C-Kit Ground Pro XRD
[X-ray Diffraction Kit]

Nov. 2022

Confocal Science Inc.

Table of contents

Contents of C-Kit Ground Pro XRD (CRT101-1)

Product name	Qty.	Description
Capillary (ϕ 0.5 mm × 47 mm)	15	Used in the CD method. 3 extra gel tubes included.
Gel tube (ϕ 1.0 mm × 1 cm)	18	
DPOC Tube	8	Used in the DPOC method.
C-Cap	16	
Silicone tubing (ϕ 1.0 mm × 50 cm)	1	Used for aspirating solution.
Sealing compound	1	Used for sealing capillaries and DPOC tubes.
Round-bottom tube (5 mL)	23	Vessel for crystallization.

Precautions and Notice

- This kit is for research purposes only. Please do not use it for any other purpose.
- When attaching the gel tube to the capillary, please be careful not to injure yourself.
- This product uses US patent 7531037 of the Japan Aerospace Exploration Agency (JAXA) under license.
- Descriptions in this handbook are subject to change without notice.

1. Introduction

1.1. Overview

C-Kit Ground Pro XRD is an equipment for protein crystallization for X-ray diffraction, developed from the know-how of crystallization experiments in the International Space Station (ISS) for decades. This kit provides experimental tools for two simple methods that do not require large-scale crystallization screening: the counter-diffusion method (CD method)[1)] and the diffusion-pair osmotic-concentration method (DPOC method)[2)]. Both methods grow crystals based on the principle of auto-searching phenomenon for optimal crystallization conditions (so-called the self-searching mechanism)[3)]. Newly developed DPOC method enables us to search for a wide range of crystallization conditions since the protein samples and crystallization solutions in the DPOC tube are osmotically concentrated due to the osmotic pressure difference between inside and outside of the DPOC tube. You can choose the better method according to the sample characteristics to grow high quality apo/complex-crystals by yourself.

The CD method equipment included in the kit was jointly developed by Confocal Science Inc. under a commission from the Japan Aerospace Exploration Agency (JAXA)[4), 5)]. In addition, Confocal Science Inc. provides C-Kit Ground Pro ND for crystallization for neutron diffraction (http://www.confsci.co.jp/product_e.html), and C-Kit Space Pro Series for high-quality crystallization under microgravity in the International Space Station (ISS).

1) García-Ruiz, J.M.; Moreno, A. Investigations on protein crystal growth by the gel acupuncture method. *Acta Crystallogr. D Struct. Biol. Commun.* 1994, D50, 484–490

2) Takahashi, S.; Koga, M.; Yan, B.; Furubayashi, N.; Kamo, M.; Inaka, K.; Tanaka, H. JCB-SGT crystallization devices applicable to PCG experiments and their crystallization conditions. *Int. J. Microgravity Sci. Appl.* 2019, 36, 360107

3) Otálora, F.; García-Ruiz, J.M. Computer model of the diffusion/ reaction interplay in the gel acupuncture method. *J. Cryst. Growth* 1996, 169, 361–367

4) Tanaka, H.; Inaka, K.; Sugiyama, S.; Takahashi, S.; Sano, S.; Sato, M.; Yoshitomi, S. A simplified counter diffusion method combined with a 1D simulation program for optimizing crystallization conditions. *J. Synchrotron Rad.* 2004, 11, 45–48.

5) JP patent 4354457, US patent 7531037

1.2. Features

- Small amount of protein sample is required: Standard amount is 8 µL (the CD method capillary) and 5 µL (the DPOC tube).
- Slow crystallization methods lead to high-quality crystals: Both the CD method and the DPOC method provide mild crystallization conditions by adjusting the concentrations of the protein sample and the crystallization reagent.
- Simple protocols: Both CD and DPOC protocols are easy to perform.
- High reproducibility and reliability: Both methods are repeatedly used in space experiments conducted by the Japan Aerospace Exploration Agency (JAXA) and Japan Manned Space Systems Corporation (JAMSS). Thus, their high reproducibility and reliability have been proven. In particular, the CD method has been applied to more than 500 protein crystallization under microgravity since 2002.
- Easy soaking in the CD method: Soaking can be done simply by transferring the capillary containing crystals to a container filled with the solution for post-soaking.
- Small amount of ligand in the DPOC method: ligands are pre-mixed with the protein solution loaded in the tube, so a small amount of ligand is enough for co-crystallization.
- Long-term stability: The crystals produced with C-Kit Pro XRD are stable for a long period in the CD capillaries with gel tube and in the DPOC tubes.
- Estimation of optimal conditions: The time-dependent changes of concentrations of the protein and the crystallization reagents in the CD capillaries and the DPOC tubes can be estimated by a one-dimensional diffusion simulation program (purchase available as **C-Kit Pro Advanced Tool** (CRT209), http://www.confsci.co.jp/product_e.html). It helps to determine the optimal conditions for crystallization.
- Please refer to “4.2. Benefits for the CD method and the DPOC method” to understand which method is better for your experiments.

2. Crystallization Methods Used in C-Kit Ground Pro XRD

2.1. The counter-diffusion method (the CD method)

C-Kit Ground Pro XRD provides a set of crystallization equipment of the GT method, which is one of the CD methods. Figure 2.1 shows a simple explanation of the standard configuration of the method and the mechanism of crystal growth.

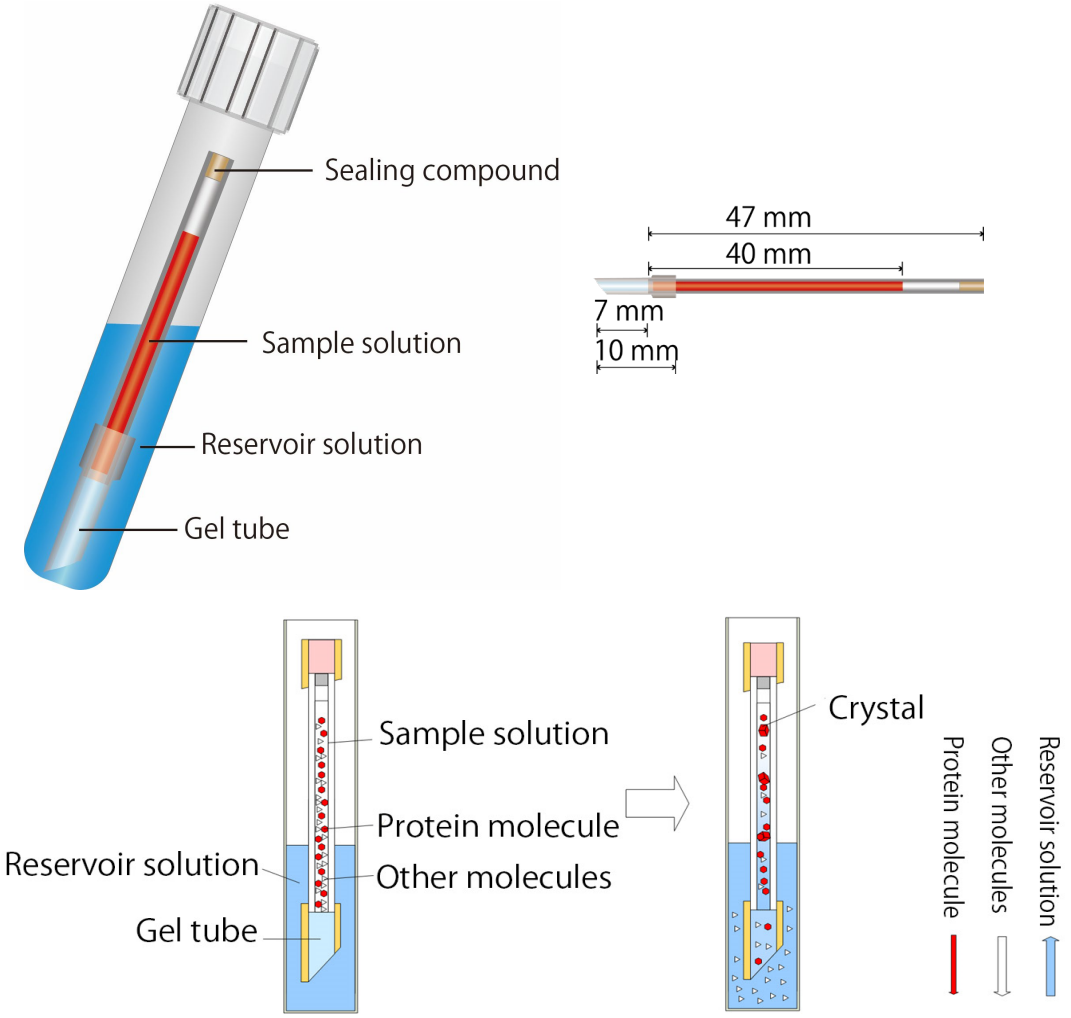

Figure 2.1 GT method illustration.
Upper left: Configuration of the GT method. Upper right: Capillary with a gel tube detail view. Bottom: Crystallization mechanism of the GT method.

