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On increasing protein-crystallization throughput for X-ray diffraction studies

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Two recent developments, a novel screening/optimization strategy that considerably reduces the number of trials required to produce diffraction-size crystals and a simple modification that doubles the screening capacity of the Douglas Instruments ORYX 1-6 protein-crystallization robot, have been implemented into a structural genomics project. The new two-step screening/optimization strategy yields diffraction-quality crystals directly from the screening process, reducing the need for further optimization. The ORYX modification involves the addition of extensions to the sample- and oil-delivery arms and software modifications that allow two plates to be set up simultaneously.

1. Introduction

A major bottleneck affecting high-throughput crystal screening processes is that the initial screen rarely produces diffraction-quality crystals (Chayen, 2002). Thus, a considerable amount of time, sample and effort is required to first screen and then optimize the crystals in order to bring them to a size and morphology that can be used for X-ray analysis. In addition, when the number of samples to be screened is high, as in case of structural genomics projects, this not only adds to the burden of experimentation but also affects processes upstream (protein production and solution preparation) and downstream (imaging and data management) by consuming protein and supplies as well as generating a substantial amount of failure data which nevertheless need to be documented and archived. Researchers at the Southeast Collaboratory for Structural Genomics (SECSG; Adams *et al.*, 2003) are currently involved in developing high-throughput procedures (see Abola *et al.*, 2000; Lesley *et al.*, 2002; Jhoti, 2001) to increase the throughput of the crystal screening and optimization processes, focusing not only on optimizing hardware but also on developing robust, reproducible and scalable processes that play a major role in influencing the overall throughput. Like other structural genomics efforts (Juarez-Martinez *et al.*, 2002; Kimber *et al.*, 2003; Page *et al.*, 2003; Wooh *et al.*, 2003), SECSG is exploring the optimization of its crystallization screens and strategies to increase their success rate and reduce costs. Analysis of the crystallization data stored in the SECSG's Crystal Monitor (Emerald Biostructures, WA, USA) database yielded the following observations, which can be exploited in increasing the success rate of the crystal screening process.

(i) Every protein has specific pH values at which it crystallizes. These pH values are termed critical pH points (CPP) and are a characteristic of a particular protein and its environment. At a CPP, the entropy of the system is at a minimum and the protein is in a conformation that favors crystallization.

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Crystallization can then be initiated by adding precipitant and/or salt.

(ii) Crystallization success is very sensitive to CPP; a change of as little as ±0.3 pH units around a CPP may fail to yield useful crystals and in many cases no crystals are observed.

(iii) The CPP for a particular protein can be changed by either varying the concentration of precipitant (and/or salt) or by changing the chemical composition of the precipitant (and/or salt). This simplifies screening for CPP, as the entire pH range need not be screened in infinite steps but can be carried out in steps of, for example, 0.5 pH unit, with any pH gaps being covered in principle by the variations in precipitant and/or salt concentration/composition as described above.

In addition to the above points, studies have also shown that proteins have a preference for the precipitants and the salts used for crystallization (see, for example, McPherson, 1989). Based on this analysis, we have designed a screening/optimization strategy (combination screens) that emphasizes the importance of pH and is aimed at producing crystals suitable in size and morphology to be tested for X-ray diffraction without optimization using the fewest possible conditions.

The combination screen requires only 132 trials to produce diffraction-quality crystals and represents a significant improvement over the 360 (288 screening plus 72 optimization) screens, based on commercial preparations, typically used by SECSG and other high-throughput centers. The resulting savings in time, effort and sample afforded by the approach described here could substantially reduce the costs associated with high-throughput structure determinations.

