There are several techniques for setting up crystallization experiments (often termed 'trials') including sitting drop vapor diffusion, hanging drop vapor diffusion, sandwich drop, batch, microbatch, under oil, microdialysis, and free interface diffusion. Here we offer an overview of these crystallization techniques.

Sitting & Hanging Drop Crystallization
Sitting and hanging drop methodologies are very popular because they are easy to perform, require a small amount of sample, and allow only a large amount of flexibility during screening and optimization.

Using the sitting drop technique (figure 1) one places a small (1 to 40 microliters) droplet of the sample mixed with crystallization reagent on a platform in vapor equilibration with the reagent. The initial reagent concentration in the droplet is less than that in the reservoir. Over time the reservoir will pull water from the droplet in a vapor phase such that an equilibrium will exist between the drop and the reservoir. During this equilibration process the sample is also concentrated, increasing the relative supersaturation of the sample in the drop.

The advantages of the sitting drop technique include speed and simplicity. The disadvantages are that crystals can sometimes adhere to the sitting drop surface making removal difficult. This disadvantage can turn into an advantage where occasionally the surface of the sitting drop can assist in nucleation. The sitting drop is an excellent method for screening and optimization. During production, if sticking is a problem, sitting drops can be performed in the sandwich box set up.

Sitting drop crystallization may be performed using Micro-Bridges® or Glass Sitting Drop Rods™ with VDX or Linbro plates. Both plates can be sealed with clear sealing tape or plain cover slides for easy viewing and access. Sitting drop crystallization may also be performed using the Cryschem Plate™. The Cryschem Plate is a specially designed plate with a post already in the center of the reservoir.

Using the hanging drop technique (figure 2) one places a small (1 to 40 microliters) droplet of the sample mixed with crystallization reagent on a siliconized glass cover slide inverted over the reservoir in vapor equilibration with the reagent. Over time the reservoir will pull water from the droplet in a vapor phase such that an equilibrium will exist between the drop and the reservoir. During this equilibration process the sample is also concentrated, increasing the relative supersaturation of the sample in the drop.

The advantages of the hanging drop technique include the ability to view the drop through glass without the optical interference from plastic, flexibility, reduced chance of crystals sticking to the hardware, and easy access to the drop. The disadvantage is that a little extra time is required for set ups.

Sandwich Drop Crystallization
The Q Plate is specially designed to allow for hanging drop, sitting drop, and sandwich drop vapor diffusion. Here we will address only the sandwich drop feature (figure 3). For sandwich drop the sample solution mixed with the precipitant is placed in the middle of a lower 18 mm siliconized glass cover slide followed by one setting a larger 22 mm siliconized glass cover slide in position along an upper edge which allows for a small amount of space between the cover slides but is close enough such that the drop is sandwiched between the glass cover slides. The advantages to the techniques are an excellent optical pathway for microscopic examination and an alternate equilibration method. The disadvantages include tedious set up and the plates’ large footprint.

Free Interface Diffusion
Free interface diffusion crystallization is less frequently used than sitting or hanging drop vapor diffusion but it is one of the methods used by NASA in microgravity crystallization experiments. Using this method one actually places the sample in liquid contact with the precipitant. When doing so one attempts to create a clearly defined interface between the sample and the precipitant. Over time the sample and precipitant diffuse into one another and crystallization may occur at the interface, or on the side of high sample/low precipitant or low sample/high precipitant. The technique allows one to screen a gradient of sample precipitant concentration combinations. The technique can readily be performed in small capillaries (figure 4).

Batch
Batch crystallization is a method where the sample is mixed with the precipitant and appropriate additives creating a homogeneous crystallization medium requiring no equilibration with a reservoir. The technique is popular with small molecule crystallographers. The advantages to the technique are speed and simplicity but the disadvantage is that only a narrow space of precipitant/sample concentration can be
sampled in a single experiment. A batch experiment can be readily performed in a capillary, small container, or plate with a small reservoir such as the Macro-Store Plate™ (HR3-116). One must be very close to the conditions which promote crystal growth in order for this technique to be successful.

MicroBatch Under Oil
In this technique a small drop of the sample combined with the crystallization reagent is pipetted under a layer of oil. For a true MicroBatch, the drop is placed under Paraffin Oil (figure 5) which allows little to no evaporation nor concentration in the drop. A Modified MicroBatch can be performed when the drop is placed under a mixture of Paraffin Oil and Silicon Oil, or straight Silicon Oil (figure 6). Such oils allow water vapor to permeate from the drop and allow sample and reagent concentration. Unless the drop is equilibrated with a reservoir, water will leave the drop until that only solids remain.

