User Guide

JBScreen Cryo Pro

Cat.-No.: CC-102

Jena Bioscience

Introduction

Crystal cryoprotection has become a fundamental tool in the repertoire of crystallographic methods for studying biological macromolecules(1). In many cases cryoprotection and subsequent data collection at low temperature are essential for obtaining a complete data set by overcoming the problem of radiation damage. Moreover, cryomethods allow crystallographers to work with small crystals, the cryomounting techniques are much gentler, and cryocooled crystals can be stored indefinitely without losing diffraction quality.

Cryoprotectants provide a means to protect macromolecular crystals from the damaging effects of ice formation during the cryocooling process. Cryoprotection usually involves immersing the crystal in a solution that forms an amorphous glass (and not crystalline ice) while being flash cooled in liquid nitrogen. The cryoprotectant solution must not destroy the crystal at the crystallization/mounting temperature, and is therefore usually composed of the same chemicals used in the crystallization experiment (at similar or higher concentrations) in addition to a cryoprotecting agent such as sugars, glycerol, ethyleneglycol, PEG or MPD.

Crystals are occasionally grown under conditions where there is no need for additional cryoprotectant; in these cases the crystals can be fished from a mother drop and cooled in liquid nitrogen without additional treatment. Usually crystals obtained in PEG solutions exceeding 21-23 % or salt conditions higher than 2.8-3.0 M can be frozen without additional cryoprotectant.

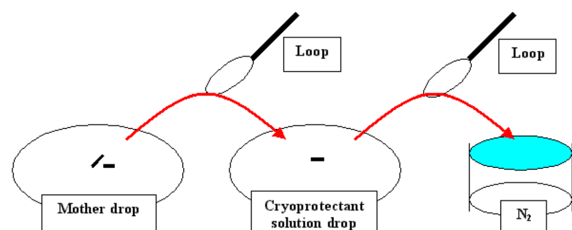

Fig. 1: *Schematic illustration outlining the major steps involved in cryocooling macromolecular crystals. The final step can involve crystal transfer to a liquid nitrogen bath or to a cryogenic gas stream (nitrogen or helium).*

Testing Your Cryoprotectant

Whether transferring your crystal directly from the mother liquor or from a cryoprotectant solution, the real measure of success is by inspection of the resulting diffraction image. Before sacrificing any precious crystals it is often wise to inspect the optical characteristics of the mother liquor or cryoprotectant solution at the target temperature of your diffraction experiment (usually between 100–130 K).

The testing phase involves transferring a nylon loop containing mother liquor or cryoprotectant to a liquid nitrogen bath or cryogenic gas stream and observing the resulting opacity of the loop's contents (Fig. 2). When transferring to a liquid nitrogen bath, be sure to keep the loop submerged if possible, or no more than 1 cm above the liquid surface.

Try to use a large loop (Ø 0.5–1.0 mm) for better visibility during the testing procedure. As a general rule, any degree of observed opacity or translucence is indicative of ice for

mation and constitutes a negative result. A transparent glassy appearence is suggestive of a potentially suitable cryoprotectant: if an X-ray source is available, collect a diffraction image to check for the presence of 'ice rings' or other undesirable features which may indicate non-ideal conditions.

An ideal cryoprotectant is one which causes minimal stress to the crystal and provides full protection against ice formation. Crystal stress may be alleviated by prolonging the equilibration period to the final cryoprotectant concentration (e.g. stepwise transfer to cryoprotectant mixtures of increasing concentration), or by a 'quick soak' approach which involves a rapid transfer of the crystal from mother liquor to the cryogenic environment via a cryoprotectant bath.

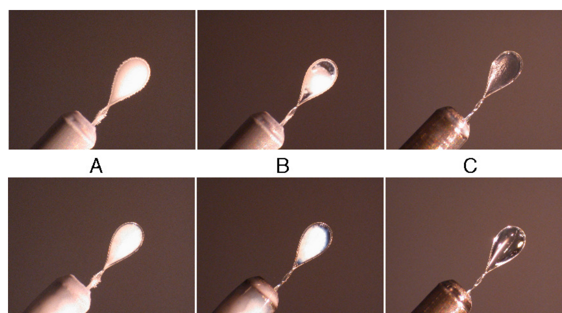

Fig. 2: *Various results of a cryoprotectant test. Images were taken at cryogenic temperatures. (A) Opaque loop indicates severe ice-formation; (B) translucent loop with a transparent perimeter is still indicative of partial ice formation; (C) transparent loop suggests suitable cryoprotection.*

The JBScreen Cryo Pro Kit

The JBScreen Cryo Pro kit is the most convenient tool on the market for producing effective cryoprotectants from your crystallization reservoir solution. The kit is divided into sugar/amino-acid-based cryoprotectants (Cryo 01–05), alcohol-based cryoprotectants (Cryo 06–11), and an oil-based cryoprotectant (Cryo 12).

