Seeding

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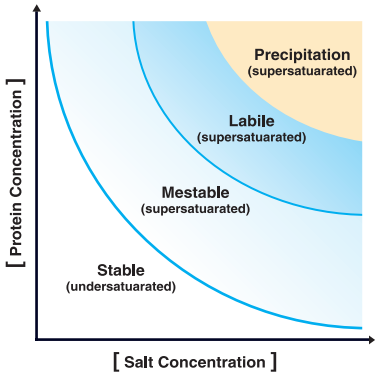
Solutions for Crystal Growth

Background

A crystallization experiment typically begins with the sample in a stabilizing solution of water and possibly other additives such as buffer, salt, reducing agent, and other chemicals. Prior to mixing the sample with crystallization reagent, this sample solution is undersaturated with respect to the biological macromolecule (protein). In an undersaturated sample solution, no crystals can nucleate, nor can crystals grow from seeds. Upon addition of a crystallization reagent the relative supersaturation of the sample is increased. Assuming the crystallization reagent decreases the solubility of the sample to increase the relative supersaturation, four events can take place (Figure 1). In the first stage, the Stable Zone, neither nucleation or growth can occur, and the drop remains clear. In the second stage of supersaturation, the Metastable Zone, spontaneous homogeneous nucleation cannot occur, but crystal growth from seeds can occur. Moving further into supersaturation, the Labile Zone, spontaneous homogeneous nucleation without seeds and crystal growth can occur. Further into supersaturation, the Precipitation Zone, precipitation of the sample from solution occurs.

Figure 1
The diagram is divided into four zones:

1. **Stable:** Undersaturated where crystal nucleation and growth is not possible; clear drops
2. **Metastable:** Supersaturated where nuclei cannot form but crystals can grow.
3. **Labile:** Supersaturated where nuclei can form and crystals can grow
4. **Precipitation:** Precipitation of sample from solution, where crystal nucleation and growth is not possible



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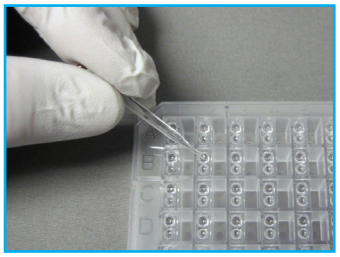
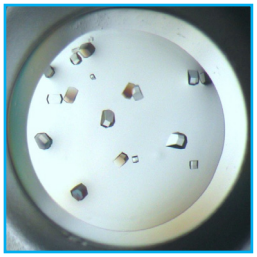
Seeding allows one to grow crystals in the Metastable Zone, where spontaneous homogeneous nucleation cannot occur, but crystal growth from seeds can occur. Why would one want to do this? For control, reproducibility, and to improve the likelihood of a successful crystallization experiment. In the Metastable Zone crystals can grow from seeds but cannot spontaneously nucleate. By placing a seed or solution of seeds in a drop which is saturated to the Metastable Zone one can use the seeds to grow larger single crystals. By controlling the number of seeds introduced into the Metastable Zone drop one can control the number of crystals grown. It is not practically possible to measure and know the number of seeds introduced to a drop, but by performing serial dilutions from a concentrated seed stock one can control the number of crystals grown in the Metastable Drop.

Seed Beads[1] are used to create a seed stock for performing subsequent seeding experiments. Seed Beads, depending upon the configuration, contain one or more beads made of either PTFE, Stainless Steel, Ceramic, or Glass, in a specially sized 1.5 ml microcentrifuge tube.

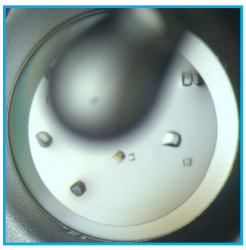
Preparing the Seed Stock – Contemporary Method

1. Position the crystallization plate and drop well containing the crystals identified for creating seed stock under the microscope. Open the drop well using an X-Acto Gripster Knife and forcep. Crush the crystals with a probe, such as the Crystal Crusher.

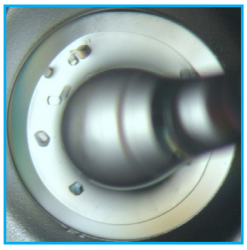


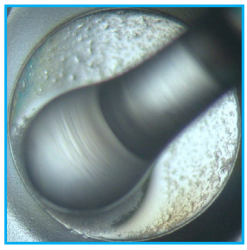


2. Working quickly, to minimize evaporation from the drop, position the Crystal Crusher above the drop well, the hemispherical end positioned down towards the drop.

