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

June. 2024

Confocal Science Inc.

Table of Contents

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

Product name	Qty.	Description
Capillary (φ0.5 mm × 47 mm)	15	Used in the CD method. 3 extra gel tubes included.
Gel tube (φ1.0 mm × 1 cm)	18	
DPOC tube	8	Used in the DPOC method.
C-Cap	16	
Silicone tubing (φ1.0 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 and developed from the know-how of crystallization experiments at 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 (CD) method [1)] and the diffusion-pair osmotic-concentration (DPOC) method [2)]. Both methods are used to grow crystals on the basis of the principle of auto-searching for optimal crystallization conditions (self-searching mechanism) [3)]. The newly developed DPOC method enables us to search for a wide range of crystallization conditions because the protein samples and crystallization solutions in the DPOC tube are osmotically concentrated owing to the osmotic pressure difference between the inside and outside of the DPOC tube. You can choose the appropriate method according to the sample characteristics to grow high-quality apo/complex crystals.

The CD equipment included in the kit was jointly developed by Confocal Science Inc., under commission from 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 the C-Kit Space Pro Series for high-quality crystallization under microgravity at the ISS.

1) García-Ruiz, J.M.; Moreno, A. Investigations on protein crystal growth by the gel acupuncture method. *Acta Crystallogr., Sect. D* 1994, 50 (4), 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 Radiat.* 2004, 11, 45–48.

5) JP patent 4354457, US patent 7531037

1.2. Features

- A small amount of the protein sample is required: The standard amount is 8 µL (CD method capillary) and 5 µL (DPOC tube).
- Slow crystallization methods lead to high-quality crystals, and both CD and DPOC methods provide mild crystallization conditions by adjusting the concentrations of the protein sample and crystallization reagent.
- Simple protocols: Both CD and DPOC protocols are easy to perform.
- High reproducibility and reliability: Both methods have been repeatedly used in space experiments conducted by JAXA and the Japan Manned Space Systems Corporation (JAMSS). Thus, their high reproducibility and reliability have been proven. In particular, the CD method has been applied to the crystallization of more than 500 proteins under microgravity since 2002.
- Easy soaking in the CD method: Soaking can be performed by simply transferring crystal-containing capillaries to a container filled with the solution for post-soaking.
- Small amounts of ligand in the DPOC method: Ligands are pre-mixed with the protein solution loaded into the tube; therefore, a small amount of ligand is sufficient 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 tubes and DPOC tubes.
- Estimation of optimal conditions: Time-dependent changes in concentrations of the protein and crystallization reagents in the CD capillaries and DPOC tubes can be estimated using a one-dimensional diffusion simulation program (available for purchase 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 of the CD and DPOC methods” to understand which method is better for your experiments.

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

2.1. The counter-diffusion (CD) method

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

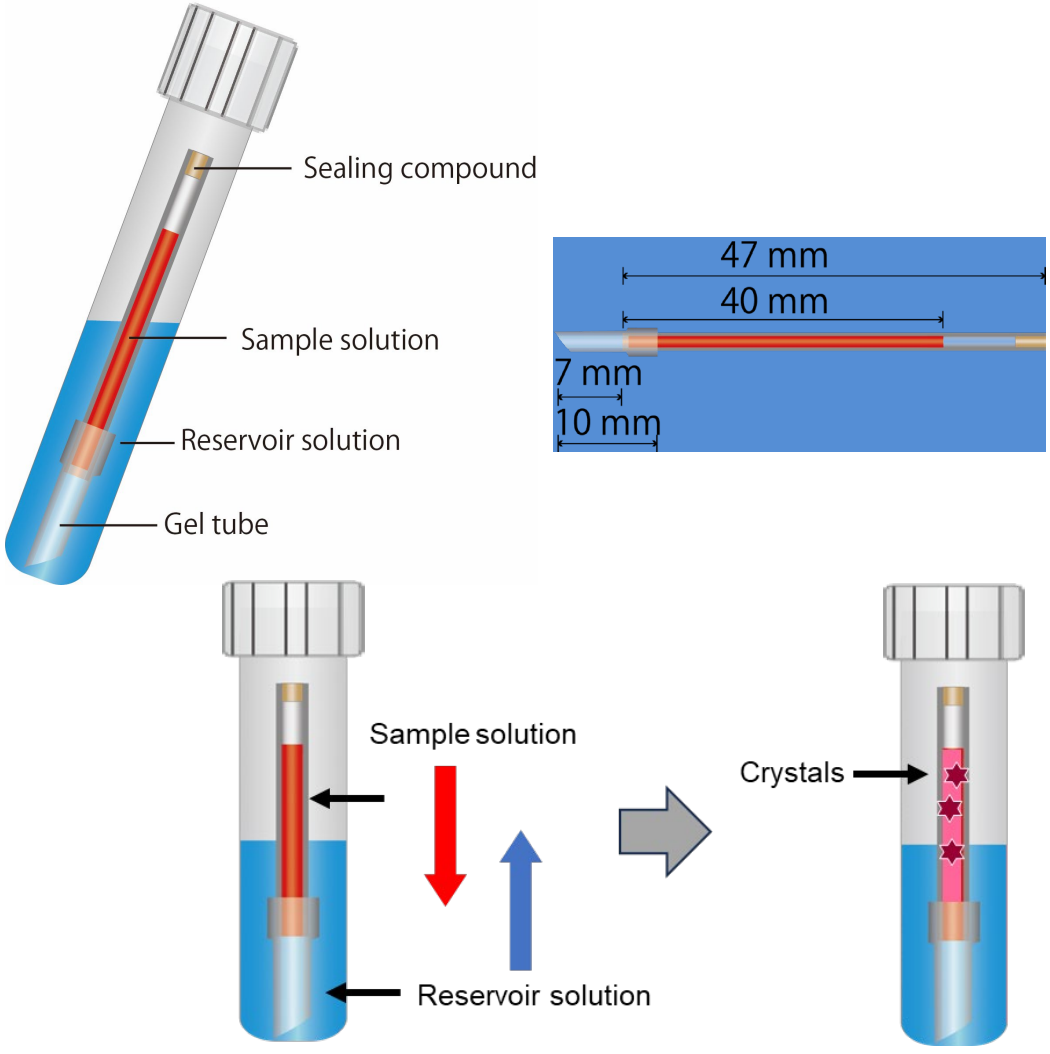


Fig. 2.1 GT method illustration.
Upper left: Configuration of the GT method. Upper right: Detailed view of a capillary with a gel tube. Bottom: Crystallization mechanism of the GT method.

A 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 the protein sample. The reservoir solution containing the 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. The protein samples and other coexisting components in the sample solution also gradually diffuse out of the capillary. The diffusion results in a continuous and wide range of concentration combinations of the crystallization reagent and sample solution within the capillary. Consequently, crystallization is triggered when the concentration combination is suitable for crystallization. This phenomenon is known as “the self-searching mechanism.” Understanding the diffusion time course of the crystallization reagent and protein sample is important for this crystallization method [4]. See “4.1. Time course of concentration changes of the solution” for more details.

