

User Guide

The Hampton Research CryoPro – Cryo-Protectant Kit consists of 36 preformulated, sterile filtered reagents for cryoprotection of biological macromolecular crystals. 35 of the 36 reagents are supplied in 10 ml volumes, a single reagent (L-(+)-2,3-butanediol) is supplied in a 0.2 ml aliquot. CryoPro is a convenient, cost-effective kit of pre-formulated novel cryoprotectants for biological macromolecular crystals.

Attention

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

CryoPro reagents are sterile filtered and do not contain preservatives nor anti-microbial agents. Sterile technique and proper storage will help to ensure the integrity and longevity of the CryoPro reagents. When possible use clean, sterile pipet tips to aspirate CryoPro solutions, especially the sugars and polymers.

Cryocrystallography Background

Low temperature X-ray diffraction methods, or 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, provide for gentler crystal mounting, and convenient, stable transport of the crystal. Cryocrystallography allows for automated sample processing at synchrotron facilities around the world for efficient transport, handling, and processing of crystals for cryocrystallographic data collection.

During cryocrystallography the crystal is cooled to cryogenic temperatures. In an effort to minimize crystal damage during cryogenic cooling, cryoprotective reagents are added to the solution 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 cryo cooling as well as a stable, diffracting crystal is the end result of a successful cryopreservation.

One approach at crystal cryopreservation is to remove nearly all external and some internal solution by transferring the crystal to an oil (Paratone) or other cryoprotective reagent without removal of excess mother liquor solvent on and about the crystal. Undesired ice nucleation can occur at the crystal surface, at the crystal solvent interface, and at fractures without removal of the native mother liquor or addition of a suitable cryoprotectant. Another approach is to prevent, minimize or slow ice formation by adding cryoprotectants to the mother liquor solution. In the instance both the surrounding solution as well as the internal solution may be cryopreserved to prevent ice formation. These procedures have been successfully applied to a broad range of biological macromolecules and is now a widespread and common procedure in biological macromolecular crystallography.

CryoPro Cryoprotectant Reagents

CryoPro contains 36 unique cryoprotectant reagents in preformulated, sterile filtered, ready to use solutions. See the accompanying insert for complete reagent and formulation details. All of the cryoprotectant reagents except for Paratone Oil, Paraffin Oil and NVH Oil are water soluble compounds.

2,3-Butanediol exists as three different stereochemical isomers, one of which, the meso form, can form hydrates which crystallize on cooling and even more rapidly

on warming. Not only the presence of certain chemical groups can influence the cryopreservative process, but the arrangement of these chemical groups can influence the water-solute hydrogen bonding network. The L-(+)-2,3-butanediol stereoisomer is supplied in a small aliquot in CryoPro due to the high cost of this material and the interest of offering a cost effective screen. Consider evaluating the 2,3-Butanediol isomer first and then as appropriate the L-(+)-2,3-butanediol stereoisomer as a cryoprotectant.

Using The CryoPro Reagents

Selecting a Cryoprotectant Reagent

As in selecting reagents for crystallization, the selection of a suitable cryoprotectant involves some trial and error as well as a 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. 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, employ the desired cooling method (for example, place the solution in a CryoLoop and place the CryoLoop in a cryostream) and observe for ice formation either visually or with X-ray diffraction. Upon cooling, a transparent drop and X-ray diffraction pattern 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 composition of the cryoprotectant serially 5 to 10% and repeat until the cooled drop remains clear while in the cryostream. Once a clear drop is achieved in the cryostream, this is typically a good starting point for cryopreservation of the crystal.

In the end, it is not 100% essential that these preliminary tests be performed. Typically the addition of 15 to 30% cryoprotectant to the mother liquor will be sufficient as a reasonable starting point.

Some crystals can simply be dipped or washed quickly in a simple cryoprotectant such as 30% glycerol for successful cryopreservation. But, when all else fails, a rational assay of each cryoprotectant with incremental increases 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.

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 begin with knowing what reagent is used to grow the crystal. Crystals grown in polymers can often be cryoprotected by simply increasing the concentration of the polymer in the mother liquor by 5 to 10%. In other instances, a different polymer might be added, such as adding PEG 600 or MPD to crystals grown in PEG 6000, or adding ethylene glycol or MPD to crystals grown in salt, or in many cases 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 acetates, chlorides, formates, nitrates, sulfates, and malonates are used for crystallization, once again, evaluate increasing concentrations of the respective salt as a cryoprotectant. Simple sugars such as sucrose, xylitol, etc can be used along 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. See "Soaking Crystals" below. In extreme cases it may be necessary to exchange the mother liquor with another crystallization reagent before successful cryopreservation is achieved.