The benefits of Crystallization Under Oil include the use of very small sample and reagent volumes with less concern for unwanted evaporation, the minimization of surface interaction with the sample, the ability to precisely control sample and reagent concentrations during the experiment, and the minimization of condensation during temperature fluctuations.

Microdialysis Crystallization
Dialysis crystallization involves placing the sample in a Dialysis Button which is sealed with a dialysis membrane. Water and some precipitants are then allowed to exchange while retaining the sample in the cell. The Dialysis Button is placed into a suitable container holding the precipitant or crystallization media (figure 7). For example, one might dialyze a sample requiring a high ionic strength for solubility against a solution of low ionic strength. The technique allows for salting in and salting out, as well as pH crystallization techniques.

Technical Support
Inquiries regarding the crystal growth techniques, interpretation of screen results, optimization strategies and general inquiries regarding crystallization are welcome. Please e-mail, fax, or telephone your request to Hampton Research. Fax and e-mail Technical Support are available 24 hours a day. Telephone technical support is available 8:00 a.m. to 5:00 p.m. USA Pacific Standard Time.
The hanging drop vapor diffusion technique is the most popular method for the crystallization of macromolecules. The principle of vapor diffusion is straightforward. A drop composed of a mixture of sample and reagent is placed in vapor equilibration with a liquid reservoir of reagent. Typically the drop contains a lower reagent concentration than the reservoir. To achieve equilibrium, water vapor leaves the drop and eventually ends up in the reservoir. As water leaves the drop, the sample undergoes an increase in relative supersaturation. Both the sample and reagent increase in concentration as water leaves the drop for the reservoir. Equilibration is reached when the reagent concentration in the drop is approximately the same as that in the reservoir.

Benefits of Hanging Drop Crystallization
- Can be cost effective.
- Sample and reagents in contact with a siliconized glass surface.
- Relatively easy access to crystals.
- Can perform multiple drops (experiments) with a single reservoir.

Using the VDX Plate
The VDX Plate is a 24 well plate manufactured from clear polystyrene. The VDX Plate is typically sealed with High Vacuum Grease (HR3-506) and Siliconized 22 mm Circle or Square Glass Cover Slides. The VDX Plate is also available pregreased. Rows of the plate are labeled A-D and columns are labeled 1-6 on the VDX Plate.

1. Apply a bead of High Vacuum Grease along the top edge of the raised reservoir A1 of the VDX Plate. It is recommended that one apply the high vacuum grease prior to pipetting the reagent. High vacuum grease may be applied by using the Grease Kit (HR3-506). Create a circular bead on the upper edge of the reservoir. Do not complete the circle. Leave a 2 mm opening between the start and finish of the circular bead. Apply the cover slide, press to relieve the air pressure and twist to close the gap. One may also use the VDX Plate Greased. These plates come pregressed.

2. Pipet 10 milliliter of crystallization reagent into reservoir A1 of the VDX Plate. (Note: Recommended reservoir volume is 0.5 to 1.0 milliliters)

3. Clean a Siliconized 22 mm Circle or Square Cover Slide by wiping the cover slide with lens paper and blowing the cover slide with clean, dry compressed air. Pipet 1 microliters of sample into the center of a Siliconized 22 mm Circle Cover Slide. (Note: Recommended total drop volume is 1 to 40 microliters)

4. Pipet 1 microliter of reagent from reservoir A1 into the drop on the cover slide containing the sample. (Note: Some people prefer to mix the drop while others do not. Proponents of mixing leave the pipet tip in the drop while gently aspirating and dispensing the drop with the pipet. Mixing ensures a homogeneous drop and consistency drop to drop. Proponents of not mixing the drop simply pipet the reagent into the sample with no further mixing).  

5. Holding the cover slide with forceps, the Pen Vac, or on the edge between your thumb and forefinger, carefully yet with out delay invert the cover slide so the drop is hanging from the cover slide.

6. Position the cover slide onto the bead of grease on reservoir A1. Gently press the slide down onto the grease and twist the slide 45° to ensure a complete seal.

7. Repeat for reservoir 2 through 24.

VDX Plate Tips
- Note the VDX Plate has a raised cover to protect the cover slides during transport and storage.
- To access a drop and/or reservoir simply grasp the edge of the cover slide with forceps or fingertips, twist and pull gently.
- VDX Plates can be stacked for convenient storage.
- One can pipet multiple drops onto the cover slide. This technique is often useful when screening additives since one can use the same reservoir with multiple drops with each drop containing a different additive. This technique can also be used to screen different drop sizes and ratios versus the same reservoir. Use care not to avoid mixing the drops during pipetting, plate transport, and plate viewing.