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

C-Kit Ground Pro XRD includes crystallization tubes for the DPOC method as another type of crystallization method. Figure 2.2 provides a simple explanation of the standard configuration of the DPOC method and mechanism of 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 protein sample to form a diffusion pair and interdiffusion for crystallization [6]. In our DPOC method, the glass capillary is replaced with a water-permeable silicone rubber tube. The solution in the DPOC tube is osmotically concentrated owing to the osmotic pressure difference between the reservoir solution outside and the solution inside. The diffusion phenomenon in the diffusion pair is similar to that observed in the CD method; however, an equal amount of the crystallization reagent solution to that of the protein sample is included in the DPOC tube, which results in a narrower range of crystallization conditions than those in the CD method. However, the effect of the osmotic concentration in the DPOC tube enables this method to search for a wider range of conditions. In addition, protein samples can be crystallized under concentrated conditions

without prior preparation. This difference from the CD method is explained in section “4.2. Benefits of the CD and DPOC methods.”

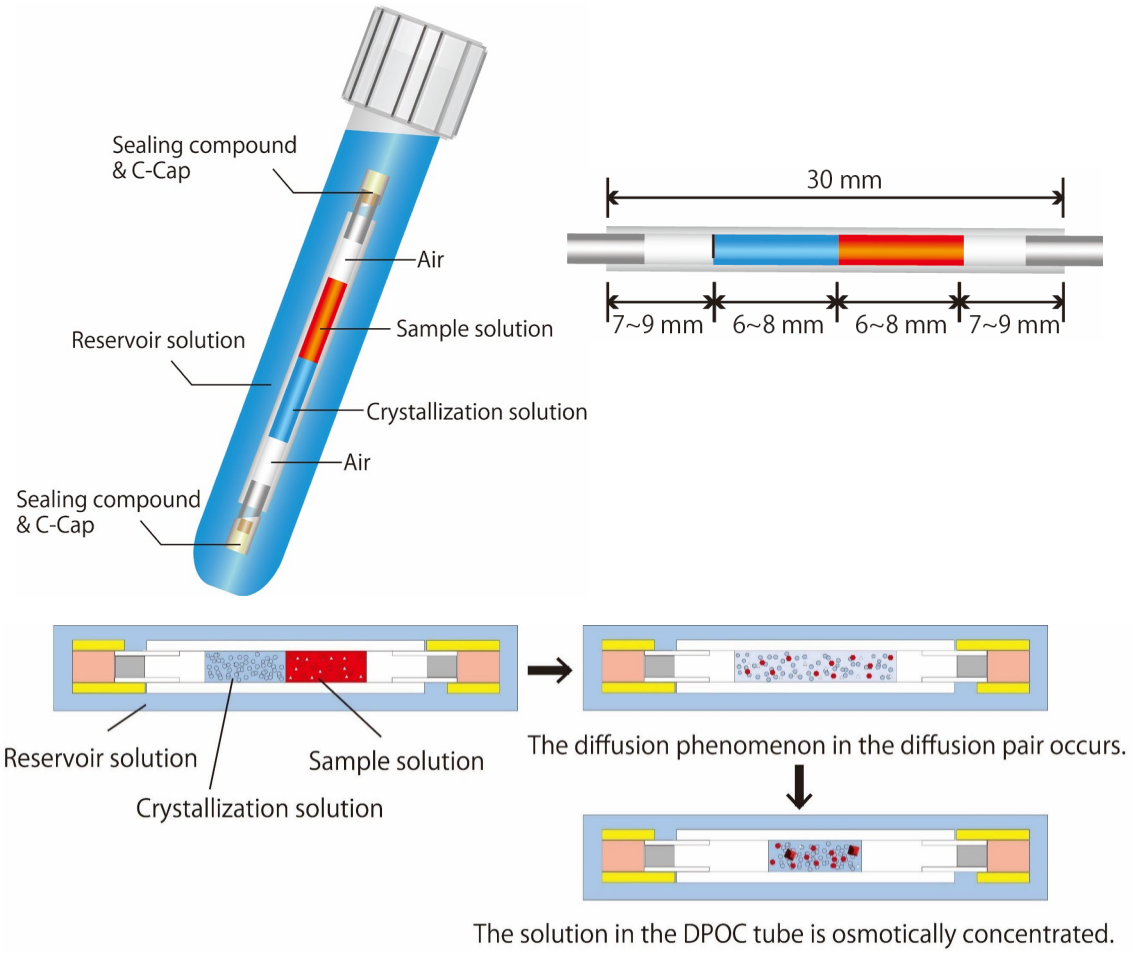


Fig. 2.2 DPOC method illustration

Upper left: Configuration of the DPOC method. Upper right: Detailed view of the DPOC tube. Bottom: Crystals are formed by interdiffusion and osmotic concentration of sample and crystallization solutions.

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 Procedure

3.1. Setting up crystallization via the CD method

3.1.1. Required items

(For each 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. Because 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 40 mm. It is desirable to prepare a little more solution for loading 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 the protein.
Gel-tube soaking solution	ca. 4 mL	It is a solution for immersing the gel tube before the start of crystallization, containing only the buffer component of the reservoir solution or its complete components. A sufficient 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 (φ0.5 mm × 47 mm)	1	Mark the upper end of sample before loading.

Gel tube (φ1.0 mm × 1 cm)	1	Gel tubes in C-Kit Ground Pro are immersed in 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 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 used for preparing solutions.
Micropipette/Tip (20 to 100 µL)	1	It is used for loading solutions into the capillary.
Surgical blade (or a cutting blade)	1	It is used for cutting the lower end of the gel tube. The cutting blade must be sharp.
Fine-point permanent marker	1	It is used for marking the end of the loaded sample on the capillary.

3.1.2. Pre-soaking of the gel tube

1. Preparation of the gel-tube soaking solution

The crystallization reagent requires time to diffuse into the gel tube. Therefore, if you want the crystals to grow faster, the gel-tube soaking solution should have the same composition as that of the reservoir solution. For crystallization under mild conditions, either the buffer component alone or that containing 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 for at least a few days before use. **Caution**: Always keep your gel tubes immersed in the liquid to prevent them from drying.

2. Time required for soaking the gel tube

According to the simulation results of the solute concentration changes 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 achieve equilibrium in the gel tube.

