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Automatic implementation of precise grid screens: the four-corners method

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Crystallization trials can be designed as a systematic gradient of the concentration of key reagents and/or pH centered on the original conditions. While the concept of the grid screen is simple, its implementation is tedious and difficult by hand. A procedure has been developed for preparing crystallization grid screens that is both efficient and achieves high accuracy because it relies on a limited number of solutions that are carefully prepared by hand. The 'four-corners' approach to designing grid screens uses the minimum and maximum concentrations of the components being varied in the grid screen as the sole stock solutions. For an N-dimensional grid only 2^N corner solutions require detailed preparation, making the screens efficient. Furthermore, by keeping the concentrations as tight as possible to the grid, the potential impact of pipette errors is minimized, creating a highly precise screen.

1. Background

The first stage of the *de novo* crystallization of a new protein includes broad screening of crystallization conditions intended to approximately locate promising conditions; typically, this includes systematic grids, incomplete factorial and sparse-matrix approaches. These promising conditions are then refined using crystal optimization trials to increase the size or X-ray diffraction quality of the crystal. These crystal optimization trials are most commonly designed as a grid screen, typically as a systematic gradient of the concentration of key reagents and/or pH centered around the original conditions (McPherson, 1999).

While the concept of the grid screen is simple, its implementation is tedious and difficult by hand. Automation techniques such as crystallization robots have been developed to offload the preparation of crystallization experiments. However, it has been our experience with crystallization robots that they are prone to pipetting errors, especially those where drops are left on the pipette tips. Additionally, as recent studies have demonstrated (Newman *et al.*, 2007), reproducibility is a significant problem in protein crystallization. Minimizing the effects of pipetting errors as described above is certainly one part of addressing this issue.

Here, we report a procedure for preparing crystallization grid screens that is efficient both for robotic instruments and multichannel hand-held pipettors, yet achieves high accuracy because it relies on a limited number of solutions carefully prepared by hand.

2. Methods

To increase the efficiency and accuracy of preparing grid screens, a 'four-corners' approach to designing grid screens has been developed. The corners, *i.e.* the combinations of the minimum and maximum concentrations of the components being varied in the grid screen (see Fig. 1), are prepared as stock solutions. A program (available as a *Microsoft Excel* spreadsheet) computes the percentage of each corner solution required to produce the target concentrations for each well in the screen. N-dimensional grids are supported across multiple trays. For an N-dimensional grid, only 2^N corner solutions require

detailed preparation, making the screens efficient. Furthermore, by keeping the concentrations as tight as possible to the grid, the potential impact of pipette errors is minimized, creating a highly precise screen.

Each dimension represents an independent gradient (*e.g.* PEG 2000 from 19% to 29% and pH from 5.5 to 8.5). The grid screen fully explores the parameter space, with every N-tuple combination being represented. If there are components that are common to all members of the grid screen (*e.g.* 50 mM sodium acetate), they are also included in each of the corner solutions. For an N-dimensional grid screen, the 2^N corner solutions are combined using mixing by proportions. The mixtures are computed as percentages of the corner solutions using

$$\text{fraction of corner } j = \frac{\prod_{i=1}^{n}[(1-b_{ij})(\max_i - c_i) + b_{ij}(c_i - \min_i)]}{\prod_{i=1}^{n}(\max_i - \min_i)}, \quad (1)$$

where b_{ij} is the ith bit of the binary representation of $j-1$, c_i is the desired concentration of the ith component and $\min_i$ and $\max_i$ are the minimum and maximum concentrations of the ith component, respectively.

While pH gradients cannot be directly designed using proportional mixing, appropriate pH-dependent proportions can be calculated *via* the Henderson–Hasselbach equation. The spreadsheet performs this calculation using buffer-specific tables (which can be precalculated given the appropriate pK_a values).

As an example, consider well *B*3 from Fig. 1. The desired solution is 23% PEG 2000, 50 mM sodium acetate, 50 mM phosphate pH 6.5. We use (1) to determine the fraction of each corner required to obtain the desired concentration. There are two variable components of this grid: (i) the PEG 2000 concentration and (ii) the pH. Therefore, $\max_1 = 29$, $\min_1 = 19$ and $c_1 = 23$.

As $\max_2$ and $\min_2$ are for the range of pH values, we use the Henderson–Hasselbach equation to compute the volume of acid or base required to achieve the desired pH in the chosen buffer system. (We use volume of base as it increases with the pH because it simplifies sign issues in the equations.) For a phosphate buffer (pK_a = 7.2), the minimum pH 5.5 ($\min_2$) requires 1.96% base, the maximum pH 8.5 ($\max_2$) requires 95.23% base and the target pH 6.5 (c_2) requires 16.63% base.

