

## Application

Water soluble cryoprotectant reagent set designed for the cryopreservation of biological macromolecular crystals.

## Description

The Hampton Research CryoPro™ cryoprotectant kit consists of 48 reagents for the cryopreservation of biological macromolecular crystals. The CryoPro reagents are supplied in 1.5 milliliter volumes. See CryoPro Formulation for reagent details. CryoPro reagents are water soluble compounds. CryoPro reagents are formulated using Type 1+ ultrapure water, 18.2 MΩ•cm resistivity at 25°C, < 5 ppb Total Organic Carbon, <1 Bacteria (CFU/ml), <0.03 Endotoxin (EU/ml).

## Attention

Do not perform crystal washes or dilutions directly in the supplied CryoPro solutions/vials. This will contaminate the stock solution. Aliquots of the reagents should be removed from the tube and assays performed in vials, plates, dishes, slides, or other appropriate platforms.

CryoPro reagents are sterile filtered and do not contain preservatives. Sterile technique and proper storage will help to ensure the integrity and longevity of the CryoPro reagents. Use clean, sterile pipette tips to aspirate CryoPro solutions.

## Storage

CryoPro reagents may be stored at -20 to 4°C. Allow the reagents to return to the desired working temperature and mix well before use.

## I'm in a Hurry and Do Not Read User Guides. What Should I Try for Cryoprotection?

### Method 1 -

#### Quick Cryo without Crystallization Reagent (Reservoir/Well Solution)

Pipette 20 µl of a CryoPro reagent onto a cover slide. Mount the crystal in a CryoLoop and transfer the crystal to the 20 µl drop of CryoPro reagent. Allow to soak for 2 seconds. Transfer crystal in CryoLoop to a liquid nitrogen source or cryostream.

### Method 2 -

#### Testing Cryo Concentration in Crystallization Reagent

Mix 4 µl of CryoPro reagent with 6 µl of crystallization reagent (reservoir/well solution). Capture this solution in a CryoLoop™ and freeze in liquid nitrogen. Inspect under the microscope. A clear, transparent solution indicates suitable cryoprotection. A milky white, opaque, translucent drop indicates ice formation and unsuitable cryoprotection. If the 40% mixture indicates suitable cryoprotection, try a mixture of 3 µl cryoprotectant with 7 µl of crystallization reagent. CryoLoop, freeze and inspect. Reduce the cryoprotectant concentration until the minimal concentration of cryoprotectant that provides a transparent solution is achieved. Then use this re-

agent for cryoprotection. Generally, a cryoprotectant concentration in the range of 5 to 30% is sufficient.

## Cryo 101

Low temperature X-ray diffraction methods, often termed cryocrystallography can minimize the rate of radiation damage to a crystal and extend crystal lifetime. Cryoprotection can stabilize a crystal, allow longer data collection times, and provide for gentler crystal mounting and convenient, stable transport of the crystal. Cryocrystallography allows for automated sample processing at synchrotron facilities for efficient transport, handling, and processing of crystals for cryocrystallography data collection.<sup>6</sup>

During cryocrystallography the crystal is cooled to cryogenic temperatures, around 100 Kelvin. In an effort to minimize crystal damage during cryogenic cooling, cryoprotectants are added to the crystallization reagent to prevent crystalline ice formation in the internal and external solution as well as at crystal-solution interfaces. Vitrification of the sample, the formation of an amorphous glass, or in essence, a clear drop after cryogenic cooling as well as a stable, diffracting crystal is the end result of a successful cryopreservation.

Without removal of the native mother liquor or addition of a suitable cryoprotectant, undesired ice nucleation can occur at the crystal surface, at the crystal solvent interface, and at fractures. One approach at crystal cryopreservation is to remove nearly all external and perhaps some internal solvent by transferring the crystal to oil such as Parabar 10312 (previously known as Paratone®), Paraffin, Santovac® or Perfluoropolyether or mixture thereof.<sup>19,35</sup> Another approach is to prevent, minimize or slow ice formation by adding cryoprotectants to the mother liquor solution (the combined crystallization reagent and protein that produce the crystal). In this instance both the surrounding solution as well as the internal solution may be cryopreserved to prevent ice formation. Both oil and miscible cryoprotection methods have been successfully applied to a broad range of crystalline biological macromolecules and are now widespread and common procedures in biological macromolecular crystallography.

## Selecting a Cryoprotectant

Just as with identifying and optimizing reagents for crystallization, the identification and optimization of a suitable cryoprotectant involves some trial and error as well as screening. A suitable cryoprotectant, when mixed with the crystal and crystallization reagent will cool to cryogenic temperature without ice formation and not damage the crystal.

