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Screening and Optimization Methods for Nonautomated Crystallization Laboratories

Terese Bergfors

Summary

Crystallization of biological macromolecules is becoming increasingly automated. However, for various reasons, many laboratories still perform at least some aspects of the work manually. A typical crystallization project entails two distinct steps: screening and optimization. The aim of the initial phase is to screen the many parameters affecting crystallization, and as broadly as possible. If any promising conditions are found, these are optimized with other protocols. This chapter describes procedures for manual screening by the vapor diffusion and microbatch methods in 96- and 24-well plate formats. For optimization, several protocols are presented, including grid and additive screens, seeding, and manipulation of the drop kinetics. The scoring of crystallization results and methods for distinguishing protein and salt crystals are also discussed in this chapter.

Key Words: Additives; crystallization; grid screens; hanging drop; microbatch; optimization; screening; seeding; sitting drop; sparse matrix; vapor-diffusion.

1. Introduction

The crystallization of biological macromolecules is usually a two-step process. The first of these is a broad sampling of the solution parameters known to affect crystallization. Useable crystals can sometimes be found by the initial search; more often however, further experiments are required to optimize the lead conditions. At present, no algorithms exist to predict which crystallization conditions will be successful for any particular macromolecule. As the crystallization databases expand with the input from structural proteomics, this situation may change. Data mining may reveal common trends for certain types of molecules *(1)* or find “hot spots” in the crystallization space. Currently though, the most used method for initial screening is a design known as the sparse matrix, which has its origin in the work of Jancarik and Kim *(2)*. Nowadays,

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many sparse-matrix crystallization kits are available commercially. **Table 1** lists sources for these as well as some other types of screens.

Kits, however, are of limited usefulness at the level of optimization because these experiments must be designed on a case-by-case basis, depending on which lead conditions are found in the screening phase. An often used optimization design is a systematic grid search varying two parameters at a time.

Structural genomics and proteomics have led to new, automated technologies for crystallization. Although many laboratories do not have the need or economic resources for automation, some of the high-throughput developments are applicable in nonautomated laboratories ***(3)***. This chapter will present manual methods of crystallization as practiced classically, but also incorporate some developments, e.g., 96-well crystallization plates from high-throughput operations (*see also* Chapter 9 for high-throughput crystallization and optimization techniques).

2. Materials

1. 96-Well crystallization plates for sitting-drop vapor-diffusion experiments, e.g., Crystal Quick™, (Greiner Bio-One, Frickenhausen, Germany, cat. no. 609171), Intelli-Plate, (Hampton Research, Aliso Viejo, CA, cat. no. HR3-297), or Corning plate (Hampton Research, cat. no. HR3-271).
2. 96-Well plates for microbatch experiments under oil (e.g., Imp@ct™, (Greiner, cat. no. 673101).
3. Optically clear sealing tape for 96-well plates (e.g., Greiner, cat. no. 676070).
4. Commercial kits (*see* **Table 1** for suppliers).
5. Paraffin oil (e.g., BDH, Poole, UK, cat. no. 29436 5H).
6. 25–250 µL Eight-channel (electronic) pipet (e.g., Biohit Proline, Helsinki, Finland, cat. no. 710220).
7. 0.2–10 µL Eight-channel (electronic) pipet (e.g., Biohit Proline, cat. no. 710300).
8. 0.2–10 µL Single-channel (electronic) pipet (e.g., Biohit Proline, cat. no. 710520).
9. Racked pipet tips for the previously mentioned pipets.
10. 24-Well tissue culture plates, e.g., Linbro dishes (Hampton Research, cat. no. HR3-110) or XRL plates (Molecular Dimensions, Cambridgshire, UK, cat. no. MD3-11).
11. Silanized glass cover slips, 0.2–0.3-mm thick, and 18–22 mm in diameter as appropriate to fit the 24-well plates.
12. High-vacuum grease.
13. Stock solution of 20% sodium azide or 0.22-µm microcentrifuge tube filter (e.g., Whatman Anopore, Kent, UK, cat. no. 6830 0202).
14. Tweezers.
15. Canned air (available from photography supply companies, Hampton Research, and others).
16. Stereo-microscope.

2.1. Optional Materials

1. 1.2–2.2 mL 96-well storage blocks (e.g., Axygen, Union City, CA 997-O-DW-20-C).

Table 1
Portfolio of Screens[a]

Name	Type of screen	Source or manufacturer
Crystal Screen HT[b], MemFac	Sparse matrix	Hampton Research (www.hamptonresearch.com)
Wizard 1 and 2[b], Cryo 1 and 2[b]	Sparse matrix	Emerald BioStructures (http://www.decode.com/emeraldbiostructures)
Flexible Sparse Matrix	Sparse matrix	Zeelen, J. (*13*)
Personal Structure Screen	Sparse matrix	Molecular Dimensions (www.moleculardimensions.com)
Crystallization Basic Kit for Proteins	Sparse matrix	Sigma Aldrich (www.sigmaaldrich.com)
JBScreen HT 1L[b] JBScreen HT 2 L[b]	Grid	Jena Bioscience (www.jenabioscience.com)
Imperial College Screen	Grid	Haire, L. (*14*)
Grid Screens™, Quik Screen, SaltRX HT[b] Sodium Malonate Screen	Grid	Hampton Research
Footprint	A solubility footprint of the protein	Molecular Dimensions
Crystool	Random	http://www-structure.llnl.gov
ZetaSol	Based on the net charge of the protein and the Hofmeister's series	Molecular Dimensions
Index HT™[b]	Combines grid, incomplete factorial, and sparse matrix features all in one screen	Hampton Research
Clear Strategy Screens	Allows full control over screening pH; contains "built-in" anomalous scatterers and cryoprotectants	Molecular Dimensions
Incomplete Factorial	A statistically efficient design	Carter, C. W., Jr. and Carter, C. W. (*15*)

[a]This list contains options available at the time of writing. New screens appear frequently.
[b]Available in a high-throughput format (96-well storage block).

2. Super-glue.
3. An animal hair or whisker.
4. Seed Bead™ (Hampton Research, cat. no. HR2-320).
5. IZIT™ dye (Hampton Research, cat. no. HR4-710).
6. Nextal (Montreal, Canada) crystallization support (cat. no. NCS-24-001).
7. Cryschem™ , Q-plate, or sitting bridge inserts (Hampton Research).
8. Acupuncture needles.
9. Centrifuge with a swing-out rotor that can accept microtiter plates.
10. Pre-Crystallization Test, PCT™ (Hampton Research, cat. no. HR2-140).
11. Low-viscosity silicone oil (BDH, cat. no. 73002 4N) or Al's oil (Hampton Research, cat. no. HR3-413).
12. A silanizing solution, e.g., Repel-Silane (Amersham Biotech, Uppsala, Sweden, cat. no. 17-1332-01) or Aqua Sil (Hampton Research, cat. no. HR4-611).

