Crystal-Tube

A Simplified Crystallization Method in the Capillary with Gel-Tube

User Manual

2010.9

Confocal Science Inc.

COSMO BIO CO., LTD.
Inspiration for Life Science

Table of Contents

1 Kit components

Catalogue No.		Description		Qty
CFS-MB2004-CRT200		**Crystal-Tube**		**1 set**
Included components	CFS-MB2004-CRT201	Screw-top test tube	16.5 x 105 , glass	24
	CFS-MB2004-CRT202	Gel-Tubing	1.0 (2.0) x 1000, silicone	1
	CFS-MB2004-CRT203	Capillary	0.3 (1.1), DURAN® glass	30
	CFS-MB2004-CRT204	Capillary	0.5 (1.1), DURAN® glass	20
	CFS-MB2004-CRT206	Capillary cutting stone		1
	CFS-MB2004-CRT207	Sample aspirator		1
	CFS-MB2004-CRT208	Sealing compound		1
CFS-MB2004-CRT209		**Starter Kit**		**1 set**

Approx I.D. (O.D.) x length (mm)

2 Introduction

Crystal-Tube is a simplified protein crystallization device. It grows crystals in a capillary for X-ray diffraction data collection by a counter-diffusion technique.

This kit can be used for optimization of crystallization conditions and for obtaining fine crystals for three-dimensional structural analysis by using capillary of different diameter.

This kit has been developed by Japan Space Utilization Promotion Center and Maruwa Foods and Biosciences Inc. subcontracted by Japan Aerospace Exploration Agency (JAXA) in the series of JAXA's project of protein crystallization experiments from 2002 to 2006 and is marketed under a licensing agreement with JAXA. (Patent: 4354457(JP) 7531037(US))

[1] Tanaka, H. et al., J. Synchrotron Rad. (2004). 11, 45-48.

3 Basic theory

The crystallization using **Crystal-Tube** is based on the counter-diffusion method. The outlook of the assembly is shown in Fig. 3-1. A capillary is filled with protein solution and a piece of gel-tubing (gel-tube) is attached to the end of the capillary. The capillary is placed into a test tube in which a reservoir solution is poured.

Protein and reservoir solutions are diffused each other from opposite direction through the gel-tube as shown in Fig. 3-2. It is called "counter-diffusion" technique [2][3]. The concentration gradients of the precipitant and the protein are formed in one

capillary from opposite direction. The gel-tube works as a diffusion barrier and the crystallization occurs gently. This type of experiment can explore a wide range of crystallization conditions in one single experiment, so that there is a higher possibility and reproducibility of obtaining high-quality crystals in a single experiment than by conventional method, such as vapor-diffusion and batch methods.

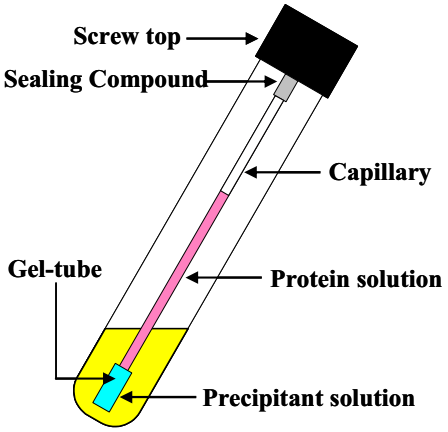


Fig. 3-1 Outlook of **Crystal-Tube** assembly

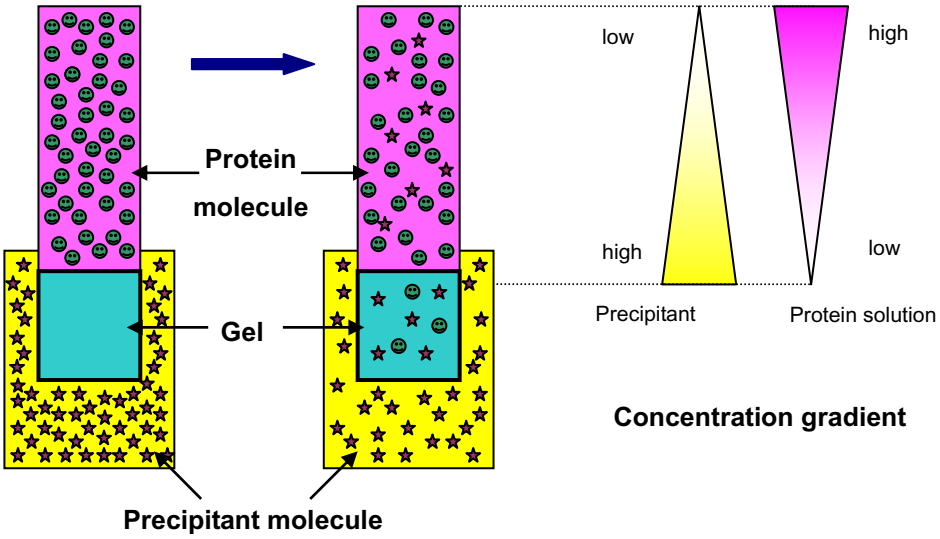


Fig. 3-2 Concentration gradient in a capillary

[2] McPherson A., Crystallization of Biological Macromolecules, Cold Spring Harbor Lab. Press (1999)

[3] Garcia-Ruiz, J.M., Moreno, A.: Acta Cryst., D50, 484-490(1994)

