C-Kit Ground Pro ND
[Neutron Diffraction Kit]

Aug. 2024

Confocal Science Inc.

Table of Contents

Contents of C-Kit Ground Pro ND (CRT101-2)

Product name	Qty.	Description
Capillary (φ0.29 mm × 29 mm)	100	Used in the batch method.
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	
LCDM (glass capillary)	6	Used in the dialysis method to grow large crystals. Dialysis membrane (MWCO 6,000–8000) is placed in the capillary.
LCDM (quartz capillary)	2	
C-Cap	10	
Silicone tubing (φ1.0 mm × 50 cm)	1	Used for aspirating solution.
Ultra-fine gel loading tip	9	Used for loading sample into LCDM.
Sealing compound	1	Used for sealing capillaries.
Round-bottom tube (5 mL)	30	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.

1. Introduction

1.1. Overview

The C-Kit Ground Pro ND is an equipment for protein crystallization that produces large crystals for neutron diffraction and has been developed from knowledge of crystallization experiments at the International Space Station (ISS) for decades. This kit provides experimental tools for three simple methods: batch, counter-diffusion (CD) [1)], and dialysis [2)]. First, the crystallization conditions are determined using batch method tools; then, the conditions are confirmed using CD method tools, and large crystals grow in a large-bore capillary with a dialysis membrane (LCDM). These steps enabled the growth of large crystals with small amounts of protein.

The CD method tools included in the kit were jointly developed by Confocal Science, under commission from JAXA [3)]. This product uses US patent 7531037 of JAXA under license [4)]. In addition, Confocal Science provides C-Kit Ground Pro XRD for crystallization for X-ray diffraction (http://www.confsci.co.jp/product_e.html) and 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) 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.

4) JP patent 4354457, US patent 7531037

1.2. Features

- A small amount of the protein sample is required: The standard amount is 1.3 µL (the batch method capillary), 8 µL (the CD method capillary), and 27 µL (the dialysis method capillary).
- Simple protocols: all protocols are easy to perform.
- High reproducibility and reliability: Both the CD and dialysis methods have been used repeatedly in space experiments conducted by JAXA and the Japan Manned Space Systems Corporation (JAMSS). Thus, high reproducibility and reliability were demonstrated. In particular, the CD has been applied to the crystallization of more than 500 proteins under microgravity since 2002.
- Long-term stability: The crystals produced using the C-Kit Pro ND were stable for a long period in the capillaries.
- Estimation of optimal conditions: Time-dependent changes in protein and crystallization reagent concentrations in the CD capillaries and LCDM were 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). This helps to determine the optimal conditions for crystallization.

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

This kit provides experimental tools for three methods: batch, counter-diffusion (CD), and dialysis. The principle of batch crystallization is simple; therefore, we explain the other two methods.

2.1. The counter-diffusion (CD) method

C-Kit Ground Pro ND provides a set of crystallization equipment for the gel tube (GT) method, which is one of the CD methods. Figure 2.1 provides a simple explanation of the standard configuration of the method and mechanism of crystal growth.

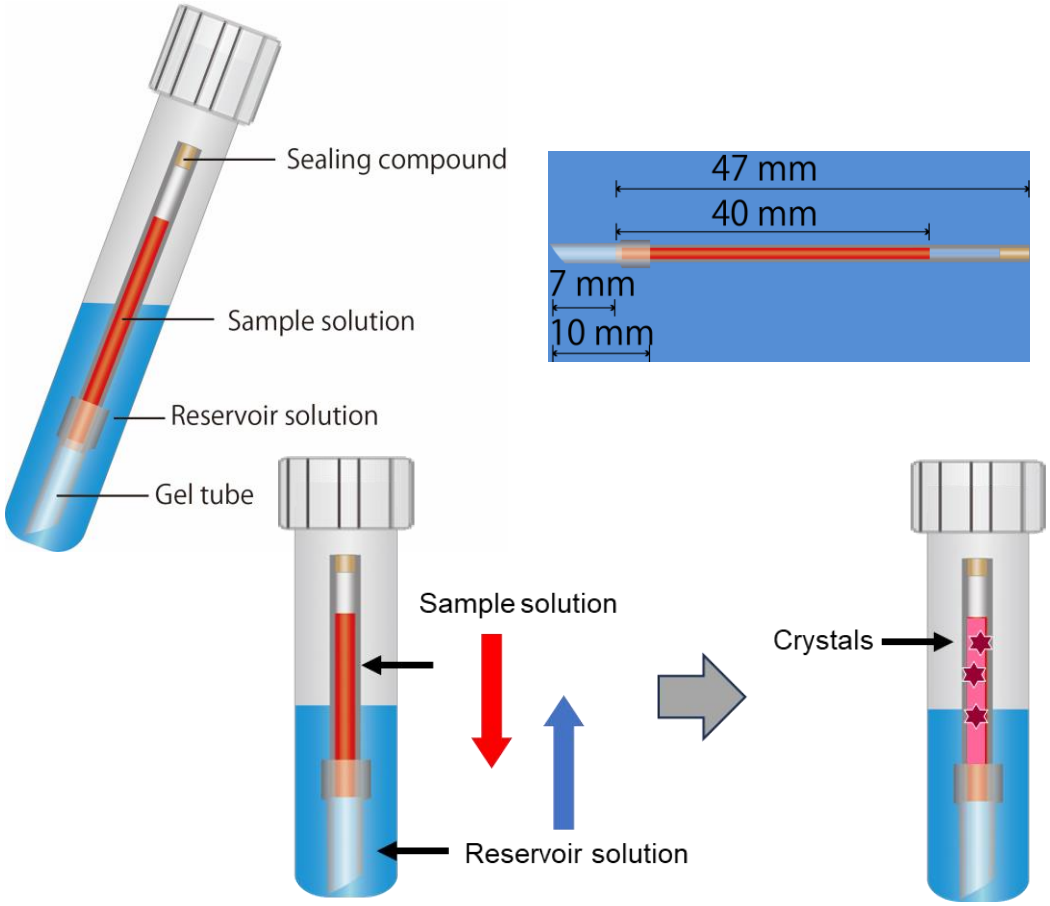

Fig. 2.1 GT (CD) method illustration.
Upper left: Configuration of the GT method. Upper right: Detailed view of a capillary with a gel tube. Bottom: Crystallization mechanism of the GT method.

A feature of this method is that a silicone tube with agarose gel, called a gel tube, 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-dependent diffusion of reagents” for more details.

2.2. The dialysis method

The C-Kit Ground Pro ND includes crystallization tools for the dialysis method as another type of crystallization method for large crystals. Figure 2.2 provides a simple explanation of the standard configuration of the dialysis method using LCDM and the crystal growth mechanism.