## Cryoprotectant Assay

To assay for the proper concentration of cryoprotectant in the reagent used to grow the crystal, one can mix the cryoprotectant with the crystallization reagent and loop a small amount of this mixture using a CryoLoop. Next, the CryoLoop containing the mixture is cooled in a bath of liquid nitro-

gen or in a cryostream. One then inspects for ice formation either visually under a microscope or with X-ray diffraction. Upon cooling, a transparent drop and X-ray diffraction pattern mostly free of powder diffraction rings or “ice rings” indicates success where the appearance of a cloudy drop or “ice rings” indicates an inappropriate cryoprotectant concentration or cryoprotectant. Incrementally increase the concentration and/or alter composition of the cryoprotectant serially, 5 to 10% and repeat the procedure until the drop remains clear when cooled. Once a cooled, clear drop is achieved, this is typically a good starting point for cryopreservation of the crystal. Next, one needs to test the stability as well as the X-ray diffraction of the crystal in the cryoprotectant. It is not essential that these preliminary diagnostic tests be performed, but they can provide useful data towards identifying an appropriate cryoprotectant and concentration for your crystal. Typically, the addition of 10 to 30% cryoprotectant to the mother liquor will be sufficient as a reasonable starting point.

Some crystals can be dipped or washed quickly (2 seconds) in a simple cryoprotectant such as 30% Glycerol for successful cryopreservation. But, when this fails, a rational assay of each cryoprotectant with incremental increases or decreases in cryoprotectant concentration as well as a test of mixtures (for example a mixture of sugars, or a sugar mixed with Ethylene glycol) may be required to determine the best cryoprotectant for a crystal.

## Where to Begin

Which cryoprotectant should be tried first? Glycerol is often tried first and appears prominently in the literature. Ethylene glycol and MPD are also very popular. However, the selection of the appropriate class of cryoprotectant or cryoprotectant begins with knowing what reagent is used to grow the crystal. Crystals grown in polymers can often be cryoprotected by increasing the concentration of the polymer in the mother liquor by 5 to 10% above the concentration used to produce crystals. In other instances, a different polymer might be added, such as adding PEG 600 or MPD((+/-)-2-Methyl-2,4-pentanediol) to crystals grown in PEG 6,000, or adding Ethylene glycol or MPD to crystals grown in salt; or in some cases, by adding increasing amounts of glycerol or Ethylene glycol to crystals grown in either polymers or salts. Where volatile organic solvents are used to grow the crystal, evaluate increasing concentrations of the volatile organic to cryopreserve the crystal. When salts such as acetate, chloride, formate, nitrate, sulfate, Tacsimate™ and malonate are used for crystallization, once again, evaluate increasing concentrations of the respective salt concentration by 5 to 10% as a cryoprotectant.

Finally, simple sugars such as sucrose can be used alone or in combination with other cryoprotectants. In some instances a solvent exchange must be performed for successful cryopreservation of the crystal. Solvent exchange can be performed either through a series of soaks or dialysis.<sup>57</sup> In extreme cases it may be necessary to exchange the mother liquor with another crystallization reagent before successful cryopreservation is achieved.

## Cryoprotection in situ

Crystallization in the presence of a cryoprotectant is another option. All Crystal Screen Cryo™, Crystal Screen Cryo™ 2 and Crystal Screen Cryo HT™ reagents contain unique crystallization reagents with added cryoprotectant at a concentration which will form the desired clear, amorphous glass upon cryogenic cooling. Although this approach sounds simple and foolproof, it is not, as cryoprotectants can behave as solubility agents for some proteins, and precipitants for others, complicating the initial crystallization screen. The cryo based crystallization screens are a reasonable strategy as long as your protein will tolerate the presence of a cryoprotectant in the crystallization reagent. But one will not know, until one tries, if this approach will work for their protein. If crystals can be obtained in a crystallization screen formulated with cryoprotectant, this is ideal and most convenient. As already stated, in some instances this is not the case and hence one must begin the quest for crystal cryopreservation based upon initial crystallization conditions in the presence of added cryoprotectants.

## Crystallization Reagents as Cryoprotectants

Crystallization reagents such as polymers (Polyethylene glycol), organic acids (malonate),<sup>9,13</sup> halide salts (Lithium chloride),<sup>7</sup> and non-volatile (MPD) and volatile (2-Propanol) organic solvents can serve as cryoprotectants for biological macromolecular crystals. Typically, the appropriate cryoprotectant concentration of these reagents is slightly higher (10 to 20%) than that used for crystallization. If a crystal is grown using one of the reagents found in CryoPro, one can first check to see if the concentration of the cryoprotectant is high enough by X-ray diffraction analysis. If ice formation is present, one can attempt to prevent ice formation by increasing the concentration of the cryoprotectant and consider adding/mixing in additional cryoprotectants.

