Critical factors for successful protein crystallization

Protein crystallization is the most widely used technique for resolving protein structures to an atomic level. Optimal conditions for growing high-quality protein crystals are extremely difficult to predict and usually must be individually determined for each protein. In the following sections, we evaluate the challenges associated with protein crystallization experiments, discuss critical factors for success in growing crystals, and provide information about our dedicated products for crystallization.

The typical crystallization workflow requires expression and purification of high-quality protein and a series of screening steps followed by upscaling and optimization to obtain high-quality crystals (Figure 2). However, a number of considerations need to be made when setting up crystallization experiments, as described in the following sections.

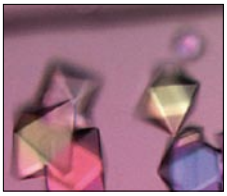


Figure 1. Crystals of proapoptotic protein.

Figure 2. Workflow for successful protein crystallization.

Protein requirements

Pure, homogeneous protein is the most critical prerequisite for successful protein crystallization. Therefore, care should be taken to optimize the protein preparation before setting up the experiment. The following methods can be used to obtain pure protein in preparative amounts:

- Affinity chromatography using, for example, His-, *Strep*, or GST- affinity tags
- Classical chromatography (e.g., ion exchange or gel filtration)

Protein purity and homogeneity can be assessed by the following methods:

- SDS-PAGE
- Size-exclusion chromatography (nonaggregated protein)
- Dynamic light scattering (polydispersity)
- Circular dichroism (protein folding)
- Mass spectroscopy (mass homogeneity; presence or absence of posttranslational modifications)

In cases where purification tags need to be removed (for example, by using QIAGEN's TAGZyme® System) to reduce flexibility of the termini, an additional purification step should be included to obtain a homogeneously processed protein sample (1, 2). Modified proteins, for example, those carrying selenomethionine, can be easily obtained using cell-free expression systems, such as QIAGEN's EasyXpress® Mega Kit.

The optimal concentration of a protein is an important variable when setting up a crystallization experiment. Concentration also varies with the chemical composition of the precipitant solution. For membrane proteins, the choice and concentration of detergent are additional important factors to consider (see the *Ni-NTA Membrane Protein Handbook;* 3). Different protein concentrations should be tested to cover the phase diagram of a given protein/precipitant combination (Figure 4). The EasyXtal® Pre-Screen Assay can be used to determine the optimal protein concentrations for a given type of precipitant.

Experimental setup

Among the many different crystallization techniques, vapor diffusion is still the most predominantly used method (Figure 3). Its setup and analysis are compatible with both manual and automated procedures. Usually, a drop of the protein solution is mixed with a drop of the screening solution. The same screening solution is pipetted into a reservoir. Since the concentration of screening solution in the protein drop is lower than in the reservoir, water diffuses over time from the protein drop into the reservoir until the concentrations in the drop and the reservoir are equal. As the concentrations of protein and precipitant increase in the protein drop, protein crystals are able to form. Vapor diffusion can be set up with drop sizes in the microliter range, for example, in 15-well plates. For initial experiments, 96-well plates are suitable as they enable screening of hundreds of conditions with smaller protein drops in the nanoliter to microliter range. For setup of crystallization trials, QIAGEN offers the NeXtal® Evolution µplate, which is highly suitable for both manual and automated procedures (Figure 5).

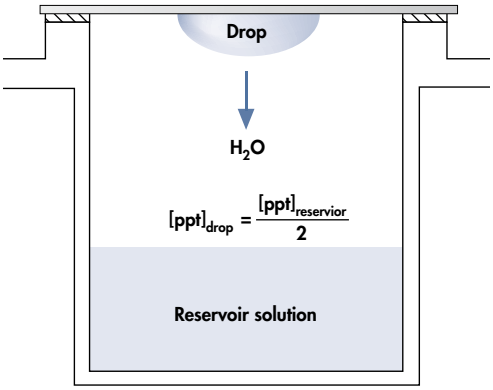


Figure 3. Principle of a vapor diffusion experiment. A mixture of the protein solution and screening solution is added into the reservoir. As water diffuses from the protein drop into the reservoir, the protein is slowly concentrated and protein crystals are able to form. **ppt**: precipitant.