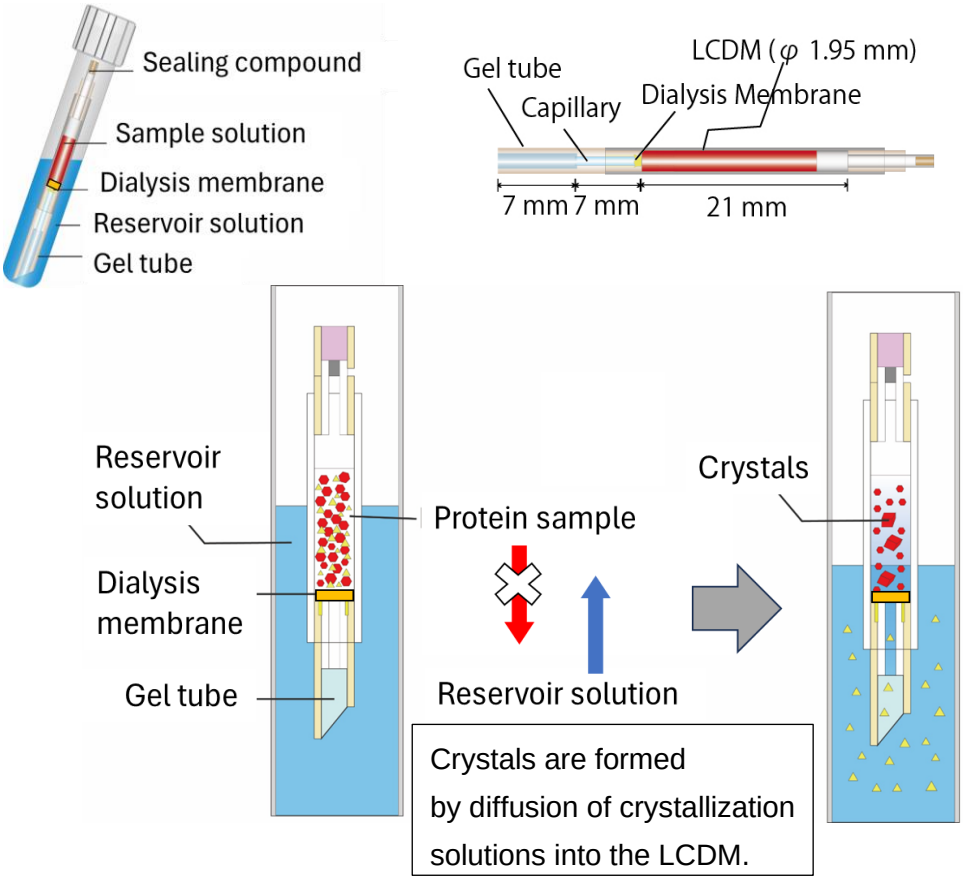

Fig. 2.2 LCDM illustration
Upper left: Configuration of the dialysis method. Upper right: Detailed view of the LCDM. Bottom: The crystal growth mechanism.

A large bore capillary loaded with the sample solution is connected to a gel tube via a dialysis membrane. The crystallization solution diffuses into the large-bore capillary through the gel tube and crystallizes when its concentration increases to an appropriate level. Understanding the diffusion time course of the crystallization reagents is important for this method. See “4.1. Time-dependent diffusion of reagents” for more details.

3. Experimental Procedure

3.1. Setting up crystallization via the batch method

3.1.1. Required items

(For each crystallization condition)

Solution	Amount	Description
Sample solution	3–5 µL	It contains a protein sample solution and crystallization reagents and is loaded into a capillary. Because the inner diameter of the capillary is 0.29 mm, the required volume is 1.3 µL for a sample length of 20 mm. It is desirable to prepare a little more solution for loading without failure.

Equipment in the kit	Qty.	Description
Capillary (φ 0.29 mm × 29 mm)	1	
Sealing compound	1	It is used for sealing both ends 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.

3.1.2. Preparation of the sample solution

Solutions containing protein samples and appropriate concentrations of crystallization reagents (and ligands, if necessary) are prepared for loading.

3.1.3. Assembling the items to start crystallization

1. Loading the sample solution

When the tip of the capillary is immersed in the sample solution, the solution is sucked into the capillary via capillary action. If this is difficult, place the capillary on its side to make it easier (Fig. 3.1).

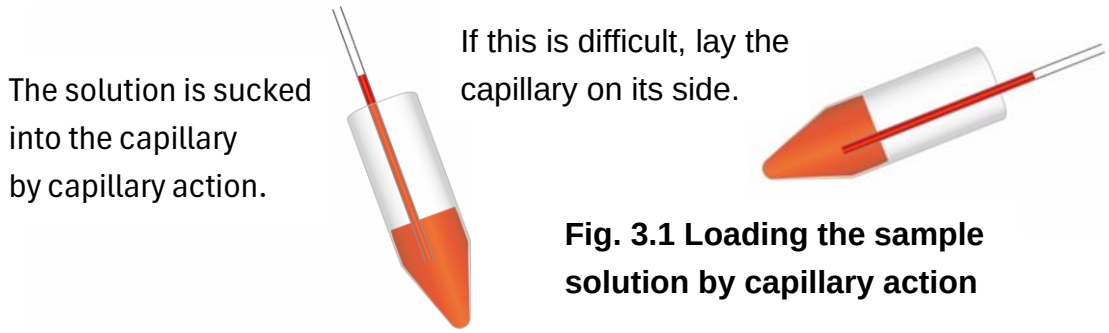

Fig. 3.1 Loading the sample solution by capillary action

When the solution does not enter by capillary action 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 insert 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 change the amount of liquid in the capillary.

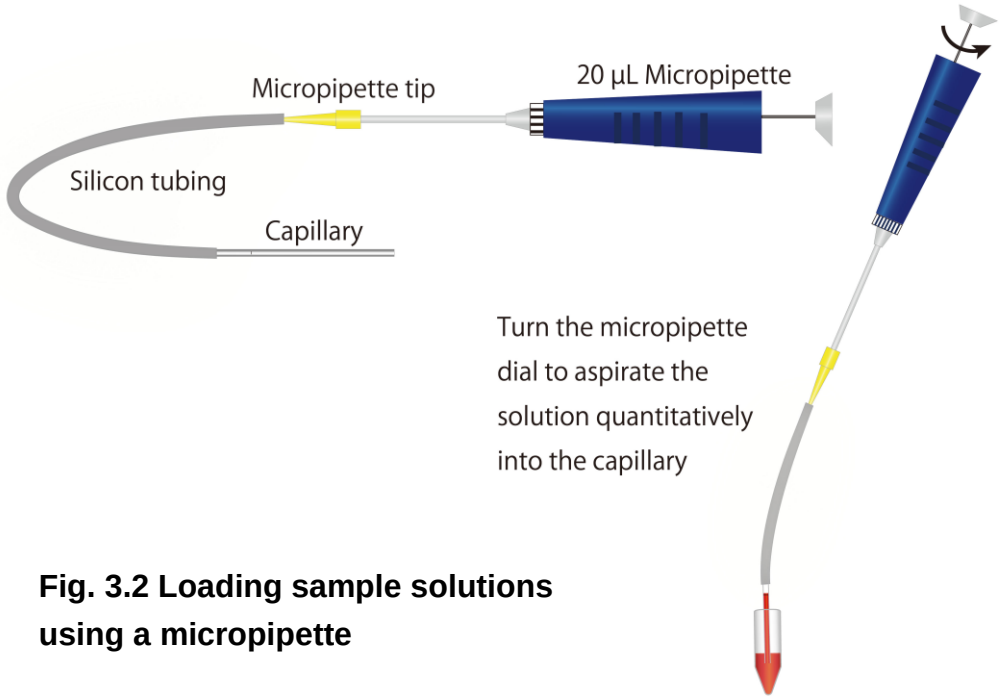

Fig. 3.2 Loading sample solutions using a micropipette

When the solution comes in contact with the sealing compound at the capillary ends, crystals sometimes grow. To prevent unwanted crystallization, the capillary should be held in the hand and slowly tilted to move the solution toward its center.

