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Trial by fire: are the crystals macromolecules?

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Protein crystallization screens frequently yield salt crystals as well as protein crystals. A simple method for determining whether a crystal is composed of salt or macromolecules is suggested. A drop containing one or more crystals is transferred to a glass cover slip and the cover slip is then passed through the flame of a Bunsen burner. Macromolecule crystals are destroyed by this treatment, while salt crystals generally remain. The test can be performed after other commonly used tests such as crushing and staining.

1. Introduction

The growth of crystals is a major bottleneck for the crystallography of macromolecules. The path to obtaining an X-ray crystal structure usually starts with systematic trials of solution conditions, pathways to equilibrium, protein concentrations and incubation temperatures. Unfortunately, salt crystals are often encountered in these screens. Several methods can be used to determine whether a crystal is composed of salt or a macromolecule (Jordanova *et al.*, 2008). However, each individual method has limits to its application or may possibly yield misleading results. A direct and definitive test is to simply mount the crystal in an X-ray beam and take an image of the diffraction pattern. Specialized equipment such as the PX Scanner (Oxford Diffraction) allows this to be performed *in situ*. However, access to an X-ray beam may not be available without significant expense and delay. In addition, the initial crystals may be small or of poor quality or may become damaged during harvesting or other handling. Thus, alternative tests to show that crystals are made of protein are needed to establish whether the results of a crystallization trial merit further study.

In the 'crush test' the crystal is poked with a fine-tipped glass rod. A crunchy crystal is likely to be salt; a soft crystal is likely to be a macromolecule. This test can be misleading as some macromolecule crystals resist crushing more than others and its performance and interpretation become more difficult as the crystal size decreases. Another test employs a dye that can visibly stain a macromolecule crystal by diffusing into its solvent channels but fails to stain salt crystals, which are closely packed. Several dyes have been used, including crystal violet (Sumner, 1918), methylene green and eosin scarlet (Jena Bioscience). Currently, the most commonly used dye is methylene blue sold as IZIT (Hampton Research) or True Blue (Jena Bioscience). Some macromolecule crystals in some solution conditions, such as low pH, stain very slowly or not at all (Eckert *et al.*, 2003). In some cases crystals disappear, as the addition of extra water with the dye solution may solubilize salt crystals but may possibly also dissolve macromolecule crystals. If too much dye is added the solution remains opaque, obscuring the result. The staining process can modify crystals and can affect the binding of any ligand and thus is considered to be destructive. In addition, the IZIT dye can form small needles or flakes under a variety of conditions (Bukrinsky & Poulsen, 2001). A dehydration test can be used, under the assumption that protein crystals are not as likely to survive the process intact as salt

