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Efficient optimization of crystallization conditions by manipulation of drop volume ratio and temperature

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Abstract

An efficient optimization method for the crystallization of biological macromolecules has been developed and tested. This builds on a successful high-throughput technique for the determination of initial crystallization conditions. The optimization method takes an initial condition identified through screening and then varies the concentration of the macromolecule, precipitant, and the growth temperature in a systematic manner. The amount of sample and number of steps is minimized and no biochemical reformulation is required. In the current application a robotic liquid handling system enables high-throughput use, but the technique can easily be adapted in a nonautomated setting. This method has been applied successfully for the rapid optimization of crystallization conditions in nine representative cases.

Keywords: protein crystallization; high throughput; optimization; temperature

The importance of obtaining a crystal for X-ray crystallographic studies is difficult to overstate; it is required to reach the goal of a three-dimensional structure of the target molecule. Producing crystals of biological macromolecules is often a challenging task. For 119,721 targets entered into TargetDB by the worldwide structural genomics centers, only 14.1% of the purified, soluble targets produced a crystal structure (Berman et al. 2000). The crystallization of biological macromolecules can be considered as a two-stage process. The first stage, "screening," determines chemical and physical conditions under which the sample has a propensity to crystallize. The second stage, "optimization," refines the chemical and physical parameters to produce crystals suitable for analysis by X-ray diffraction. Optimization methods have not been brought to high-throughput contemporaneously with screening (Chayen and Saridakis 2002).

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Jancarik and Kim (1991) used a sparse matrix approach to design a set of solutions (cocktails) to screen and identify crystallization conditions; these solutions are readily available in commercial kits. An expansive collection of chemicals known to crystallize macromolecules appears in the literature. It is not practical or feasible to exhaustively screen all of the combinations of these chemicals. These cocktails incorporate a range of pH, chemical species, and concentrations. A sparse matrix design is used to formulate a reasonable number of cocktails to survey the vast chemical landscape. When even a single experiment produces a crystalline outcome, the screening experiments are considered successful.

Optimization makes use of information derived from the screening experiments to produce crystals of sufficient size and quality for diffraction. Both environmental variables (such as temperature) and chemical variables (type and concentration of chemicals and the solution pH) are refined. It is advantageous when multiple experiments produce outcomes suitable for optimization from different cocktails. The crystals produced from these chemically distinct solutions often have different physical properties, which

can be exploited to provide alternatives if difficulties are encountered downstream during optimization, X-ray diffraction, or structure solution and refinement.

A widely used optimization strategy, the grid screen, arrays chemical variables identified during screening experiments to refine the chemical conditions and produce crystals of increased volume (Cox and Weber 1988). The concentration of a precipitating agent (the chemical species that is primarily responsible for driving the macromolecule to a state of supersaturation) and solution pH are varied in a regular fashion from an initial coarse screen to finer grids in a series of successive experiments. Another experimental design, first reported to crystallize *Bacillus stearothermophilus* tryptophan tRNA synthetase, refines crystallization solutions using an incomplete factorial to distribute crystallization variables in a randomized and balanced manner (Carter and Carter 1979). Using stepwise multiple-regression analysis, scores of crystal quality are correlated to design the next series of experiments.

Both the grid and incomplete factorial approaches produce a common product. They describe the next generation of cocktails that will be used to optimize crystallization conditions. Regardless of the method used to design these solutions, time and materials must be expended to prepare them. The majority of the time required to set up optimization experiments is devoted to formulating and then producing cocktail solutions. A significant time-savings and potential increase in reproducibility would be realized if solutions which produced crystals in the screening experiments could be directly employed for optimization without the need to reformulate.

The use of temperature to control the level of supersaturation in crystallization experiments is well-established for small molecules, but remains an underutilized variable for the crystallization of macromolecules (Rosenberger 1986; Landsberg et al. 2006). Despite its limited use, temperature has been shown to be an important and generally applicable variable for the crystallization of biological macromolecules.