2. Sealing both ends of the capillary

Seal the ends 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. Turn the capillary left and right 2–3 times and then pull it out (Fig. 3.3). Repeat this process until approximately 2 mm of the sealing compound is packed into the top of the capillary.

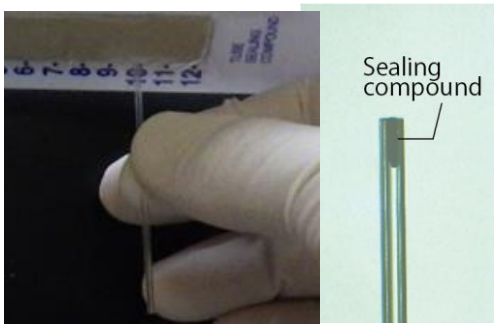

Fig. 3.3 Sealing the capillary

3. Starting crystallization

The capillary is then gently placed in a round-bottom tube and capped. It is then placed vertically at an appropriate temperature.

3.2. Setting up crystallization via the CD method

3.2.1. 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	1–2 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.

3.2.2. 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.

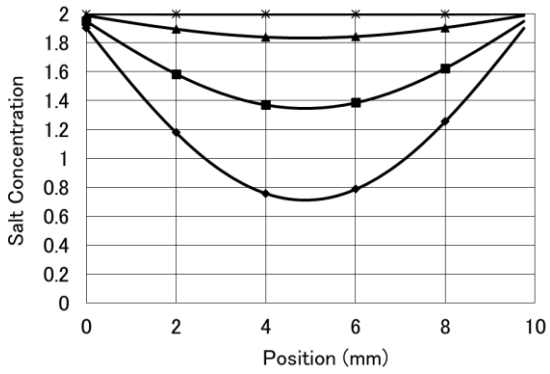

Fig. 3.4 Simulation results of internal NaCl concentration of a gel tube immersed in 2 M NaCl solution.

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.

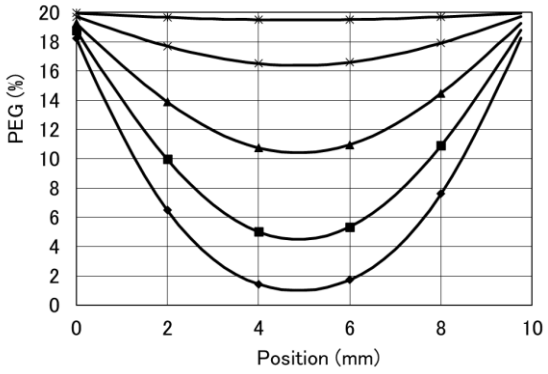

Fig. 3.5 Simulation results of internal PEG4000 concentration of a gel tube immersed in 20% PEG4000 solution.

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.

3.2.3. 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.

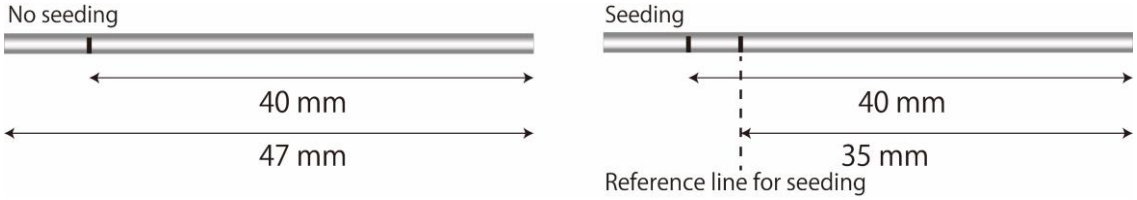

Fig. 3.6 Reference lines on the capillary

3.2.4. 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 to make it easier. Ensure that you do not lift the capillary out of the sample solution during loading to avoid air bubbles in the capillary.

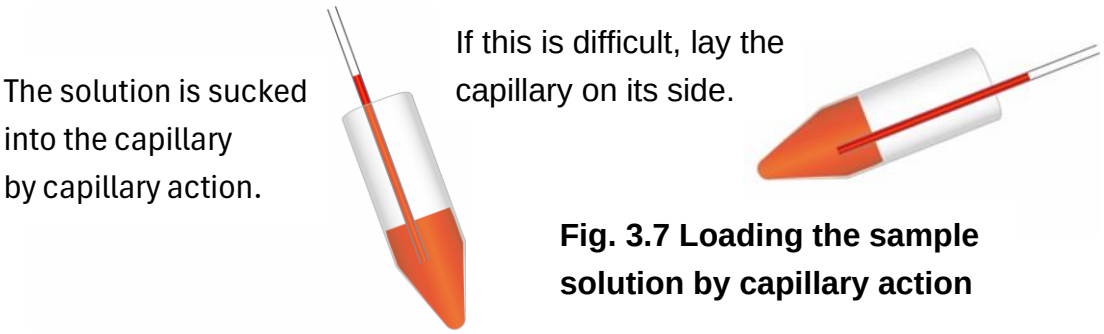

Fig. 3.7 Loading the sample solution by capillary action

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.

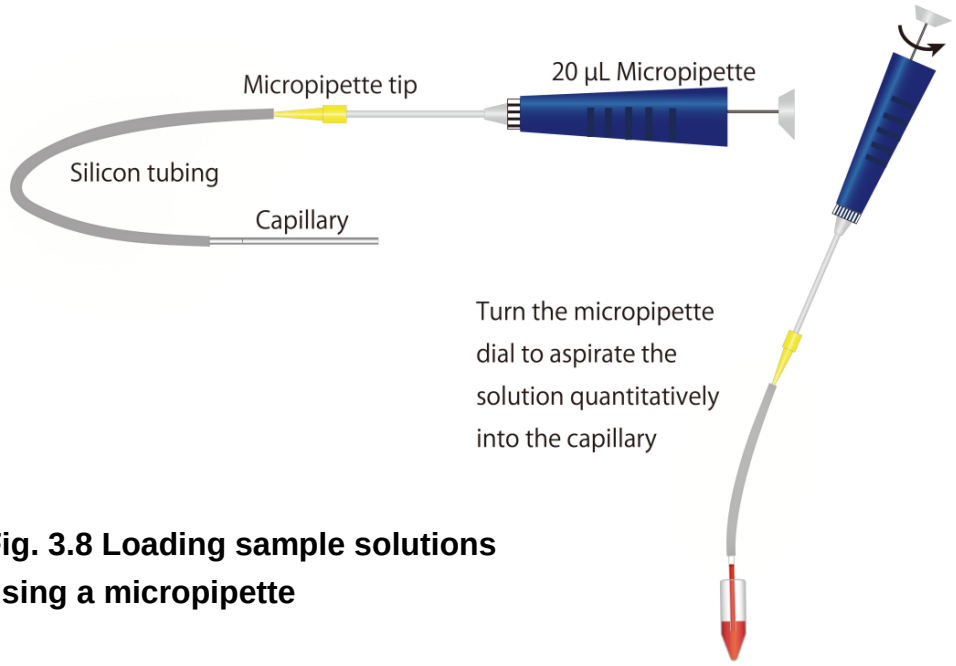

Fig. 3.8 Loading sample solutions using a micropipette

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.