4 Advantages of **Crystal-Tube**

- Small amount of protein sample: 2μl (for optimization), 10μl (for X-ray diffraction experiment)
- Favorable crystallization condition: The timing of crystallization can be controlled by the lengths of the gel-tube, amount of protein solution in a capillary, and the concentrations of protein and precipitant solution.
- Easy set-up: The Gel-tubing help you assemble **Crystal-Tube** easily.
- High reproducibility and reliability: This device is a spin-off of JAXA-GCF space experiment. High reproducibility and reliability is proven after crystallizing over 400 different kinds of proteins.
- Easy post-soaking: Post-soaking is a common technique to bind ligands to protein molecules in a crystal. You just exchange the precipitant solution in the test tube to the ligand solution for post-soaking.
- Long-term crystal stability: The crystals in the capillary is stable for a long time.
- Membrane protein crystallization: Phase separation due to concentrated detergent in the solution does not occur.
- Optimization of the crystallization condition: 1-dimensional simulation program (option) can help you estimate the time-course of the solution conditions in the capillary.

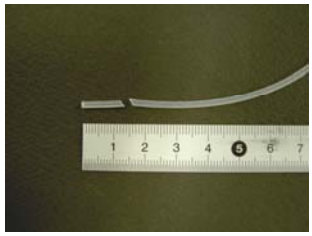
5 Preparation

Crystallization device	**Crystal-Tube**
Protein solution	Concentration: 5~10mg/ml Amount: 2~8μl/capillary
Precipitant solution	Concentration: see chapter 10 Amount: 1~2ml/test tube
Others	Razor blade, pipette, etc.

6 Experimental procedures

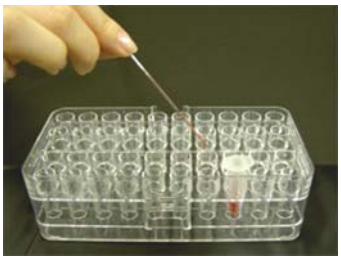
1. Cut a piece of a silicone tubing (gel-tube) in appropriate length

   - Low molecular weight precipitant (salt, organic compound, etc.) : Gel-tube length 13-17mm
   - High molecular weight precipitant (polyethylene glycol etc.) : Gel-tube length 9-11mm
   - Keep the gel-tube in buffer solution or water to protect it from drying.
   - Keep the gel-tube in precipitant solution if the protein sample precipitates at low ionic strength (a few days before set-up, if possible).



2. Load protein sample in a capillary

- For optimization of crystallization condition, use capillary of 0.3mm in diameter and load protein sample up to 30~40mm length in a capillary (sample ~2-micro L).
- For growing crystals for X-ray diffraction analysis, use capillary of 0.5mm in diameter and load protein sample up to 40mm length in a capillary (sample 10-micro L).
- For obtaining larger crystal, use a capillary of larger diameter.



- Longer sample length can scan more crystallization conditions.
- Better quality crystal grows in the upper position of the capillary.
- We advise you to practice loading sample in a capillary beforehand.

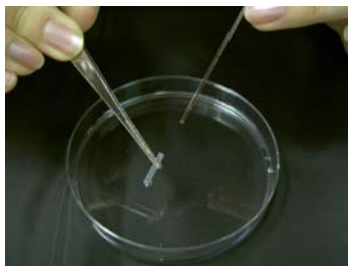
3. Seal the upper end of the capillary with sealing compound

- If the sealing is incomplete, osmotically-evoked pressure may cause increase/decrease of protein solution in the capillary and disturb crystallization.

4. Attach the gel-tube at the end of the capillary

- Insert 3-5mm of the capillary in the gel-tube.

  Avoid air bubble between the gel-tube and the capillary. Cut the protruding gel crosswise from the gel-tube.
- The gel-tube length in the silicone tubing determines the time-course of the protein and precipitant diffusion.
- For low molecular weight precipitant (salt, organic compound, etc.), gel length is 8-12mm.
- For high molecular weight precipitant (polyethylene glycol etc.), gel length is 4-6mm.



5. Pour reservoir solution in a test tube with a micropipette

- 1～2ml is enough.

6. Place the capillary with the gel-tube in the test tube

7. Close the screw top test tube. Let it stand for crystallization.

Observe the crystallization from time to time. Observe once a day right after sample loading. Observe once in a few days after a few weeks. Record the position of crystals, precipitation, oil, etc.

For low molecular weight precipitant, it takes from two weeks to one month to come to equilibrium. For high molecular weight precipitant, it takes more than two months to come to equilibrium. For controlling time-course of the concentration change, one-dimensional simulation program is available (option).

7 Harvesting crystals

1. Preparation of a harvest solution

Especially for high molecular weight precipitant, it sometimes takes 2 to 3 months to come to equilibrium. In this case, prepare a harvest solution whose concentration is the same or a little higher than the solution around the target crystal. For estimation of the solution condition, one-dimensional simulation program is available. We advise to check if it doesn't cause any damage to the crystal in harvest solution of several concentrations using spare crystal beforehand.

2. Cutting the capillary

Cut the capillary with razor blade 1-2mm apart from the target crystal. (Fig. 7-1).

Fig. 7-1 Cutting the capillary

3. Harvesting a crystal

The most suitable way to take the crystal out is using gentle water flow which does not cause any damage to the target crystal (Fig. 7-2). If the crystal is attached to the capillary wall, manipulating the crystal with a nylon wire is effective (Fig. 7-3) (Fig. 7-4).