The feature of this method is that a silicone tube with agarose gel, called a gel tube (GT), is connected to the end of the capillary loaded with protein sample. The reservoir solution containing crystallization reagents gradually diffuses into the capillary through the gel tube, forming a concentration gradient of the components of the reservoir solution inside the capillary. Protein samples and other coexisting components in the sample solution also gradually diffuse out of the capillary. The diffusion brings about a continuous and wide range of concentration combinations of the crystallization reagent and the sample solution within the capillary. As a result, crystallization is triggered at the point where the concentration combination is suitable for the crystallization. This phenomenon is called “the self-searching mechanism”. Understanding the diffusion time-course of the crystallization reagent and protein sample is important in this crystallization method[4]. See “4.1. Time course of concentration change of the solution“ for the details.

2.2. The diffusion-pair osmotic-concentration method (DPOC method)

C-Kit Ground Pro XRD includes the crystallization tubes for the DPOC method as another type of crystallization method. Figure 2.2 shows a simple explanation of the standard configuration of the DPOC method and the mechanism of its crystal growth.

The conventional diffusion-pair method of crystallization has been known for a long time: by successively loading a glass capillary with a crystallization reagent and a protein sample to form a diffusion pair and interdiffusion for crystallization[6]. In our DPOC method, the glass capillary is replaced with the water-permeable silicone rubber tube. The solution in the DPOC tube is osmotically concentrated due to the osmotic pressure difference between the reservoir solution outside and the solution inside. The diffusion phenomenon in the diffusion pair is common with the CD method, but the equal amount of the crystallization reagent solution to that of the protein sample is included in the DPOC tube, which results in searching for a narrower range of crystallization conditions than the CD method. However, the osmotic concentration effect in the DPOC tube enables the method to search a wider range of conditions. In addition, protein samples can be crystallized under concentrated conditions without prior preparation. The

difference from the CD method will be explained in “4.2. Benefits for the CD method and the DPOC method”.

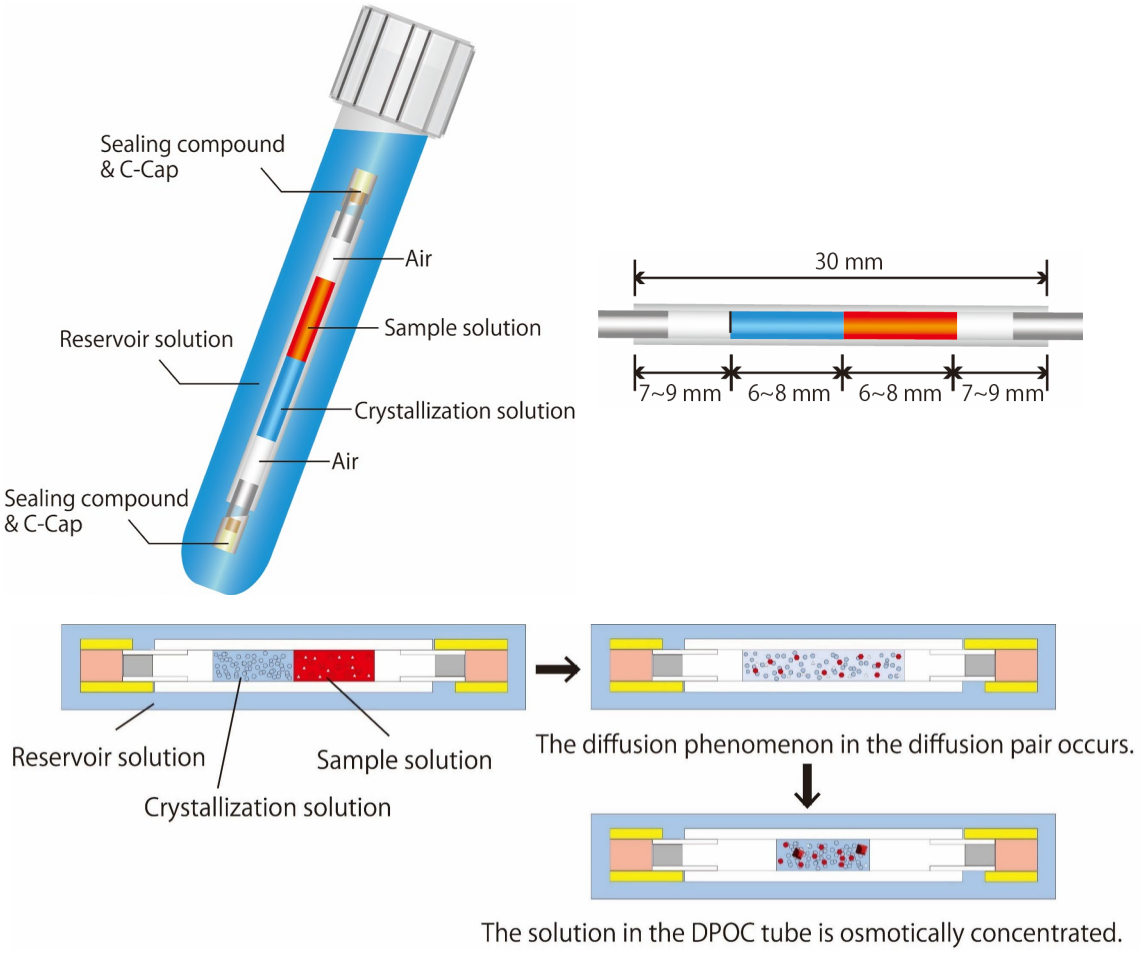

Figure 2-2. DPOC method illustration
Upper left: Configuration of the DPOC method. Upper right: DPOC tube detail view. Bottom: Crystals are formed by interdiffusion and osmotic concentration of sample solution and crystallization solution.

6) Salemme, F.R. A free interface diffusion technique for the crystallization of proteins for X-ray crystallography. *Arch. Biochem. Biophys.* 1972, 151, 533-539.

3. Experimental Manual

3.1. Setting up crystallization by the CD method

3.1.1. Required items

(For one crystallization condition)

Solution	Amount	Description
Sample solution	6～8 μL	It contains a protein sample solution, which may also contain a ligand or crystallization reagents, and is loaded into a capillary. Since the inner diameter of the capillary is 0.5 mm, the required volume is 6 μL for a sample length of 30 mm, and 8 μL for that of 40 mm. It is desirable to prepare a little more to load it without failure.
Reservoir solution	ca. 1 mL	It contains crystallization reagents and buffer solution, which may also contain ligand components. It diffuses into the capillary through a gel tube to crystallize protein.
Gel tube soaking solution	ca. 4 mL	It is a solution to immerse the gel tube before starting crystallization, containing only the buffer component of the reservoir solution or its full components. Enough amount of liquid to immerse the gel tube (10 mm in length) is required.
Seed crystal	A few μL	Prepare for seeding, if necessary.

Equipment in the kit	Qty.	Description
Capillary (ϕ 0.5 mm × 47 mm)	1	Mark the upper end position of sample before loading.
Gel tube (ϕ 1.0 mm × 1 cm)	1	Gel tubes in C-Kit Ground Pro are immersed in a 0.04% NaN_3 solution.

Sealing compound	1	It is used for sealing the upper end of the capillary.
Silicone tubing	1	It is used if suction is required when loading solutions into the capillary.
Round-bottom tube (5 mL)	1	A vessel for the crystallization.

Required equipment	Qty.	Description
Tube with lid	1	A tube with a height of 40 mm and a capacity of about 5 mL It is used for pre-soaking the gel tube.
Micropipette / Tip	1 each	It is for preparing solutions.
Micropipette / Tip (20 to 100 µL)	1	It is for loading solutions into the capillary.
Surgical blade (Or a cutting blade)	1	It is for cutting the lower end of the gel tube. Its cutting blade must be sharp.
Fine-point permanent marker	1	It is for marking the end position of loaded sample on the capillary.