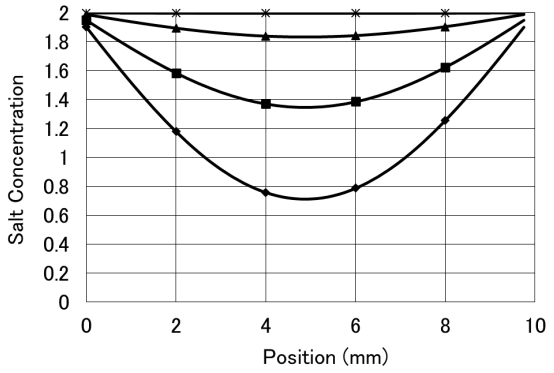


Fig. 3.1 Simulation results of internal NaCl concentration of a gel tube immersed in 2 M NaCl solution.
The horizontal axis is the distance from the end of the gel tube, and the vertical axis is the NaCl concentration. The curves show, from the bottom, NaCl concentrations after 0.05, 0.1, 0.2, and 0.5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

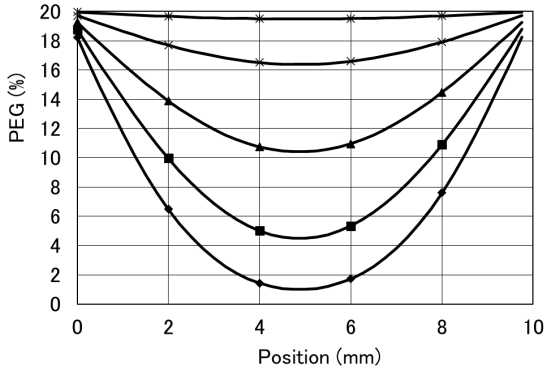


Fig. 3.2 Simulation results of internal PEG4000 concentration of a gel tube immersed in 20% PEG4000 solution.
The horizontal axis is the distance from the end of the gel tube, and the vertical axis is the PEG4000 concentration. The curves show, from the bottom, PEG4000 concentrations after 0.25, 0.5, 1, 2, and 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 the crystallization reagents, ligands, etc., mix them before loading them into the capillary.

2. Seed solution

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

3. Reservoir solution

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

4. Capillary with a marked line

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

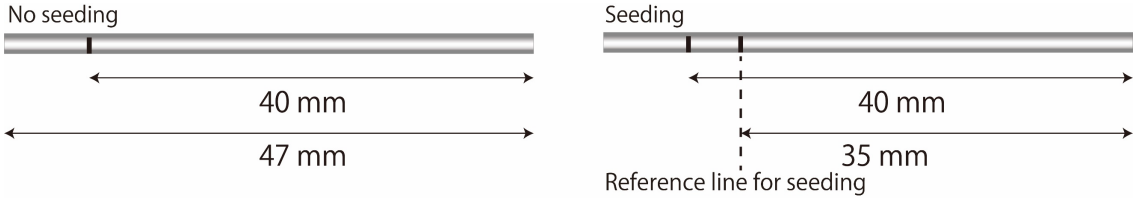


Fig. 3.3 Reference lines on the capillary

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 into the capillary by capillary action. If this is difficult, lay the capillary on its side. Ensure that you do not lift the capillary out of the sample solution during loading to avoid air bubbles in the capillary.

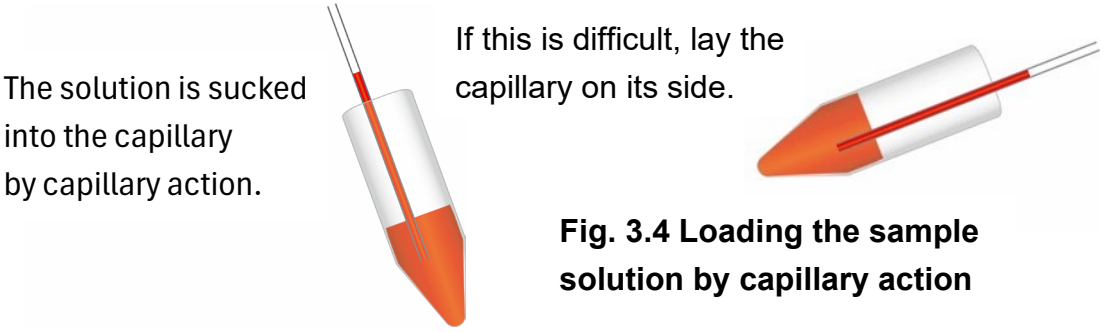


Fig. 3.4 Loading the sample solution by capillary action

For seeding, fill the sample solution up to the first reference line, 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 capillary action (e.g., in case of a highly viscous solution); conversely,

the solution immediately fills the capillary (in case of solutions that include detergents and organic solvents). 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, taking care not to affect the amount of liquid in the capillary.

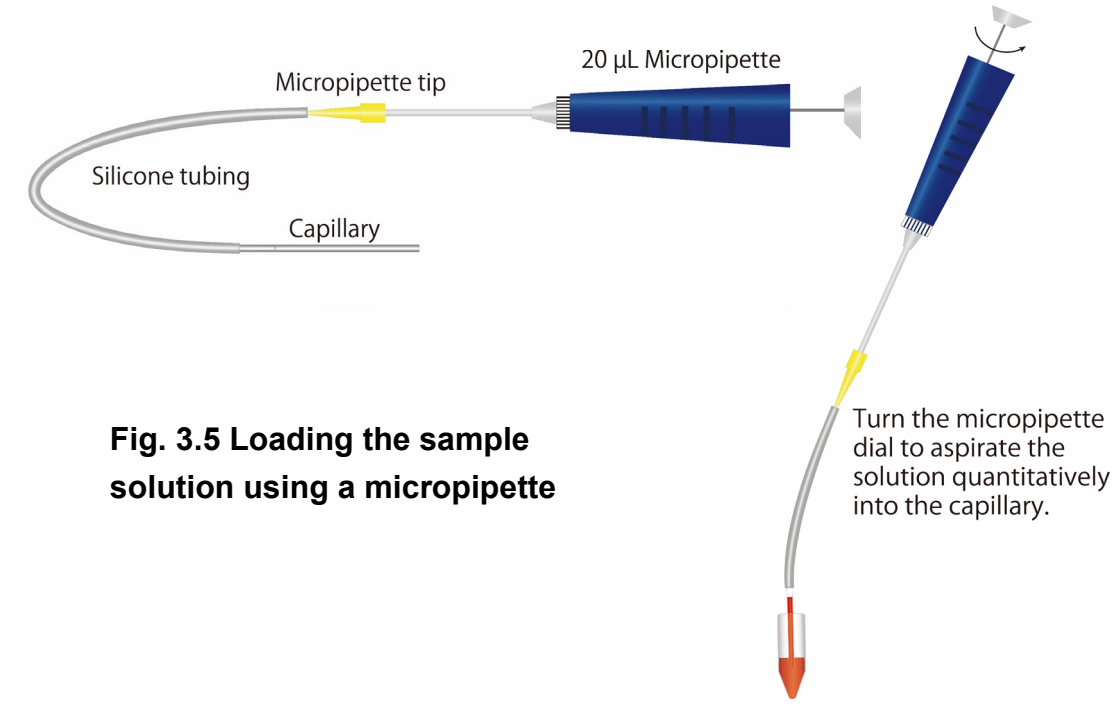


Fig. 3.5 Loading the sample solution using a micropipette

For seeding, fill the sample solution up to the first marked line, 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.

