Second Edition **September 2010**

NeXtal® CubicPhase Handbook

For *in meso* crystallization of membrane proteins

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NeXtal DWB CubicPhase I Suite (96 x 1.5 ml screening suite solutions)	1
NeXtal DWB CubicPhase II Suite (96 x 1.5 ml screening suite solutions)	1
Handbook	1

NeXtal CubicPhase 1w Mo µplate (10)	**130803**
1-well NeXtal Evolution µplates with monoolein	10
Handbook	1

NeXtal CubicPhase 1w Mo µplate (100)	**130805**
1-well NeXtal Evolution µplates with monoolein	100
Handbook	1

NeXtal CubicPhase 2w Mo µplate (10)	**130814**
2-well MRC µplates with monoolein	10
Handbook	1

NeXtal CubicPhase 3w Mo µplate (10)	**130816**
3-well IntelliPlate low profile µplates with monoolein	10
Handbook	1

NeXtal CubicPhase 1w Mo/C 8% µplate (10)	130822
1-well NeXtal Evolution µplates with monoolein/8% cholesterol	10
Handbook	1

NeXtal CubicPhase 2w Mo/C 8% µplate (10)	130824
2-well MRC µplates with monoolein and 8% cholesterol	10
Handbook	1

NeXtal CubicPhase 3w Mo/C 8% µplate (10)	130826
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NeXtal DWB CubicPhase I Suite	130928
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Piercing Tool	1
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NeXtal DWB	1
Piercing Tool	1
Adhesive Foil	1 sheet
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Storage

NeXtal CubicPhase µplates should be stored at –20°C. NeXtal DWB CubicPhase I and II Suites should be stored at room temperature (15–25°C). Kit

components can be stored under these conditions for at least 6 months without any reduction in performance.

Product Use Limitations

NeXtal CubicPhase products are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding NeXtal CubicPhase products or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of NeXtal CubicPhase products is tested against predetermined specifications to ensure consistent product quality.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

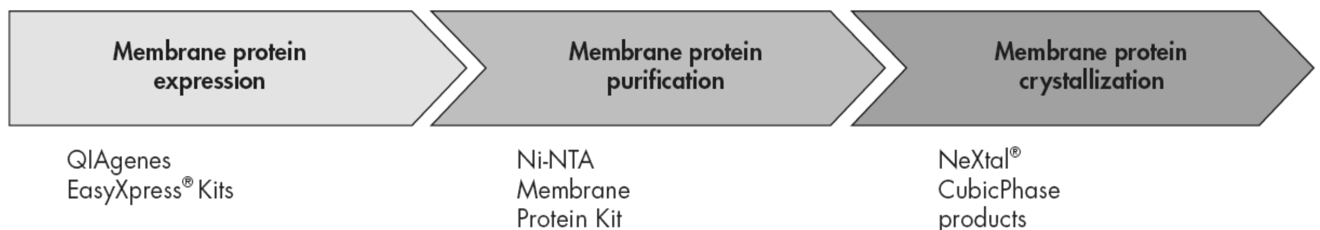
Proteins can be separated into 2 general classes according to their location — proteins that are associated with cellular membranes, so-called membrane proteins and proteins that are not associated with cellular membranes (soluble proteins).

Approximately 30% of a mammalian genome encodes for membrane proteins, which are one of the most important protein classes in that they receive, differentiate, and in some cases, transmit intra- and intercellular signals. Some examples of membrane proteins include cell-adhesion molecules, translocases, and receptors in signaling pathways.

Defects in membrane proteins cause a number of serious diseases including neurodegenerative disorders, autoimmune conditions, and metabolic diseases. Therefore, membrane proteins constitute approximately 50% of possible targets for novel drugs. However, despite their essential functions, the information available on membrane protein structures is, to-date, very limited.

Due to their interaction with biological lipid bilayers, membrane proteins by nature are hydrophobic and have a tendency to aggregate. They are also often oligomers with a high molecular weight. These features account for some of the technical challenges in the study of membrane proteins.

QIAGEN offers a complete range of products to express, purify, and crystallize membrane proteins (see below). For a complete overview, visit www.qiagen.com/protein.



In meso crystallization

Structural biology, and especially X-ray crystallography, is a very powerful technique that is used to gain insights into protein function and to serve as a reference for structure-guided inhibition (e.g., for rational drug design). However, the structural information available for membrane proteins is scarce, especially for therapeutically relevant human membrane proteins. In addition to the difficult expression and purification steps, crystallization of membrane proteins remains one of the greatest challenges of structural biology. Recently, crystallization in meso phase has yielded crystals where other techniques have failed (1). Meso phase mimics the lipid bilayer environment of the plasma membrane; this bilayer extends three-dimensionally through space and separates 2 systems of water channels providing space for accommodation of hydrophobic and hydrophilic domains (Figure 1). It provides close to natural conditions so that the membrane protein is correctly folded.

In meso crystallization describes the crystallization of membrane proteins in the different phases that aqueous and lipidic compounds can adopt when mixed in different ratios. These phases are named based on their crystallographic characteristics. Of special interest are the sponge phase, the lipidic cubic phase (LCP), and the lamellar phase (1). In the sponge phase and LCP, protein moves in the three dimensions defined by the particular structure adopted by the membrane, while in the lamellar phase, only two-dimensional diffusion of embedded protein is possible, since it is a two dimensional, membrane-like environment (Figure 1). Crystallization can occur in any of the meso phases described above.

QIAGEN's NeXtal CubicPhase products use monoolein (Mo) alone or in combination with 8% cholesterol (C). Mo provides a lipid environment which imitates the natural lipid bilayer of a cell. In eukaryotic cells, cholesterol plays a very important role by increasing the flexibility of the membrane. This lipid may interact with many membrane proteins and increase their stability in the lipidic environment. NeXtal CubicPhase products enable the setup of experiments with or without cholesterol as an additional experiment variable — providing greater flexibility. Addition of cholesterol means that the incubation temperature of the experiment can be reduced from 22°C to 18°C, which could be a critical experimental factor, especially for the most temperature-sensitive membrane proteins.