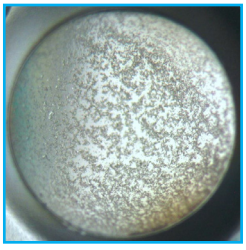


3. Lower the Crystal Crusher into the drop and begin crushing the macro crystals into micro crystals with a gentle up and down motion throughout the entire drop. Keep the Crystal Crusher in the drop, this is no time to get silly and splash or spread the drop you brute.





4. In a matter of seconds, your macro crystals will be crushed into micro crystals. Remove and set the Crystal Crusher aside. Seal the experiment using HR4-508, 0.75 inch wide Crystal Clear Mini Sealing Tape.



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If necessary add reservoir solution to the drop to minimize and compensate for evaporation from the drop, depending upon the time spent crushing the crystals and the drop size. If the drop well contains only a few small crystals, consider combining several drop wells to increase the seed crystal concentration. Read Observations, Notes and Suggestions #12 about combining drops.

5. Place a Seed Bead (tube contain a bead or beads) into a bucket of ice. Pipet 5 to 10 µl of crystallization reagent from the reservoir, and add it to the drop well containing the crushed crystals. Aspirate and dispense the drop several times. Use the pipet tip to dislodge crystals stuck to the plate. Pipet the mixture from the drop well to the Seed Bead tube on ice.

6. Repeat step 5 for a total of five to ten times until all of the crushed crystals have been transferred, and there are about 50 µl of solution containing crushed crystals in the Seed Bead tube. Be sure to remove all crystals that might be sticking to the plate.

7. Vortex the Seed Bead tube for three minutes, stopping every 30 seconds to cool the tube on ice. This is your seed stock.

8. Use this undiluted seed stock for Microseed Matrix Screening (MMS) The contemporary method uses a higher seed concentration than the classical method, is amenable to automation due to the smaller volume of seed stock and can produce more hits.

9. Manual MMS Set Up: 1.5 µl of protein, 1 µl of reservoir, and 0.5 µl of seed stock. Before pipetting the seed stock, agitate the tube in case the suspended crystals have settled in the tube.

10. Automated Contact Dispensing MMS Set Up: 0.3 µl of protein, 0.2 µl of reservoir, and 0.1 µl of seed stock. Before pipetting the seed stock, agitate the tube in case the suspended crystals have settled in the tube.

11. Before storing the seed stock, proceed with Serial Dilutions for seeding now, up to 1 in 100,000. Fresh seeds are better than old seeds when creating stocks. Use these diluted seed stocks in later experiments if too many crystals are obtained. Freeze all seed stocks immediately at -80°C (or -20°C if not available).

Preparing the Seed Stock - Classical Method

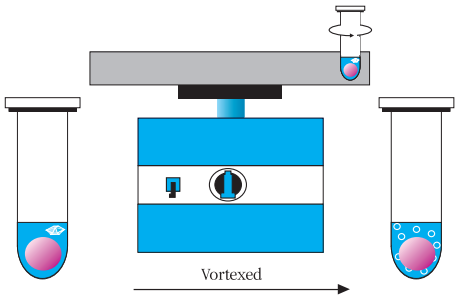
1. Pipet 50 microliters of crystal stabilizing solution into the microcentrifuge tube with the Seed Bead. The stabilizing solution is a mixture of sample and crystallization reagent in which the crystal will not dissolve nor continue to grow, but is a solution which will support the stability of the crystal. A solution closely approaching that of the drop from which the crystal is removed is a good starting point for the stabilizing solution. The simplest option is to use the crystallization reagent (reservoir solution) that produced the crystals. A more complex option is to perform some empirical experimentation to determine the reagent composition of the stabilizing solution. The stabilizing solution will be a reagent composition somewhere between that of the reservoir used to obtain the crystal and that of the drop at the initial mixing stage.

2. Remove crystals from a drop using a Mounted CryoLoop or pipet. Do not leave the seed exposed to the air for any longer than absolutely necessary. Macromolecular crystals have a high solvent content and can be damaged or destroyed by evaporation of water from about the crystal.