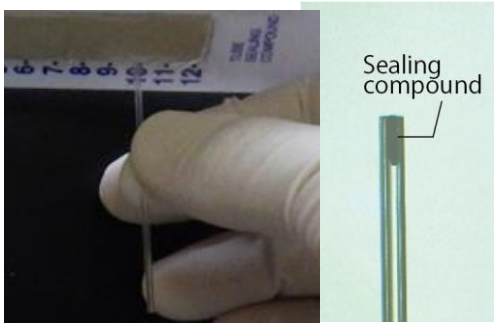

Fig. 3.9 Sealing the capillary

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.

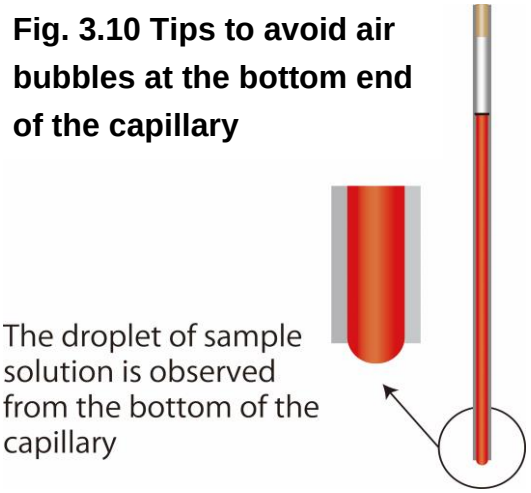

Fig. 3.10 Tips to avoid air bubbles at the bottom end of the capillary

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.

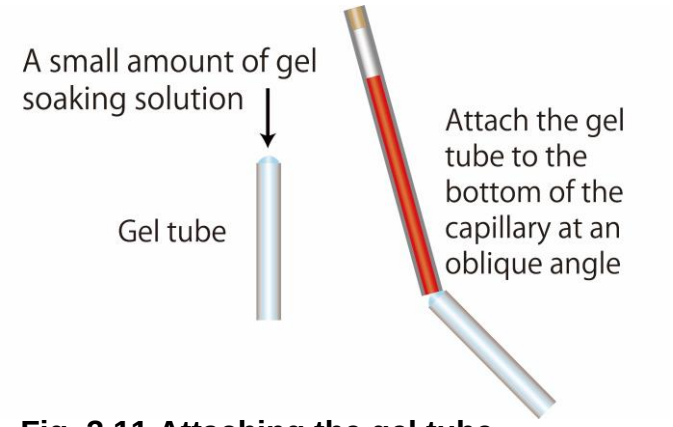

Fig. 3.11 Attaching the gel tube

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.

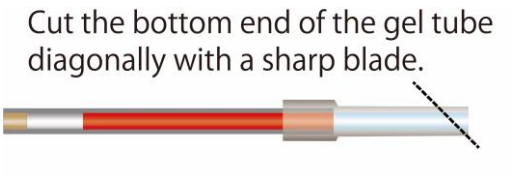

Fig. 3.12 Cutting the bottom of the gel tube

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, when the crystallization reagent enters the gel tube by diffusion into 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.

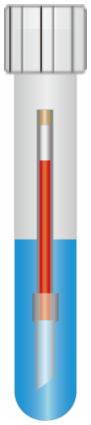

Fig. 3.13 Place vertically and start crystallization

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

3.3. Setting up crystallization via the dialysis method

3.3.1. Required items

(For each crystallization condition)

Solution	Amount	Description
Sample solution	ca. 27 µL	It contains a protein sample solution for loading into an LCDM tube. Because the inner diameter of the LCDM tube is 1.95 mm, the required volume is 27 µL for a sample length of 9 mm. It is desirable to prepare a little more solution for loading without failure.
Reservoir solution	ca. 1 mL	It interdiffuses with the sample solution in the LCDM to crystallize the protein. It contains crystallization reagents and buffers, which may also contain ligands if required.

Equipment in the kit	Qty.	Description
LCDM tube (φ1.95 mm × 29 mm)	1	It has a gel tube at one end.
C-Cap	1	It is a stopper to plug the capillaries of the LCDM.
Sealing compound	1	It is used to seal the end of LCDM.
Silicone tubing	1	It is used to load solutions into the LCDM.
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 µL)	1	It is used for loading solutions into an LCDM.
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.

3.3.2. Pre-soaking of the LCDM

1. Pre-soaking solution

LCDMs in the C-Kit Ground Pro are immersed in a 0.04% NaN_3 solution. Protein samples may be damaged by rapid changes in salt concentration if LCDM with low ionic conditions is used directly for protein crystallization without pre-soaking. The crystallization reagent requires time to diffuse into the LCDM. Therefore, the crystals grow faster, when the LCDM has been presoaked in a solution with 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 pre-soaking solution. Add approximately 4 mL of the solution to the tube with a lid and immerse the LCDM for at least a few days before use. The entire LCDM, not only the gel tube, should be immersed to equilibrate at the upper end of the LCDM.

2. Time required for soaking LCDMs

According to the simulation results of the solute concentration changes in the LCDM immersed in the soaking solution, it takes approximately 1 day for the NaCl solution and approximately 2 weeks for the PEG4000 solution to achieve equilibrium in the LCDM.

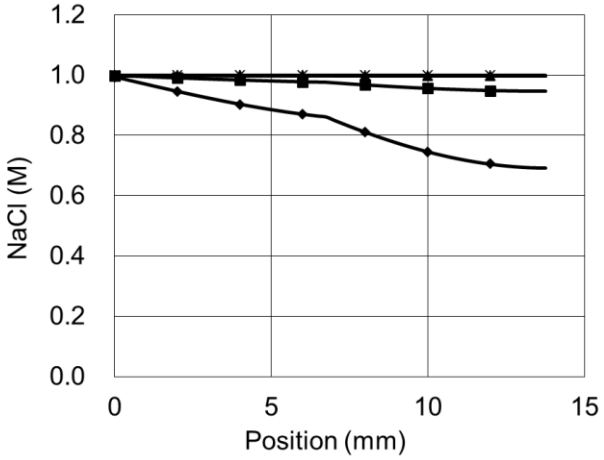

Fig. 3.14 Estimation of NaCl concentrations in an LCDM.
The horizontal axis is the distance from the end of the gel tube, and a dialysis membrane is placed at 14 mm. The vertical axis is the NaCl concentrations. The curves show, from the bottom, NaCl concentrations 0.5, 1, and 2 days after soaking in 1M NaCl.

