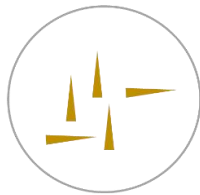


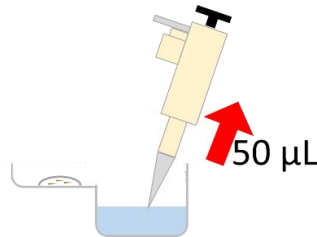
Procedure for Making the Seed Stock

- Any crystalline protein material can be used for microseeding, including fine needles, “spherulites”, microcrystals, and irregular poorly-formed crystals.
- Note that the microcrystals in the seed stock are not stable because the seed stock contains very little protein. Therefore the seed stock should be kept on ice and frozen as soon as possible, preferably at -80°C .
- If you have plenty of crystals, use the Seed Bead from Hampton Research, HR2-320, see <http://hamptonresearch.com>.
- If you have only a few small crystals, modify the procedure below by crushing the crystals with a probe and making a smaller volume of seed stock (e.g. 15 μL) without using the Seed Bead.

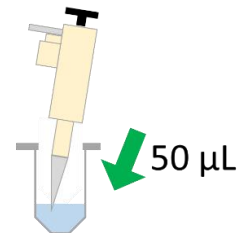
The method is based on the method of D’Arcy et al., adapted from Luft and DeTitta, references below.



1. Identify crystals.



2. Open well and remove 20-50 μL of reservoir solution.



3. Place reservoir solution in seed bead tube and keep on ice.



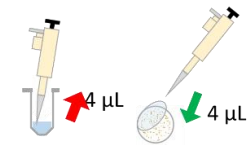
4. Crush crystals with crystal crusher for ~ 30 seconds.



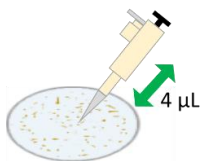
5. Crystals should break easily as in image above. Check crystals are not cross linked) You can feel a click if they are salt crystals.



6. Wash crystal crusher in cooled reservoir solution in seed bead tube to collect seeds from crystal crusher



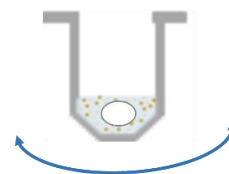
7. Take 2-6 μL of cooled reservoir solution from seed bead tube and add to drop containing crystals.



8. Dispense and suck several times in drop well.



9. Transfer the mixture back to the Seed Bead tube. Repeat the process until no crystals are visible in the drop.



10. Vortex seed bead 4 x 30 seconds and keep on ice. Use seed stock immediately or freeze at -80°C . flick the seed stock tube immediately before use to ensure seeds have not fallen to bottom.

11. Use this (undiluted) seed stock for rMMS microseeding (seeding into a random screen). For rMMS not dilute 1:100 as instructed in the Hampton Research Seed Bead instructions. *The more crystals there are in your seed stock the more hits you will obtain.* Recommended volumes to use are 0.3 μL of protein, 0.2 μL of reservoir, and 0.1 μL of seed stock for robotic work. Before using the seed stock, agitate it in case the suspended crystals have settled in the tube.
12. However, we suggest that you make a dilution series straight away, up to 1 in 10,000. 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).
13. rMMS can be performed by hand (increase the volume) or with a robot that uses contact dispensing.
14. Immediately after use, freeze the undiluted seed stock at -80°C / -20°C .

Strategic Considerations

Step 1: find as many new hits as possible

The choice of seed-crystals used for microseeding experiments will vary depending on the objective of the experiment. Near the beginning of a project it is helpful to find several crystallization hits that can provide alternative starting points for crystal optimization. (These alternative hits can, for example, be used in "combinatorial" experiments for recombining the ingredients identified.) rMMS also greatly reduces the need for crystal optimization because good-quality crystals are more likely to grow in the metastable zone of the phase diagram.

We therefore suggest using rMMS routinely as soon as the first crystals are obtained (or, more accurately, as soon as the first crystals stop growing). For this initial round of rMMS, a seed stock should be made with as much crystalline material as possible. If only one well is available that contains crystals, or if the crystals are small, it may be helpful to set up say 10 repetitions of the original hit (without seeding) to increase the supply of crystals. If, however, several different hits are obtained, seed crystals can be harvested from several conditions and mixed together. To avoid phase separation, crystals grown in high-salt conditions should be harvested separately from crystals grown in e.g. high-PEG conditions. If crystals from several wells are mixed, a reservoir solution that is less likely to give salt crystals should be selected to suspend the seed crystals. For example, high concentrations of phosphate, sulfate, calcium, magnesium etc. should be avoided.