3. Place the seed crystals in the microcentrifuge tube containing 50 microliters of stabilizing solution and Seed Bead. Close the microcentrifuge tube.

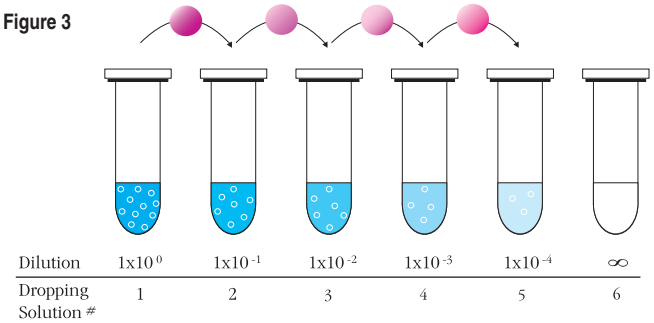
4. Vortex the microcentrifuge tube containing the seed crystal and the Seed Bead for 3 minutes. Alternatively, one may choose to sonicate the microcentrifuge tube containing the seed crystal and the Seed Bead for 3 minutes. See Figure 2 below.

Figure 2



5. Pipet 450 microliters of the stabilizing solution into the microcentrifuge tube containing the vortexed crystal and Seed Bead. This is your seed stock.

Figure 3



Preparing Serial Dilutions for Seeding

- **Dropping Solution 1:** Undiluted seed stock in stabilizing buffer. Dilution 1x10 ^{0}.
- **Dropping Solution 2:** 45 microliters of stabilizing buffer containing no protein or crystals plus 5 microliters of Dropping Solution 1. Dilution 1x10 $^{-1}$.
- **Dropping Solution 3:** 45 microliters of stabilizing buffer containing no protein or crystals plus 5 microliters of Dropping Solution 2. Dilution 1x10 $^{-2}$.

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- **Dropping Solution 4:** 45 microliters of stabilizing buffer containing no protein or crystals plus 5 microliters of Dropping Solution 3. Dilution $1x10^{-3}$.
- **Dropping Solution 5:** 45 microliters of stabilizing buffer containing no protein or crystals plus 5 microliters of Dropping Solution 4. Dilution $1x10^{-4}$.
- **Dropping Solution 6:** 45 microliters of stabilizing buffer containing no protein or crystals plus 5 microliters of Dropping Solution 5. Dilution $1x10^{-\infty}$.

Serial dilution of the seed stock can be performed if seeding experiments using the seed stock produce too many crystals in the drop. When preparing a number of serial dilutions of the seed stock, one should reserve a portion of each serial dilution for future crystallization experiments. What follows is an example of how to perform a serial dilution to prepare dropping solutions for seeding. One may prepare fewer or more dilutions depending upon how many drops are to be set. Also, one may change the dilution from 1:10 to some other ratio such as 1:2, 1:5, 1:20, and so on. Be certain to mix or vortex the seed stock prior to performing each dilution. Failure to vortex mix can lead to inconsistency. See Figure 3.

Setting the Drops - Seeding with the Seed Stock

Set sitting or hanging drops over reservoir solutions of reagent composition identical to that used to obtain the initial seed crystals. Do not add reservoir solution to the drops unless one wishes to further dilute the drops (Note: this may dissolve the seeds). To slow vapor diffusion equilibration one may dilute the reservoir solution. To speed vapor diffusion equilibration one may use a more concentrated reservoir solution.

Example 1. Original crystals grown using 2.0 M Ammonium sulfate, 0.1 M HEPES pH 6.8. Stabilizing solution is 2.0 M Ammonium sulfate, 0.1 M HEPES pH 6.8. Seed crystals from step 5 are composed of 500 ml of 2.0 M Ammonium sulfate, 0.1 M HEPES pH 6.8 and crystals, vortexed.

For the crystallization experiment, pipet 2.0 M Ammonium sulfate, 0.1 M HEPES pH 6.8 into the reagent well (reservoir). For the drop, pipet 1 part of protein plus 1 part of seed stock. The drop will equilibrate from 1.0 M to 2.0 M Ammonium sulfate.

Example 2. The results of Example 1 produced numerous, small crystals after only 24 hours. In an effort to reduce the number of crystals, increase crystal size and slow the experiment one can reduce the concentration of the reservoir to 70% of the original.

