



Exercises - instructions

SEEDING LAB EXERCISES

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In this lab session we will be doing two exercises:

1. Instantaneous streak seeding varying the protein concentration.
2. Comparing the effects of adding different amounts of seeds.

1. What is seeding?

The use of an existing nucleus (usually a small crystal or crystal fragment), introduced into a new drop, where it acts as a growth site.

2. Why seed?

- To separate nucleation from growth and bypass the need for spontaneous nucleation. It is easier to add onto an already existing nucleus than create one *de novo*. See Fig. 1. Seeding and the phase diagram.
- Improve the size of the crystals or control the number of crystals
- Get more consistent results when crystals don't always appear in known conditions
- Speed up results if spontaneous nucleation is slow

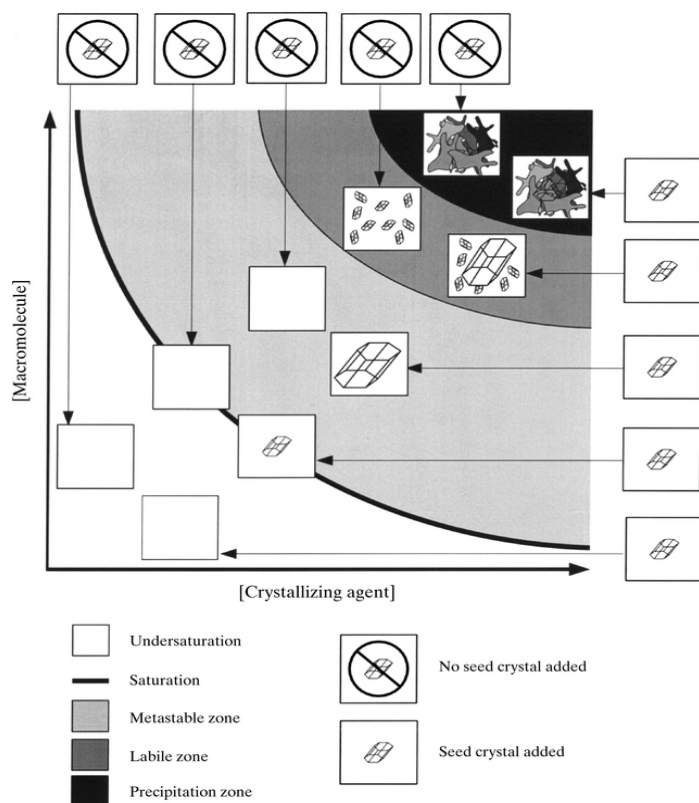
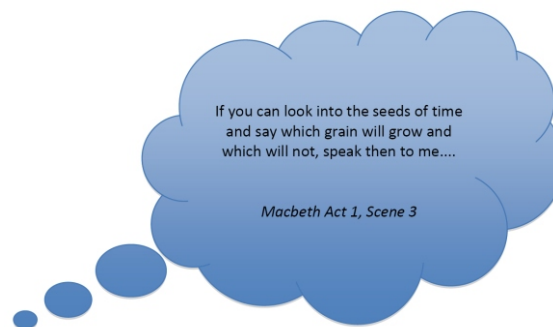


Figure 1. Seeding and the phase diagram. From Luft and DeTitta, Acta Cryst. (1999) D55, 988-993.



- To obtain a wider range of crystal forms (polymorphs) by seeding into totally different precipitants

3. What are the types of seeding?

- **Macro seeding** is the transfer of a single, pre-grown, washed crystal.
- **Micro seeding** is the transfer of microscopic crystals, crushed up into fragments.
- **Streak seeding** is a form of micro seeding that transfers the microseeds by a stroking motion with a whisker or hair of some sort. See Fig. 2. **Streak seeding.** Can be used on its own or in combination with microseeds in a dilution series.
- **Jab seeding** is another variation on micro seeding where the new drop is "innoculated" with a single jab of the seed transfer tool.
- **Matrix micro seeding** is when microseeds are placed into a screen of conditions totally unrelated to the ones where the seed crystals originated.