3.1.2. Gel-tube pre-soaking

1. Preparation of gel tube soaking solution

It takes time for the crystallization reagent to diffuse in the gel tube. Therefore, if you want to grow crystals in a short time, the gel tube soaking solution should have the same composition as the reservoir solution. For crystallization under mild conditions, either the buffer component only or that contains the required amount of the crystallization reagent is available for the solution. Add about 4 mL of the solution to the tube with a lid, and immerse the gel tubes at least for a few days before use.

2. Time required for soaking the gel tube

According to the simulation results of the solute concentration change in the gel tube immersed in the gel soaking solution, it takes about 0.5 days for the NaCl solution and about 4 days for the PEG4000 solution to complete the solution equilibrium in the gel tube.

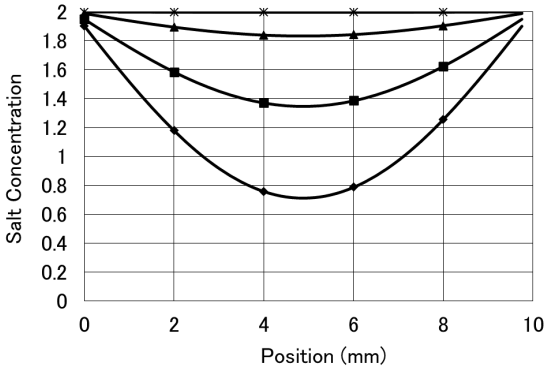

Fig. 3.1 Simulation results of internal NaCl concentration of a gel tube immersed in a 2 M NaCl solution. The horizontal axis is the distance from the end of the gel tube, and the vertical axis is the NaCl concentration. The curves show, from the bottom, NaCl concentrations after 0.05, 0.1, 0.2, 0.5 days.

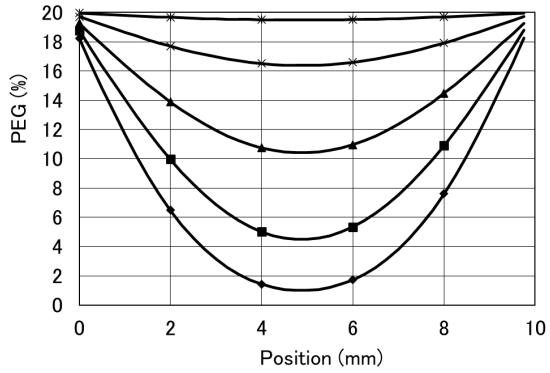

Fig. 3.2 Simulation results of internal PEG4000 concentration of a gel tube immersed in a 20% PEG4000 solution. The horizontal axis is the distance from the end of the gel tube, and the vertical axis is the PEG4000 concentration. The curves show, from the bottom, PEG4000 concentrations after 0.25, 0.5, 1, 2, 4 days.

3.1.3. Preparation of solutions and a marked capillary

1. Sample solution

Prepare the required amount of protein sample solution for loading. If it is necessary to mix crystallization reagents, ligands, etc., mix them before loading into the capillary.

2. Seed solution

If you are planning micro-seeding or macro-seeding, prepare seed crystals in a suitable solution.

3. Reservoir solution

Prepare the required amount of reservoir solution for filling in a 5 mL round-bottom tube.

4. Capillary with the marked line

Use a permanent marker to write a mark showing the end position of loaded sample on the capillary. Put a mark at 40 mm from the bottom edge when loading the sample solution of 8 μL, and mark two lines at 35 and 40 mm, when seeding.

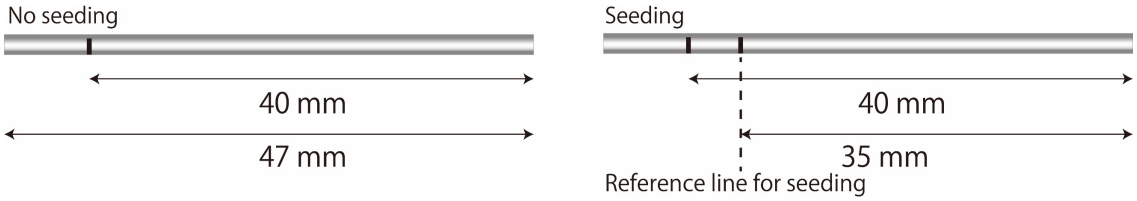

Fig. 3.3 Reference lines on the capillary

3.1.4. Assembling the items to start crystallization

1. Loading the sample solution to the marked line

When the tip of the capillary is immersed in the sample solution, the solution is sucked up into the capillary by the capillary action. If it is difficult, lay the capillary on its side to make it easier. Take care not to lift the capillary out of the sample solution during loading to avoid air bubbles in the capillary.

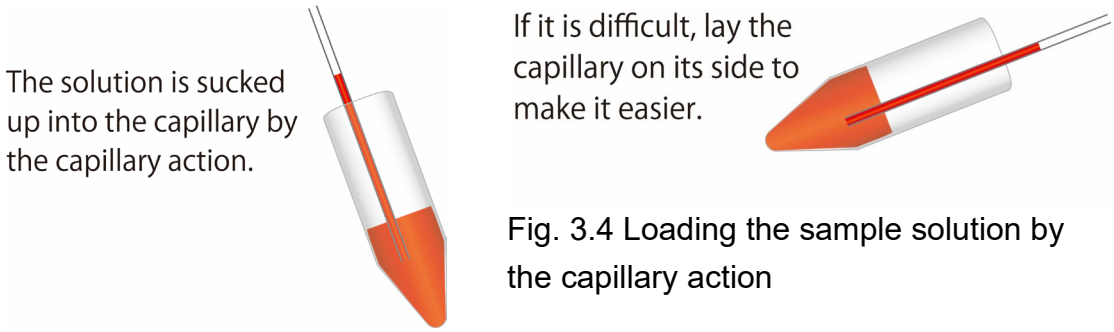

Fig. 3.4 Loading the sample solution by the capillary action

For seeding, fill the sample solution up to the first reference line, then move the capillary into the seed solution and fill the seed solution until it reaches the second reference line.

Depending on the properties of the sample solution, the solution does not enter by the capillary action (in case of highly viscous solution, etc.), or conversely, the solution immediately fills the capillary (in case of solutions including detergents, organic solvents, etc.). In these cases, use a micropipette for quantitative loading as follows.

Attach a silicone tubing to a 20 μL micropipette tip, and attach its other end to the top of the capillary. Adjust the micropipette to the filling volume, and immerse the bottom end of the capillary in the sample solution for aspiration. Alternatively, turn the micropipette dial to aspirate the solution quantitatively into the capillary. After loading the required amount, remove the silicone tubing gently with taking care not to affect the amount of liquid in the capillary.

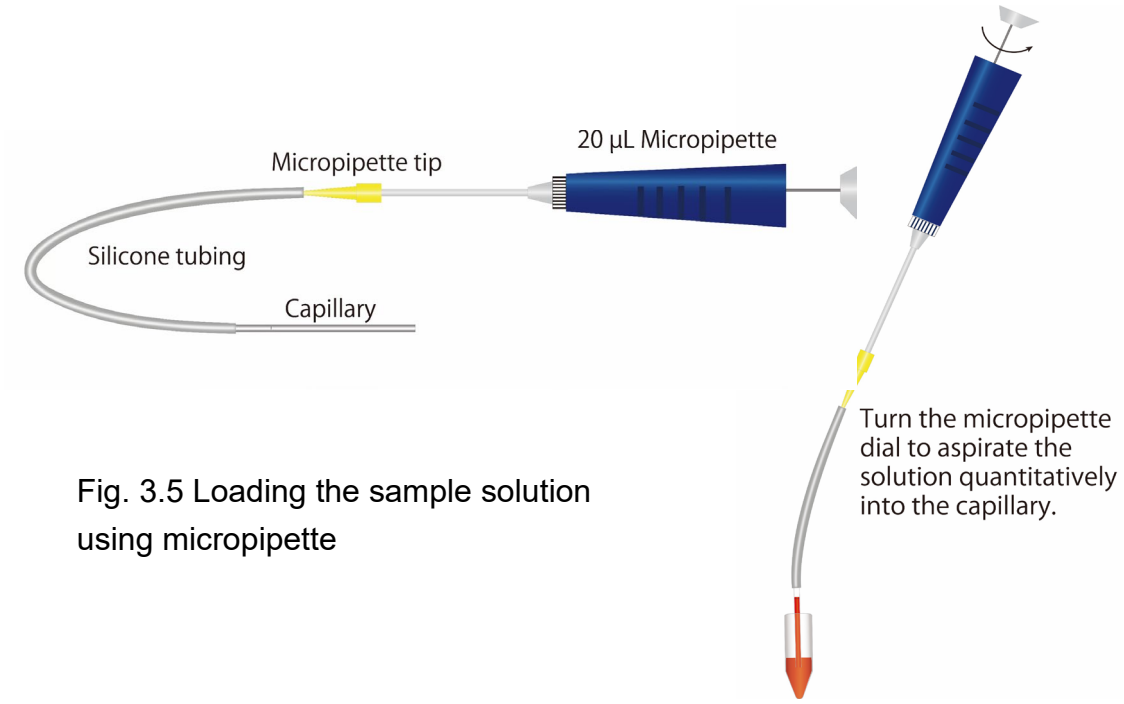

Fig. 3.5 Loading the sample solution using micropipette

For seeding, fill the sample solution up to the first marked line, then move the capillary into the seed solution and turn the dial of the micropipette so that the sample solution reaches the second line.