For the crystallization experiment, pipet 1.4 M Ammonium sulfate, 0.07 M HEPES pH 6.8 into the reagent well (reservoir). For the drop, pipet 1 part of protein plus 1 part of seed stock. The drop will now equilibrate from 1.0 M to 1.4 M Ammonium sulfate.

Example 3. The results of Example 2 still produced too many, small crystals after 24 hours. In an effort to reduce the number of crystals and increase crystal size, one can use a different serial dilution seed stock. From Preparing Serial Dilutions for Seeding, use Dropping Solution 2 for the seed stock.

For the crystallization experiment, pipet 1.4 M Ammonium sulfate, 0.07 M HEPES pH 6.8 into the reagent well (reservoir). For the drop, pipet 1 part of protein plus 1 part of Dropping Solution 2. The drop will now equilibrate from 1.0 M to 1.4 M Ammonium sulfate, but with fewer seeds.

Microseed Matrix Seeding (MMS)

Microseed Matrix Seeding is the method where seed crystals are systematically added to a crystallization screen.[3,4,12,13] By adding seeds instead of protein:

1. It is likely to greatly increase the number of crystallization hits that are observed;
2. It is likely that good quality crystals will grow, because the crystals often grow at lower levels of saturation;
3. Reagents can be used where no spontaneous nucleation takes place, so that the number of crystals can be controlled by determining the number of nuclei that are added to the well (by diluting the seed stock).

Setting the Drops for Microseed Matrix Seeding (MMS)

Pipet the crystallization screen reagent into the reagent well (reservoir). To create the drop, pipet 0.2 µl of crystallization screen reagent (reservoir), 0.1 µl of seed stock from step 5 and 0.3 µl of protein solution. As a starting point, use the same protein concentration here as used to produce the seeds in a previous screen. Repeat for the remaining reagents.

Observations, Notes, and Suggestions

1. The 3.0 mm PTFE Seed bead has a density of 2.2 g/cc. The 1.6 mm Stainless Steel Beads have a density of 7.9 g/cc. Use a magnet, such as a magnetic stir bar, to remove the Stainless Steel Beads from the tube within 24 hours after generating the seed stock to prevent oxidation of the beads in solution. The 1.0 Zirconium Silicate Ceramic Beads have a density of 3.8 g/cc. The 1.0 Glass Beads have a density of 2.5 g/cc.
2. When seeding, one would prefer to have an initial sample / reagent composition in the drop that will not produce crystals without the addition of a seed. This will prevent nucleation secondary to the introduced seeds as well as excessive nucleation.
3. If, after performing the seeding experiment with a particular set of dilutions, one still observes excessive nucleation and small crystals, repeat the seeding experiment with further dilution of the Seed Stock/Dropping Solutions.
4. Use a new, clean Seed Bead and microcentrifuge tube each time one is generating a new seed stock. This will prevent contamination and carryover.
5. Vortexing the Seed Bead in the presence of detergents and/or other chemical additives which can foam is not recommended. In the pres-

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ence of detergents or other chemical additives which can foam, it is recommended one use sonication to disrupt the seed using the Seed Bead.