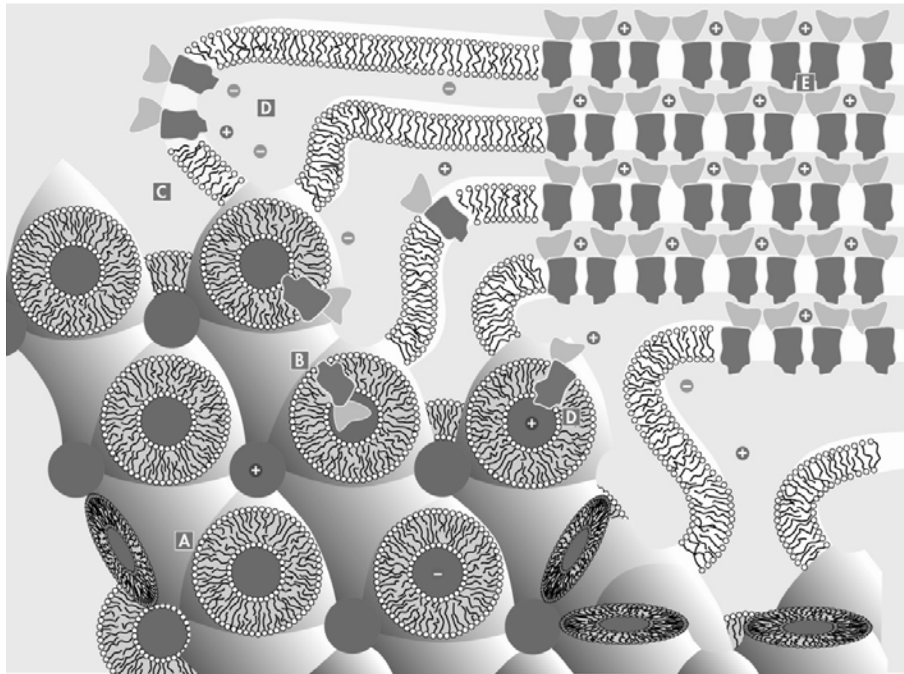


Figure 1. The theory of *in meso* crystallization. A Lipids (e.g., monoolein [Mo]) mixed with aqueous solution form a three-dimensional network of lipid bilayer and aqueous channels, also know as a LCP. B When membrane proteins are added into this mix, they insert into the lipid bilayer and can diffuse in its three-dimensional structure. C Upon dehydration and with the influence of precipitant, the LCP rearranges itself and becomes a lamellar phase D where lipid bilayers and proteins are stacked on top of each other E. Adapted from Caffrey (2).

Reaching different meso phases (sponge, cubic, and lamellar)

The transition between the different phases is dependent on the ratio of water and lipid in the mixture (Figure 2). While the experiment starts as sponge phase with an excess of water, the water content of the mixture is gradually reduced because of vapor diffusion. This dehydration leads to formation of LCP, and eventually to the lamellar phase (which is also driven by the precipitant added to the solution). Sponge phase, LCP, and lamellar phase may yield crystals; however like any other crystallization experiment, these are experimental variables which must be tested for each membrane protein.

To reach any of the meso phases, it is necessary to allow vapor diffusion from the protein well to the reservoir. The rate of vapor diffusion depends on the difference in ionic strength between these 2 compartments. The higher the difference, the more vapor will diffuse. In a typical vapor diffusion experiment, 1 unit of protein solution is mixed with 1 unit of crystallization screening solution (e.g., 1 µl + 1 µl). At the beginning of the experiment, the concentration of the crystallization screening solution in the protein well is half its concentration in the reservoir. Therefore, the vapor will diffuse from the protein well to the reservoir, leading to an increase in the concentration of the screening solution in the protein well.

However, to reach the cubic phase and subsequently the lamellar phase, more diluted crystallization screening solution must be added to the protein well compared to the reservoir, typically a ratio of 1:14. To achieve this, a pre-dilution of the crystallization screening solution is performed. While the reservoir solution remains undiluted, a 1 in 7 dilution (125 µl of screening solution + 875 µl of water in a new deep-well block) of the crystallization screening solution used in the protein well is performed. The diluted screening solution is then mixed in equal parts with the protein solution in the protein well to finally result in a 1 in 14 dilution.

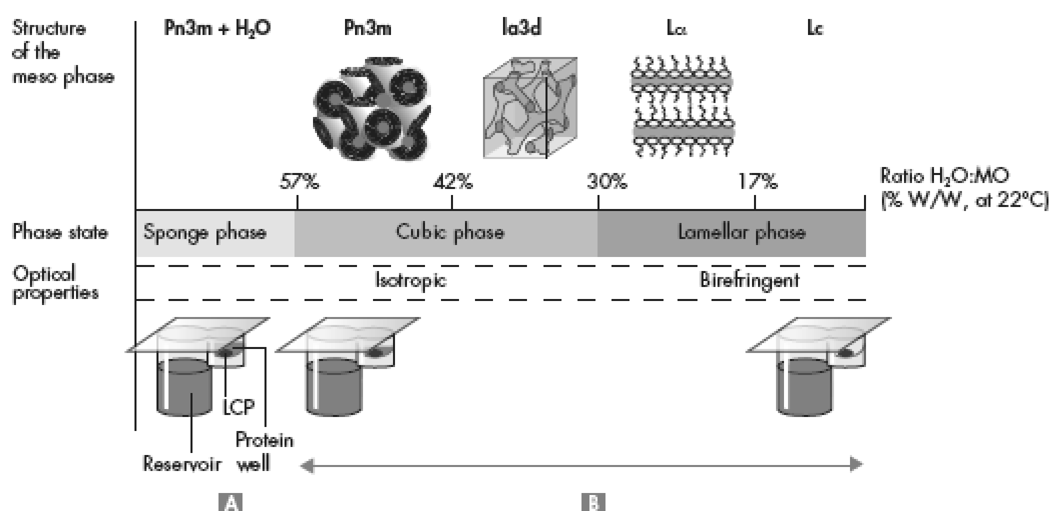


Figure 2. Hypothetical events occurring during *in meso* crystallization experiments. Phase transformations in meso phase experiments. **A** Lipids such as monoolein have the property to form complex phases with aqueous solutions, dependent on temperature and the water:monoolein ratio. These phases are named based on the crystallographic characteristics. The starting point of the crystallization experiment is a mixture of monoolein and excess aqueous solution (protein and precipitating agent) called the sponge phase. **B** The vapor diffusion from the protein well to the reservoir increases the concentration of protein and precipitant within the drop and also 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 achieved will be anywhere from sponge phase to LCP to lamellar phase. It is possible to distinguish between the phases by examining the optical properties of the protein well. The sponge and LCP structure are isotropic, while the lamellar phase displays birefringent properties. Adapted from Caffrey (2).

