Sample Preparation for Crystallization

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The Sample

The sample is the single most important variable in the crystallization experiment.[9,10] Begin with a pure, homogeneous, stable, active sample. The sample should be as pure as possible, 95 to 98%, assayed by Coomassie stained SDS-PAGE. A homogeneous, active sample, free of contaminants, aggregates, and minimal conformational flexibility is desired. Dynamic Light Scattering (DLS) can be used as a diagnostic for sample homogeneity, measuring the polydispersity of the sample, pointing out aggregation, which can be a deterrent to crystallization.[1-4, 10] DLS can be also used to screen and identify sample buffer components such as buffer, pH, ionic strength, excipients, additives, and other chemical variables, as well as temperature, towards optimization of the sample buffer formulation to maximize sample homogeneity. Differential Scanning Fluorimetry (DSF or Thermofluor®) can be used as a diagnostic for sample stability, measuring the temperature stability of the sample in the presence of chemical variables such as pH, buffer, ionic strength, excipients, and additives.[5-7]

Homogeneity Not Heterogeneity

Absolute homogeneity is essential for optimal crystallization as well as crystallographic analysis. An awareness of possible heterogeneity combined with methods and efforts to avoid and remove heterogeneity in the sample preparation should be a priority. Possible sources of sample heterogeneity include the following.[16]

- Presence, absence, or variation in a bound prosthetic group, ligand, cofactor, or metal ion
- Variation in composition of carbohydrate on a glycoprotein
- Unintentional proteolytic modification
- Oxidation of sulfhydryl groups
- Reaction with heavy metals
- Presence, absence, or variation in post-translational side chain modification (methylation, amidation, phosphorylation, glycosylation, or lipidation)
- Variation in amino of carboxy terminus, or modification of the terminus
- Variation in aggregation or oligomer state
- Conformational flexibility or instability due to the dynamic nature of the sample
- Incomplete or incorrect refolding or partial denaturation
- Combining different preps or purifications

Buffer

The sample buffer should be the simplest formulation possible that maintains the solubility, stability, activity, and homogeneity of the sample. Often times the selected sample buffer is the purification buffer or a "because that's what we've always used before" buffer. The best buffer for purification may not be the best buffer for crystallization. Using diagnostic tools such as DLS and DSF, the sample buffer formulation, with regard to buffer, pH, ionic strength, excipients, and additives can be refined to promote sample stability, solubility, homogeneity, and crystallization.

Choose a buffer such that the desired sample pH falls well within the effective buffering capacity of the buffer. A review of the literature, Protein Data Bank, and Biological Macromolecule Crystallization Database will reveal Good's Buffers and organic acids are frequently used sample buffers with good success in crystallization experiments.[11-15] The buffer molecule itself can be a significant sample variable. For example, at pH 7.0, Phosphate, Cacodylate, MES, Bis-Tris, ADA, Imidazole, BIS-TRIS propane, MOPS, and HEPES are reasonable buffers to consider. But one or more buffers may perform better than the others with regard to sample stability, solubility, and homogeneity, again, assayable by DLS (solubility & homogeneity) and DSF (stability).

Ionic strength, often in the form of Sodium chloride, should be of high enough concentration for optimal sample solubility, stability & homogeneity, and no more, as excessive salt and ionic strength can be an issue with crystallization and cryo preservation of the protein. Again, assayable by DLS and DSF.

Additives or excipients such as ligands, substrates, co-factors, inhibitors, metals, polyols, sugars, detergents, salts, polyamines, linkers, chaotropes, and other small molecules should be considered, evaluated and assayed for possible inclusion in the sample buffer. These can also be added at a later time to the crystallization experiment during optimization.

Reducing agents (anti-oxidants) may be included to protect free cysteines. Free cysteines can form intra- or intermolecular disulfide bridges that can lead to sample heterogeneity or aggregation. In general, the volatile Beta-mercaptoethanol (BME) is the shortest lived (hours to 3 days), Dithiothreitol (DTT) of intermediate (up to 7 days), and Tri (2-carboxyethyl) phosphine hydrochloride (TCEP) longest lived (weeks) anti-oxidant. The stability of the reducing agent can change with pH and temperature. The anti-oxidant L-cysteine can precipitate in the presence of oxygen, as well as form hexagonal crystals in crystallization reagents and should likely be avoided.

Characterization

There are plenty of advantages to cloning, expressing and purifying the protein yourself, including the knowledge, control, and documentation of the experimental variables. One can also learn a great deal about the sample's behavior, solubility, and stability doing the work. However, it is often the case where someone else does the work leading up to and including the purification, and one might be handed the sample for crystallization. Either way, it is a good idea to characterize the protein before crystallization screening. Some variables to consider if you're handed a sample for crystallization include the following.

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- What is the sample buffer?
- Was phosphate used at any time during the prep and purification?
- Are there disulfides or free cysteines?
- What ligands, substrates, co-factors, inhibitors, or metals are present or needed
- Are protease inhibitors present, is the sample sensitive to proteolysis?
- Has the protein or a similar protein previously been crystallized?
- At what pH range is the sample stable and unstable?
- At what temperature range is the sample stable or unstable?
- Is the protein glycosylated, methylated, phosphorylated?
- Are detergents present and if yes, what concentration?
- Is the sample a complex, dimer, trimer or ...?
- Is the sample membrane associated or a membrane protein?

