

## An automated microseed matrix-screening method for protein crystallization

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A microseed-matrix procedure has been established with the aim of influencing the nucleation event in standard crystallization screens. The method is based on the original description of matrix seeding described by Ireton & Stoddard (2004, *Acta Cryst. D* **60**, 601–605). Seed stocks are produced using a simple 'seed-bead' method. The protein, reservoir solutions and seed stocks are pipetted simultaneously using a three-bore dispensing tip in drops of 0.6 µl total volume. The number and type of hits produced with the proteins tested in this study has been increased and it is believed that this method could be generally applicable to proteins where little or no nucleation is normally observed.

### 1. Introduction

Obtaining crystals in the first screening process can often prove to be a very frustrating process. It is not uncommon that proteins which can be readily purified and concentrated to very high levels produce no or only a few hit conditions in initial screens. We have observed with such proteins that many drops in the screens remain clear: this may be because sufficient levels of supersaturation cannot be reached for spontaneous nucleation to occur. If seeds are introduced into a crystallization drop, the level of supersaturation required for nucleation and subsequent crystal growth is much lower. Seeding has now become a well established strategy during the optimization of crystallization conditions. An excellent review has been published by Bergfors (2003) which describes most of the commonly used methods of seeding and cites the most pertinent references in the field. Ireton & Stoddard (2004) described what they refer to as 'microseed matrix screening', a method where poorly diffracting crystals were used to seed into similar but non-identical conditions, resulting in a much improved crystal form with a 10% reduction in the unit cell. The resulting structure showed that the altered packing was a consequence of the noncovalent cross-linking of three independent subunits through the coordination of a single bound calcium ion; these crystals could not be reproduced without seeding. The authors also observed similar effects with other nonrelated proteins in the presence of various metal ions.

The aim of the present study was to establish a protocol for introducing seeds as part of the screening process in cases where spontaneous nucleation was low or the crystal morphology was poor. This would allow us to evaluate the effect on the number of screening hits or the improvement in crystal morphology. In order to simplify the procedure, we have incorporated the addition of seeds into the screening procedure using a standard crystallization robot.

### 2. Materials and methods

The proteins that were selected for these studies were all proteases associated with ongoing in-house structure-based drug-discovery programmes: matrix metalloprotease 12 (MMP12), a ubiquitin-specific processing protease (USP7), a bifunctional viral protein (BVP), porcine pancreatic elastase (PPE) and bovine trypsin.

## 2.1. Protein preparation and purification

MMP12 was purified essentially as described by Bertini *et al.* (2005); prior to crystallization, the protein was concentrated to 22 mg ml<sup>-1</sup> in 10 mM Tris pH 7.2, 10 mM calcium chloride, 0.1 mM zinc acetate and 100 mM acetohydroxamic acid. USP7 was purified as described by Hu *et al.* (2002); the protein in 10 mM Tris pH 8.0, 200 mM sodium chloride, 5 mM 1,4-dithiothreitol and 5% glycerol was concentrated to 25 mg ml<sup>-1</sup> for crystallization. BVP was purified using nickel chelate and size-exclusion chromatography. The protein was concentrated to 7 mg ml<sup>-1</sup> in 50 mM Tris pH 7.5, 10% glycerol, 1 M sodium chloride and 5 mM 1,4-dithiothreitol.

PPE was obtained from Roche Diagnostics (catalogue No. 1 027 905) and the powder was resuspended in distilled water to a final concentration of 50 mg ml<sup>-1</sup>. Trypsin from bovine pancreas was purchased from Sigma–Aldrich (catalogue No. T8003) and was solubilized in distilled water to give a final concentration of 90 mg ml<sup>-1</sup>. All proteins were stored as 50 µl aliquots at 193 K.