Step 2: optimize the seed stock

Later on in a project it may be important to look for crystals with different unit cells in order to improve diffraction, avoid twinned crystals, or obtain crystals that are suitable for binding ligands. At this stage only the most suitable crystals (e.g. those that diffract best) should be used to make the seed stock. Sometimes repeated rounds of microseeding are required, where only the "best" crystals are used to make the seed stock for the subsequent round. It may be helpful to maintain a "library" of seed stocks with different unit cells etc. If possible, a "neutral" precipitant such as PEG 3000 should be used to suspend the seed crystals to encourage novel crystal contacts and to crystallize complexes that may be unstable in high-salt solutions [see Shaw Stewart *et al.* ref. below].

Step 3: grow crystals for data collection, soaking etc.

Finally, classical seeding experiments, where a single crystallization condition is used, are often helpful near the end of a project. It is often necessary to dilute the seed stock in order to get the desired number of crystals per drop. A "combinatorial" experiment (where a series of seed stocks of increasing dilution are added to a crystallization condition) is a quick method for finding the optimal dilution of the seed stock. This approach is described below. (This is a different use of the combinatorial experimental lay-out from the use mentioned in step 1, above, for reshuffling ingredients.)

Notes

1. Crystals can be crushed in their wells using a rounded glass probe. Such a probe can be made from a glass rod, pipette or capillary using a blow-torch or Bunsen flame. Solid glass rods or thick-walled capillaries are stronger than thin-walled tubing, but Pasteur pipettes can be used. First heat the glass rod near the middle until it becomes soft, then quickly remove the rod from the flame and draw it out by pulling apart the ends. (If you keep the rod in the flame while pulling it will generally break.) Aim to pull the glass down to a diameter below 0.25 mm. Break the glass at the point where it is around 0.25 mm, and briefly plunge the broken end into the flame. Repeat this until a blob of glass with a diameter of about 0.5 mm is formed on the end. This probe is

- useful for crushing crystals because it is easier to hit the crystals, and because it does not damage the plastic bottom of the well.
2. Several groups have reported that, for some proteins, only *fresh* crystals work. Crystals that have been in the lab for a few weeks may not work, even though the crystals still diffract. Make a seed stock as soon as possible after the crystals stop growing.
4. If required, the volume of seed stock added to each drop can be reduced to as little as 10 nl using any of the Oryx systems. This means that only about 1.5 μ l of seed stock is required for 96 wells. It is helpful to dispense a few microlitres of reservoir solution "on top of" the seed stock to prevent air bubbles from being sucked up.
5. Seed crystals of membrane proteins are particularly unstable. Since the reservoir of a membrane protein crystallization experiment does not normally contain detergent, seed crystals may dissolve if the normal procedure is followed. Also, fewer crystals of membrane proteins may be available to make the seed stock. We recommend that seed crystals of membrane crystals are crushed in their wells and harvested in the mother liquor (the solution in the drop, which includes protein) without any additions. The small quantity of seed stock used by Oryx is an important advantage here. Make a note of any crystals that can be seen in the drops immediately after dispensing them.
6. If you are attempting to crystallize a complex, you should avoid high salt conditions (see Radaev and Sun ref. below). Try suspending crystals in e.g. 40% PEG 3000 instead of the reservoir solution. To find out if the seed crystals will be stable in PEG, incubate uncrushed crystals in PEG for 24 hours. If the crystals do not crack, shatter or dissolve the PEG solution can almost certainly be used to prepare a seed stock.
7. Similarly, high salt conditions can be unhelpful for heavy atom derivatization and small molecule complexes, so you may also wish to suspend seeds in PEG in these cases. Moreover, this approach will reduce the number of salt crystals obtained.
8. Some groups recommend combining as many hits as possible to make the seed stock. For example, combine the drops from all the hits in high-PEG conditions to make a seed-stock, and the drops from all the hits in high-salt conditions to make another. (If you mix high-PEG and high-salt conditions you may get two phases and crystals are more likely to dissolve [see Shaw Stewart *et al.* ref. below].)
9. "Preseeding the protein stock" (suspending crushed crystals in the normal protein stock) can be beneficial but it is less effective than using a separate seed stock.
10. Seed stocks can be obtained by harvesting crystals from microfluidic devices and capillaries.
11. rMMS experiments can be dispensed by hand. Typically the volumes dispensed will be increased slightly to e.g. 1.5 μ l of protein, 1 μ l of reservoir solution, and 0.5 μ l of seed stock (dispensed in that order). The seed stock can easily be dispensed with a 10 to 100 μ l Hamilton gas-tight syringe with a blunt needle (e.g. Point Style 3). After dispensing the seed stock to a drop, clean the needle by passing it through the reservoir of the next well before dispensing the next drop.

For more information on points 4 - 10 request a preprint from Douglas Instruments for the reference by Shaw Stewart *et al.* below.

For References please visit

http://www.douglas.co.uk/MMS_proc.htm