crystals (McPherson, 1982). The interpretation of the results of this test may be difficult, as we have observed protein crystals mounted on nylon loops that visually appear to survive days of room-temperature storage. This raises the possibility of a false negative that results in a protein crystal being misidentified as salt. Dehydration may also allow mother-liquor components to crystallize. Protein crystals can be cross-linked with glutaraldehyde (Quiocho & Richards, 1964; Lusty, 1999). The cross-linked crystals can then be put into a low ionic strength solution where salt crystals should dissolve. Glutaraldehyde must be used with care as it can damage tissues, especially mucous membranes. Note that free amines such as ethanolamine, Tris buffer or ammonium ions will interfere with this assay. Crystals may be harvested, washed in mother liquor and analysed by gel electrophoresis (Bergfors, 2007). However, this method requires a significant amount of material for detection and the protein could be lost during the procedure. Cross-polarization uses the anisotropic nature of crystalline materials to refract light and produce birefringence (Bodenstaff *et al.*, 2002; Echalier *et al.*, 2004). Birefringent crystals appear as rainbow-colored objects against a dark background. This aids the detection of crystals in the presence of precipitate and can identify twinned crystals. However, not all macromolecule crystals are birefringent (Lowe & Amos, 1998). Organic and inorganic materials present in crystallization screens can also form birefringent crystals, resulting in false positives. By using specialized equipment, protein crystals can be identified by virtue of their infrared spectra (Chan *et al.*, 2009) or their intrinsic fluorescence (Judge *et al.*, 2005). However, the intrinsic fluorescence of proteins varies with amino-acid composition and noncrystalline material can have significant fluorescence, possibly limiting the sensitivity of detection. Chemical modification of a protein prior to crystallization by attaching a fluorescent probe (Sumida *et al.*, 2001; Forsythe *et al.*, 2006) has been used to detect protein crystals. Like glutaraldehyde cross-linking mentioned above, this covalent modification method is not compatible with nonprotein free amines. Some proteins may be difficult to modify owing to solubility problems in the solutions used for the modification reaction. The covalently modified protein may have altered biological or physical properties. However, the use of trace amounts of modified protein mitigates this risk and crystal growth can be repeated with unmodified protein. In addition to visibly colored dyes, the fluorescent molecules SYBR Gold and ANS (1,8-ANS) have been used to identify macromolecule crystals (Groves *et al.*, 2007; Kettenberger *et al.*, 2006). Fluorescent or colored dye can be added to a protein sample prior to crystallization to facilitate high-throughput crystallization trials (Cosenza *et al.*, 2007; Groves *et al.*, 2007). The fluorescence of ANS is strongly enhanced when bound to protein crystals (Groves *et al.*, 2007), providing good contrast. However, staining of previously grown crystals with ANS has not been demonstrated. This may not be of concern as prior addition reduces handling. It may be possible to use an alternative fluorescent dye such as bromophenyl blue, fluorescein, trypan blue or rhodamine if staining after crystal growth is desired. As with the use of visibly colored dyes, effects of fluorescent dyes on the properties of the protein are possible. Of course, crystal growth can be repeated without the dye.

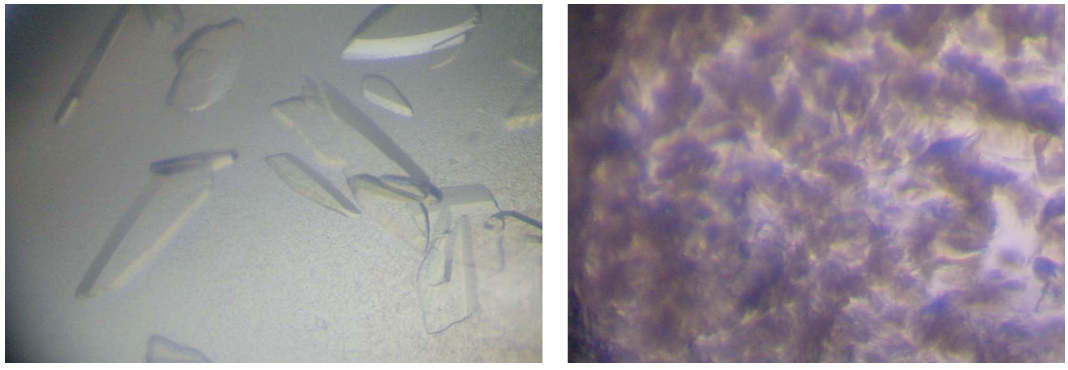


Figure 1
Protein crystals are destroyed by the melt test. Crystals of EpsG protein before (left) and after testing (right). The largest crystals were approximately 0.6 × 0.6 × 0.2 mm in size.