Be sure to not only ask questions, but also document the answers and anything you learn about the protein, before and after you begin experiments.

Measure the concentration of the protein, run an SDS-PAGE, consider running a native PAGE if the protein has been refolded, and if sample and time permits, add isoelectric focusing (IEF), analytical gel filtration, dynamic light scattering, and mass spectroscopy. Such characterization can provide insight following crystallization screening and optimization, especially when crystallization does not occur or optimization of crystals, for size, X-ray diffraction, or other metric, results in less than desired results.

Concentration

The range of concentration in which proteins have been crystallized has been quite wide (1 - 300 mg/ml). For most soluble proteins, 5 to 25 mg/ml in a sample buffer that promotes the sample stability, homogeneity and monodispersity is a reasonable starting point for an initial crystallization screen. A pre-crystallization test such as the PCT (HR2-140 or HR2-142) can be used to better determine the appropriate protein concentration for crystallization screening. A dilute sample can be concentrated using centrifugation filter devices, or in a dialysis device against 30% w/v Polyethylene glycol 20,000.

To remove amorphous material and large aggregates, the protein can be filtered using a 0.22 micron, low protein binding filter or centrifugation at 15,000 RCF for 15 minutes. Some prefer not to filter or centrifuge the sample prior to screening, believing amorphous material might promote nucleation.

Handling

Use care when handling the protein; be kind and gentle. Do not shake or vortex the protein and avoid doing anything that might foam the protein. When experiment time calls, thaw the small sample aliquot promptly, in warm hands, and place the thawed sample in an ice bath. Avoid exposing the protein to unnecessary temperature fluctuations. Unless the protein is stable at room temperature, maintain the protein in an ice batch once thawed.

Storage

Proteins can be stored at 4°C, -20°C, or -80°C, but the stability, homogeneity, and activity of the protein must be assayed to ensure the optimal storage temperature. Repeated freeze thawing of the sample should be avoided. Store the protein in multiple, small aliquots. Rapid, flash cooling by pipetting 20 to 50 µl of protein into liquid nitrogen will generate small pellets that can be collected and stored individually in small (PCR) tubes.[4,6] Glycerol in the sample may help the protein tolerate freezing but should be avoided when possible for crystallization samples. Glycerol can be difficult to remove by dialysis and the glycerol can be a crystallization variable, influencing sample solubility and homogeneity.

Label samples clearly with the identification, batch identification, and date of storage. For easy organization and identification, nest the sample, storing the sample in batches of PCR tubes in 10 ml or 50 ml centrifuge tubes and organize them by batch or sample. It is prudent to document and hold onto detailed notes concerning the purification, storage, and handling of the sample.

Combining Batches

Avoid combining different preps and purifications of the sample. Expression, purification, and concentration conditions and procedures are never identical so each batch should be screened separately for crystallization.

Ammonium Sulfate Precipitation

Perhaps less utilized today than during the primordial days of protein crystallization, avoid using Ammonium sulfate precipitation as a final purification and/or concentration step. It can be difficult to completely remove all the Ammonium sulfate by a desalting column or dialysis. The remaining trace amounts of ammonium sulfate can interfere with crystallization screening results, create reproducibility problems, and in some reagents lead to false positive salt crystals. It is not uncommon for trace amounts of ammonium sulfate in the sample to cause precipitation or excessive nucleation in screen conditions containing Polyethylene glycol and salt.

Lyophilization

Avoid lyophilization. Even though there are many examples of proteins which crystallize after lyophilization (lysozyme, thaumatin, and catalase), lyophilization is to be avoided when possible. If the protein is lyophilized, the sample should be dialyzed before crystallization to remove buffers, salts, and excipients that may have been added prior to lyophilization.

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To Azide or Not

Don't, but if you must, read on. Sodium azide (NaN_3) is an anti-microbial preservative that is sometimes used to protect samples and crystallization reagents from microbial contamination. Sodium azide is toxic and should be handled with care. Typical Sodium azide concentrations are around 1 mM (0.02% - 0.1% w/v). If you choose to use Sodium azide remember that it is toxic to humans and critters, as well as microbes, it is an inhibitor for some proteins and may become an unintentional ligand for your sample, it can interfere with heavy atom derivatization, some metal azides are explosive, and there are reports where eliminating sodium azide from the experiment improved crystallization. Alternatives to Sodium azide include thymol and Thimerosal.

An alternative to the use of antimicrobials is the use of proper sterile technique and materials. Sterile filter all samples and reagents into sterile containers. Store samples and reagents at 4 degrees Celsius or colder. Use sterile pipet tips. Keep your work area clean. Develop a sterile technique with your crystallization set ups. With common sense, sterile reagents and sample, good technique, and sterile pipet tips, one can successfully avoid the use of chemical antimicrobials in the crystallization lab.

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