## 2.2. Crystallization

The seed preparations were made using the 'seed-bead' kit from Hampton Research, as described by Luft & DeTitta (1999). Briefly, crystals obtained either from initial screens or using published conditions were placed in 50 µl of their respective reservoir solution and mechanically homogenized on a standard laboratory vortex for 3 min at full speed. These seeds were stored as 50 µl aliquots and frozen at 193 K. Dilutions of the seed stocks (between tenfold and 500-fold) were also made with the respective reservoir solutions and stored in the same manner.

Vapour-diffusion crystallization experiments and automated seeding were performed using an Oryx-8 crystallization robot (Douglas Instruments). A 'matrix-seeding script' was written by Douglas Instruments for the Oryx-8 robot. This allowed the simultaneous dispensing of protein, reservoir solutions and seeding stocks.

In control experiments (no seeds added), 0.3 µl screening solution was added to 0.3 µl protein solution in 96-well Intelliplates (Robbins Instruments); the reservoir wells contained 90 µl of the screen solution. For screens where seeding was performed, 0.2 µl screening solution and 0.1 µl microseeds were added to 0.3 µl protein solution using the same system as described above. The original (concentrated) seed stocks were used for preliminary experiments and in some cases were diluted for subsequent experiments based on the results of initial seeded screens. The screening solutions used for the experiments were either Index from Hampton Research or the PEGs Suite from Qiagen.

The plates were sealed with clear plastic tape (Hampton Research) and incubated at 296 K. Images of all drops were collected after a period of four weeks using a Crystal Score imaging system (Diversified Scientific Inc.). In order to discriminate between protein and salt crystals, images were also collected on the Crystal Score system using a DUVI 204 UV-light source (PLS-Design GmbH) with transmission filters at 260–395 or 270–320 nm. Protein crystals will fluoresce when excited with UV light because of the presence of tyrosine and tryptophan residues and the high protein concentration

**Table 1**

Number of hit conditions with no seeds added and with seeds (concentrated seed stocks) using either Index Screen or Nextal PEG Suite.

Protein	No. of hits from 96 conditions without seeding	No. of hits from 96 conditions with seeding
MMP12	2 (Index)	33 (Index)
BVP	1 (Nextal)	63 (Nextal)
USP7	4 (Index)	23 (Index)
Trypsin	9 (Index)	22 (Index)
PPE	7 (Index)	21 (Index)

within the crystal. This is not the case with salt crystals and the method therefore allows us to eliminate false positives. The crystallization plates are illuminated on the XY stage of the Crystal Score imager using a fibre-optic light source. A more detailed description of this technique can be found at [http://www.hamptonresearch.com/stuff/ppt\\_files/RAMC2005T5.ppt#1](http://www.hamptonresearch.com/stuff/ppt_files/RAMC2005T5.ppt#1).