NeXtal CubicPhase crystallization products

Current methods for the setup of meso phase experiments include numerous manual steps. The NeXtal CubicPhase system enables fully automatable, high-throughput membrane protein crystallization setup. Easy manual setup of the experiments is also possible using a multichannel pipet (see protocols on pages

25, 26, 39, and 40). The system utilizes the advantages of vapor diffusion crystallization together with those of *in meso* crystallization, which targets the phase transformation point from cubic to lamellar phase, as well as crystallization in the presence of excess water or in the sponge phase. NeXtal CubicPhase crystallization products consist of a range of crystallization microplates, screens, and a starter kit. Features can be found on Table 1, page 14.

Table 1. Features of various NeXtal crystallization microplates

Plate	Cat. no.	Number of wells	Reaction volume (protein + screening solution)	Coating	Incubation temp.
NeXtal CubicPhase 1w Mo µplate (10)	130803	1	450 nl + 450 nl	Monoolein	22°C
NeXtal CubicPhase 1w Mo µplate (100)	130805	1	450 nl + 450 nl	Monoolein	22°C
NeXtal CubicPhase 2w Mo µplate (10)	130814	2	100 nl + 100 nl	Monoolein	22°C
NeXtal CubicPhase 3w Mo µplate (10)	130816	3	100 nl + 100 nl	Monoolein	22°C
NeXtal CubicPhase 1w Mo/C 8% µplate (10)	130822	1	450 nl + 450 nl	Monoolein + 8% Cholesterol	18°C
NeXtal CubicPhase 2w Mo/C 8% µplate (10)	130824	2	100 nl + 100 nl	Monoolein + 8% Cholesterol	18°C
NeXtal CubicPhase 3w Mo/C 8% µplate (10)	130826	3	100 nl + 100 nl	Monoolein + 8% Cholesterol	18°C

The NeXtal CubicPhase Kit is recommended when you first start using this technology. It comprises of 2 components which can be also purchased separately:

- Two NeXtal CubicPhase 1w Mo μplates: Extra evaporation-tight, NeXtal Evolution μplates delivered prefilled with monoolein for automated setup of the meso phase experiment upon hydration with membrane protein solution
- Two sets of 96 dedicated screening solutions optimized for *in meso* experiments and successfully used for membrane protein crystallization:

  NeXtal CubicPhase I Suite: This set of screening solutions contains 96 variations of buffered solutions with different added salts. The solutions contain no other components such as organics or PEGs. The pH variation and the ionic strength are optimized for meso phase experiments.

  NeXtal CubicPhase II Suite: This set of screening solutions uses different molecular weight PEGs at set pH as precipitating agents.

It is possible to use the NeXtal CubicPhase μplate with other screening solutions (3).

Protein requirements

Membrane proteins used for a crystallization experiment should be homogenous and as pure as possible, although purity is less critical for crystallization compared to other systems since meso phase crystallization has a higher tolerance for impurity (8). Purity and homogeneity can be determined by various methods (e.g., size exclusion chromatography, dynamic light scattering experiments, etc.). For the purification of membrane proteins, QIAGEN recommends the Ni-NTA Membrane Protein Kit (cat. no. 30610) or a combination of QIAGEN Ni-NTA matrices with suitable detergents (see ordering information, page 56).

The recommended protein concentration depends on the concentration of the screening solution. Diluted or undiluted screening solutions can be used as described in the protocols detailed in this handbook (also see pages 11–12). When using diluted screening solutions, the protein concentration should be in the range of 2–5 mg/ml; when using undiluted screening solutions, the protein concentration should be from 5–20 mg/ml. As both detergent and membrane protein may be dissolved in the meso phase, their ratio represents an experimental parameter that may require optimization (3).

Principle and procedure

The NeXtal CubicPhase Kit enables crystallization of membrane proteins in meso phase for structural studies by a unique combination of *in meso*

crystallization and sitting-drop vapor diffusion using standard liquid-handling robotics.

By mixing the protein with the reservoir solution (precipitating agent), both components are diluted. In the vapor diffusion experiment, the subsequent equilibration against the reservoir reduces the water content in the protein well. Therefore, the concentration of protein and screening solution is gradually increased.

NeXtal CubicPhase μplates allow setup of meso phase crystallization experiments (Figure 3). The different lipidic structures in the meso phases accommodate the membrane protein by separation from the detergent, and allow the three-dimensional diffusion of the protein under almost natural conditions. To enhance entry of membrane proteins into the meso phase, it is of critical importance to start with an initial excess of water. It is recommended to first add the protein solution to the dried monoolein. The critical parameter is the total volume of the initial drop (see protocols).

Note: The required volume depends on the amount of evaporation during dispensing which is directly related to the speed of the setup (see troubleshooting guide, page 50).

The concentration of the precipitant (reservoir solution) within the initial droplet determines the endpoint of the vapor diffusion experiment, which is either cubic phase or lamellar phase formation (Figure 2).

By testing both methods, the chances of achieving the optimum crystallization conditions for a given protein are increased.

The general workflow for using the NeXtal CubicPhase μplates is described in Figure 3.