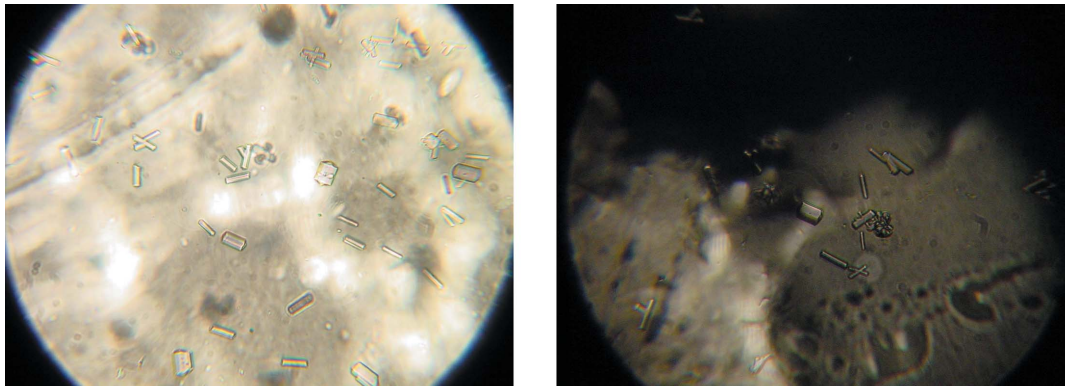


Figure 2
Salt crystals survive the melt test. Crystals found in a solution based upon Emerald Wizard Screen II condition 21 (30% methylpentanediol, 1 *M* sodium acetate pH 4.5, with the addition of 300 m*M* ammonium sulfate) mixed with an equal volume of protein solution in 50 m*M* sodium phosphate pH 7, 300 m*M* sodium chloride before (left) and after the test (right). The largest crystal is approximately 0.1 mm in length.

Each test for the detection of macromolecule crystals has strengths and weaknesses and the tests vary in degree of difficulty, time required and expense. Here, we present a simple method for determining whether a crystal is composed of protein or salt that is based upon the stability of the crystal when it is briefly exposed to heat from the flame of a Bunsen burner. Macromolecule crystals are destroyed by this treatment, while salt crystals generally remain. The test can be performed after other commonly used tests such as crushing and staining.

2. Methods

2.1. Preparation of protein crystals

EpsG protein was concentrated to 40 mg ml^{-1} in 15 m*M* Tris pH 7.5, 15 m*M* NaCl. Crystals were grown by hanging-drop vapor diffusion against a reservoir containing 6 m*M* zinc sulfate, 60 m*M* MES pH 6.5, 15% PEG MME 550 as described by Jens *et al.* (2009). EpsH protein was concentrated to 15 mg ml^{-1} in 15 m*M* Tris pH 7.5, 100 m*M* NaCl. Crystals of EpsH were grown by hanging-drop vapor diffusion against a reservoir containing 7% PEG 4000, 90 m*M* sodium acetate pH 4.6, 10 m*M* Tris–HCl and 175 m*M* sodium formate as described by Raghunathan *et al.* (2009).

2.2. The melt test

Solution (2 µl or less) containing at least one crystal is transferred with a micropipette or a nylon loop from a crystallization trial to a siliconized circular cover slip (Hampton Research). Successful transfer is verified under a microscope. A stereo microscope used for evaluating crystallization trials can be employed. Alternatively, a standard light microscope with higher resolution may be used to evaluate smaller crystals. The cover slip is then held by tweezers and passed rapidly across the low flame of a Bunsen burner with the drop side on top, much like the method used for heat-fixing bacterial smears to a slide prior to staining. Boiling is avoided to ensure that heating is not excessive. In most cases, the heating is stopped just as all of the liquid on the cover slip is vaporized. Viscous conditions such as a high PEG or glycerol solution may not allow the full evaporation of the liquid to occur and four or five passes resulting in a visible reduction of drop volume should be sufficient to destroy protein crystals (Fig. 5). Cracking of the glass around the edges of the cover slip can occur just after removal from the flame, indicating that excessive heat may have been employed. Care should be taken to avoid this, but it does not affect the test results for stable salt crystals. Additionally, care should be taken when flammable chemicals such as 2-propanol are present, as the drop may catch fire (Fig. 4). Goggles should be worn. After cooling, the material on the cover slip is again observed under the microscope. Photographs taken before and after the melt test are recommended to allow comparison.

3. Results

We have developed a simple 'melt test' to distinguish between macromolecule and salt crystals. As test cases we have used the EpsG