6. Sonication using the Seed Bead allows one to use smaller volumes than the vortex method.
7. If using sonication do not leave the sample in the ultrasonic bath too long since this can heat the sample.
8. To prevent splashing when vortexing, grasp the tube in the middle while vortexing. Should drops of the sample appear on the upper sides of the tube or in the lid, place the tube in a centrifuge for 3 to 5 seconds to sediment the liquid. Do not centrifuge for any longer since this will "pellet" the seeds.
9. Any crystalline protein material can be used for microseeding, including fine needles, spherulites, microcrystals, irregular poorly-formed crystals and even granular looking precipitate. Seed anything that might be crystalline. Skins do not seem to work for seeding.
10. When performing iterative seeding during optimization, be more selective about which seeds to include in the seed stock, identifying and choosing the best crystals; do not mix the good, bad and the ugly, leave that to Sergio Leone.
11. Microcrystals in the seed stock are not stable because of the lower protein concentration in solution. The seed stock should be kept on ice and frozen as soon as possible, preferably at -80ºC when not being used.
12. It has been observed that, for some proteins, only fresh crystals work for seeding. Crystals that have been in the lab for a few weeks may not work for seeding, even though the crystals still diffract. Make a seed stock as soon as possible after the crystals stop growing.
13. One may consider combining as many hits as possible to make the seed stock. But be careful to avoid creating solutions that could result in salt crystals or phase separation. One could try to combine the drops from all the hits in PEG based conditions to make one seed-stock, and the drops from all the hits in salt based reagent to make another seed stock. Avoid mixing metals and salts such as calcium and phosphate as this can produce salt crystals. Avoid mixing high salt and high PEG concentration as this can produce phase separation.
14. MMS experiments can be dispensed by manually. The volumes dispensed will be increased slightly to approximately and in the following order, 1.5 µl of protein, 1 µl of reservoir solution, and 0.5 µl of seed stock.
15. When using automation for MMS, contact dispensing appears to be favored as non-contact dispensing can be prone to clogging.
16. Be careful not to optimize salt crystals. Salt crystals are a side effect of MMS due to the random mixing of reagents.
17. The potential of seeding and MMS. Increase hit rate in crystallization screens. More reagents to choose from for ligand soaking and heavy atom derivatives. New space groups. Use apo form to seed for ligands and inhibitors. Avoid twinning. Larger, more ordered, better diffracting crystals. Cross seeding between complexes.

What is Streak Seeding?

During streak seeding one touches a fine tool to crystalline material to dislodge, remove, and transfer small crystals (seeds) to a drop that will support the growth of potentially larger and more perfect crystals. [14-18]

Why do Streak Seeding?

A seed can provide a template on which additional macromolecules can assemble and under the proper conditions, grow to form a large single crystal. Using seeding can avoid problems associated with growing crystals from spontaneous nucleation because seeds can grow into larger crystals in the metastable region of the solubility curve, which is a region of lower relative supersaturation (Figure 1). In the Metastable Region the sample and reagent concentration are such that seed crystals may grow larger, yet crystals cannot nucleate. Nucleation occurs in a region of the solubility curve termed the Labile Region.[1]

Metastable Region = Crystals can grow larger from seeds. Crystals cannot nucleate
Metastable Region = Lower protein and reagent concentration than labile region
Metastable Region = Less aggregation events, reversible or irreversible

Labile Region = Crystal can grow larger from seeds. Crystal can nucleate
Labile Region = Higher protein and reagent concentration than metastable region
Labile Region = More aggregation events, reversible or irreversible

Figure 1 - Seeding and the phase diagram

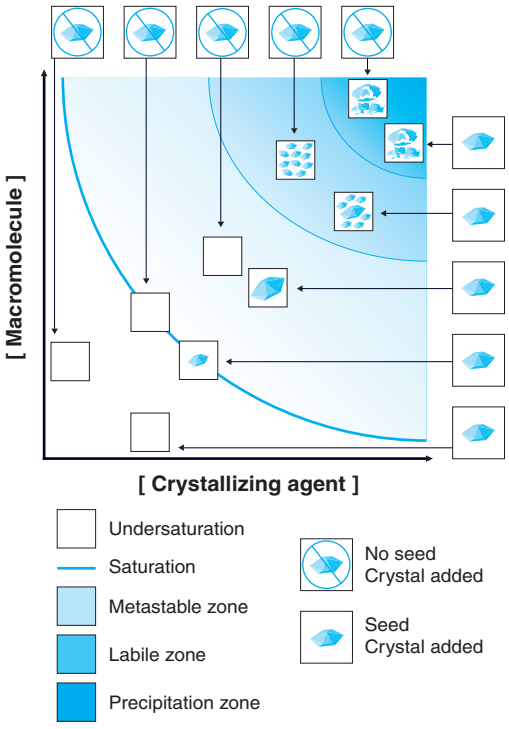

Figure 1 courtesy of Luft and De Titta (Acta Cryst. (1999) D55, 988-993.)

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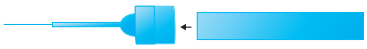
Take Home Lesson:
There is a better chance of nucleation in the labile region where the protein and reagent concentration are high. It is better to seed into the metastable region where lower protein and reagent concentration favor growth, not nucleation.