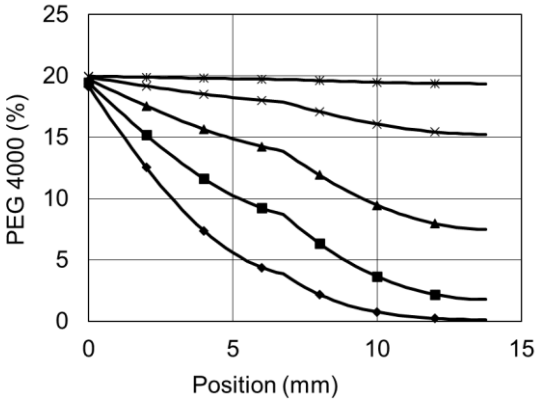

Fig. 3.15 Estimation of PEG 4000 concentrations in an LCDM.
The curves show, from the bottom, PEG 4000 concentrations 1, 2, 4, 8, and 16 days after soaking in 20% PEG 4000. Others are same as in Fig. 3.14.

3.3.3. 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 them into the LCDM.

2. Crystallization solution

Solution that crystallizes proteins by interdiffusion with the sample solution in the LCDM, contains crystallization reagents and buffers, which may also contain ligands if required.

3.3.4. Assembling the items to start crystallization

1. LCDM

Remove the LCDM from the soaking solution and aspirate the liquid inside using an ultrafine gel-loading tip.

2. Sample loading to LCDM

Load the sample solution into the LCDM using a 20 µL micropipette with an ultra-fine gel-loading tip. Load approximately 27 µL sample solution and leave at least 1 mm of air at the top of the LCDM.

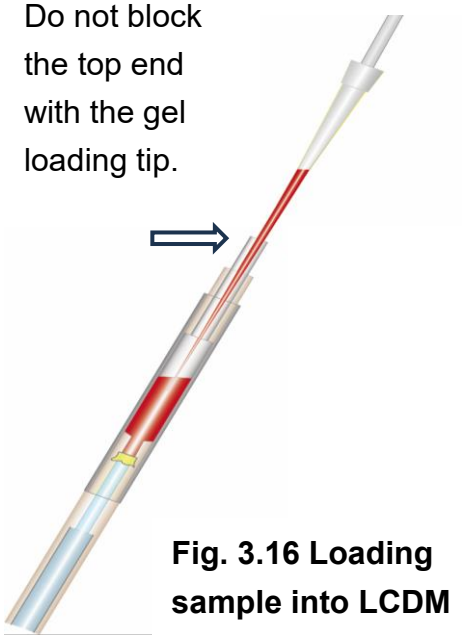

Fig. 3.16 Loading sample into LCDM

3. Sealing the LCDM

Seal the upper end of the LCDM with the sealing compound. Then, attach the C-Cap to protect the sealing from the osmotic pressure. Insert the top of LCDM into the diagonal cut side of the C-Cap. Push the plastic plug inside the C-Cap while covering the other end of the C-Cap with your finger to prevent the plug from popping out. When the plastic plug is pushed to the end of the other side of the C-cap, installation is complete.

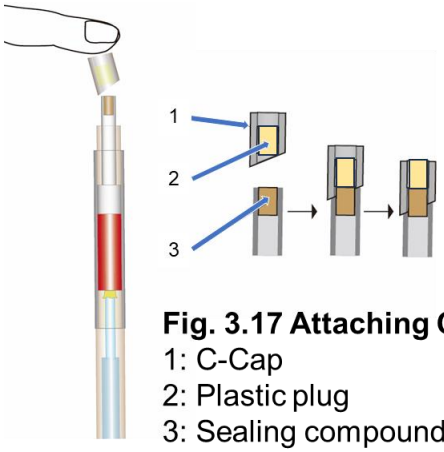

Fig. 3.17 Attaching C-Cap
1: C-Cap
2: Plastic plug
3: Sealing compound

4. Cutting the bottom of LCDM

The bottom of each gel tube is then cut diagonally using a sharp blade. This enables the reservoir solution to easily diffuse into the LCDM, even if the tip of the gel tube is in close contact with the bottom of the round-bottom tube.

Fig. 3.18 Cutting the bottom of LCDM

5. Starting crystallization

Approximately 4 mL of the reservoir solution is added to the round-bottom tube, the LCDM is gently placed with the gel tube side down, and the round-bottom tube is capped. It is placed vertically at the desired temperature. In many cases, the crystallization solution has a higher density than the sample solution. Therefore, when the crystallization reagent diffuses into the LCDM with the gel tube side down, the density of the lower part of the LCDM increases, which suppresses the solution movement in the LCDM due to density-driven convection.

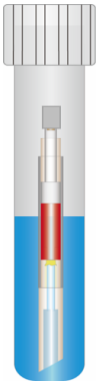

Fig. 3.19 Place vertically and start crystallization

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

3.4. Harvesting crystals from the LCDM

If there is a single crystal in a suitable location in the quartz capillary, it is possible to remove the solution from the capillary with a gel-loading tip and directly perform a neutron diffraction experiment. However, in some cases, harvesting the crystals may be necessary. The following is an explanation of how to achieve this:

If you would like to remove a crystal from a CD method capillary, please refer to the description in the instruction manual for the C-Kit Ground Pro XRD, CRT101-1, "3.3. Harvesting crystals from a CD method capillary" (http://www.confsci.co.jp/images/C-kit%20Ground%20Pro%20XRD%20Manual%20Eng%20240613.pdf).

3.4.1. Desirable harvesting solution

To harvest crystals while maintaining good quality, the harvested crystals should be stored in a harvesting solution with the same composition as the solution in the LCDM, where the target crystals are located. The solution composition in the LCDM does not vary significantly depending on its location in the LCDM; however, it does vary depending on the duration of the crystallization reaction. The crystallization reagent concentration in the LCDM takes about one month to reach equilibrium if salts are diffused, and several months or more if PEGs are diffused ("4.1. Time-dependent diffusion of reagents"). Therefore, if one plans to harvest crystals before equilibrium is reached, one must predict and prepare a harvesting solution containing crystallization reagent components at approximately the same concentration as the crystallization reagent in the LCDM. If the concentrations are inappropriate, the crystals may dissolve or break because of osmotic pressure differences.

For the prediction of the concentration, please refer to the description in "4.1. Time-dependent diffusion of reagents" In addition, to ensure reliable crystal harvesting, we recommend preparing and testing several solutions with concentrations above and below the predicted concentrations. The prepared harvesting solutions are placed on the depressions of cavity glass slides.