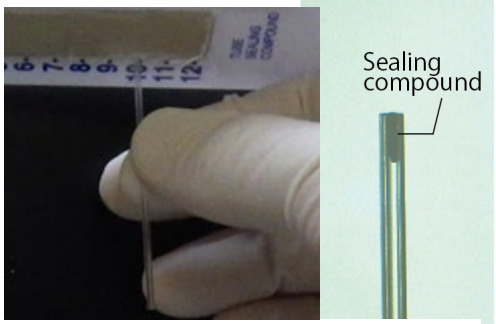
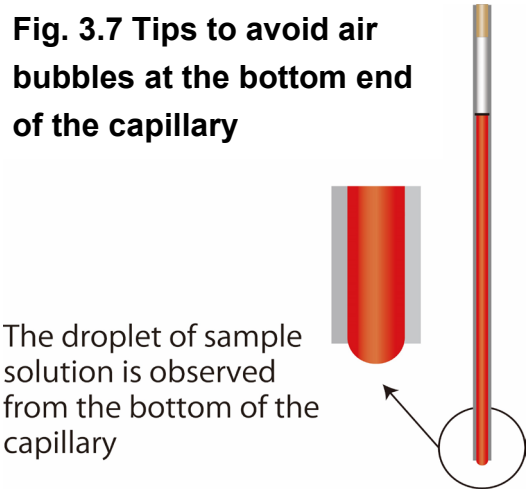


Fig. 3.6 Sealing the capillary

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

Fig. 3.7 Tips to avoid air bubbles at the bottom end of the capillary



3. Attaching the gel tube

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

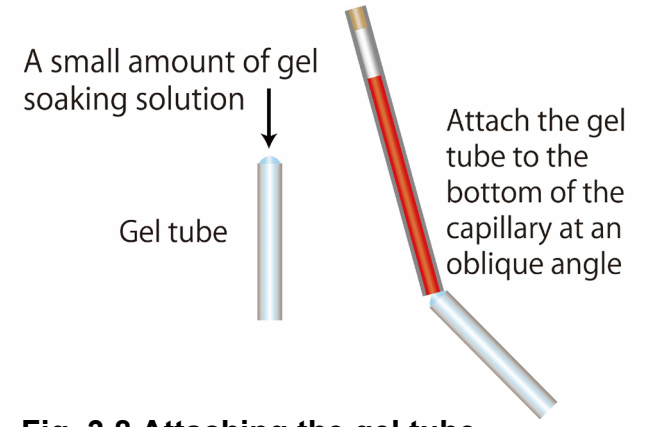


Fig. 3.8 Attaching the gel tube

Next, cut the bottom of the gel tube diagonally with a sharp blade. This 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.

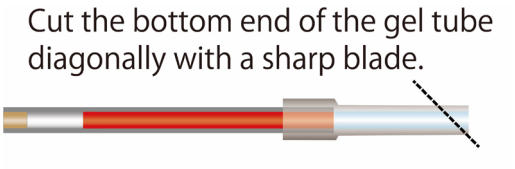


Fig. 3.9 Cutting the bottom of the gel tube

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 at the appropriate 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 towards the bottom of the capillary, suppressing density-driven convection in the capillary. As a result, we can expect to obtain better quality crystals owing to the similar effects of convection suppression in a microgravity environment.

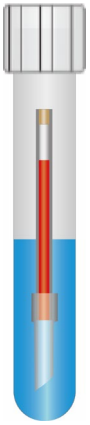


Fig. 3.10 Place vertically and start crystallization

Note: The capillary must be placed horizontally during observation, but this is not a problem for short periods.

3.2. Setting up crystallization via the DPOC method

3.2.1. Required items

(For each crystallization condition)

Solution	Amount	Description
Sample solution	5–7 µL	It contains a protein sample solution for loading into a DPOC tube. Because 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 8 mm. It is desirable to prepare a little more solution for loading 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 buffers as the crystallization solution. No ligands or seeds are required. It is used to immerse the DPOC tube.

Equipment in the kit	Qty.	Description
DPOC tube (φ1.0 mm × 30 mm)	1	It has short capillaries at both ends.
C-Cap	2	It is a stopper to plug the capillaries of the DPOC tube.
Sealing compound	1	It is used to seal 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 crystallization.

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

3.2.2. Pre-soaking of the DPOC tube

1. Reservoir solution

This is the solution outside the DPOC tube settled in the round-bottom tube, with the same concentrations of crystallization reagents and buffer as in the crystallization solution in the DPOC tube. No ligand components or seeds were required.

2. Marking the DPOC tube

Mark a line on the DPOC tube for loading solution by 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.

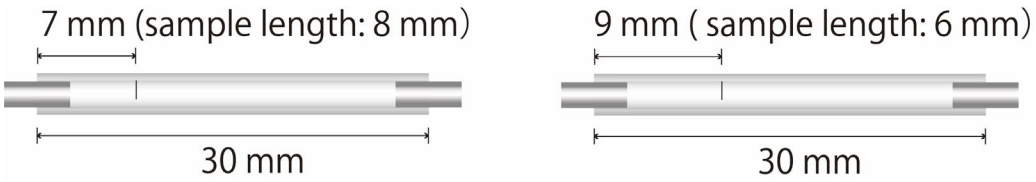


Fig. 3.11 Reference lines on the DPOC tube

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, mix them before loading into the DPOC tube.

2. Crystallization solution

A solution that crystallizes proteins by interdiffusion 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 an adequate amount to the crystallization solution.

