CRYSTAL GROWTH & DESIGN

ARTICLE

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Combining Counter-Diffusion and Microseeding to Increase the Success Rate in Protein Crystallization

Published as part of the Crystal Growth & Design virtual special issue on the 13th International Conference on the Crystallization of Biological Macromolecules (ICCBM13).

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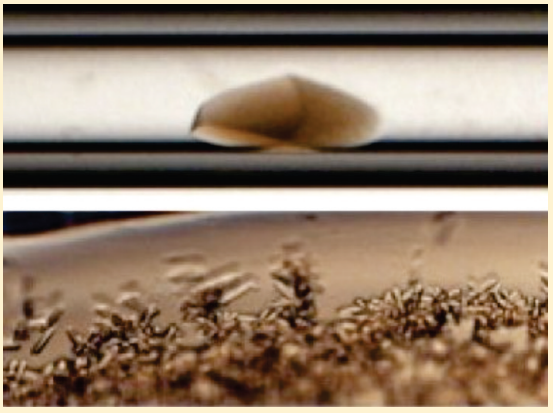
Supporting Information

ABSTRACT: A new method of increasing the success rate in protein crystallization screening experiments by combining microseeding with counter-diffusion crystallization in capillaries (SCD) is presented. We have investigated the number of crystallization hits obtained with and without microseeding with 10 model proteins. For the cases studied, SCD generally increases the number of hits and is particularly useful when only relatively low protein concentration stocks are available, either because the stocks were prepared for, e.g., vapor diffusion experiments, or because the protein is poorly soluble. In either case, the addition of seeds becomes necessary to overcome the nucleation energy barrier so that crystal growth can take place even when the wave of supersaturation that passes along the capillary is insufficient to promote nucleation.



1. INTRODUCTION

In the past, several methods have been developed to improve the quality and size of protein crystals, with the aim of determining their three-dimensional structures. Among these, microseeding[1−4] and counter-diffusion (ref 5 and references therein) have been shown to work independently in a very efficient manner, although with two very different approaches. On the one hand, the seeding technique is based on the decoupling of nucleation and growth[6−8] (the introduction of seeds eliminates the nucleation energetic barrier), and therefore, the system has to be in the metastable region of the phase diagram prior to the introduction of the seeds. This avoids undesirable nucleation events while promoting crystal growth. On the other hand, counter-diffusion experiments self-screen for the best crystallization conditions in a convective-free environment by visiting a wide area of the phase diagram in the course of an individual experiment.[5,9] The counter-diffusion technique is a very convenient method of achieving near-ideal conditions for crystal optimization. As the precipitant concentration slowly rises along the capillary, a few nuclei are formed. The crystals formed begin to grow, reducing the concentration of protein around them, so that further nucleation is prevented in that region. At the same time, the slow increase in precipitant causes crystals to grow slowly and to be well-ordered.

The proteomic era has boosted the automation and simplification of crystallization techniques with the aim of increasing the success rate while minimizing the amount of sample required. This array of developments has also brought seeding and counter-diffusion to a new level of utility. Screening crystallization conditions with the capillary counter-diffusion technique is now feasible for high-throughput laboratories by using capillaries of 0.1 mm inner diameter and 30 mm length, in combination with reduced crystallization kits comprising only 24 conditions. In spite of its advantages, there is, however, a reason why nucleation difficulties may limit the use of the counter-diffusion method, namely, that protein stocks are often prepared for vapor diffusion, where lower concentrations are generally used.

Microseeding has been popular for many years as an optimization technique. The microseeding technique has also been combined with crystal screening experiments in the matrix microseed

Received: October 15, 2010
Revised: March 24, 2011
Published: April 01, 2011

Table 1. Proteins Used in This Study[a]