3.4.2. Disassembly of LCDM container

The thin capillary on the C-Cap side of the LCDM can be removed by pulling it out, taking care not to apply excessive force. The thin capillary on the gel tube

side cannot be removed because it has an attached dialysis membrane; therefore, the end of the LCDM container is cut (broken) with a diamond file.

3.4.3. Harvesting

The prepared harvesting solution is placed on a cavity glass slide. A short piece of the cut LCDM is then immersed in the solution and held with forceps. It can be easily held by using the stand with reverse action tweezers (sold separately, (http://www.confsci.co.jp/images/C-Kit%20Pro%20Advanced%20Tool_2208_Eng.pdf). While observing under a stereomicroscope, pour the harvesting solution into the fixed LCDM from the C-Cap side. Pipette the solution around the crystal to force it out of the capillary and into the harvesting solution. It is recommended to pour the crystals out through the broken side with the larger opening.

Some crystals may adhere to the glass surface of the LCDM. 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; it drives the crystals out of the capillary into the harvesting solution.

In practice, it is recommended to prepare lysozyme crystals using the CD method to familiarize oneself with the procedure for harvesting crystals. As lysozyme crystals easily adhere to the inner surface of the capillary, they are suitable for training.

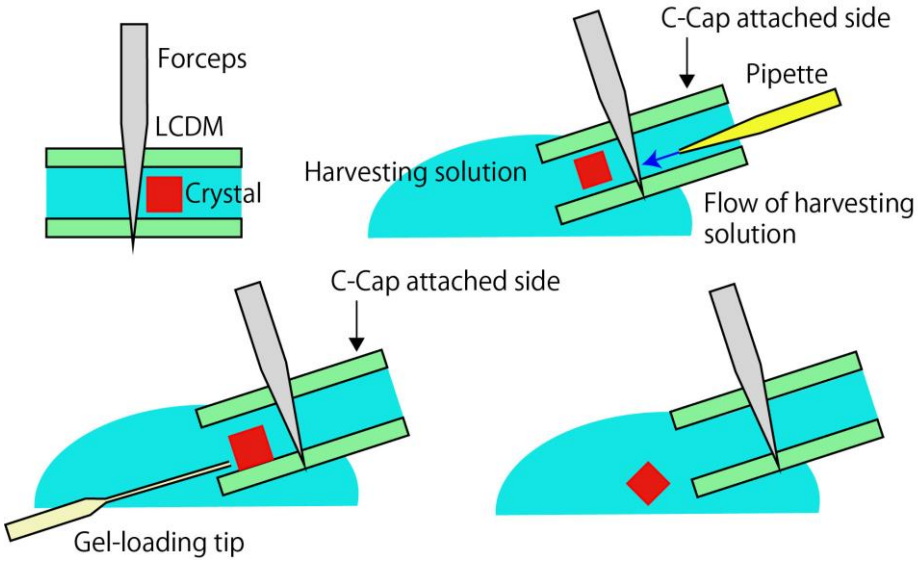

Fig. 3.20 Harvesting

Upper left: Fix the cut LCDM with forceps so that you can work with your hands freely while observing under a stereomicroscope.
Upper right: Pipette the solution into the LCDM and push the crystal gently.
Lower left: If the crystal adheres to the LCDM, touch it gently with a gel-loading tip and move it a little. The broken side with the larger opening is easier to touch.
Lower right: The crystal is pushed into the harvesting solution.

4. Technical Notes

4.1. Time-dependent diffusion of reagents

Protein crystallization can be expected when the concentrations of the protein sample and crystallization reagent are in a suitable combination. Therefore, it is important to understand time-dependent diffusion of the proteins and crystallization reagents in the CD method capillary and LCDM.

4.1.1. Change of reagent concentrations in the CD method capillary

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 reagent, NaCl are shown below.

Example 1

Sample solution: 40 mm length in a capillary

Reservoir solution: 1 M NaCl

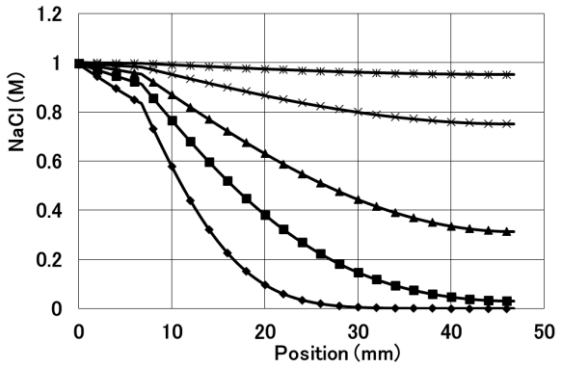

Fig. 4.1 Estimation of NaCl concentrations in a capillary.
♦, ■, ▲, x, and ж show NaCl concentrations 0.25, 1, 3, 8, and 16 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and the vertical axis indicates the NaCl concentrations at the location.

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. Thus, its diffusion time course is almost the same.

4.1.2. Change of reagent concentrations in the LCDM

Example 2

Sample solution: 9 mm length (27 μL) in the LCDM

Reservoir solution: 1 M NaCl

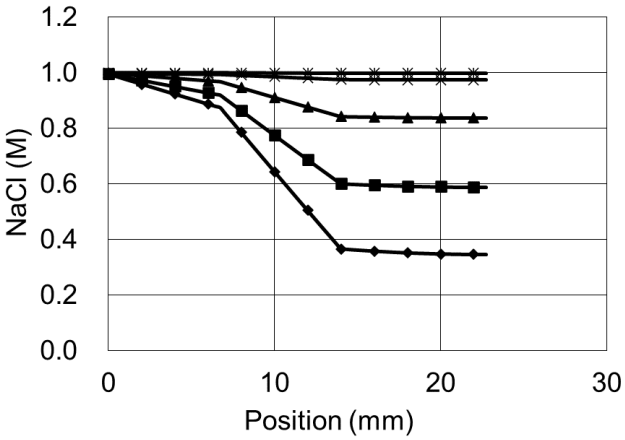

Fig. 4.2 Estimation of NaCl concentrations in the LCDM. ♦, ■, ▲, x, and ж show NaCl concentrations 4, 8, 16, 32, and 64 days after loading, respectively. The horizontal axis shows the distance from the end of the gel tube, and a dialysis membrane is placed at 14 mm. The vertical axis indicates the NaCl concentrations at the location.

The NaCl concentration in LCDM reaches equilibrium in 32 days. Ammonium sulfate, which is often used as a salt, has a diffusion coefficient similar to that of NaCl, so the time lapse is similar.

The dialysis membrane in LCDM has a MWCO of 6,000-8,000, but our actual measurements show that even PEG4000, which has a lower molecular weight, permeates very slowly. PEGs, which has a large molecular weight and high viscosity, are useful as crystallization reagents. Although, when using it as a crystallization solution of the dialysis method, it takes a long time, such as several months, to grow crystals. In that case, we recommend that a component that cannot permeate the dialysis membrane (such as high molecular weight PEGs) should be added to the protein sample in advance.

