cations) ¹¹³	Metal ions sometimes stabilize the protein structure, help the folding and may promote intermolecular interactions	May cause protein aggregation and precipitation	Concentration comparable with that of the protein
Anions such as bromide, fluoride, formate, malonate, acetate and citrate, phosphate and thiocyanide ^{114–116}	Bind mainly to basic residues. Decrease the protein solubility, easing crystallization. Bromide, iodide and anions containing heavy atoms allow single/multiwavelength anomalous diffraction phasing ^{117–119}	Excessive insolubilization of the protein might lead to amorphous precipitate	Any concentration from 5 to 200 M or even higher, depending on the anion and on the protein
Detergents such as β-octylglucoside (β-OG)	Are reported to promote single crystal formation, to improve reproducibility and crystal quality ^{79,81}	Makes more difficult the handling of the solutions in which it is present (foam formation)	The initial concentration of detergent in the drop should be only slightly higher than the critical micellar concentration (CMC). It may prove advantageous to mix two detergents or add NaCl (50–150 mM) along with the detergent. Salt concentration strongly affects the CMC of the detergent ^{120,121} Amphiphiles such as heptanetriol act as cosurfactants and should be used for fine-tuning
Spermine, cadaverine, spermidine and putrescine	Counterions for negatively charged groups shield electrostatic repulsions	Ligands to metal cofactors might displace metal ions from the protein	1–50 mM

(vi) cocrystallizing with partner proteins/domains or with ligands, (vii) changing the crystallization setup (e.g., from hanging or sitting drop to microbatch), (viii) resorting to DNA shuffling to introduce random mutations.

70| If all trials prove unsuccessful, it is worth trying to isolate small crystals or clusters and ask for beamtime on a synchrotron microfocus beamline in order to obtain diffraction from a single domain of a multiple crystal.

71| When microcrystalline precipitate is observed and any one of the above optimization strategies has produced useful crystals, evaluate seeding techniques^{5–7}. Several seeding techniques are available and are described in Steps 72–98 outlined next.



Optimization of crystallization conditions—crystal nuclei transfer techniques (seeding techniques) ● TIMING 60 h–4 d

General aspects

72| Start preparation of crystallization plates with pre-equilibrated protein solution drops (Step 74) before starting the preparation of crystal seeds.

73| Transfer the seeds (for any technique used) in the pre-equilibrated plates in duplicate by using both 24 and 48 h pre-equilibrated plates.

74| With regard to the preparation of pre-equilibrated protein drops for seeding, ideally, the seeds should be introduced in a protein solution lying in the metastable zone, but determining the metastable zone in the protein phase diagram is very demanding in terms of experimental time and protein supply. The practical alternative to the determination of the phase diagram for the seeding is to prepare, in a 24-wells sitting drop plate, a series of 12 drops (the drops are obtained by mixing 1 μL of protein solution with 1 μL of the appropriate precipitant solution) in which the protein concentration is lowered in steps of 4–5% with respect to the conditions where crystals appeared (i.e., if crystals have been obtained from a 10 mg ml^{-1} protein solution, prepare 12 drops where the final protein concentration goes from 10 mg ml^{-1} to 4–5 mg ml^{-1}). The precipitant concentration in the wells remains constant and the same as that used to obtain the initial crystals.

75| Prepare the other 12 drops series by keeping the protein concentration constant (i.e., the same as that used to produce the initial crystals) and by lowering the concentration of precipitant in steps of 2–3% with respect to the conditions from which the initial crystals were grown. For example, if crystals were initially obtained from 30% wt/vol PEG 4000, prepare drops where the final PEG concentration in the equilibrated drop will go from 30% wt/vol to approximately 18% wt/vol.

76| Duplicate the plate.

■ **PAUSE POINT** Let the drops equilibrate at constant temperature for 24 and 48 h.

77| Depending on the initial trial results, finer screening grids for seeding can be prepared. Alternative experiments for seeding can be designed⁴⁵.

Macroseeding (also called seed transfer) ● TIMING 24 h

78| Two different precipitant concentrations for seed washing should be found: (i) a condition in which seed crystals are stable for approximately 15 min, but not more than 1 h, and (ii) a condition in which seed crystals are stable indefinitely^{7,44}. With this aim in mind, set up a series of precipitant concentrations, going from approximately 50% to approximately 150% of the precipitant concentration needed for obtaining the starting crystals, in steps of 10%⁴⁴.

79| Fill the sitting drop bridges (only the bridge!) with approximately 40 μL of the above precipitant solution. Fill the well, around the bridge holding the drop, with water in order to avoid rapid drop evaporation as depicted in **Figure 2**.

80| Find a sufficient number of crystal seeds that are free of defects (10–20 very thin plates or rods, single prismatic crystals, etc., which are free from evident twinning or any other crystallites).

