



Crystallization Basic Kit for Membrane Proteins

Product Number **73513**

Store at 4 °C

TECHNICAL BULLETIN

Application

Crystallization Basic Kit for Membrane Proteins is a rapid empirical screening method to determine the best conditions for the crystallization of membrane proteins and biological macromolecules. This Kit can also be used for determining the solubility of a certain macromolecule, screening a wide range of pH and different types and concentration of precipitants.

Membrane proteins are often difficult to crystallize. The hydrophobic nature of these proteins leads to amorphous aggregation in aqueous solutions, rather than crystal formation. These proteins are routinely purified with a detergent, which provides the best protein stability and activity in solution. The presence of detergents increases the number of variables critical to crystallization for membrane proteins compared to soluble proteins. The reagents in this kit are specifically formulated for screening of crystallization conditions in conjunction with the screening of crystallization detergents.

The reagents of this kit vary in pH, along with changes in the concentration and content of buffer, salt, and precipitant. The buffers, sodium acetate, sodium citrate, ADA, HEPES sodium salt, and Tris-HCl, allow testing at five different pH values, 4.6, 5.6, 6.5, 7.5, and 8.5, respectively. Individual volatile agents, non-volatile agents, and salts, along with combinations of these three, comprise the four groups of precipitating agents.

Quality of reagents – a 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 experiences 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 4 °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.

Sample Preparation Instruction

The sample has to be as pure as possible and free of amorphous material or other particles. The purity should be at least >90% when stained with coomassie on an SDS gel. Amorphous material or precipitates should be removed by centrifugation or micro-filtration (2, 3, 4, 5).

For the storage of membrane protein it is recommended to have a high concentration, for example 5 mg/ml. The temperature depending 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 direct into liquid nitrogen (5).

The recommended membrane protein concentration is 10-20 mg/ml in water or sample buffer. The sample buffer concentration should be 10-20 times lower than the buffer concentration used in the well. (5-10 mM). The salt concentration should be as low as possible. Drops with PEG will increase in size when there is too much salt in the protein solution. The typical detergent concentration should only be about 2-3 times above the critical micelle concentration (CMC).

Procedures

The following procedure describes the use of Crystallization Basic Kit for Membrane Proteins with the Sitting Drop Vapor Diffusion method. It's also usual to use the Hanging Drop method for the Crystallization Kit for Membrane Proteins. Directions for the Hanging, Sitting Drop and other crystallization methods are available from Fluka Techservice.

1. Prepare a Cryschem Plate (from Emerald BioStructures) or MVD/24 plate (from Charles Supper Company) for Sitting Drop Vapor Diffusion. If using cover slides 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. There is no need for cover slides if sealing tape is used. 50 wells are to be prepared for a complete Crystallization Basic Kit for Membrane Proteins. See figure 1 and 2.
2. Pipet 800 μ l of each reagent into the fifty wells. *Use always a clean pipet tip for each reagent!*
3. Pipet 2 μ l of the sample into the dents of the crystallization post of the plate. See figure 2.
4. It's possible to pipet the detergent directly into the sample, or dilute the detergent in the reservoir and then pipet the solution of the reservoir into the drop of the sample. Because of the prior equilibration with the reservoir, the crystallization screening detergent concentration in the drop should be 1 to 3 times the critical micelle concentration. When the detergent is directly placed into the drop, pipet 1 μ l of the corresponding crystallization screening detergent (suggested stock detergent concentration equal to ten times the critical

micelle concentration) into the 2 μ l sample drop.



Figure 1

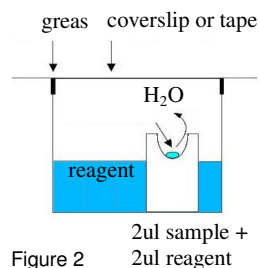


Figure 2

5. Pipet 2 μ l 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.
6. Repeat steps 3. to 5. for all reagents.
7. Either seal the entire plate with clear sealing tape (Stratech, Molecular Dimension Ltd) or seal the individual wells with cover slides and sealants.
8. A recommendable possibility is to accomplish the Crystallization Kit both 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.