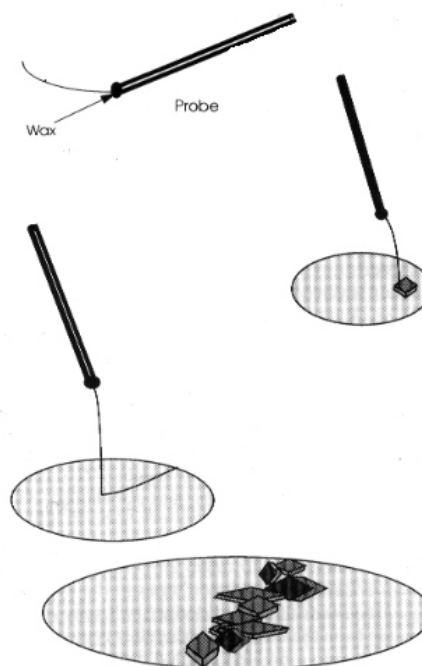


Figure 2. Streak seeding from Enrico Stura, Published in "Crystallization of Nucleic Acids and Proteins" Edited by Ducruix and Giege, 1992, Oxford University Press.

Experiment 1: Instant streak seeding

Reference: This exercise has been adapted from **Enrico Stura's** chapter "Seeding" in **Protein Crystallization: Strategies, Techniques, and Tips** edited by T. Bergfors 1999 International University Line, La Jolla Ca.

Purpose of this experiment:

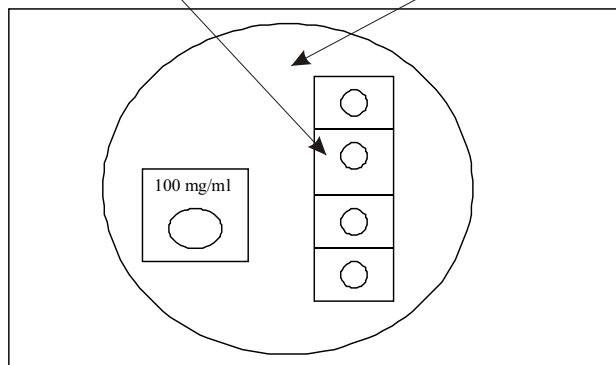
1. To learn how to generate new seeds by the easy, fast, and simple method of streak seeding.
2. To observe the effects of decreasing protein concentration on the nucleation rate.

Materials needed:

- a crystal wand — available from Hampton Research (HR8-133) or you can make your own from a cat whisker, human hair or horse tail hair, super glue or wax to affix the whisker to a yellow pipette tip, and a razor blade to cut the whisker.
- lysozyme 100 mg/ml stock solution.
- A dilution series of lysozyme at 80, 60, 40 and 20 mg/ml (already prepared for you.)
- 30% polyethylene glycol (PEG) 5000 or 6000 in 1 M NaCl, 50 mM Na acetate, pH 4.7 = the precipitant (ready-mixed for you today).
- A surface for making the drops, e.g., a Petri dish lid, cover slip, etc.

Method:

1. On the Petri dish lid, pipette 10 μ l of lysozyme (100 mg/ml). Now add an equal volume (i.e., 10 μ l) of the precipitant (= 30% PEG 6000 in 1M NaCl, 50 mM Na acetate, pH 4.7).
2. Watch the drop under the microscope. The first thing you should see is a phase separation. After that, the crystals should nucleate in 5-15 minutes. The fresher the lysozyme the longer time it will take to nucleate, so the time will vary. It can take some practice to recognize the nucleation in its initial stages. Note: If the protein precipitates immediately, the protein concentration is too high. Redo with a slightly lower protein concentration; try 80 mg/ml.
3. Once the parent crystals have formed in the drop with 100 mg/ml, you can set up the 10 μ l drops of lysozyme at 80, 60, 40, and 20 mg/ml on the inside of a Petri dish lid.