Fig. 7-2 Making water flow

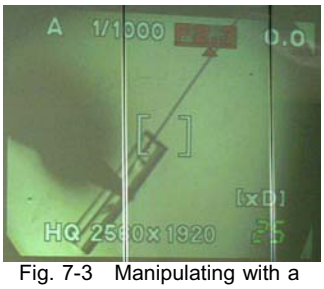


Fig. 7-3 Manipulating with a nylon wire

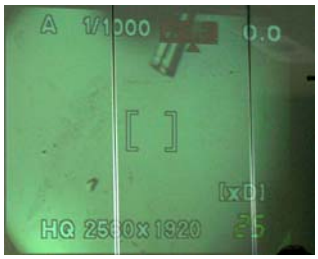


Fig. 7-4 Harvested crystal

4. Collecting a crystal with a nylon loop

The target crystal in the harvest solution is collected using a nylon loop for X-ray diffraction data collection.

8 Cryoprotecting crystals

Cryoprotection method is crucial for X-ray diffraction experiment in the synchrotron facility. For cryoprotection, it is necessary to prepare the cryoprotectant solution containing precipitant of the same concentration as the harvest solution with cryoprotectant of whose concentration does not cause ice-ring when it is flash-cooled.

Glycerol is the most frequently-used cryoprotectant. About 40 % glycerol solution can avoid creating ice-ring. Polyethylene glycol (PEG) solution which is frequently used as crystallizing reagent can also be cryoprotectant. About 40 % PEG solution can also avoid creating ice-ring. If PEG of high concentration is used as a precipitant solution, crystals can be directly cryocooled. If PEG of lower concentration is used, glycerol can be added to the precipitant solution for cryoprotection. Appropriate total concentration of glycerol plus PEG is about 40%. Trehalose and PEG 400 can also be used as a cryoprotectant.

Crystals are usually scooped with a nylon loop and briefly soaked in the cryoprotectant solution. However, in this method, crystals are sometimes cracked by osmotic pressure. In this case, it is recommended to soak crystals into the cryoprotectant gradually. Using Crystal-Tube, it is easy to soak in the cryoprotectant solution gradually if the outer solution is changed from the precipitant solution to the cryoprotectant solution.

Crystals are usually flash-cooled in the nitrogen gas stream. It is not recommended to soak in the liquid nitrogen directly, because nitrogen gas bubbles are attached to the surface of the crystals and interfere cooling efficiency.

9 Diffusion time-course in a capillary

For ensured crystallization, it is helpful if you can estimate the time-course of diffusion of protein and precipitant. The following explanation is for the efficient usage of Crystal-Tube kit. For more information, it is recommended to use 1-dimensional simulation program (option).

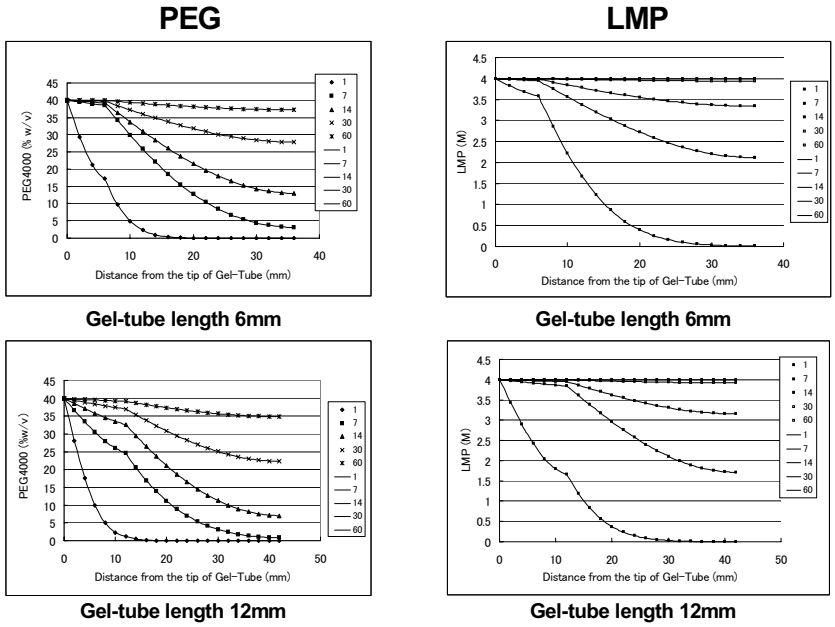


Fig. 9-1 Relationship between the gel-tube length and the diffusion

The length of the gel-tube is the key to control the diffusion. The Fig. 9-1 shows the time-course of the diffusion of precipitant, such as polyethylene glycol 4000 (PEG) and low molecular weight precipitant (MW is about 120) (LMP), in the case of different gel length (6 mm and 12 mm). The horizontal axis means the length from the tip of the gel-tube and the longitudinal axis means the concentration of the precipitant. Each line shows the concentrations of the precipitant in the capillary on the day 1, 7, 14, 30 and 60, respectively. The longer the gel-tube is, the slower the precipitant diffuses. In the case of the high molecular weight precipitant, the diffusion is slower. The length of the gel-tube is recommended to be 6 to 8 mm in the case of PEG as a precipitant, and 10 to 12 mm in the case of LMP.