In addition to improvements made to the initial crystal screening process described above, we have designed a simple modification to the Douglas Instruments ORYX 1-6 crystallization robot that almost doubles its screening capacity. The ORYX 1-6 is well suited for carrying out crystallization screening trials using the microbatch-under-oil (Chayen *et al.*, 1990) technique. The ORYX, as factory-configured, uses 72-well HLA plates (labelled *a* in Fig. 1) for the crystallization setups (1 µl drops) and a standard 96-well plate (labelled *b* in Fig. 1) to hold the various screening solutions. In screening mode, a two-bore tip (labelled *c* in Fig. 1) is used to dispense protein and the crystallization solutions simultaneously; the drop is immediately overlaid with 10 µl paraffin oil using the Gilson tip (labelled *d* in Fig. 1) to prevent dehydration of the drop during setup. Once the plate loading is complete, 4 ml of a 70/30 paraffin/silicone oil mixture is layered over the wells to seal the plate. The paraffin/silicone oil mixture allows the drop to slowly dehydrate over time in a manner somewhat similar to a vapor-diffusion setup.

In our applications, the ORYX has proven to be robust and dependable. The 1 µl drop volume allows the production and harvesting of diffraction-size (0.1–0.5 mm) crystals from the initial screening well. However, the 55 min required to set up a plate is slow compared with other commercial systems and represented another bottleneck in the high-throughput crystal screening process. This prompted us to investigate whether modifications to either the robot's hardware or control software could be made to increase the speed of plate setup. An observation that the *XY* stage (labelled *e* in Fig. 1) of the robot holding the HLA and screening-solution plates could accom-

Table 1
Proteins tested using combination screens.

ORF/gene	MW (kDa)	Conc. (m*M*)	Buffer	ORF annotation
Pfu-757388	48.1	1.00	20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 2 m*M* DTT	NDP-sugar dehydrogenase
Pfu-619484	35.6	1.35	20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* DTT	Aspartate carbamoyltransferase catalytic subunit
Pfu-1673561	18.6	0.84	20 m*M* Tris–HCl pH 7.0, 100 m*M* NaCl, 2 m*M* DTT	LSU ribosomal protein L30P
Pfu-715081	21.4	1.23	20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 2 m*M* DTT	NAD(P)H oxidase
Hum-Q9BZB2	11.5	0.51	20 m*M* Tris–MOPS pH 7.5, 100 m*M* KCl, 0.2% sodium cholate, 10% glycerol	Short coiled-coil protein
Pfu-997397	17.2	0.41	20 m*M* Tris–HCl pH 7.4, 300 m*M* NaCl, 2 m*M* DTT	Conserved hypothetical protein
Pfu-1249026	31.7	0.71	20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* DTT	Hydrogenase subunit γ
Pfu-981072	48.4	1.01	20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* DTT, 2 m*M* EDTA	Malate oxidoreductase (malic enzyme)
Pfu-1147304	17.9	0.55	20 m*M* Tris–HCl pH 7.4, 300 m*M* NaCl, 2 m*M* DTT	Conserved hypothetical protein
Pfu-355681	48.3	0.80	20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* DTT	*S*-Adenosylhomocysteinase