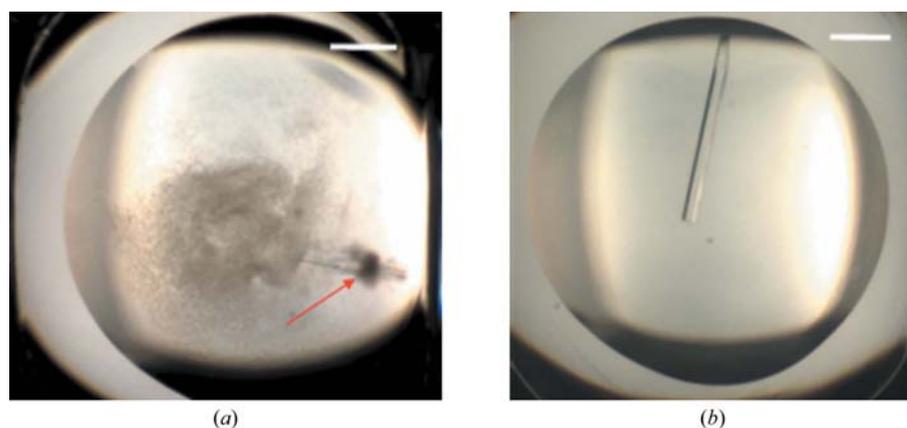
## 3. Results

### 3.1. MMP12

MMP12 was initially crystallized as described by Bertini *et al.* (2005), with the protein in complex with 100 mM acetohydroxamic acid and using 30% PEG 6000, 100 mM Tris pH 8.0 as the reservoir. Very few crystals were obtained under these conditions and we postulated that inducing nucleation by introducing seeds might have an influence in this case. When the protein was screened using the Index Screen, only two hits were observed. A seed stock was made using a single crystal grown under the conditions described above; using this concentrated seed stock, the number of conditions producing crystals increased considerably to 32 (Table 1 and Figs. 5a and 5b).

### 3.2. BVP

When screening for initial crystallization conditions with BVP, a single crystal was observed in one Nextal PEG Suite condition containing 25% PEG monomethyl ether 2000, 100 mM MES pH 6.5 (Fig. 1a). The crystal was of sufficient size to be used for streak-seeding (Stura & Wilson, 1991) into an identical condition in multiple drops. These experiments produced enough crystals to make a seed-



**Figure 1**  
BVP crystals. (a) Original screen hit without seeding, grown in 25% PEG monomethyl ether 2000, 100 mM MES pH 6.5. (b) Best crystals in an identical screen after seeding, grown in 20% PEG 3350, 200 mM trisodium citrate dihydrate using a 200-fold diluted seed stock. The solid bar represents 200 µm.

bead stock, which was used in subsequent experiments in the Nextal PEG Suite. The number of hits obtained in this screen increased from one without seeds to 65 when seeds were added (Table 1 and Figs. 5c and 5d). When the seed stock was diluted 200-fold, we observed a corresponding reduction in the number of crystals and, in certain conditions, an improvement in crystal morphology (Fig. 1b). These crystals could subsequently be reproduced in multiple drops using the

same seed concentration and crystallization condition (20% PEG 3350, 200 mM trisodium citrate dihydrate).

### 3.3. USP7

USP7 often produced crystals with twinned morphology under the published conditions (Hu *et al.*, 2002) using 20% PEG 1000, 100 mM Tris pH 7.0. Crystals grown under very similar conditions (30% PEG 3350, 100 mM HEPES pH 7.0) were taken to produce a seed-bead stock, which was used for the seeded Index Screen (Fig. 2a). The seeded screens again produced an increase in the number of hits observed, from four with seeds to 23 when seeds were added (Table 1 and Figs. 5e and 5f). A second screen with USP7 using a seed stock diluted 500-fold produced fewer crystals per drop and a number of crystals showed much improved morphology (Fig. 2b).

### 3.4. Bovine trypsin

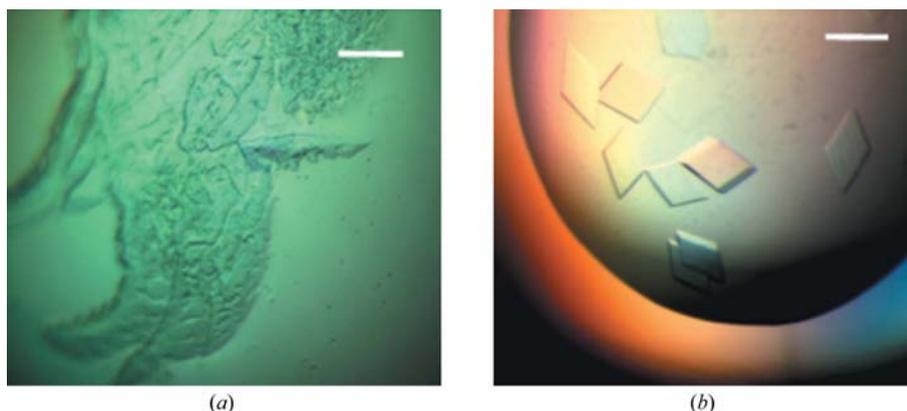
When complexed with an inhibitor, bovine trypsin crystallizes more readily than the native protein. An inhibited trypsin was therefore used to generate a sufficient number of crystals to produce seed stocks to be used for the crystallization of the apo protein to establish whether the number of hits could be increased. The seed crystals were grown in 25% PEG 3350, 200 mM lithium sulfate, 100 mM HEPES pH 7.5. The non-inhibited trypsin produced crystals in only eight out of 96 conditions without seeds in the Index Screen, whereas 22 hits were observed in the seeded screen (Table 1 and Figs. 5g and 5h)

