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**Crystallization Cryo Kit for Proteins**
Product Number **75403**
Store at 2-8 °C

# TECHNICAL BULLETIN

## Application

The Crystallization Cryo Kit for Proteins is a rapid empirical screening method to determine the best conditions for the crystallization of biological macromolecules like proteins, etc. in the presence of cryoprotectant. Crystallization Cryo Kit for Proteins is also effective in determining the solubility of a macromolecule in a wide range of precipitants and pH.

The Crystallization Cryo Kit for Proteins is an easy, modified screen to find crystallization conditions based upon the original screening method of Jancarik and Kim (1). The difference from the original screening method is the presence of glycerol as a cryoprotectant.
The reason for using a cryoprotectant is to prevent radiation damage to protein crystals during data collection. Crystals are frozen and maintained at –173°C under a stream of nitrogen. To prevent ice crystals from forming during the process of freezing it is necessary to soak crystals in a series of solutions with increasing cryoprotectant concentration. This kit contains reagents and solutions formulated to form an amorphous glass upon cooling, the drop containing the protein crystal and mother liquor will remain clear and no “ice rings” appear in the diffraction pattern.
The solution and crystallization conditions are empirically derived based on known or published crystallization conditions of various proteins in the past, so as to sample as large a range of buffer, pH, precipitant (salts, polymers, volatile organics, and non-volatile organics) and cryoprotectant variables as possible, using small amounts of proteins.

## Quality of reagents – the key to success

Crystallization Cryo Kit for Proteins reagents are formulated using high purity reagents (mostly Biochemika ultra/MicroSelect from Fluka). These reagents are specially purified and analyzed to ensure the absence of any significant traces of ions or other impurities. This enables the reliable and precise formulation of crystallization conditions as required for best results. There are many instances where the Biochemika ultra/MicroSelect chemicals have successfully been used for different crystallization methods. All solutions are sterile filtered using 0.22 micron filters.
The kit contains 10 ml of each component, but all solutions are available separately as 100 ml bottles. Larger quantities are available on request.

## Precautions and Disclaimer

This product is for laboratory research use only. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

## Storage/Stability

It is recommended that the reagents of this kit be stored at 2-8 °C. Storage at –20 °C will not adversely affect the kit reagents and the reagents as supplied are stable at room temperature for short-term storage. Kit reagents should not be set under ultraviolet light to protect them from microorganisms. Since kit component 80924 (HEPES-Na pH 7.5; 0.075M – Sodium phosphate monobasic 0.6M – Potassium phosphate monobasic 0.6M – Glycerol 25% (v/v) solution) show the tendency to form crystals during storage at 2 –8°C, it is recommend to warm the solution for a couples of minutes up to about 30 – 40°C until a clear solutions is created.

## Sample Preparation Instruction

The sample has to be as pure as possible and free of amorphous material or other particles. The purity should be >90% when stained with Coomassie on an SDS gel. Amorphous material

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can be removed by centrifugation or micro-filtration ( 2, 3, 4, 5). For the storage of protein it is recommended to have a high concentration, for example 5 mg/ml. The temperature depends on the protein, generally cells or bacteria tolerate freezing at –70 °C better than purified proteins. A method to do so is to pipet the sample directly into liquid nitrogen (5).

Sample concentrations used in practice vary widely, but concentrations of 5 to 10 mg/ml have been used most frequently and may be useful as a starting point. If possible the sample should just contain water and the macromolecule. Unnecessary additives can falsify the result. In case disturbing ions, reducing agents, ligands or other additives are present, they may be removed by dialysis of sample against water. The purification or concentration by ammonium sulfate precipitation is a poor choice because after this step it is nearly impossible to remove the salt by dialyzing or with a desalting column.

*Note:*
The follow Ions could be responsible for inorganic crystals forming due to the presence of bivalent cations: $CO_3^{2-}$, $BO_3^{3-}$, $PO_4^{3-}$.
Example of bivalent cations: $Zn^{2+}$, $Ca^{2+}$, $Mg^{2+}$
Concentrations of less than 10 mM are not a problem.