What to Seed From?
Seed from an existing crystal in an attempt to improve size, morphology or quality. Seed from any solid phase such as precipitate in an attempt to identify if the precipitate is crystalline in nature. Seed from a liquid phase such as phase separation or oil in attempt to identify if the liquid phase may produce crystals when seeded into a drop equilibrated to the metastable zone.[2]

What Methods to Use?
Streak seeding can be performed with hanging and sitting drop vapor diffusion as well as microbatch. Free interface diffusion using capillaries is also possible but placement of the seeds into the capillary can be tedious.

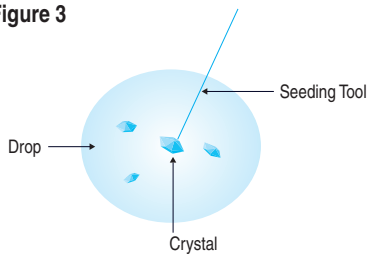
Streak Seeding Technique
First assemble the Seeding Tool by removing the cylindrical tube cover and inserting the cover in the back side of the Seeding Tool to create an extended handle (Figure 2). Or simply use the short handle without the extension in place if preferred.

Figure 2



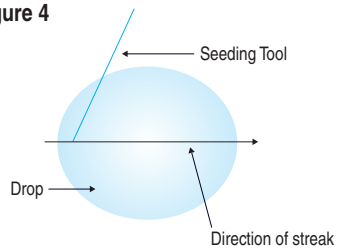
1. Collect the seeds. Touch the end of the probe to the donor crystal. If streak seeding from microcrystalline material or precipitate, drag the tip of the probe through the donor microcrystalline material or precipitate (Figure 3). Note: Some of the very small seed crystals will remain attached to the probe but you will likely not be able to see these small seeds under 10-100x magnification. So do not expect to see the donor seeds when you are streak seeding.

Figure 3



2. Deposit the seeds. Run the tip of the probe in a straight line across the middle of the recipient drop containing the sample and reagent. The tip of the probe should touch the bottom of the drop during the streak. Tip: The time interval between collecting the seeds and depositing the seeds should be quick, between 1 and 30 seconds. Having a prepared reagent, sample and plate set up and minimizing the distance between the location of seed collection and deposition will help minimize the time interval. Tip: Be consistent in the direction of your streak line. For example, always streak from 12:00 to 6:00 or from 9:00 to 3:00 (Figure 4 on page 2). This will make it simple to remember where to look for crystals growing along the streak line.

Figure 4



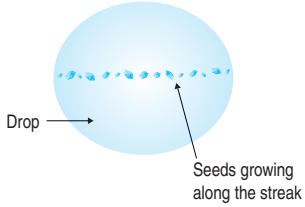
3. Seal the crystallization plate. Crystals should appear along the streak line.

Evaluating the Results and Refining the Streak
Seed crystals deposited along the streak line in the donor drop will either remain seeds, grow into larger crystals, or dissolve into the solution.

If the seed crystals remain as seeds, one will not be able to see the seeds under the microscope (10-100x). No crystals will appear along the streak line. Donor drops in subsequent drops should have a higher relative supersaturation to support seed growth. Increase the sample and/or reagent concentration in the drop by allowing the drop to pre equilibrate longer before streak seeding or increase the sample/reagent concentration. In general, it is better to change one variable at a time in order to understand the impact of changing that variable, so it is recommended to change only protein concentration or reagent concentration and not both at the same time.

If crystals appear along the streak line, the streak seeding has been successful (Figure 5). If the crystals are too small for X-ray diffraction analysis or demonstrate an undesirable morphology, performing iterative streak seeding (perform streak seeding again from the crystals grown along the streak line in the donor drop into a new donor drop) may help to improve the crystals.

Figure 5



Crystals appearing away from the streak line in the donor drop are likely self nucleating. This is an indication the relative supersaturation of the drop at the time of seeding was too high for an ideal seeding environment. This means the sample and/or reagent concentration was too high at the time of seeding. Nucleation in the donor drop can be prevented by reducing the sample and/or reagent concentration.

The appearance of precipitate indicates the sample and/or reagent concentration is too high. Reduce the sample and/or reagent concentration.

Cleaning the Probe

Probes can be cleaned by rinsing with deionized water and wiped dry. The probes can also be cleaned using 10% v/v methanol, isopropanol, or ethanol followed with rinsing in deionized water and wiped dry. The probes are natural fibers and will wear with use. When performance of the Seeding Tool diminishes discard the Seeding Tool.