3.2.4. Assembling the items to start crystallization

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

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

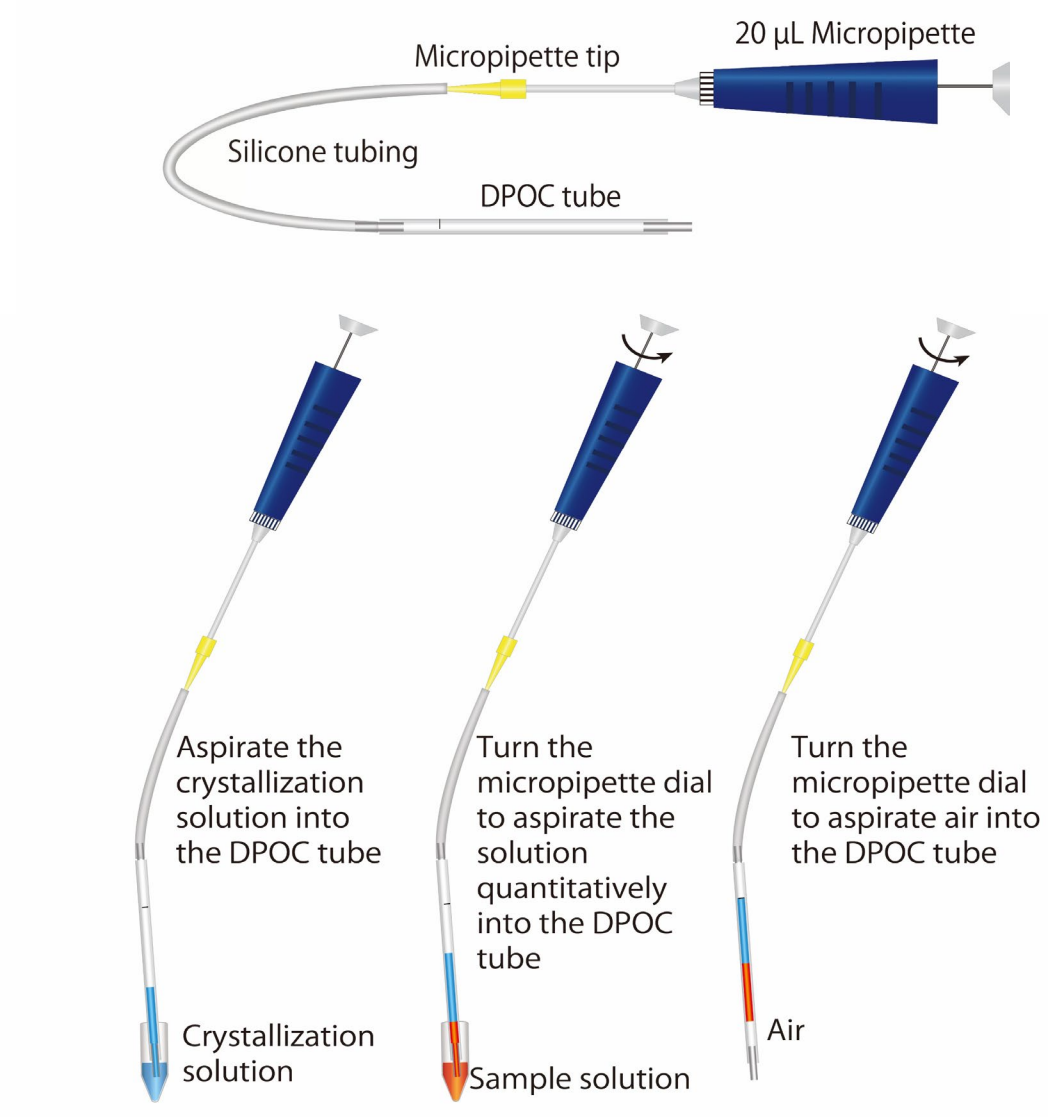


Fig. 3.12 Loading solutions into the DPOC

2. Loading solutions

Place the other end of the DPOC tube into the crystallization solution, and aspirate 4.7–6.3 µL of the solution. Next, move the end of the DPOC tube into the sample solution and aspirate the same amount of solution 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

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

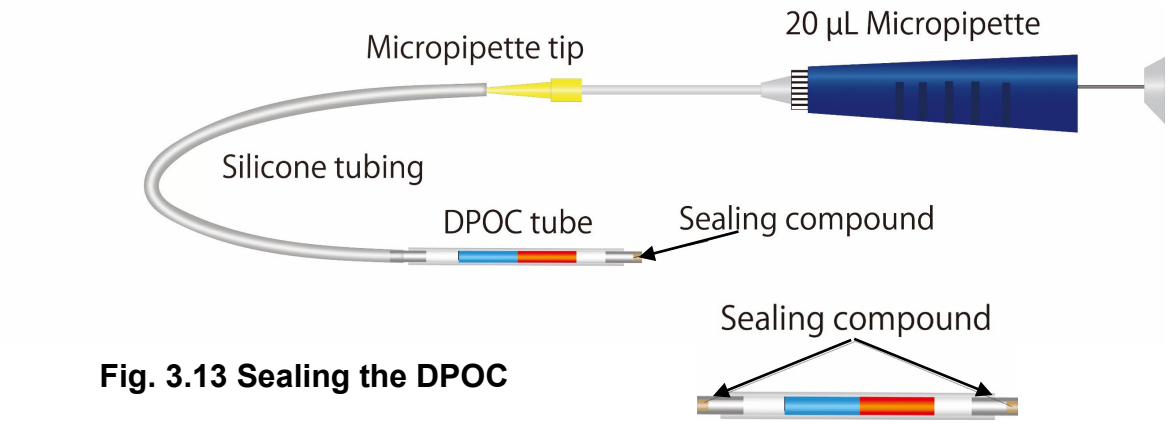


Fig. 3.13 Sealing the DPOC

4. Attaching C-Caps

To protect both outer ends of the DPOC tube from excessive osmotic pressure, plug each outer end of the capillary with C-Caps. Insert the edge of the capillary in 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. Hold the plug with your finger such that it does not protrude from the opposite end. The installation is completed when the plug is pushed to the end of the C-Cap.

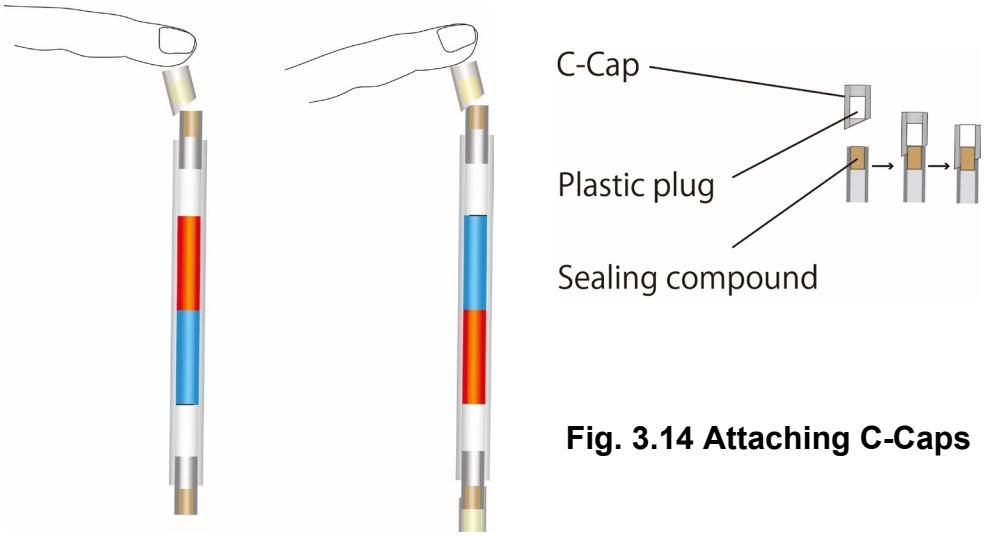


Fig. 3.14 Attaching C-Caps

5. Starting crystallization

Add about 4 mL of reservoir solution to the round-bottom tube, gently place the DPOC tube in it with the crystallization solution side down, and cap the round-bottom tube. Ensure that the entire DPOC tube is immersed in the reservoir solution in the round-bottom tube, and place it vertically at 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 towards the bottom, which suppresses the movement of the solution in the DPOC tube owing to density-driven convection. As a result, we can expect suppression of solution convection in a microgravity environment to yield better-quality crystals.