81| Pick up a crystal with a cryo-loop (for description of cryo-loops see for example: <http://www.hamptonresearch.com/products/Product.aspx?sid=136>), and deposit one crystal in each of the drops prepared in Step 79.

82| Observe at periodic intervals up to 12 h. Note the ‘survival time’ of the crystal in the drop and the precipitant concentration allowing indefinite, 1 h and 15 min survival of the crystal, respectively.

▲ **CRITICAL STEP** Do not leave your plate on the microscope platform to do this as the lamp will warm up the plate, thus interfering with the experiment.

83| Perform a trial wash series, transferring a single crystal from high to low concentration of precipitant, starting with the concentration at which the seed crystals are indefinitely stable, ending with a concentration at which seeds are only stable for 15 min to 1 h. Modify, if necessary, on either end of the concentration range, to keep your crystal intact. Seeds are washed in a slightly dissolving solution to remove the top layer of protein, which contains possible defects, from the surface of the seed without causing excessive etching or cracking.

84| Transfer the seed to a pre-equilibrated drop prepared as in Steps 74–76.

? **TROUBLESHOOTING**

Microseeding ● TIMING 24 h

85| To prepare a microseed suspension, wash seeding material as in Steps 78–83, to remove amorphous precipitate.

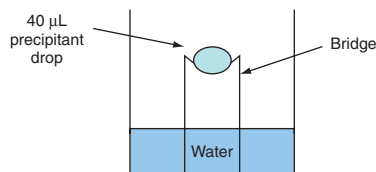


Figure 2 | Schematic representation of the sitting drop setup for finding the two precipitant conditions of Step 78.

PROTOCOL

86| Stabilize the seeding material by suspending it in a nondissolving precipitant solution (usually 10–20% above the precipitant concentration from which initial crystals appeared) and by working at a temperature that does not dissolve it.

87| Prepare a seed stock suspension by crushing the seeding material (crystals) in the above solution. For crushing, put the solution in an Eppendorf tube together with glass spheres and use a Vortex mixer.

■ **PAUSE POINT** This seed stock solution can be stored at constant temperature (i.e., temperature that does not dissolve the crystals) for later use. The seed stock solution is likely to be stable for at least 12 h.

88| Perform serial dilutions of the seed stock solution with the precipitant solution used in Step 86, to reach final dilutions of 1 in 1,000 to 1 in 10,000,000. Sequential dilutions are absolutely necessary, since the stock seed solution contains too many nuclei to be useful in the nucleation of only a small number of crystals.

89| Introduce microseeds from different dilutions into new pre-equilibrated (24–48 h) drops either by pipetting the nuclei from the stock as a small aliquot (0.1–1 μ l) or by transferring them with a seeding wand that is dipped into the microseed mixture to pick up seeds and then touched, stirred or streaked across the surface of the new drop⁶. Observe the seeded drops every 24 h.

? TROUBLESHOOTING

90| If nucleation appears to be independent of the dilution of the seed solutions, lower the precipitant concentration in the pre-equilibrated solutions.

91| If crystals do not appear in any of the pre-equilibrated drops, increase the precipitant concentration in the solutions to be equilibrated.

92| If going from a pre-equilibrated condition to the next, there is a sudden decrease in the number of crystals obtained, design a finer grid around these conditions.

Streak seeding ● TIMING 3 h

93| Mount an animal whisker (not commercially available) on the end of a wand with melted wax.

94| Degrease the whisker with ethanol/methanol, wash in water and dry. Alternatively, wash a previously used seeding tool with water and dry, or use the seeding tool from Hampton Research (for the seeding tool description see Hampton Research cat. no. 8-133).

95| Touch with the whisker (seeding tool) a crystal obtained in the optimization of crystallization section: seeds will be dislodged by friction.

96| Streak the whisker (seeding tool) across the centre of pre-equilibrated (24–48 h) drops (Steps 74–76). A good whisker should be able to transfer seeds evenly to up a dozen drops. This process can be repeated seven to ten times in order to improve the quality and size of the crystal^{46,47}.

▲ **CRITICAL STEP** Keep the distance the whisker travels in air to a minimum, as in this process the seeds may dehydrate and degrade. Also, the time lapse for which the pre-equilibrated drop wells are open, should be kept to a minimum.

▲ **CRITICAL STEP** Sitting drops provide better performance than hanging drops for streak seeding, as they do not dry out as much while the cover slip is off.

97| Along with streak seeding tries, evaluate also the effect that different additives have in the protein sample drop. Additives to consider include salts, polyols, divalent cations, detergents, chaotropes, organic solvents, ligands and cofactors.