3. Methods

3.1. Preparation of the Sample for the Initial Screen

The protein or other biological macromolecule is the single most important component in the crystallization trials. The sample purity, homogeneity, and stability are major determinants of the outcome of the crystallization experiment.

1. Assay the purity of the sample by gel electrophoresis or other methods. At a minimum this means a SDS gel, stained with Coomassie blue. The protein should appear at least 90% pure. Run native and isoelectrofocusing gels if possible. Archive the gel(s) for comparison with future batches of the protein and to monitor possible changes in the protein over time. For similar reasons, archive 5–10 µL of the protein by flash-freezing it in liquid nitrogen. Store this aliquot at –80°C.
2. Exchange the purification buffer for 10 m*M* HEPES, pH 7.0 or 10 m*M* Tris-HCl, pH 8.0. Substitute other buffers as necessary if HEPES or Tris are inappropriate for a particular protein (*see* **Note 1**). The ionic strength of a 10 m*M* buffer may be too low to keep the protein in solution. NaCl should then be included (begin with 25–150 m*M*). Reducing agents, cofactors, or detergents may also be required by certain proteins.
3. Determine the protein concentration by standard procedures. The concentration of protein to use in the crystallization trial will depend on how soluble the molecule is. For a poorly soluble protein, this may be as low as 2–4 mg/mL (*see* **Note 2**). For highly soluble proteins, begin screening at 20–40 mg/mL. These values should be considered as rough guidelines only (*see* **Note 3**). As a rule of thumb, the more soluble the protein, the more concentrated it should be for the purpose of initial screening.
4. The protein solution should be protected from bacterial growth. Add 0.02% sodium azide or pass the protein through a 0.22-µm filter (*see* **Note 4**). Buffers and precipitants used in the crystallization trials should be filtered.
5. Use freshly purified protein when possible. Otherwise, avoid repeated freezing and thawing of the entire protein stock. Divide the protein solution into 100-µL

aliquots. Store them at a temperature appropriate for the protein, usually –80°C. Before setup of the crystallization trials, thaw one aliquot and centrifuge it at 16,000*g* for 5 min to pellet dust, aggregated molecules, and so on. The supernatant should be free of any turbidity before use in the crystallization drops.

3.2. Principles of the Vapor-Diffusion Method

Crystals can only grow from supersaturated solutions; therefore, the protein must be brought to supersaturation. This chapter describes two methods for doing this: vapor-diffusion and microbatch experiments. In these approaches, a solution containing buffer and precipitating agent is prepared. The precipitant can be a salt, polymer, organic solvent, or combinations thereof. Additional components, e.g., dithiothreitol, azide, detergents, and so on, may also be included; collectively all these ingredients constitute what is called the mother liquor, i.e., the solution from which the crystals grow.

In the vapor-diffusion experiment, a droplet of the undersaturated protein solution is mixed with a droplet of the mother liquor. This mixture is then equilibrated against a much larger reservoir containing only mother liquor. The concentration difference between the reservoir and the droplet causes water to leave the droplet; it diffuses as a vapor into the reservoir. As this happens, the protein and mother liquor both become more concentrated. This leads to a supersaturated state of protein while at the same time, the increased precipitant concentration lowers the protein's solubility. Under favorable conditions, this combination of events drives the protein out of solution as a highly ordered solid phase, namely a crystal.

The geometry of the vapor-diffusion experiment has several variations: the two most common ones are sitting drops and hanging drops. Examples of these two will be described in the next sections.

3.2.1. Procedure for Sitting-Drop Vapor-Diffusion Setup in 96-Well Plates

1. Select one of the commercially available screens sold in a 96-well storage block format. Alternatively, prepare your own screen in a similar storage block.
2. Using the larger volume (25–250 µL) multichannel pipet, transfer 50–100 µL of each of the 96 solutions from the storage block to the reservoirs of the 96-well crystallization plate for sitting drops (**Fig. 1A**).
3. Set the single-channel pipet on its multidispensing mode to aspirate 8 µL of protein solution from a microcentrifuge tube (**Fig. 1B**). Dispense the 8 µL as 1-µL aliquots into the first row of sitting drop wells. Repeat for the remaining rows (*see* **Note 5**).
4. With the smaller volume multichannel pipet (0.2–10 µL), transfer 1 µL from the plate reservoirs to the sitting drop wells corresponding to them. This droplet should be delivered so that it merges with the 1-µL protein droplet. Stirring or mixing of the combined droplets is optional (*see* **Note 6**).