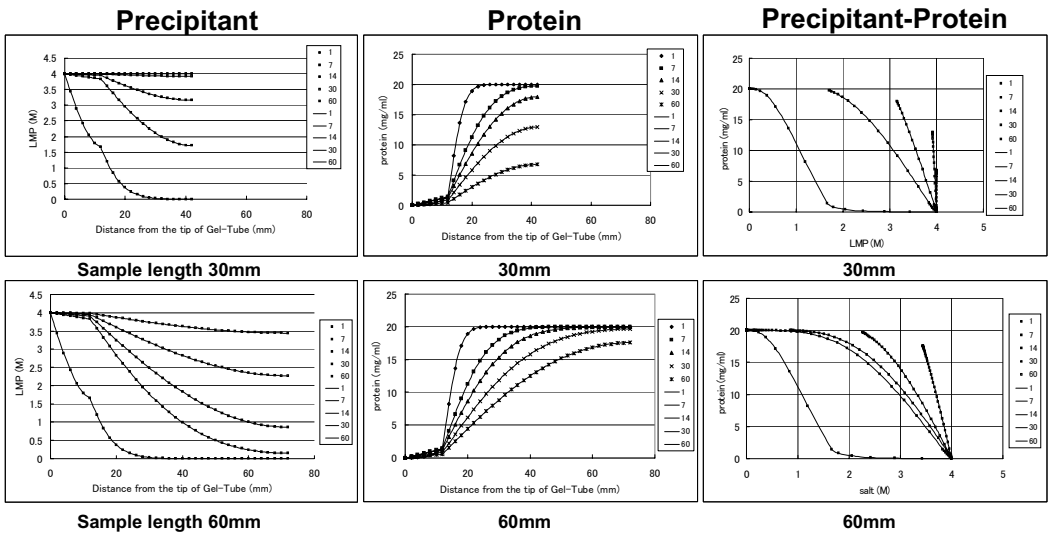


Fig. 9-2 Relationship between the length of the protein sample and the diffusion (low molecular weight precipitant)

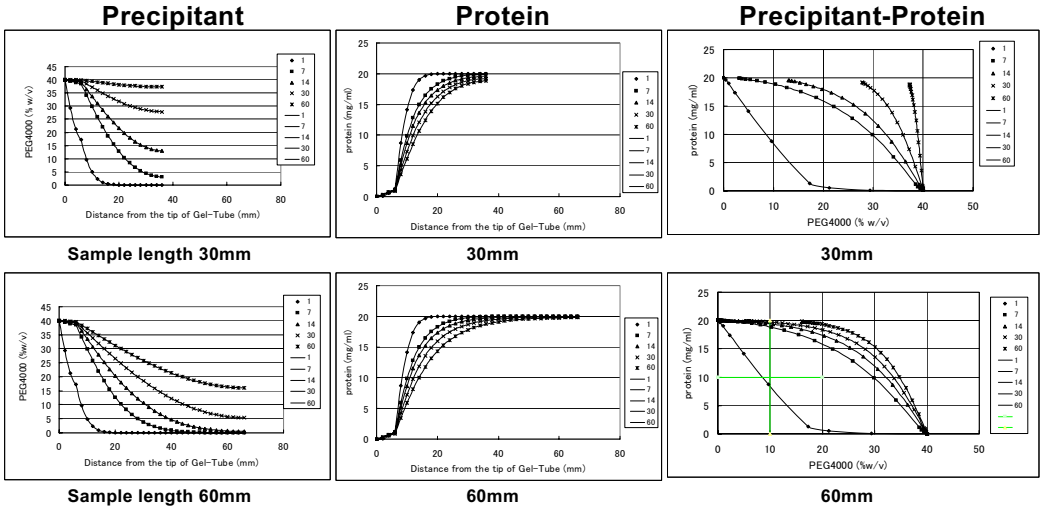


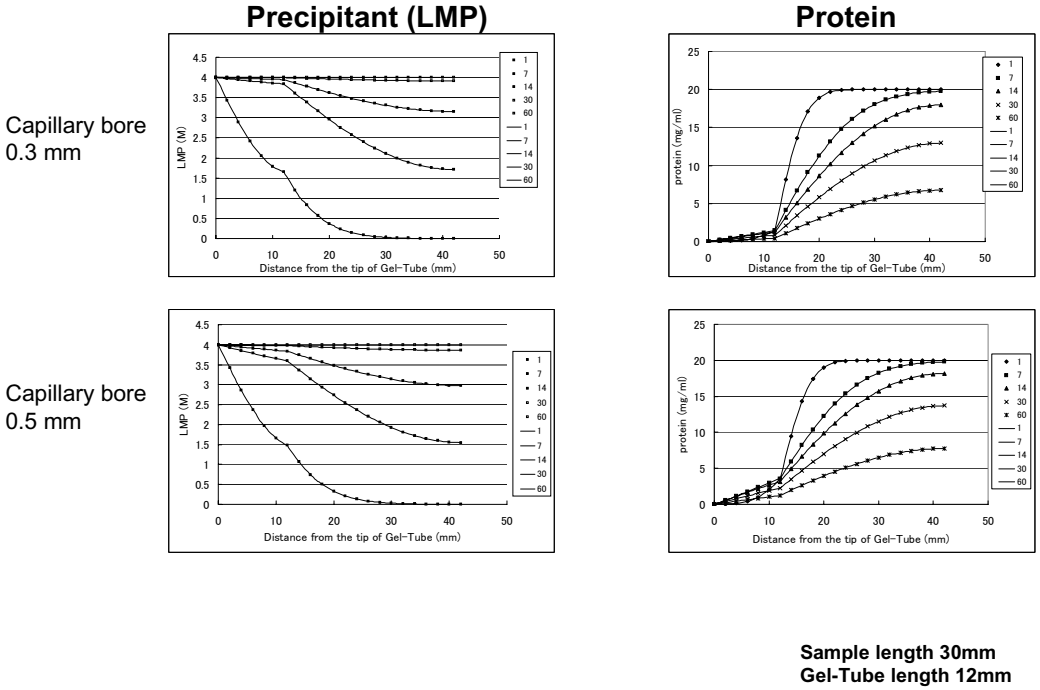
Fig. 9-3 Relationship between the length of the protein sample and the diffusion (polyethylene glycol)