**Procedures**

The application method described below is the most common method of crystallization: the Hanging Drop Vapor Diffusion method. Other methods like the Sitting Drop, Microdialysis, MicroBatch. and Sandwich Drop methods are also applicable for the Crystallization Cryo Kit for Proteins. The sitting drop method is being used more and more. It will probably gain further importance since the hanging drop procedure is difficult to use with automated procedures. Directions for the Hanging, Sitting Drop and other crystallization methods are available from Fluka Technical Service.

1. Take three 24-Well plates (from Stratech, ICN Biomedicals, Costar, Falcon, Molecular Dimensions Ltd.). It is recommended that on each upper edge of the 24 wells should be put a thin film of grease. This ensures that the cover slide doesn't shift. Prepare fifty wells for one full screen of a protein. See figure 1.

2. Pipet 800 µl of each reagent into the fifty wells. *Use always a clean pipet tip for each reagent!*

3. 2 µl of sample are put into the middle of a clean, siliconized 22 mm (18 mm if you use Costar plate) diameter cover slide (Molecular Dimensions Ltd, Stratech). See figure 1. It is also possible to take a square cover slide.

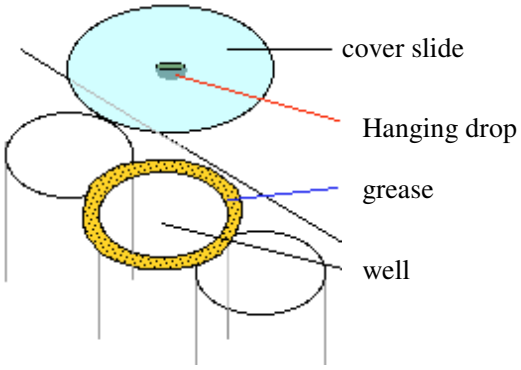


figure 1: well plate

4. Pipet 2 µl of the corresponding crystallization reagent from each well into the sample droplet. Mix with caution by dispensing and aspirating the droplet with the pipet. Avoid foaming by keeping the tip in the drop.

5. Invert the cover slide and droplet directly over the well containing the appropriate reagent and place the cover slide on top of the well. Make sure the grease seal is complete between the cover slide and the edge of the well.

6. Repeat steps 3. to 5. for all reagents.

7. A recommended practice is to perform the crystallization screen at 4 °C and at room temperature if there is enough sample. Incubate and store the plates in a place with stable temperature and free of vibration.

Sample solubility is also temperature dependent. Although most crystallizations have been achieved at room temperature, in many cases different temperatures have led to success. Comparison of results of screening at two different temperatures (4 °C and room temperature) helps determine the magnitude of temperature effects on sample solubility. Temperature may be an important parameter in the optimization procedure.

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## Observations

Drops are typically observed by a stereo microscope at 10 to 100X. Record all observations by scanning every droplet on the slides.

Scan the focal plane for small crystals and record observations for all droplets. Scan the first time shortly after the screen is set up. Then for the first 5-10 days, information may be recorded daily and, thereafter, on a weekly basis. Records should include the clarity of the droplet (clear, precipitate, or crystals), along with descriptive phrases and a numerical scale. The following are possible examples (see also observation sheet):
10(= crystal grown 1 D) shower of needles, yellow
6(= gelatinous protein precipitate) red/brown
1(= drop is clear), green
7 (= fully precipitated dark color) dark green
It can also be useful to write down the largest crystal size!

## Results and Interpretation

A clear drop may be an indication that the drop has not yet reached its final state. If the drop remains clear after 2 to 4 weeks, the relative sample and reagent supersaturation may be too low. If a majority of drops remain clear, consider repeating the entire screen using a protein sample at higher protein concentration.