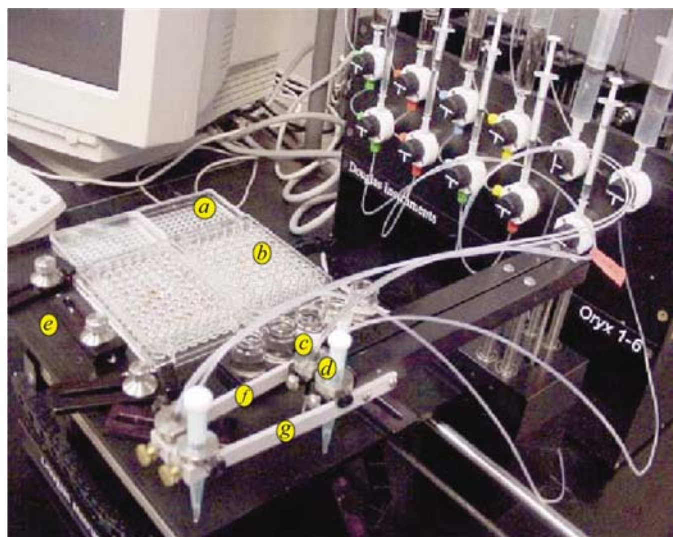

Figure 1
A photograph of the Douglas Instruments ORYX 1-6 crystallization robot, showing the modifications (gray) to its sample-dispensing (*f*) and oil-dispensing (*g*) arms (black). The modifications include extensions to the original sample- and oil-dispensing arms, replacing syringe 5 in the ORYX syringe bank with a second 10 ml oil-dispensing syringe and modifications to the ORYX control program.

Table 2
Combination screens currently being used at SECSG.

pH is controlled using the Hampton Research Stock Option buffer kit.

Screen name	Precipitant	Salt	pH control range
SECSG I	PEG 400	NaCl	3.2–9.4
SECSG II	PEG 1000	NaCl	3.2–9.4
SECSG III	PEG 3000	NaCl	3.2–9.4
SECSG IV	PEG 3350	NaCl	3.2–9.4
SECSG V	PEG 4000	NaCl	3.2–9.4
SECSG VI	PEG 6000	NaCl	3.2–9.4
SECSG VII	PEG 8000	NaCl	3.2–9.4
SECSG VIII	PEG 10 000	NaCl	3.2–9.4
SECSG IX	MPD	NaCl	3.2–9.4
SECSG X	Ammonium sulfate	NaCl	3.2–9.4
SECSG XI	Glycerol	NaCl	3.2–9.4
SECSG XII	2-Propanol	NaCl	3.2–9.4

modate a second set of plates has led to a simple and inexpensive modification that has doubled the throughput of the robot.

2. Experimental

2.1. Sample preparation

All proteins used in the analyses were chosen at random from a set of SECSG *Pyrococcus furiosus* structure-determination targets. The molecular weights for these proteins range from 11 to 48 kDa (see Table 1). Proteins were prepared as follows: the individual ORFs/genes were amplified from genomic DNA using PCR and cloned into a modified pET-24 vector with a six-residue N-terminal His tag. *Escherichia coli* BL21(DE3)Star containing the vector and supplemented with pRIL plasmid was grown in 2YT medium (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl) at 310 K to an OD_{600} of 0.6–0.8. The cultures were then induced with 0.4 m*M* IPTG and incubated overnight at 291 K. Cells were harvested by centrifugation and resuspended in 20 ml 50 m*M* phosphate buffer pH 7.0, 500 m*M* NaCl and 10 m*M* imidazole. Cells were lysed by sonication and the crude extract loaded onto Ni-affinity columns. After elution, the proteins were concentrated, passed through a Superdex 75 gel-filtration column and concentrated to a volume of 500 µl. Quality control was performed using SDS–PAGE, mass spectroscopy and metal analysis.

2.2. Combination screens

12 combination screens (Table 2; also see supplementary material[1]) were designed using *Design Expert* 5 software (Stat-ease Inc, MN, USA) based upon the top 12 precipitants identified from data mining of the Crystal Monitor database using its ‘Query’ tool. Response-surface methodology (RSM; see Carter, 1990) was used to determine the optimum coverage of crystallization space and to determine the actual pH step size. RSM is the method of choice for optimizing various processes as well as individual steps within a process. It is routinely used in a wide variety of fields to improve processes in terms of efficiency, output and variability. Of the various methods available for carrying out RSM, the central composite design (CCD) method is the most popular, primarily because of its simplicity in terms of experiment design, execution and analysis. In addition, CCD reduces the number of experiments so that optimizations can be performed quickly and reliably allows for rapid decision making during the process. For the RSM calculations, three

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference PU5048). Details for accessing these data are given at the back of the journal.