Batch crystallization methods, including microbatch-under-oil (Chayen et al. 1992), combine an aliquot of protein and cocktail solution for crystallization. The two solutions merge to form an experiment drop. Microbatch-under-oil differs from traditional batch methods because it uses oil to containerize and to retard the rate of dehydration of the aqueous experiment drop. Batch methods are in general well-characterized (Koszelak et al. 1996), can be used with small solution volumes, and can be incubated at a variety of temperatures (Chayen et al. 1990). Manipulating the concentration of the protein and precipitating agent by varying their ratios to each other has been demonstrated to be an effective and efficient way to produce high-quality crystals using batch methods (Rayment 2002).

The microbatch-under-oil technique has been used for both screening and optimization experiments during this study. Crystallization outcomes are presented for eight different proteins that have been optimized for crystal volume using a high-throughput Drop Volume Ratio/Temperature (DVR/T) method. This method can be setup rapidly with a small volume of protein solution. There is no need to reformulate protein or cocktail solutions from those used for the screening experiments. DVR/T experiments sample temperature simultaneously with the concentrations of the protein and cocktail solutions.

Results

Eight biological macromolecules submitted to the Center for High-Throughput Structural Biology were used for this study. These represented typical problems and ranged from 25- to 75-kDa molecular weight (Table 1; Fig. 1A–I). Screening experiments for these samples at 23°C produced outcomes ranging in quality from needles and highly twinned plates to small single crystals. The effects of changing the experiment drop composition (volume of protein to crystallization cocktail) and temperature on the outcomes of these experiments are readily observed (Fig. 1).

A simultaneous, microscopic assessment of the outcomes shows the incubation temperature affected all of these samples. Examples where solubility is directly (Fig. 1A) and inversely related to temperature (Fig. 1D) are observed by comparing the number of clear (undersaturated) outcomes to the number of supersaturated outcomes (phase separation, precipitate or crystals) for each group of 64 experiments. The experiment drop chemistry plays an important role in the relationship between a protein's solubility and temperature. Interestingly, the solubility dependence on temperature can be reversed by changing the chemistry of the cocktail solution for the same protein, (Fig. 1A,B). Sample P6306 (Fig. 1A) is more soluble with an increase in temperature using a cocktail solution of 100 mM Na Acetate pH 5.0, 100 mM NH_4SCN, 20% (w/v) PEG

Table 1. *Protein solutions used for optimization*

Figure 1	Protein	[Protein] mg/mL	Protein solution
A	P6306	20	10 mM Tris pH 7.4, 100 mM NaCl
B	P6306	20	10 mM Tris pH 7.4, 100 mM NaCl
C	P5687	4	20 mM HEPES pH 7.6, 100 mM NaCl,5% (v/v) glycerol, 1 mM DTT, 1 mM EDTA
D	P6334	9	50 mM Tris pH 8.5, 500 mM NaCl
E	P6127	35	20 mM Tris pH 9.0
F	P6512	20	10 mM Tris pH 7.5, 0.02% (w/v) Na Azide
G	P6510	10	50 mM Tris pH 7.6, 1 mM EDTA
H	P6102	11	50 mM K Phosphate pH 7.4, 100 mM KCl
I	P6893	10	20 mM Tris pH 7.6