Crystallization in a cryoprotective reagent is another option. Crystal Screen Cryo from Hampton Research contains 50 unique crystallization reagents with added cryoprotectant at a concentration which will form an amorphous glass upon cryo cooling. However, cryoprotectants can behave as solubilization agents for some crystallization samples and precipitation agents for others, possibly complicating the initial crystallization screen. If crystals can be obtained in a crystallization screen preformulated with cryoprotectant, this is ideal and most convenient. However, in many instances this is not the case and hence one must begin the quest for crystal cryopreservation based upon initial crystallization conditions and added cryoprotectants.

Cryoprotectant Procedures Using Oil

Treating a crystal with oil (Paratone, Paraffin and NVH) can remove water from the surface of the crystal which will prevent destructive ice formation in the aqueous layer, at the crystal surface, and in fractures. The oils supplied in CryoPro have low optical distortion, no crystal formation during cooling and low scattering. These oils are also free of Si and F which have relatively high scattering. In an oil based cryo procedure a crystal is placed in a small drop of oil using a CryoLoop. In some instances it might be easier to place a drop of mother liquor containing the crystal into 500 microliters of oil on a glass cover slide. The water can be removed using a Paper Wick, filter paper, MicroWick, or needle by teasing the crystal away from the liquid, or the liquid away from the crystal. Once the water is removed from the surface of the crystal, the oil coating protects the crystal from loss of internal water and excessive dehydration. However, in some instances the oil can absorb water and dehydrate the crystal when the crystal is left in the oil for an extended period of time. Typically it is not necessary to remove the oil from the crystal for diffraction analysis. Crystals with low mechanical strength can break during oil treatment. Proteins and crystals with extreme hydrophilic properties can make surface water removal difficult. For fragile and hydrophilic crystals, attempt cryoprotection by modifying the solution with a cryoprotective reagent. Cracking of the crystal during the oil based procedure can be an indication of rough handling, excessive mother liquor removal or crystal drying.

Crystallization Reagents as Cryoprotectants

Crystallization reagents such as polymers (polyethylene glycols), organic acids (malonates), halide salts in high concentration, and non-volatile (MPD) and volatile (isopropanol) organic solvents can serve as cryoprotectants for biological macromolecular crystals. Typically, the appropriate cryoprotectant concentration of these reagents is slightly higher 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. Ideally, one might wish to refer to "Selecting a Cryoprotectant Reagent" above to determine the "best" cryoreagent and cryoreagent concentration for the crystallization reagent, but

more and more often, researchers are resorting to the "wash" or "quick dip" in a cryoprotectant solution. Initial washes should be performed on expendable crystals, when available since inappropriate cryoprotectant conditions can damage the crystal. Save the best crystals for optimized washing 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 seconds to less than a minute. In 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, but time is allowed for equilibration between the internal crystal solvent and the cryoprotectant. The time required for a given soak is dependent upon several variables:

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

Cryoprotectant soaks follow the same basic guidelines as other soaks (heavy atoms, ligands, inhibitors). 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, a post in a Cryschem plate, or the ledge of a 96 well crystallization plate. Observe the crystal for morphological changes (dissolution or cracking). If no changes appear, or even if they do, test the crystal for diffraction (beauty is sometimes only skin deep for crystals – ugly crystals can diffract!). Soaks should be performed at the same temperature as crystallization. Soaks can require minutes or hours. For example, one may move incrementally through steps of 5 to 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 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 each step or the percent increment in cryoprotectant adjusted slightly. In some cases, extreme soaks (days or weeks) can be used to improve crystal diffraction by dehydration, but this is beyond the scope of this discussion. Soaks at 4 degrees Celsius 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 what

ever the crystal will tolerate) of the cryoprotectant until a concentration is found that does not cause morphological changes. If the crystal will not tolerate this serial soak procedure, try dialysis using Microdialysis Buttons. Microdialysis will allow for slower equilibration of the cryoprotectant concentration within the crystal. Another advantage of microdialysis is that the cryopreservation reagent can be removed, added, or altered without direct handling of the crystal. If microdialysis does not work, then the cryo procedure should be started anew with a different cryoprotectant. The final measure of success is crystal diffraction with minimal increase in mosaicity.

Crystal Crosslinking and Cryopreservation

If serial soaking and microdialysis with a wide variety of cryoprotective reagents 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 procedure(s).

Troubleshooting

Non-isomorphism is often 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.

Paratone, oils, and 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 cryocooling.

Paratone, oils, and viscous polymers can deform thin needles and blades during cryocooling. Consider alternative, less viscous cryoprotective reagents for these crystal morphologies.

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 during transport or storage, simply warm the solution to slightly above room temperature to solubilize the reagent. Often times this can be effected with a pair of warm hands rolling closed tube in hands for 5 to 10 minutes time.

References and Readings

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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 5:00 p.m. USA Pacific Standard Time.

Individual CryoPro reagents can be ordered from Hampton Research through the CustomShop lab. Contact Hampton Research Technical Support or Customer Service for additional ordering information.

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