protein	supplier (part code)	protein buffer	SC	HC
catalase	Sigma Aldrich (C-3155)	30 mM sodium phosphate pH 6.1	7 mg/mL	40 mg/mL
concanavalin A	Sigma Aldrich (L-7647)	50 mM sodium acetate pH 7.0	5 mg/mL	15 mg/mL
glucose isomerase	Hampton Research (HR7-102)	0.1 M Hepes pH 7.0	10 mg/mL	30 mg/mL
hemoglobin	Sigma Aldrich (H-2500)	50 mM sodium acetate pH 7.0	60 mg/mL	90 mg/mL
α-lactalbumin	Sigma Aldrich (L-6010)	Distilled water	5 mg/mL	30 mg/mL
myoglobin	Sigma Aldrich (M-1882)	50 mM sodium phosphate pH 5.3	5 mg/mL	80 mg/mL
ribonuclease-A	Sigma Aldrich (H-2500)	0.1 M sodium acetate pH 6.1	50 mg/mL	240 mg/mL
thaumatin	Sigma Aldrich (T-7638)	0.1 M sodium phosphate pH 7.0	10 mg/mL	90 mg/mL
trypsin	Sigma Aldrich (T-7418)	2% (*w*/*v*) benzamidine	30 mg/mL	90 mg/mL
xylanase	Macro Crystal Oy	0.2 M sodium phosphate pH 7.0, 43% glycerol	36 mg/mL	56 mg/mL

[a] For each protein, the standard protein concentration (SC) was identified as the concentration that was appropriate for vapor diffusion experiments. At this concentration, roughly half the wells contained precipitate, and half were clear in standard sitting drop experiments. The high protein concentration (HC) was the highest protein concentration that could easily be obtained.

screening (MMS) technique[10,11] and automated using different hardware to simplify the liquid handling.[12−15] The automation of microseeding has helped to find new crystallization conditions, producing crystals of higher quality (i.e., the protein crystals listed in the references above). Since crystal improvement could be biased by the chemical cocktail present in the mother-liquor,[16] Villaseñor and co-workers developed a method to deliver nanoliter amounts of seed stock into protein drops using acoustic waves and demonstrated that the seeds are, by and large, more effective in increasing the number of hits than the chemical bias introduced by the mother-liquor.[17]

We have applied new microseeding methodology to 10 commercial model proteins using a minimal screen (24 conditions) in capillaries of 0.2 mm inner diameter. On the basis of the successful results obtained, we propose the use of microseeding in combination with counter-diffusion to boost nucleation because it yields crystals in conditions where they would not normally occur and is especially helpful when high-concentration protein stocks are not available.

2. MATERIALS AND METHODS

2.1. Preparation and Use of Seed Stocks. Seed crystals were grown by the vapor diffusion, batch or counter-diffusion methods. Full descriptions of the methods used to obtain the initial seed can be found in the Supporting Information, section 1. A protein stock containing seed crystals was prepared by thoroughly crushing seed crystals in the well of a vapor diffusion experiment. A microscope was used to ensure that all large crystals were broken up. Four microliters of protein stock was added to the well, and the resulting mixture was transferred to a test tube containing an additional 40 μL of protein and a Seed Bead (Hampton Research), and vortexed for 2 min. For seeds stocks prepared from counter-diffusion experiments, a 4 μL drop of protein solution was placed on a glass plate, and crystals were pushed out of the capillary with a fine wire, crushed in the solution, and transferred and vortexed as described above. This preseeded protein stock was loaded into capillaries in the normal way.

The seed stock in which to dip the capillaries was prepared by thoroughly crushing crystals in the wells in which they had grown with a glass probe or by extracting crystals from the capillary into a 4 μL drop of the mother-liquor. The capillaries were briefly dipped in this suspension so that the solution moved up the capillary about 1 mm. Immediately after this, standard protein (with no seed crystals) was loaded.

2.2. Seeding Counter-Diffusion (SCD) Setup. All experiments were carried out in the GCB boxes of the CSK-24 screening kit (Triana Science & Technology), with capillaries of 0.2 mm inner diameter (CP-02-100). Following the manufacturer's instructions, protein was loaded into the capillaries by capillary action, and capillaries were plugged at one end with putty and pressed through the agarose layer at the top of each box so that the open end of the capillary was in the screening solution (the open end is plugged with agarose in the process).

Each protein was set up in one GCB and four capillaries as follows: (1) standard protein concentration was used with no seeding; (2) high protein concentration was used with no seeding; (3) standard protein concentration was preseeded with crushed crystals (Mix protocol); and (4) the fourth capillary was dipped in crushed crystals before loading standard protein concentration (Touch protocol) (see Table 1). Any crystallization cocktail that produced single crystals or crystalline material, which could be further improved, was considered a hit. All experiments were incubated at 20 °C and the development followed for 6 weeks.

3. RESULTS AND DISCUSSION

We have selected 10 commercial model proteins to study the effect of seeding in capillary counter-diffusion screening experiments using a minimal set of 24 conditions (GCB-CSK-24, Triana Science and Technology).