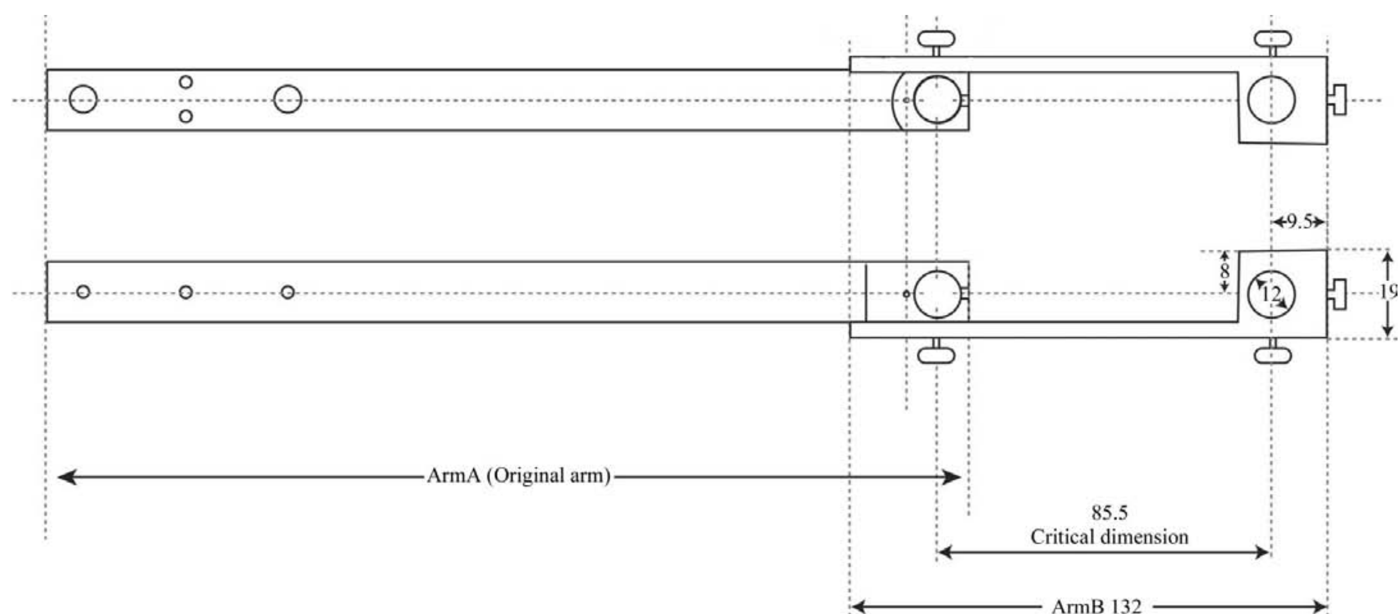

Figure 2
An engineering drawing of the aluminium extensions (ArmB) made for the ORYX 1-6 sample- and oil-dispensing arms (ArmA). The extensions were made in the University of Georgia Machine Shop.

components (precipitant, salt and pH) were varied in each screen so that the spread was optimized, covered adequate crystallization space and was statistically significant.

A single salt additive, NaCl, was selected in order to minimize the number of crystallization conditions in each individual screen. Each screen contained 72 trials (65 unique conditions plus seven repeats for statistical analysis; see supplementary material), consisting of a unique precipitant, sodium chloride and pH control from 3.2–9.4. Screens were prepared using the Hampton Research StockOptions buffer-solutions kit. Conditions for all 12 combination screens can be found in the supplementary material.

In addition, a pre-screen was prepared in order to identify those combination screens that had the highest chance, from the presence of all protein phases in the drop, of crystallizing a particular protein. The pre-screen consisted of the 12 precipitants listed in Table 2 sampled at five (10, 15, 20, 25 and 30%) different concentrations, giving a total of 60 conditions (see supplementary material). Combination screens were then selected based on those conditions from the pre-screen where precipitate was observed at a precipitant concentration of 25% or higher. Thus, the total number of screens needed is 132: 72 for the participant screen and 60 for the combination screen.