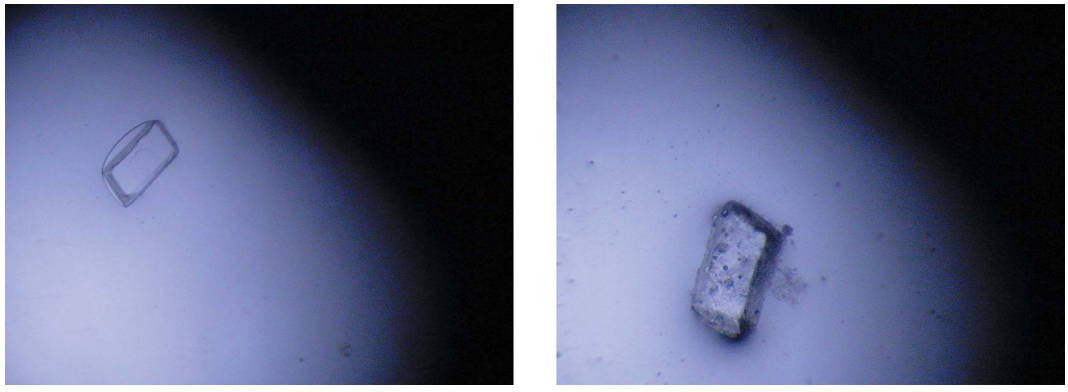


Figure 3
Salt crystals survive the melt test. Crystal found in Hampton Crystal Screen II condition 25 (1.8 *M* ammonium sulfate, 100 m*M* MES pH 6.5, 10 m*M* cobalt chloride) mixed with an equal volume of protein solution in 15 m*M* bis-tris pH 6.5, 100 m*M* NaCl before (left) and after the test (right).

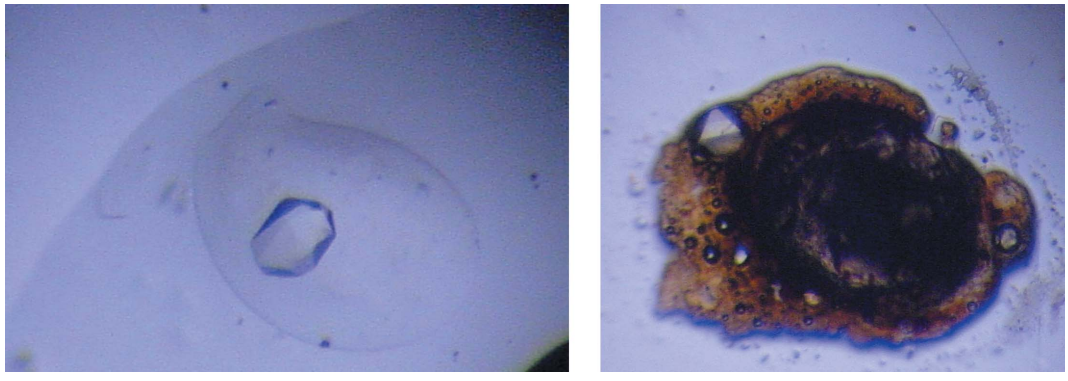


Figure 4
Salt crystals survive the melt test. Crystal found in Hampton Crystal Screen condition 8 (30% 2-propanol, 100 m*M* sodium cacodylate pH 6.5, 200 m*M* sodium citrate) mixed with an equal volume of protein solution in 15 m*M* bis-tris pH 6.5, 100 m*M* sodium chloride before (left) and after the test (right). The crystal survived despite the solution catching fire.

and EpsH proteins, which are pseudopilin proteins required for type II secretion by the bacterial pathogen *Vibrio cholerae*. Since these proteins are insoluble in their full-length mature forms, we worked with recombinant proteins with truncations of 25 or 26 N-terminal amino acids, respectively. In addition, each of the recombinant proteins has its C-terminus appended with an eight-residue His tag, LEHHHHHH. A melt test performed upon crystals of the EpsG protein is shown in Fig. 1. The protein crystals are destroyed by this simple heating procedure. Similar results were obtained in tests of other protein crystals including the EpsH protein and lysozyme (not shown). In contrast, salt crystals survive a melt test, as shown in Figs. 2, 3 and 4. The left panel of Fig. 5 shows an EpsG crystal that was previously stained blue with IZIT dye following the manufacturer's recommended procedure (Hampton Research). The right panel shows the results of a melt test performed upon the stained protein crystal. The blue-stained EpsG crystal was destroyed, showing the utility of the melt test in conjunction with a staining test.