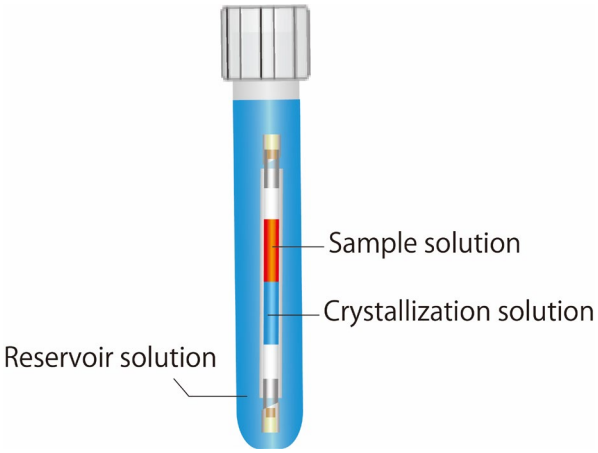


Fig. 3.15 Place vertically and start crystallization

Note: The DPOC tube must be placed horizontally during the observation; however, this is not a problem for short periods.

3.3. Harvesting crystals from the CD capillary

3.3.1. Harvest solution

To harvest crystals while maintaining 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 inappropriate, the crystals may dissolve or break because of osmotic pressure differences.

The concentrations of the components in certain parts of the solution in the CD capillary depend on the position in the capillary and duration of crystallization reaction. For successful crystal harvesting, we recommend preparing the harvest solution with estimated component concentrations. Our simulation program, sold separately, will be helpful for estimating the component concentrations in CD capillaries (available for purchase as **C-Kit Pro Advanced Tool** [CRT209], http://www.confsci.co.jp/product_e.html). If, unfortunately, your harvested crystal is deteriorated in the solutions, please prepare and try multiple solutions with component concentrations close to the initial estimated values.

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, pull both ends of the capillary to break it away from the scratch. The capillary will be cut at the exact position.

3.3.3. Harvesting crystals

Place the prepared harvest solution in a cavity glass slide. Then, a short piece of the cut capillary is immersed in the solution and held with forceps. While observing with a stereo microscope, pipette the harvest solution around the crystal and pour the crystal out of the capillary into the harvest solution.

Some of the crystals may adhere to the glass surface of the capillary. In such cases, carefully touch and move them with a thin, blunt tool, such as a gel-loading tip (QSP 124-R204). Pipette the solution again around the crystal. The solution drives the crystals out of the capillary into the harvest solution. Scoop out the crystals in the harvest solution with a cryoloop, immerse in a cryoprotectant solution if necessary, and then use for cryocooling. The details are provided in “3.5. Cryocooling crystals.”

For practice, it is recommended to prepare lysozyme crystals by using the CD method to familiarize yourself with the procedure for picking out crystals. Because lysozyme crystals easily adhere to the inner surface of the capillary, they are suitable for training.

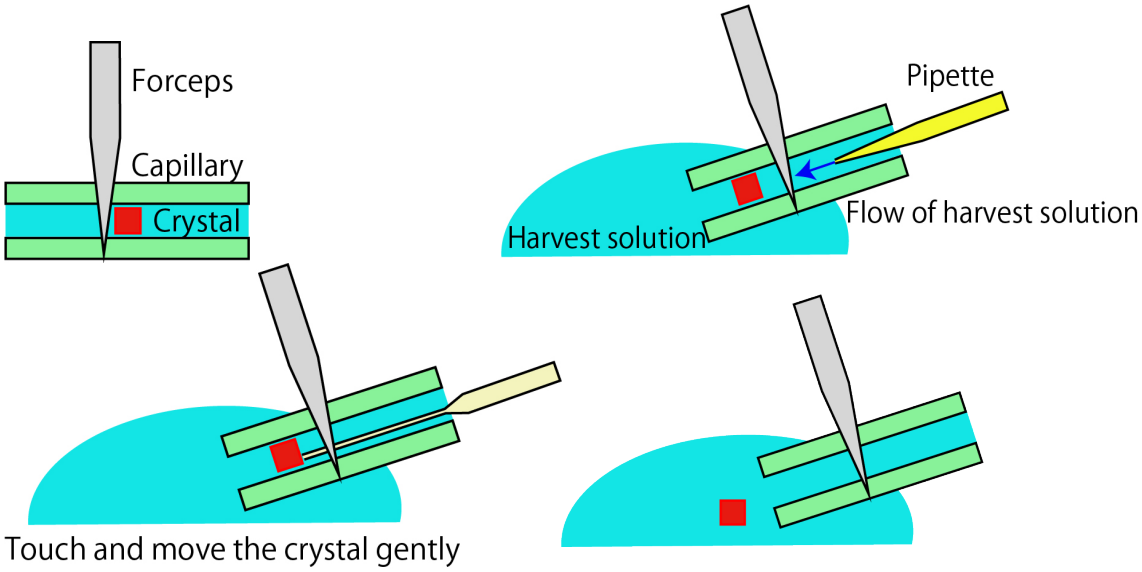


Fig. 3.16 Harvesting crystals

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 pushed 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 PEG solutions and sample solution reaches equilibrium around 10 days. However, the concentration of a sample solution by an external reservoir solution requires several weeks. The actual concentration in the DPOC tube can be estimated from the length of the solution in the DPOC tube during harvesting. Based on this estimation, prepare a harvest solution by diluting the crystallization solution to the same concentration as that of 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 (or a sharp knife for delicate work). When cutting, care should be taken not to deform the DPOC tube or crush the crystals.

3.4.3. Harvesting crystals

Add the prepared harvest solution to a cavity glass slide. Then, immerse a short piece of the DPOC tube in the solution with forceps. While observing with a stereo microscope, pipette the harvest solution around the crystal and pour the crystal out of the DPOC tube into the harvest solution. Scoop the crystals in the harvest solution out with a cryoloop, immersed in a cryoprotectant solution if necessary, and then use for cryocooling. The details are provided in “3.5. Cryocooling crystals.”

For practice, we also recommend that you prepare lysozyme crystals by using the DPOC method and familiarize yourself with the procedure for harvesting crystals.