The crystallization phase diagram

The crystallization event can be best described using the phase diagram. A phase diagram describes the behavior of a mix of protein and precipitant at different concentrations (Figure 4). For example, at low concentrations of both protein and precipitant, the drop remains clear (condition 1 in Figure 4). As the concentration of both components increases during the vapor diffusion experiment (Figure 3), they pass through the metastable phase to the nucleation phase, where first tiny crystals can form (condition 2 in Figure 4). As these crystals grow, they use up protein, thereby decreasing the protein concentration and reverting back to the metastable phase, where no new crystals are nucleated, but the existing small crystals grow larger (condition 3 in Figure 4). If the concentration of protein and precipitant is too high, precipitates form immediately without forming crystals (condition 4 in Figure 4).

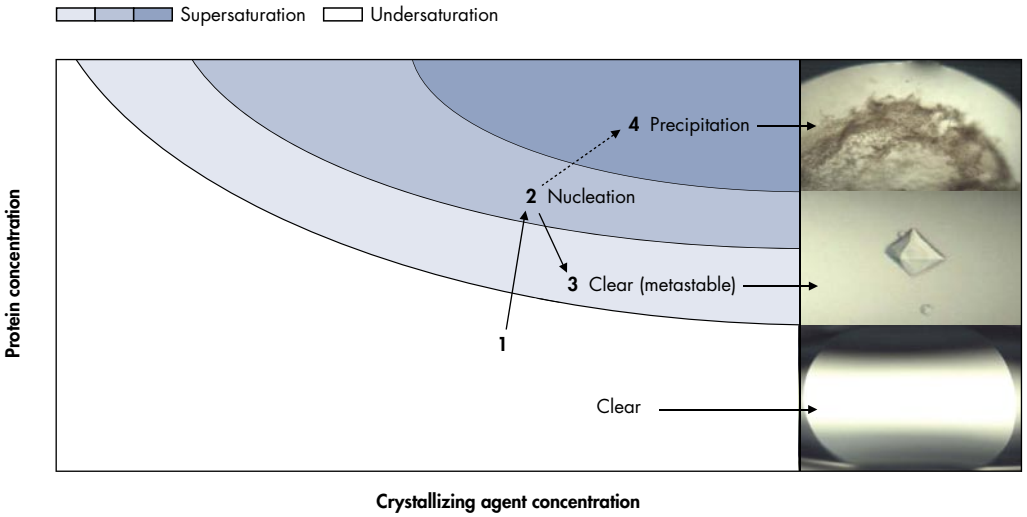


Figure 4. The crystallization phase diagram.

Evaporation from the vapor diffusion vessel has a considerable effect because it pushes the drop to the precipitation phase (condition 4 in Figure 4). This effect is usually uncontrollable and difficult to reproduce. The extent of evaporation depends on the composition of the screening solution, as well as on the plastic and sealing equipment used. Reproducibly low evaporation can be achieved with the NeXtal Evolution µplate (Figures 5 and 6). Its broad rims enable a tight interaction with the sealing tape, and even after individual wells have been cut open to harvest a crystal, the remaining wells can be incubated further (Figure 5).

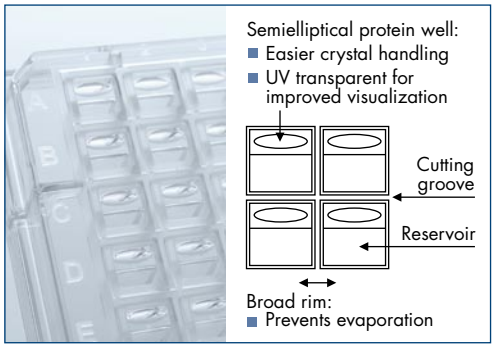


Figure 5. The NeXtal Evolution µplate and important technical features.