The melt test is faster than the IZIT staining test and gives more definitive results than the dehydration test. The left panel of Fig. 6 shows crystals of EpsH protein that were treated with IZIT dye for about 4 h while simultaneously being dehydrated. The crystals appear to remain intact and have not been stained by the blue dye. Thus, they could be misidentified as salt crystals. The melt test was performed on these dehydrated EpsH crystals and, as shown in the right panel, the protein crystals were destroyed.

A potential artefact in this test is the possible formation of new salt crystals as the sample is heated. We performed the melt-test procedure on solutions corresponding to Crystal Screen I and II (Hampton Research) to test for such salt crystals. Each solution was mixed with an equal volume of buffer (50 m*M* sodium phosphate pH 7.0, 300 m*M* NaCl). When 6 µl drops were used the drops vaporized more slowly and salt crystals (verified by the crush test) were observed in a few of the tests. However, with drop sizes of 1–2 µl none of the 96 conditions tested showed salt-crystal formation.

Organic salts are generally less stable than inorganic salts. Crystals of sodium citrate survived a melt test as shown in the upper panels of Fig. 7. However, as shown in the lower panels, excessive heating destroyed these organic salt crystals.

4. Discussion

Salt crystals can form when macromolecules in phosphate buffers are mixed with crystallization trial solutions containing divalent cations. The melting points of these inorganic salt crystals are generally greater than 873 K, while the melting points of macromolecule crystals are below 373 K. The large difference in this fundamental property of the two types of crystals is exploited in a simple test for determining whether a crystal is composed of salt or macromolecules. A drop containing crystals is transferred to a glass cover slip and the cover slip is then passed through the flame of a Bunsen burner. Crystals of macromolecules are destroyed by this treatment, while salt crystals remain. As boiling is avoided and the cover slip is removed from the flame just as the liquid disappears, the temperature

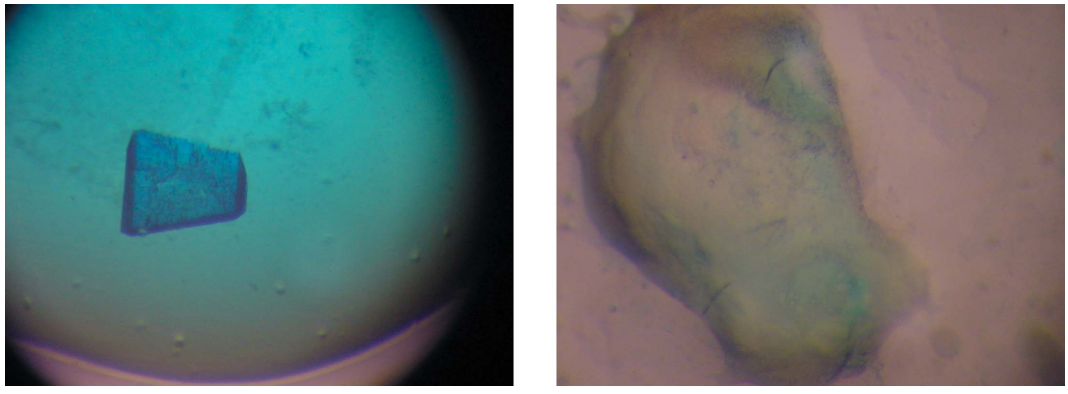


Figure 5
Staining does not interfere with the melt test. EpsG protein crystal dyed with IZIT (left) and destroyed after the melt test (right). The crystal was approximately 0.6 × 0.4 × 0.2 mm in size. Note that the high concentration of PEG 550 makes complete evaporation of the drop impractical.