Preparing the Recipient Drop

Seeds need to be added to drops with the sample and reagent concentration well below their supersaturation points. Seeds should not be added to drops where the relative supersaturation will allow the formation of crystal nucleation. This may result in the growth of the seeds but one will also see the formation of additional nucleic (crystals) and/or precipitate which can interfere with the quality of the growth of the seed crystals. Seeds need to be added to a drop which is in the metastable region of the solubility phase diagram, a point where crystals can grow but cannot nucleate. In essence, the reagent and sample concentration required to nucleate crystals has a higher relative supersaturation than the reagent and sample concentration required to grow a crystal.

One should seed into a drop which has a lower sample and reagent concentration than required for nucleation. Your seeding source material likely was produced in a drop where the sample and reagent concentration is too high for the best seeding results. One will need to lower the sample and reagent concentration in the recipient seeding drop for ideal seeding results. Determining the best concentration of sample and reagent for the drop and reservoir is empirical and will require some experimentation.

The following suggestions are offered as guidelines for such experimentation. Seed into a drop with the sample and reagent concentration at approximately 85% to 98% of that required to produce the original donor crystals. For example, crystals grown in a drop containing 10% PEG 3,350 over a reservoir containing 20% PEG 3,350 might be seeded into a drop containing 85% or 8.5% PEG 3,350 and a reservoir of 17% PEG 3,350. If no crystal growth is observed in the recipient drop, try increasing the concentration of PEG 3,350 in the drop to perhaps 9% and 18% for the reservoir. If, on the other hand, too many crystals appear along the seed line or additional nucleation is observed aside from the seed line, decrease the PEG 3,350 concentration in the drop to 7% and 14% in the reservoir. Too expedite the procedure one can set multiple drops and vary the concentration of drop and well components over a series of drops and reservoirs.

One may also hold the reagent concentration constant and dilute the sample concentration in the drop. Dilute the initial sample concentration in the drop by 50% (i.e. from 20 to 10 mg/ml) and streak seed. Based on the results, adjust the sample concentration as required. The appearance of no crystals will require one to increase the sample concentration. The appearance of too many crystals will require one to dilute the sample concentration further.

Another variant to evaluate in determining the ideal drop and well concentration is to set the drop containing sample and reagent over the reservoir, seal and leave overnight (approximately 24 hours) to allow for partial equilibration of the drop with the reservoir, then perform the streak seeding into the partially equilibrated drop. Although highly empirical, and sensitive to reagent type, drop and reservoir volume, and plate type, this quick and easy method does some times work.

Different outcomes may be observed if the sample concentration is lowered and the reagent concentration maintained, or if the sample concentration is maintained and the reagent concentration lowered. Therefore, consider sample and reagent concentrations as individual variables when optimizing seeding. The sensitivity of the seeds to sample and reagent concentration (overall relative supersaturation) depends on the size of the metastable zone. Small metastable zones will be more sensitive to smaller changes in sample and reagent concentration.

Another method to try when streak seeding is rather than seed into a single drop, pass the Seeding Tool with seeds successively through several droplets (serial seeding), thereby decreasing the number of seeds transferred to later drops. If too many crystals form along the seeding line, reduce the length of the streak line through the drop or simply dip the Seeding Tool into the recipient drop, touching the bottom of the slide or well without drawing a line in order to deposit fewer seeds.

Another variation of seeding is sequential or iterative seeding where one repeats the seeding procedure up to 7 to 10 times in order to obtain the desired results. Here, streak seeding is performed from the donor seed into the recipient drop. After crystals grow along the streak line in the recipient drop, the streak seeding is performed from these crystals into a new recipient drop. This process can be repeated 7 to 10 times in order to improve the quality and size of the crystal.

Seeding In Other Situations

When working with mutants or variants of a sample to be crystallized, try seeding from crystals of the native sample into sample drops containing the mutant or variant to stimulate the growth of crystals of the mutant or variant form of the sample.

Seed to increase the size and volume of the crystal. Iterative streak seeding can produce crystals with sizes and volumes 10 to 1,000 fold larger than the seed donor crystals.[3]

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Using the Seeding Tool, stir old drops with precipitate but no crystals to see if the kinetic energy of the mixing or disruption of the precipitate can induce nucleation and crystal growth.