Using the Q Plate
1. Pipet 10 milliliter of crystallization reagent into reservoir A1 of the Q Plate. (Note: Recommended reservoir volume is 0.5 to 10 milliliters)

2. Clean a Siliconized 18 or 22 mm Circle Cover Slide by wiping the cover slide with lens paper and blowing the cover slide with clean, dry compressed air. Pipet 2 microliters of sample into the center of a Siliconized 18 or 22 mm Circle Cover Slide. (Note: Recommended total drop volume is 1 to 40 microliters)

3. Pipet 2 microliters of reagent from reservoir A1 into the drop on the Cover Slide containing the sample. (Note: Some people prefer to mix the drop while others do not. Proponents of mixing leave the pipet tip in the drop while gently aspirating and dispensing the drop with the pipet. Mixing ensures a homogeneous drop and consistency drop to drop. Proponents of not mixing the drop simply pipet the reagent into the sample with no further mixing).

4. Holding the cover slide with forceps, Pen Vac, or on the edge between your thumb and forefinger, carefully yet with out delay invert the cover slide so the drop is hanging from the cover slide.

5. Place the siliconized cover slide with the drop onto the step inside reservoir A1 of the Q Plate. (Note: An 18 mm cover slide rests on the lower step while a 22 mm cover slide rests on the upper step. Since the 18 mm cover slide is closer to the reservoir, vapor equilibration occurs at a higher rate than when using 22 mm cover slides).

6. Repeat steps 1 through 5 for the remaining 23 reservoirs.

7. Seal the Q Plate plate with 3 strips of Clear Sealing Tape (HR4-510).

Using the Q Plate II
1. Pipet 10 milliliter of crystallization reagent into reservoir A1 of the Q Plate II. (Note: Recommended reservoir volume is 0.5 to 10 milliliters)
2. Clean a Siliconized 18 mm Circle Cover Slide by wiping the cover slide with lens paper and blowing the cover slide with clean, dry compressed air. Pipet 2 microliters of sample into the center of a Siliconized 18 mm Circle Cover Slide. (Note: Recommended total drop volume is 1 to 40 microliters)

3. Pipet 2 microliters of reagent from reservoir A1 into the drop on the Cover Slide containing the sample. (Note: Some people prefer to mix the drop while others do not. Proponents of mixing leave the pipet tip in the drop while gently aspirating and dispensing the drop with the pipet. Mixing ensures a homogeneous drop and consistency drop to drop. Proponents of not mixing the drop simply pipet the reagent into the sample with no further mixing).

4. Holding the cover slide with forceps, Pen Vac, or on the edge between your thumb and forefinger, carefully yet with out delay invert the cover slide so the drop is hanging from the cover slide.

5. Place the siliconized cover slide with the drop onto the ledge inside reservoir A1 of the Q Plate II.

6. Repeat steps 1 through 5 for the remaining 23 reservoirs.

7. Seal the Q Plate II plate with 2 strips of Clear Sealing Tape (HR4-510).

Q Plate & Q Plate II Tips
• Use Crystal Clear Sealing Tape (HR4-510). Other Brands are optically inferior and the adhesive will turn opaque with certain crystallization reagents.
• To access a drop and/or reservoir of a Q Plate or Q Plate II sealed with tape simply make a circular incision in the tape using the inside of the reservoir as a guide. Use a sharp blade to cut the tape and hold the incised piece of tape with forceps. The opening can be sealed with another strip of tape.
• One can pipet multiple drops onto the cover slide. This technique is often useful when screening additives since one can use the same reservoir with multiple drops with each drop containing a different additive. This technique can also be used to screen different drop sizes and ratios versus the same reservoir. Use care not to avoid mixing the drops during pipetting, plate transport, and plate viewing.
• The Q Plate can also be used for hanging drop and sandwich drop vapor diffusion experiments. The Q Plate II can also be used for hanging drop vapor diffusion experiments.
• Use care when transporting and viewing Q Plate and Q Plate II’s. A bump to the plate can toss the cover slide out of position or onto the tape. In very dry, high static environments one may prefer to treat the plates and slides with a static removing device to prevent the glass slides from “jumping” onto the tape.

Other Plates
The Linbro® Plate and the Costar™ 3424 Plate are also used for hanging drop vapor diffusion crystallization.