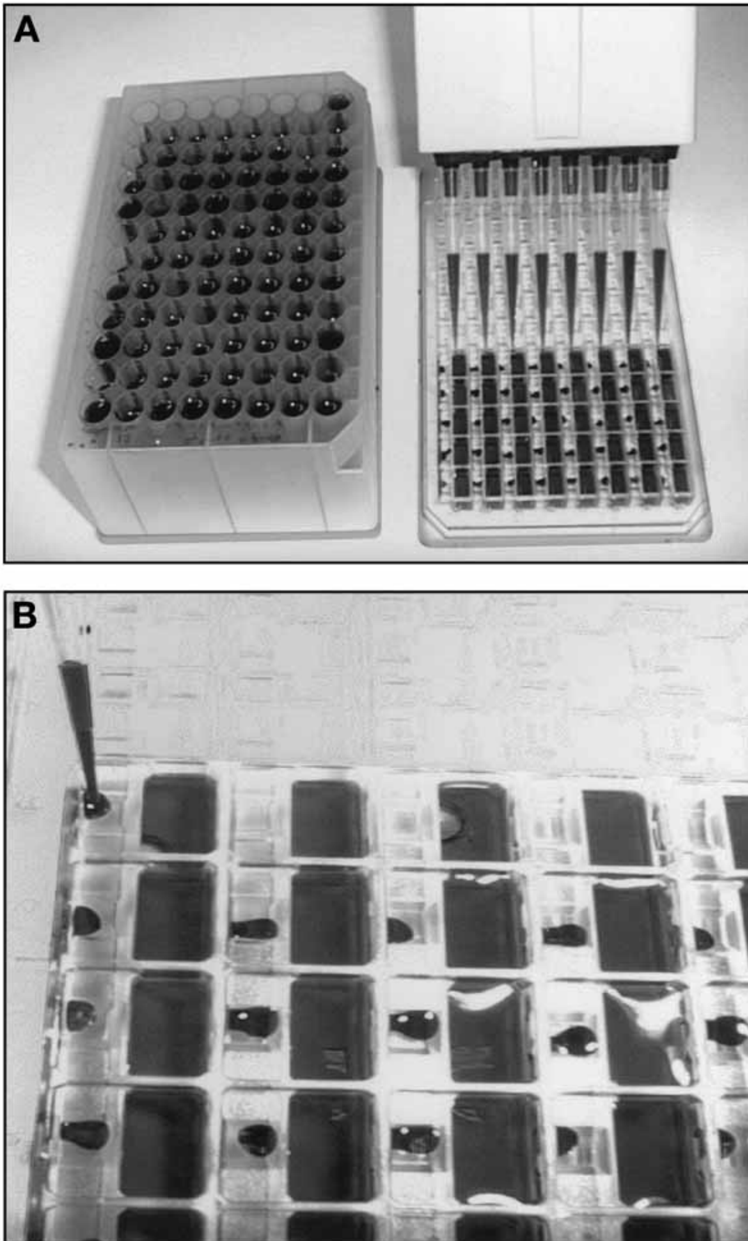


Fig. 1. Setup in a 96-well plate for sitting-drop vapor-diffusion experiments. **(A)** A multichannel pipet transfers aliquots of the screening solutions (left) to the reservoirs of a Crystal Quick™ plate (right). **(B)** Delivery of the protein solution with a repeating, single-channel pipet to the shallow depressions (close-up view). The solutions have been dyed to enhance visibility in the photograph.

5. Seal the experiment with a transparent tape or optically clear film, such as that used for PCR plates.
6. Examine the droplets under the microscope. If there are air bubbles or the two 1-µL droplets have not coalesced, centrifuge the plate at 300*g* for 5 min. (Any rotor for microtiter plates will be able to accept the crystallization dishes.)
7. Store the plate at a constant, controlled temperature.
8. Repeat with a different screen, temperature, or method, e.g., microbatch instead of vapor diffusion, if there is sufficient protein.

3.2.2. Procedure for Hanging-Drop Vapor Diffusion in 24-Well Plates

The setup with 96-well plates using multichannel pipets is compact, quick, and reduces the need for reagents to a minimum. However, the most frequently used item of plasticware for vapor diffusion in the last decade has been the 24-well disposable tissue-culture plate. Next is a description of how to perform vapor-diffusion setups with hanging drops in these plates (*see* **Fig. 2** and **Note 7**).

1. Choose a 24-well plate type with thick rims around the reservoirs, e.g., Linbro or XRL plates.
2. Grease the raised rims with high-vacuum grease. The grease can be smeared onto the rim with a fingertip or a small painting brush, or dispensed from a 10-cc syringe filled with grease.
3. Pipet 0.5–1.0 mL of each kit reagent (bought commercially or prepared in-house) into a well of the plate.
4. Place a cover slip on the bench-top. Remove any dust on it with a puff of canned air.
5. Carefully pipet 1–5 µL of protein solution onto the cover slip. Try to form a drop that is as spherical as possible (*see* **Note 8**).
6. Add 1–5 µL of the reservoir solution from the first well to the protein droplet on the cover slip. Avoid the formation of air bubbles in the two droplets. Remove any such bubbles by a quick poke with an acupuncture needle.
7. With a pair of tweezers, carefully invert the cover slip without disturbing the droplet.
8. Suspend the hanging drop over the reservoir by placing the cover slip onto the greased rim. Press gently to ensure a tight seal between cover slip and rim.
9. Prepare the rest of the plate in the same manner.
10. Inspect the seals at low magnification under the microscope for air gaps between the rim and cover slip. Press out any air bubbles or, failing this, pry up the cover slip and recoat the rim with a thicker layer of grease. Gaps will lead to dehydration of the experiment.
11. Gently transfer the plate to the crystallization room without shaking, bumping, or splashing the drops (*see* **Note 9**).

3.3. Principles of Crystallization by Microbatch

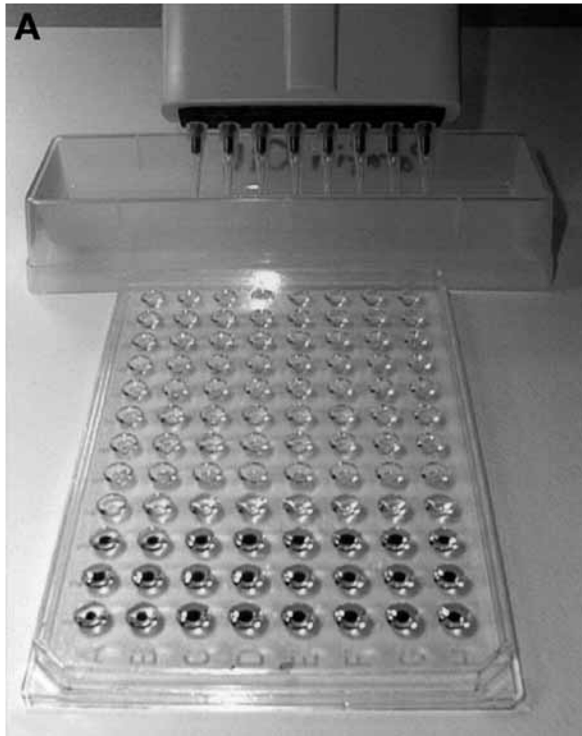
Microbatch can be used instead of vapor diffusion or in parallel with it (*see* **Note 10**). In microbatch, the protein and mother liquor components are mixed at high concentrations to achieve supersaturation directly. By contrast, the protein

Fig. 2. Hanging-drop vapor-diffusion setup in 24-well tissue culture plates.
The hanging drop, suspended on a cover slip, is about to be sealed on the greased rim. The well contains 1 mL of the screening solution. Solutions have been dyed to enhance visibility in the photograph.

solution in a vapor-diffusion experiment is initially undersaturated, but becomes supersaturated during the course of the trial. The microbatch experiment is done under oil to prevent evaporation of the drop components. There is no equilibration with a reservoir.