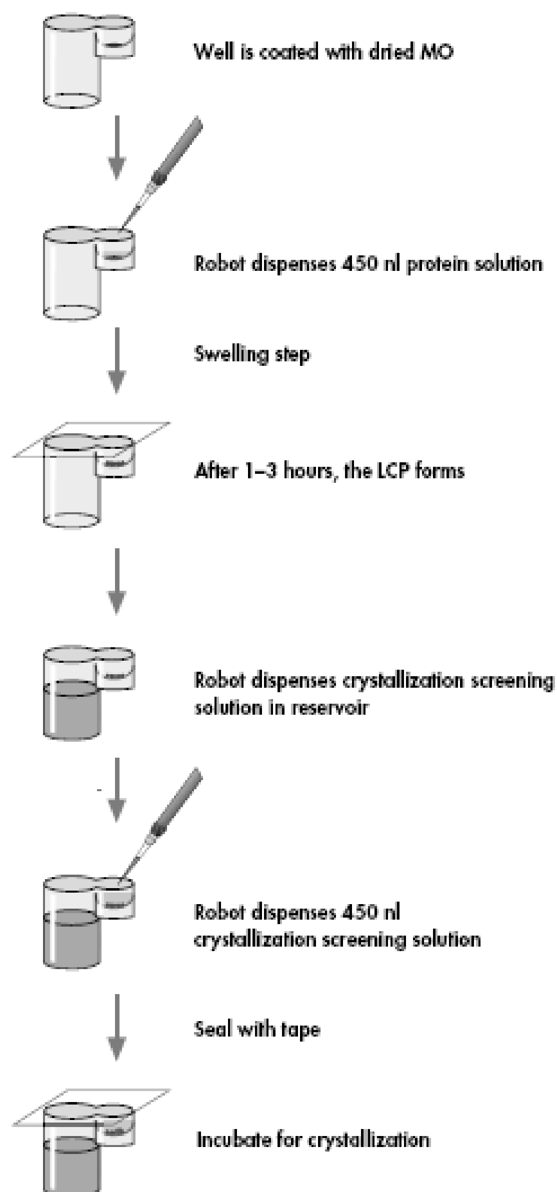


Figure 3. General workflow for NeXtal CubicPhase µplates. An example of a µplate coated with monoolein (MO) is shown here. The volumes indicated in the example refer to the LCP protocol using a liquid-handling robot and 1-well µplates. Other protocols are described in this handbook for µplates coated with MO + 8% cholesterol, as well as for 2- and 3-well µplates, and for setup using a multichannel pipet. Although incubation times and volumes may differ from this example, the general guidelines remain the same.

The NeXtal CubicPhase principle involves passive mixing of the protein solution with the monoolein coated on the µplate. This system does not rely on active mixing of the protein solution with the lipids, therefore, it will not heat up the solution which can lead to protein precipitation. The formation of the meso phase occurs during the swelling step (step 3 in Figure 3). Diffracting crystals — similar to those obtained in standard meso phase experiment using active

mixing of lipids and protein solution — are obtained using NeXtal CubicPhase µplates (Figure 4).

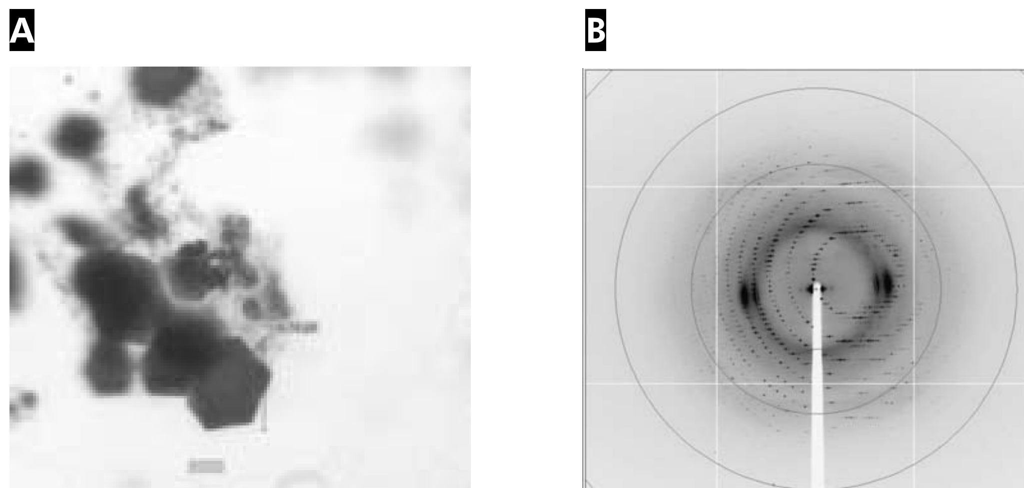


Figure 4. Protein crystals obtained using the NeXtal CubicPhase µplate. **A** Crystals of bacteriorodhopsin obtained in the NeXtal CubicPhase µplate. **B** Diffraction pattern of the bacteriorodhopsin crystals at 1.5Å.

Protocols for liquid-handling robots

Table 2 provides an overview of the different protocols available for NeXtal CubicPhase µplates. Please choose the suitable protocol carefully. Several variables such as those listed below need to be taken into account:

- Automated setup using a liquid-handling robot or manual setup with a multichannel pipet
- Volume (450 nl or 100 nl) suitable if using a liquid-handling robot
- Incubation temperature of crystallization plates (22°C or 18°C)
- Use of monoolein alone or monoolein together with 8% cholesterol
- Number of protein wells/reservoirs required
- Sponge phase experiments or lipidic cubic phase experiments (note that both protocol types are available for all plates/pipetting techniques)

Table 2. Protocol types and pipetting techniques

Cat. no.	Pipetting technique/volumes: Liquid-handling robot		Pipetting technique/volumes: Multichannel pipet	Incubation temp. (°C)		No. of wells			Protocol no.
	100 nl	450 nl	800 nl	22	18	1	2	3	
130803*		■	■	■		■			1, 2, 3, 4
130805†		■	■	■		■			1, 2, 3, 4
130814‡	■			■			■		5, 6
130816¶	■			■				■	7, 8
130822§		■	■		■	■			9, 10, 11, 12
130824**	■				■		■		13, 14
130826††	■				■			■	15, 16

* NeXtal CubicPhase 1w Mo µplate (100).

† NeXtal CubicPhase 2w Mo µplate (10).

‡ NeXtal CubicPhase 2w Mo µplate.

¶ NeXtal CubicPhase 3w Mo µplate (10).

§ NeXtal CubicPhase 1w Mo/C 8% µplate (10).

** NeXtal CubicPhase 2w Mo/C 8% µplate (10).

†† NeXtal CubicPhase 3w Mo/C 8% µplate (10).

Important Notes

- Incubation temperature is a very important experimental factor. The temperature should be maintained at 22°C for plates containing Mo (monoolein) alone and reduced to 18°C for plates containing a combination of monoolein and cholesterol.
- The composition of the crystallization solutions used to perform the screen is another key factor to take into account. NeXtal CubicPhase Suites have been tested and approved for compatibility with meso phase crystallization.

Protocol 1: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 1w Mo μplates (for 450 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 450 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 1w Mo μplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 μl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 μl water to each well of the same deep-well block.

  Note: This step can also be performed manually.

  Note: Diluted screening suite solution should be stored at room temperature.
- **Pipetting program 2**: This program setting is to enable transfer of 450 nl protein stock solution to each protein well on the NeXtal CubicPhase 1w Mo μplate.
- **Pipetting program 3**: This program setting is to enable transfer of 70 μl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 1w Mo μplate.