Technical Support
Inquiries regarding the hanging drop crystallization method, interpretation of screen results, optimization strategies and general inquiries regarding crystallization are welcome. Please e-mail, fax, or telephone your request to Hampton Research. Fax and e-mail Technical Support are available 24 hours a day. Telephone technical support is available 8:00 a.m. to 5:00 p.m. USA Pacific Standard Time.

Hampton Research
27632 El Lazo Road, Suite 100
Laguna Niguel, CA 92677-3913 U.S.A.
Tel: (949) 425-1321 • Fax: (949) 425-1611
Technical Support e-mail: tech@hrmail.com
Internet: www.hamptonresearch.com

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Experiments
Viewing Crystallization
Crystallization experiment.

Figure 4
Typical observations in a crystallization experiment.

Clear Drop

Skin/Precipitate

Precipitate

Precipitate/Phase

Quasi Crystals

Diffractable Crystals

Microcrystals

Needle Cluster

Plates

Rod Cluster

Single Crystal

Observing the Experiment
Gently set the plate onto the observation platform. If the platform is smooth and free of protrusions one may simply slide the plate in the X and Y directions on top of the viewing platform to view each of the drops. Use low magnification to view and center the drop in the field of view. Scan the drops at 20 to 40x magnification. When something suspicious appears increase the magnification to 80 or 100x for a better view. Scan the entire depth of the drop using the fine focus control of the microscope. Sometimes crystals will form at different depths of the drop because different areas of the drop can equilibrate at different rates. Also, crystals sometime forms at the top of a drop and as the crystal gains mass it will fall to a lower portion of the drop. Scrutinize everything until you are familiar with the differences between crystals, microcrystals, precipitate, and sweater fuzz. True crystals will feature edges. Precipitate does not have edges. Crystals can appear as needles, blades, walnuts, spherulites, plates, and various geometric shapes. Crystals vary in size anywhere from a barely observable 20 microns to 1 or more millimeters but most seem to fall in the range of 0.05 to 0.5 mm. Figure 1 shows typical examples of what one might observe in a crystallization experiment.

Differentiating Between Microcrystals & Precipitate
Microcrystals (less than 0.02 mm) can be difficult to differentiate from precipitate, especially under low power or with a low to medium quality microscope. Differentiate microcrystals from amorphous precipitate by looking for birefringence (light colored shiny spots under a polarizer in dark field mode = crystals). Other tests to differentiate crystals from precipitate include streak seeding, or the use of a small amount (1 microliter) of colored, low molecular weight water soluble dye (crystal violet and methylene blue will often penetrate the solvent channels of macromolecular crystals and color them, where as precipitate will not be colored).

Precipitate can appear as clumps, fine wispy clouds, or anything in between and can range in color from white to yellow, brown or rust. During screening and very preliminary optimization one may wish to observe the drops immediately after set up, or rust. During screening and very preliminary optimization one may wish to observe the drops immediately after set up, or one day later and each day thereafter for the first week. Observations may be performed once a week thereafter until the drops turn into a crust of deceased sample and reagent. Never throw plates away until the drop is dead. Why? Most crystallization plates are made from polystyrene which allows for some evaporation over time. Evaporation leads to increased relative supersaturation and maybe crystals over time. Time also can lead to changes in the protein (denaturation of less stable forms, proteolytic cleavage, and other changes) which might promote crystallization. Take careful notes during observations and be especially conscious of changes that occur between observations. Most crystallization observations are done at room temperature since this is where one will find most microscopes and it is most comfortable.

Cold Experiments
4°C experiments may be observed in a cold room by moving the microscope into the cold room. Allow time for the microscope to equilibrate to 4°C to prevent fogging of the optics as well as unnecessary temperature transfer from the warm microscope to the cold experiment. Wear a warm jacket with gloves to stay as comfortable as possible in the cold room. Excessive moisture in a cold room can be very destructive to a microscope so check with your maintenance group to keep the cold room as dry as possible. If a cold room is unavailable one is forced simply to work fast, moving plates from an incubator to the microscope carefully and making rapid yet thorough observations. Move only one plate at a time and gently close the incubator door between transfers since slamming the door will cause vibrations which can influence crystallization. Cold experiments tend to fog up rapidly especially if the light source is hot (if no infrared filters or light pipes are used). This is difficult to avoid and is one reason researchers prefer working in cold rooms.

Technical Support
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Hampton Research
27632 El Lazo Road, Suite 100
Laguna Niguel, CA 92677-3913 U.S.A.
Tel: (949) 425-1321 • Fax: (949) 425-1611
Technical Support e-mail: tech@hrmail.com
Internet: www.hamptonresearch.com

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