Reagent Formulation and Handling

HAMPTON RESEARCH
Solutions for Crystal Growth

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Good crystallization results depend on being able to replicate experiments, and experiments cannot be replicated unless the reagents are made correctly and consistently. Be consistent with sources, methods, and techniques. Maintain accurate and detailed records; your future self and perhaps others, will thank you later.

Whether formulating reagents for screening, for optimization, or in reproducing a hit from a commercial screen or kit, one should use high purity deionized water and quality chemicals, along with good laboratory technique when handling, weighing, formulating, titrating, filtering, and filling reagents into containers. Quality, accuracy, and precision, together with consistent protocols and techniques will help avoid late night (early morning) head scratching associated with the inability to generate the desired results in screening, optimization, and reproducing an experiment.

Water

Water is the most commonly used solvent in crystallization experiment and can constitute up to 99% of the mass of a crystallization reagent. The quality of water used in the crystallization lab is therefore critical for a successful crystallization experiment.

Natural (tap) water contains organic and inorganic ions, particulate and colloids, microbes and their byproducts, all of which need to be removed from water for crystallization experiments. Hence the need for Type 1 ultrapure water from a quality lab water purification system.

Table 1
Different water types and specifications

Contaminant	Parameter and Unit	Type 3	Type 2	Type 1
Ions	Resistivity (MΩ•cm @ 25°C)	> 0.05	> 1.0	> 18.0
Organics	TOC (ppb)	< 200	< 50	< 10
Pyrogens	(EU/mL)	N/A	N/A	< 0.03
Particulates	Particulates > 0.2 µm (units/mL)	N/A	N/A	< 1
Colloids	Silica (ppb)	< 1000	< 10	< 10
Bacteria	Bacteria (cfu/mL)	< 1000	< 100	< 1

All Hampton Research reagents are formulated using Type 1+ ultrapure water: 18.2 megaohm-cm resistivity at 25°C, < 5 ppb Total Organic Carbon, bacteria free (< 1 Bacteria (CFU/ml)), pyrogen free (< 0.03 Endotoxin (EU/ml)), RNase-free (< 0.01 ng/mL) and DNase-free (< 4 pg/µL). The exceptional grade of water not only requires a superb lab water purification system, but also requires the user to properly maintain the system, as well as handle the purified water properly. Depending upon the water system, regular maintenance can involve the scheduled and timely replacement of cartridges, UV lamps, and filters.

Be sure to follow the lab water purification system manufacturer's recommended maintenance schedule. Not replacing a cartridge or filter on schedule can affect the quality of the water which in turn may affect the outcome or reproducibility of a crystallization experiment. If maintenance is ignored for extended periods, the pH of the water can change significantly. As well, microbes can develop in poorly maintained systems, and these microbes can secrete proteases that can proteolytically modify or destroy the crystallization sample in a crystallization experiment.

Different water systems and different methods of water purification produce different specs of water. For example, glass distilled water typically has a pH of 5.5 while reverse osmosis and deionization systems typically produce water with a pH of 7.

Use water freshly dispensed from the lab water purification system. Purified water has extremely high purity, and exposure to a metal or glass container can dissolve significant (relative to its purity) amounts of metal and degrade water quality. Deionized water can leach zinc, lead, copper, iron, aluminum and other substances from glass or plastic storage containers. Purified water stored in plastic containers for several weeks has nearly the same level of total organic carbon (TOC) as tap water. Do not try to revive water with an autoclave. An autoclave can leave a wide variety of contaminants; the water may be sterile, but not pure.

Chemical & Buffer Selection

Like water, the quality and consistency of chemicals, including buffers, can be a significant variable in the crystallization experiment. Select and purchase chemicals of at least ACS reagent grade, and if possible, analytical grade, which is generally the purest and well characterized. Suppliers sometimes use trade names to denote grade, so in these instances one should look to the definition of the grade, application, and certificate of analysis to best understand the quality of the chemical or buffer. A lot specific Certificate of Analysis is available for Hampton Research Optimize™ reagents (Polymers, Organics, Salts, Buffers, etc). As well, descriptions, specifications, purity, assay, and other information when appropriate and available is presented for each reagent on the Hampton Research web site (hamptonresearch.com).