2.3. Modifications to the ORYX 1-6

The efficiency of the ORYX 1-6 robot was doubled by the addition of a pair of extensions to the sample-dispensing (labelled *f* in Fig. 1) and oil-dispensing (labelled *g* in Fig. 1*g*) arms that allows the setting up of two screening plates simultaneously. The arm extensions were constructed in a local mechanical shop based on the drawings shown in Fig. 2. The ORYX syringe bank was also modified by replacing the 100 µl Hamilton syringe at position 5 with a 10 ml syringe in order to supply the second oil-dispensing tip. Finally, the necessary modifications were made to the ORYX control program (*WASP*) to allow the simultaneous setup of two plates. The modified control program is available from Douglas Instruments (http://www.douglas.co.uk).

Figure 3
Optimization of Pfu-1862794 using response-surface methodology (RSM) and central composite design (CCD). (*a*) Conditions that produced micro-crystals obtained during coarse screening (288 conditions). (*b*) Crystals produced from the RSM/CCD-predicted conditions based on 13 RSM/CCD experiments. Images were recorded using the DeCode Genetics imaging system supplied with Crystal Monitor.

Table 3
Optimization of Pfu-1862794 using response-surface methodology.

Two factors (monobasic ammonium phosphate and glycerol) were varied to improve crystal size. A central composite design for the experiment was constructed and 13 experiments were performed as suggested by *Design Expert* 5. The outcome of each experiment in terms of crystal size was recorded and used to predict the best condition for growing crystals.

Trial	Monobasic ammonium phosphate (m*M*)	Glycerol [%(*v*/*v*)]	Crystal size (µm)
1	255	20	100
2	400	10	50
3	400	30	66
4	750	6	66
5	750	34	16
6	750	20	75
7	750	20	66
8	750	20	75
9	750	20	75
10	750	20	75
11	1100	10	117
12	1100	30	17
13	1245	20	33

3. Results and discussion

3.1. Combination screens

The following example, screening and optimization of Pfu-1862794 (a putative 27.9 kDa extragenic suppressor protein) from *P. furiosus*, illustrates a common approach in use by other groups and the SECSG prior to the development of the combination screen reported here. The Pfu-1862794 sample was first concentrated in 20 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl by centrifugation (Millipore, 5 kDa cutoff) to produce the 18 mg ml^{-1} solution used in the crystallization trials. A total of 288 conditions from Crystal Screen I, Crystal Screen Cryo, MEMFAC, PEG–Ion screens (Hampton Research) and Wizard I and II screens (Decode Genetics) were screened to find initial hits. Crystals (Fig. 3*a*) were observed using Hampton Research Crystal Screen Cryo condition No. 48 containing 80 m*M* Tris–HCl buffer pH 8.5 containing 1600 m*M* monobasic ammonium phosphate and 20%(*v*/*v*) glycerol. Once crystals are observed, optimization is generally carried out using a 72-condition grid screen (usually pH and precipitant) centered on the conditions that produced the initial crystals. Thus, the screening/optimization process using this approach requires 360 (288 screening plus 72 optimization) conditions.

Another optimization strategy commonly used, which is more efficient, uses RSM to construct a central composite design with, in this example, precipitant and cryoprotectant as variables in order to design a small set of experiments whose results can in turn be used to predict the optimal concentrations of the precipitant and cryoprotectant for crystal growth. In the Pfu-1862794 example, *Design Expert* 5 was used to construct the central composite design based upon the hit observed in the initial screen. 13 experiments (Table 3) were carried out as indicated by *Design Expert.* Based on the results (crystal size) of the 13 experiments, *Design Expert* predicted the following optimal concentrations of the precipitant and cryoprotectant for crystal growth: 1200 m*M* monobasic

ammonium phosphate, 10%(v/v) glycerol. This prediction was validated experimentally as illustrated in Fig. 3(b). Using the RSM/CCD approach, the total number of screening and optimization trials is reduced to 302 (288 screening plus 13 RSM plus one predicted best). This is somewhat better than the 360 conditions used in our original screening strategy, but still requires more than double the number of conditions compared with the combination screens reported here.