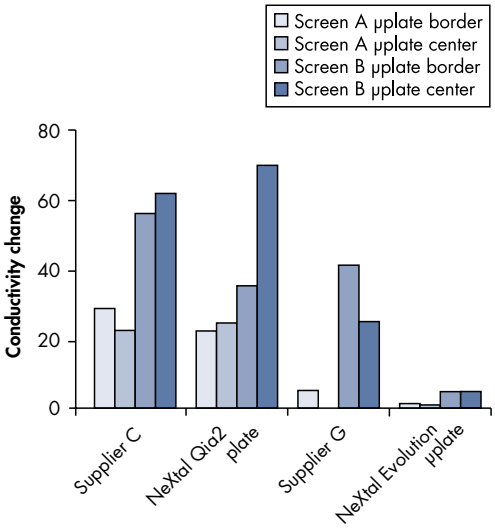


Figure 6. Evaporation data comparing the NeXtal Evolution µplate with other models. The NeXtal Evolution µplate ensures superior results compared to those obtained with plates from other suppliers. Screen A: 0.1 M Tris, pH 8.5, 35% MPD, 0.2 M ammonium sulfate; Screen B: 0.1 M sodium cacodylate, pH 6.5, 35% isopropanol, 0.2 M magnesium chloride. Solutions were pipetted on central or border wells of the microplate.

Initial screening

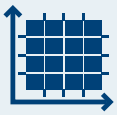
When starting a crystallization experiment, screening conditions should cover a large part of the chemical space to enhance crystal formation. Crystallization suites are usually either based on experimental conditions outlined in literature references and databases or based on a rational combination of chemical conditions. Although past experience can be used to deduce starting conditions for crystallization setup, it does not guarantee success.

Screening strategies and precipitant types

The general function of precipitants in protein crystallization experiments is to decrease the solubility of the protein. Polymers such as PEG, salts, and organic solvents are the most commonly used precipitants. There are 3 main screening strategies, as discussed in the next section.

Sparse matrix

Sparse matrix (or incomplete factorial) strategies typically map a wide sector of the chemical space using many different reagents. Sparse matrix screens are ideally suited for first screening trials.



Grid screens

Grid screens systematically evaluate two factors, such as a precipitant in different concentrations at various pH values. Usually, grid screens are used for refinement of successful conditions.

Ionic sampling

Ionic sampling uses the effect that the ionic strength of a solution has on a macromolecule (i.e., its effect on the surface charge and therefore solubility). This strategy keeps one component, for example pH, constant while varying a second component, for example different salts. An ionic sampling screen ensures further refinement of successful conditions.

Choice of crystallization screens

Even though screening thousands of different conditions increases the chances of growing crystals, the amount of protein available for a given crystallization experiment is usually the limiting factor. Choosing the right conditions for an initial screening is therefore a challenge. The Joint Center for Structural Genomics (JCSG) has statistically evaluated all commercially available screening solutions in more than half a million experiments (4). The results of this work are contained in JCSG Core Screens, which together form 384 unique screening conditions in a sparse matrix screen. If enough protein is available to screen 500 conditions, we recommend combining the JCSG Core I–IV Suites with the PACT Screen (5) for initial experiments. If only 200 conditions can be screened, the JCSG+ Suite from the same group, in combination with the PACT Suite has been shown to be effective (6). All screening suites are available as 10 ml tubes and as automatable 1.5 ml deep-well (DW) blocks. Table 1 summarizes the screens recommended for initial screening.

Special applications

For special applications such as crystallization of protein–protein complexes or nucleic acids, dedicated screens have been compiled based on database entries. These have been shown to work for a range of proteins; however, it is better to combine them with other initial screens (e.g., JCSG Core I–IV Suites and PACT Suite) for greater coverage of the chemical space.

