CRYS TAL LI ZA TION OF MEM BRANE PRO TEINS IN MICROBATCH


An in creas ing num ber of mem brane pro teins in a vari ety of dif fer ent de ter gents have been crys tal lised in microbatch un der oi l. Some of these had failed to crys tal lise by all meth ods other than microbatch. Dis pens ing is quick and sim ple even when per formed man u ally and the drops in oil do not spread out as they do in vapour di f fu sion over the si li con ised coverslips. Using robots thousands of microbatch trials can be dis pense d in high through put mode in nano litre vol umes.

The microbatch can be used for both screen ing and for op ti mi sa tion of mem brane pro teins. The pro to col for set ting up microbatch ex per i ments con tain ing mem brane pro teins is iden ti cal to that de scribed in ex er cise 2.

Ex er cise 3: Harves ting and moun ting crys tals from microbatch


Harves ting crys tals from microbatch is slightly more dif fi cult than harves ting from coverslips or from stan dard sit ting drops. How ever after some prac tice it can be achieved easily.

Two al ter na tive ways of harves ting crys tals from microbatch:

Ma teri als re quired
- Cryoprotectant so lu tion
- Precip i tate so lu tion at ~ 5 % higher con cen tra tion than that in the drops
- Micro tools (Hampton Re search)
- Stan dard pi pette
- Scalpel
- Loops
- Depres sion plates

Method
1. Add a few microlitres of cryo-protectant so lu tion to the drop con tain ing the crys tals.
2. Af ter several min utes check that the crys tals are not cracked or di ssolved by look ing at them under the mi cro scope. If they crack/di ssolve, adjust the con cen tra tion of cryo-protectant or change cryo- protectant.
3. Take the crys tals di rectly out of the oil using a loop and freeze.

If the above pro to col proves tricky, har ves t in the fol low ing way:
1. Add har ves t so lu tion (of ~5% higher con cen tra tion of precip i tant than that in the drop) into the well con tain ing the crys tals. If you have a 1 μl drop, add 5-10 μl of har ves t so lu tion.
2. Wait a short while (up to 15 min utes) to al low the crys tals to equi lib rate.
3. With draw the en larged drop using a stan dard 10-100 μl pi pette which had its tip cut off with a scalpel in or der to widen its bore.
4. If the crys tals stick to the ves sel, loosen them gently inside the drop using mi cro tools or very thin strips of filter paper (the edge of the strip that will touch the crys tal is best torn rather than ‘cleanly’ cut with scis sors).
5. Trans fer the drop into a de pres sion well con tain ing more har ves t so lu tion.
6. From this stage on wards, han de the crys tals as you would be from a stan dard di f fu sion trial.

Ex er cise 4: Con ta i ner less crys tal li zati on


Pur pose of this ex per i ment: (1) to aid har ves ting (2) to re duce the amount of nu cle ation in a trial by elim i nat ing the ef fects of sur face con tact be tween the crys tal li za tion trial and its sup port ing ves sel.

Ma teri als re quired
- “Gelled Sur face” kit from Mo lec u lar Di men sions, UK (figure c)
- Para ffin oil
- Lysozyme 40 mg/ml in Na ac e tate, pH 4.7, 12% NaCl from the pre vi ous ex per i ments

Pro ce dure
- Pi pette the para ffin oil into the wells of the Gelled-Sur face plate.
- Pre pare the pro tein/pre cip i tant drops as de scribed for microbatch ex per i ments.
- In sert the pi pette tip into the well, under the sur face of the para ffin oil, and dis pense the drop.
- Ex per i ment with dif fer ent size drops e.g. 1-20 μl.

You can also try dis pens ing a drop un der oil (figure a) and com pare with a drop dis pend ed be tween two oils of dif fer ent den si ties (Figure b).