There are several reasons for precipitation in a drop. A precipitate can indicate that the sample or precipitant concentration is too high (precipitation within 1 day) or it is not the preferred crystallization condition (within a few days). In the case of too high concentration repeat the screen with lower protein concentration. If more than a majority of drops contain a precipitate with no crystals present, consider a dilution of the sample and repeating the entire screen. In the case of precipitation problems for several screens, it may be useful to dilute the precipitant in the reagent. Precipitation may also be an indication that the target protein has denatured. It may be necessary to take steps to stabilize the target protein. These could include the addition of additives like salts, reducing agent, glycerol, ligands, non-detergent sulfobetaine or other appropriate stabilizing reagents.
If you should have only denatured protein left, you may use the Renaturation Basic Kit for Proteins (96827) to find appropriate renaturation conditions. Sample purity may also cause precipitation. Low sample purity, aggregation, or a heterogeneous preparation may be responsible for precipitation. In these cases, further sample purification is required. It is possible that a crystal may form out of a precipitate. Crystals can grow extremely fast, in few minutes, or may require much more time, up to a few months. This is the reason that crystallization plates should never be trashed, or a drop disregarded too early. Store and record the plates until the drops are dried out.

It is recommended that a high power microscope be used to examine the precipitate between crossed polarizing lenses. True amorphous precipitates do not glow. Birefringent microcrystalline precipitates can glow as a result of the plane of light polarization. It may be possible to use streak seeding to produce larger crystals from microcrystalline precipitates.

Screens, which produce crystals, provide the first clues regarding conditions for crystallization. It may be necessary to optimize these conditions to produce crystals with the proper size and quantity for analysis. The following parameters should be considered during optimization: pH, salt type and concentration, precipitant type and concentration, temperature, sample concentration, and other additives.

## Considerations for the Concentration of Cryoprotectant

The ideal cyroprotected crystallization reagent will form an amorphous glass at –173 °C, without ice damage to the crystal or observed “ice bands”. If the crystal breaks or the drop has a milky appearance upon freezing, try to increase concentration of cryoprotectant in the reagent. Alternatively, adjust the concentration of the screen reagent components. You may have to evaluate different crystallization reagents and test other cryoprotectants. The following small molecules are also frequently used as cryoprotectants:

| Fluka | cryoprotectant |
|---|---|
| 49769 | Glycerol |
| 03747 | Ethylene glycol |
| 45670 | Erythritol |
| 49140 | Glucose |
| 18965 | 2R,3R-(-)-Butane-2,3-diol |
| 81170 | PEG-400 |
| 95369 | Paraffin |
| 59304 | 2-propanol |
| 68338 | 2-Methyl-2,4-pentandiol (MPD) |
| 84099 | Sucrose |

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The appropriate cryoprotectant to use, amount of soak time, final cryoprotectant concentration, and the rate at which the cryoprotectant is increased must be determined by the user by exposing a frozen loop containing the final cryoprotectant solution but no crystal.

**References**

1. Sparse Matrix Sampling: a screening method for crystallization of proteins. Jancarik, J. and Kim, S.H. J. Appl. Oryst., 24, 409-411, 1991.
2. Crystallization of nucleic acids and proteins, Edited by A. Ducruix and R. Giege, The Practical Approach Series, Oxford Univ. Press, 1992.
3. Current approaches to macromolecular crystallization. McPherson, A. Eur. J. Biochem. 189,1.23, 1990.
4. Protein and Nucleic Acid Crystallization. Methods, A Companion to Methods in Enzymology, Academic Press, Volume 1, Number 1, August 1990.
5. Garman, E.F. and Mitchell, E.P., J. Appl. Cryst. (1996) 29, 584-587.
6. Riboldi-Tunnicliffe, et al., Cryocrystallography with oil - an old idea revived. J. Appl. Cryst. 32:1003 (1999)
7. Panjikar, et al., Use of dry paraffin oil and Panjelly in the xenon derivatization of protein crystals. J. Appl. Cryst. 35:117(2002)
8. Sack S., et al., Crystallization and Preliminary X-Ray Diffraction Analysis of UDP- N-acetylglucosamine Enolpyruvyltransferase of Enterobacter cloacae., J. of Structural Biol., 117, 73-76 (1996)

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# 75403 Crystallization Cryo Kit for Proteins Observation Sheet

Sample description: _____ Date: _____
concentration: _____ Incubation Temperature: _____
buffer: _____ Reservoir Volume: _____
1 Drop contains : Crystallization Reagent_____ul Sample_____ul Additive (name)_____(volume)_____ul

1 drop is clear
2 drop contains non-protein particles

**precipitate without birefringent and edges**
3 mostly clear drop
4 fully precipitated dark colour
5 gelatinous protein precipitate
6 phase separation