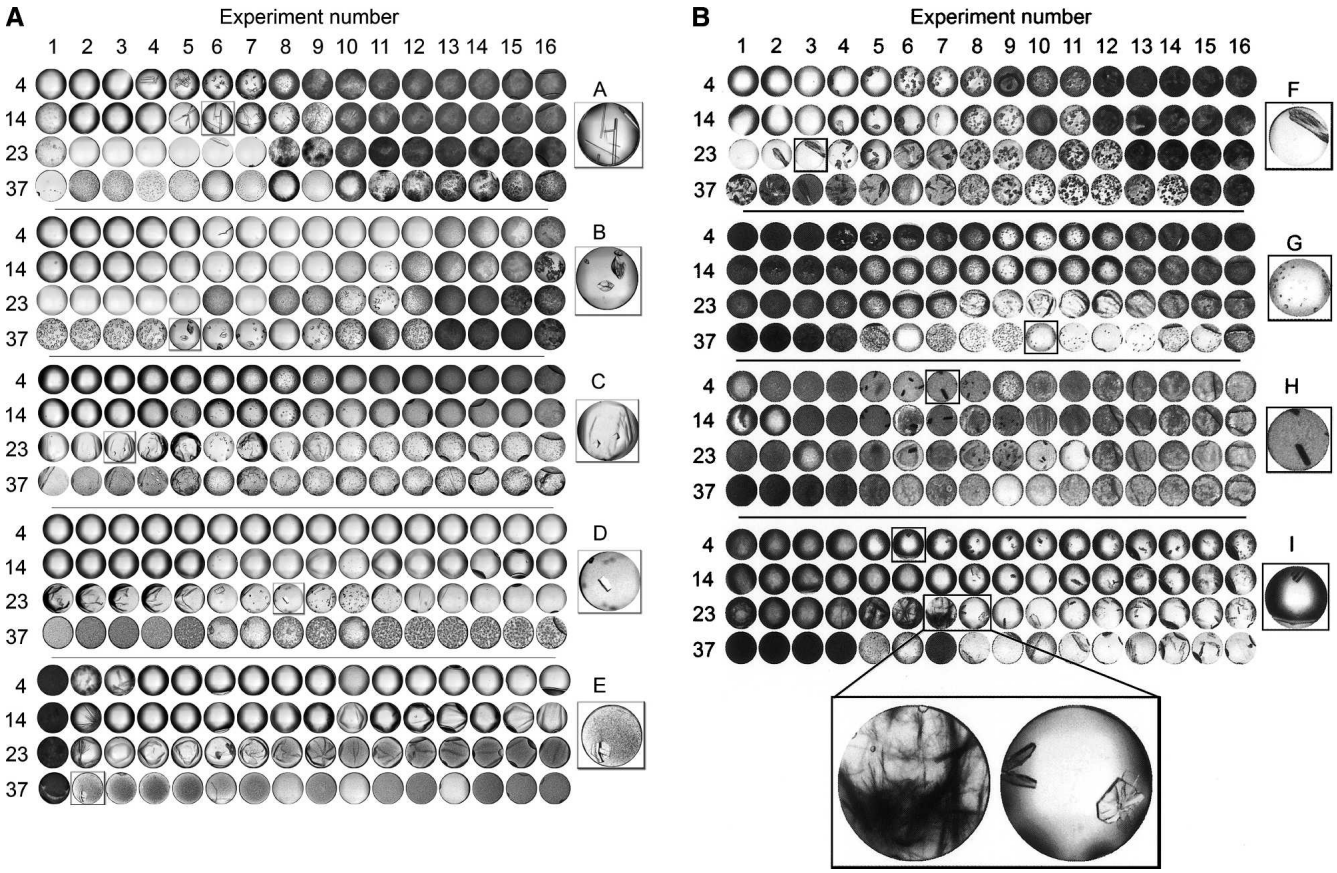

Figure 1. Outcomes of DVR/T experiments. All of the images have a well diameter (circle) of 0.9 mm. The highlighted thumbnail image is considered a "good outcome" and is enlarged to the *right* of the array. Each array of 64 images (4 rows × 16 columns) is a single-protein and cocktail solution. The numbers 1 through 16 correlate with the volume of protein and cocktail solution used to construct the experiment drop. A gradual progression of inversely related volume ratios takes place in the series of images. Column 1 has the highest volume of protein and lowest volume of cocktail solution; column 16 has the highest volume of cocktail and lowest volume of protein solution. The four different temperatures used to incubate the plates (4°C, 14°C, 23°C, and 37°C) appear along the *left* side of each array. All images were recorded three weeks after the experiment was set up. The protein and cocktail solutions (*A–I*) are listed in Tables 1, 2, respectively.

4000. P6306 is less soluble with increasing temperature (Fig. 1B) using a cocktail solution of 100 mM MOPS pH 7.0, 100 mM NH_4Br, 80% (v/v) PEG 400. In most cases there is a readily identified, optimum temperature for crystallization. The optimum temperature for this group of proteins (highlighted by the thumbnails of "good" outcomes) varies and is distributed across all four temperatures.