## Washing Crystals in Cryoprotectants

In some instances, successful cryoprotection of a crystal can be achieved following a brief wash in the crystal mother liquor with added cryoprotectant. Initial washes should be performed on expendable crystals, since inappropriate cryoprotectant conditions can damage the crystal. Save the best crystals for optimized wash protocols. In a wash procedure, the solvent layer at the surface of the crystal, where ice formation may start, is either removed or modified with a cryoprotectant. In this procedure a crystal is removed from the mother liquor drop which produced the crystal and transferred to a drop of mother liquor with added cryoprotectant. The transfer can be performed using a CryoLoop. The wash time can vary but typical wash times are brief, on the order of a single second. In a wash, the objective is to remove surface solvent, not soak the cryoprotectant into the crystal. A longer wash time would qualify as a soak.

## Soaking Crystals in Cryoprotectants

During a soak, the cryoprotectant is used not only to remove the surface solvent layer from the crystal, but with time there can be equilibration between the internal crystal solvent and the external cryoprotectant. The time

required for a given soak is dependent upon several variables, including the following:

- Concentration of cryoprotectant
- Nature of the crystal's solvent channels
- Molecular weight of cryoprotectant
  - Temperature
  - Size of the crystal
- Diffusion property of the cryoprotectant
  - Physical stability of the crystal
  - Hydration state of the crystal
- Nature of the crystallization reagent
  - Nature of solvent channels

Cryoprotectant soaks follow the same basic guidelines as soaks for heavy atoms, ligands, and inhibitors but may also add osmolality as a variable. Specific procedures may vary for each crystal. Initial soaks should be performed on expendable crystals, when available, since a non-optimized soaking procedure can damage the crystal. Save the best crystals for optimized soaking protocols. Soaking experiments are typically undertaken when brief washes fail to provide sufficient cryopreservation of the crystal. To begin, place the crystal in the desired cryoprotectant and crystallization reagent mixture. This can be as simple as placing a drop of the mixture on a siliconized glass slide or an empty drop well in a sitting drop crystallization plate. Observe the crystal for morphological changes (dissolution or cracking). If there are no visual changes, and perhaps even if there are changes (beauty is sometimes only skin deep for crystals—ugly crystals can diffract beautifully), test the crystal for diffraction. Soaks should be performed at the same temperature as the original crystallization. Soaks can require seconds or hours. For example, one may move incrementally through steps of 10% with successive soaks 1 to 2 minutes in length. In such an instance the crystal may be exposed to cryoprotectant for no more than 15 minutes during the entire serial soak. If, during this procedure isomorphism is uncovered during X-ray diffraction, or visible morphological changes take place, the soak time may need to be increased to say, 30 minutes for equilibration as the cryoprotectant concentration is adjusted. In some cases, extreme soaks (days or weeks) can be used to improve crystal diffraction by dehydration.<sup>36</sup> Soaks at 4°C can require twice as much time for equilibration compared to soaks at room temperature.

If, during the soak the crystal shows morphological changes (viewed microscopically), move the crystal to higher and higher concentrations in 5% increments (or whatever increment the crystal will tolerate) of the cryoprotectant until a concentration is found that does not result in morphological changes. If the crystal will not tolerate this serial soak procedure, try dialysis<sup>37</sup> using Dialysis Buttons™. Microdialysis will allow for slower equilibration of the cryoprotectant concentration within the crystal. Another advantage of microdialysis is that many cryoprotectants can be removed, added, or composition and concentration altered without direct handling of

the crystal. If microdialysis does not work, then the cryoprotection procedure should be started anew with a different cryoprotectant. The final measure of success is crystal diffraction with minimal increase in mosaicity.

### Crosslinking for Cryo

If serial soaking and microdialysis with a wide variety of cryoprotectants does not lead to a successfully cryopreserved crystal, one might consider stabilizing the crystal by chemically crosslinking the protein crystal before the cryopreservation procedure. The crystal can be crosslinked using glutaraldehyde or other suitable protein crosslinking reagent and then processed through the aforementioned cryopreservation procedures.<sup>29</sup>

