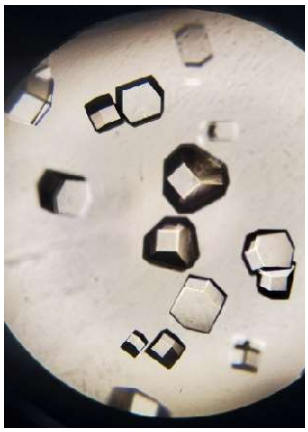
Lysozyme Protein Crystallography Workshop

INTRODUCTION



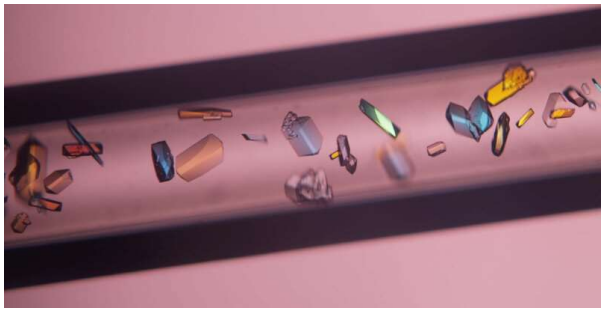


Figure 1. LEFT: Lysozyme protein crystals from the first STARS at GT crystallography workshop (January 20th, 2024). STARS members responsible: Diego Gonzalez and Selina Huang. Photo taken two days after the event. RIGHT: Protein crystals from Axiom Space (https://www.axiomspace.com/research/protein-crystallization)

As you can probably see, the best crystals are the ones that are perfect single crystals. These single crystals are needed for X-ray diffraction and structure determination.

What is structure determination and why does it even matter? Structure determination is solving the structure of a macromolecule (e.g. protein, DNA) by taking diffraction data on it and by building and refining a molecular model for it. This can be useful for designing therapeutics to treat diseases.

Recall Watson, Crick and Rosalind Franklin. They used X-ray crystallography to solve the DNA structure, and in 1962, James Watson, Francis Crick, and Maurice Wilkins were awarded the Nobel Prize for their contributions.

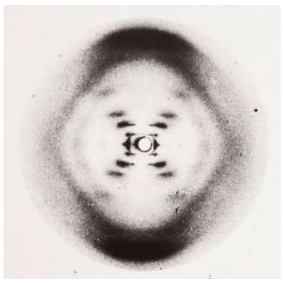


Figure 3. The diffraction of DNA crystal. James Watson (bottom left) and Francis Crick (right) and their DNA model, through the analysis of the diffraction patterns. (https://www.sciencehistory.org/education/scientific-biographies/james-watson-francis-crick-maurice-wilkins-and-rosalind-franklin/; https://undsci.berkeley.edu/the-science-checklist-applied-solving-dnas-double-helix/)

A lot of important research is related to structural biology. In fact, 56 Nobel Prize laureates were awarded in the last 125 years for research that was implicated with crystallography (https://www.iucr.org/people/nobel-prize).

THEORY

If we want to grow rock candy (sugar crystals), how can you grow it? You can (1) make an undersaturated solution and wait for the water to evaporate and for the crystals to form, (2) make a saturated solution and run the risk of having microcrystals already present in the solution, or (3) make a supersaturated solution through heating up a solution and then letting it cool back to room temperature.

Options (2) and (3) would be great for making sugar rock candy, but for the purposes of crystal-growing, option (1) would be best.

How would we do it? We can use the sitting-drop vapor diffusion technique to grow our protein crystals:

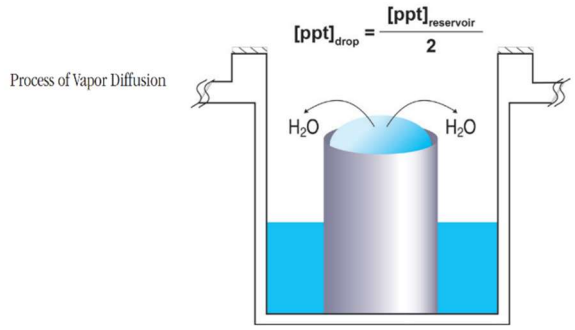


Figure 4. Sitting drop vapor diffusion technique, as illustrated in “Crystal Growth 101: Sitting Drop Vapor Diffusion Crystallization” by Hampton Research.