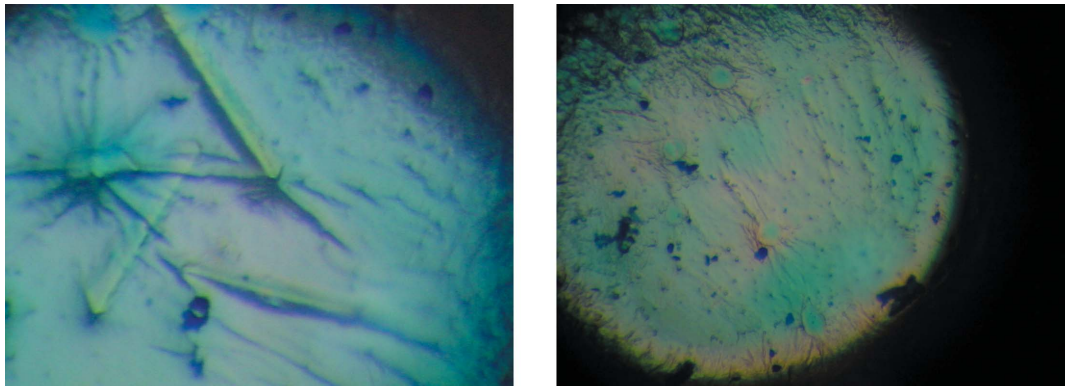
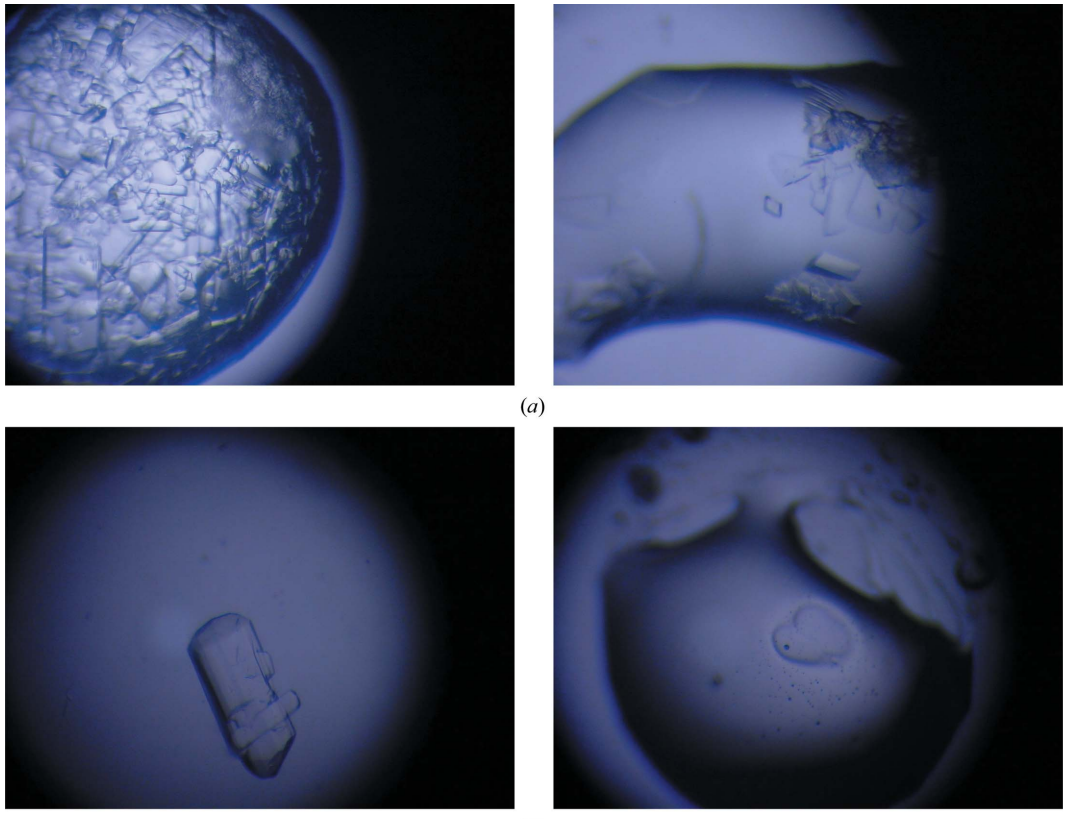


Figure 6
The melt test is faster than staining and more definitive than a dehydration test. EpsH protein crystals after treatment with IZIT dye and dehydration (left) and destroyed after the melt test (right). The largest crystal was approximately 0.8 × 0.2 × 0.2 mm in size.