### 3.5. PPE

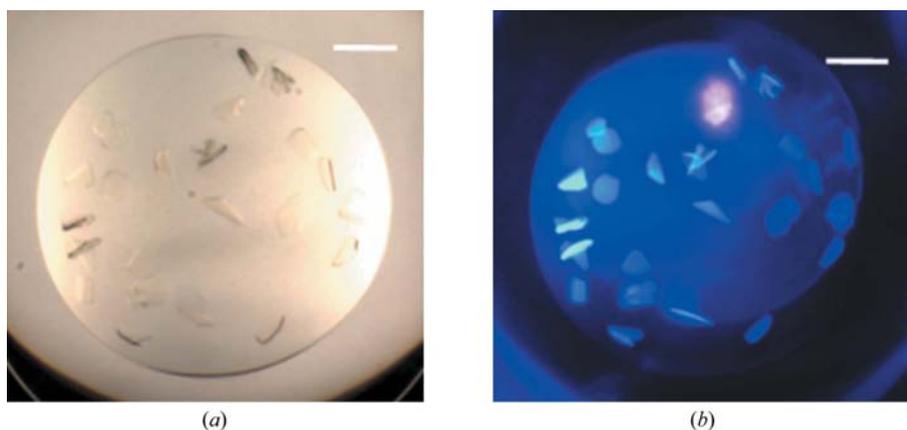
PPE crystallizes readily in standard screens and was selected to determine whether a greater number of or different hit conditions could be obtained. Crystals which grew in 20% PEG monomethyl ether 5000, 100 mM bis-Tris pH 6.5 from the initial screen were used to produce a concentrated seed stock. The non-seeded Index Screen produced seven hit conditions, all of which were in solutions containing PEG 3350 with various salts over the pH range 3.5–8.5. The seeded screen produced 21 conditions, four of which did not contain any PEG (Table 1 and Figs. 5i and 5j).

### 3.6. Summary of screening results

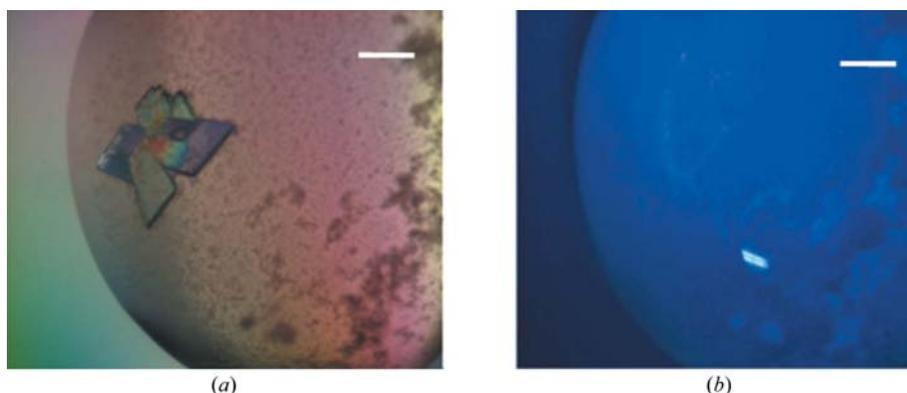
Images from all drops on screening plates were examined using both visible and ultraviolet light; hits were confirmed as protein crystals if the fluorescence appeared higher than the background. An example of protein crystals analysed under visible light is shown in Fig. 3(a) and the same drop illuminated with UV light is shown in



**Figure 2**  
Comparison of the USP7 crystals used for seeds grown in 30% PEG 3350, 100 mM HEPES pH 7.0 (a) and crystals after seeding in 20% PEG 3350, 200 mM magnesium hexahydrate (b). The solid bar represents 200  $\mu$ m.