4. **Add 10 μ l of the precipitant to each of these 4 new drops and stir. Important!** These drops also need precipitant, not just protein, or the experiment does not work. Students often forget this step.

5. Now you can transfer the nuclei (from the parent drop) by streak seeding. Dip the seeding wand once into the drop of parent crystals to pick up the seeds, then streak the wand across the four new drops. You do not need to re-dip the wand into the parent drop for each new drop.

What to look for or think about:

- See if you can get the crystals to grow along a streak line. Because of the high protein concentrations you will also get spontaneous nucleation but many crystals will form preferentially along the streak line. The crystals will be extremely small because they grow so rapidly. In this experiment the protocol has been optimized for speed, rather than crystal size.

1. The purpose of this experiment is to demonstrate the method and the principles. In the next experiments, a more refined version will be presented.

2. Recover the 20 microliter drop of 100 mg/ml lysozyme (the parent drop). Place the 20 microliters (or as much as you can recover) into an eppendorf tube and fill to about 50 microliters with the precipitant (30% peg, etc). This will be your Seed Stock in the next experiment.

Experiment 2: Comparing the effects of adding different amounts of seeds.

Purpose of this experiment:

- **To illustrate the effect of adding different amounts of seeds.**
- **To illustrate a very fast and easy way of creating a dilution series of seeds.**

In contrast to experiment 1 above, this experiment will be allowed to equilibrate overnight to permit slower growth of the crystals after seeds are added. Three rows of identical drops will be prepared. Seeds will be added by either *jabbing* the drops or *streak seeding* them. These drops will be compared to a control where no seeds have been added. An entire row of drops will be seeded sequentially to create a dilution of the seeds. Thus, the first drop will have the most seeds and the last drop in that row will have the least.

Materials required:

1. two 20 ml Petri dishes (the large size)
2. 5 ml of 6% NaCl in 50-100 mM sodium acetate buffer, pH 4.7
3. 5 ml of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7
4. 100 microliters of 20 mg/ml lysozyme, dissolved in water
5. a seeding wand (you can use the same one from the previous experiment.)
6. two eppendorf tubes

Method:

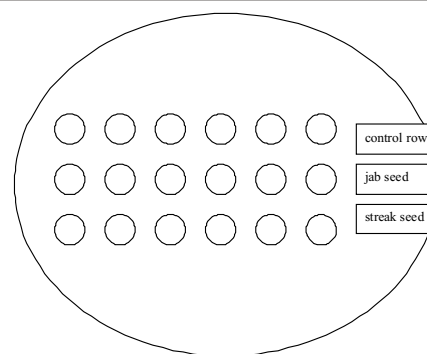
1. In an eppendorf tube, mix 50 microliters of lysozyme (20 mg/ml) with 50 microliters of 6% NaCl in 50-100 mM sodium acetate buffer, pH 4.7 = MIXTURE A.



2. In another eppendorf tube (mark them, so you don't mix them up!), mix 50 microliters of lysozyme (20 mg/ml) with 50 microliters of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7 = MIXTURE B.
3. In one Petri dish, pour approximately 5 ml of the buffered 6% NaCl solution in the bottom = PLATE A.
4. Repeat for the other Petri dish, using buffered 12% NaCl instead = PLATE B.
5. Using MIXTURE A, pipette 3 rows of 6 drops each onto the lid of PLATE A. Each drop will contain 5 microliters. See picture.
6. Repeat for MIXTURE B and PLATE B.
7. Row 1 will be the control.
8. Row 2 will be *jab seeded*. Using the seed transfer tool, dip or stir it into the parent drop in the previous experiment (experiment 1) to pick up seeds.
9. Now jab all the drops in row 2 with the seed transfer tool. To do so, barely touch the outermost edge of the drop with the tool = a quick jab. Rinse the tool in the reservoir solution between each jab to create a dilution series of the seeds.
10. Row 3 will be *streak seeded*. Rinse the seed transfer tool thoroughly and wipe it off with a tissue. Dip or stir it into the parent drop in the previous experiment (experiment 1) to pick up a new batch of seeds.
11. Now streak seed the drops in row 3 with the seed transfer tool. Rinse the tool in the reservoir solution between each streak to create a dilution series of the seeds.
12. Invert the lids of the Petri dishes over the correct, respective reservoirs and seal the rims with parafilm.
13. Wait 24 hours to observe the results.