Table 1. Screening suites from QIAGEN

	Description	Suite
Core screens for initial investigations	Statistically evaluated solutions	JCSG Core Suites (I–IV)*
	Systematic effect of pH, anions, and cations	PACT Suite*
	Using an optimized sparse matrix	JCSG+ Suite
	Using classic solutions	Classics, Classics II, Classics Lite, and Cryos Suites
	Rapid analysis of polymers, alcohols, and salts	ComPAS Suite
Screens analyzing single precipitant types	Varying salts and pH	Anions and Cations Suites, pHClear and pHClear II Suites
	Using polyethylene glycols of varying molecular weights	PEGs and PEGs II Suites
	Using ammonium sulfate	AmSO4 Suite
	Using 2-methyl-2,4-pentanediol (MPD)	MPD Suite
Screening conditions for specialized applications	For membrane proteins in cubic phase or sponge phase experiments	CubicPhase I and CubicPhase II Suites†
	For membrane proteins in classical vapor diffusion experiments	MbClass and MbClass II Suites
	For protein–protein complexes	Protein Complex Suite
	For protein–nucleic acid complexes	Nucleix Suite
Rapid optimization of initial crystallization hits	Salt additives at different concentrations and pH	Opti-Salts Suite

* Recommended for a standard initial screen of 500 conditions.

† To be used with NeXtal CubicPhase µplates.

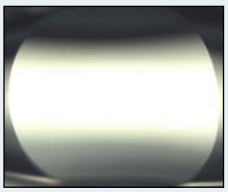
Interpretation of results

While the setup of a vapor diffusion experiment is straightforward, interpretation of results requires more expertise. In many cases, salt crystals form in the protein drop rather than protein crystals. Differentiating between protein and salt crystals is very difficult using standard light microscopy methods. A possible alternative is to use a microscope equipped with a UV light source. Proteins can show epifluorescence at 340 nm due to tryptophan residues, which can be detected. However, if the protein does not contain any tryptophan residues, or if fluorescence quenching occurs, this method cannot be applied. Alternatively, the absorption of aromatic residues at 280 nm (which is also commonly used for protein quantification) can be used for visualization of protein crystals. This requires a camera for detecting UV light and suitable filters. The NeXtal Evolution µplate is highly UV transparent and allows for both visualization methods (Figure 7).

Figure 7. Crystal detection in the NeXtal Evolution µplate.

Interpretation of results

The following section provides typical examples of the most commonly observed experimental results.



Clear drop

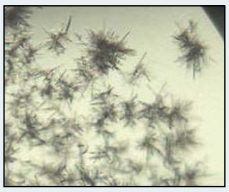
If the drops stay clear, the initial concentrations of protein and/or precipitate are too low to draw any conclusions. QIAGEN recommends increasing the concentration of protein and/or precipitant or decreasing the incubation temperature in the next round of screening.

Precipitate

If a precipitate is obtained, it usually means that the protein and/or precipitant concentrations are too high for crystal formation. There are two types of precipitates — those that consist of denatured protein and those that contain native protein and can be resolubilized. Conditions that result in the denaturation of protein should be avoided in the future. If the protein remains in its native conformation, protein and/or precipitant concentrations can simply be decreased. An easy way to find out if the precipitate contains native or denatured protein is to set up the condition again using QIAGEN's EasyXtal 15-Well Tool (Figure 10). When using this tool, the reservoir solution can be diluted (or even replaced by water) and the experiment can be incubated for longer. If the protein is still in its native conformation, it will dissolve into a clear drop after some time. If not, it is irreversibly damaged (see the *EasyXtal and NeXtal Protein Crystallization Handbook* for more details).

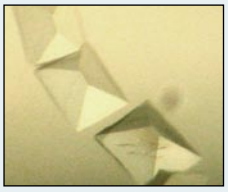
Phase separation

Phase separation leads to small oily areas that form in the drop. These areas have increased protein concentration. This state favors crystallization; however, further optimization may be required. In parallel to optimization, the original drop should be further incubated because it may lead to crystal growth over time.



Microcrystals and needles

If microcrystals or needles are obtained, the screening conditions are probably suitable, but the concentration of the protein and/or precipitant is only slightly too high and the experiment is stuck in the nucleation phase. QIAGEN recommends slightly decreasing the concentrations of protein and/or precipitant or seeding into a fresh drop of protein solution. Setting up the experiment at a lower temperature may also lead to larger crystal growth due to altered growth kinetics.