Varying the volume ratio used to compose the experiment drop can lead to a dramatic change in crystal morphology. An example of this effect, at a constant temperature of 23°C, appears in Figure 1I. In this series of experiments, fibrous, dendrite crystals abruptly change to plate morphology. Dendrites form at a higher concentration of protein (hereafter designated [protein]) and lower [cocktail] in the experiment drop. The demarcation takes place when the cocktail volume is held constant at 200 nL, while the protein volume decreases from 250 nL (experiment number 7, 23°C) to 200 nL (experiment number 8, 23°C). This decreases the [protein] and increases the [cocktail] sufficiently to cause the dramatic shift in morphology. These trends in morphology continue for both dendrites and plates in sequential progression away from this boundary.

Crystals that were judged to be of good optical quality appeared across the range of drop volume ratios used for these experiments. An example where the best crystals form near one volume ratio extreme ($V_{protein} > V_{cocktail}$) is P5687 (Fig. 1C). It should be noted that the starting concentration of P5687 was relatively low (4 mg/mL). This makes the higher volume ratio of protein to cocktail producing the best crystals somewhat intuitive. It demonstrates crystal quality can be improved by changing the volume ratio without the need to reformulate (concentrate) the protein solution.

The 1536 well microassay plates used to set up these experiments have narrow, conical wells (0.9 mm diameter at the bottom of the well); crystal retrieval from these plates is not easily achieved. As such, there is no X-ray

data to demonstrate the quality of diffraction. These small-volume batch experiments are designed to quickly identify optimized crystallization conditions. Scale-up for X-ray diffraction analysis is the next challenge.

Discussion

Every crystallization protocol will take a unique path through the phase diagram influencing the experiment's outcome (Ries-Kautt and Ducruix 1992). The DVR/T method uses exactly the same microbatch-under-oil crystallization protocol for both screening and optimization. Using the same protocol improves reproducibility and eliminates the complication of converting crystallization conditions from one method used for screening to another for optimization (Chayen 1998). The optimized conditions will need to be reproduced in a container that is better suited for retrieving the crystals; this may require an increase in solution volumes. The method has another practical advantage; it makes use of the same cocktails for screening and optimization. This prevents batch differences caused by reformulation. Cocktails, especially those containing PEGs, undergo chemical changes over time (Jurnak 1985). The high-throughput crystallization screening experiment uses some variety of polyethylene glycol (PEG) in >1000 of the 1536 cocktail solutions. Aged PEG solutions have been reported to produce crystals of RNase A minor trimer that could not be obtained using freshly prepared solutions of PEG (Liu et al. 2002). This aging of the cocktails can cause considerable difficulty when attempting to reproduce and optimize results from the screening experiments. If the optimization experiments are set up shortly after the screening experiments, then using exactly the same solutions for screening and optimization can eliminate this aging effect. If the cocktails are not freshly prepared prior to setting up the screening experiments, the DVR/T approach may improve reproducibility by making use of these same ''aged'' screening cocktails for optimization.

The DVR/T method has proven to be extremely effective in optimizing initial hits. While simple to perform and analyze, the optimization protocol is deceptively multiparametric but appropriately so for the task at hand. Carter and Yin (1994) explored Harden-Sloane response-surface designs to optimize crystallization conditions. They found it was advantageous to sample the effects of protein concentration and supersaturation simultaneously. The DVR/T technique implicitly samples supersaturation space. This is an effective way to detect the presence of optimal conditions that lie well outside the range of supersaturation investigated in traditional optimization methods.

In the microbatch-under-oil method, the initial hit is a logical point to start the optimization. Rather than following a complicated path to reach a nucleation point, the initial conditions are well known; they are not dependent on time or geometry. Figure 2 shows a simplified phase diagram for crystallization. The initial crystallization condition is known to be at a metastable state (nucleation was heterogeneous) or a labile state (nucleation was spontaneous and homogeneous). To optimize this condition the supersaturation has to be decreased such that nucleation still occurs while approaching the lower level of supersaturation that is ideal for crystal growth. This promotes a small number of crystal nuclei and prolongs the growth of those into large crystals. The DVR/T experiments sample different volume ratios with a midpoint (equal volume of protein and cocktail solution) mimicking the initial crystallization condition. Depending on the location of this condition on the solubility diagram, the DVR/T path may first sample a local maximum of supersaturation; however, it will also sample points where there is a decrease in the supersaturation. The DVR/T technique should be centered on the temperature of the initial crystallization hit (23°C for the experiments presented in this paper) to ensure that the starting point falls in the labile or metastable zone.