Labware for Reagent Formulation

A detailed guide to materials, equipment, and technique for the general laboratory is beyond the scope of CG101. It is well worth spending time becoming familiar with, practicing, and mastering good laboratory practices as this can have a direct correlation to the level of accuracy, precision, and data quality in the crystallization experiment.[1,2]

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Accuracy is the degree to which the result of a measurement conforms to the correct value or standard. Precision is the degree to which repeated measurements reproduce the same result. In other words, accuracy is shooting a single bullseye, where precision is repeatedly shooting a bullseye.

To help achieve and ensure both accuracy and precision when formulating stock solutions of chemicals and buffers, one uses quantitative methods and labware with the highest tolerance. The accurate measurement of liquid volume can be difficult, and there are a number of different types of containers for making volume measurements. Tubes, Erlenmeyer flasks, beakers, graduated cylinders, and volumetric flasks each have a specific purpose and associated level of accuracy and precision. The volume contained and delivered by any container is never the exact volume indicated by the markings; the volume is guaranteed to be within some prescribed tolerance. Disposable centrifuge tubes are a popular container for storing reagents but offer poor accuracy and precision in determining volume and should only be used when absolute uncertainty is the formulation goal. Erlenmeyer flasks and beakers are accurate to 5 - 10%, graduated cylinder to 1 - 5%, Class B volumetric flask about 0.06 - 0.08%, and Class A volumetric flask about 0.01 – 0.03%. Class A or other specialized volumetric flasks are the most accurate and precise container for quantitative volumetric measurement. For higher accuracy and precision one could turn to quantitative gravimetric measurement.

Liquid Handling

On a smaller scale, perhaps 10 milliliters or less, liquids, as well as chemical and buffer stocks can also be measured and formulated using manual and electronic pipettes, as well as liquid handling automation (robots). Such liquid handling is associated with reproducing hits, and formulating custom, or optimization screen experiments with reagent well (reservoir) volumes around 50 - 1,000 µl. Quality pipettes and robots with matching tips, as well as regular (annual) calibration, maintenance, and proper use, can help to ensure experimental accuracy and precision. There is more to pipetting than jamming on generic tips, turning dials, and pushing plungers or buttons. Find, read, follow, and keep nearby the user guide for your pipettes. Meanwhile, here are some pipetting basics.

For adjustable manual pipettes with a volume lock control, unlock to adjust the volume, and then lock, to aspirate and dispense. To eliminate errors due to mechanical backlash: when setting the desired volume, first turn the knob 1⁄3 turn above the desired volume. Then turn the knob slowly clockwise until the desired volume is displayed. Always dial down to the desired volume.

Pipettes and tips are often designed together as a pipetting system and can deliver better results than generic misfit options.

Don't dip too deep into the reagent or sample. For example, a 2 - 10 µl pipette tip should have an immersion depth of 1 - 2 mm, a 200 - 2,000 µl tip, 3 - 6 mm.

Pipette vertically, or within 20° of vertical. Don't invert or lay the pipette flat with liquid in the tip.

Use a new, sterile tip for each unique reagent or buffer. When aspirating, first press the plunger to the first stop and hold it. Hold the pipette vertical or within 20 degrees of vertical, place the tip into the solution to the proper depth, relax your thumb and allow the plunger to slowly rise. Do not let go of the plunger or release the plunger too quickly, or this will result in inaccurate measurement. Pause in the solution, especially viscous solutions such as Polyethylene glycols and Glycerol, to ensure the full volume of solution is drawn into the tip. Withdraw the tip slowly from the solution, allowing the surface tension of the liquid in the container to wick away solution from the outside of the tip. When dispensing, touch the tip to the sidewall of the container, depress the plunger slowly to the first stop, wait 1-3 seconds for the liquid to move down the inside of the tip, then depress to the second stop (blowout) and hold for 1-3 seconds to ensure a complete dispense. Still holding the plunger, withdraw the tip while sliding along the side of the container to remove any stubborn solution from the tip. Gently release the plunger, discard the tip, and repeat the cycle as needed, with a fresh tip (to prevent carry-over).