  Note: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable transfer of 450 nl of the diluted screening suite solution to each protein well on the NeXtal CubicPhase 1w Mo μplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 µl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 µl water to each well.**
3. **Unpack the NeXtal CubicPhase 1w Mo µplate and dispense 450 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo µplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 22°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 70 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo µplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 450 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 1w Mo µplate using the liquid-handling robot.**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 1w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 2: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 1w Mo μplates (for 450 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 450 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 1w Mo μplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable transfer of 450 nl of protein stock solution to each protein well on the NeXtal CubicPhase 1w Mo μplate.
- **Pipetting program 2**: This program setting is to enable transfer 70 μl of screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 1w Mo μplate.

  Note: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 450 nl from each reservoir well of the NeXtal CubicPhase 1w Mo μplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo μplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 1w Mo μplate and dispense 450 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo μplate using pipetting program 1.**
3. **Seal with tape.**
4. **Incubate at 22°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 70 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 450 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 1w Mo µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 1w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 3: Manual Setup of a Lipidic Cubic Phase Experiment using a Multichannel Pipet and NeXtal CubicPhase 1w Mo plates

Equipment and reagents to be supplied by the user

- Set of appropriate multichannel pipets capable of dispensing volumes given in the protocol
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Prepare a 1:7 dilution of screening suite solutions. Transfer 125 µl of each screening suite solution into the corresponding well of a new deep-well block. Add 875 µl of water to each well of the same deep-well block.**
3. **Unpack the NeXtal CubicPhase 1w Mo µplate and dispense 800 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo µplate.**
4. **Seal with tape.**
5. **Incubate at 22°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and membrane protein solution form the LCP during the incubation.
6. **Dispense 70 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo µplate.**
7. **Dispense 800 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 1w Mo µplate.**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 1w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature. Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 4: Manual Setup of a Sponge Phase Experiment Using a Multichannel Pipet and NeXtal CubicPhase 1w Mo µplates

Equipment and reagents to be supplied by the user

- Set of appropriate multichannel pipets capable of dispensing volumes given in the protocol
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 1w Mo µplate and dispense 800 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo µplate.**
3. **Seal with tape.**
4. **Incubate at 22°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.
5. **Dispense 70 µl of each screening suite solution into the corresponding reservoir well of the NeXtal CubicPhase 1w Mo µplate.**
6. **Dispense 800 nl screening suite solutions from the reservoir wells to the corresponding protein wells of the NeXtal CubicPhase 1w Mo µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 1w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 5: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 2w Mo µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 2w Mo µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 µl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 µl water to each well of the same deep-well block.

  Note: This step can also be performed manually.

  Note: Diluted solution can be stored at room temperature.
- **Pipetting program 2**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 2w Mo µplate.
- **Pipetting program 3**: This program setting is to enable transfer of 50 µl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 2w Mo µplate. **Note**: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable transfer of 100 nl of the diluted screening suite solution to each protein well on the NeXtal CubicPhase 2w Mo µplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 2w Mo µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 µl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 µl of water to each well.**
3. **Unpack the NeXtal CubicPhase 2w Mo µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 2w Mo µplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 22°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 50 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 2w Mo µplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 100 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 2w Mo µplate using the liquid-handling robot**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 2w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 6: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 2w Mo μplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 2w Mo μplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 2w Mo μplate.
- **Pipetting program 2**: This program setting is to enable transfer of 50 μl screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 2w Mo μplate.

  Note: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 100 nl from each reservoir well of the NeXtal CubicPhase 2w Mo μplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 2w Mo μplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 2w Mo μplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 2w Mo μplate using pipetting program 1.**
3. **Seal with tape.**
4. **Incubate at 22°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 50 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 2w Mo µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 100 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 2w Mo µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 2w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 7: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 3w Mo µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 3w Mo µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 µl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 µl water to each well of the same deep-well block.

  Note: This step can also be performed manually.

  Note: Diluted solution can be stored at room temperature.
- **Pipetting program 2**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 3w Mo µplate.
- **Pipetting program 3**: This program setting is to enable transfer of 50 µl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 3w Mo µplate.

  Note: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable transfer of 100 nl of the diluted screening suite to each protein well on the NeXtal CubicPhase 3w Mo µplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 3w Mo μplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 μl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 μl water to each well.**
3. **Unpack the NeXtal CubicPhase 3w Mo μplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 3w Mo μplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 22°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 50 μl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 3w Mo μplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 100 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 3w Mo μplate using the liquid-handling robot**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 3w Mo μplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 8: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 3w Mo µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 3w Mo µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 3w Mo µplate.
- **Pipetting program 2**: This program setting is to enable transfer of 50 µl screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 3w Mo µplate.

  Note: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 100 nl from each reservoir well of the NeXtal CubicPhase 3w Mo µplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 3w Mo µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 3w Mo µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 3w Mo µplate using pipetting program 1.**
3. **Seal with tape.**
4. **Incubate at 22°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 50 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 3w Mo µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 100 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 3w Mo µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 3w Mo µplate at 22°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 9: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 1w Mo/C 8% µplates (for 450 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 450 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 1w Mo/C 8% µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 µl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 µl water to each well of the same deep-well block.

  Note: This step can also be performed manually.

  Note: Diluted solution can be stored at room temperature.
- **Pipetting program 2**: This program setting is to enable transfer of 450 nl protein stock solution to each protein well on the NeXtal CubicPhase 1w Mo/C 8% µplate.
- **Pipetting program 3**: This program setting is to enable transfer of 70 µl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 1w Mo/C 8% µplate.

  Note: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable transfer of 450 nl of the diluted screening suite to each protein well on the NeXtal CubicPhase 1w Mo/C 8% µplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 µl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 µl of water to each well.**
3. **Unpack the NeXtal CubicPhase 1w Mo/C 8% µplate and dispense 450 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 18°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.
   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 70 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo/C 8% µplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 450 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate using the liquid-handling robot**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 1w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 10: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 1w Mo/C 8% µplates (for 450 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 450 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 1w Mo/C 8% µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable transfer of 450 nl protein stock solution to each protein well on the NeXtal CubicPhase 1w Mo/C 8% µplate.
- **Pipetting program 2**: This program setting is to enable transfer of 70 µl screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 1w Mo/C 8% µplate. **Note**: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 450 nl from each reservoir well of the NeXtal CubicPhase 1w Mo/C 8% µplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 1w Mo/C 8% µplate and dispense 450 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate using pipetting program 1.**
3. **Seal with tape.**