In a DVR/T experiment, a range of pH values will be sampled. The protein and cocktail solutions typically contain different buffers. The $[\text{Buffer}]_{\text{cocktail}} > [\text{Buffer}]_{\text{protein}}$ so that the pH of the cocktail solution dominates the experiment drop. As the ratio of protein to cocktail solutions changes, pH is effectively used as a variable during the screening experiments. Fine screening of pH as a single variable has been successfully used for optimization (McPherson 1995). The range sampled in the DVR/T method will depend in part upon the type and concentration of the buffers used in the cocktail and protein solutions. It will also depend on the volume of

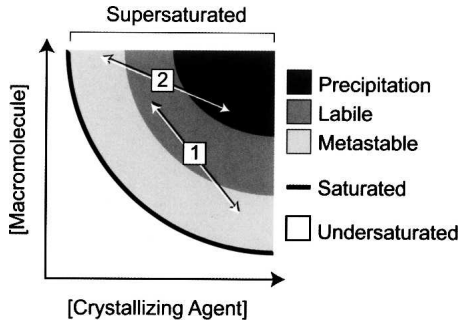

Figure 2. Drop volume ratio effect on supersaturation. Crystals are observed in the screening trials with a 1:1 ratio of protein to cocktail solution (1) or (2). Holding all other variables constant, we assume this experiment falls someplace in the labile zone where spontaneous homogeneous nucleation will occur. Varying the volume ratio of protein to cocktail solution (in a series of batch crystallization experiments) will sample points that lie roughly along a path indicated by the arrows on the graph. Different areas in the solubility diagram where the [protein] is higher and the [precipitating agent] is lower, and where the [precipitating agent] is higher and the [protein] is lower will be sampled.

cocktail and protein solution in the experiment drop. Using P5687 as an example, the cocktail buffer is 100 mM Citrate, pH 4.0 and the protein buffer is 20 mM HEPES, pH 7.6. The cocktail buffer will dominate the pH if the solutions are combined in an equal volume ratio (50 mM Citrate, 10 mM HEPES). However, at the extreme of the volume ratio (23% cocktail, 77% protein) the protein's buffer will significantly affect the final pH of the experiment drop (23 mM Citrate, 15 mM HEPES). This sampling of pH will be a volumetric titration of the $Buffer_{cocktail}$ and $Buffer_{protein}$. The pH of DVR/T experiments will also depend upon the incubation temperature, in particular the temperature coefficient of the buffers. This effect can be significant when experiments are being incubated in a range of temperatures from 4°C to 37°C.

The experiments presented here show that temperature is a very significant variable. Studies have shown that temperature influences protein solubility, affecting both nucleation and crystal growth (Giegé and Mikol 1989). It is an important, and yet often ignored, variable for crystallization that will have some effect on solubility for the majority of samples (Christopher et al. 1998). The effects of temperature are not always obvious. Within a narrow 5°C temperature range three different growth mechanisms were reported for crystals of $tRNA^{PHE}$ (Ng et al. 1997). Calculations predict the existence of a highly concentrated layer of metastable liquid–liquid protein phase separation on the surface of the growing crystal. This layer has an effect on both the kinetics of nucleation and the growth of macromolecular crystals and is critically dependent on temperature (Haas and Drenth 2000). Temperature induction (brief incubation of the crystallization experiment at an elevated temperature from subsequent growth) was required to crystallize GrpE from *Thermus thermophilus* (Kitano et al. 1998). Temperature change has also been used to reduce microheterogeneity (Leinala et al. 2002) and has been shown to cause conformational changes in the structure even after crystals have formed (Dunlop et al. 2005). Samples which do not show a temperature-dependent solubility in one condition may well be sensitive in others. The DVR/T method does not separate specific temperature-related effects but does make use of temperature during optimization. The relationship between temperature and solubility is readily observed in the results, guiding the choice of a preferred crystallization condition.