Gravimetric Measurement

The gravimetric measurements for Hampton Research reagents are always performed to an exact mass, each and every time, since 1991, and never a mass range. This is one small step in of many, that helps ensure reagent accuracy, precision, and consistency.

Analytical balances measure masses to a high degree of precision. Depending upon the type and quality of the balance, readability is up to 0.01 mg (0.00001 gram). Follow the manufacturer's user guide for proper use and care of the balance.

The balance should be located in a suitable area, free of traffic, temperature, humidity, and pressure changes. Be sure the balance is properly calibrated and tared. Handle each sample appropriately. Weigh hygroscopic samples quickly. Place the container and sample in the center of the balance, not off to the side. Do not lean on the bench while weighing. Check the level indicator bubble on the balance to confirm the balance is properly leveled before weighing. Do not handle tare containers with bare hands since fingerprints add mass as well as contaminants. One can use an anti-static device (Zerostat) to remove static electricity, dust, and lint from film, glass, and plasticware. Use a brush to clean spills in the weighing area. Keep the balance and work area clean and spotless. This is particularly important in order to avoid contamination of reagents.

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pH Measurement

Temperature and pH

The pH of all Hampton Research reagents and buffers is measured at 25.0 degrees Celsius. The temperature of the solution should always be considered when measuring pH. The most common cause of error in pH measurement is temperature, because temperature variations can affect the electrode slope, reference element drift, temperature sensor errors, and the calibration buffer and sample.

Different buffers and chemicals have different temperature dependence, and Tris is a notorious example, pH measuring 7.5 at 0 degrees Celsius, 7.0 at 25 degrees Celsius, and 6.1 at 100 degrees Celsius. For this reason, it is a necessity to use a pH probe and meter that can measure temperature during pH measurement. Some pH probes and meters offer temperature compensation, but this is an approximation and best practice is to maintain and measure pH at the exact, desired temperature, typically 25.0 degrees Celsius. One can use an ice bath and warming plate to achieve and maintain a constant temperature during pH measurement. During the formulation and titration of some reagents, the temperature can decrease or increase significantly, at which point one must wait for the temperature to return to and stabilize at the desired temperature before final titration or pH measurement.

pH Probe Care

It is important to properly handle, maintain, clean, and calibrate the pH probe to ensure optimal performance, accuracy, and precision. This, in turn, will help ensure the crystallization experiments will be reproducible. Handle the probe with great care. Regularly inspect the probe for cracks, salt crystal buildup, and membrane/junction deposits. Ensure the probe has the appropriate volume of fill solution, which will need to be replenished from time to time per the manufacturer's user guide. When not in use, the probe is to be stored in electrode storage solution, not water, nor calibration buffer. Use fresh buffers for calibration and choose calibration buffers that are 1 to 4 pH units apart. Use a two-point calibration for precise measurements, bracketing the pH to be measured. For example, use a pH 4 and 7 standard to calibrate the probe to measure pH 6. When one needs to measure pH 8.5, the probe will need to be recalibrated using pH 7 and 10 standards. Maintain a uniform and reasonable stir rate during pH measurement. No whirlpools. Between pH measurements, rinse the electrode with deionized water. Gently touch the probe with a lint free wipe to remove excess water. Avoid wiping and rubbing the probe as this can produce errors due to polarization. Clean the probe following the manufacturer's guidelines. Common junction cleaning can typically be accomplished using 0.1 M HCl with moderate stirring for 30 minutes. Remove and replace the probe fill solution after cleaning. Check the millivolt (mV) reading of the probe to ensure the measurement is within specification. If slow response or drifting continue after cleaning and refill, replace the probe. Even with proper care and cleaning, typical probe life is 1 - 3 years.