2. Sealing the capillary

Seal the upper end of the capillary with the sealing compound. Turn the sealing compound container upside down, and push it onto the upper end of the capillary until the capillary reaches the bottom plate of the sealing compound container.

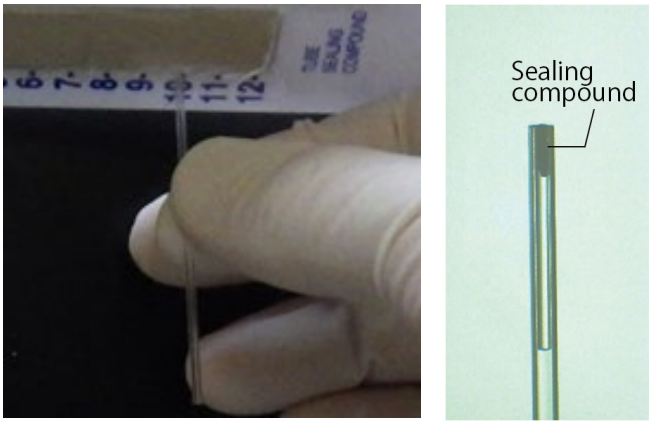

Fig. 3.6 Sealing the capillary

Air bubbles sometimes enter the lower end of the capillary at the loading. Air bubbles prevent the reservoir solution from diffusing in, so stuff the sealing compound again and again from the top of capillary to push out the air from the bottom. Allow the droplet of sample solution to be observed from the bottom of the capillary.

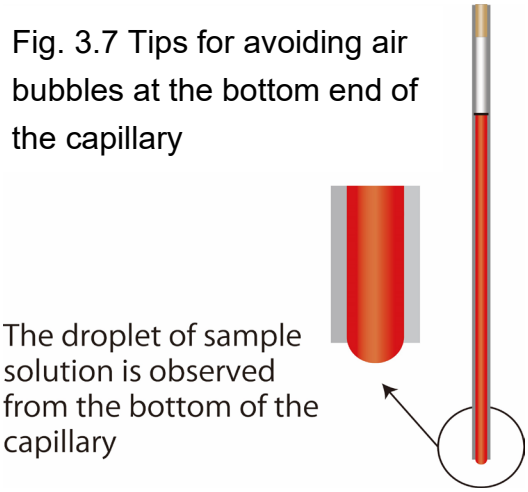

Fig. 3.7 Tips for avoiding air bubbles at the bottom end of the capillary

3. Attaching the gel tube

Drip a small amount of gel soaking solution on the capillary end of the gel tube before attaching the capillary to prevent air between the capillary and the gel tube. Then, attach the gel tube to the bottom end of the capillary. A small amount of the gel will be pushed out from the bottom end of the gel tube after the connection.

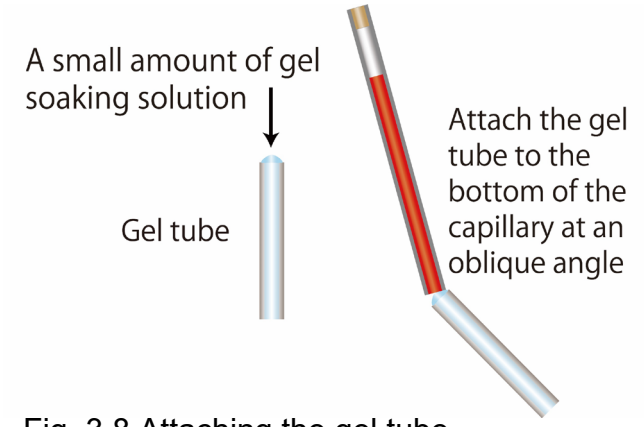

Fig. 3.8 Attaching the gel tube

Next, cut the bottom end of the gel tube diagonally with a sharp blade. It enables the reservoir solution to easily diffuse into the capillary, even if, the tip of the gel tube is in close contact with the bottom of the round-bottom tube.

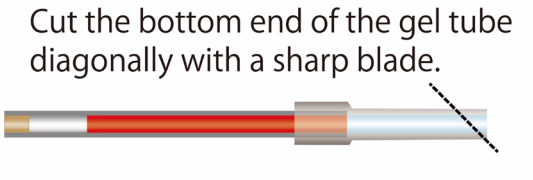

Fig. 3.9 Cutting the bottom end of the gel tube

4. Starting crystallization

Gently place the capillary into the round-bottom tube containing the reservoir solution and cap it. Place it vertically with the gel tube facing down under adequate temperature. In many cases, the reservoir solution containing the crystallization reagent has a higher density than the sample solution. Thus, in the capillary with the gel tube side down, the density of the solution increases toward the bottom of the capillary, suppressing the density-driven convection in the capillary. As a result, we can expect to obtain better quality crystals due to the similar effects of convection suppression in a microgravity environment. **Note**: the capillary must be placed horizontally during observation, but there is no problem for the short periods.

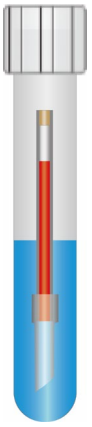

Fig. 3.10 Place vertically and start crystallization

3.2. Setting up crystallization by the DPOC method

3.2.1. Required items

(For one crystallization condition)

Solution	Amount	Description
Sample solution	5～7 μL	It contains a protein sample solution to be loaded into a DPOC tube. Since the inner diameter of the DPOC tube is 1.0 mm, the required volume is 4.7 μL for a sample length of 6 mm, and 6.3 μL for that of 8 mm. It is desirable to prepare a little more to load it without failure.
Crystallization solution	5～7 μL	It contains crystallization reagents and buffers, which may also contain ligands if required. It interdiffuses with the sample solution in the DPOC tube to crystallize the protein. When seeding, it also contains the seed crystals.
Reservoir solution	ca. 4 mL	It contains the same concentrations of crystallization reagents and the buffers as the crystallization solution. No ligands and seeds are required. It is used to immerse the DPOC tube.

Equipment in the kit	Qty.	Description
DPOC tube (ϕ 1.0 mm × 30 mm)	1	It has short capillaries on both ends.
C-Cap	2	It is a stopper to plug the capillaries of the DPOC tube.
Sealing compound	1	It is used for sealing the ends of the capillary.
Silicone tubing	1	It is used to load solutions into the DPOC tube.

Round-bottom tube (5 mL)	1	A vessel for the crystallization.

Required equipment	Qty.	Description
Micropipette / Tip	each 1	It is for preparing solutions.
Micropipette / Tip (20 to 100 µL)	1	It is for loading solutions into DPOC tubes.
Fine-point permanent marker	1	It is for marking the end position of loaded sample on the DPOC tube.

3.2.2. DPOC tube pre-soaking

1. Reservoir solution

This is the solution outside the DPOC tube settled in the round-bottom tube, containing the same concentrations of the crystallization reagents and the buffer as the crystallization solution filled in the DPOC tube. No ligand components, seeds, etc. are required.

2. Marking on DPOC tube

Mark the line on DPOC tube for loading solution using a permanent marker. If the sample volume is 4.7 µL (sample length 6 mm), mark at 9 mm from the end of the container; if the sample volume is 6.3 µL (sample length 8 mm), mark at 7 mm, these loading volumes enable air bubbles to enter both ends of the loaded solution.

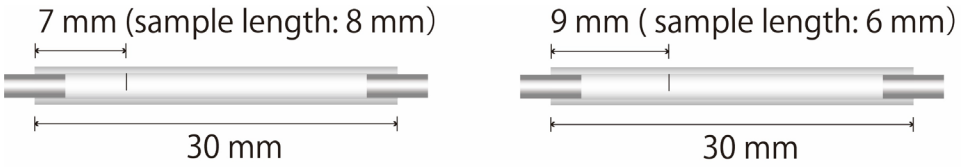

Fig.3.11 Reference lines on DPOC tube

3. Pre-soaking the DPOC tube

Immerse the DPOC tube in the reservoir solution for one to several days to remove the air adsorbed on the tube material and allow it to absorb moisture.