4. **Incubate at 18°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 70 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo/C 8% µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 450 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 1w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 11: Manual Setup of a Lipidic Cubic Phase Experiment using a Multichannel Pipet and NeXtal CubicPhase 1 w Mo/C 8% µplates

Equipment and reagents to be supplied by the user

- Set of appropriate multichannel pipets capable of dispensing volumes given in the protocol
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Prepare a 1:7 dilution of screening suite solutions. Transfer 125 µl of each screening suite solution into the corresponding well of a new deep-well block. Add 875 µl of water to each well of the same deep-well block.**
3. **Unpack the NeXtal CubicPhase 1w Mo/C 8% µplate and dispense 800 nl of protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
4. **Seal with tape.**
5. **Incubate at 18°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.
6. **Dispense 70 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
7. **Dispense 800 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
8. **Seal with tape.**

9. **Incubate the NeXtal CubicPhase 1w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 12: Manual Setup of a Sponge Phase Experiment Using a Multichannel Pipet and NeXtal CubicPhase 1w Mo/C 8% µplates

Equipment and reagents to be supplied by the user

- Set of appropriate multichannel pipets capable of dispensing volumes given in the protocol
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Procedure

1. **Equilibrate the NeXtal CubicPhase 1w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 1w Mo/C 8% µplate and dispense 800 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
3. **Seal with tape.**
4. **Incubate at 18°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.
5. **Dispense 70 µl of each screening suite solution into the corresponding reservoir well of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
6. **Dispense 800 nl screening suite solutions from the reservoir wells to the corresponding protein wells of the NeXtal CubicPhase 1w Mo/C 8% µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 1w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week is recommended.

Protocol 13: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 2w Mo/C 8% µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 2w Mo/C 8% µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 µl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 µl of water to each well of the same deep-well block. Diluted solution can be stored at room temperature.
  Note: This step can also be performed manually.
- **Pipetting program 2**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 2w Mo/C 8% µplate.
- **Pipetting program 3**: This program setting is to enable transfer of 50 µl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 2w Mo/C 8% µplate.
  Note: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable transfer of 100 nl of the diluted screening suite to each protein well on the NeXtal CubicPhase 2w Mo/C 8% µplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 2w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 µl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 µl of water to each well.**
3. **Unpack the NeXtal CubicPhase 2w Mo/C 8% µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 2w Mo/C 8% µplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 18°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 50 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 2w Mo/C 8% µplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 100 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 2w Mo/C 8% µplate using the liquid-handling robot**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 2w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 14: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 2w Mo/C 8% µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 2w Mo/C 8% µplate for the first time.

Pipetting programs

- **Pipetting program 1**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 2w Mo/C 8% µplate.
- **Pipetting program 2**: This program setting is to enable transfer of 50 µl screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 2w Mo/C 8% µplate. **Note**: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 100 nl from each reservoir well of the NeXtal CubicPhase 2w Mo/C 8% µplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 2w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 2w Mo/C 8% µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 2w Mo/C 8% µplate using pipetting program 1.**
3. **Seal with tape.**

4. **Incubate at 18°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 50 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 2w Mo/C 8% µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 100 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 2w Mo/C 8% µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 2w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 15: Setup of a Lipidic Cubic Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 3w Mo/C 8% µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1 x empty deep-well block (e.g., Corning, cat. no. 3961)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 4 pipetting programs. This step is only required before using the NeXtal CubicPhase 3w Mo/C 8% µplate for the first time

Pipetting programs

- **Pipetting program 1**: This program setting is to enable the following steps: (1) Preparation of a 1 in 7 dilution of screening suite solutions to obtain suitable pipetting volumes for the liquid-handling robot. (2) Transfer of 125 µl of each screening suite solution into the corresponding well of a new deep-well block. (3) Addition of 875 µl water to each well of the same deep-well block. Diluted solution can be stored at room temperature. **Note**: This step can also be performed manually.
- **Pipetting program 2**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 3w Mo/C 8% µplate.
- **Pipetting program 3**: This program setting is to enable transfer of 50 µl undiluted screening suite solution from each well of the original deep-well block to the corresponding reservoir on the NeXtal CubicPhase 3w Mo/C 8% µplate.

  Note: This step can also be performed manually.
- **Pipetting program 4**: This program setting is to enable Transfer 100 nl of the diluted screening suite to each protein well on the NeXtal CubicPhase 3w Mo/C 8% µplate.

Procedure

1. **Equilibrate the NeXtal CubicPhase 3w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.

2. **Using pipetting program 1, prepare a 1:7 dilution of the screening suite solutions using the liquid-handling robot. Transfer 125 µl of each screening suite solution to the corresponding well of an empty deep-well block. Add 875 µl of water to each well.**
3. **Unpack the NeXtal CubicPhase 3w Mo/C 8% µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 3w Mo/C 8% µplate using pipetting program 2.**
4. **Seal with tape.**
5. **Incubate at 18°C for 3 h.**

   Note: It is crucial to incubate the plate at this temperature.

   Monoolein and the protein solution form the LCP during the incubation.

6. **Dispense 50 µl of the undiluted screening suite solutions into the reservoir wells of the NeXtal CubicPhase 3w Mo/C 8% µplate using pipetting program 3.**
7. **Using pipetting program 4, dispense 100 nl of the diluted screening suite solutions into the corresponding protein well of the NeXtal CubicPhase 3w Mo/C 8% µplate using the liquid-handling robot**
8. **Seal with tape.**
9. **Incubate the NeXtal CubicPhase 3w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Protocol 16: Setup of a Sponge Phase Experiment Using a Liquid-Handling Robot and NeXtal CubicPhase 3w Mo/C 8% µplates (for 100 nl volumes)

Equipment and reagents to be supplied by the user

- Liquid-handling robot capable of pipetting 100 nl volumes
- 2 x sealing tapes (e.g., cat. no. 132105)
- 1x deep-well block filled with 96 x 1.5 ml screening suite solutions (QIAGEN recommends NeXtal DWB CubicPhase I Suite or NeXtal DWB CubicPhase II Suite supplied with the kit or available separately)

Things to do before starting

- Prepare the liquid-handling robot with 3 pipetting programs. This step is only required before using the NeXtal CubicPhase 3w Mo/C 8% µplate for the first time.