Concentration Units & Definitions

Molarity

Molarity is the number of moles of solute per liter and represented as M, such as 3.0 M Ammonium sulfate. Molarity is the ratio between the moles of dissolved solute (solid stuff) and the volume of solution (liquid stuff) in liters. The accepted volume of the solution is 1 L, so a 1 M (molar) solution would be 1 M = 1 mole of solute/1 L solution. Molarity is a way of determining the concentration of a solution. Dilute solutions are typically expressed in terms of millimolarity (mM) where 1 mM = 0.001 M. Typically, in crystallization we are asked to make something like a 3.5 M solution of Ammonium sulfate. To do this we need to know the molecular weight (M_r) of Ammonium sulfate (132.14 g/mole), the volume of solution to make (let's make 500 ml or 0.5 L), and the desired concentration (3.5 M). Then we calculate:

grams required = (desired Molarity)(formulation volume in liters)(M_r)
grams required = (3.5 mole/liter)(0.5 liter)(132.14 g/mole) = 231.25 g

To formulate the 3.5 M Ammonium sulfate we then weigh 231.25 g of Ammonium sulfate and add deionized water to dissolve the Ammonium sulfate and then adjust the final volume to 0.5 liter (500 ml). Do not simply add 500 ml of water to 231.25 g of Ammonium sulfate. Ideally one should use the most precise measuring instrument possible such as a class A volumetric flask. A less desirable instrument would be a graduated cylinder and the least desirable would be a beaker. Molarity is typically used as a concentration unit for salts, 1,6-Hexanediol, detergents, and some additives.

% w/v

% w/v (percent weight/volume) is often used when formulating high molecular weight Polyethylene glycols (PEGs) which are typically solids as well as some additives in solid form. % w/v is the weight of a solute in a given volume. % w/v = gram per 100 ml. For example, let's make 1,000 ml of a 50% w/v PEG 4,000.

grams required = (desired concentration in g/100 ml)(formulation volume in milliliters)
#grams required = (50 g/100 ml)(1,000 ml) = 500 grams

50% w/v PEG 4,000 is 500 g of PEG 4,000 in a final volume of 1,000 ml and not 500 g of PEG 4,000 plus 500 g of water. To make 1 liter of a 50% w/v solution of PEG 4,000, weigh 500 grams of PEG 4,000 into a volumetric flask and bring the final volume to 1 liter with water. Do not make the mistake of adding 500 grams of PEG 4,000 to 500 ml of water and believing you have made a 50% w/v solution.

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% v/v

% v/v (percent volume/volume) is often used when formulating liquid, low molecular weight polymers (PEG 400), organics (MPD), and organic solvents (2-Propanol) into stock solutions. % v/v is the ratio of the volume of solute in 100 ml of solution. For example, a 100 ml solution of 50% v/v 2-Propanol contains 50 ml of the solute (100% 2-Propanol). Formulating reagents using % v/v can be done more precisely and accurately using the mass of the solute being formulated instead of the volume of the solute. To do this we need to know the density of the solute. For example, let's formulate 500 ml of 30% v/v (±)-2-Methyl-2,4-pentanediol (MPD). The density of MPD is 0.925 g/ml at 25° Celsius.

#grams required = (desired % v/v) (desired formulation volume in ml) (density of solute in g/ml)
#grams required = (30 ml of solute/100 ml of solution) (500 ml) (0.925 g/ml) = 138.75 grams

30% of 500 ml is (0.3)(500) = 150 ml. To account for the difference in density of MPD we multiply 150 ml by 0.925 g/ml and obtain 138.75 grams. To formulate a 30% v/v MPD solution by mass we would then add 138.75 grams of MPD to a volumetric flask and bring the final volume to 500 ml at 25° Celsius with deionized water.