Crystals

If crystals form, their quality should be checked by X-ray diffraction before further optimization. If small but well-diffracting crystals form, upscaling and/or optimization of crystal conditions should be performed to grow larger crystals.

Refinement and optimization

Several approaches can be taken in order to fine-tune successful hit conditions and to grow larger and/or better-diffracting crystals (Figure 8). One strategy involves choosing suites using chemicals that worked best in the initial screening for a second, more defined round of screening using dedicated precipitants. Another easy optimization strategy involves use of the Opti-Salts Suite, which comprises of premixed salt additive solutions at different pH available in deep-well blocks. The 96 conditions from this suite are mixed with the original hit condition (available separately as an EasyXtal Refill-Hit) to yield 96 new conditions that form an optimization grid around the originally successful condition (Figure 9). This method has been successful in many cases and can be performed in 96-well format (Figure 5).

Optimization conditions can also be prepared using QIAGEN's NeXtal Stock Solutions that contain exactly the same chemicals as the original screening solutions. This optimization step can be performed in a 96-well format, but usually a larger format is used to limit the number of solutions to be prepared and to grow larger crystals. The EasyXtal 15-Well Tool is highly suited for optimization experiments because the original hit can be placed in the center well as a control and the variation grid can be set up around the central position (Figure 10).

If no hit conditions are obtained even after evaluating many crystallization experiments, variations of the protein should be generated. For example, these modifications can involve removal of flexible loops, reduction of protein surface charge (7–8), or expression and purification of only subdomains of the protein. Alternatively, homolog proteins from other organisms can be tested.

Upscaling

In cases where the crystals grown in small drops in 96-well format are not large enough for data collection, upscaling to a larger drop size can be attempted. The EasyXtal 15-Well Tool is highly suitable for setting up drops in the microliter range. Converting an experiment from smaller to larger volumes has an impact on the surface-to-volume ratio and further optimization is often required to achieve similar results to those obtained in the nanoliter range.

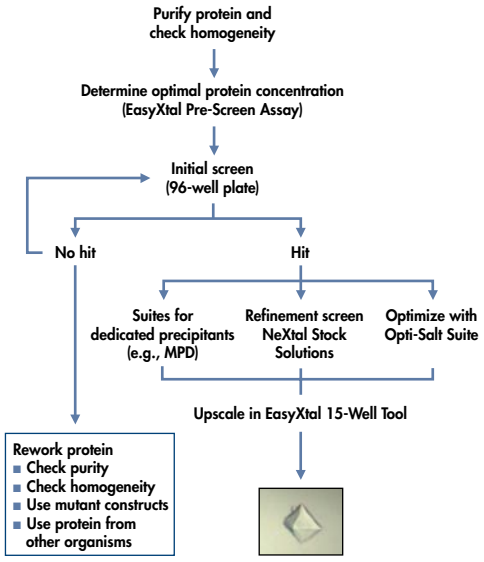


Figure 8. Decision tree for optimization experiments. Several approaches can be taken when a hit condition is identified and these can be performed in conjunction with each other or separately.

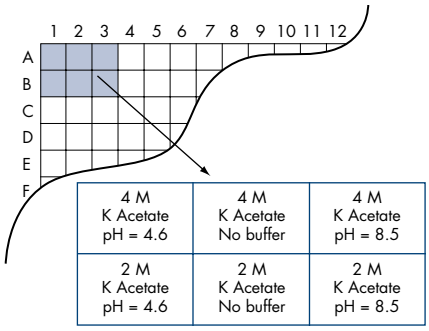


Figure 9. Principle of Opti-Salt optimization grids. The method involves screening of 16 different chemicals in grids of 6, where concentration of the additive and pH are varied compared to the original hit condition.

A

B

C

Figure 10. A EasyXtal 15-Well Tool B Standard and C DropGuard Crystallization Supports used with the EasyXtal 15-Well Tool.