Computing the fraction of corner 1 ($j = 1$, $b_{11} = 0$, $b_{21} = 0$) (1) reduces to

$$\frac{[(\max_1 - c_1)(\max_2 - c_2)]}{[(\max_1 - \min_1)(\max_2 - \min_2)]}$$

or

$$\frac{[(29-23)(95.23-16.63)]}{[(29-19)(95.23-1.96)]} = 0.5056.$$

Similarly, corners 2 through 4 reduce to the following:

$$\text{corner } 2_{j=2,b_{12}=1,b_{22}=0}: \frac{[(c_1 - \min_1)(\max_2 - c_2)]/}{[(\max_1 - \min_1)(\max_2 - \min_2)]} = 0.3371,$$

$$\text{corner } 3_{j=3,b_{13}=0,b_{23}=1}: \frac{[(\max_1 - c_1)(c_2 - \min_2)]}{[(\max_1 - \min_1)(\max_2 - \min_2)]} = 0.0944,$$

$$\text{corner } 4_{j=4,b_{14}=1,b_{24}=1}: \frac{[(c_1 - \min_1)(c_2 - \min_2)]}{[(\max_1 - \min_1)(\max_2 - \min_2)]} = 0.0629.$$

Combining the corner solutions in the proportions above produces a 23% PEG 2000, 50 mM sodium acetate solution at pH 6.5.

Two-dimensional grids using these formulae have been implemented in *Excel* in two forms. One produces printable instructions for hand-pipetting for 24-well or 96-well crystallization trays. The other form produces instructions that can be executed by a Tecan Freedom EVO pipetting robot running proprietary *Gemini* 4.2 software.

Follow-on optimization trials are finer gradients as they typically further focus on a smaller range of values. The follow-on corner solutions can also be created from combinations of the original corner solutions using the proportional mixing procedure described above, thereby reducing the effort required.

The software includes an additional parameter to design a user-specified level of redundancy (onefold, twofold or fourfold redundancy) into the screen. Our initial rationale for this parameter was to address issues we have observed with automated pipetting robots designed to transfer screens from deep-well blocks to crystallization experiment trays. It is not uncommon for one or more tips of these multi-channel low-volume transfer pipettors to become clogged.

The redundancy parameter defines the number of times every condition is duplicated on the tray. The tray is divided into equal quadrants (top and bottom for twofold redundancy and four equal-sized quadrants spanning from the corner to the middle of the plate for fourfold redundancy). The set of experiments are rotated and translated as a block between the quadrants. This places identical conditions at different areas of the tray while maintaining the logical and spatial relationships between the conditions within the block.

We have also found the technique useful for roughly estimating the reproducibility of crystallization results. Recent studies have suggested that reproducibility is a significant problem in protein crystallization (Newman *et al.*, 2007). With this technique, we can roughly judge whether a particular result will be easily reproduced by comparing the results of duplicate conditions in the other areas of the tray.

Senger and Mueser have reported a similar technique for creating a simple gradient screen by proportionally mixing two end points (Senger & Mueser, 2005). There are only two end points in their method, even when the concentration of multiple components is varied, effectively limiting the search to one dimension; this limits the breadth of

	1: 19%		2: 21%		3: 23%		4: 25%		5: 27%		6: 29%	
A pH 5.5	100% C1 0% C3	0% C2 0% C4	80% C1 0% C3	20% C2 0% C4	60% C1 0% C3	40% C2 0% C4	40% C1 0% C3	60% C2 0% C4	20% C1 0% C3	80% C2 0% C4	0% C1 0% C3	100% C2 0% C4
B pH 6.5	84.3% C1 15.7% C3	0% C2 0% C4	67.4% C1 12.6% C3	16.9% C2 3.1% C4	50.6% C1 9.4% C3	33.7% C2 6.3% C4	33.7% C1 6.3% C3	50.6% C2 9.4% C4	16.9% C1 3.1% C3	67.4% C2 12.6% C4	0% C1 0% C3	84.3% C2 15.7% C4
C pH 7.5	30.7% C1 69.3% C3	0% C2 0% C4	24.5% C1 55.5% C3	6.1% C2 13.9% C4	18.4% C1 41.6% C3	12.3% C2 27.7% C4	12.3% C1 27.7% C3	18.4% C2 41.6% C4	6.1% C1 13.9% C3	24.5% C2 55.5% C4	0% C1 0% C3	30.7% C2 69.3% C4
D pH 8.5	0% C1 100% C3	0% C2 0% C4	0% C1 80% C3	0% C2 20% C4	0% C1 60% C3	0% C2 40% C4	0% C1 40% C3	0% C2 60% C4	0% C1 20% C3	0% C2 80% C4	0% C1 0% C3	0% C2 100% C4

Screen: 19–29% PEG 2000, 50 m*M* sodium acetate, 50 m*M* phosphate buffer pH 5.5–8.5
C1: 19% PEG 2000, 50 m*M* sodium acetate, 50 m*M* phosphate buffer pH 5.5
C2: 29% PEG 2000, 50 m*M* sodium acetate, 50 m*M* phosphate buffer pH 5.5
C3: 19% PEG 2000, 50 m*M* sodium acetate, 50 m*M* phosphate buffer pH 8.5
C4: 29% PEG 2000, 50 m*M* sodium acetate, 50 m*M* phosphate buffer pH 8.5

Figure 1
Example of four-corners grid mixing. Shown here are mixing proportions for the indicated grid, in a 24-well format for legibility. The proportions for the more common 96-well format are similar.