3.3.1. Procedure for Microbatch Under Oil in 96-Well Plates

1. Dispense 25–30 mL of paraffin oil (*see* **Note 11**) into a reagent basin (**Fig. 3A**).
2. With a multichannel pipet, transfer 10–20 μL of the oil from the reagent basin into the depressions of the microbatch plate.
3. Using the single-channel pipet, dispense the protein solution into the oil-filled wells. The protein will sink because it is heavier than the oil.



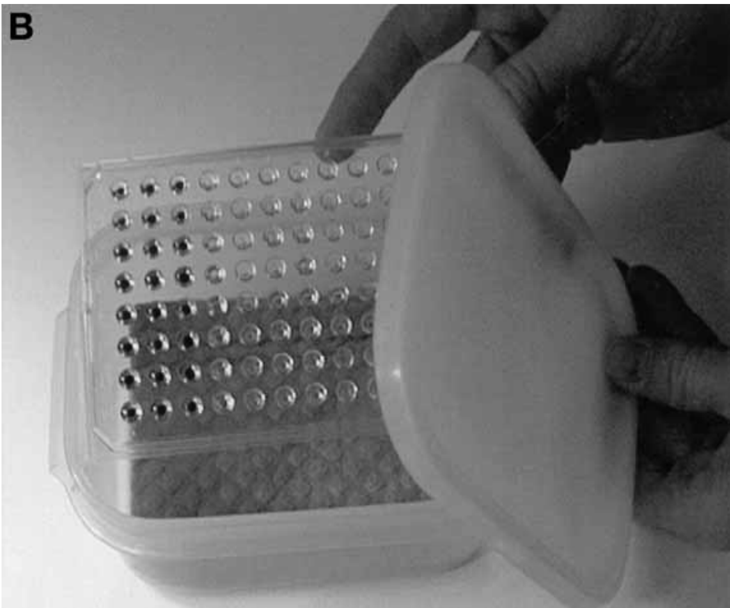


Fig. 3. Microbatch in 96-well plates. **(A)** Transfer of oil from the reagent basin to the Imp@ct^{TM} microbatch plate with a multichannel pipet. The first three rows at the bottom of the photograph show coalesced droplets of the protein and screening solutions under the oil. **(B)** Storage of the plate in a plastic sandwich box with a wetted sponge towel.

4. Select or prepare a 96-well storage block of screening solutions.
5. With the smaller volume multichannel pipet (0.2–10 µL), transfer 1 µL of each of the 96 solutions from the storage block to the microbatch dish. The droplet of screening reagent will also eventually sink to the bottom of the oil-filled well and coalesce with the protein droplet. To speed up the coalescence, centrifuge the plates for 5 min at 300*g* (optional).
6. Place the microbatch experiment inside a plastic sandwich or freezer box, e.g., Tupperware™ with some wetted paper towels at the bottom. This will supplement the effect of the oil layer in preventing drying-out of the experiment. Close the box with its close-fitting plastic lid (**Fig. 3B**).

3.4. Examination of the Crystallization Drop Results

1. Examine the drops under a stereomicroscope immediately after setup, the next day, then after 1 wk.
2. If the overwhelming majority of the drops in the screen have remained clear after 1 wk, repeat the screen with at least double the protein concentration.
3. If more than 80% of the drops have precipitated after 1 wk, the protein concentration may be too high. Reduce the protein concentration by half and repeat the screen (*see* **Note 12**).
4. Give each drop a score (*see* **Table 2**). Look for any trends that emerge with respect to the mother liquor components, e.g., pH or precipitant type.
5. Optimize the promising conditions. However, first verify that any crystals or crystalline precipitates are indeed protein and not salt (*see* **Table 3**).
6. Continue to monitor the experiment on a weekly basis for 1 mo and thereafter monthly or until the experiment dries out.

3.5. Optimization

Optimization can be carried out in many ways, e.g., by:

1. Fine-tuning the crystallization conditions found by the initial screen. This can include adjustments or changes in the pH, temperature, or protein/precipitant concentrations.
2. The introduction of low-molecular weight additives, substrates, or ligands.
3. The use of macro- or microseeding.
4. Varying the kinetics of the experiment.
5. Combinations of **steps 1–4**.

3.5.1. Optimization Protocol With Grid Screens: Three Examples

Because optimization is a different kind of sampling problem than initial screening, the search design is also different. There are many sophisticated experimental designs for efficient optimization, e.g., the orthogonal array, Box-Behnken, central composite, and Hardin–Sloane approaches. However, a systematic grid screen of two parameters is the simplest way to begin optimization because the design is intuitively obvious.

Table 2
Scoring System for Results in Crystallization[a]

Score	Description
0	Clear drop
1.	Denatured protein. Sometimes accompanied by formation of a skin over the drop surface.
2.	Heavy amorphous precipitate. Most of the protein in the drop has precipitated and the drop is full of grayish, grainy, sand-like particles.
3.	Light amorphous precipitate.
4.	Sporadic precipitation. Nonamorphous.
5.	Crystalline precipitate. Glassy, scintillating, more transparent than amorphous precipitate. Crystalline precipitates will exhibit birefringence under polarized light whereas amorphous ones do not.
6.	Spherulites. Round and chunky, semi-ordered. Sometimes needle-shaped crystals begin to grow out from these.
7.	Oils. This a semi-liquid phase of highly concentrated protein. Oils can be distinguished from spherulites by probing them with a needle.
8.	Gels. Gelatinous protein appears as irregular, transparent patches.
9.	Phase separation. Often appears as hundreds of small droplets or a mixture of droplets and a few big ones.
10.	Crystals: sea-urchins or needle clusters. Thin needle-like crystals originating from a single site or sometimes from spherulites.
11.	Crystals: needles growing singly.
12.	Crystals: plates. Thin, two-dimensional.
13.	Crystals: three-dimensional but growing on top of each other or in inseparable clusters.
14.	Crystals: single, three-dimensional, distinct edges. Check diffraction quality: do not assume that there is any relationship between optical appearance and internal crystalline order.

[a]Pictures of the different phenomena can be found at http://xray.bmc.uu.se/terese/crystallization/library.html.

The protocols listed next will use the following hypothetical example as the case to be optimized. The initial screen performed at 20°C has produced hundreds of promising, but too small, needle-shaped crystals. The reservoir consisted of 20% polyethylene glycol (PEG) 4000, 0.2 *M* unbuffered sodium acetate, and 0.1 *M* Tris-HCl buffer, pH 8.0. The protein concentration was 20 mg/mL and 1 µL of it was mixed with 1 µL of the reservoir solution in a sitting drop, then equilibrated against the reservoir in a vapor-diffusion setup.