Cross-seeding and epitaxial ● TIMING 24 h

98| To implement cross-seeding, use as nucleation starter-crystals of proteins in some way related to the protein being probed, such as mutants. Different mineral samples (i.e., mica and apophyllite) may instead be used as epitaxial nucleants. Recent studies demonstrate that porous materials, such as porous silicon, with porous sizes comparable to the size of the protein molecules, display nucleation-inducing properties. Specific experimental methods for cross-seeding and epitaxial are not different from the ones already detailed, therefore apply the approach described in Steps 74–97^{48–51,106}.

Examination of the crystallization experiments in the final stages of optimization

99| When we are aiming for highly ordered, relatively large crystals (see INTRODUCTION for crystal size definition), it is better not to disturb the crystal growth. Wait for few days or even 1 or 2 weeks before checking the seeding experiments.

● TIMING

Preliminary sample preparation and characterization: 30 h–5 d

Protein solubility screens—selection of the appropriate protein concentration for crystallization: 4 h–4 d

Fast screening of crystallization conditions: 2 h for manual preparation of 96 different conditions
 Optimization of crystallization conditions—chemical and physical variables: 24 h–48 h
 Optimization of crystallization conditions—crystal nuclei transfer techniques (seeding techniques): 60 h–4 d
 Note that the above mentioned time intervals are rough estimates. They depend on the operator skill and refer exclusively to the time needed to accomplish the practical aspects of the protocol and do not involve the time needed to observe the outcome of the different experiments performed.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
14	Loss of protein	Adsorption of protein on to membrane surfaces	Choose appropriate membranes with low binding capacity (polyethersulfone membrane are often appropriated)
15	Nonspecific aggregation during concentration process	Conformational changes due to variations of the environment	Select an appropriate protein buffer pH ^{6,65}
20	Protein denaturation. The tendency of the protein solution to foam upon gentle stirring can be a sign of denaturation	Foaming and air bubbles formation may create an oxidizing environment	Minimize shaking and use special care on handling the macromolecular solution
28	Crystals can sometimes adhere to the drop holder surface making the removal difficult. This disadvantage can turn into an advantage where occasionally the surface of the drop holder can assist the nucleation	Heterogeneous nucleation and growth that produce crystals sticking tenaciously to the support	Use a crystal probe to remove the crystal
28	Protein characterized by low solubility (concentration lower than 3 mg ml ⁻¹). Low protein concentration may produce very small crystals	Depends on the physical properties of the protein. Whatever crystallization method is used, it requires high concentrations of protein as compared to normal biochemical operations	Set up screening experiments with drops containing protein solution/precipitant solution ratio greater than 1:1. The increase of protein amount and drop size may produce larger crystals. Bigger drops can give bigger crystals, because there is more protein in the drops and the rate of equilibration is slower
35	Most of the drops have a heavy amorphous precipitate	The protein sample is heterogeneous and impure, not only in terms of contaminating agents, but also in terms of sequence integrity and conformation	Consider diagnostic testing (i.e., SDS–polyacrylamide gel electrophoresis, mass spectroscopy, isoelectric focusing, activity, light scattering, circular dichroism and nuclear magnetic resonance (NMR) as in PRELIMINARY SAMPLE PREPARATION AND CHARACTERIZATION) to measure the homogeneity and purity of the protein
54	The optimization process is often more complex and difficult than one might expect	It is a nonlinear process and one variable may be coupled to another	Carefully plan additional trials with matrices encompassing a parameter range spanning the conditions that have provided the 'hit'. Vary pairs of the above parameters or factors following the order reported in Step 55
84	Dissolving seeds	Too much of your final wash solution added to the sitting drop; concentration of precipitant too low in final wash; precipitant concentration too low in either drop or reservoir	Remove excess liquid with a small thin strip of filter paper or a very thin capillary. Lightly increase the precipitant concentration in final wash. Increase the precipitant concentration



TABLE 4 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Too many nucleation sites	Improperly washed seed that generates microseeds or unwanted nuclei in the drop being seeded, precipitant concentration too high, rate of equilibration too high	Increase the series of washes, slightly reduce precipitant concentration and reduce equilibration rate by a decrease in reservoir/drop volume ratio (see Step 66)
89	Microseeding does not work, too many nucleation sites	Microseed solution has been stored at 4 °C for too long. Microseed solution contains too many nuclei and/or two last points in Step 84 above; protein drop concentration is too high	Use only freshly prepared microseed solution ⁶ . Operate with more diluted microseed solution till 10 ⁻⁷ and/or according the above Step 84 solutions; seed drops at variable protein concentration

ANTICIPATED RESULTS

The following is an example of a grid designed upon observing precipitate formation in a screen from our laboratory: Grid for OXA-48 enzyme. At a protein concentration of 6 mg ml⁻¹ a precipitate was observed with PEG 4000 20% wt/vol and pH 8.5 in Tris-HCl buffer: PEG 4000% (wt/vol) 4, 6, 8, 10, 12, 14, 16, 18, 20 each concentration tested versus pH values: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 (overall a 9 × 9 grid).