Pipetting program

- **Pipetting program 1**: This program setting is to enable transfer of 100 nl protein stock solution to each protein well on the NeXtal CubicPhase 3w Mo/C 8% µplate.
- **Pipetting program 2**: This program setting is to enable transfer of 50 µl screening suite solution from each well of the deep-well block to the corresponding reservoir on the NeXtal CubicPhase 3w Mo/C 8% µplate. **Note**: This step can also be performed manually.
- **Pipetting program 3**: This program setting is to enable transfer of 100 nl from each reservoir well of the NeXtal CubicPhase 3w Mo/C 8% µplate to the corresponding protein well.

Procedure

1. **Equilibrate the NeXtal CubicPhase 3w Mo/C 8% µplate to room temperature (15–25°C) by incubation for at least 10 min.**

   Note: Do not unpack the plate before reaction setup.
2. **Unpack the NeXtal CubicPhase 3w Mo/C 8% µplate and dispense 100 nl protein solution onto the dry monoolein in each protein well of the NeXtal CubicPhase 3w Mo/C 8% µplate using pipetting program 1.**
3. **Seal with tape.**

4. **Incubate at 18°C for 1 h.**

   Note: It is crucial to incubate the plate at this temperature.

5. **Dispense 50 µl of the 96 screening suite solutions into the reservoir wells of the NeXtal CubicPhase 3w Mo/C 8% µplate using pipetting program 2.**
6. **Using pipetting program 3, transfer 100 nl from each reservoir into the corresponding protein wells of the NeXtal CubicPhase 3w Mo/C 8% µplate.**
7. **Seal with tape.**
8. **Incubate the NeXtal CubicPhase 3w Mo/C 8% µplate at 18°C until crystals appear.**

   Note: It is crucial to incubate the plate at this temperature.

   Time for crystal formation varies; we recommend daily inspection during the first week.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Crystalline structure obtained that is not protein	
Monoolein crystals have formed	Check the incubation temperature. It should be at least 22°C for pure monoolein or 18°C for mixtures of monoolein with cholesterol.
Many small protein crystals obtained	
Slow down nucleation/crystal growth	Overlay the reservoir solution with an evaporation barrier of silicone oil, paraffin oil, or a mixture of both oils to slow down the vapor diffusion experiment or reduce the protein concentration.
LCP is not formed completely after incubation	
	Wait for at least a day and then check again. The time taken to pipet the protein solution onto the monoolein using the robot is too long. The water component of the protein solution is very important. Water is required to form the cubic phase. If the water evaporates during pipetting, a larger volume must be pipetted. For example, if the liquid-handling robot takes 15 min to pipet the entire plate, 450 nl water is needed to form the cubic phase. Protocols for manual setup require use of 800 nl volumes to compensate for the slower setup procedure.

Appendix: Optimization of *in meso* crystallization experiments

As with every crystallization experiment, there are multiple parameters that can be varied to optimize crystal formation and growth. These include:

- Protein concentration
- Protein purity and homogeneity
- Composition of crystallization solution
- Temperature

In addition to these, there are other parameters that can be varied for *in meso* crystallization experiments. These are discussed in the following sections.

The speed of equilibration between the protein well and the reservoir within the reaction chamber is crucial. Equilibration that is too fast can lead to formation of the lamellar phase too quickly. Too slow an equilibration can mean that the experiments do not reach the desired *in meso* phase (lamellar or LCP) at all, or only after a long period of time. The speed of equilibration can be influenced by the following factors:

- Ratio of the precipitant in the reservoir to that in the protein well (concentration of precipitant in reservoir to concentration of screening solution in protein well)
- Influence of the protein solution (e.g., added salts, detergents, or buffers)
- Inclusion of an equilibration barrier in the reservoir well to slow down evaporation (e.g., paraffin oil, silicone oil, or mixtures of both)

Visit www.qiagen.com/Crystallization/CubicPhase/FAQ for more information and visualization of different stages of the *in meso* crystallization experiment.

Certain chemicals (additives) favor the transition to sponge, cubic, and lamellar phases, whereas others can disturb this process. Additives in suitable concentrations can be added to the reaction setup in the protein well to enhance formation of the meso phases leading to crystallization success. See references, page 55, for more information.

As for all crystallization experiments, some conditions within the crystallization screen may not be compatible with your individual protein and lead to precipitation. These can only be determined experimentally.

In meso phases

Details on this can be found in the sections below.

Difference between sponge, cubic, and lamellar phase

Sponge, cubic, and lamellar are all meso phases. The difference between them is the ratio of aqueous solution to lipid, and therefore the degree of organization. The water to lipid ratio decreases from sponge phase to cubic phase to lamellar phase

Any of these phases might yield crystals. Please refer to appropriate protocols in this handbook for crystallization experimental setup in sponge phase and LCP.

Identifying the correct phase

Sponge and cubic phase are isotropic phases, while lamellar phase is anisotropic and will refract polarized light. The other difference between sponge and cubic phase is that sponge phase contains more water, and if crystals are obtained, they will tend form at the top of the drop.

Note that it is possible to obtain crystals in any of the meso phases.

During the vapor diffusion experiment, the equilibrium is shifted from the starting phase (e.g., LCP phase) to the target phase (e.g., lamellar phase). This endpoint of the equilibrium is another experimental variable, just like the protein concentration or the screening solution. An advantage of NeXtal CubicPhase μplates is that a combination of meso phase crystallization and vapor diffusion can be used, enabling testing of different phases as the water evaporates from the drop.

Protein requirements

Protein purity and concentration are key factors to consider in crystallization experiments. Details can be found in the following section.

Protein purity

In general, protein crystallization requires highly pure proteins, typically >95% (as evaluated by SDS PAGE or gel filtration chromatography). However, protein crystallization in lipidic cubic phase was reported to tolerate impurities (e.g., protein and lipid contaminants from the cell lysate) to up to 50% of the protein sample volume (8).

Protein in solution

The solution in which the protein is maintained is another experimental factor that needs to be tested to ensure protein stability in solution.

The protein solution may contain an unknown amount of detergents, salt, lipids, and maybe additional additives (e.g., DTT). The pH might also vary.

Note that organic solutions (e.g., ethanol) at a higher concentration are not compatible with the NeXtal CubicPhase system. Lower concentrations may be tolerated, however, this needs to be determined experimentally.

Protein concentration

As with any protein, the concentration to use for protein crystallization experiments depends on several factors, however, as a rule of thumb, crystallization in lipidic cubic phase requires lower concentration than used in standard vapor diffusion experiments. Concentrations as low as 2–5 mg/ml are a good starting point for experiments. If enough protein is available, it is also possible to use a much higher concentration (20 – 30 mg/ml).