**precipitates shows birefringent or has edges**
7 sperulites or small structures maybe edges
8 crystal grown 1 D
9 crystal grown 2 D
10 crystal grown 3 D

| No. | Fluka No. | Name | Date: | Date: | Date: | Date: | Date: |
|---|---|---|---|---|---|---|---|
| 1. | 92599 | Ca-chloride 0.02M, Na-acetate (pH 4.3) 0.1M, 2-Methyl-2,4-pentanediol 30% | | | | | |
| 2. | 96864 | K-,Na-tartrate 0.26M, Glycerol 35% | | | | | |
| 3. | 96347 | $NH_4$-phosphate 0.26M, Glycerol 35% | | | | | |
| 4. | 94030 | $NH_4$-sulfate 1.5M, TRIS-HCl (pH 8.5) 0.075M, Glycerol 25% | | | | | |
| 5. | 93949 | Na-citrate 0.2M, HEPES Na-salt (pH 7.5) 0.1M, 2-Methyl-2,4-pentanediol 30% | | | | | |
| 6. | 93189 | Mg-chloride 0.16M, TRIS-HCl (pH 8.5) 0.08M, PEG 4000 24%, Glycerol 20% | | | | | |
| 7. | 79973 | Na-acetate 0.98M, Na-cacodylate (pH 6.5) 0.07M, Glycerol 30% | | | | | |
| 8. | 74614 | Na-citrate 0.14M, Na-cacodylate (pH 6.5) 0.07M, 2-Propanol 21%, Glycerol 30% | | | | | |
| 9. | 77437 | $NH_4$-acetate 0.17M, Na-citrate (pH 5.6) 0.085M, PEG 4000 25.5%, Glycerol 15% | | | | | |
| 10. | 77582 | $NH_4$-acetate 0.17M, Na-acetate (pH 4.6) 0.085M, PEG 4000 25.5%, Glycerol 15% | | | | | |
| 11. | 72871 | $NH_4$-dihydrogenphosphate 0.7M, Na-citrate (pH 5.6) 0.07M, Glycerol 30% | | | | | |
| 12. | 72872 | Mg-chloride 0.18M, HEPES Na-salt (pH 7.5) 0.09M, 2-Propanol 27%, Glycerol 10% | | | | | |
| 13. | 80055 | Na-citrate 0.2M, TRIS-HCl (pH 8.5) 0.1M, PEG 400 30% | | | | | |
| 14. | 80556 | Ca-chloride 0.19M, HEPES Na-salt (pH 7.5) 0.095M, PEG 400 26.6%, Glycerol 5% | | | | | |
| 15. | 80074 | $NH_4$-sulfate 0.17M, Na-cacodylate (pH 6.5) 0.085M, PEG 8000 25.5%, Glycerol 15% | | | | | |
| 16. | 80084 | Li-sulfate 1.125M, HEPES Na-salt (pH 7.5) 0.075M, Glycerol 25% | | | | | |
| 17. | 80091 | Li-sulfate 0.17M, TRIS-HCl (pH 8.5) 0.085M, PEG 4000 25.5%, Glycerol 15% | | | | | |
| 18. | 80093 | Mg-acetate 0.16M, Na-cacodylate (pH 6.5) 0.08M, PEG 8000 16%, Glycerol 20% | | | | | |
| 19. | 74613 | $NH_4$-acetate 0.16M, TRIS-HCl (pH 8.5) 0.08M, 2-Propanol 24%, Glycerol 20% | | | | | |
| 20. | 80053 | $NH_4$-sulfate 0.16M, Na-acetate (pH 4.6) 0.08M, PEG 4000 20%, Glycerol 20% | | | | | |
| 21. | 79974 | Mg-acetate 0.2M, Na-cacodylate (pH 6.5) 0.1M, 2-Methyl-2,4-pentanediol 30% | | | | | |
| 22. | 73803 | Na-acetate 0.17M, TRIS-HCl (pH 8.5) 0.085M, PEG 4000 25.5%, Glycerol 15% | | | | | |
| 23. | 76525 | Mg-chloride 0.2M, HEPES Na-salt (pH 7.5) 0.1M, PEG 400 30% | | | | | |
