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Fragment screening: how to produce apo-crystals with microseeding

Fragment screening requires a reliable and reproducible source of apo-crystals. The fragment library consists of 800 compounds in a single plate, which are transferred acoustically to the target plate containing apo-crystals. In an ideal 3-well SBS plate, there would be apo-crystals in all 288 drops. Typically though, there are only 10 - 20 drops that contain crystals.

This is because spontaneous nucleation is a stochastic event. There is no guarantee that it will happen, even in presumably identical drops. (See Newman et al., 2007, for her example with lysozyme. Even a robust crystallizer like lysozyme does not crystallize in every drop.)

To ensure a steady and reliable supply of apocrystals, use microseeding. Seeding bypasses the waiting time and unreliability of spontaneous nucleation. The seeds are the nuclei, so they jumpstart the process of crystal growth.

Preparing a supply of apo crystals with seeding

1. Identify a condition that has produced apo-crystals. Make a 10- to 50-ml solution of it. (You may need it for future plates and this is more reproducible than making a new solution each time.)
2. Fill the reservoirs of a 96-well SBS-plate with this solution. Set the plate aside and go to step 3.
3. Prepare the seed stock, as described below. Seed stock works best when freshly prepared (Shaw Stewart et al., 2011), but you may use frozen seed stocks for convenience or if fresh seeds are not available.
4. Put the plate from step 2 onto your robot deck. Have your robot program dispense your apo protein, mix it with the reservoir solution, and add the seeds. There is no need to pre-equilibrate the drops before seeding them as described in earlier literature.

How to prepare the seed stock (estimated time: 30 min)

1. Select the best quality crystals you can afford to use from your previous experiments. If you have a choice, use crystals grown from PEG rather than a salt, to avoid an increased risk of salt crystals. If there are no crystals are available, use any crystalline material, even precipitates. (Precipitates are often microcrystals.) Take as many crystals as possible to generate the seed stock, as a higher initial concentration of seeds ensures the seeding will work. You can optimize the number of seeds in a second round if there are initially too many.

2. Assuming the seed crystals are in a 200 nl sitting drop, begin by adding 5 µl of the reservoir solution to the drop containing them.

3. The crystals need to be crushed thoroughly. This can be done in the drop with a **crystal crusher**, or in an Eppendorf tube with a **seed bead**, or a combination of both. A **crystal crusher** is a rounded glass probe made from a glass pipette or capillary stretched during heating, **Fig. 1a**.

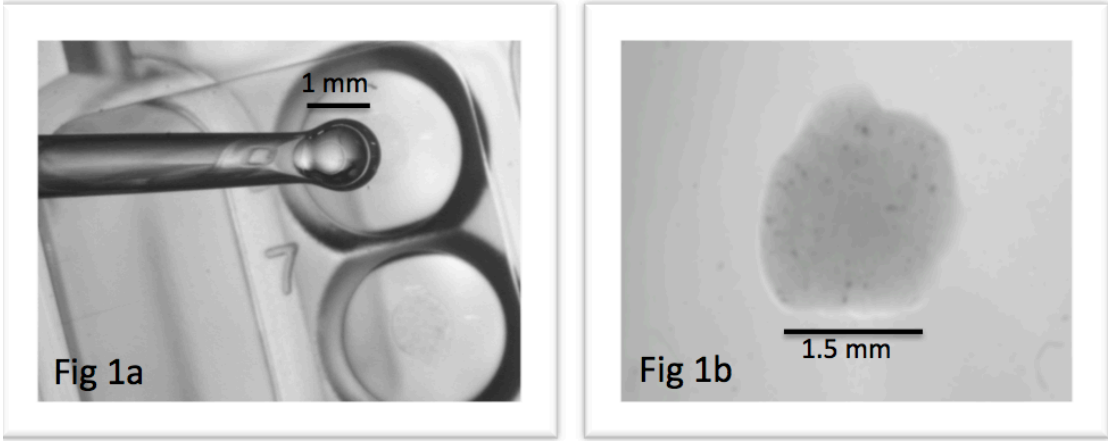

Figure 1a. Rounded glass probe for smashing crystals in the drop. The probes are easy to make yourself, but are also available on request from Douglas Instruments, UK or Hampton Research HR4-216. How to make one yourself is described here: https://www.douglas.co.uk/MMS_proc.htm

Fig 1b.The crystals are thoroughly smashed now. (Drop volume shown here is only 1 µl. I recommend you use about 5 µl). The drop may not always look like this concentrated; it depends on how many crystals it contains before you smash them. The point is that the crystals should be turned into a slurry or well-homogenized soup, however you decide to do that (glass probe, seed bead, or combination of both).

4. Recover the seed crystals (**Fig. 1b**) in your pipette tip and transfer them into an Eppendorf tube.

5. Add another 10µl reservoir solution to the drop and mix thoroughly to recover more seeds crystals, then add this amount into the Eppendorf tube.

6. Continue in this way until you have about 50µl in the seed bead tube. This ensures a high recovery of crushed seeds.

7. Add an additional 50µl of reservoir solution for a total volume of 100µl = this is your **seed stock**.

8. Vortex the **seed stock** for 1-2 minutes to further crush the seeds. Don't let it get heated up; place on ice intermittently during the vortexing. Vortexing with a **seed bead** in the tube ensures even and thorough crushing of the seeds, but you can also skip the beads, depending on how well you have already crushed the crystals. Beads are available in teflon (**HR2-320 Hampton Research**) or stainless steel (**HR4-780).**

9. Take 1 µl of the vortexed **seed stock** and dilute it 100x with the same reservoir solution = the **100x dilution.**

10. You now have two tubes of seeds: **the stock** and a **1:100 dilution**. Set up drops and seed them using your crystallization robot. A good ratio is: 100 nl protein + 100 nl reservoir solution + 30 nl seeds. Try both tubes of seeds if you have enough protein. If protein amount is limited, try with the more concentrated stock first. It's better to have too much nucleation than too little. You can optimize the amount of seeds later.

11. Store the concentrated and diluted seed stocks at -80°C.

12. Wait for crystals to appear. If there are too many crystals, repeat with the seed dilution that you stored at -80°C for this purpose. The 1:100 dilution might still give too many crystals. Try 1:1000 or 1:10,000 if necessary.

13. Now you have a plate of apo crystals ready for fragment screening.

Seeds prepared in this way are usually stable in the original reservoir solution and are a useful backup if you do not have access to freshly made seeds. The frozen seed stocks (both concentrated and diluted) can undergo a few cycles of freezing and thawing before they lose their ability to nucleate crystallization. An in-depth study on the stability of seed crystals has been made by Shaw Stewart et al. (2011).

He has also made a video that shows how make the seed stocks:
https://youtu.be/tXGmX81sc0A

References
This protocoll has been adapted from the original one in:
D'Arcy, Bergfors et al. 2014, Acta Cryst F70, 1117-1126.

References cited above:
Newman, J. et al. 2007, Acta Cryst D63, 826-832.
Shaw Stewart et al. 2011, Cryst.Growth Design 11, 3442-3441.