Influence of Detergent

The Crystallization Basic Kit for Membrane Proteins allows for sparse matrix sampling for screening of crystallization reagents with a given crystallization detergent. Important properties to be considered during the selection of detergents for use in crystallization screening are the CMC, micelle size, molecular weight, and the hydrophobic tail of the detergent molecule, which binds to the hydrophobic regions of the protein. These properties can change several parameters (protein-protein, protein-detergent, protein-

solvent, and detergent-detergent interactions) of the sample liquid dynamics.

The detergent dimension in solution (micelle size) is a parameter critical in membrane protein crystallization. It is recommended to start screening with detergents, which have the smallest micelle sizes and progress to those with larger micelle sizes. A screen of the reagents in this kit should be performed for each detergent screened. For example, the following order would be suggested:

ethylene glycol (Fluka 03747)
 2-methyl-2,4-pentandiol (Fluka 68338)
 1,2,3 heptanetriol (Fluka 51845, 51846)
 hexyl- β -D-glucopyranoside (Fluka 53180)
 nonyl- β -D-glucopyranoside (Fluka 74420)
 6-cyclohexylhexyl- β -D-maltoside (Fluka 29396)

Another concern in crystallizing membrane proteins is phase separation caused by the presence of detergents in salt solutions. The presence of salts may raise the ionic strength of the solution to a point where the detergent will partition into a separate phase. The hydrophobic protein will migrate into the detergent rich phase and denature. The use of amphiphiles like in table 1 (0.5 to 1.0%) may help prevent phase separation:

The use of crystallization detergents is not entirely harmless in all cases. At too high concentrations, they can denature the target protein. Myoglobin can be denatured by detergents, either by solubilizing the heme pocket or by solubilizing the heme itself.

Product	cmc [mM]	MW [g/mol]	density [kg/l]
benzamidine HCl (Fluka 12073)		156.62	solid
CTAB (Fluka 52365)	1	364.46	solid
6-cyclohexylhexyl- β -D-maltoside (Fluka 29396)	0.56	508.61	solid
1,2 dimethoxyethane (Fluka 38569)		90.12	0.87
Dodecyl- β -D-maltoside (Fluka 44205)	0.1-0.6	510.6	solid
ethylene glycol (Fluka 03747)		62.07	1.113
1,2,3 heptanetriol (Fluka 51845, 51846)		148.2	solid
1,6 hexanediol (Fluka 52800)		118.18	solid
hexyl- β -D-glucopyranoside (Fluka 53180)	250	264.32	solid
LDAO (Fluka 40234)	1-2	272	solid
2-methyl-2,4-pentandiol (Fluka 68338)		118.18	0.922
nonyl- β -D-glucopyranoside (Fluka 74420)	6.5	306.4	solid
n-octyl- β -D-glucoside (Fluka 75083)	80	292.38	solid
Zwittergent 3-10 (Fluka 30694)	25-40	307.49	solid

Table 1 Amphiphiles, their critical micelle concentration (cmc), molecular weight and density (out of literature)



Observation

Drops are typically observed by a stereo microscope at 10 to 100X. Record all observation 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 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 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 membrane 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 favorable crystallization condition (within a few days). In the case of too high concentration repeat the screen with lower membrane protein concentration. If more than a majority of drops contain a precipitate with no crystals present, consider also a dilution of the sample and repeating the entire screen. In case of precipitation problem for several times it may be useful to try out as a last essay to dilute the precipitant in the reagent.

Precipitation may also be an indication that the target membrane protein has denatured. It may be necessary to take steps to stabilize the target membrane 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 by some means just have denatured protein left, you may use the Renaturation Basic Kit for Proteins (96827) to find out sufficient

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 accrue out of a precipitate. Crystals can grow extremely fast in few minutes or may require much more time up to a few months. This are the reasons that crystallization plates should never been trashed or disregard a drop too early. Store and record the plates until the drops are dried out.