It is important to remember that when concentrating the protein solution, the concentration of other components in the protein solution will also increase, (e.g., lipids). This is another factor to take into account.

In general, it is recommended to try several protein concentrations.

Crystallization plates

Extra wells in the NeXtal CubicPhase 2w and 3w μplates can be used to test various experimental conditions such as:

- Different protein concentrations
- Different screening solution dilutions
- Protein ligands/inhibitors
- Different detergents
- Different protein samples
- Duplicates (in 2-well μplates) or triplicates (in 3-well μplates)

NeXtal CubicPhase 2w and 3w μplates can also be used to set up control experiments (e.g., buffer only, no screening solution).

Temperature used for crystallization experiments

Temperature is the most important factor in crystallization experiments. In fact, meso phase crystallization is very temperature sensitive. The working range is 20–24°C; however, 22°C is the optimal temperature.

Below this range, Mo crystal-like structures will form which are difficult to distinguish from protein crystals. Also the chance of obtaining protein crystals will be severely reduced. Lower temperatures can be used if the lipidic phase

contains, for example, monoolein **and** 8% cholesterol. In this case, temperatures as low as 18°C can be used successfully

Screening solutions compatible with lipidic cubic phase crystallization

Some molecules may prevent proper formation of the lipidic cubic phase. Specific crystallization screens have been developed to work specifically with this system, and other screens are being evaluated for their compatibility. See www.qiagen.com/Crystallization/CubicPhase/FAQ for more information.

Working with diluted or undiluted screens

Two approaches can be taken with regards to screening solution — either undiluted screens or diluted screens may be used. See page 11 for details

The dilution of the screening solution is an experimental factor that needs to be optimized. As a starting point, we recommend using a 1 in 7 dilution, however, 1:1, 1:4, and 1:8 dilutions may also be tested.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Ordering Information

Product	Contents	Cat. no.
NeXtal CubicPhase Kit	For crystallization of membrane proteins: 2 x 96-well NeXtal Evolution µplates coated with monoolein and 2 deep-well blocks containing 96 x 1.5 ml solutions for crystallization of membrane proteins	130807
NeXtal CubicPhase 1w Mo µplate (10)	For crystallization of membrane proteins: 10 x 96-well NeXtal Evolution µplate with 1 protein well/reservoir, coated with monoolein	130803
NeXtal CubicPhase 1w Mo µplate (100)	For crystallization of membrane proteins: 100 x 96-well NeXtal Evolution µplates with 1 protein well/reservoir, coated with monoolein	130805
NeXtal CubicPhase 2w Mo µplate (10)	For crystallization of membrane proteins: 10 x 96-well (MRC) µplates with 2 protein wells/reservoirs, coated with monoolein	130814
NeXtal CubicPhase 3w Mo µplate (10)	For crystallization of membrane proteins: 10 x 96-well (IntelliPlate low profile) µplates with 3 protein wells/reservoirs, coated with monoolein	130816
NeXtal CubicPhase 1w Mo/C 8% µplate (10)	For crystallization of membrane proteins: 10 x 96-well NeXtal Evolution µplates coated with monoolein and cholesterol 8%	130822
NeXtal CubicPhase 2w Mo/C 8% µplate (10)	For crystallization of membrane proteins: 10 x 96-well (MRC) µplates with 2 protein wells/reservoirs, coated with monoolein and cholesterol 8%	130824
NeXtal CubicPhase 3w Mo/C 8% µplate (10)	For crystallization of membrane proteins: 10 x 96-well (IntelliPlate low profile) µplates with 3 protein wells/reservoirs, coated with monoolein and cholesterol 8%	130826

Ordering information

Product	Contents	Cat. no.
NeXtal DWB CubicPhase I Suite	96 x 1.5 ml solution for crystallization of membrane proteins	130928
NeXtal DWB CubicPhase II Suite	96 x 1.5 ml solution for crystallization of membrane proteins	130929
Related products		
NeXtal DWBlock Opti-Salt Suite	96 x 0.5 ml Opti-Salt Suite solution for rapid optimization of initial crystallization hits	130921
NeXtal Evolution µplate (10)	10 x 96 well plates for protein crystallization	132045
NeXtal Evolution µplate (100)	100 x 96 well plates for protein crystallization	132046
QIAgenes™ Kits — For optimized expression of recombinant human proteins in *E. coli* or insect and mammalian systems		
QIAgenes Expression Kit *E. coli*	QIAgenes Expression Construct *E. coli*, TNFα Positive Control, Penta·His Antibody (BSA-free), 4 Ni-NTA Spin Columns	Varies
QIAgenes Expression Kit Insect/Mammalia	QIAgenes Expression Construct Insect/Mammalia, CDC2 Positive Control, Penta·His Antibody (BSA-free), Ni-NTA Magnetic Agarose Beads	Varies
EasyXpress® Insect Kit II — For high yields of in vitro synthesized posttranslationally modified eukaryotic proteins		
EasyXpress Insect Kit II (20)	For 20 x 50 µl reactions: *Spodoptera frugiperda* insect cell extract, reaction buffers, in vitro transcription reaction components, RNase-Free water, gel-filtration columns, and positive-control DNA	32562
Ni-NTA — For purification of 6xHis-tagged proteins		
Ni-NTA Agarose (25)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210

Ordering Information

Product	Contents	Cat. no.
Ni-NTA Superflow Cartridges 5 x 5 ml	5 cartridges pre-filled with 5 ml Ni-NTA Superflow: for automated purification of His-tagged proteins using liquid chromatography systems	30761
Ni-NTA Superflow (25)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Ni-NTA Membrane Protein Kit — For standardized solubilization and purification of membrane proteins		
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
Detergent DM	2 g *n*-Decyl-ß-D-maltopyranoside (DM)	34114
Detergent DDM	2 g *n*-Dodecyl-ß-maltoside (DDM)	34124
Detergent OG	5 g *n*-Octyl-ß-D-glucopyranoside (OG)	34134
Detergent LDAO	2 g *N,N*-Dimethyldodecylamine-*N*-oxide (LDAO)	34144
Detergent NG	2 g *n*-Nonyl-ß-D-glucopyranoside (NG)	34154
Detergent FOS-choline-16	1 g FOS-choline-16 (FOS)	34164
Detergent Cymal 6	2 g Cymal 6 (Cy6)	34174

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