A summary of the preliminary work required for the production of seed crystals, which were obtained in a variety of qualities (well-shaped crystals, microcrystalline material, and spherulites) and by a variety of techniques (batch, vapor diffusion, counter-diffusion, and previous seeding experiments) can be found in Supporting Information. Four capillaries were set up for each protein as described above. The results of the seeding experiments (capillaries 3 and 4) of each protein have been compared to capillary 1, which gives us an estimate of the maximum number of possible hits, and to capillary 2, which is recommended to fully exploit the benefits of the counter-diffusion technique.

3.1. Concanavalin A. Concanavalin A seed crystals were obtained by the sitting drop vapor diffusion method. As expected, the use of the standard protein concentration solution (5 mg/mL) in the crystallization screening experiments produced fewer hits (10) than the high protein concentration solution (15 mg/mL), which almost doubled the number of hits (19). The use of seeds increased the number of hits to 18 (Mix protocol) and to 23 (Touch protocol). The Touch protocol was so effective that it produced three times more hits than the more concentrated solution without seeds.

3.2. Catalase. Catalase seed crystals (abundant, elongated and small) were obtained by the sitting drop technique using the

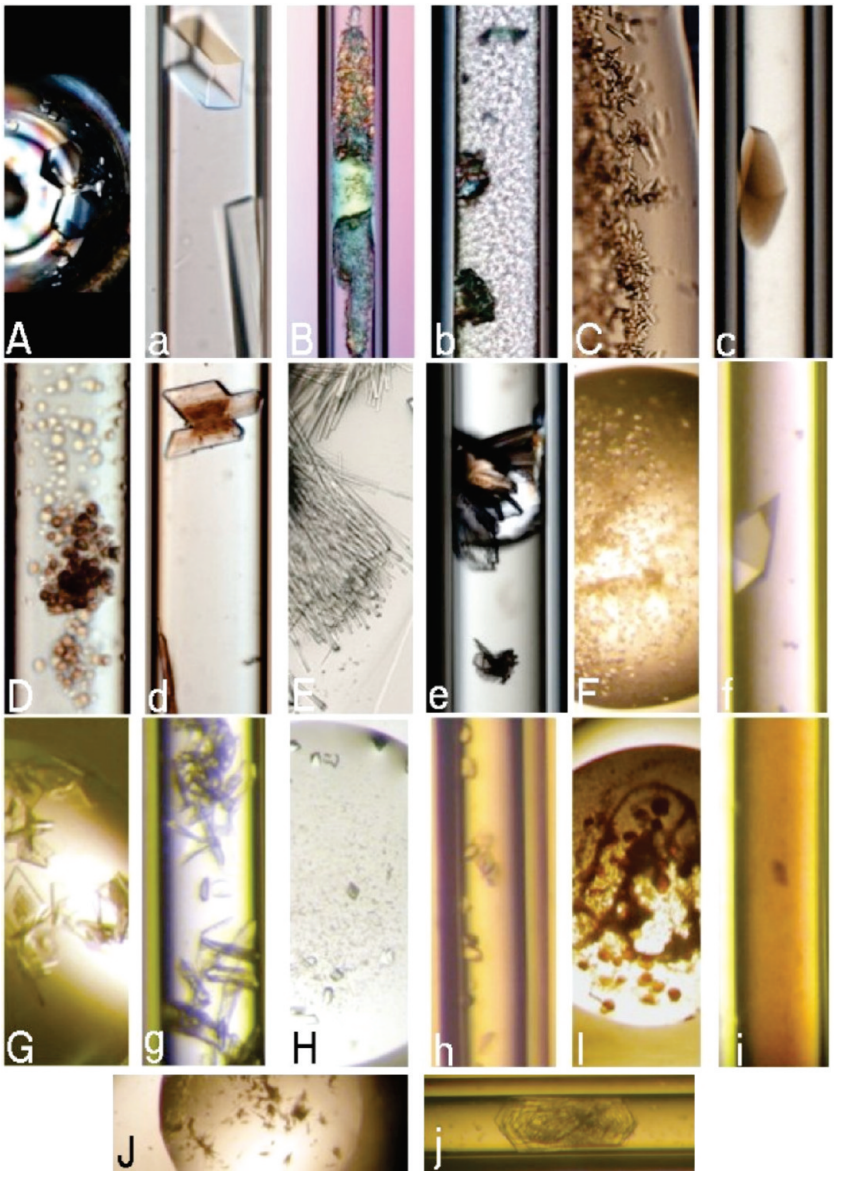


Figure 1. Microphotographs of protein crystals. Capital letters indicate seed crystals, while lowercase letters reflect crystals grown in seeding experiments. (A) glucose isomerase seed crystals and (a) glucose isomerase crystals after seeding at 10 mg/mL in condition C2; (B) ribonuclease-A crystalline material and (b) ribonuclease-A crystallized at 50 mg/mL in condition C21; (C) catalase needle crystal and (c) catalase crystal at 7 mg/mL in condition C8; (D) myoglobin crystalline aggregate and (d) myoglobin crystallized at 5 mg/mL in condition C1; (E) α-lactalbumin thin needles and (e) α-lactalbumin crystallized at 5 mg/mL in C13; (F) thaumatin crystals and (f) thaumatin crystal from condition C7; (G) xylanase crystal plates and (g) xylanase crystals obtained at 36 mg/mL in condition C3; (H) concanavalin crystals and (h) concanavalin crystallized at 5 mg/mL in condition C11; (I) hemoglobin crystalline material and (i) hemoglobin crystallized at 60 mg/mL in condition C14; and (J) trypsin crystallized at 30 mg/mL and (j) trypsin at 30 mg/mL in condition C9.

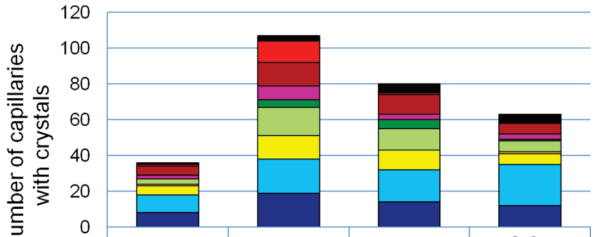