It is recommendable to use a high power microscope 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.

References

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- Practical Approach Series. Oxford Univ. Press 1992.
8. Cudney, B. et al, Acta Cryst, D50, 414-423, (1994).
 9. Bergfors, T. The crystallization lab manual. 1993.
 10. McPherson, A., Koszelak, S., Axelrod, H., Day, J., Robinson, L., McGrath, M., Williams, R., and Cascio, D. The effects of neutral detergents on the crystallization of soluble proteins. J. Cryst. Growth. 76:547-553, 1986.

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Crystallization Basic Kit for Membrane Proteins Observation Sheet

Sample description: _____

Date: _____

concentration: _____

Incubation Temperature: _____

buffer: _____

Reservoir Volume: _____

1 Drop contains : Crystallization Reagent _____ ul Sample _____ ul Additive

(name) _____ (volume) _____ ul

- | | | |
|---------------------------------------|---|---|
| | precipitate without birefringent and edges | precipitates shows birefringent or has edges |
| 1 drop is clear | 3 mostly clear drop | 7 spherulites or small structures maybe edges |
| 2 drop contains non-protein particles | 4 fully precipitated dark colour | 8 crystal grown 1 D |
| | 5 gelatinous protein precipitate | 9 crystal grown 2 D |
| | 6 phase separation | 10 crystal grown 3 D |

No.	Fluka No.	Name	Date:	Date:	Date:	Date:	Date:
1.	80070	Na-chloride 0.1M, Na-acetate (pH 4.6) 0.1M, 2-Methyl-2,4-pentanediol 12%					
2.	74102	Zn-acetate 0.1M, Na-acetate (pH 4.6) 0.1M, PEG 4000 12%					
3.	80361	NH ₄ -sulfate 0.2M, Na-acetate (pH 4.6) 0.1M, PEG 4000 10%					
4.	82178	Na-chloride 0.1M, Na-acetate (pH 4.6) 0.1M, 2-Propanol 12%					
5.	80877	Na-acetate (pH 4.6) 0.1M, PEG 4000 12%					
6.	92189	NH ₄ -sulfate 1M, Na-acetate (pH 4.6) 0.1M					
7.	82851	Mg-sulfate 1M, Na-acetate (pH 4.6) 0.1M					
8.	86303	Mg-chloride 0.1M, Na-acetate (pH 4.6) 0.1M, PEG 400 18%					
9.	94783	NH ₄ -dihydrogenphosphate 1M, Li-sulfate 0.1M, Na-acetate (pH 4.6) 0.1M					
10.	96904	Na-chloride 0.1M, Na-acetate (pH 4.6) 0.1M, PEG 6000 12%					
11.	94588	Mg-chloride 0.1M, Na-acetate (pH 4.6) 0.1M, PEG 6000 12%					
12.	96299	Na-chloride 0.1M, Na-citrate (pH 5.6) 0.1M, PEG 400 18%					
13.	76527	Li-sulfate 0.1M, Na-citrate (pH 5.6) 0.1M, PEG 4000 12%					
14.	80088	Na-citrate (pH 5.6) 0.1M, 2-Propanol 10%					
15.	73347	Na-chloride 0.1M, Na-citrate (pH 5.6) 0.1M, 2-Methyl-2,4-pentanediol 12%					
16.	82767	Mg-sulfate 1M, Na-citrate (pH 5.6) 0.1M					
17.	85242	Na-chloride 0.1M, Na-citrate (pH 5.6) 0.1M, PEG 4000 12%					
18.	80807	Li-sulfate 0.1M, Na-citrate (pH 5.6) 0.1M, PEG 6000 12%					
19.	71236	Mg-chloride 0.1M, Na-citrate (pH 5.6) 0.1M, 2-Methyl-2,4-pentanediol 4%					
20.	91150	Na-chloride 0.1M, Na-citrate (pH 5.6) 0.1M					
21.	94293	Li-sulfate 0.1M, Na-citrate (pH 5.6) 0.1M, PEG 400 4%					
22.	72639	NH ₄ -sulfate 1M, ADA (pH 6.5) 0.1M					
23.	89468	Li-sulfate 0.1M, ADA (pH 6.5) 0.1M, 2-Propanol 2%, PEG 4000 12%					
24.	93751	NH ₄ -dihydrogenphosphate 1M, ADA (pH 6.5) 0.1M					
25.	86974	Mg-chloride 0.1M, ADA (pH 6.5) 0.1M, PEG 6000 12%					
26.	79971	ADA (pH 6.5) 0.1M, 2-Methyl-2,4-pentanediol 12%					
27.	80059	Mg-sulfate 1M, Li-sulfate 0.1M, ADA (pH 6.5) 0.1M					
28.	95773	Li-sulfate 0.3M, ADA (pH 6.5) 0.1M, PEG 400 4%					
29.	86952	K-,Na-hydrogenphosphate each 0.5M, NH ₄ -sulfate 0.1M, HEPES Na-salt (pH 7.5) 0.1M					
30.	89729	Na-chloride 0.1M, HEPES Na-salt (pH 7.5) 0.1M, PEG 4000 10%					
31.	85852	Mg-chloride 0.1M, HEPES Na-salt (pH 7.5) 0.1M, PEG 400 18%					
32.	79049	K-,Na-tartrate 1M, HEPES Na-salt (pH 7.5) 0.1M					
33.	85901	NH ₄ -sulfate 0.1M, HEPES Na-salt (pH 7.5) 0.1M, PEG 400 18%					
34.	76565	NH ₄ -sulfate 0.1M, HEPES Na-salt (pH 7.5) 0.1M, PEG 4000 10%					
35.	89805	Na-citrate 0.1M, HEPES Na-salt (pH 7.5) 0.1M, 2-Methyl-2,4-pentanediol 12%					
36.	92684	Na-citrate 1M, HEPES Na-salt (pH 7.5) 0.1M					