3.2.3. Preparation of solutions

1. Sample solution

Prepare the required amount of protein sample solution for loading. If it is necessary to mix ligands etc., mix them before loading into the DPOC tube.

2. Crystallization solution

A solution that crystallizes proteins by interdiffusing with the sample solution in the DPOC tube. Add not only crystallization reagents and buffers, but also ligands, if required.

When seeding, prepare micro seeds or macro seeds from seed crystals in advance, and add adequate amount of them to crystallization solution.

3.2.4. Assembling the items to start crystallization

1. Attaching the DPOC tube to a silicone tubing and a micropipette

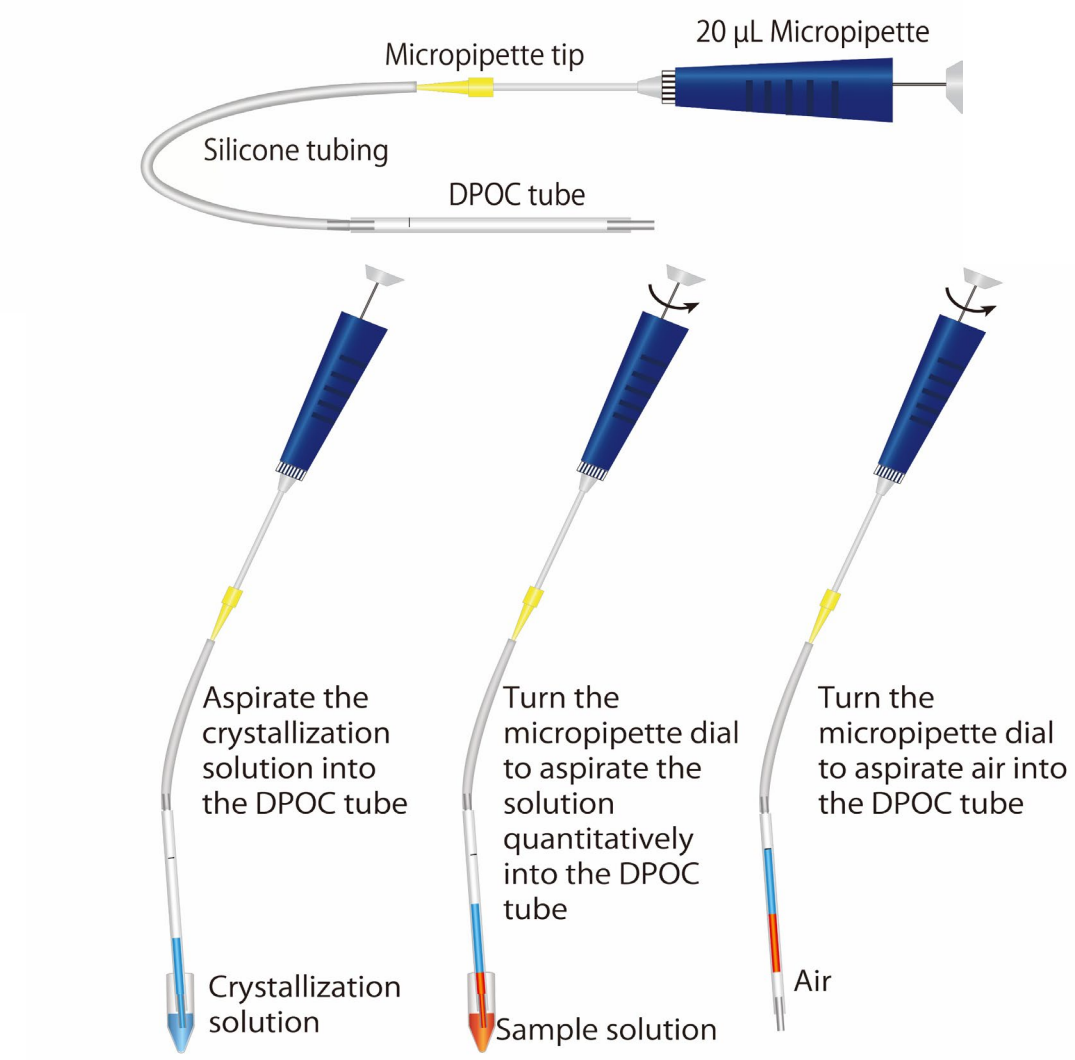

Fig. 3.12 Loading solutions into the DPOC tube

Pick the pre-soaked DPOC tube from the reservoir solution, and connect its marked-side end to a micropipette via a silicone tubing. If there remains reservoir solution in the DPOC tube, remove it by pipetting.

2. Loading solutions

Place the other end of the DPOC tube into the crystallization solution, and aspirate the solution of 4.7-6.3 µL. Next, move the end of the DPOC tube into the sample solution and aspirate the solution of the same amount as the crystallization solution. Then, remove the end of the DPOC tube from the solution, and load air by turning the dial of the micropipette until the surface of the crystallization solution reaches the marked line on the DPOC tube.

3. Sealing the DPOC tube

Stay the DPOC tube connected to the silicone tubing and seal the free end of the DPOC tube with sealing compound. Then remove the silicone tubing and seal the opposite end of the DPOC tube with the sealing compound.

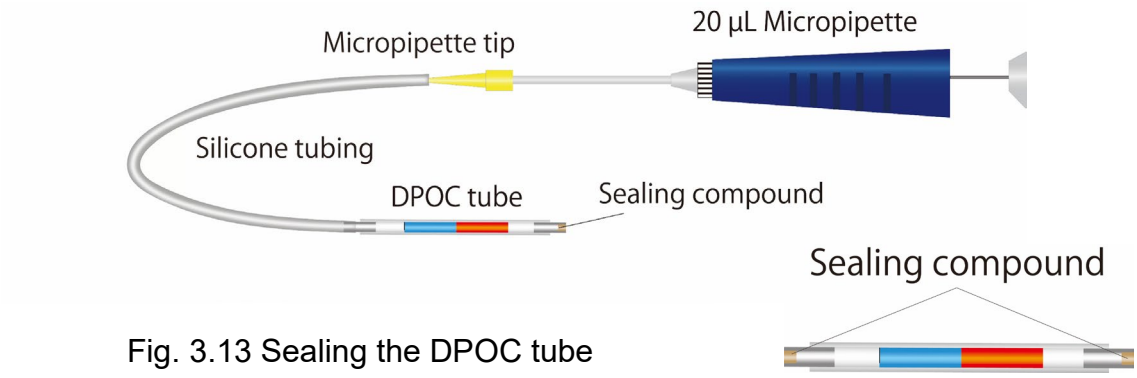

Fig. 3.13 Sealing the DPOC tube

4. Attaching C-Caps

In order to protect the both outer ends of the DPOC tube from excessive osmotic pressure, plug each outer ends of the capillaries by C-Caps. Insert the edge of the capillary of the DPOC tube to the diagonally cut end of the C-Cap. There is a plastic plug inside the C-Cap, so push it in together. At this time, hold it with your finger so that the plug does not protrude from the opposite end. The installing is complete when the plug is pushed all the way to the end of the C-Cap opposite side.

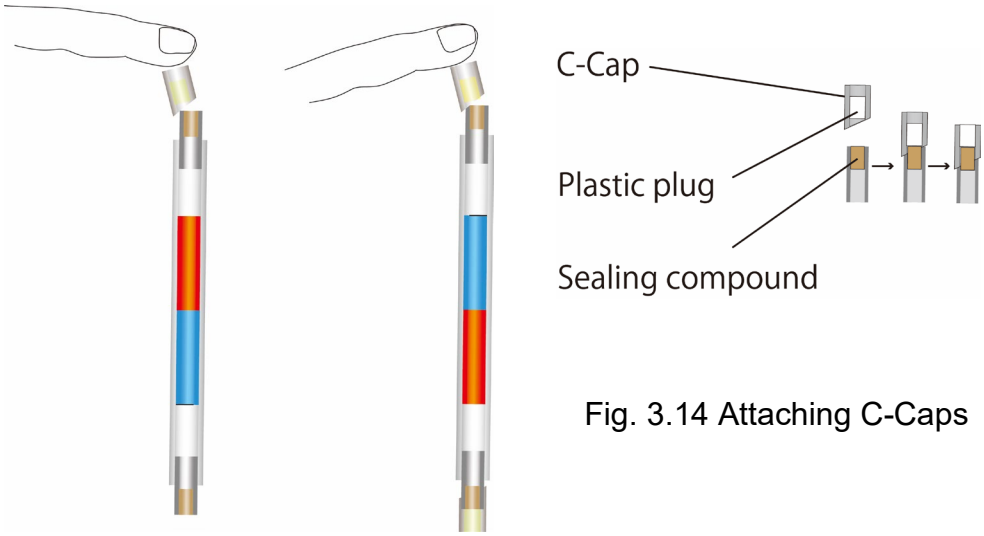

Fig. 3.14 Attaching C-Caps

5. Starting crystallization

Add about 4 mL of reservoir solution to the round-bottom tube, gently place the DPOC tube in it with crystallization solution-side down, and cap the round-bottom tube. Make sure that the entire DPOC tube is immersed in the reservoir solution in the round-bottom tube, and place it vertically under the desired temperature. In many cases, the crystallization solution has a higher density than the sample solution. Thus, when the crystallization-solution side is facing down, the density increases toward the bottom, which suppresses the movement of the solution in the DPOC tube due to the density-driven convection. As a result, we can expect suppression of solution convection as that under a microgravity environment to obtain better quality crystals. **Note**: the DPOC tube must be placed horizontally during observation, but there is no problem for the short periods.