1. Perform at least one of the following setups at a temperature different from that used in the initial screen, e.g., instead of 20°C try 4 or 8°C.
2. The protocols are described for vapor-diffusion setups; microbatch can also be used.

Table 3
Salt or Protein? Methods for Testing the Crystals[a]

Method	Description
X-ray diffraction	This is the definitive method for determining if a crystal is salt or protein. If the crystal can be mounted, check the diffraction pattern on an in-house X-ray source. The answer will be immediately obvious because the diffraction spots for salt are at high resolution and far apart, whereas they are close together for macromolecular crystals. As a result, collect a 3–5° oscillation picture with the detector close to the crystal.
Snap test	With the drop at low magnification under the microscope, use an acupuncture needle or other sharp object to break the crystal. Salt crystals are extremely hard and can even be heard to snap upon breaking. Most protein crystals crumble and smash easily upon probing them.
IZIT™ dye	Hampton Research sells a proprietary blue dye, IZIT, which binds to most proteins. The dye will concentrate in the crystals if they are protein and turn them blue, although there are reports of "false-negatives," i.e., protein crystals that failed to turn blue. The IZIT dye itself can also crystallize, usually appearing as extremely long thin needles that form within minutes. Instructions for using the dye accompany the product.
Run an SDS gel on the crystals.	1. Collect the crystals in an excess of mother liquor (e.g., 50 µL) and place them in a microcentrifuge tube. A single large crystal (0.5 mm^3) usually contains enough protein to be visible on a SDS gel developed with silver staining. Otherwise, pool together as many small crystals as possible. 2. Centrifuge 5 min at 16,000*g* to pellet the crystal(s). 3. Remove the supernatant (save it for the gel) and resuspend the crystals in 50 µL fresh mother liquor; centrifuge again. Repeat twice. The washing steps are necessary to remove traces of protein still in solution, i.e., not incorporated into the crystals. At the same time, it is important that the mother liquor itself does not cause the crystals to dissolve. Check that the crystals are visible at the bottom of the tube after the final wash step. 4. Remove the supernatant after the last wash and dissolve the crystals directly in 5–20 µL of SDS gel-loading buffer. 5. Run the washing supernatants on the same gel as the crystals along with a sample of the protein solution as a control. If the washing has been done properly, the gel may show some protein in the first washes, but there should be none visible in the final wash.

[a]Many of the components commonly used for crystallizing protein can also give rise to salt crystals. Therefore no crystal should be celebrated until it is known if it is salt or protein.

3.5.1.1. Grid Screen 1: PEG and Protein Concentrations

1. Set up three rows of PEG 4000 at concentrations of 10, 15, 20, and 25% in the reservoirs. Include the buffer previously listed (0.1 *M* Tris-HCl, pH 8.0) and the 0.2 *M* sodium acetate. Use a reservoir volume appropriate for the plate type, i.e., 100 µL in a 96-well plate and 500–1000 µL in a 24-well plate.
2. Prepare dilutions of the protein solution at 10, 15, and 20 mg/mL.
3. Make a droplet (1–5 µL protein and 1–5 µL reservoir solution) as described in the screening section and equilibrate it against its corresponding reservoir.
4. Repeat for the four PEG concentrations at all three protein concentrations to give a total of 12 drops.

3.5.1.2. Grid Screen 2: pH

1. In this optimization, prepare a reservoir solution consisting of 20% PEG 4000 and 0.2 *M* sodium acetate but substitute different buffers for the 0.1 *M* Tris-HCl, pH 8.0. A broad scan of pH using only four points could be made with:
   a. 0.1 *M* sodium acetate or citrate buffer, pH 4.5.
   b. 0.1 *M* cacodylate or MES, pH 6.0.
   c. 0.1 *M* HEPES or Tris-HCl, pH 7.5.
   d. 0.1 *M* Bis-Tris propane or glycine-NaOH, pH 9.0.
2. The range of pH values should be selected based on what is already known about the protein's solubility with respect to pH. The pH can be screened more finely, e.g., every 0.5–1.0 pH units, if there is sufficient protein.
3. Material permitting, the grid can be enlarged to screen pH while simultaneously varying the concentrations of protein and PEG.

3.5.1.3. Grid Screen 3: Different PEGs

Substitute other molecular weight PEGs for the PEG 4000. For example, try PEG 1000, monomethyl ether PEG 5000, and PEG 10,000 at four concentrations each, e.g., 5, 10, 15, and 20%.

3.5.2. Optimization With an Additive Screen

Small molecules such as chaotropes, cosmotropes, cations, detergents, amphiphiles, polyamines, chelators, linkers, polyamines, and others are often used as additives in crystallization.

1. Select one of the additive/detergent kits from suppliers like Hampton Research and Molecular Dimensions or refer to the literature for suggestions.
2. For the optimization previously mentioned example, prepare 25 mL of 20% PEG 4000, 0.1 *M* sodium acetate, and 0.1 *M* Tris-HCl, pH 8.0. Preparing all 25 mL at once will ensure that the reservoirs are identical.
3. Pipet 1 mL into each of the wells in a 24-well tissue culture plate. (There will be 1 mL left over.)
4. Prepare droplets consisting of 2.5 µL of the protein and 2.5 µL of reservoir solution.

5. Add 0.5 µL of the kit additive to this 5 µL droplet. (It is not necessary to include the additive in the 1-mL reservoir.)
6. As a control, substitute 0.5 µL of buffer for additive.
7. Equilibrate against the reservoir as usual.
8. Rate the results with a simple scoring system:
   a. Same as the control, the additive has no effect (=).
   b. Crystals have improved, e.g., they are bigger (+).
   c. Worse than the control, the crystal morphology has deteriorated, the protein has precipitated, or the drop remains clear (–).
9. Usually one or more additives will cause some improvement to the crystal quality (at least visually, this does not necessarily mean the diffraction quality has improved). However, even additives that have negative effects are of interest because they are obviously perturbing the system. Repeat the experiments for these additives at one-tenth of their original concentrations.
10. Additives can dramatically change the rates of nucleation and growth, so be patient.