% saturation

% saturation is the concentration of material in solution as a percent of the maximum concentration possible at the given temperature. A saturated solution is one where there is equilibrium between undissolved solute and dissolved solute. To make a saturated solution, a salt is added to water and often warmed to enhance solubilization. Complete dissolution is desired. Upon cooling, some of the solute (salt) will crystallize out and leave behind a saturated solution. The actual concentration of a saturated stock depends upon the temperature of the solution. For example, at 0°C, 127.5 g of Potassium iodide can be dissolved into 100 ml of water, but at 20°C, 144 g of potassium iodide can be dissolved into 100 ml of water. Therefore, depending upon whether the solution is kept at room temperature or in the cold, the salt concentration will be different. % saturation is a rather old school way to make salt solutions for crystallization. However, since we often perform crystallization at different temperatures, the actual concentration in the bottle, reservoir, or drop can be very different. Exact reproduction of a % saturation stock solution not only depends upon careful mass and volume measurement, but also temperature. Keep life simple, avoid reproducibility problems and stick with M, % w/v and % v/v when formulating solutions.

Milligram per milliliter (mg/ml)

Milligram per milliliter (mg/ml) is typically used to express or determine protein concentration. To make a 20 mg/ml lysozyme solution we would weigh 20 mg of lysozyme and simply add 1 ml of buffer (20 mg plus 1 ml). However, others might weigh 20 mg of lysozyme and add 980 µl (0.98 ml) (20 mg in 1 ml). Be sure you document which method was used to avoid a slight concentration inconsistency and potential reproducibility problems later.

Dilutions

Dilute 1:10 means adding one part of diluate (reagent) to nine parts of diluent (water). Said another way, a 1:10 dilution means 1 part of the reagent plus 9 parts of water. Deionized water is the most frequently used diluent in crystallization experiments. Formulating a 0.1 M solution from a 1.0 M solution is a 1:10 dilution. To make 10 ml of 0.1 M solution from a 1.0 M solution, mix 1 ml of the 1.0 M solution with 9 ml of diluent.

A Bit About Buffers

All Hampton Research buffers are titrated using either HCl or NaOH. All buffer titrations are performed at 25.0 degrees Celsius.

Pay attention to buffer details and whether the buffer is the free acid or base. If Tris is indicated, do not substitute with Tris hydrochloride. Tris is titrated with HCl, where Tris hydrochloride is titrated with NaOH. A 1.0 M Tris pH 8.5 will have a conductivity of approximately 20 mS/cm at 25°C, where a 1.0 M Tris hydrochloride pH 8.5 will have a conductivity of approximately 60 mS/cm at 25°C. This different level of ionic strength can affect the outcome of the crystallization experiment, especially in a low ionic strength formulation.

The pH of a 1.0 M buffer will change when diluted in water to a final concentration of 0.1 M. The final pH of the reagent will also change due to the presence of other reagents, such as salt, polymers, and additives. Pay particular attention to the indicated reagent formulation. In most screens, the buffer pH is that of a 1.0 M stock prior to dilution with water and other reagent components. For example, the reagent 0.1 M Tris pH 8.5, 2.5 M Ammonium sulfate has a final measured pH of 8.1. 8.5 is the measured pH of the 1.0 M Tris 8.5 before dilution to 0.1 M and in the presence of 2.5 M Ammonium sulfate. An exception to this generalization are the Grid Screens, where the pH is titrated after all components are in solution.

Inspect stored buffers before use, swirling to check for settled contaminants; if they appear cloudy or discolored, do not use them. Such solutions may have microbial contamination or may have become chemically unstable. One exception to this is MOPS, which sometimes appears slightly yellow.

Some buffers, including ADA, CAPS, CAPSO, and CHES do not go into solution until titrated with the appropriate acid or base.

Sterile Filtration of Reagents

All Hampton Research reagents, unless noted otherwise are sterile filtered using 0.22 or 0.45 µm filters into sterile containers. Detergents are formu-

lated into sterile filtered deionized water, and not filtered following formulation, as micelles can be trapped in the filter, altering the final concentration of the detergent. All crystallization reagents save for detergents should be sterile filtered. Many crystallization reagents are susceptible to microbial growth if contaminated, and most crystallization experiments take place at room temperature, a cozy place for microbes to thrive.

