Seed Bead™

HAMPTON RESEARCH
Solutions for Crystal Growth

Overview

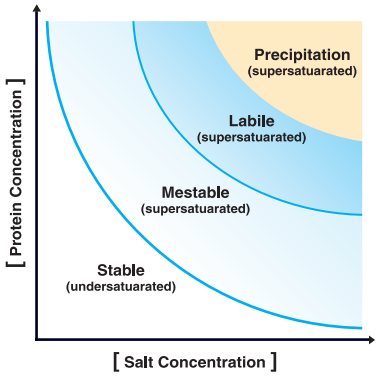
This publication describes the use of the Seed Bead to create a seed stock for performing subsequent seeding experiments. Seed Bead is based upon the research of Joseph R. Luft and George T. DeTitta at the Hauptman Woodward Medical Research Institute.[3] The Seed Bead Kit contains 24 Seed Beads manufactured from PTFE, individually contained in a special 1.5 milliliter microcentrifuge tube and instructions.

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 macromolecule in question (sample). 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, three events can take place. In the first stage of supersaturation, the Metastable Zone, spontaneous homogenous nucleation cannot occur, but crystal growth from seeds can occur. Moving further into supersaturation, the Labile Zone, spontaneous homogeneous nucleation and crystal growth can occur. Further into supersaturation, the Precipitation Zone, precipitation of the sample from solution occurs. See Figure 1 below.

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 nucleic can form and crystals can grow
4. **Precipitation:** Precipitation of sample from solution, where crystal nucleation and growth is not possible



Seeding

Seeding allows one to grow crystals in the Metastable Zone, where spontaneous homogenous 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.

Preparing the Seed Stock – Contemporary Method

1. Place the Seed Bead (tube containing a bead) into a bucket of ice.

2. Open the drop well containing the crystals identified for creating seed stock. Crush the crystals with a probe. 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.

3. 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.

4. Repeat step 3 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.

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

6. 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.

7. 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.

8. 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.

9. 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

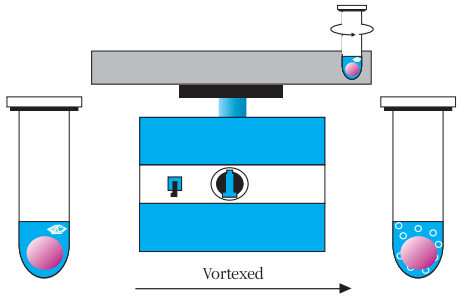
is a good starting point for the stabilizing solution. The simplest option is to use the crystallization reagent (reservoir solution) that produced the crystals. A more complex option is to perform some empirical experimentation to determine the reagent composition of the stabilizing solution. The stabilizing 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.

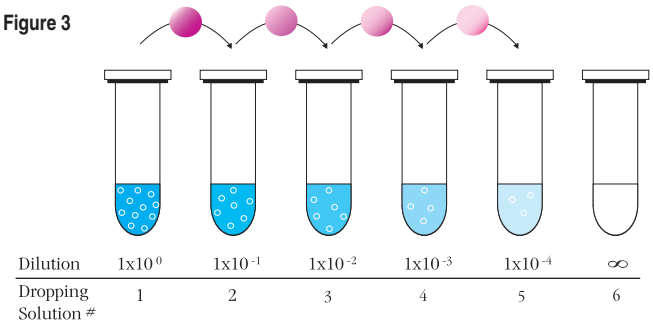
4. Vortex the microcentrifuge tube containing the seed crystal and the Seed Bead for 3 minutes. Alternatively, one may choose to sonicate the microcentrifuge tube containing the seed crystal and the Seed Bead for 3 minutes. See Figure 2 below.

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 $^{-2}$.
- **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.[5-8] 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.
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 presence 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.

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Related Products

HR4-780 Seed Bead Steel kit - 24 tubes with Steel Seed Beads

HR4-781 Seed Bead Ceramic kit - 24 tubes with Ceramic Seed Beads

HR4-782 Seed Bead Glass kit - 24 tubes with Glass Seed Beads

HR4-216 Crystal Crusher - 5 pack

HR8-133 Seeding Tool - 5 pack

HR4-217 CrystalProbe - 12 pack

Technical Support

Inquiries regarding the Seed Bead kit and general inquiries regarding crystallization are welcome. Please e-mail tech@hrmail.com.

Hampton Research
34 Journey
Aliso Viejo, CA 92656-3317 U.S.A.
Tel: (949) 425-1321 • Fax: (949) 425-1611
Technical Support e-mail: tech@hrmail.com
Website: www.hamptonresearch.com