No.	Fluka No.	Name	Date:	Date:	Date:	Date:	Date:
37.	96327	Mg-sulfate 0.6M, HEPES Na-salt (pH 7.5) 0.1M, PEG 400 4%					
38.	92635	Mg-sulfate 0.6M, HEPES Na-salt (pH 7.5) 0.1M, 2-Methyl-2,4-pentanediol 4%					
39.	80566	K-,Na-tartrate 0.1M, Li-sulfate 0.1M, HEPES Na-salt (pH 7.5) 0.1M					
40.	72989	Na-chloride 0.15M, TRIS-HCl (pH 8.0) 0.1M, PEG 6000 12 %					
41.	80092	Na-chloride 0.1M, TRIS-HCl (pH 8.0) 0.1M, 2-Methyl-2,4-pentanediol 12%					
42.	80428	Li-sulfate 0.1M, TRIS-HCl (pH 8.5) 0.1M, 2-Methyl-2,4-pentanediol 12%					
43.	80429	K-, Na-hydrogenphosphate each 0.5M, NH ₄ -hydrogenphosphate 0.1M, TRIS-HCl (pH 8.5) 0.1M					
44.	88411	Na-acetate 0.1M, TRIS-HCl (pH 8.5) 0.1M					
45.	86782	Na-chloride 0.1M, TRIS-HCl (pH 8.5) 0.1M					
46.	90173	NH ₄ -hydrogenphosphate 0.1M, TRIS-HCl (pH 8.5) 0.1M, PEG 6000 12%					
47.	81855	K-,Na-tartrate 0.1M, Mg-sulfate 0.4M, TRIS-HCl (pH 8.5) 0.1M					
48.	87772	Li-sulfate 0.2M, TRIS-HCl (pH 8.5) 0.1M					
49.	77927	NH ₄ -sulfate 0.5M, TRIS-HCl (pH 8.5) 0.1M					
50.	77484	Na-citrate 0.1M, TRIS-HCl (pH 8.5) 0.1M, PEG 400 5%					