Expected results:

1. After 24 hours, the control row in PLATE A will probably be still clear (but crystals will grow there after 48 hours, so it is best to examine the plates before that).



Petri dish for experiment 2.

In PLATE B, you probably will see sea urchin-type crystals by 24 hours.

2. The rows with jab seeding and streak seeding will give different effects. Jab seeding works best if the drop is very large, because there is a dilution effect across the surface of the drop. The drops here are only 5 μ l. Once back home, try the effect on large sitting drops (40 microliters) to make it more pronounced.
3. Jab and streak seeding several drops in a row (serial seeding) is a fast and easy way of making a dilution series of seeds in the new drops. The drops seeded last (by either method) should show the least number of crystals compared to the first drops in the row.

See an example in Figure 3.

- 4 **How to make a seed dilution series to control more exactly the number of seeds you add (things to do back home):** If you have a crystallization robot, it is easy to deliver different amounts of the seeds to your drops, creating a *de facto* dilution series that way. You can also make a dilution series manually in eppendorf tubes and then take an aliquot of seeds to the hanging/sitting drops containing protein/precipitant. The protocol is found in **D'Arcy, Bergfors et al., Acta Cryst (2014) F70, 1117-1126, section 2.1. Producing seed stocks and dilutions.**

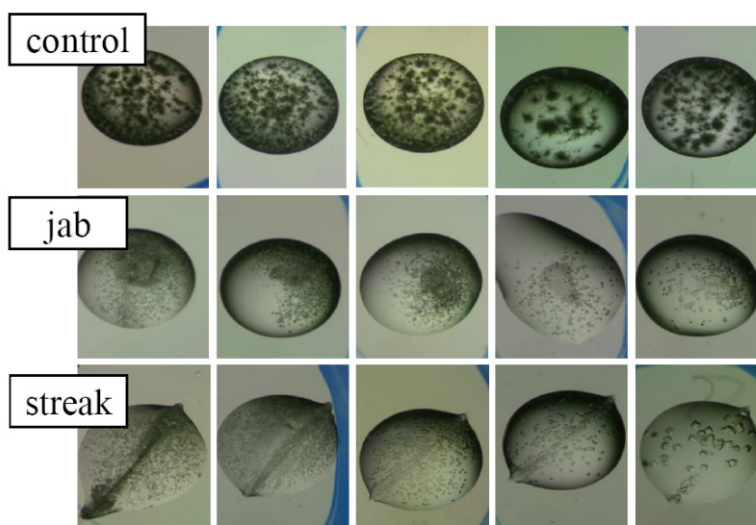


Figure 3. Comparison of drops that have been serially seeded with a control. These results were photographed 48 hours after setup. Row 1 is a control and has not been seeded. The lysozyme crystallizes in the form of sea urchins or spherulites. Row 2 has been jab seeded. The needle inoculated with seeds was jabbed into the center of the drops. Row 3 has been streak seeded. Notice that crystals tend to form along the seed line, but there is much spontaneous nucleation even outside the streak line in the first drops. However, the final drop in the row (drop 5 in the series) has nice, large crystals. This effect was achieved because the number of seeds added to the drop was diluted by passing the streak wand through the first drops in the row. (Photos by Terese Bergfors).