Try the method using your prob lem pro teins that are giving you many small unus able crys tals.
Exercise 5: Filtration experiments


Purpose of this experiment: Filtration will remove particles (dust, protein aggregates, fungi, bacteria, etc) and thus reduce the amount of nucleation in the trials leading to the growth of fewer larger crystals.

Materials required:
- Centrifugal filters: 0.2 µm 0.1µm
- http://www.milipore.com/catalogue/item/ufc30vv25
- http://www.millipore.com/catalogue/item/ufc30vv00
- Bench centrifuge.
- 40 mg/ml lysozyme stock
- 12% NaCl

Procedure:
1. Mix 150 µl of lysozyme at 40 mg/ml with 150 µl precipitant (12% NaCl in 10 mM acetate buffer pH 4.7) in a microcentrifuge tube.
2. Divide the mixture into 3 aliquots of 100 µl each:
   (a) leave one aliquot unfiltered
   (b) filter the second aliquot through a 0.22 µm filter
   (c) filter the third aliquot through a 0.1 µm filter
   Filtration is performed by placing the filters in a bench centrifuge at 2-9 g for 1 – 2 minutes.
3. Dispense droplets of each aliquot for crystallization under the oil.

Expected results:

You will get a number of crystals in the unfiltered solution, a smaller number of larger crystals in the solution which was filtered with a 0.22µm filter, and very few or no crystals at all in the solution which was filtered through the 0.1 µm filter.

Warning:
- You need to filter a minimum of 40 µL in order not to lose any protein.
- Do not use the filtration method if your protein is sticky

Exercise 6: Improving crystal quality by separating nucleation and growth in hanging drops (not using oils!)


This is a method to get nucleation “going” and then “back off” before the nucleation becomes excessive. It can be used for optimisation when small useless crystals are formed, and improvement can not be obtained neither by fine-tuning the concentrations of the protein and precipitating agents nor by addition of additives. In practice, cover-slips holding the drops are incubated for some time over reservoir solutions that normally give many small crystals and after given times the cover-slips are transferred over reservoirs with lower precipitant concentrations that would normally yield clear drops. This method can also be used for screening.

Example for optimisation:

Materials needed:
Trypsin from porcine pancreas(Sigma catalogue no: T-0134)dissolved in de-ionised water at a concentration of 40 mg/ml.
1 M Tris pH 8.4
3M ammonium sulphate
Mineral oil (viscosity of ~5) to put round the edge of the Linbro plates for sealing the cover-slips (not the thick Vaseline usually applied as a seal). Standard Linbro plates or pre greased plates and siliconised cover-slips or EasyXtal Tools (From Qiagen). Pasteur pipette with rubber squeezer or plastic squeezer pipettes.

Procedure:
1) Set up 6 trials under conditions that would give you low quality crystals, in this example it is reservoir solutions containing 1.7M ammonium sulphate and 0.1M Tris pH 8.4. Dispense those into 6 wells of a plate.
2) Prepare 6 reservoirs with solutions containing precipitant concentration that would result in producing a clear drop if crystallization drops were set up and left to incubate under these conditions. In this example it is reservoir solutions containing 1.3M ammonium sulphate and 0.1M Tris pH 8.4. Dispense those into 6 wells of another row in the same plate.
3) Set up hanging drops by taking equal volumes (recommended 1 microlitre + 1 microlitre) of the 40mg/ml protein solution and mixing it with the well solutions containing the 1.7 M ammonium sulphate.

4) After 2 hours transfer one of the cover-slips from over the wells containing the 1.7M ammonium sulphate and just place it over one of the wells which contain 1.3M ammonium Sulphate (Figure). The transfer should only take 1-2 seconds.

5) Repeat the same with the other drops after 3, 4 and 6 hours.

6) Leave 2 drops at the high concentration (1.7M) as controls

7) set two drops at the low concentration (1.3M) as controls.

8) Observe the results each day for two weeks.

**Expected results:**

The drops which are left over the reservoirs containing 1.7 M ammonium sulphate will have clusters of crystals overnight. The drops hung over the 1.3M should be clear. The drops transferred after 4 or 6 hours should produce large single crystals after 8-14 days.