The length of the protein solution in the capillary determines the concentration range of protein and precipitant as a function of time. The Fig. 9-2 shows time-course of the protein of MW 15,000 and the LMP in the case of different protein solution length in the capillary (30 mm and 60 mm). It can scan larger range of the concentration of protein

and precipitant in the longer protein solution length even though some of the protein solution diffuses out to the precipitant solution.

In the case of LMP precipitant, it is recommended that the protein solution length is at least 60 mm (0.3 mm capillary diameter, 4.2 µl protein solution) because of the fast diffusion.

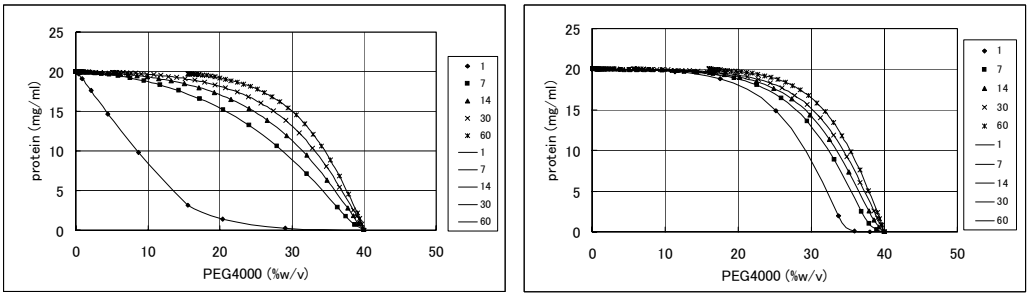
As shown in Fig. 9-3, in the case of PEG as a precipitant, the diffusion of protein solution out to the precipitant solution is suppressed because PEG reduces the diffusion of the protein solution. So, 30 mm length of the protein solution in the capillary is enough to scan wide range of the precipitant solution. The longer length of the protein solution rather makes the diffusion of the PEG slow, so that it takes longer time to crystallize.



Sample length 30mm
Gel-Tube length 12mm

Fig. 9-4 Relationship between the capillary bore and the diffusion

The capillary of 0.3mm diameter is recommended for the optimization of the crystallization condition to reduce the amount of the sample. On the other hand, the capillary of 0.5mm diameter is recommended for growing larger crystals for X-ray diffraction data collection. In the case of larger diameter of the capillary, the time-course of the diffusion is changed as shown in Fig. 9-4.



pre-saturation (-) **pre-saturation (+)**

Capillary bore 0.5mm
Gel-Tube length: 6mm

Fig. 9-5 Relationship between the pre-saturation of the gel-tube and the diffusion

As shown in Fig. 9-5, to accelerate the diffusion, it is helpful to pre-saturate the gel-tube in the precipitant solution before the crystallization set-up.

10 Concentration and amount of sample

As shown in the chapter 9, only one capillary can scan almost all range (from 0 to the full concentration) of the crystallization conditions. The upper right area (protein and precipitant concentration are both high) is the only blind spot of this scan (show the diagram below).