**Figure 3**  
Example of the same protein crystals illuminated with visible light (a) and UV light (b) to confirm protein crystal hits. The solid bar represents 200  $\mu$ m.



**Figure 4**  
Example of salt crystals illuminated with visible (a) and UV light (b). The solid bar represents 200  $\mu$ m.

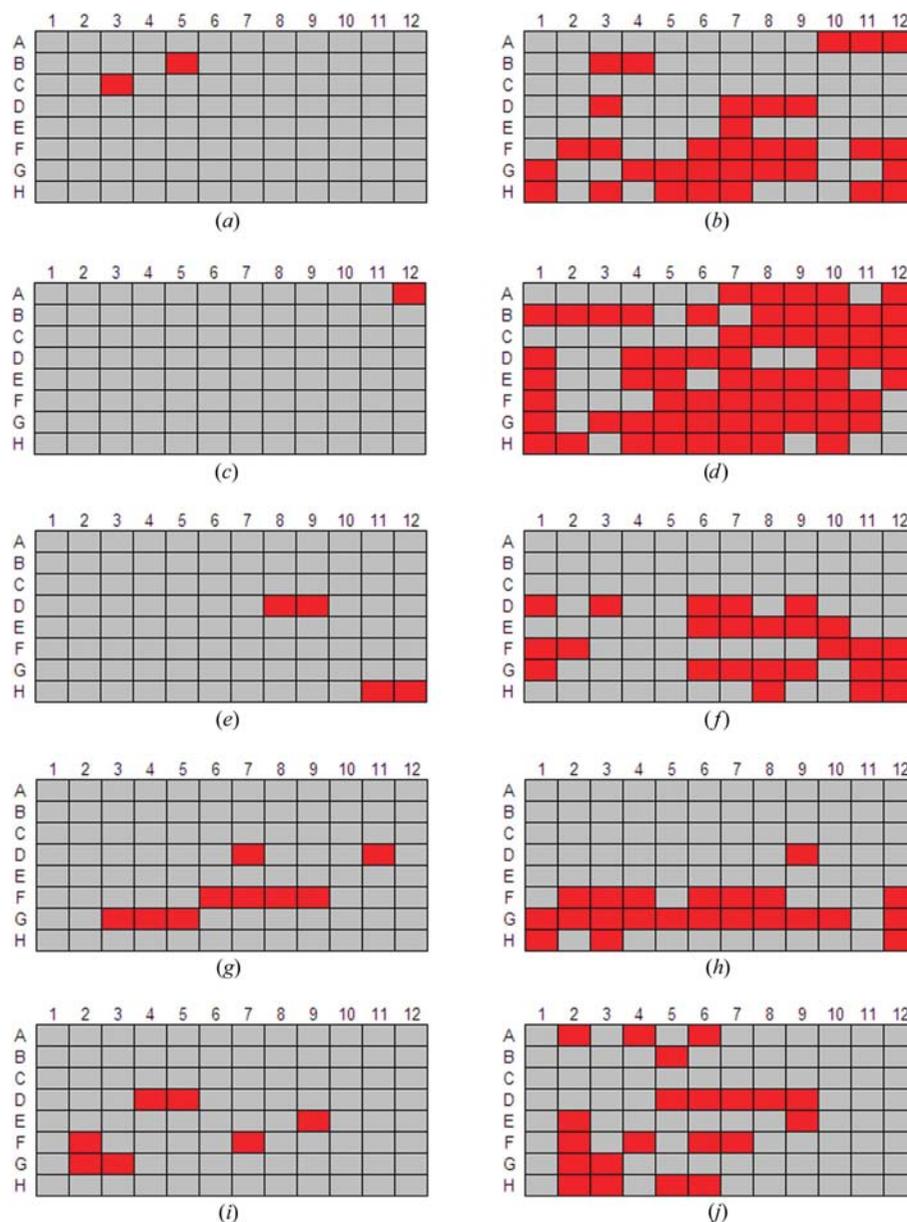
Fig. 3(b) (the pink object in this picture is an artefact generated by reflection of the UV light by the plastic of the crystallization tray). The lack of fluorescence for a salt crystal is illustrated in Fig. 4. The total number of hits with and without seeding for each protein is summarized in Table 1; concentrated seed stocks were used for all the seeding experiments shown in this table.