When no crystals can be grown after exhaustive screening, streak seed from drops with precipitate into clear drops. Desperate times call for desperate measures.

Try streak seeding from bundles of crystals or needle crystals to change the crystal morphology.

Try streak seeding and at the same time evaluate different additives in the sample drop. Additives to consider include salts, polyols, divalent cations, detergents, chaotropes, organic solvents, ligands and co-factors.

If one experiences repeated dissolution of seeds no matter how the reagent and sample concentration are varied, try cross linking the seeds or donor crystals with glutaraldehyde before steak seeding to see if cross linking the crystal will prevent crystal dissolution.

Streak Seeding Lab Exercise[17]

The following exercise will demonstrate the streak seeding technique and also allow one to observe the effect of decreasing sample concentration on the nucleation rate.

Materials

- Seeding Tool
- Lysozyme stock. Make fresh the day of the experiment. 100 mg/ml in 0.1 M Sodium acetate trihydrate pH 4.6
- Crystallization Reagent: 30% PEG 3,350, 1.0 M Sodium chloride, 0.1 M Sodium acetate trihydrate pH 4.6.
- Siliconized cover slides and VDX Plate with sealant or your favorite crystallization plate.

Procedure

1. On a siliconized cover slide pipet 10 microliters of 100 mg/ml lysozyme and 10 microliters of Crystallization Reagent. Pipet the lysozyme then the reagent. Gently aspirate and dispense the drop 5 to 10 times to mix the viscous crystallization reagent with the sample. Avoid making bubbles and denaturing the protein. Keep the tip in the drop during aspiration and dispensing.

2. Crystals will nucleate in 5 to 15 minutes. A freshly prepared stock of lysozyme will take longer to crystallize than an old stock. If the lysozyme precipitates immediately and does not solubilize with mixing, dilute the lysozyme stock to 80 mg/ml with 0.1 M Sodium acetate pH 4.6 and repeat step 1. If the lysozyme still precipitates immediately and does not solubilize with mixing, dilute the lysozyme stock to 60 mg/ml with 0.1 M Sodium acetate trihydrate pH 4.6 and repeat step 1.

3. To evaluate the effect of protein concentration on nucleation rate as well as the size and number of crystals perform the following experiment. Dilute the 100 mg/ml lysozyme stock to create stocks of 80, 60, 40, 20 and 10 mg/ml (Table 1 below).

Table 1

Final lysozyme concentration (mg/ml)	100	80	60	40	20	10
µL 100 mg/ml lysozyme	500	400	300	200	100	50
µL 0.1 M sodium acetate pH 4.6	0	100	200	300	400	450

Set two experiments for each of the six concentration of lysozyme. In the first set of experiments using 100, 80, 60, 40, 20 and 10 mg/ml lysozyme, pipet 10 microliters of lysozyme and 10 microliters of crystallization reagent. Gently aspirate and dispense the drop 5 to 10 times to mix the viscous crystallization reagent with the sample. In the second set of experiments using 100, 80, 60, 40, 20 and 10 mg/ml lysozyme, pipet 10 microliters of lysozyme and 10 microliters of crystallization reagent. Gently aspirate and dispense the drop 5 to 10 times to mix the viscous crystallization reagent with the sample. After preparing each drop apply streak seeding to each of the second set of drops by touching a parent crystal from the experiment in step 1 and streak seed across the drop.

4. Can you get crystals to grow across a streak line? Do you see fewer, but larger crystals as you dilute the lysozyme concentration? How long does nucleation and growth take in the drops with no streak seeding versus the drops with streak seeding? Can you find a concentration of lysozyme where crystals only grow with streak seeding?

5. For variations, try decreasing the reagent concentration and keeping the lysozyme concentration constant (i.e. 100 mg/ml or other constant concentration). Try equilibrating the drops over night and then performing streak seeding. How do the results of these experiments compare to drops where seeding was performed without equilibration?

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12 x 20 mg plus 12 x 1 ml solubilization buffer

HR2-805 15-Minute Lysozyme Crystallization Reagent
30% w/v PEG MME 5,000, 1.0 M Sodium chloride,
0.05 M Sodium acetate trihydrate pH 4.6, 100 ml

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