Crystallization is a complex process and the DVR/T embraces this as an optimization method. As described above variables exploited by the nature of the method include [protein], [precipitating agent], pH, temperature, and even the kinetics of supersaturation. All of these factors influence the outcomes of the crystallization experiments (Fig. 1). These variables are highly correlated and not sampled in fine-enough detail to gain theoretical insight but do provide an expansive empirical overview of a sample's crystallization behavior. Extracting information on the contribution of an individual parameter to the crystallization would require a more sophisticated optimization screen taking into account the correlation between variables and the nonlinearity of response to a change in any individual variable.

Optimization takes place by a qualitative visual assessment of the resulting crystals. X-ray analysis of the resulting crystals is the only method that provides a quantitative evaluation of the optimization. This is a logical next step for the final round of optimization before X-ray structural data collection takes place. However, as it stands the DVR/T method is directly applicable to neutron diffraction studies. Neutrons are uniquely sensitive to protonation state or hydrogen positions, key to many biological mechanisms. Typically, structures derived from X-ray data already exist and crystallization conditions are known. Neutron sources are far less intense than the equivalent synchrotron X-ray source and neutrons are weakly scattered. Therefore, a key step to enabling successful neutron diffraction is maximizing the volume of those crystals (Snell et al. 2006). DVR/T naturally achieves this by providing a fine sampling around the X-ray optimized conditions. As the crystal is already known to diffract, visual results on volume and volume trends from DVR/T can be immediately used to enable neutron studies.

Perhaps the most striking result of the method is that a small change can have a dramatic effect on the result. Conditions that would otherwise not be considered as a starting point for optimization are strikingly close to the conditions required for producing visually perfect single crystals. This offers a potential method to address the 86% of soluble proteins that do not form crystals. Using high-throughput methods, the DVR/T technique could be used as a secondary hit screen. With a minimal amount of sample the top 24 initial hits could be run through the DVR/T method instead of a single initial condition. The technique could also be used to explore an often overlooked problem in initial condition screening. A perfect crystallization condition results in a large single crystal. Nucleation is a stochastic process with inherent variability. In the perfect case this variability can produce a range of zero, one, or several nucleation points that grow into crystals. A perfect crystallization condition may not be identified as such, i.e., a clear drop could result. One could replicate the number of experiments to try and exclude this at the expense of sample needed. However, the DVR/T method provides a visual indication of the crystallization space around a condition. A clear drop surrounded by a sea of drops containing small crystals can be immediately identified and that condition revisited. This does not preclude missing a condition in the initial hit screen and the application of DVR/T to that case is an area of future research.

While DVR/T is a powerful method, a note of caution needs to be sounded. The DVR/T experiments are not ideal, static batch experiments; they will dehydrate over time even

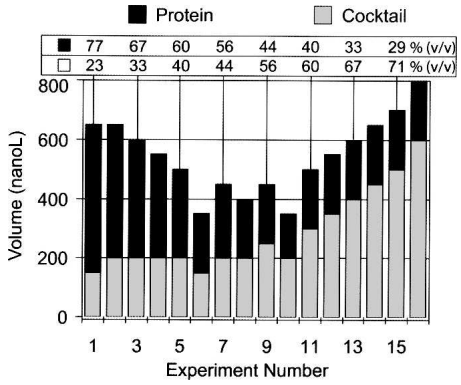

Figure 3. Experiment drop composition. This graph illustrates the volumes of protein and cocktail solutions used to construct the experiment drops. Progressing from experiment 1 through 16, there is a sequential decrease in the volume percent (and correlated concentration) of the protein solution, in the experiment drop ranging from a high of 77% (v/v) to a low of 25% (v/v). The inverse relationship exists for the cocktail solution; its volume percent progressively increases from 23% (v/v) to 75% (v/v). The final drop volume is not held constant, ranging from 350 to 800 nL. This was done to reduce the amount of protein solution required to set up these experiments.

when covered by oil. The rate of dehydration is not constant. Plates incubated at 4°C will dehydrate more slowly than those stored at 37°C. The drop volume will in part determine the impact of this dehydration. At constant temperature, the dehydration will occur at approximately the same rate for drops of different volumes. When the same volume of water evaporates, the relative increase in concentration of nonvolatile components is roughly double for a 350-nL compared to an 800-nL drop. This affects the rate at which the protein solution supersaturates, an influential variable on the outcome of crystallization experiments (Luft and DeTitta 1997). Even in cases where DVR/T may fail to produce crystals of sufficient quality it will provide considerable insight on the effects of temperature and chemistry on the sample's solubility. This knowledge will aid a more detailed approach to optimizing these recalcitrant samples.