Option (1) was to create an undersaturated solution of protein and let the water evaporate away. Since the system must be in a closed, controlled environment, water in the system cannot evaporate out of the system. We can take advantage of water vapor diffusion off the droplet and into the precipitant to produce the same effect in the system. This will decrease the amount of water available to the protein solution and lead to an increased protein concentration in the droplet to cause the proteins to crystallize.

How do we ensure vapor diffusion in the correct direction occurs? We dilute the concentration of the precipitant (or essentially the well solution) when we place it onto the “island” with equal volumes to the protein already there. Now we have effectively halved the concentration of the precipitant in the droplet. The water in the droplet will now want to migrate from its high water potential (in the droplet) down to a lower water potential (in the reservoir) to equilibrate the two precipitant concentrations, decreasing the one in the well and increasing the one in the droplet until they are equal. In this process, water leaves the water droplet, causing the protein concentration there to increase, leading to the formation of protein crystals.

YOUR AIM FOR THIS EXPERIMENT

In this experiment, you will be growing lysozyme protein crystals with crystal-growing conditions of your choosing.

EXPERIMENT OVERVIEW

Each plate has four rows (rows A - D) and six columns (columns 1 - 6).

You will be varying the buffer pH (4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6) along the columns and the salt concentration along the rows.

(1) Choose your pH values:

Column 1: _____, Column 2: _____, Column 3: _____, Column 4: _____, Column 5: _____, Column 6: _____

(2) **Choose you salt identity:** NaCl / $NaNO_3$? (circle one)

If you chose NaCl, use concentration range 0.40 M to 1.20 M. If you chose $NaNO_3$, use concentration range 0.30 M to 0.60 M.

Before you write down the values that you want to use for the salt, open the Excel sheet and go to the Template tab. Put your four desired salt concentrations into the highlighted rows to generate the volumes required of stock solution in the green table underneath. Are the volumes you need to micropipette nice numbers to work with? If yes, write your desired salt concentrations below. If no, adjust the salt concentrations until the micropipette volumes are cleaner numbers to pipette.

(3) Choose your salt concentrations:

Row A: _____ Row B: _____ Row C: _____ Row D: _____

Each well for rows A, B, and C (such as wells A1, B4, C3, etc.) will have the precipitant solution that consists of (1) pH buffer (stock: 0.625 M) at final concentration of 0.1 M, (2) salt (NaCl or $NaNO_3$) (stock: 3 M) at your desired final concentrations, and (3) DI water. These three components, after being mixed together in each of the wells, constitutes your precipitant in each well.

Each row D well will have the pre-prepared precipitant from Hampton Research (which is 30% w/v PEG MME 5,000, 1.0 M NaCl, 0.05 M Sodium acetate trihydrate pH 4.6).

Now, verify the desired concentrations of each of the components in the Excel file to automatically generate the volumes of pH buffer and salt stock solutions you need.

In the Excel file, you had put your desired salt concentrations in the three highlighted yellow rows for rows A, B, C, D in the “Template plate set-up” tab.

In the green table below, you will see the volumes of (1) pH buffer stock solution, (2) salt stock solution, and (3) DI water that you will use to make your precipitant for each well. These calculations were made through $M_1V_1 = M_2V_2$ calculations (which are also embedded in the Excel sheet). The total volume of each precipitant solution in each well is 500 µl.

The volumes required of the stock solutions stay the same across a given row, they are different between row to row. (BEWARE: though your pH buffer stock volume stays the same across a given row, the identity of your pH buffer stock differs based on the column you are working on, make sure to double check your stock labels and your chart each time before dispensing liquid into your well plate).

Record your volumes for each row:

Row A: Volume of pH buffer stock: _____ µl

Volume of salt solution stock: _____ µl

Volume of DI water: _____ µl

Row B: Volume of pH buffer stock: _____ µl

Volume of salt solution stock: _____ µl

Volume of DI water: _____ µl

Row C: Volume of pH buffer stock: _____ µl

Volume of salt solution stock: _____ µl

Volume of DI water: _____ µl

Row D: Volume of HR precipitant: __300_ µl

Now that you have all your calculations ready, you are good to go for your experiment!