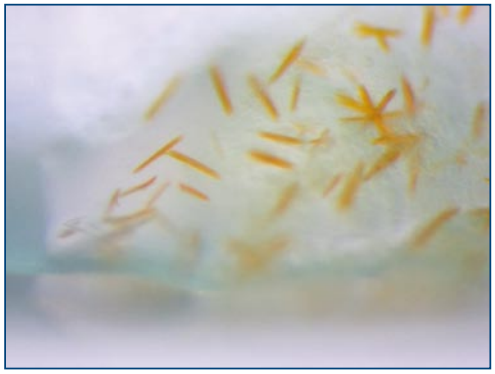


Figure 11. Crystals of Sensory Rhodopsin (SR) at microscopic magnification.

The EasyXtal 15-Well Tool also allows for a number of other techniques. These include:

- Screening of cryoprotectants
- Screening of ligands
- Seeding experiments

The DropGuard crystallization support accommodates 3 drops of 2 µl total volume (1 µl protein + 1 µl precipitant) or 3 drops of a 4 µl total volume. Wells are numbered for easy identification, drop spreading is prevented, and visualization of the drops is enhanced due to the unique optical properties of the well (Figure 10). X-Seal crystallization supports contain X-Seal, which is made from an advanced O-ring material that minimizes evaporation and prolongs the reuse of reservoir solutions.

Membrane protein crystallization

Crystallization of membrane proteins is especially challenging. Membrane proteins consist of hydrophobic (membrane-spanning) regions and can only be kept in solution in aqueous environments in the presence of detergents forming a micelle around the hydrophobic regions. Although membrane proteins can be crystallized under such conditions (9), they still remain in an unphysiological state as they lack the lipid bilayer, which is the natural environment for membrane proteins. Lipidic Cubic Phase (LCP) (10) has been developed as a method for crystallization of membrane proteins under conditions imitating those found in their natural environment. QIAGEN has adapted the classical LCP batch method and developed the NeXtal CubicPhase crystallization product range. These products enable fully automatable, high-throughput crystallization of membrane proteins in meso phase (10), in a vapor diffusion mode, using standard liquid-handling robots. NeXtal CubicPhase crystallization products include NeXtal Evolution µplates (Figure 5) prefilled with a thin coating of monoolein (MO) on the crystallization wells (Figure 13). A set of two dedicated crystallization screens (NeXtal DWB CubicPhase I and II Suites) can be used to set up initial screening experiments (Figures 12 and 14).

The LCP experimental setup is very easy to perform using NeXtal CubicPhase products (Figures 12–14). The protein solution is pipetted on the monoolein layer in the protein well, and the cubic phase is formed by passive diffusion of water and protein into the lipid phase. After a 1–3 hour incubation time, the screening solution is added to the protein well and the reservoir to start the experiment. In this vapor diffusion experiment, concentration of the drop leads to a structural change of the meso phase, enabling protein crystallization. A new bacterial 7- transmembrane domain receptor was crystallized using NeXtal CubicPhase products (Figure 11).