Crystallization Mushroom device (Triana Science and Technology) with a protein concentration of 36 mg/mL. Here again, the use of high protein concentration (40 mg/mL) yielded more hits (19) than the standard protein concentration (7 mg/mL), which only afforded crystals in 8 conditions. The Mix and Touch seeding protocols increased the number of conditions producing crystalline material to 14 and 12 hits, respectively.

3.3. Ribonuclease A. In the case of ribonuclease A, the seeds were grown by capillary counter-diffusion technique using a protein concentration of 240 mg/mL. The same protein concentration was used as high protein concentration (8 hits). Lowering this concentration six times reduces the number of hits four times. Both seeding protocols increased the number of hits to 3 producing single well-faceted crystals along the capillary.

3.4. α-Lactalbumin. Seed crystals of α-lactalbumin were obtained by the hanging drop method from acicular homogeneous crystals surrounded by clear solution. The reduction of protein concentration from 30 mg/mL to 5 mg/mL decreased the number of hits from 16 to 3. For the seeding experiments, the Mix procedure (12 hits) was significantly more successful than the Touch protocol (6 hits).

3.5. Myoglobin. In the case of myoglobin, amorphous precipitate or polycrystalline spherulites were obtained in all the conditions tested. It is well known that the quality of the initial

dx.doi.org/10.1021/cg101380j |*Cryst. Growth Des.* 2011, 11, 2122–2126



	S.C., no seeding	H.C., no seeding	S.C., Mix seeding	S.C., Touch seeding
Xylanase	1	3	5	5
Trypsin	1	12	1	0
Thaumatin	5	13	11	6
Ribonuc. A	2	8	3	3
Myoglobin	0	4	5	1
α-Lactalb.	3	16	12	6
Hemoglobin	1	0	0	1
Gluc. Isom.	5	13	11	6
Concan. A	10	19	18	23
Catalase	8	19	14	12

Figure 2. Number of hits in the protein crystallization study in capillaries by the combination of counter-diffusion and microseeding techniques (SCD).

crystal seed can be crucial to get improved crystals, but it has also been reported that spherulites or other precipitates with short-range order can work.[8] In the case of myoglobin, we used spherulites to obtain our initial seed stock. To avoid the formation of any undesirable precipitate (observed during the seed preparation experiments), we decreased the protein concentration 16 times, from 80 mg/mL to 5 mg/mL, but no hits were obtained. The use of seeds (Mix protocol) dramatically improved the number of hits to 5 compared to the 4 obtained with the high protein concentration solution. Only the Mix protocol produced well-faceted crystals. The Touch protocol only produced one hit.