4.2. How to make a large crystal

4.2.1. The amount of required sample protein

Large amounts of proteins are required to create large crystals. The amount can be estimated as follows:

For simplicity, the crystal is assumed as a cube with each side measuring “a mm.” The following number of protein molecules will be present in this cube.

(V_m is the Matthews Coefficient, and M is the molecular weight).

$$\frac{a^3}{1000 \times V_m \times M \times 10^{-24}} \quad (1)$$

Number of moles of the protein present is:

$$\frac{a^3}{V_m \times M \times 6.02 \times 10^2} \quad (2)$$

The mass (g) of the protein present is:

$$\frac{a^3}{V_m \times 6.02 \times 10^2} \quad (3)$$

If the concentration of the protein sample is C mg/mL, and the solubility (protein concentration at which crystals no longer grow) is C_e mg/mL, the required volume of the protein solution (μL) is as follows:

$$\frac{a^3 \times 1660}{V_m \times (C - C_e)} \quad (4)$$

For example, for lysozyme (PDB: 3IJV), V_m is 1.84. Assuming a crystal size of 1 mm^3, protein concentration of 35 mg/mL, and solubility of 4 mg/mL, the required solution volume is approximately 29.1 μL. In the case of α-amylase (PDB: 6TAA), V_m is 2.18; assuming that other conditions are the same, the required volume is 24.6 μL.

4.2.2. Number of crystals

The amount of the protein sample estimated in the previous section is for one crystal. If three crystals are formed, the size of each crystal may be reduced by one-third.

The probability of protein crystal nucleation can be estimated using the following two equations [5].

$$\frac{dL(t)}{dt} = A_1(C(t) - C_e). \qquad (5)$$

$$I(t) = C(t)A_2 exp\left(-\frac{A_3}{ln\left(\frac{C(t)}{C_e}\right)^2}\right) \qquad (6)$$

where *C(t)* and *L(t)* are the concentration of the protein and the characteristic length of the crystal, respectively, assumed to be a cube at time *t*. *I(t)* is the nucleation probability at time *t*. C_e, A_1, A_2, A_3 are constants experimentally determined for each crystallization.

In our previous result [5], the four constants were estimated by applying the experimental results, the dependence of the crystal growth rate, and the number of nuclei formed on the protein concentration. The nucleation probability *I(t)* (number of nucleation per unit time and unit volume) at time *t* was then calculated, as shown in Fig. 4.3. This indicates that the probability increases approximately linearly above a certain protein concentration. According to the results, the number of lysozyme crystals after 24 h in the experiment with the 30 μL crystallization vessel is 5.9, 32.8, 62.5, and 91.9, for protein concentrations of 10, 15, 20, and 25 mg/mL, respectively. This analysis shows that the protein concentration has a significant effect on the number of crystals obtained.

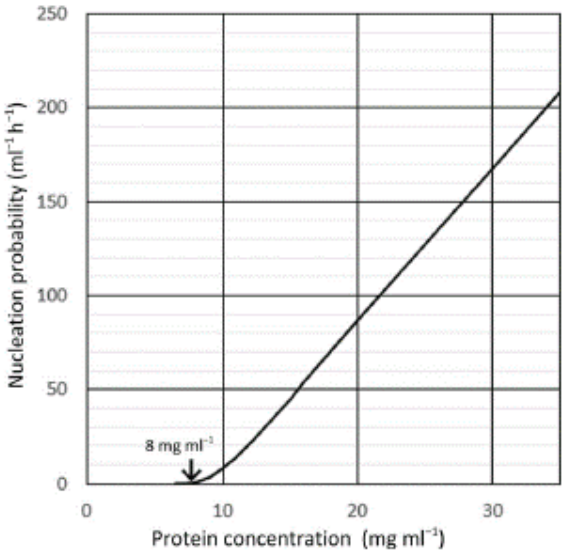

Fig. 4.3 Nucleation probability on protein concentration [5]. Lysozyme was crystallized in 0.4 M NaCl, 15% PEG4000, 50 mM acetate buffer (pH 4.5) at 20°C. Based on the experimental results of crystal formation, parameters were estimated, and the protein concentration dependence of the nucleation probability was calculated based on the equation (6).

In principle, it is possible to estimate the specific protein concentration required to grow one crystal: the first crystal nucleates and grows to reduce the protein concentration in the vessel; thus, the probability of nucleation is reduced, making it impossible to grow the second crystal. However, this type of

crystallization requires long incubation times using large volumes of low-concentration samples, and estimating the protein sample concentration is quite difficult.

5) Nakamura, H., Takahashi S., Inaka K., Tanaka H. Semi-empirical model to estimate ideal conditions for the growth of large protein crystals. *Acta Cryst.* 2020, D76, 1174–1183.

4.2.3. How to design crystallization experiments for large crystals

We recommend the following procedure for determining conditions for growing large crystals.

1. Understanding the overview using the batch method

Set up crystallization using the batch method. The sample solutions contain a constant protein concentration and varying concentrations of crystallization reagents. Observe for approximately 1–2 weeks. Identify the highest concentration of crystallization reagent at which crystals are not obtained (C_L) and the lowest concentration at which crystals are obtained (C_H). For example, in the experiment shown in Fig. 4.3, batch crystallization with NaCl concentrations in the range of 100–1000 mM, in increments of 100 mM, is recommended.

2. Understanding the dependence of nucleation probability on crystallization reagent concentration

Prepare the crystallization reagents at several concentrations between C_L–C_H (determined in the previous section) and conduct various batch crystallization reactions. In general, the number of growing crystals increases over time, eventually reaching a constant value. In an actual experiment, it is difficult to observe the time course in detail; therefore, it is sufficient to observe the approximate time and number. The probability of nucleation at each time point during the crystallization process is calculated by dividing the number of newly appeared crystals per unit time by the volume of the solution.

For example, a crystallization experiment was conducted under the following conditions (Fig. 4.4): 20 mg/mL lysozyme, 50 mM acetate buffer (pH 4.5), 10% PEG4000, and NaCl concentrations of 200–400 mM (in 20 mM intervals), temperature 4°C.