### Cryo Tips

- If a quick wash or soak does not produce the desired results, consider spending the time uncovering the optimal cryoprotectant concentration with which to permeate the crystal, followed by the evaluation of oils to remove and replace the external liquid around the crystal with immiscible oil. Successful cryoprotection is the fine line between too little cryoprotection (ice formation) and too much cryoprotection (crystal damage).
- Non-isomorphism is sometimes caused by insufficient equilibration between the cryoprotectant and the crystal. Allow more time for equilibration during the serial wash, serial soak, or microdialysis.
- Crystal dissolution during washing or soaking is an indication of too low relative supersaturation. Increase protein and/or crystallization reagent concentration to maintain the relative supersaturation of the mother liquor during addition of cryoprotectant.
- Viscous polymers can form large drops and introduce extraneous material into the X-ray beam and lead to excessive background scattering as well as CryoLoop movement in the stream due to the excessive mass on the loop. Remove excess drop material prior to cryogenic cooling.
- Viscous polymers can deform thin needles and blades during cryogenic cooling. Consider alternative, less viscous cryoprotectants for more delicate crystal morphologies.
- Smaller crystals are generally more tolerant and easier to cryoprotect than larger crystals.
- Anneal the crystal in the cryostream. In a nutshell, interrupt/block the cryostream for 30 seconds, or remove the crystal from the cold stream and then replace the crystal in the cold stream.<sup>33,34</sup>
- Move quickly, the CryoLoop mounted crystal from crystallization drop to cryoprotectant. Move quickly, the CryoLoop mounted crystal from the cryoprotectant to the liquid nitrogen or cryostream.
- Short soak the crystal in cryoprotectant. Pass the CryoLoop mounted

crystal through the cryoprotectant solution, waiting 2 seconds or less before moving the crystal into a liquid nitrogen bath or the cryostream.

- Long soak the crystal in cryoprotectant. If the crystal is damaged after a short soak, try leaving the crystal in a cryoprotectant for 15, 30, or 60 minutes, or even 24 hours. Damaged crystals can sometimes be recovered.<sup>32</sup>

- Dilution & Equilibration. In an attempt to minimize osmotic shock to the crystal, bring the crystal to the final, desired cryoprotectant concentration in small steps. If the final cryoprotectant concentration is 30%, formulate 6 reagents with increasing concentrations of cryoprotectant; 5, 10, 15, 20, 25, and 30%. Mount the crystal in a CryoLoop and transfer to the 5% cryoprotectant reagent. Seal to minimize evaporation. After 30 minutes move to the next higher concentration of cryoprotectant reagent (10%). Repeat this procedure until the crystal is in the 30% cryoprotectant reagent. Transfer the crystal to the liquid nitrogen bath or cryostream.<sup>36</sup>

- When single component cryoprotectant reagents do not produce the desired result, evaluate different mixtures of miscible cryoprotectants, such as 5% Glycerol, 5% Glucose, 5% Xylitol, 5% Ethylene glycol.<sup>35</sup>

### Formulation Note

Some CryoPro reagents are formulated very near their point of saturation at room temperature (approximately 25 degrees Celsius). If precipitate or crystalline material appears in the tube following transport or storage, warm the solution up to 50 degrees Celsius to solubilize the reagent. Often time reagent solubility can be achieved with a pair of warm hands rolling closed tube in hand for 5 to 10 minutes time.

### Ten Things about Cryoprotection

1. For any and all crystallization reagents try adding glycerol to your crystallization condition such that the final concentration is between 5 and 30% v/v.
2. For any and all crystallization reagents try dragging the crystal through oil (Perfluoropolyether Cryo Oil, Paraffin Oil, Santovac Cryo Oil, Parabar 10312 (Paratone), or NVH).
3. For Polyethylene glycol (PEG) based crystallization reagents where the  $M_r < 5,000$  try increasing the concentration of the PEG or adding a low molecular weight PEG such as PEG 400.
4. For Polyethylene glycol (PEG) based crystallization reagents where the  $M_r > 5,000$  try adding a low molecular weight PEG such as PEG 400.
5. For Crystal Screen reagents look at the same reagent number in Crystal Screen Cryo and go there.
6. For Crystal Screen 2 reagents see reference 12 and go there.
7. For MPD based crystallization reagents try increasing the concentration of MPD.
8. For salt based crystallization reagent try increasing the concentration of salt (particularly malonate, formate and other organic acids, as well as the salt lithium sulfate).
9. When nothing else works, try 20% 2,3-Butanediol. If that does not work try oils (Perfluoropolyether Cryo Oil, Paraffin Oil, Santovac Cryo Oil, Parabar 10312 (Paratone), or NVH). If that does not work, collect data at room temperature in a quartz capillary.
10. If you cannot achieve the desired X-ray diffraction resolution with various cryogenic methodologies, you might try checking how well the crystal diffracts at room temperature using a capillary mount. If room temperature diffraction is also poor, the problem may be the crystal, not the cryoprotectant.

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### Technical Support

Inquiries regarding CryoPro reagent formulation, interpretation of screen results, optimization strategies and general inquiries regarding crystallization are welcome. Please e-mail, fax, or telephone your request to Hampton Research. Fax and e-mail Technical Support are available 24 hours a day. Telephone technical support is available 8:00 a.m. to 4:30 p.m. USA Pacific Standard Time.

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