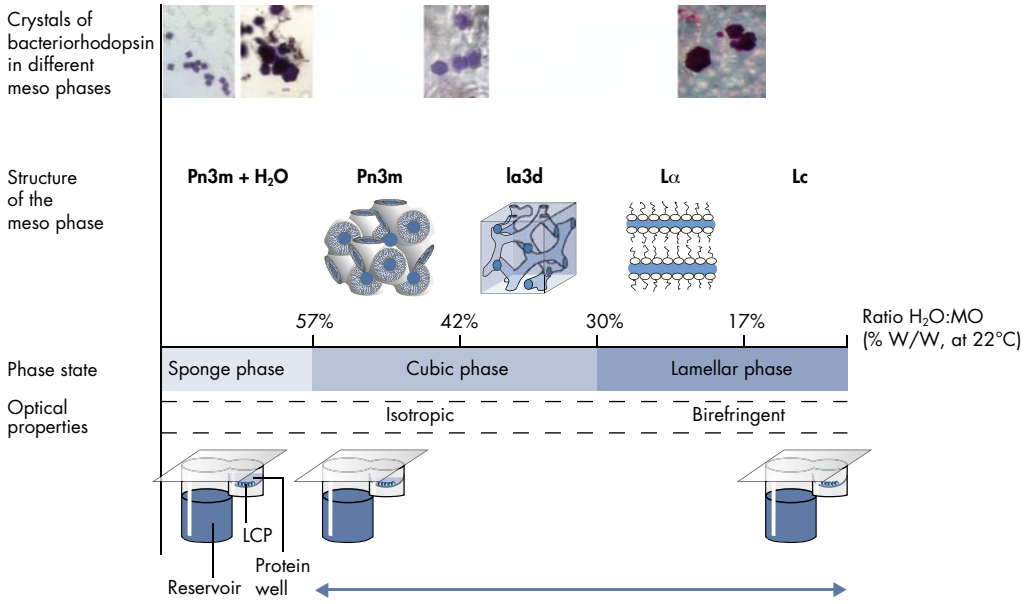


Figure 12. Phase transformations in meso phase experiments. Lipids such as monoolein (MO) have the ability to form complex phases with aqueous solutions, dependent on temperature and the ratio of water to MO. These phases are named based on the crystallographic characteristics. The starting point of the crystallization experiment is a mixture of MO and excess aqueous solution (protein and precipitating agent) called the sponge phase. The vapor diffusion from the protein well to the reservoir increases the concentration of protein and precipitant within the drop and triggers a transformation of the meso phase. Depending on when the water pressure equilibrium between the protein well and the reservoir is reached, the structure of the meso phase reached will be anywhere from sponge phase to lipidic cubic phase (LCP) to lamellar phase. It is possible to distinguish which phase is reached by examining the optical properties of the protein well. The sponge and LCP structure are isotropic, while the lamellar phase displays birefringent properties (10). Protein crystals can form in any of the phases as shown for bacteriorhodopsin.

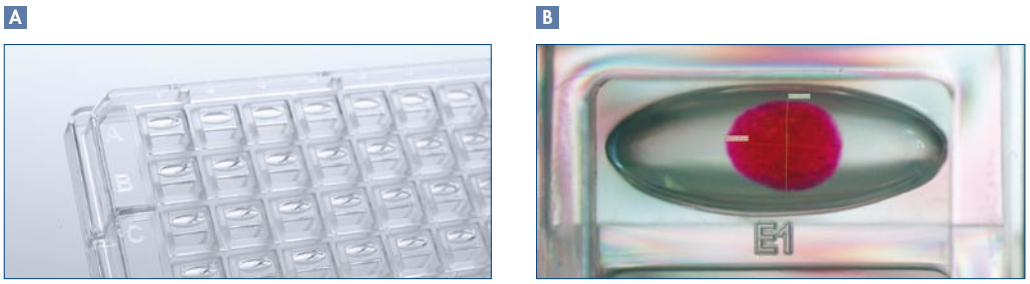


Figure 13. NeXtal CubicPhase µplate. A Design of precoated NeXtal CubicPhase µplate. B Top view of the oval-shaped protein well coated with monoolein (MO). For better visualization, the MO has been colored with a red dye.

