Seeding Tool

HAMPTON RESEARCH
Solutions for Crystal Growth

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.

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

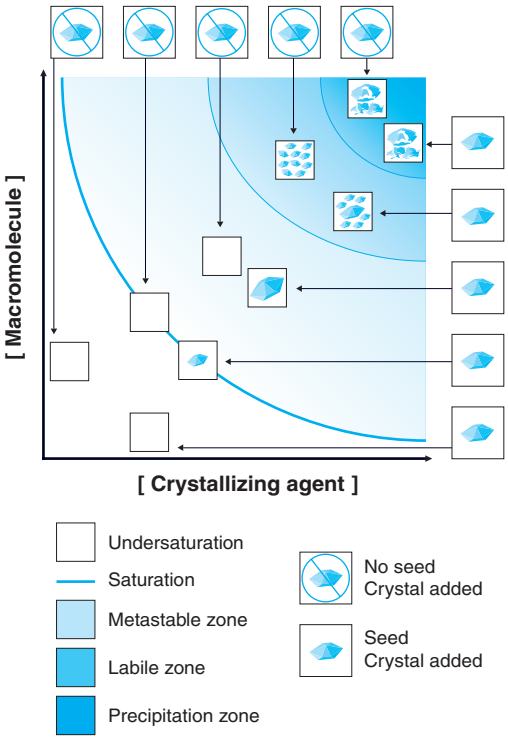


Figure 1 courtesy of Luft and De Titta (Acta Cryst. (1999) D55, 988-993.)

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]

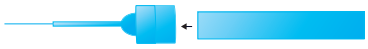
What Methods to Use?

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

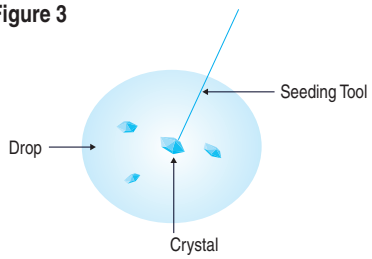
First assemble the Seeding Tool by removing the cylindrical tube cover and inserting the cover in the back side of the Seeding Tool to create an extended handle (Figure 2). Or simply use the short handle without the extension in place if preferred.

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.

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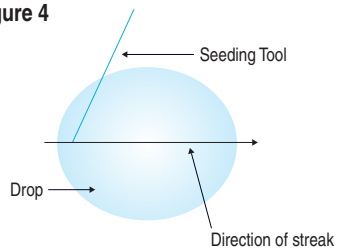
Figure 4



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

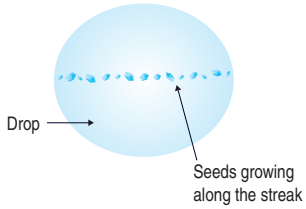
Evaluating the Results and Refining the Streak

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.

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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]

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.

References:

1. Crystallization of nucleic acids and proteins, a Practical Approach. Edited by A. Ducruix and R. Giege. (1992) Oxford University Press. ISBN 0-19-963246-4.
2. Protein Crystallization, Techniques, Strategies, and Tips. A Laboratory Manual. Edited by Terese Bergfors. (1999). International University Line. ISBN 0-9636817-5-3.
3. Repeated seeding technique for growing large single crystals of proteins. C. Thaller et al. (1981) J. Mol. Biol. 147, 465-469.

Streak Seeding Lab Exercise[1]

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 3350, 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.

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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?

References

1. Protein Crystallization, Techniques, Strategies, and Tips. A Laboratory Manual. Edited by Terese M. Bergfors. Chapter 14. Seeding. By Enrico A. Stura. Pages 141-153. (1999).

Other Seeding & Crystal Manipulation Tools

HR4-217 Crystal Probe - pack of 12
HR2-320 Seed Bead kit
HR4-811 Micro-Tool set
HR4-837 Micro-Tool 2 set

Further Reading on Seeding

Seeds to Crystals. Terese Bergfors (2003), Journal of Structural Biology, Volume 142, Number 1, pages 66-76.

Blundell, T.L. & Johnsom, L.N. (1976) Protein Crystallography, Academic Press, London.

McPherson, A. (1976) Methods Biochem. Anal. 23, 249-345.

D'Arcy A, Mac Sweeney A, Haber A., Using natural seeding material to generate nucleation in protein crystallization experiments. Acta Crystallogr D Biol Crystallogr. 2003 Jul;59(Pt 7):1343-6.

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"Seeding techniques", E.A. Stura and I.A. Wilson in Crystallization of Nucleic Acids and Protein, A Practical Approach. Pages 99-126. Edited by A. Ducruix and R. Giege. Oxford University Press, 1992.

Luft, J.R. and DeTitta, G.T., "A method to produce microseed stock for use in the crystallization of biological macromolecules". Acta Cryst. (1999) D5, 988-993.

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