3.5.3. Protocol for Optimization by Seeding

Nucleation is the formation of the first ordered aggregates of molecules. These ordered aggregates are the templates on which more of the molecules preferentially accumulate, eventually building the crystal. The probability that some molecules will meet and form an ordered aggregate is greater the more there are of them; thus, the higher the level of supersaturation, the more likely it is that nucleation will occur. These high levels of supersaturation, however, tend to lead to the formation of too many crystals.

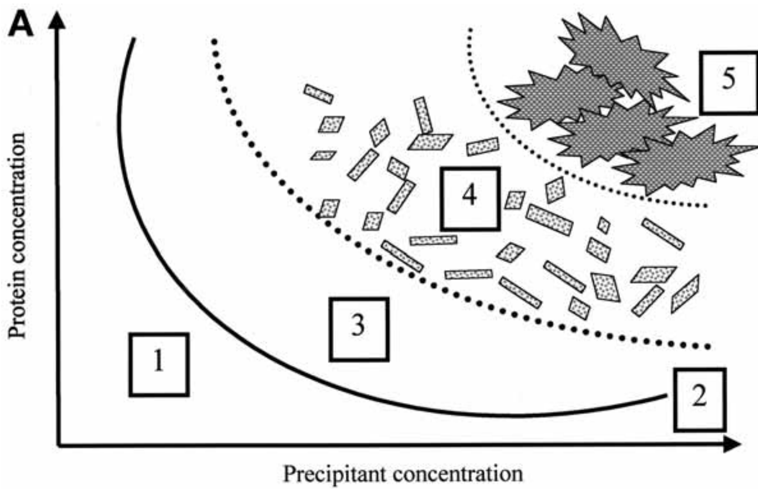
Nonetheless, these small crystals can be used as seeds. They are put into a new experiment set up at a lower level of supersaturation, i.e., the metastable zone in the phase diagram (**Fig. 4A**). This procedure, known as seeding, bypasses the need for spontaneous nucleation because the seed serves as the ready-made nucleus for growth. By limiting the number of seeds that are introduced, ordered growth of just a few, and, therefore, larger, crystals is possible.

The drawback of this method is that a phase diagram for the protein is rarely available. The concentrations of protein and precipitant corresponding to the metastable zone must be determined empirically by lowering one or the other, or both.

Many seeding protocols for macro- and microseeding exist. One easy and fast variant of microseeding is streak seeding ***(4)***.

3.5.3.1. Protocol for Streak Seeding

1. Either microbatch or vapor diffusion can be used (*see* **Note 13**). The microbatch oil will not affect the seeds. Equilibrate the drops overnight vs the reservoirs if using vapor diffusion.



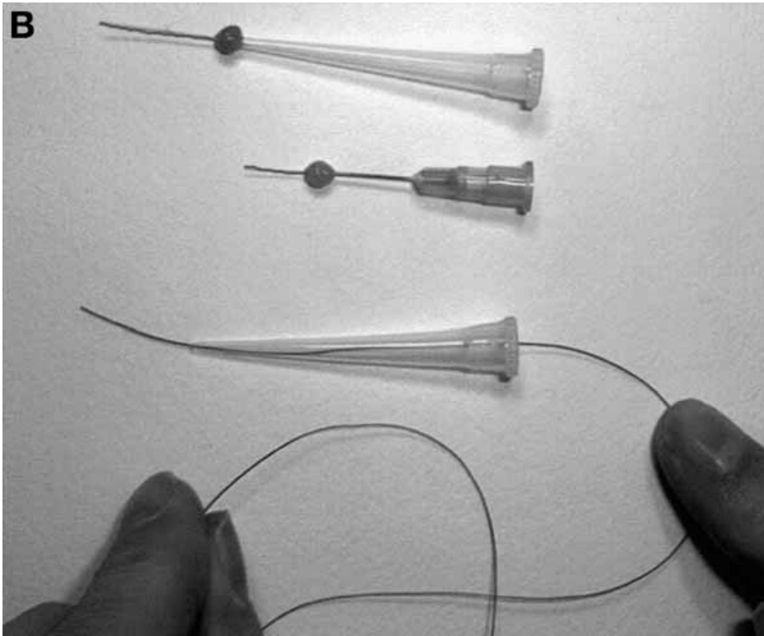


Fig. 4. Seeding. **(A)** Hypothetical phase diagram. (1) Undersaturated zone; no solid phase of the protein is possible. (2) The dark thick line indicates the limit of solubility. (3) Metastable region; the level of supersaturation will support crystal growth but is not high enough to initiate spontaneous nucleation. This is the best region in which to place a seed crystal. (4) Labile zone; spontaneous nucleation occurs in this region and the crystals form. (5) At high levels of supersaturation the protein precipitates. **(B)** Streak-seeding wands made with horsetail hair. The horsetail hair is threaded through a pipet tip or hypodermic needle and fixed into place with a drop of super-glue or wax. A scalpel blade should be used to trim the hair to a working length of 1–2 cm. *(Figure continues)*

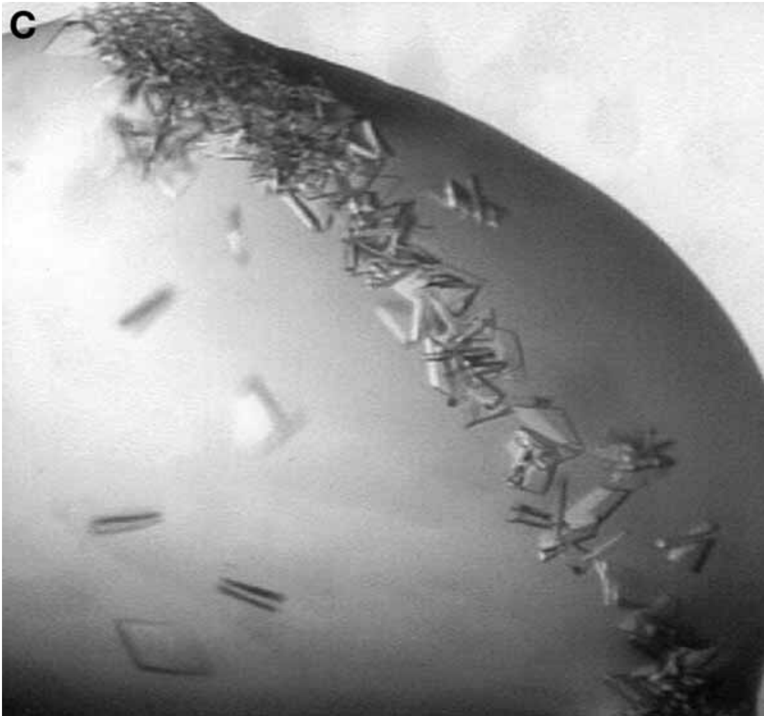


Fig. 4. *(continued)* **(C)** Result of a streak-seeding experiment showing crystals growing along the streak line.