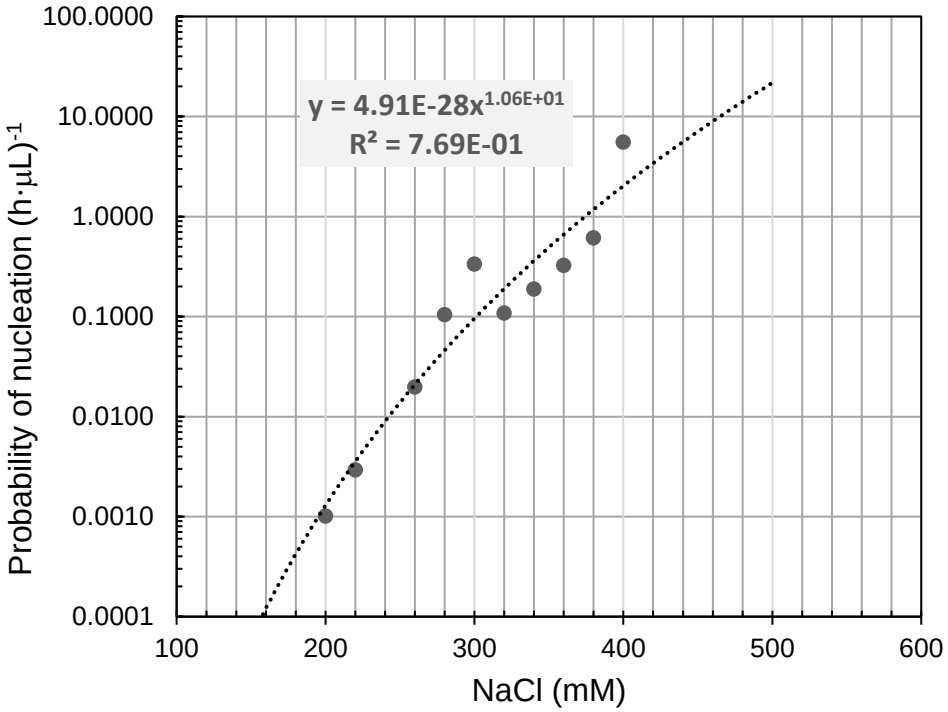

Fig. 4.4 Analysis of probability of nucleation

Based on these results, we will attempt to obtain a large crystal. It is important not to grow crystals one after another, so we will determine the conditions to obtain the first crystal approximately 10 days after starting crystallization.

When the solution volume is 30 μL, the nucleation probability for obtaining the first crystal on the 10th day is 1/240 h/30 μL = 0.00014. The NaCl concentration is estimated to be 163 mM using the approximate formula shown in the graph above.

3. Confirmation by counter diffusion (CD) method

Confirm the estimated conditions on a small scale using the CD method. Set up crystallization using the CD method with a crystallization reagent concentration (e.g., 180 mM) slightly higher than that estimated in the previous section (163 mM) and observe the progress. If the crystals grow faster and in greater numbers than expected, change the concentration of the crystallization reagent slightly lower. If the opposite is true, set it slightly higher.

4. Crystallization by the dialysis method

If crystallization proceeds over a favorable time course using the CD method, attempt crystallization using LCDM. As discussed in "4.1. Time-dependent diffusion of reagents", the increase in the concentration of crystallization reagents in the LCDM, is approximately half than that in the CD method capillary. First, use the dialysis method under the same conditions as the CD method.

4.2.4. For crystallization reagents containing multiple components

In most cases, crystallization reagents used for protein samples consist of (1) a high concentration of the main crystallization reagent, (2) a low concentration of the secondary crystallization reagent, and (3) a buffer. Examples of (1) include several molars of ammonium sulfate and PEGs at concentrations of several tens of percent. Examples of (2) include tens to hundreds of millimolars of NaCl. The nucleation probability described in the previous section depends not only on the concentration of the main crystallization reagent but also on the concentration of the secondary crystallization reagent.

The crystallization conditions can be easily controlled by changing the concentration of the crystallization reagent component, which has a milder effect on the nucleation probability. Therefore, we examine how the concentrations of the main (e.g., ammonium sulfate) and secondary (e.g., NaCl) crystallization reagents affect the number of crystals formed using the batch crystallization. The results will determine which crystallization reagent component is the key component that has a milder effect on the number of growing crystals. The concentration of this key component is then analyzed, as shown in Fig. 4.4, to determine the appropriate concentration for growing a single crystal.

Next, crystallization is performed using the CD method under the following condition: 1) the crystallization reagent component with fixed concentration is added to both the protein sample and reservoir solution, 2) the tested key component is further added to the reservoir solution at a concentration slightly higher than that estimated in the previous test, and then 3) its crystallization progress is observed. After the desired crystallization process is confirmed using the CD method, it is scaled up for crystallization in the LCDM.

Care must be taken when PEG is the main crystallization reagent. PEGs, which have large molecular weights, do not pass through the dialysis membrane of the LCDM (see "4.1. Time-dependent diffusion of reagents"). Therefore, to achieve the desired crystallization, it is essential to add PEGs to the protein sample in advance and adjust the concentration of the secondary crystallization reagent as a key component.

4.2.5. In the cases of inverse concentration dependence

In some cases, increasing the concentration of the crystallization reagent may decrease the probability of nucleation without any precipitation. For protein crystallization with such crystal-formation characteristics, it is also possible to determine conditions for growing large crystals.

Here, we explain the case where PEG is the main crystallization reagent, and many crystals are obtained with additional 50 mM NaCl but not at 150 mM. Using the batch method, the number of crystals formed is measured using several concentrations of the crystallization reagent, ranging from the concentration at which crystals can be obtained (e.g., NaCl 50 mM) to the concentration at which crystals are difficult to obtain (e.g., NaCl 150 mM), and a graph of the concentration dependence of the crystal formation probability (similar to Fig. 4.4) is created. Next, from the graph, the crystallization reagent concentration at which one crystal can be obtained in approximately 1–2 weeks is estimated. For the purposes of the following explanation, we will assume that the concentration is estimated to be 75 mM from the graph.

In the CD experiment, a protein sample is mixed with PEG, a buffer solution, and the high concentration of NaCl (150 mM NaCl in the example), and then loaded in a capillary. The reservoir solution contains the same concentration of PEG, buffer, and 75 mM NaCl. Under these conditions, crystallization is confirmed using the CD method, and then that for large crystals is attempted using the dialysis method.

As in the case, crystallization, in which the concentration of a specific component is gradually reduced to crystallization, is unique to the CD and dialysis methods.

4.2.6. Quality of protein samples

To optimize crystallization conditions using the experiments described so far, it is important that the nucleation probability shows a simple behavior with respect to concentration change of crystallization reagents, and reproducibility for the specific crystallization conditions. For the desired nucleation probability, the protein molecules should be well dispersed in the solution as monomolecules in a uniform state. If the sample is slightly denatured over time and forms multimers, these will act as nuclei and promote crystal formation, making it difficult to control the nucleation probability.

The following indicators can be used to determine whether a protein sample is appropriate.

 A single band of the desired molecular weight is detected by SDS-PAGE.

 The single peak in ion exchange chromatography, and the concentration of the NaCl gradient during elution roughly is consistent with the calculated charge density.

 The single band in native-PAGE and its migration distance roughly matches the number of charges.

 Dynamic light scattering shows monodisperse signals with reasonably narrow peak widths, indicating that the particle size is an integer multiple of the molecular weight.

Note that the nucleation probability may vary depending on the sample preparation lot. Therefore, it is desirable to do everything from examining the conditions using the batch method to crystallization in LCDM using the same lot sample. Thus, we recommend the preparation of a sufficient amount of protein samples.

Furthermore, online technical support (paid) is available via email or video meeting from experts in both methods: C-Kit Ground Pro e-mail support (CRT101-3), C-Kit Ground Pro video support (CRT101-4, (http://www.confsci.co.jp/images/C-kit%20Ground%20Pro_220908E.pdf) will surely help you to achieve highly challenging protein crystallization.

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