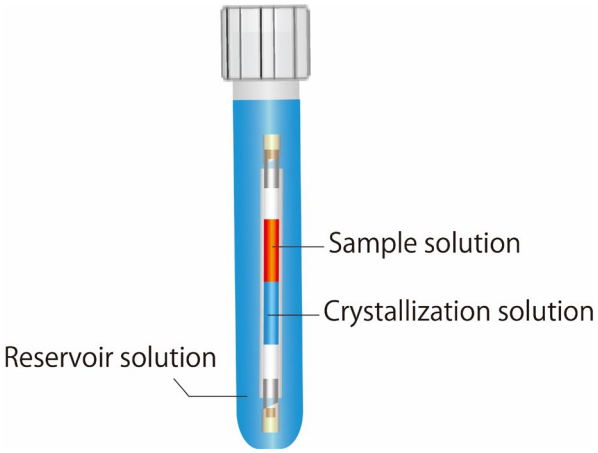

Fig. 3.15 Place vertically and start crystallization

3.3. Harvesting crystals from the CD capillary

3.3.1. Harvest solution

To pick out crystals with keeping their good quality, the harvested crystals should be stored in a harvest solution with the same composition as the solution in the capillary where the target crystals are located. If the concentrations are not appropriate, the crystals may dissolve or break due to the osmotic pressure difference.

The concentrations of the components in a certain part of solution in a CD capillary depends on its position and crystallization time. It is necessary to utilize a harvest solution whose composition is almost identical to the solution around the target crystal in the capillary. For a successful crystal harvesting, we recommend to prepare several harvest solutions whose concentrations of components are close to the estimated values. Our simulation program, separately sold, will be helpful for the estimation of component concentrations in the CD capillaries (http://www.confsci.co.jp/product_e.html).

3.3.2. How to cut the capillary

Cut the capillary 3-4 mm apart from either side of the target crystal. Make a sharp scratch on the capillary glass with a diamond file or capillary cutter, bend it in the direction to open the scratch, and apply a pulling force to cut it.

3.3.3. Harvesting crystals

Place the prepared harvest solution in the hole slide glass. Then, a short piece of capillary is immersed in the solution and held with forceps. While observing with a stereo microscope, make a flow of harvest solution around the crystal by pipetting, and pour the crystal out of the capillary into the harvest solution.

Some crystals may adhere to the glass surface of the capillary. In such cases, carefully touch and move with a thin, blunt tool such as a gel loading tip (QSP 124-R204). Add a stream of solution by pipetting around the crystal again. The flow of solution drives the crystals out of the capillary into the harvest solution. The crystals in the harvest solution are scooped with a cryoloop, immersed in a cryoprotectant solution if necessary, and then used for X-ray diffraction experiments. Details will be explained in “3.5. Cryocooling crystals”.

For practice, it is recommended to prepare lysozyme crystals by the CD method and use them to familiarize yourself with the procedure for picking out

crystals. Because lysozyme crystals easily adhere to the inner surface of the capillary, they will be the good crystals for training the operation.

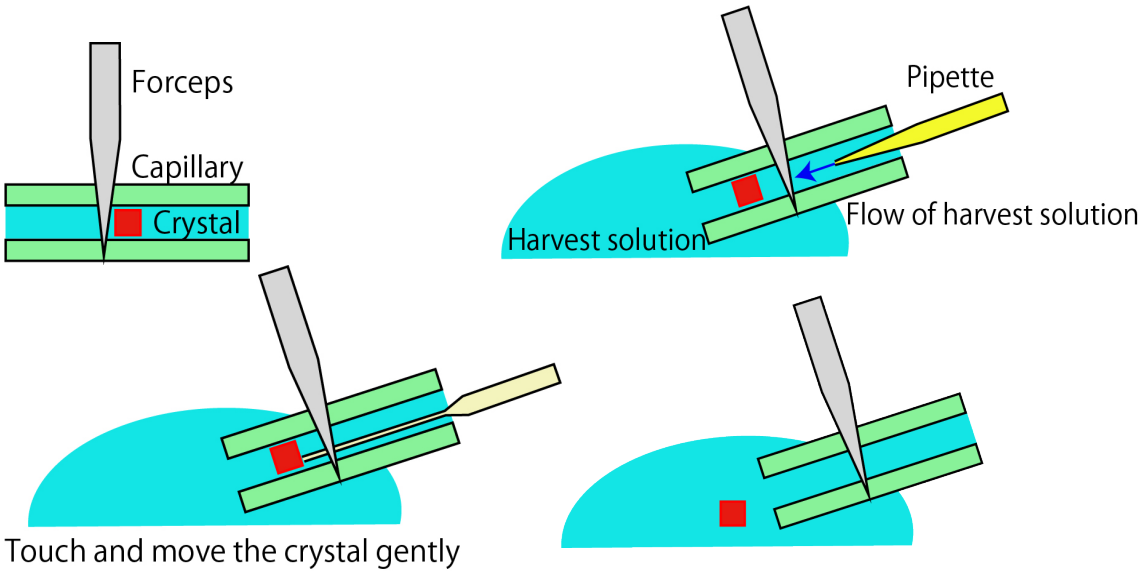

Fig. 3.16 Harvesting crystals
Upper left: Fix the cut capillary with forceps so that you can work with your hands free while observing with a stereo microscope
Upper right: Pipette the solution into the capillary and push the crystal gently
Lower left: If the crystal adheres to the capillary, touch it gently with a gel loading tip and move it a little.
Lower right: The crystal is poured into the harvest solution

3.4. Harvesting crystals from the DPOC tube

3.4.1. Harvest solution

Diffusion of the crystallization reagent inside the DPOC tube is rapid, and even the diffusion pair of PEGs and sample solution reaches equilibrium around 10 days. However, osmotic concentration of sample solution by an external reservoir solution takes several weeks. The actual concentration in the DPOC tube can be estimated by the length of solution in the DPOC tube when harvesting. Based on that estimation, prepare a harvest solution by diluting the crystallization solution to the same concentration as the solution around the crystal.

3.4.2. How to cut the DPOC tube

Cut the DPOC tube a few mm apart from either side of the target crystal with a surgical blade (such as a sharp knife for delicate work). When cutting, be careful not to deform the DPOC tube and crush the crystal inside.

3.4.3. Harvesting crystals

Place the prepared harvest solution in the hole slide glass. Then, a short piece of DPOC tube is immersed in the solution and held with forceps. While observing with a stereo microscope, make a flow of harvest solution around the crystal by pipetting, and pour the crystal out of the DPOC tube into the harvest solution. The crystals in the harvest solution are scooped with a cryoloop, immersed in a cryoprotectant solution if necessary, and then used for X-ray diffraction experiments. Details will be explained in “3.5. Cryocooling crystals”.

For practice, it is also recommended for you to prepare lysozyme crystals by the DPOC method and familiarize yourself with the procedure for harvesting crystals.

3.5. Cryocooling crystals

In X-ray diffraction using synchrotron radiation, crystals must be cryocooled to protect from radiation damage. In that case, the water around the crystal should freeze into amorphous ice. If not, crystalline ice would damage the protein crystals and a good X-ray diffraction image would not be obtained. To prevent the formation of crystalline ice during freezing, scoop the crystals out of the harvest solution with a cryoloop, immerse them into the cryoprotectant solution for a few seconds to replace the solution around the crystals with a cryoprotectant, and then, cryocool them rapidly.