2. With the previous example as the model again, set up a 3 × 4 grid screen as previously described. The reservoirs can be 5, 10, 15, and 20% PEG 4000, 0.1 *M* Tris-HCl, pH 8.0, and 0.2 *M* unbuffered sodium acetate. The protein concentration can be 5, 10, and 15 mg/mL. Equilibrate overnight.
3. Make a seeding wand with horsetail hair or an animal whisker, e.g., from a cat or rabbit (**Fig. 4B**).
4. To pick up the seeds for transfer to the new drops made in **step 2**, touch or scratch the surface of the parent crystal with the seeding wand (*see* **Note 14**).
5. Draw a line with the seeding wand through all the new drops in one row. There are enough microcrystals trapped on the wand to inoculate four to six drops without retouching the parent crystal to pick up more.
6. Wait 2–7 d and then examine the drops. In conditions that are supersaturated, the seed crystals will grow along the streak line (**Fig. 4C**). Drops that remain clear indicate that the conditions are undersaturated, which has caused the seeds to dissolve. Try to find the concentrations where the protein appears to be only slightly supersatured, i.e., no spontaneous nucleation occurs but inserted seeds grow.

3.5.3.2. Protocol for Seeding With a Dilution Series of Seed Stock

Further refinements can be introduced to provide better control over the number of seed nuclei that are transferred.

1. Use the streak-seeding protocol in **Subheading 3.5.3.1.** to find the concentrations of protein and precipitant that correspond to the metastable zone of the phase diagram (**Fig. 4A**).
2. Set up a row of five identical drops within this metastable zone. Equilibrate overnight if using the vapor-diffusion method.
3. Crystals grown in the previous streak-seeding experiments or from the initial screen can be used as seeds. Crush or pulverize the crystal(s) in the parent drop with a needle, tissue homogenizer, glass rod, Seed Bead, or similar tool.
4. Flood the drop of crushed crystals with 50–100 µL of mother liquor to make recovery of the seeds easier.
5. Transfer the seeds to a microcentrifuge tube. Vortex to ensure an even dispersion of the seeds, then immediately take 10 µL of seed stock and add it to a new tube containing 90 µL mother liquor (= a 10X dilution). Repeat in this manner to make the series of five dilutions (i.e., 10^2, 10^3, 10^4, and 10^5) of the seed stock.
6. From the first of these tubes (the 10X dilution), remove a 0.1- to 0.5-µL aliquot and add it to one of the new drops made in **step 2**. Repeat with the second drop for the second (100X) dilution and so on. Use a fresh pipet tip for each drop (*see* **Note 15**).
7. Save the seed dilutions at the temperature at which they grew. Wait 2–7 d, then examine the drops to find which dilution gave the optimum number of seeds.
8. Having once determined the optimum dilution, the same seed stock can be used many times (*see* **Note 16**).

3.5.4. Optimization by Varying the Kinetics of the Experiment

The rate at which supersaturation occurs can greatly affect the outcome of the crystallization experiment and even determine if crystals appear or not. There are many ways of manipulating the kinetics and some possibilities are given here:

1. Vary the mixing ratio of protein and reservoir solution in the droplets. Instead of 1 µL protein and 1 µL of the reservoir solution, try ratios of 3:1 and 1:3.
2. Increase the size of the droplet. Try 10 µL protein plus 10 µL reservoir instead of 1 µL plus 1 µL.
3. If the initial screen used hanging drops, try sitting drops and vice versa.
4. Try a 96-well plate instead of a 24-well plate, or vice versa.
5. Change the method, e.g., use microbatch instead of vapor diffusion and vice versa (*see* **Note 17**).
6. To slow down vapor-diffusion experiments in 24-well plates, cover the 1000-µL solution in the reservoir with 100–500 µL of paraffin oil ***(5)***.
7. To speed up microbatch experiments, exchange the 100% paraffin oil for a mixture of 50% paraffin:50% silicone oil ***(6)***.

3.6. What to Do if There are No Crystals to Optimize or the Optimization is Unsuccessful

1. Recheck the drops in the original screen. Crystals have been known to appear after many months. Have a colleague also check the drops; they may see things that have been missed.
2. Is the protein concentration set high enough? (*See* **Note 18.**)
3. Is the protein pure enough? Consider a further purification step or a different fraction from the current purification. Rescreen.
4. Check the stability of the protein. Is it degrading during the course of the crystallization trials? Run a gel on the protein from the drop and compare it with the archived material.
5. If there are no crystals, look for the next best results, e.g., spherulites, crystalline precipitates, or phase separation, and optimize around these conditions.
6. Add a ligand, substrate, or cofactor to the protein and rescreen. Binding of a ligand, etc., can completely change the conformation of the molecule, making it more amenable to crystallization.
7. Vary the type of search strategy for initial screening. Try a design other than the sparse matrix (**Table 1**).
8. Consider modifying the molecule if extensive screening is unsuccessful. Examples of possible modifications are removing or moving affinity tags (e.g., from the N- to the C-terminus), making truncations, chemically or genetically modifying residues, deglycosylating the protein, or expressing it in a different system.
9. Some researchers report that dynamic light scattering is a useful diagnostic method for determining if a protein solution is likely to crystallize or not *(7,8)* (*see* Chapter 6).

4. Notes

1. Many proteins have been successfully crystallized in phosphate buffer but it will easily give rise to inorganic salt crystals, e.g., calcium phosphate. Citrate is frequently used as a buffer but keep in mind that it chelates metal ions. Cacodylate is another common crystallization buffer, handle it with caution because it is an arsenic compound.
2. Glycerol (try 10–30%) will increase protein solubility. If hydrophobic interactions between molecules are the cause of aggregation, neutral detergents can improve the solvation properties. β-octyl glucoside (0.25–0.5%) and CHAPS (0.1–0.3%) are frequently used.
3. Hampton Research sells a product called PCT™, Pre-Crystallization Test, for finding an appropriate protein concentration for use in their Crystal Screen.
4. Microcentrifuge tube filters, such as Whatman's Anopore, have an advantage over syringe filters because there is no hold-up loss. Anopore is an inert substance with low protein-binding properties. Nonetheless the careful researcher will confirm the protein concentration again after filtration. Sodium azide can be used to prevent bacterial growth but beware of this compound's toxicity. Moreover, it is a ligand for some proteins and may later appear in electron-density maps.