3.6. Thaumatin. Thaumatin seed crystals were obtained from sitting drop setups. The use of the standard protein concentration solution (10 mg/mL) in the crystallization screening experiments also produced fewer hits (5) than the high protein concentration solution (90 mg/mL), which nearly tripled the number of hits (13). The Mix protocol doubled the number of hits (11) in comparison to the low concentration without seeds, while the Touch protocol also increased the number of hits to 6.

3.7. Xylanase. Xylanase seed crystals were obtained from sitting drop setups. Both seeding protocols (5 hits) worked better than the high concentration experiment (3 hits). They were also much more effective than the low concentration experiment without seeds, which produced only one hit.

3.8. Trypsin. Trypsin seed crystals were obtained from sitting drop setups. For this protein, seeding had no apparent beneficial effect producing only one hit (Mix protocol, 30 mg/mL), while the higher protein concentration (90 mg/mL) dramatically increased the hit rate to 12.

3.9. Glucose Isomerase. Glucose isomerase seeds were prepared from well-faceted crystals obtained from a batch experiment. We observed a drastic reduction in the number of hits, from 13 to 5, when going from high (30 mg/mL) to standard (10 mg/mL) protein concentration. Adding seeds to the low concentration protein solution (Mix protocol) increased the number of hits to 11, while the Touch protocol produced 6 hits, only 1 more than the low protein concentration solution.

3.10. Hemoglobin. Hemoglobin seed crystals were obtained from sitting drop setups. Crystals could only be obtained after three months in sitting drop wells. These crystals were used to prepare a seed stock, which produced crystals in sitting drops overnight. These sitting drop crystals were in turn used to generate seed stocks for counter-diffusion. Only the standard protein concentration (60 mg/mL) and the Touch protocol produced 1 hit while the high protein concentration (90 mg/mL) and the Mix protocol did not produce any crystals.

Figure 1 shows a selection of seed crystals used and the crystals grown in seeding experiments. In most cases, an improvement in crystal morphology can be observed. Since crystals are more likely to be grown in the metastable region of the phase diagram where crystals grow more slowly, they are likely to be more ordered.

In counter-diffusion experiments, it is recommended that protein stock solutions should be as concentrated as possible. The results clearly show that the high protein concentration sample gave many more hits than any other method. If, however, high protein concentration stocks are not available, either because stocks have been prepared for vapor diffusion or because the protein is insufficiently soluble, microseeding can be used to increase the number of hits (Figure 2). Both seeding protocols (Mix and Touch) nearly doubled the number of hits in comparison to that with the setup with standard protein concentration. In three cases (33%), the number of hits using seeds was higher than those obtained with high protein concentration solution. The seeding protocols also found some hits that were not picked up by the protocol that used high protein concentration. At this point, it is also important to notice that the chemical bias effect[16] is almost negligible in the capillary counter-diffusion technique because the ratio of protein to precipitant volume is in the order of 10^4. Moreover, seeding in counter-diffusion ensures the stability of the seeds since they are initially mixed at the highest concentration of protein available, typically the same protein concentration used to produce the seeds, and they are never diluted, contrary to what occurs with any other crystallization technique.

In general, the Mix protocol works better than the Touch protocol, probably due to the fact that the latter did not always ensure that seed crystals were carried along the capillary. Only in one case, concanavalin A, did the Touch protocol produce more hits than the Mix protocol, even more hits than the high protein concentration solution. This result indicates that the high protein concentration solution was not high enough to fully exploit the 24 screening conditions. The same conclusion can be drawn from the xylanase data.

In two cases, ribonuclease A and xylanase, both seeding protocols worked equally well, whereas in two cases (trypsin and hemoglobin), only one of the protocols produced a hit. In the case of hemoglobin, the use of seeding did not improve the success rate since one hit was also obtained using the low concentration protein solution. Moreover, the case of hemoglobin is unusual because the solution with high protein concentration did not produce any hits. We did not try to explain this result further because hemoglobin appears to be very reluctant to crystallize.

Two proteins, glucose isomerase and catalase, were crystallized in two very distinguishable crystal forms (probably two polymorphs) at the stage of generating the initial seeds. Although we selected only one of the crystal forms as seed stock, we found that both forms could be obtained from the same seeds (Figure 1A-a and C-c). Similar results have been obtained recently with human arginase,[18] but since we have not yet characterized those crystals by X-ray diffraction, we will not discuss this observation further.