The combination screens described here are an attempt to streamline the crystal screening process with the goal of producing useable crystals, *e.g.* crystals that can be screened for their diffraction quality, using as few screening conditions as possible. Using the combination-screen approach, proteins are first pre-screened using a 60-condition precipitant screen to identify which of the 12 combination screens is best suited for the particular sample (protein plus buffer) under study. Once the proper combination screen or screens has been identified, the sample is screened against the 72 conditions that make up the chosen screen. Thus, the combination screen approach requires only 132 conditions (60 pre-screen plus 72 combination screen), which represents a marked improvement over the other screening/optimization approaches described above.

To test the combination-screen approach, a random sample of ten proteins (Table 1) from our structural genomics project were selected as described above. Based on the results of the pre-screen, each protein was screened using the appropriate combination screen. Of the ten proteins selected for the test, eight proteins showed crystals in at least one well that were suitable in size and morphology to be tested for X-ray diffraction (Fig. 4), although one protein, Pfu-1673561, had visible twinning defects. The two proteins that failed to crystallize (Pfu-1249026 and Pfu-981072) using the combination screens had also failed to produce any crystals when screened previously using the standard SECSG 360-condition screen as described above.

The pre-screen is essential to the success of the combination screen. By choosing a precipitant that precipitates a protein at a concentration of 25% or higher, all phases for protein interaction with a precipitant are covered (Mikol & Giegé, 1989; DeMattei & Feigelson, 1991; Stura *et al.*, 1992, 1994; Jancarik *et al.*, 2004). This maximizes the phase space covered and increases the chances of crystallization. The precipitant screen also eliminates the chances of a protein being present only in a single phase, soluble or insoluble, either of which could result in the failure of a trial.

It should also be stressed that any statistical approach to crystallization experiments is only as good as the precision and accuracy of solution preparation (Carter & Yin, 1994). This is especially true when designing experiments using RSM since the screens must in most cases be prepared in-house. Special attention is also required when pipetting highly viscous PEG solutions either manually or using robots, since this is a potential area where the precision and accuracy of the delivered volumes can be compromised, resulting in inconsistent data for the RSM analysis, poor reproducibility and failure to produce usable crystals.

The combination screens reported here were designed to produce 0.1–0.5 mm crystals for diffraction analysis. However, diffraction quality is dependent not only on crystal size and morphology, but also on other factors such as mosaicity and crystal packing, which are beyond the scope of this work. In