Formulations:

Cryo 01 – Sucrose/Xylitol
Cryo 02 – Sucrose/Glucose
Cryo 03 – Sucrose
Cryo 04 – Xylitol
Cryo 05 – Taurine

Cryo 06 – Glycerol
Cryo 07 – Pentaerythritol propoxylate (5/4 PO/OH)
Cryo 08 – Pentaerythritol ethoxylate (15/4 EO/OH)
Cryo 09 – Ethylene glycol
Cryo 10 – MPD ((±)-2-Methyl-2,4-pentanediol)
Cryo 11 – PEG 400

Cryo 12 – Mineral Oil

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Utilization

Cryo 01–05

Microtubes containing the formulations Cryo 01–05 consist of predispensed 10 mg samples (crystalline or syrup concentrate) suitable for dilution with your crystallization reservoir solution. Three samples of each formulation are provided. Selecting one microtube from each of the different formulations in this series, pipette a 40 µl volume of crystallization reservoir solution to the cryoprotectant sample. Mix thoroughly by repeated aspirations with the pipette, vortex and centrifuge. The five individual microtubes will now contain ~50 µl of the reservoir-solution/cryoprotectant mixture. Load mixture (5-20 µl) onto the sample depression of the accompanying microscope slide. With a cryoloop, transfer a crystal from the mother liquor to the cryoprotectant mixture, allow to soak for 1-2 seconds, and then transfer the crystal to a liquid nitrogen bath or cryogenic gas stream. This method has been tested and used successfully with crystals obtained under a wide range of salt concentrations (0.4-3.0 M) and polymeric precipitants (PEG, PEG-MME, etc.).

Cryo 06–11

Microtubes containing the formulations Cryo 06–11 contain 50 µl of ultra-pure liquid sample. Mix 3 µl of cryoprotectant with 7 µl of reservoir solution. Test the mixture on a loop to see if it provides efficient cryoprotection (refer to the section 'Testing Your Cryoprotectant' above). If the 30% cryoprotectant mixture prevents ice formation, try reducing the cryoprotectant concentration: mix 2.5 µl cryoprotectant with 7.5 µl reservoir solution and test again. Continue to reduce the cryoprotectant concentration in this manner until a minimal protective concentration is established. Usually a concentration of 5-25% cryoprotectant is sufficient, depending on the composition of the reservoir solution.

Cryo 12 (Mineral Oil)

Prior to using, oil should be dehydrated if possible on a SpeedVac: pipette 20 µl of oil into a microtube, leave the cap open, and spin in a SpeedVac for 10 minutes. Add 20 µl of reservoir solution to the dehydrated oil and vortex to saturate the oil with reservoir solution. Centrifuge the mixture to separate the oil and water phases. Take 10 µl of the oil phase and test for cryoprotecting abilities. When transferring a crystal from the crystallization droplet to the oil mixture, care must be taken to minimize the amount of mother liquor transferred along with the crystal[2].

Overcoming crystal stress during cryosoaking

The benefits of cryoprotection can sometimes be outweighed by the damaging effects of the cryoprotectant mixture on your crystals during the treatment process. Cryoprotectant may protect your crystallization mother liquor or reservoir solution from ice formation, but may also destroy your crystals due to large perturbations in the ionic or osmotic composition of the soaking solution. Crystals may exhibit physical damage (such as cracking) during the cryosoaking step, or may display significantly reduced diffraction quality when compared to a capillary-mounted crystal. If crystal degradation seems to be a problem during the cryosoaking procedure, better results can often be achieved by:

a) Shorter soaking time

Often a quick pass of the loop/crystal through the cryoprotectant solution is sufficient to protect the crystal from ice formation without damaging the crystal lattice. Aim for a soaking time of 1 second or less.

b) Prolonged soaking time

Leave cracked or damaged crystals in cryosolution for a longer duration, observing the crystals under the microscope after 10, 30, 60 minutes, or perhaps even overnight. Damaged crystals can occasionally recover and reanneal if left undisturbed for a while[3].

c) Controlled equilibration

Try to reduce osmotic shock by bringing crystals to the final cryoprotectant concentration in small increments. For example, if a final cryoprotectant solution contains 30% glycerol, prepare 6 cryoprotectant solutions with increasing concentrations of glycerol: 5%, 10%, 15%, 20%, 25% and 30%. Transfer the crystal stepwise from lowest to highest glycerol concentration, allowing 10-20 minutes soaking time at each concentration. Be sure to cover the solutions during the soaking time to prevent evaporation.

Tips:

- smaller crystals tend to tolerate cryoprotection and freezing better than larger ones.

- annealing the crystal in the cryostream can often result in better diffraction quality[4-5]: with the crystal mounted on the goniometer head and placed in the cold gas stream, block the gas stream briefly (2-5 seconds) with a piece of aluminum foil, allowing the crystal to thaw. Following this, unblock the stream to allow the crystal to re-freeze. The unblocking motion should be performed rapidly to avoid temperature gradient fluctuations around the crystal during the re-freezing event. Compare the diffraction image with the previous one and conduct a repeat soaking/annealing step if desired[6].

- crystal damage associated with using high cryoprotectant concentrations can be reduced by using a lower concentration of cryoprotectant in combination with oil[7].

References:

(1) Garman *et al.* (1997) Macromolecular Cryocrystallography. *J. Appl. Cryst.* **30**:211.

(2) Riboldi-Tunnicliffe *et al.* (1999) Cryocrystallography with oil – an old idea revived. *J. Appl. Cryst.* **32**:1003.

(3) López-Jaramillo *et al.* (2002) Soaking: the effect of osmotic shock on tetragonal lysozyme crystals. *Acta Cryst.* **D58**:209.

(4) Harp *et al.* (1998) Macromolecular crystal annealing: Overcoming increased mosaicity associated with cryocrystallography. *Acta Cryst.* **D54**:622.

(5) Kriminski *et al.* (2002) Flash-cooling and annealing of protein crystals. *Acta Cryst.* **D58** :459.

(6) Samygina *et al.* (2000) Improving the X-ray resolution by reversible flash-cooling combined with concentration screening, as exemplified with Ppase. *Acta Cryst.* **D56**:595.

(7) Kwong *et al.* (1999) Use of cryoprotectants in combination with immiscible oils for flash cooling macromolecular crystals. *J. Appl. Cryst.* **32**:102.