**Tip:** The time of transfer is selected by reference to the time in which it took to see the first crystals in the trials producing the poor crystals. In the case of trypsin, crystals appeared within 24 hours meaning that nucleation would have occurred anytime between set up of the experiments to several hours before the crystals appeared. Hence transfer was done at intervals of 2 hours after set up. Trials that are transferred too soon will produce clear drops while those that are transferred too late will yield low quality crystals. In cases of other proteins where crystals take a few days to appear, the transfers should be performed at longer time intervals e.g. every 12 hours or so.

**For screening:**

3D structure screen by (Molecular Dimensions, UK, MD1-13)

This screen consists of two sets of solutions: 24 contain sparse matrix screening conditions; the other 24 are a 70% dilution of the precipitants in those solutions (buffer and additive concentrations remain the same). Using this screen, the hanging drops are first incubated for 3-6h over the solutions at high concentrations. The cover-slips holding the drops are then transferred over the reservoirs at 70% dilution.

**Exercise 7: What to do when you get no crystals?**


This is a screening technique used in vapour diffusion hanging drops when trials remain persistently clear.

**Materials required:**

1. EasyXtal Tools Made by Qiagen
2. Screens of your choice
3. Your protein solution

1. Set up your screening as you would normally do
2. Loosen the screw caps of drops that remained clear after 2 weeks by 90° (see diagram). The angle of cap allows variable amounts of evaporation without exposing the drops
3. Observe the drops every 2 hours in the first day and re-seal the caps when the drops visibly shrink.

**Expected results:**

By loosening the cap you are allowing evaporation for a limited time. What you are effectively doing is inducing nucleation which you then arrest when re-sealing. This will drive the system into supersaturation and hopefully give you a hit which you can then proceed to optimise using the technique of your choice.
The method can also be used for optimization as described in Govada, L. and Chayen, N.E. (2009) Crystal Growth & Design 9, 1729-1732.

INTRODUCING NUCLEANTS INTO CRYSTALIZATION TRIALS


First, determine the metastable zone as follows:

1. Note the conditions in a ‘hit’ of a screen that have yielded poor crystals or a crystalline precipitate.
2. Dispense (manually or by a robot) 10-24 trials using a crystallization method of your choice, varying the protein and precipitant concentrations in steps (as instructed in point 3 below) on a matrix grid.
3. The concentrations of protein and precipitating agents should be above and below the concentrations that gave the conditions of the hit. Alternatively the pH, temperature, or another parameter to which solubility is sensitive can be varied.
4. Plot the results and you will obtain the supersolubility curve. e.g. diagram below. For example, if a screen produced ‘hits’ at conditions of 10-22 mg/ml of protein and 1-1.5 M Ammonium sulphate, the experiments are set at concentrations ranging from 5-25 mg/ml protein versus 0.5-2.2 M ammonium sulphate, thus covering a range of conditions above and below the ‘hits’. The area just below the curve is the metastable zone. X represent conditions obtained from screening ‘hits’, giving low quality crystals or crystalline precipitate. △ represent clear drops; ▲ represent precipitate.

Insert your nucleant at conditions just below the curve (where the heads of the arrows are pointing) using fine tweezers and let the trial incubate as you would normally do.

Nucleant are used mostly for optimization but can also be used for screening. When nucleants are placed in supersaturated conditions, crystals will usually appear faster.

References on crystallization methods in microbatch and vapour diffusion (and some in microgravity)


**Books**

*Protein Crystallisation* Bergfors, T.M. ed. 2009 La Jolla, International University Line, USA.


Methods and Results in crystallization of Membrane Proteins. 2003 Iwata, S. ed La Jolla, International University Line, USA.


*Protein Crystallization Strategies for Structural Genomics*. 2007 Chayen, N. E. ed La Jolla, International University Line, USA.