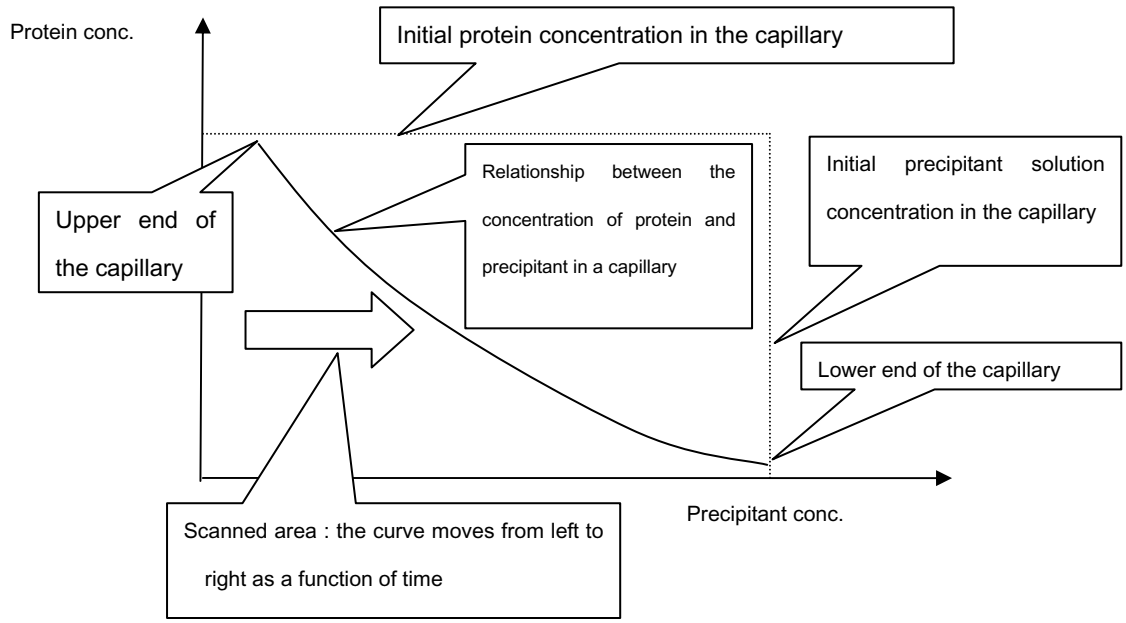


Fig. 10-1 Phase diagram of concentration of protein and precipitant in a capillary

In the case of the vapor-diffusion method, crystallization occurs at high concentration of protein and precipitant. Therefore, in the case of the counter-diffusion method, to start crystallizing within several days, the starting solution of the protein and the precipitant should be highly concentrated.

To concentrate sample usually have a risk to loose a certain amount of the sample. And the sample sometimes precipitates at high concentration. If the amount of the sample is small, it is not preferable to concentrate the protein sample.

In that case, it is easier to concentrate the precipitant in the reservoir solution. To increase the possibility of crystal growth, it is preferable to concentrate the precipitant solution several 10s % higher. In this case, only the precipitant, not the buffer, is to concentrate. For example, if the optimal crystallization condition in the vapor-diffusion method is 10 % PEG 8000, 100 mM sodium acetate and 2 mM calcium chloride, the crystallization condition for the counter-diffusion method is preferable to be 15~20 % PEG 8000, 100 mM sodium acetate and 2 mM calcium chloride. But it is not

recommended to use more than 40~50 % of PEG, because higher PEG may damage the crystals.

In the case of low molecular weight precipitant, such as salt and organic compound, the preferable gel-tube length is 10 to 12 mm. In the case of high molecular weight precipitant, such as high molecular weight PEG, the preferable gel-tube length is 6 to 8 mm. The 1-dimensional simulation of the diffusion in the capillary may help choose the right length of the gel-tube.

The 1-dimensional simulation of the diffusion can also be applied for the optimization of the crystallization condition. After checking the time and the position of the crystallization in a capillary, these data will be applied to the 1-D simulation which will supply better starting condition of the crystallization. According to the simulation, the concentration of the precipitant and the gel-tube length can be changed for the better time-course of the crystallization.

If there is no crystal growth but precipitant/oil were observed, the concentration of the precipitant may be too high and the time-course of the crystallization may be too fast. It is recommended to decrease the concentration of the precipitant and/or to use longer length of the gel-tube. If the precipitant solution stays clear and no crystal appears, the concentration of the precipitant may be too low. It is recommended to increase the concentration of the precipitant and to use shorter length of the gel-tube. It is also recommended to add a reservoir solution to a protein solution as 1:1 before crystallization set-up, like vapor-diffusion method, since such premixing can increase the precipitant concentration in the protein solution from the starting point and sometimes enhances the nucleation.

If you apply commercial screening kits for **Crystal-Tube**, the concentration of the precipitant may be too low because the kit is usually fixed for the vapor-diffusion method or the batch method. In that case, it is recommended to increase a precipitant component several 10s % higher.

11 Effect of a salt when PEG is used

Although PEG is the most widely used precipitant in protein crystallization, the concentration of co-existing salt in the solution has not been well discussed. To determine the optimum salt concentration range, we crystallized several kinds of protein in a 30 % PEG 4000 solution at various NaCl concentrations with various pH levels with using **Crystal-Tube**. Results are shown in following table.

Protein		Lysozyme		Alpha-amylase			Glucose isomerase	
pH		4.5	7.0	5.5	7.0	9.0	7.0	9.0
Inonic strength of buffer (mM)		17.0	11.0	42.0	11.0	5.0	11.0	5.0
NaCl (mM)	0	x	x	x	x	x	x	x
	100	x	x	crystal	x	x	x	x
	200	x	x	crystal	crystal/oil	oil	crystal/precipitate	crystal
	300	x	crystal	crystal	crystal/oil	oil	crystal/precipitate	crystal/precipitate
	400	crystal	crystal	crystal/oil	oil	oil	crystal/precipitate	crystal/precipitate
	500	crystal	crystal	-	oil	oil	crystal/precipitate	crystal/precipitate
	600	crystal	crystal	-	oil	oil	crystal/precipitate	crystal/precipitate
	700	crystal	crystal	-	oil	oil	crystal/precipitate	crystal/precipitate
Marginal ionic strength (mM)		417	311	142	211	205	211	205
Calculated pI		10.7		4.4			5.0	
Vm / PDB ID		2.08 / 1bwh		2.18 / 6taa			2.78 / 1xib	
Charge density (mM)		654	455	265	362	462	258	338