EXPERIMENT PROCEDURE

Materials and stock solutions for experiment:

- **Micropipettes** (GT Boggs Biology Prep Lab)
- **Micropipette tips** (GT Boggs Biology Prep Lab)
- **Staircase centrifuge tube racks** (GT Boggs Biology Prep Lab)
- **VDXm sitting-drop Crystallography plates** (Donation from Hampton Research)
- **Sodium acetate pH buffer (0.625 M)** (pHs: 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6) (2 Is it 2 µm filtered / sterile-filtered; prepared by STARS Atlanta Branch)
- **NaCl salt solution (3 M)** (2 µm filtered / sterile-filtered; prepared by STARS Atlanta Branch)
- **$NaNO_3$ salt solution (3M)** (2 µm filtered / sterile-filtered; prepared by STARS Atlanta Branch)
- **DI water** (GT Boggs Biology Prep Lab)
- **15-crystallization reagent** (Donation from Hampton Research)
- **Lysozyme protein solution (25 mg/ml)** (solubilization buffer: 0.02 M sodium acetate pH 4.6 buffer) (2 µm filtered / sterile-filtered; prepared by STARS Atlanta Branch)
- **Sealing tape** (Donation from Hampton Research)
- **Thin Sharpies** (Boggs Biology Prep Lab)

PPE (Personal Protective Equipment):

- **Safety glasses** (each student participant)
- **Lab coats** (each student participant)
- **Gloves** (Boggs Biology Prep Lab)

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GT Open Biology Lab

HAMPTON RESEARCH

DECTRIS

Steps:

- Getting ready
    - Have the listed materials and stock solutions ready
- Annotate the crystal-growing conditions on the hard-plastic cover of your crystallography tray
    - Record your name and your partner's name, date, and protein: lysozyme (25 mg/mL)
    - Along the top, record the column buffer pH values
    - Along the left side, record the row salt concentration values
    - On the left side, record the salt identity
    - Draw a box around Row D. Write "HR reagent" on the left side
- Remove the tray lid
- Prepare the precipitant solutions in the wells for a row or two rows
    - (You can optimize the micropipetting procedure however you would like to minimize the number of micropipette tips used. Suggested types: (1) Seal one row immediately after working on it or (2) Seal two rows immediately after working on both of them (Just make sure to work fast to avoid evaporation))
    - Add pH buffer stock solution to the well(s)
    - Add salt stock solution to the well(s)
    - Add DI water to the well(s)
        - When adding water to the wells, if you need to use two micropipettes, deliver the smaller volume first and then deliver the larger volume, so that you can use the larger volume pipette to begin mixing the precipitant well solution without needing a new tip (this is an example of optimizing the pipetting procedure)
        - To avoid making bubbles and risking the chance of getting solution stuck in the mechanism of the micropipette, while you are pipetting up and down the solution (also known as aspirating the solution) in circles around the "island," make sure to keep the micropipette tip completely submerged under the solution and to pipette up and down slowly (so the solution doesn't jump)
- Prepare the droplet on the "island" for a row or two rows
    - Add 1 µl of protein solution on top of the "island"
        - Ensure that you can see the small droplet of protein on top of the "island"
    - Using the same tip (this is a special case), micropipette 1 µl of the precipitant solution of the same well's reservoir and inject the 1 µl of precipitant solution into the protein droplet in the same well's "island"
        - Now you should have a 2 µl droplet on top of the "island" for that well
- Sealing one or two rows

    - ONLY IF YOU ARE SEALING ROWS ONE BY ONE: Take one piece of sealing tape and cut it along the length of the tape in half
    - Approximate the location of where to put down the tape so that the upper and lower edges of the horizontal tape will be at least extend more than 2 mm over the top and bottom rims of the wells in the row or rows and will have a .05 to 1 inch over hang to the right.
    - Gently place down the tape onto the row or rows so that all the wells can be securely covered, without stretching the tape. Gently tap on the tape and then start using a thumb to press on the tape and secure the tape on the crystallization plate so that it has no more bubbles left.
    - Take the flap on the right, and bend over the tape so that there is a tab on the right now.
- Prepare other rows
    - Make sure to check the identities of the pH buffers that you are adding, along with the volumes of stock solutions and DI water that you are adding
- Put the hard-plastic cover on top of your crystallography tray and check that the crystal-growing condition values and the identifying names and date are visible from above
- Place the crystallography tray into the designated incubator area.
- Congratulations! You have successfully set up your own tray of protein crystals!
    - STARS branch will be checking up on your crystals at our next club meeting. Feel free to come by to check on your crystals!
    - Thank you for participating in this workshop event! We will send you a feedback form. Please complete it to let us know how we did!