PEG-based crystallization reagents themselves have a cryoprotective effect. So, if the harvest solution containing 35-40% PEG, crystals can be cryocooled without adding extra cryoprotectant. If the main component of the harvest solution is PEG-based, but the concentration is lower, adding the same kind of PEG to the harvest solution is a reliable method to prepare the cryoprotectant solution. Alternatively, glycerol can be added to make a cryoprotectant solution. In that case, the glycerol concentration together with the PEG concentration will be adjusted approximately 40%.

When salts are used for the crystallization reagents, glycerol is the most common cryoprotectant used as an additive to the harvest solution. A glycerol concentration of approximately 40% would suppress the formation of crystalline ices. trehalose, ethylene glycol, PEG400, etc. are also used as cryoprotectants.

Before applying your cryoprotectant solution to the target crystals, it is recommended to check whether the solution can suppress the formation of crystalline ice: the droplet of good cryoprotectant solution will freeze into an amorphous ice when scooped with a cryoloop and frozen quickly.

However, problems such as cracks in the crystal may sometimes occur due to the large difference in osmotic pressure between the liquid in the crystal and the cryoprotectant solution. This tends to occur especially with using low-molecular-weight cryoprotectants such as glycerol. In that case, it is recommended to gradually increase the concentration of cryoprotectant in the cryoprotectant solution, which is easy if the crystallization is performed by the CD method (see “4.2.4. Easy soaking (the CD method)”).

As a cryocooling method, please use a method that you already have experienced, such as flash cooling or direct immersion in liquid nitrogen.

4. Technical Notes

4.1. Time course of concentration change of the solution

Protein crystallization can be expected when the concentrations of the protein sample and the crystallization reagent are in the proper combination. To obtain crystals, it is important to understand the time course of concentration change of protein solution and crystallization reagent in the CD method capillary and in the DPOC tube.

4.1.1. Concentration changes of the CD method sample solution

One-dimensional diffusion simulation estimates the time course of the concentration of the crystallization reagent in the CD capillary. A simulation program (purchase available as **C-Kit Pro Advanced Tool**, CRT209, http://www.confsci.co.jp/product_e.html). makes it easy to estimate diffusion profiles under various conditions. Examples of diffusion simulations for commonly used crystallization reagents such as NaCl and PEG4000 are shown below.

Example 1

Sample solution: 40 mm length in a capillary

Reservoir solution: 1 M NaCl

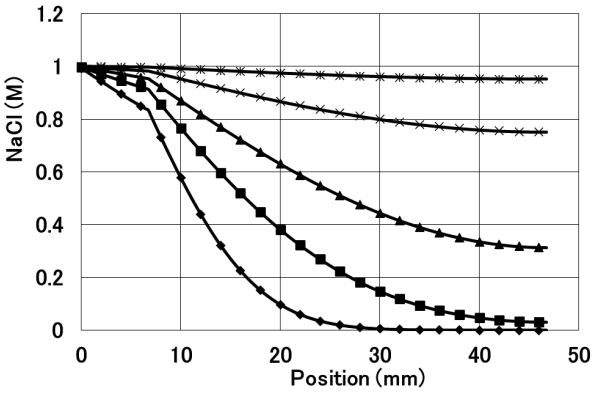

Fig. 4.1 Estimation of NaCl concentration in a capillary. ◆, ■, ▲, ×, and ✳ show NaCl concentrations 0.25,1,3,8 and 16 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis the NaCl concentration at the location.

The NaCl concentration in the capillary reaches near equilibrium in 16 days. Ammonium sulfate, which is often used as a crystallization reagent, has almost the same diffusion coefficient as NaCl, so the diffusion time course is almost the same.

Example 2

Sample solution: 40 mm length in a capillary

Reservoir solution: 25% PEG4000

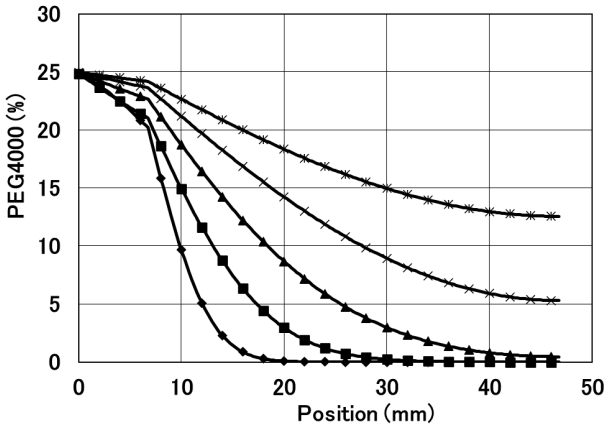

Fig. 4.2 Estimation of PEG 4000 concentration in a capillary.

◆, ■, ▲, × and ✳ indicate PEG 4000 concentrations 1, 4, 12, 32 and 64 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis the PEG 4000 concentration.

Even after 64 days, the PEG4000 concentration in the capillary only increased to about half of the reservoir solution concentration at the distal end from the gel tube. To obtain crystals in a short period, either the higher concentration of PEG4000 in the reservoir solution or premixing an appropriate concentration of PEG4000 with the protein sample in the capillary are recommended. Since the diffusion coefficient of PEGs is approximately inversely proportional to the 1/2 power of its molecular weight[7], equilibration of the PEG concentration is much slower when using higher-molecular-weight PEGs.

7) Luo, Z.; Zhang, G. Scaling for sedimentation and diffusion of poly (ethylene glycol) in water. *J. Phys. Chem. B* 2009, 113, 12462–12465

Example 3

Sample solution: containing 12.5% PEG 4000, 40 mm length in a capillary

Reservoir solution: 25% PEG4000

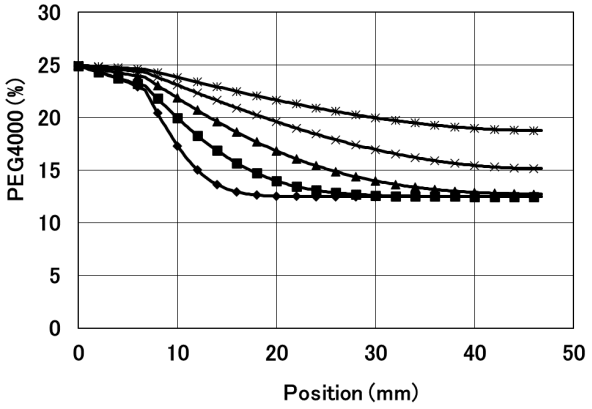

Fig. 4.3 Estimation of PEG4000 concentration in a capillary. Symbols are the same as in Figure 4.2.

PEG4000 was added to the sample solution in the capillary in Fig. 4.3, so the PEG concentration rises quickly than that in Fig. 4.2.

4.1.2. Concentration changes of the solution in the DPOC tube

The time-course of interdiffusion of protein solution with crystallization solution in the DPOC tube can also be estimated by the one-dimensional diffusion simulation.

Example 4

Sample solution (8 mm) and 1M NaCl solution (8 mm) in a DPOC tube
Reservoir solution : 1M NaCl

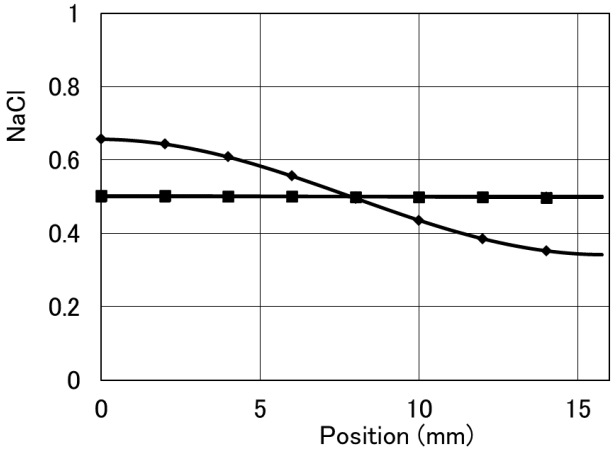

Fig. 4.4 Estimation of NaCl concentration in a DPOC tube.
◆ and ■ indicate NaCl concentrations 0.25 and 1 day after loading. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis the NaCl concentration at the location.

The NaCl concentration in the capillary reaches near equilibrium only in one day.