When using a reagent that has been sitting around for more than a couple of weeks, give the container a swirl and inspect for signs of microbial growth such as a subtle, faint white, off white or yellow to brown precipitate coming off the bottom and swirling into solution. Sometimes partially precipitated reagents will resemble microbial growth to the untrained eye. To help differentiate precipitate from microbial growth, try warming the solution in your hands or a temperature incubator up to 50 degrees Celsius for 60 minutes. If the precipitate disappears it might well be precipitated reagent. Microbial growth will not dissolve.

Labware for Crystallization Reagent Storage

All labware used to contain and store crystallization reagents must be sterile, free of particulate, of a material that is compatible with the reagent, minimize gas and vapor permeability, and where practical, offer optical clarity for content inspection. Crystallization reagents should be protected from light to promote chemical stability. This is the reason Hampton Research kits and reagents are packaged in reclosable boxes.

Polypropylene offers compatibility with crystallization reagents and offers good protection against vapor permeability (evaporation). Hampton Research kits are filled into sterile polypropylene tubes and blocks. Some of the Optimize™ reagents are filled into polypropylene for optimal compatibility. Most of the Optimize™ reagents are compatible with Polyethylene terephthalate glycol-modified (PETG), and this material offers better optical clarity than polypropylene as well as being a better barrier to gas permeability. Polystyrene is inferior to polypropylene and PETG with regard to compatibility, gas and vapor permeability, and should not be used to store crystallization reagents.

Be sure labware is properly closed and sealed before stowing away reagents. Inspect labware (caps and containers) for cracks, and replace as needed. Date all filled containers with the date of receipt so one can readily know the age of the reagent.

Reagent Storage, Stability & Lifetime

Follow the reagent maker's guidelines for reagent storage and expiration. Most crystallization reagents are best if used within 12 months of receipt.

Safety

Always wear the appropriate safety equipment when handling chemicals. This might include, but not be limited to safety goggles/glasses/eye ware/face shield, nitrile exam gloves, lab coat and/or safety apron, and non-slip, closed toe footwear.

When working with acids, always measure water first and remember, add acid into water.

Work in a fume hood when handling acids and bases and volatile chemicals and all chemicals where the SDS recommends handling in a fume hood.

Reagent Formulation Examples

Following are examples of how to reproduce 1,000 µl volumes of Hampton Research crystallization screen reagents. Scale to the desired volume. Always add water first to promote the solubility of subsequently added reagents.

Grid Screen Salt HT Reagent 52 (E4)
0.8 M Sodium / Potassium phosphate pH 6.9

- 800 µl Deionized water
- 70 µl 4.0 M Sodium phosphate monobasic monohydrate
- 130 µl 4.0 M Potassium phosphate dibasic
- Make no pH adjustment

Crystal Screen reagent 17 (B5)
0.2 M Lithium sulfate monohydrate, 0.1 M TRIS hydrochloride pH 8.5, 30% w/v Polyethylene glycol 4,000

- 200 µl Deionized water
- 100 µl 1.0 M TRIS hydrochloride buffer
- 100 µl 2.0 M Lithium sulfate monohydrate
- 600 µl 50% w/v Polyethylene glycol 4,000
- Make no pH adjustment

Crystal Screen Cryo reagent 12 (A12)
0.18 M Magnesium chloride hexahydrate, 0.09 M HEPES sodium pH 7.5, 27% v/v 2-Propanol, 10% v/v Glycerol

- 440 µl Deionized water
- 100 µl 1.0 M HEPES sodium pH 7.5
- 90 µl 2.0 M Magnesium chloride hexahydrate
- 100 µl 100% Glycerol
- 270 µl 100% 2-Propanol
- Make no pH adjustment

GRAS Screen 2 reagent 93 (H9)
8% v/v Tacsimate™ pH 7.0, 20% w/v Polyethylene glycol 1,000

- 520 µl Deionized water
- 80 µl 100% Tacsimate pH 7.0
- 400 µl 50% w/v Polyethylene glycol 1,000
- Make no pH adjustment

References and Readings

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