of the crystals will not be raised far above 373 K. Note that avoidance of cover-glass cracking also provides a control against excessive heating. The large gap between the melting points of macromolecule crystals and many salt crystals permits loose temperature control. Thus, clear results are commonly obtained from this procedure. However, some salts, such as organic salts formed from the buffer, mother liquor or added ligands, could potentially confound the results of this test, as they may form crystals with lower melting temperatures. If this is a concern then control experiments in which all components except the protein are added can be performed to test for growth of salt crystals. In addition, if a particular salt with a known melting point is suspected, a control salt with a known lower melting point can be tested on the same cover slip with periodic observation under a microscope (Fig. 7). Although temperature is controlled loosely in this test, it is critical. The initial heating must be performed rapidly, to favour salt precipitation over possible salt-crystal formation, and the temperature must not become too high, as even stable inorganic crystals can be melted by the heat of a Bunsen burner (up to 2173 K). In short: heat fast, but not too much. Some mother-liquor solutions, such as those containing high concentrations of high-molecular-weight polyethylene glycol, leave behind a significant residue in this procedure, making small crystals of salt more difficult to detect. As the drop evaporates in a melt test, salts in the solution will precipitate. When working with a crystal screen solution that uses salt as the precipitating agent it may be necessary to work fast, as any evaporation that occurs during handling might allow new salt crystals to form. The salt precipitate may obscure the view of the target crystal and cloud the result. However, because heating is rapid no additional salt crystals are likely to grow. In addition, because the test is rapid salt crystals are unlikely to dissolve. Thus, the observation of crystals after the melt test clearly indicates a negative result: the crystals are not protein. On the other hand, if the initial crystals were small and significant residue or precipitate is generated, any remaining salt crystals may be difficult to observe. The melt test may then be scored as inconclusive or give a false-positive result. A melt test performed upon a salt crystal with a low melting point will also produce a false-positive result. Thus, the melt test may give a false-positive result that would inevitably be discovered to be salt but is unlikely to give a false-negative result for an actual protein crystal.

The melt test is fast, inexpensive and easily performed. It is faster and gives more definitive results than the dehydration test. Unlike the dehydration test, new salt crystals do not form to potentially confound the results. We confirmed this with melt tests of 96 different screening solutions mixed with a buffer that contained both phosphate and sodium chloride. The melt test is less time-consuming than post-crystallization staining, cross-linking or gel-electrophoresis tests and requires no special equipment. The test can be performed after other commonly used tests such as crushing and staining. As the test is clearly very destructive it should be performed last. For example, a potential protein crystal could be examined for intrinsic fluorescence or birefringence and then crushed with a glass rod; the remaining crystal particles could then be stained with a colored or fluorescent dye and finally a sample of the material could be transferred to a cover slip for a melt test. Thus, this rapid and simple test is compatible with many other tests for macromolecule crystals, including *in situ* high-throughput tests, and may be a useful extension to them. It may be especially useful in conjunction with a staining test. Among seven proteins that were crystallized in the presence and absence of IZIT



(*a*)

(*b*)

Figure 7
Crystals of sodium citrate used as a melt-test control. (*a*) Sodium citrate crystals before (left) and after (right) teh melt test. (*b*) Sodium citrate crystals before (left) and after (right) the melt test. The crystals in (*a*) survive the melt test, while crystals in (*b*) were destroyed, indicating excessive heating. Salts with a higher melting point than sodium citrate are likely to survive a melt test conducted as in (*a*). The crystals were obtained from a saturated stock solution of sodium citrate.

dye, only four produced blue crystals when grown in the presence of the dye (Cosenza *et al.*, 2007). Any crystals that fail to stain might be judged to be salt crystals. If these crystals do not survive the melt test then they should be studied further as they could in fact be protein crystals. If they do survive the melt test then they are not protein crystals. While this test is very destructive, with the 'innocent' macromolecule crystals not surviving the trial, it should be noted that many of the other tests for salt crystals are also destructive. Of course only a portion of a sample need be tested; some can be saved for seeding experiments or other characterization.

Note added in proof: recent papers in this journal evaluate the use of intrinsic fluorescence (Gill, 2010) and show that using higher excitation wavelengths can overcome problems caused by opacity of the containment hardware to UV-light (Dierks *et al.*, 2010).

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