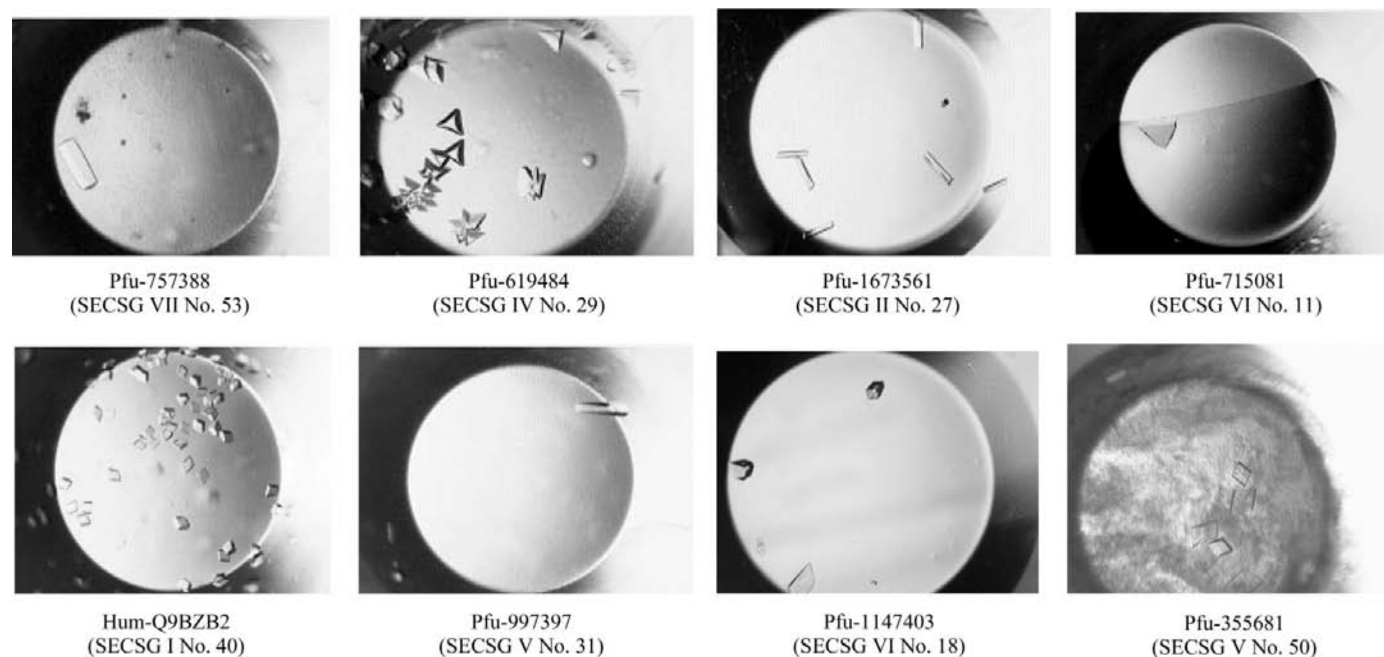

Figure 4
Crystals of the eight proteins produced using the combination screens. The screen used and the condition that gave the best crystals are indicated in parentheses. For comparison, the diameter of the inner well is approximately 1.4 mm. Images were recorded using the DeCode Genetics imaging system supplied with Crystal Monitor.

cases where the diffraction quality of the crystals produced by the combination screen needs improvement, other techniques such as the use of additives and linkers (Cudney *et al.*, 1994), reductive methylation of lysines (Rypniewski *et al.*, 1993), surface mutagenesis (Garrard *et al.*, 2001) and, as in the case of *P. furuosis* proteins, heat treatment can be used.

Eight out of ten proteins tested in the above example produced usable crystals by screening only 132 conditions compared with the 302 conditions required using the RSM/CCD optimization or the 360 screens required for the more traditional screening/optimization method described above. The end result is that less time, effort and protein are needed to produce diffraction-quality crystals. The strength of the combination screens comes from finer pH control and the facts that the crystallization space sampled is statistically more significant and that the pre-screening step reduces the number of conditions to be screened. The power of fine pH sampling is evidenced by the fact that crystals suitable for X-ray diffraction screening were obtained for eight out of ten randomly selected proteins using the 132-condition combination screens; although the sample size is small, its random nature implies that the combination screens should be generally applicable to most proteins.

3.2. Modifications to the ORYX 1-6

The reproducibility and performance of the modified ORYX system was verified by a set of tandem crystallization experiments using two structural genomic proteins, Pfu-81500 and Pfu-1385827, from *P. furiosus*.

Pfu-81500 is a putative alcohol dehydrogenase with a molecular weight of 43.8 kDa. For crystallization trials, the protein was concentrated to 125 m*M* in 20 m*M* Tris–HCl buffer pH 8.0, 200 m*M* NaCl and 2 m*M* DTT (dithiothreitol) using a Millipore centrifugal concentrator (5 kDa cutoff). Commercial sparse-matrix screens (Jancarik & Kim, 1991; McPherson, 1992) were then set up in microbatch mode using the modified ORYX system. Each 1 µl drop, consisting of 0.5 µl protein concentrate and 0.5 µl screening condition, was overlaid with 10 µl paraffin oil immediately after setup in order to prevent dehydration. Once the plate setup was complete, the whole plate was covered with 4 ml of 70/30 paraffin/silicon oil mixture to allow slow evaporation of the drop. The plates were incubated at 291 K and observed twice a week for four weeks.