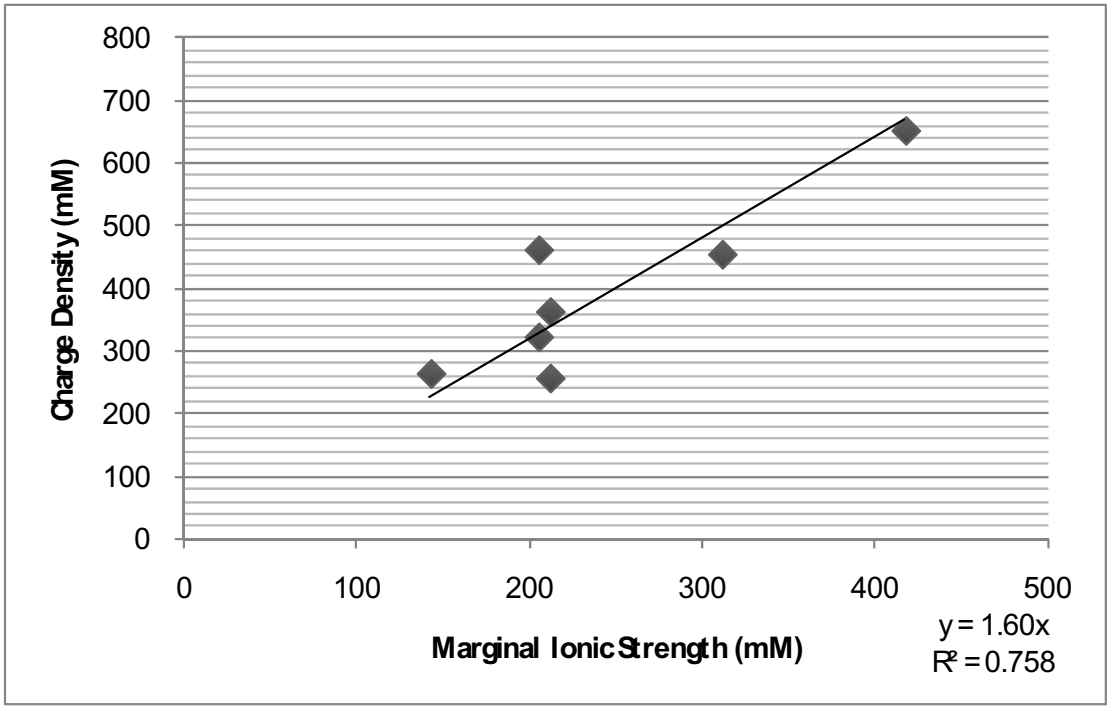
As is shown in the table, no crystal appeared without NaCl, whereas too high concentration of NaCl could cause precipitate or oil, indicating that applying optimum concentration range of NaCl is important.

The lowest effective salt concentration (that is, marginal concentration) depended on the pH of the protein solution and the pI of the protein molecule. When the difference between pH and pI was larger, protein was charged more intensely, and the minimum concentration of NaCl for obtaining crystals was higher. It was consistent with the fact that, among three proteins, lysozyme has the largest net charge and required the highest concentration of NaCl. Bonneté (2007) reported that a salt has some relation to the electrostatic screen effect between the protein molecules.

Matsushima and Inaka (2007, The 7th Annual Meeting of the Protein Science Society of Japan) showed that the Charge Density defined by the following equation corresponds to the minimum concentration of salt:

$$Charge\ Density(M) = \frac{Number\ of\ charges}{Volume\ for\ one\ protein} = \frac{Number\ of\ charges \times 10^{27}}{V_M \times MW \times 6.02 \times 10^{23}}$$

As is shown in the following figure, the Charge Densities of these proteins calculated using pH, amino acid profile and V_M registered in PDB, are plotted against the value of the ionic strength of the solution.



A clear linear relationship, the coefficient of which was 1.61 (R2=0.76), was found. Using this relationship, the lowest concentration of the salt in the PEG 4000 solution can be predicted prior to performing crystallization experiments. A scheme for the prediction is followings:

1. Calculating number of charges of a protein molecule using its amino acid profile and pH of the solution.
2. Estimating V_M value from the histogram of V_M values of PDB entries (Kantardjieff & Rupp, 2003)
3. Using equation above to estimate the charge density.
4. Using linear relation above to estimate plausible concentration of NaCl for obtaining crystals.

Our results can also provide a tip for converting crystallization condition from vapor-diffusion method. One of the significant differences between counter-diffusion and vapor-diffusion is the concentration change of salt in a crystallization capillary. In the vapor-diffusion method, a protein solution and a reservor solution are usually mixed in a drop at a 1:1 ratio. Then crystallization occurs in the drop in which the components are concentrated through water loss. Even if reservoir solution does not have enough salt, the salt concentration in the drop may reach the marginal concentration level when the original protein solution contains some salt. But in the case of counter diffusion, the salt concentration in the capillary does not exceed that of reservoir solution. Considering this difference, it is necessary to fix proper amount of salt in the reservor solution.

12 FAQ

1. Q. What is the recommended concentration of protein solution at the beginning?

A. For screening wide range of the crystallization condition in a short time, it is recommended to use higher concentration of the protein solution. But if the precipitation occurs in the high concentration protein solution, it is better to use the protein concentration such as 5 to 10 mg/ml which is usually used for the vapor-diffusion method and batch method.

2. Q. What is the recommended concentration of the precipitant at the beginning?

A. For screening wide range of the crystallization condition in a short time, it is recommended to use higher concentration of the precipitant. It is preferable to use several tens % higher concentration than the vapor-diffusion method and the batch method. If it is impossible to increase the concentration of the precipitant, please use as high concentration as possible.