3.5. Cryocooling crystals

For X-ray diffraction using synchrotron radiation, the crystals must be cryocooled to protect them from radiation damage. The water around the crystals needs to freeze into amorphous ice through the freezing process. If not, growing crystalline ice would damage the protein crystals, and a good X-ray diffraction image would not be obtained. To prevent the crystalline ice from forming during freezing, the crystals must be frozen in a cryoprotectant solution. Scoop the crystals out of the harvest solution with a cryoloop, immerse them into a cryoprotectant solution for a few seconds to replace the solution around the crystals with the cryoprotectant, and then cryocool them rapidly. For cryocooling, please use a method that you have already used, such as flash cooling or direct immersion in liquid nitrogen.

PEG-based crystallization reagents have a cryoprotective effect. Therefore, if the harvest solution contains 35–40% PEGs, the crystals can be cryocooled without the addition of extra cryoprotectants. If the main component of a harvest solution is PEG-based but at a low concentration, adding the same type of PEG to the harvest solution to adjust PEG concentration to approximately 40%. It is a reliable method for preparing cryoprotectant solutions. When salts are used as crystallization reagents, glycerol is the most common cryoprotectant used as an additive in the harvest solution. A glycerol concentration of approximately 40% would suppress the formation of crystalline ice. Trehalose, ethylene glycol, PEG4000 have also been used as cryoprotectants.

Before applying a cryoprotectant solution to the target crystals, we recommend you to test whether the solution can suppress crystalline ice formation. A droplet of a good cryoprotectant solution will freeze into amorphous ice when scooped with a cryoloop and frozen quickly.

However, cracking of the crystal may sometimes occur, due to the large osmotic pressure difference between the liquid inside the crystal and the cryoprotectant solution. This tends to occur when using low-molecular-weight cryoprotectants such as glycerol. A solution to this problem is to sequentially soak the crystals in several solutions containing intermediate concentrations of the cryoprotectant. For example, if the harvesting solution contains 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, prepare three solutions: (A) 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, 10% glycerol, (B) 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, 20% glycerol and

(C) 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, 30% glycerol. Then, soak the crystal in the solutions in the order of (A), (B) and then (C) to gradually increase the cryoprotectant concentration around the crystal. Please refer to the later section, “4.2.4. Easy soaking” for tips.

4. Technical Notes

4.1. Time course of concentration changes in the solution

Protein crystallization can be expected when the concentrations of the protein sample and crystallization reagent are in a suitable combination. To obtain crystals, we recommend that understanding the time course of concentration changes in the protein solution and crystallization reagents in the CD method capillary and DPOC tube and optimizing concentrations of the solutions to occur suitable combinations.

4.1.1. Concentration changes in the CD method sample solution

A one-dimensional diffusion simulation can be used to estimate the time course of crystallization reagent concentration in the CD capillary. A simulation program (available for purchase as **C-Kit Pro Advanced Tool**, CRT209, http://www.confsci.co.jp/product_e.html) makes it easy to estimate the 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

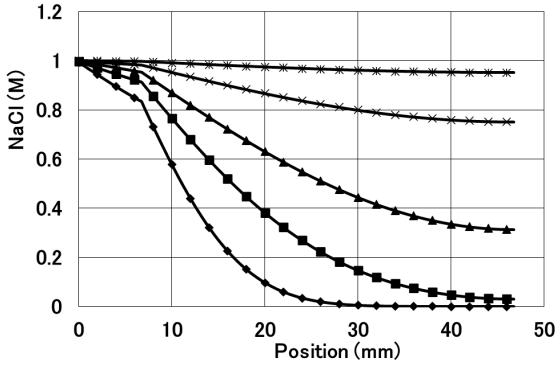


Fig. 4.1 Estimation of NaCl concentration in a capillary.
♦, ■, ▲, x, and ж show NaCl concentrations 0.25, 1, 3, 8, and 16 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis indicates the NaCl concentration at the location.

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

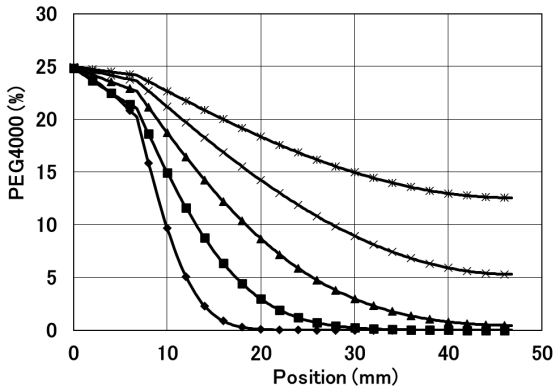


Fig. 4.2 Estimation of PEG 4000 concentration in a capillary.
♦, ■, ▲, x, and ж indicate PEG 4000 concentrations 1, 4, 12, 32, and 64 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis indicates the PEG 4000 concentration.

Even after 64 days, the PEG4000 concentration in the capillary increased to approximately half of the reservoir solution concentration at the distal end of the gel tube. To obtain crystals within a short period, either a higher concentration of PEG4000 in the reservoir solution or premixing an appropriate concentration of PEG4000 with the protein sample in the capillary is recommended. Because the diffusion coefficient of PEGs is approximately inversely proportional to the 1/2 power of their 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

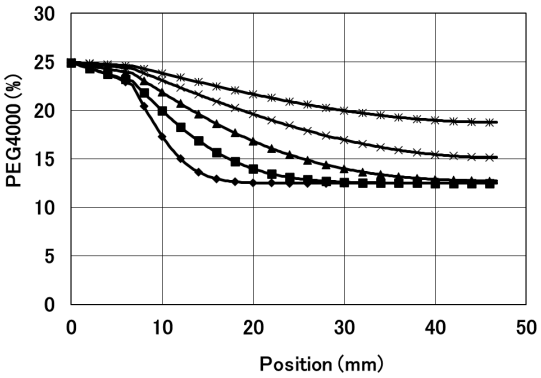


Fig. 4.3 Estimation of PEG4000 concentration in a capillary.
Symbols are the same as in Figure 4.2.

PEG4000 was added to the sample solution in the capillary, as shown in Fig. 4.3; thus, the PEG concentration increased faster than that shown in Fig. 4.2.

4.1.2. Concentration changes in the DPOC tube solution

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

Example 4

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

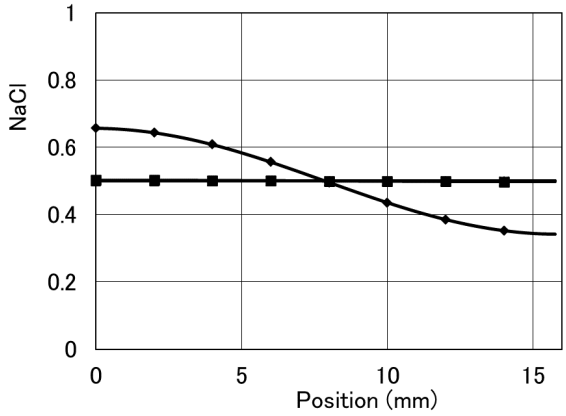


Fig. 4.4 Estimation of NaCl concentration in the DPOC tube.
♦ and ■ indicate NaCl concentrations 0.25 and 1 day after loading, respectively. The horizontal axis shows the distance from the end of the tube, and the vertical axis indicates the NaCl concentration at the location.