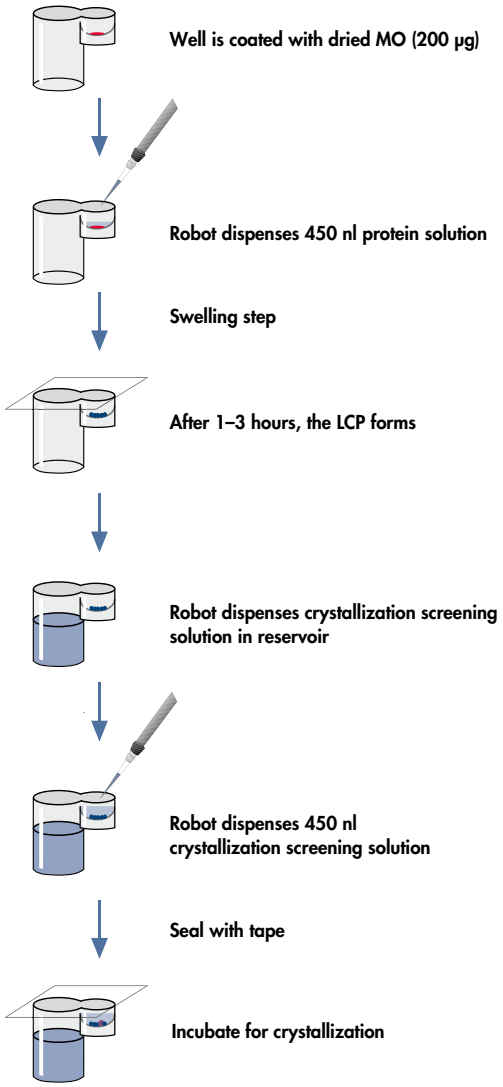


Figure 14. Workflow for setting up a crystallization experiment using the NeXtal CubicPhase µplate. The membrane protein solution is dispensed directly onto the dry lipid (monoolein). The protein solution diffuses into and rehydrates the lipid, resulting in the passive formation of the lipidic meso phase.

Advanced solution for protein crystallization

Analysis of a protein's three-dimensional structure is essential to elucidate its function. Until recently, identifying the chemical conditions that lead to highly diffracting protein crystals has been a tedious and time-consuming process. To overcome such challenges, QIAGEN has developed the NeXtal and EasyXtal product lines, which offer unparalleled quality, convenience, and flexibility in protein crystallization. No matter what your application, benefit from QIAGEN's expertise and trust our specialized products for your crystallization experiments!

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Ordering Information

Product	Contents	Cat. no.
Ni-NTA Superflow (25 ml)*†	For purification of 6xHis-tagged proteins: 25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Strep-Tactin Superflow Plus (10 ml)*†	For purification of *Strep*-tagged proteins: 10 ml *Strep*-Tactin-charged Superflow (max. pressure: 140 psi)	30004
Glutathione Superflow (10 ml)*†	For purification of GST-tagged proteins: 10 ml Glutathione Superflow	30900
Ni-NTA Membrane Protein Kit	For 5 detergent screenings and 5 affinity purifications: 7 detergents, buffers, Ni-NTA Superflow, Penta·His Antibody, disposable columns	30610
EasyXpress Protein Synthesis Mega Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o methionine, methionine, RNase-free water, gel-filtration columns, and reaction flasks	32516
EasyXtal Pre-Screen Assay	1 x 24-well DropGuard Crystallization Tool prefilled with 24 screening solutions	130222
EasyXtal 15-Well Tools (20)*	20 empty 15-well plates with standard crystallization supports	132006
EasyXtal 15-Well Tool X-Seal (20)*	20 empty 15-well plates with X-Seal crystallization supports	132008
EasyXtal 15-Well DG-Tool (20)*	20 empty 15-well plates with DropGuard crystallization supports	132106
EasyXtal 15-Well DG-Tool X-Seal (20)*	20 empty 15-well plates with DropGuard X-Seal crystallization supports	132108
NeXtal Evolution µplate (10)*	10 microplates for protein crystallization trial setup	132045
NeXtal Tubes	96 x 10 ml tubes containing crystallization screening solutions	Varies
NeXtal DWBlocks	Piercable deep-well block containing 96 x 1.5 ml crystallization screening solutions, piercing tool, adhesive foil	Varies
NeXtal DWBlock Opti-Salt Suite	Piercable deep-well block containing 96 x 0.5 ml suite solution, piercing tool, adhesive foil	130921
EasyXtal Refill-Hits	50 ml of individual crystallization suite solution	Varies
NeXtal Stock Solutions	50 or 200 ml of crystallization stock solution	Varies
NeXtal CubicPhase Kit	2 x 96-well plates coated with monoolein and 2 deep-well blocks containing 96 x 1.5 ml solutions for crystallization of membrane proteins	130807
NeXtal CubicPhase µplate (10)*	10 x 96-well plates coated with monoolein for crystallization of membrane proteins	130803

* Other kit sizes and/or formats available; see www.qiagen.com.

† Also available as a prefilled cartridge.

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