In order to illustrate the number and type of hits obtained, an overview with and without seeds for the five different proteins is given in Figs. 5(a)–5(j). Each block represents the 96 wells on the crystallization plate (using Index Screen or PEGs Suite); wells coloured in grey indicate conditions which produced no crystals and red for drops where crystals were observed and confirmed as protein.

#### 4. Conclusions

We have described a simple method to perform matrix microseeding in which crystals grown in one set of conditions are seeded into a secondary screen of 96 crystallization solutions (Index Screen or PEGs Suite). The seed stocks can be stored at 193 K and can survive many cycles of freezing and thawing without a decrease in the nucleation effect observed. This technique has great potential for improving hit rates in early stages of screening for crystallization conditions. The success of this method will allow the selection of more starting points for optimization. In all cases tested an improvement in the number of hits was observed, which varied from a 2.7-fold to a 65-fold increase. In some cases, the nucleation could be controlled and the crystal morphology considerably improved by simply using a more diluted seed stock. This was the case for MMP12, where conditions containing 25% PEG 3350, 200 mM NaCl and 100 mM Tris pH 8.5 produced the most promising crystals from the seeded screen. These conditions could be repeated in six identical drops with the seed stock diluted 50-fold. Fewer but larger crystals were obtained in these drops. In the case of BVP a 1:200 dilution of the seed stock also produced the best crystals. Interestingly, with some proteins crystals could be obtained after matrix seeding in conditions that differed from those where the initial crystals were chosen for seed stocks. For example, PPE seeds were isolated from 20% PEG monomethyl ether 5000, 100 mM bis-Tris pH 6.5, but subsequently generated crystals in drops containing 2 M ammonium sulfate or sodium/potassium phosphate. Similarly, MMP12 seeds produced from 30% PEG 6000, 100 mM Tris pH 8.0 induced crystals in 3.0 M sodium chloride pH 6.5–8.5.

This could prove to be important when choosing the conditions best suited for inhibitor-soaking studies, where the type of stabilizing solution or ionic strength could influence the solubility or binding of the small molecule in the crystal. Further crystallographic analysis



**Figure 5**

Comparison of hits in seeded and nonseeded crystallization screens. Grey, no crystals; red, confirmed protein crystals. (a) MMP12, no seeds, (b) MMP12, with seeds; (c) BVP, no seeds, (d) BVP, with seeds; (e) USP7, no seeds, (f) USP7, with seeds; (g) apo trypsin, no seeds, (h) apo trypsin, with seeds; (i) PPE, no seeds, (j) PPE, with seeds. Nextal PEG Suite was used for BVP; Index Screen was used in all other cases.

will be needed to establish whether polymorphs can be generated with improved diffraction properties. The hit rates could potentially be improved even further if specific secondary screens are designed using a single precipitating agent with a wide range of metal ions, as was described in the original paper by Ireton and Stoddard. Our present study illustrates the importance of the nucleation event during the initial screening process and demonstrates the potential of the method to improve output rather than throughput in the crystallization process.

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