5. Volumes larger or smaller than 1 µL can also be used.
6. Stirring or mixing of the droplet will increase any nucleation that occurs. This effect may or may not be desirable, depending on whether the protein nucleates too easily (giving rise to too many crystals) or hardly at all (drops remain clear).
7. The 24-well plates can be converted for sitting-drop setups by the insertion of commercially available plastic bridges that are placed into the reservoirs. There are also 24-well plates specifically made for sitting drops, e.g., Cryschem™ and Q Plate.
8. Protein wets glass and spreads all over the cover slip, especially if detergents are present. To avoid this, cover slips should be made of plastic or silanized if made of glass. Already silanized cover slips can be bought from the suppliers given in **Table 1** or they can be silanized in-house with a silanizing solution, e.g., Repel-Silane (Amersham Biotech) or AquaSil (Hampton Research).
9. For hanging-drop vapor-diffusion experiments at 4°C, place the crystallization plates inside a Styrofoam box. This is to minimize local fluctuations in temperature that can give rise to condensation on the cover slips. Sitting drops and microbatch are less vulnerable to condensation problems.
10. Vapor diffusion and microbatch create supersaturation of the protein in quite different ways. In a comparison of the two methods on six proteins, 30% of the successful conditions were unique for each technique, respectively ***(9)***.
11. Paraffin oil is a highly effective barrier, permitting little evaporation. The experiment will not dry out for at least 1 mo. Diluting the paraffin with silicone oil will greatly increase the rate of evaporation. This can be an advantage during screening because results will be obtained more rapidly ***(10)***. However, the experiments dry out in a matter of days. A 50:50 mixture of paraffin:silicone oil is commercially available (Al's oil, Hampton Research) or can be made in-house. 100% silicone oil will lead to dryness overnight in the Imp@ct plates and therefore is not suitable in this low-volume type of microbatch plate.
12. To determine if the precipitated protein is denatured or simply too highly supersaturated, a simple test can be performed. Try to redissolve the precipitate by flooding the drop with mother liquor from the reservoir, buffer, or just water. Denatured protein will not redissolve.
13. A plate designed for seeding into hanging drops is manufactured by Nextal (www.nextalbiotech.com). It consists of greaseless crystallization supports that can easily be opened and resealed again.
14. The same seeding wand can be reused a few (5–10) times.
15. The seeds can be transferred by streak seeding instead of as aliquots. Wipe the seeding wand clean between each drop with a Kimwipe.
16. The seeds will settle rapidly to the bottom of the tube. It is therefore important to vortex the tube immediately before use. Under the microscope, check an aliquot of the seed stock to confirm that the seeds are still present. Seeds can dissolve because of temperature changes or bacterial contamination of the storage solution.
17. Changing the method from vapor diffusion to microbatch, or vice versa, will have a greater effect on the outcome than variations of the same method, like substituting sitting drops for hanging drops ***(11)***.

18. Although there are proteins that have been crystallized at only 1 mg/mL, in general, poorly soluble proteins are poor candidates for crystallization. Nucleation is favored by high levels of supersaturation, which in turn requires high protein concentrations. If simpler methods (*see* **Note 2**) do not improve solubility, mutations can be introduced. One of many examples where this has worked is HIV-1 integrase *(12)*. Substitution of a single amino acid made it possible to reach a concentration of 25 mg/mL and eliminated the detergent needed to keep the wild-type protein soluble.

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References

1. Hennessy, D., Buchanan, B., Subramanian, D., Wilkosz, P. A., and Rosenberg, J. M. (2000) Statistical methods for the objective design of screening procedures for macromolecular crystallization. *Acta Crystallogr. D. Biol. Crystallogr.* **56,** 817–827.
2. Jancarik, J. and Kim, S.-H. (1991) Sparse matrix sampling: a screening method for crystallization of proteins. *J. Appl. Cryst.* **24,** 409–411.
3. Villaseñor, A., Sha, M., Thana, P., and Browner, M. (2002) Fast drops: a high-throughput approach for setting up protein crystal screens. *BioTechniques* **32,** 184–189.
4. Stura, E. and Wilson, I. (1991) Applications of the streak seeding technique in protein crystallization. *J. Crys. Growth.* **110,** 270–282.
5. Chayen, N. (1997) A novel technique to control the rate of vapour diffusion, giving larger protein crystals. *J. Appl. Cryst.* **30,** 198–202.
6. Chayen, N. (1997) The role of oil in macromolecular crystallization. *Structure* **5,** 1269–1274.
7. Bergfors, T. (1999) Dynamic Light Scattering. In: *Protein Crystallization: Techniques, Strategies, and Tips,* (Bergfors, T., ed.), International University Line, La Jolla, CA, pp. 29–38.
8. Zulauf, M. and D'Arcy, A. (1992) Light scattering of proteins as a criterion for crystallization. *J. Cryst. Growth* **122,** 102–106.
9. Baldock, P., Mills, V., and Shaw Stewart, P. (1996) A comparison of microbatch and vapour diffusion for initial screening of crystallization conditions. *J. Crystal Growth* **168,** 170–174.
10. D'Arcy, A., MacSweeney, A., Stihle, M., and Haber, A. (2003) The advantages of using a modified microbatch method for rapid screening of protein crystallization conditions. *Acta Crystallogr. D. Biol. Crystallogr*. **59,** 396–399.
11. Rupp, B. (2003) Maximum-likelihood crystallization. *J. Structural Biol.* **142,** 162–169.
12. Dyda, F., Hickman, A., Jenkins, T., Engelman, A., Craigie, R., and Davies, D. (1994) Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. *Science* **266,** 1981–1985.

13. Zeelen, J. (1999) A flexible sparse matrix screen. In: *Protein Crystallization: Techniques, Strategies, and Tips,* (Bergfors, T., ed.), International University Line, La Jolla, CA, pp. 77–90.
14. Haire, L. (1999) Imperial College grid screen. In: *Protein Crystallization: Techniques, Strategies, and Tips,* (Bergfors, T., ed.), International University Line, La Jolla, CA, pp. 125–129.
15. Carter, C. W., Jr. and Carter, C. W. (1979) Protein crystallization using incomplete factorial experiments. *J. Biol. Chem.* **254,** 12,219–12,223.