In our view, myoglobin represents a good example of the general case in structural biology laboratories in which protein concentrations are kept at low concentrations trying to maximize the number of experiments. In these cases, only the combination of some initial crystalline material with the minimum matrix screening will produce well-faceted crystals. When this combination is applied in capillary counter-diffusion experiments, the protein solution contained in the capillary will feed the seeds to produce crystals of adequate size for X-ray characterization.

4. CONCLUSIONS

The use of seeding coupled in capillary counter-diffusion experiments significantly increases the probability of finding new crystallization conditions. Mixing the protein solution with the seed stock produces a homogeneous distribution of seeds along the capillary and is the most effective protocol. The solution containing the seed crystals is never diluted, preventing seed dissolution. The counter-diffusion technique allows the introduction of seeds into a system where the mass transport is controlled by diffusion and in which saturation increases slowly enough to allow the growth of several seeds along the capillary, often far enough apart for the crystals to grow completely independently.

The combination of seeding with capillary counter-diffusion seems to work in two parallel ways: (i) it increases the nucleation rate when protein concentration is not high enough to trigger nucleation, and (ii) it acts as a crystal improvement methodology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Seed production and preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This work was funded by the OptiCryst project of the VI European Framework Programme and partly by Douglas Instruments Ltd. This research is a product of the "Factoría Española de Crystalización", a project Consolider- Ingenio 2010 of the Ministerio de Innovación y Ciencia (MICINN) of Spain.

■ REFERENCES

(1) Thaller, C.; Weaver, L. H.; Eichele, G.; Wilson, E.; Karlsson, R.; Jansonius, J. N. *J. Mol. Biol.* **1981**, *147* (3), 465–469.
(2) Stura, E. A.; Wilson, I. A. *J. Cryst. Growth* **1991**, *110*, 270–282.
(3) Zhu, D.-Y.; Zhu, Y.-Q.; Xiang, Y.; Wang, D.-C. *Acta Crystallogr., Sect. D* **2005**, *61*, 772–775.
(4) Ren, J.; Nettleship, J. E.; Sainsbury, S.; Saunders, N. J.; Owens, R. J. *Acta Crystallogr., Sect. F* **2008**, *64*, 247–251.
(5) Otálora, F.; Gavira, J. A.; Ng, J. D.; García-Ruiz, J. M. *Prog. Biophys. Mol. Biol.* **2009**, *101*, 26–37.
(6) McPherson, A.; Shlichta, P. *Science* **1988**, *239*, 385–387.
(7) Stura, E. A.; Wilson, I. A. *Methods* **1990**, *1*, 38–49.
(8) Bergfors, T. *J. Struct. Biol.* **2003**, *142* (1), 66–76.
(9) Garcia-Ruiz, J., M. *Methods Enzymol.* **2003**, *368*, 130–154.
(10) Ireton, G. C.; Stoddard, B. L. *Acta Crystallogr., Sect. D* **2004**, *60*, 601–605.
(11) Obmolova, G.; Malia, T. J.; Teplyakov, A.; Sweet, R.; Gilliland, G. L. *Acta Crystallogr., Sect. D* **2010**, *66*, 927–933.
(12) D'Arcy, A.; Mac Sweeney, A.; Haber, A. *Acta Crystallogr., Sect. D* **2003**, *59*, 1343–1346.
(13) Newman, J.; Pham, T. M.; Peat, T. S. *Acta Crystallogr., Sect. F* **2008**, *64*, 991–996.
(14) Walter, T. S.; Mancini, E. J.; Kadlec, J.; Graham, S. C.; Assenberg, R.; Ren, J.; Sainsbury, S.; Owens, R. J.; Stuart, D. I.; Grimes, J. M.; Harlos, K. *Acta Crystallogr., Sect. F* **2008**, *64*, 14–18.
(15) Khurshid, S.; Haire, L. F.; Chayen, N. E. *J. Appl. Crystallogr.* **2010**, *43*, 752–756.
(16) St John, F. J.; Feng, B.; Pozharski, E. *Acta Crystallogr., Sect. D* **2008**, *64*, 1222–1227.
(17) Villasenor, A. G.; Wong, A.; Shao, A.; Garg, A.; Kuglstatter, A.; Harris, S. F. *Acta Crystallogr., Sect. D* **2010**, *66*, 568–576.
(18) Newman, J.; Pearce, L.; Lesburg, C. A.; Strickland, C.; Peat, T. S. *Acta Crystallogr., Sect. F* **2011**, *67*, 90–93.