Both plates showed similar results, producing a number of hits under identical conditions on each plate. The best crystals in terms of morphology and size were observed from the Hampton Memfac screen condition No. 5 (Fig. 5, top), which contained 100 m*M* sodium acetate buffer pH 4.6 and 12%(*v*/*v*) PEG-4000.

Pfu-1385827 is a 16.7 kDa conserved hypothetical protein. For crystallization trials, the protein was concentrated to 0.90 m*M* in 20 m*M* Tris–HCl buffer pH 7.4, 300 m*M* NaCl and 2 m*M* DTT. Crystallization screens were then set up using the modified ORYX system as described previously for Pfu-81500. Both plates again showed similar results, with the best crystals observed for condition No. 26 of the Wizard II (Emerald Biostructures) screen, which contained 100 m*M* CHES–NaOH buffer pH 9.5 and 30%(*v*/*v*) PEG 400 (Fig. 5, bottom). The two test cases show that the modified system performs as anticipated and that the two dispensing units give very similar results. In addition, the total dispensing time required for two plates (50 trials each) has been reduced from 55 min for the ORYX system as factory-configured to 28 min for the modified system described here, almost doubling the throughput.

It is important to point out that the ORYX modification also required the reorientation of the stock-solution plate from its initial portrait (six columns by 12 rows) configuration to a landscape (12 columns by six rows) configuration in order to accommodate the additional plate on the robot's *XY* stage. This change affected steps both upstream (dispensing of solutions into wells on the stock plate) and downstream (data entry and observation recording into the Crystal Monitor database) of the process. Although these adjustments were minor and were offset by the increased throughput, this example illustrates that even simple modification of instrumentation or protocols can affect other system subprocesses. Thus, a careful analysis of the impact of the proposed modification on the entire process should be carried out prior to its implementation. This is especially true for high-throughput applications.

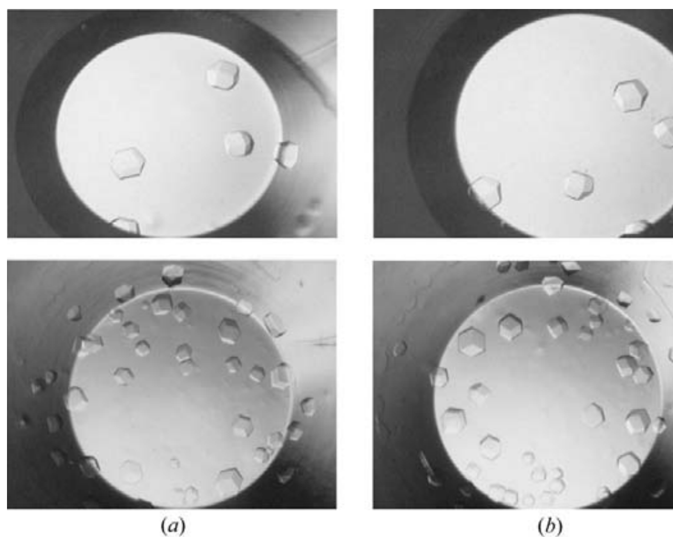

Figure 5
Crystals of Pfu-81500 (top) and Pfu-1385827 (bottom) produced by (*a*) the original arms (ArmA) and (*b*) the modified arms (ArmB) of the ORYX 1-6 robot grown under similar crystallization sonditions. In both cases crystals in the two photographs appear similar in size and shape, indicating that the arm extensions are performing as anticipated. Images were recorded using the DeCode Genetics imaging system supplied with Crystal Monitor.

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