3. Q. What is the optimal length of the gel-tube? Is there any caution to handle the gel-tube?

A. It is recommended to use 10 to 12 mm of the gel-tube for low molecular weight precipitant, such as ammonium sulfate, and 6 to 8 mm of the gel-tube for high molecular weight precipitant, such as high molecular weight polyethylene glycol. The gel-tube is stocked in distilled water. It is usually no problem to use the gel-tube directly from the distilled water. However, if the protein can easily precipitate at lower ionic strength, soak the gel-tube in the same buffer as the protein solution for a few days before crystallization set-up.

4. Q. How can I use the gel-tube for the screening of the crystallization condition?

A. At first, please use a capillary of 0.3 mm diameter for the screening of the crystallization condition. 2 micro-L of the protein solution is necessary. Commercial screening kit of crystallization condition is available. If the commercially available

screening kit is not used, it is recommended to use following scheme.

① Choose a plausible buffer with a pH 1~2 departed from the pI of the target protein.

② Prepare the following reservoir solutions:

・Buffer + 4M $(NH_4)_2SO_4$

・Buffer + 30 % PEG 4000 + 100 mM ~ 1 M NaCl

③ Crystallization can sometimes be accelerated if the protein and the reservoir solutions are mixed 1:1 prior to crystallization set-up.

Notice that there is an optimum salt concentration for PEG as the precipitant, as shown in the table in Chapter 11. If too low, crystals will not grow. If too high, precipitate or oil will be obtained. In addition, shorter length of the gel-tube can also reduce time.

Please check the time when the crystallization starts and the position where the crystallization occurs in a capillary. These data will be applied to the 1-D simulation which will supply the starting condition of the optimized crystallization.

If there is no crystal growth but precipitant/oil, the concentration of the precipitant may be too high and the time-course of the crystallization may be too fast. It is recommended to decrease the concentration of the precipitant and/or to use longer length of the gel-tube. If the precipitant solution stays clear and no crystal appears, the concentration of the precipitant may be too low.

5. Q. How can I reduce time for crystallization?

A. Please add low concentration of the precipitant in the protein solution in the capillary beforehand. Shorter gel-tube can also reduce time for crystallization. Premix a protein solution and reservoir solution is also recommended.

6. Q. After the crystallization set-up, precipitation occurs all over the capillary. What can I do?

A. Addition of the precipitant to the protein solution sometimes makes the protein molecule soluble and more stable.

13 Tips for further usage of **Crystal-Tube**

There are tips for the usage of the **Crystal-Tube**. These may help the customers who are used to use the vapor-diffusion method or the batch method and not familiar with the counter-diffusion method. The followings are know-how we have obtained during the technical verification of the **Crystal-Tube**.

- It is recommended to adjust pH of the protein solution to that of the precipitant solution.
- If the precipitation occurs much faster than the diffusion of the precipitant in the capillary, it is recommended to add low concentration of the precipitant or salt in the protein solution. This precipitation may occur because of the low ionic strength in the protein solution.
- If the gel-tube which is soaked in the distilled water is used, precipitation sometimes occurs at the bottom of the capillary because of the low ionic strength in the protein solution. To avoid this, soak the gel-tube in the same buffer solution as the protein solution for a few days prior to the crystallization experiment.
- If the additive to the protein solution is crucial for crystallization, add the same concentration of the additive to the precipitant solution.
- If the solubility of the protein in the precipitant solution is high, decrease the amount of the precipitant solution as possible (about a few hundred micro-L).
- High concentration of organic compound may cause degradation of the protein molecule. Cryoprotectant such as ethylene glycol may cause the same. Polyethylene glycol may not cause damage to the protein molecule.

14 Possibility of **Crystal-Tube**

Application of the crystallization of membrane proteins

Crystal-Tube may work effectively in the crystallization of membrane proteins. Detergent is often used for the crystallization of membrane proteins. In the case of vapor-diffusion method, the detergent is added to the drop so that the concentration of the detergent increases in the drop when vapor diffuses. And phase separation may occur easily in the drop and sometimes crystal can not grow.

On the other hand, in the case of the **Crystal-Tube**, if the same concentration of the detergent is added both to the protein solution and the precipitant solution, the concentration of the detergent does not increase and only the concentration of the precipitant can be increased in the capillary. If the detergent is very expensive, please decrease the amount of the precipitant solution as possible.

Easy soaking

In the **Crystal-Tube**, if you want to soak a fragment to the protein crystal, you just add it to the precipitant solution and it can diffuse into crystals in the capillary to form

protein-fragment complexes. If cryoprotectant is added to the precipitant solution, it can diffuse slowly to crystals and no damage for the cryoprotectant may occur. If very high concentration of precipitant solution is used at the beginning of the crystallization, it can induce nucleation of the crystal (pulse-diffusion method).

15 Precaution

- This kit is for research use only. It is forbidden to use it for purposes other than those stated.
- Please refer to the articles [1] and [3] if you submit your paper.
- Please handle the capillary with care.

Crystal-Tube is marketed under a licensing agreement with Japan Aerospace Exploration Agency. (Patent: 4354457(JP)　7531037(US))

Manufactured by Confocal Science Inc.

COSMO BIO CO., LTD.
Inspiration for Life Science
TOYO 2CHOME, KOTO-KU, TOKYO, 135-0016, JAPAN
http://www.cosmobio.co.jp/index_e.asp　E-mail: export@cosmobio.co.jp
Phone : +81-3-5632-9617　　FAX : +81-3-5632-9618