The NaCl concentration in the capillary reaches near equilibrium in only 1 day.

Example 5

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

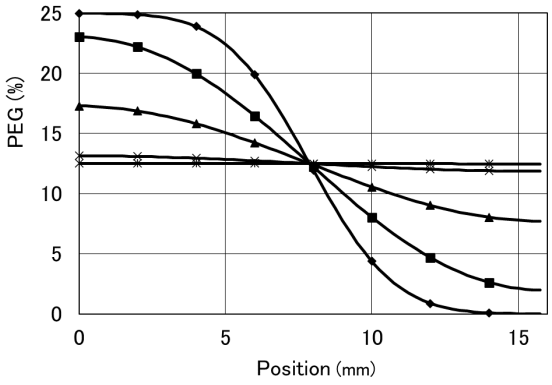


Fig. 4.5 Estimation of PEG4000 concentration in a DPOC tube.
♦, ■, ▲, x, and ж indicate PEG4000 concentrations 0.25, 1, 3, 8, and 16 days after loading, respectively. The horizontal axis shows the distance from the end of the tube, and the vertical axis indicates the PEG4000 concentration at the location.

PEG4000 in the capillary reaches equilibrium 8 days after loading.

Furthermore, the solution in the DPOC tube was slowly concentrated by the osmotic concentration caused by the concentration difference between the solution in the DPOC tube and the reservoir solution; therefore, the concentration process was measured as shown 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

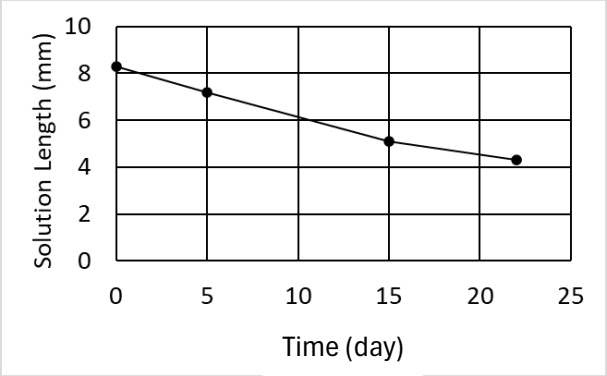


Fig. 4.6 Time-course of solution concentration indexed by the solution length in a DPOC tube.

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 of the CD and DPOC methods

The advantages and limitations of the CD and DPOC methods have been provided below. These differences should be considered when selecting the optimal crystallization method.

4.2.1. Crystals with high resolution (the CD method)

In general, higher the concentration of the main crystallization reagent (e.g., PEGs or ammonium sulfate), higher is the diffraction resolution. In the conventional vapor diffusion method, crystallization under these conditions tends to cause clustering or precipitation. In contrast, in the CD method, a higher concentration of crystallization reagents can be used to obtain crystals that diffract X-rays at a high resolution, since concentration of the reagents in the CD capillary increases slowly.

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 from the purification process. Some of these compounds may inhibit crystallization. A typical component that inhibits crystallization is high concentrations of NaCl. Contaminating NaCl may cause protein precipitation when a crystallization reagent is added.

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

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

4.2.3. Small amounts of ligands (the DPOC method)

During co-crystallization with the 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 greater than μM requires the addition of the ligand to the reservoir solution.

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

4.2.4. Easy soaking (the CD method)

Soaking crystals with ligands using the CD method is very easy. After the crystals are obtained, the CD capillary is simply transferred to a fresh reservoir solution containing the ligand and left for a period of time for the ligand to diffuse in.

You can also apply this procedure to solution exchange for cryoprotection of the crystals. Crystals may deteriorate sometimes when they are dipped in a cryoprotectant solution, since concentration of the cryoprotectant component around the crystals increases too rapidly. To avoid the rapid change, soak the crystals in cryoprotectants, such as high concentrations of PEGs, ethylene glycol, and glycerol. It enables slowly exchange of the solution around the crystals. For example, if the reservoir solution contains 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, prepare a protectant solution containing 10 mM $MgCl_2$, 1.5 M ammonium sulfate, 50 mM acetate buffer, 30% glycerol, and transfer the CD capillary containing the crystals into it. After the solutions inside and outside the capillary reach equilibrium, the crystals are harvested from the capillary using this protectant solution and frozen.

However, in the DPOC method, the crystals must be removed from the tube and transferred to a soaking solution.

4.3. Design of crystallization conditions

4.3.1. Proteins with unknown crystallization conditions

Commercially available screening kit reagents (e.g., https://hamptonresearch.com/cat-1.html) are typically used to screen crystallization conditions. In these experiments, the self-searching mechanism of the CD and DPOC methods allows a wider range of combinations of concentration conditions to be searched in a single capillary/tube than the commonly used batch and vapor diffusion methods.

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 the main crystallization reagent. Calculate the pI value of your protein sample. Then, select 2 reagent bottles from each group: one with 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 using the CD method. Prepare 2 sample solutions for each reagent: one containing only the protein sample and the other containing the protein sample mixed 1:1 with the selected reagents. Use the corresponding 4 reagents as reservoir solutions. Then, test the protein crystallization with the 8 crystallization conditions.
3. Crystallization experiments using the DPOC method are recommended using the 4 selected screening kit reagents as both crystallization and reservoir solutions.
4. When no crystals are obtained after a maximum of 1 month, the possible reasons and countermeasures may be as follows:

CD method	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 found only in the sample solution.

5. If crystals are not obtained even after 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, we often observe that the crystals cannot be reproduced. A possible cause is the slight difference in the salt and buffer concentrations included in your protein preparation. Therefore, the CD method is preferable in these cases because it is less affected by these molecules in protein.

In 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 in the batch or vapor diffusion method, so that you can obtain crystals more efficiently from your own preparation.

Easy protocols to screen crystals are as follows:

1. Select several crystallization conditions reported in papers or PDB.
2. Then, crystalize with the CD method by using the reported crystallization solution as the reservoir solution. Prepare 2 sample solutions: one is the reported protein solution and the other is the protein solution mixed 1:1 with the reported crystallization solution.
3. Setup crystallization experiment by using the DPOC method.
4. If no crystals are obtained after a maximum of 1 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 from experts 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 you to achieve crystallization.

Confocal Science Inc.

5-14-15 Fukasawa, Setagaya-ku, Tokyo 158-0081

TEL: +81-3-5809-1561 FAX: +81-3-6411-6261

E-mail: info@confsci.co.jp http://www.confsci.co.jp/