Example 5

Sample solution (8 mm) and 25% PEG4000 solution (8 mm) in a DPOC tube

Reservoir solution：25% PEG4000

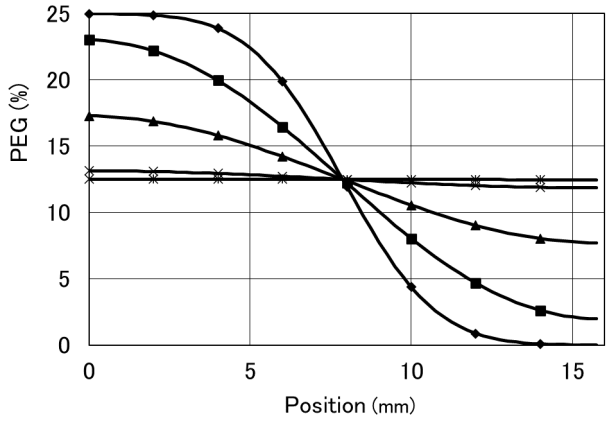

Fig. 4.5 Estimation of PEG4000 concentration in a DPOC tube. ◆, ■, ▲, × and ✳ indicate PEG4000 concentrations 0.25, 1, 3, 8 and16 days after loading. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis the PEG4000 concentration at the location.

PEG4000 in the capillary reaches equilibrium 8 days after loading.

Further, the solution in the DPOC tube would be slowly concentrated by osmotic concentration caused by the concentration difference between the solution in the DPOC tube and the reservoir solution, so the concentration process was measured in Fig. 4.6.

Example 6 Sample solution (4 mm) and 20% PEG4000 solution (4 mm) in a DPOC tube

Reservoir solution：20% PEG4000 containing 600 mM NaCl

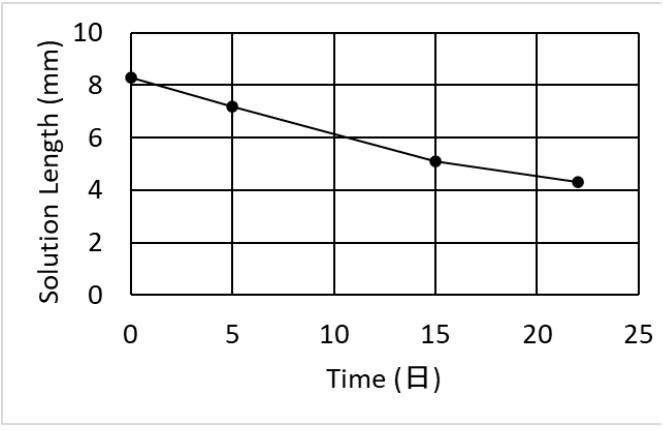

Fig. 4.6 Time-course of solution concentration indexed by the solution length in a DPOC tube.

After 22 days, the solution volume in the DPOC tube is concentrated by half and reaches equilibrium.

Fig. 4.6 shows that the time to reach equilibrium is approximately 3 weeks.

4.2. Benefits for the CD method and the DPOC method

The advantages and limitations of CD method and DPOC method are described as follows. Consider these differences to select the optimal crystallization method.

4.2.1. Crystals with high resolution (the CD method)

In general, the higher the concentration of the main crystallization reagent (e.g., PEG or ammonium sulfate), the higher the diffraction resolution tends to be. In the conventional vapor diffusion method, crystallization under such conditions tends to cause clustering or precipitation. On the other hand, in the CD method, a higher concentration of crystallization reagents can be applied to obtain crystals that diffract X-rays to high resolution.

4.2.2. Subcomponent elimination from protein sample (the CD method)

Protein samples usually contain low molecular weight compounds such as salts, EDTA, or buffers that are resulting from the purification process. Some of these may inhibit crystallization. A typical component inhibiting crystallization is high concentrations of NaCl. Contaminating NaCl may cause precipitation when the crystallization reagent is added, or it may suppress nucleation and crystallization.

The CD method is good for crystalizing such protein samples. In the CD method with a gel tube, the concentration of the crystallization reagent in the capillary increase over time as shown in the previous section. At the same time, low-molecular-weight components in the capillary diffuse out, and their diffusion rates are faster than those of protein samples. As a result, low-molecular-weight components in the capillary will decrease, allowing crystallization without the effects of components that inhibit crystallization.

On the other hand, osmotic concentration in the DPOC method increases the concentrations of inhibitory components in protein samples, making their crystallization more difficult.

4.2.3. Small amounts of ligands (the DPOC method)

In the co-crystallization with ligand, the added free compound diffuses out of the capillary in the CD method. Therefore, preparation of complex crystals with a ligand whose K_D value is higher than μM, requires addition of the ligand into the reservoir solution.

On the other hand, in the case of the DPOC method, loss of ligands from sample solution would not occur, and further, the ligand solution would be concentrated during crystallization period. Thus, small amount of ligand added only to the sample solution is required for the DPOC method, and it enables you to obtain the complex crystals with a low-affinity ligand.

4.2.4. Easy soaking (the CD method)

Post-soaking crystals with ligands is very easy in the CD method. After crystals are obtained, the capillary of the CD method is only transferred to a reservoir solution containing the ligand and stand for a sufficient time to allow the ligand diffusing in. You can also soak crystals in cryoprotectants such as high concentrations of PEGs, ethylene glycol and glycerol by just adding them into the reservoir solution.

However, in the DPOC method, picking out crystals from the tube, and then transferring them to a soaking solution is required.

4.3. Design of crystallization conditions

4.3.1. Proteins with unknown crystallization conditions

Commercially available screening kit reagents will be usually applied (ex., https://hamptonresearch.com/cat-1.html). The self-searching mechanism of the CD method and the DPOC method allows a wider range of combinations of concentration conditions to be searched in a single capillary/tube than the batch method and vapor diffusion method that are commonly used.

Easy protocol for screening crystallization condition is as follows.

1. Choose 2 groups of screening kit reagents: one group containing salts (e.g., ammonium sulfate) and the other containing PEGs (e.g., PEG4000) as a main crystallization reagent. Calculate the pI value of your protein sample. Then, select two reagent bottles from each group: one bottle having a preset pH above the pI (+1 to +2) and the other below the pI (-1 to -2) of the protein sample.
2. First, perform crystallization experiments by the CD method. Prepare two sample solutions for each reagent: one contains only the protein sample and the other contains the protein sample mixed 1:1 with the selected reagents. Use the corresponding 4 reagents as the reservoir solutions. Then, test your protein crystallization with the 8 crystallization conditions.
3. Then, crystallization experiments by the DPOC method are recommended, using the 4 selected screening kit reagents as both the crystallization solution and the reservoir solution.
4. When no crystals are obtained after a maximum of one-month, possible matters and countermeasures might be as follows.

The CD method	The DPOC method	Countermeasure
No change		The protein molecule may have a large charge. Retest with a reservoir solution containing an additional 100-150 mM NaCl.
Gradual precipitation		Close to the conditions for obtaining crystals. Retest using a reservoir solution with reduced concentration of one component of the kit solution. Reduce the concentration of

		the supporting component of the crystallization reagent.
Crystals grow once on the gel tube side of the capillary, but disappear later.	—	Sample solution may contain something required for crystallization. Retest the CD method by adding components to the reservoir solution that are only contained in the sample solution.

5. If crystals cannot be obtained even after taking these countermeasures, try several other reagents with a similar pH in the screening kit.

4.3.2. Proteins with known crystallization conditions

Even in the case of protein samples for which crystallization has been reported so far, we often experience that the crystals cannot be reproduced. In that case, possible cause is difference in salts and buffers included during the preparation of protein samples. Therefore, the CD method is preferable in those cases, because it is less affected by those differences as mentioned before.

Of course, in the both CD and DPOC methods, the self-searching mechanism allows a wider range of combinations of concentration conditions to be searched in a single capillary/tube than the batch method or vapor diffusion method, so that you can obtain crystals from your own preparation more efficiently.

Easy protocols to screen crystals are as follows.

1. Select several crystallization conditions reported in papers or PDB.
2. Then, crystalize by the CD method using the reported crystallization solution as the reservoir solution. And prepare two sample solutions: one is reported protein solution, and the other is the protein solution mixed 1:1 with the reported crystallization solution.
3. Setup crystallization experiment with the DPOC method.
4. If no crystals are obtained after a maximum of one-month, please refer to the previous section (“4.3.1. proteins with unknown crystallization conditions”) for countermeasures.

Furthermore, on-line technical support (paid) is available via email or video meeting with whom expertized in both methods: **C-Kit Ground Pro e-mail support** (CRT101-3 http://www.confsci.co.jp/product_e.html), **C-Kit Ground Pro video support** (CRT101-4 http://www.confsci.co.jp/product_e.html) will surely help your success in crystallization.

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