The case of the CutA1 protein from *Escherichia coli*

CutA1 is a small protein of approximately 12 kDa that is widespread in nature from bacteria to plants, animals and humans. The protein was originally identified in a gene locus of *Escherichia coli* called *cutA* that is known to be involved in divalent metal ions tolerance, but its specific role in the different organisms was not clear. In order to shed light on the function of the protein, we started a structural determination project of one representative protein from the bacteria (*E. coli* CutA1) and one from mammals (rat CutA1)⁶⁷.

The *E. coli* protein used for the initial crystallization screenings was highly pure and well folded, as indicated by mass spectra and NMR spectroscopy, respectively, and the concentrated solutions appeared to be stable for several days.

Despite the favorable data from the preliminary sample characterization, the initial crystallization screenings of *E. coli* CutA1 provided only amorphous precipitates. Similarly, every attempt to vary the parameters that are usually the most important effectors of crystallization (pH, precipitant type and temperature) led to complete failures.

The key to the successful crystallization of the *E. coli* CutA1 was the exploration of the idea that the Cys residues present in the *E. coli* sequence were not engaged in disulfide bridges, as originally assumed, but in fact exposed to solvent. These residues would thus be subject to oxidation with formation of intermolecular aggregates, which would precipitate during the crystallization trial. Under this hypothesis, we treated the protein solution with *p*-hydroxy-mercury benzoic acid (an organomercury compound where the Hg atom can exchange his OH group with a SH group from a Cys residue) in a protein/additive ratio of 1/3 (one Hg atom for each of the three Cys present in the sequence) and repeated the crystallization screen.

Success was immediate, and we obtained high-quality crystals (1.5 Å diffraction limit). As revealed by the crystal structure, the *p*-hydroxy-mercury benzoate molecule protects the Cys sulfidryl group from reacting. At the same time, this mercury-protein adduct provides an excellent heavy atom derivative, which we used for the successful structure determination by multiwavelength anomalous diffraction measurements at the Hg edge⁶⁷. The crystal structure (PDB code 1NAQ) revealed a trimeric quaternary structure for CutA1 with all Cys exposed to solvent covalently modified by the Hg-compound.

Please note that the material safety data sheet (MSDS) for *p*-hydroxy-mercury benzoic acid is available at <https://fscimage.fishersci.com/msds/71467.htm>. However, the same general precautions related to every organomercury compound apply.

The case of *Bacillus subtilis* superoxide dismutase

While facing the difficult task to crystallize a superoxide dismutase-like protein from *Bacillus subtilis* (BsSOD) we ran some NMR experiments, which revealed that BsSOD was only partially structured in solution^{61,107}. The NMR data demonstrated the existence of secondary structure elements that fluctuate over several different conformations in solution. We still proceeded with the screening procedure outlined in this protocol, and one of the screens revealed that the protein readily precipitated as amorphous material when a zinc chloride solution was added. This observation alerted us that the Zn(II) ion should cause protein aggregation and precipitation. We then set up a screen by using Zn(II) concentration as a variable and eventually we were able to grow well diffracting crystals of the protein. The crystal structure revealed that the zinc ion is responsible of the formation of a BsSOD dimer by establishing intermolecular coordination bonds with properly positioned Asp and His side chains



located at the surface of the protein⁶¹. The coordination bonds promote a transition from a disordered structure to a completely folded structure of BsSOD. The experiment, illustrated earlier, suggests that engineering such sites at the surface of a protein might provide an additional tool in the hands of protein crystallographers able to promote the folding of protein lying in a molten-globule-like state²⁵. A research project to explore the possibility to use this method to crystallize such proteins has been started in our laboratory.

The case of yeast copper thionein

The story of yeast copper metallothionein (Cu-MT) provides an example of the use of information from NMR spectroscopy to achieve successful crystallization of a difficult protein²⁴.

Cu-MT contains 53 aa, out of which 12 are Cys and 6–8 copper (I) ions. Crystallization attempts of Cu-MT date back to the protein's discovery in 1975 (ref. 108) and have always failed. NMR measurements on Cu-MT solutions provided evidence that 4 residues at the N-terminus and 13 residues at the C-terminus were disordered and that two Cys located in the disordered C-terminal part were not involved in copper coordination.

These results suggested that the observed disorder could be responsible for the failures of Cu-MT crystallization and a minimal construct was produced lacking the first 4 and the last 13 residues of yeast MT. Subsequent extensive NMR analysis showed that, whatever their structure, the seven copper ions are arranged *identically* within this minimal frame as they are in the native Cu₇-MT¹⁰⁹. After several attempts, small but suitable crystals for x-ray analysis could be indeed obtained from this construct, and diffraction data of good quality were measured at a synchrotron radiation source²⁴.

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