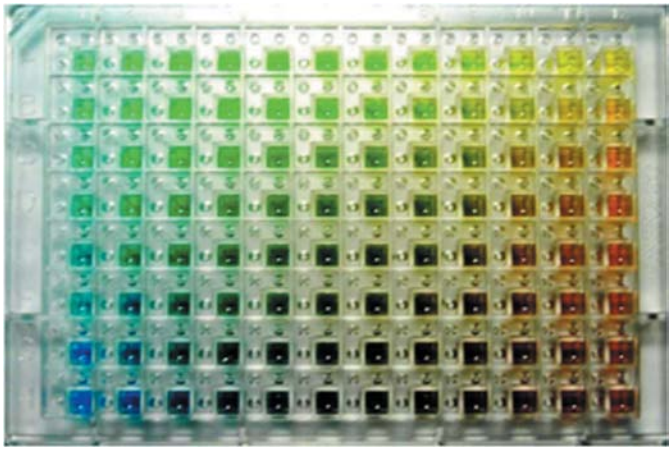

Figure 2
An example gradient using the four-corners mixing calculations implemented using an eight-channel hand-held electronic pipettor. Each of the four corner solutions contained a different color food coloring; both gradients were smooth gradients from 0 to 100% of each of the corners.

the combinations explored. Furthermore, Senger and Mueser do not employ the Henderson–Hasselbach equation to calculate mixing proportions, which makes it very difficult to obtain uniform pH gradients. Our approach overcomes these difficulties; we therefore feel it is more generally applicable.

3. Results and discussion

The four-corners method is designed to provide two primary advantages in preparing grid screens: precision and efficiency. Precision is increased by bounded errors and better starting solutions. By keeping the concentrations as tight as possible to the grid, the potential impact of pipette errors is confined. For instance, assume for the example shown in Fig. 1 that a drop is left on the pipette tip when dispensing solution corner 1 into the well of *B*3 that introduces a pipetting error of ∼10%. Because the concentrations of both of the carefully calibrated corner solutions being mixed are by design relatively close to the desired concentration of the final well solution, the impact of such a change in volume is minimized. As such, using the methods described above, the estimated pipetting error would only change the concentration of PEG 2000 from 21% to 21.14% and the pH from 6.5 to 6.4.

A more careful test was performed using our Tecan pipetting robot to prepare multiple putatively identical solutions followed by measurements of the pH of the individual solutions. They were reproducible to within better than 0.01 pH units. It should be noted that larger systematic errors of as much as 0.2 pH units were observed for some buffer solutions. They were highly reproducible (to within a pH of ±0.01); we suspect they arise from non-ideal behavior of the buffers under the specific conditions tested, leading to apparent errors in the associated pK_a values. These systematic errors are likely to be an issue primarily if it becomes necessary to translate crystallization conditions from one buffer system to another.

The four-corners approach is also more efficient because of the reduced number of pipetting steps and hand-prepared mixtures that are required to create a grid screen. Assuming a 96-well plate and an electronic multi-channel hand-held pipette for each of the three solutions per well (the base solution common to all wells, the solution for the first gradient and the solution for the second gradient), it would take one aspiration and one dispense for each of 96 wells, requiring 3 (solutions) × 2 (aspirate and dispense) × 96 (wells) = 576 steps. Some efficiency could be realised by using the ability of the electronic pipette to perform a single large aspiration with multiple variable-sized dispenses across an entire row or column, reducing the number of pipetting steps to 312.

The four-corners method can be prepared in as few as 80 steps by (i) preparing the corners in large quantities, (ii) distributing the corner solutions to create the outside columns (again in fairly large quantities) and (ii) distributing the outside columns to the inner wells. Each corner would require the three solutions to be aspirated and dispensed [4 (corners) × 3 (solutions) × 2 (operations) = 24 steps]. Again using the electronic pipette's multiple variable-sized dispense capabilities, the first and last columns require one aspiration and seven dispenses of each of the corner solutions [(7 + 1) × 4 (corner solutions) = 32 steps]. Finally, the first and last columns need to be distributed to the other wells [1 (aspiration) + 11 (variable-sized dispenses to other columns) × 2 (first and last column) = 24 steps], leading to a total of 80 steps.

The reduction of 312 pipetting steps to 80 results in an efficiency gain of over 74%. Furthermore, this does not include the efficiency gained from the reduced number of titrations, volume adjustments and measurements that were simplified out of the above estimates. Fig. 2 shows a qualitative illustration of the effectiveness of this procedure.

Our practical experience with this procedure is limited because it is new. We have primarily used the four-corners method for the preparation of broad grid screens for developing response curves for pH and precipitating agent conditions on some very difficult proteins. While the success rate of these crystallizations has been low (not unexpectedly owing to the nature of the proteins being studied), the technique described here significantly reduced the effort required to set up customized grids; it also reduced errors. We have noted an apparent reduction in the rate of failures attributable to pipetting errors, such as drops left on the pipette tips. Furthermore, there has been marked increase in the willingness of those who actually perform the pipetting to set up gradient-based grids because the tedium of doing so is greatly reduced.

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