DVR/T optimization experiments can be set up in any laboratory that crystallizes biological macromolecules. The method makes use of the initial hit and the biochemical conditions associated with that hit alone. It is highly suited to automation yet robotic techniques are not needed (but they do speed up the process). In the case of the Center for High-Throughput Structural Biology, a significant community of 700 researchers makes use of the high-throughput screening facilities. This laboratory, located at the Hauptman-Woodward Medical Research Institute, screens 200 samples each month to identify crystallization conditions. Data tracking and image packaging software needs to be developed and refined before the DVR/T method can be offered to this community of researchers to optimize crystallization conditions identified during screening. This will add significantly to the automation of the pipeline from macromolecule to structure.

Materials and Methods

The samples used for the crystallization experiments were provided by investigators through the Center for High-Throughput Structural Biology (www.chtsb.org). These were soluble proteins of unknown structure. A high-throughput infrastructure was used to screen for initial crystallization conditions (Luft et al. 2003), thereby identifying suitable cocktails for optimization. Eight proteins that produced results, ranging in quality from needles and highly twinned plates to small single crystals, were randomly selected for the optimization experiments. The protein solutions used for both the screening and optimization are listed (Table 1). None of the samples had been crystallized previously. The screening facility utilizes source/destination plate protocols with the TANGO™ liquid handling system (Matrix Technologies Corp.) to set up microbatch-under-oil crystallization experiments. The experiments were set up in 1536-well experiment plates (Greiner BioOne) using USP-grade mineral oil (Sigma). Upon completion, each experiment plate contained a single-protein solution combined with an equal volume of 1536 different crystallization cocktails under mineral oil; each well held a unique experiment drop (200 nL protein + 200 nL cocktail). An automated imaging system recorded the experiments' outcomes immediately after the addition of the protein

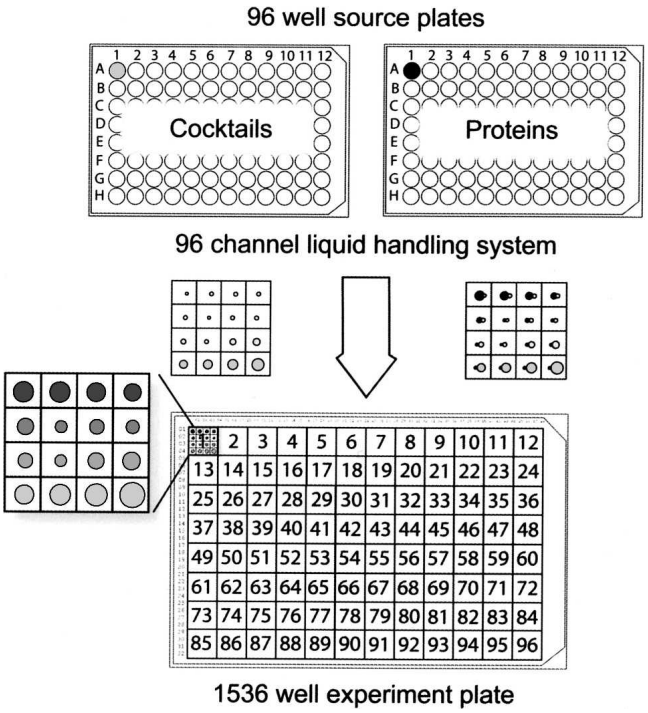

Figure 4. High-throughput DVR/T optimization. An entire source plate holding as many as 96 cocktail solutions is simultaneously aspirated and dispensed into an oil-filled, 1536-well experiment plate using a 96-channel liquid handling system. The same process is used to deliver protein solution to the experiment plate. The 96 syringes in the liquid handling system are arranged in an 8 × 12 array. The syringes are geometrically centered on every fifth well of a 1536-well plate. Each syringe dispenses solution into a 4 × 4 array of 16 adjacent wells in the experiment plate. Four replicate plates are prepared and separately incubated at temperatures of 4°C, 14°C, 23°C, and 37°C. Individual experiment plates hold as many as 96 different proteins and cocktails at 16 different volume ratios each.

solution, and weekly thereafter for one month. Outcomes were reviewed to identify those that have potential in optimization trials.