| 24. | 73806 | Ca-chloride 0.14M, Na-acetate (pH 4.6) 0.07M, 2-Propanol 14%, Glycerol 30% | | | | | |
| 25. | 71591 | Na-acetate 0.7M, Imidazole (pH 6.5) 0.07M, Glycerol 30% | | | | | |
| 26. | 76658 | $NH_4$-acetate 0.2M, Na-citrate (pH 5.6) 0.1M, 2-Methyl-2,4-pentanediol 30% | | | | | |
| 27. | 80384 | Na-citrate 0.14M, HEPES Na-salt (pH 7.5) 0.07M, 2-Propanol 14%, Glycerol 30% | | | | | |
| 28. | 80387 | Na-acetate 0.17M, Na-cacodylate (pH 6.5) 0.085M, PEG 8000 25.5%, Glycerol 15% | | | | | |
| 29. | 76659 | K-,Na-tartrate 0.52M, HEPES Na-salt (pH 7.5) 0.065M, Glycerol 35% | | | | | |
| 30. | 80803 | $NH_4$-sulfate 0.17M, PEG 8000 25.5%, Glycerol 15% | | | | | |
| 31. | 87429 | $NH_4$-sulfate 0.17M, PEG 4000 25.5%, Glycerol 15% | | | | | |
| 32. | 89252 | $NH_4$-sulfate 1.5M, Glycerol 25% | | | | | |
| 33. | 81892 | Na-formate 3.6M, Glycerol 10% | | | | | |
| 34. | 81893 | Na-formate 1.4M, Na-acetate (pH 4.6) 0.07M, Glycerol 30% | | | | | |
| 35. | 80924 | Na-,K-dihydrogenphosphate each 0.6M, HEPES Na-salt (pH 7.5) 0.075M, Glycerol 25% | | | | | |
| 36. | 89725 | TRIS-HCl (pH 8.5) 0.065M, PEG 8000 5.2%, Glycerol 35% | | | | | |
| 37. | 82216 | Na-acetate (pH 4.6) 0.07M, PEG 4000 5.6%, Glycerol 30% | | | | | |
| 38. | 89734 | Na-citrate 1.26M, HEPES Na-salt (pH 7.5) 0.09M, Glycerol 10% | | | | | |
| 39. | 80864 | $NH_4$-sulfate 1.7M, HEPES Na-salt (pH 7.5) 0.085M, PEG 400 1.7%, Glycerol 15% | | | | | |
| 40. | 91959 | Na-citrate (pH 5.6) 0.095M, 2-Propanol 19%, PEG 4000 19%, Glycerol 5% | | | | | |
| 41. | 82809 | HEPES Na-salt (pH 7.5) 0.085M, 2-Propanol 8.5%, PEG 4000 17%, Glycerol 15% | | | | | |
| 42. | 83617 | K-dihydrogenphosphate 0.04M, PEG 8000 16%, Glycerol 20% | | | | | |
| 43. | 90213 | PEG 1500 24%, Glycerin 20% | | | | | |
| 44. | 91638 | Mg-formate 0.1M, Glycerol 50% | | | | | |
| 45. | 83198 | Zn-acetate 0.16M, Na-cacodylate (pH 6.5) 0.08M, PEG 8000 14.4%, Glycerol 20% | | | | | |
| 46. | 84461 | Ca-acetate 0.16M, Na-cacodylate (pH 6.5) 0.08M, PEG 8000 14.4%, Glycerol 20% | | | | | |
| 47. | 83196 | $NH_4$-sulfate 1.6M, Na-acetate (pH 4.6) 0.08M, Glycerol 20% | | | | | |
| 48. | 84796 | $NH_4$-dihydrogenphosphate 1.6M, TRIS-HCl (pH 8.5) 0.08M, Glycerol 20% | | | | | |
| 49. | 81696 | Li-sulfate 0.8M, PEG 8000 1.6%, Glycerol 20% | | | | | |
| 50. | 81713 | Li-sulfate 0.4M, PEG 8000 12%, Glycerol 20% | | | | | |

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