For the optimization step, microbatch-under-oil crystallization experiments were set up using a PlateMate2x2 liquid handling system equipped with a 96-channel syringe head (Matrix Technologies Corp.). Oil (5 μL) was delivered to each well of a 1536-well microassay plate. Next, two separate 96-well source plates were prepared. The first contained 25 μL aliquots of as many as 96 different protein solutions; the second held aliquots of crystallization cocktails arrayed to match the proteins. The 96 cocktail solutions were simultaneously aspirated from the source plate (once) and dispensed into an oil-filled 1536-well experiment plate (16 times) at the volumes shown (Fig. 3). Each of the 96 syringes dispensed one cocktail solution at volumes ranging from 0.15 to 0.60 μL into a 4 × 4 array in the experiment plate (Fig. 4). This process was repeated to prepare four replicate plates. The protein solutions were delivered in a volume range of 0.15–0.50 μL into the same four experiment plates that contained the cocktails. These deliveries were completed in <1 h. Plates were centrifuged at low speed to merge the cocktail and protein solutions. A single 25-μL aliquot of protein was sufficient to prepare four replicate 16 protein:cocktail drop volume ratio trials. The four plates were separately incubated at temperatures of 4°C, 14°C, 23°C, and 37°C. These values were chosen to sample a range of temperatures nearing the extremes of the range for crystallization (4°C through 37°C); this includes values near commonly (4°C and 23°C) and infrequently (14°C and 37°C) sampled temperatures (Charles et al. 2006). All of the crystallization experiments (screening and optimization) were set up and imaged weekly at 23°C. It required 25 min to image each plate; plates were returned to an incubator immediately after imaging. After setting the screening experiments, protein solutions were stored at −80°C for several months before being used for optimization trials. The same mineral oil, 1536-well plates, protein solutions (Table 1), and cocktail solutions (Table 2) were used in both the screening and optimization trials.

Table 2. *Cocktail solutions used for optimization*

Figure 1	Cocktail solution
A	100 mM Na Acetate pH 5.0, 100 mM NH_4SCN, 20% (w/v) PEG 4,000
B	100 mM MOPS pH 7.0, 100 mM NH_4Br, 80% (v/v) PEG 400
C	100 mM Na Citrate pH 4.0, 100 mM NaCl, 60% (v/v) PEG 400
D	100 mM CAPS pH 10.0, 100 mM K_3PO_4 20% (w/v) PEG 4,000
E	Crystal Screen HT™ E12 (Hampton Research, Aliso Viejo, CA, USA), 100 mM Na Acetate pH 4.6, 100 mM $CdCl_2$, 30% (v/v) PEG 400
F	100 mM Na Citrate pH 4.0, 100 mM $(NH_4)_2HPO_4$, 40% (w/v) PEG 1,000
G	100 mM HEPES pH 7.0, 100 mM $MgCl_2$, 20% (w/v) PEG 4,000
H	Index™ G12 (Hampton Research, Aliso Viejo, CA, USA), 100 mM HEPES pH 7.5, 200 mM $MgCl_2$, 25% (w/v) PEG 3350
I	Crystal Screen HT™ B6 (Hampton Research, Aliso Viejo, CA, USA), 100 mM Na Cacodylate pH 6.5, 200 mM Mg Acetate, 20% (w/v) PEG 8,000

Verification that the crystals were protein was accomplished either by observing color in the crystals (chromophores) or through the addition of Izit dye to the experiment drop (Hampton Research, Aliso Viejo, CA, USA). If the dye was absorbed by the crystals, they were considered to be composed of protein.

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