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Crystallization of Clusterin Mutant TL1 (Human Clu- Δ (214–238))

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Andreas Bracher¹, Patricia Yuste-Checa¹, Jerome Basquin^{2,3}, Elena Conti^{2,3}, F Ulrich Hartl¹

¹Department of Cellular Biochemistry, Max Planck Institute of Biochemistry, Martinsried, Germany;

²Department of Structural Cell Biology, Max Planck Institute of Biochemistry, Martinsried, Germany;

³Crystallization Crystallography Core Facility, Max Planck Institute of Biochemistry, Martinsried, Germany



Patricia Yuste-Checa

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We use this protocol and it's working

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Abstract

This protocol details how to set up crystallization experiments with Clusterin mutant TL1 (human Clu- Δ (214–238)) and to screen for crystallization conditions. Crystal harvesting and mounting for cryodiffraction experiments is also presented.

Materials

Equipment needed:

- Phoenix crystallization pipetting robot (Art Robbins Instruments) for 100-500 nl + 100-500 nl drop size crystallization screening in 96-well sitting-drop plates
- Tecan Evo 200 with 8 needles liquid handling workstation with integrated sealer for screen making and aliquoting
- MRC 2 lens crystallization 96-well plates (SWISSCI UVXPO-2LENS)
-  Polyolefin foil **HJ-BIOANALYTIK GmbH Catalog #900 360**
- Stereo light microscope with camera and optional polarizing filters
-  Mounted CryoLoop - 10 micron **Hampton Research Catalog #HR4-995**
-  CryoCaps with Data Matrix **Molecular Dimensions Catalog #MD7-401**
-  CrystalWand Magnetic, Straight **Hampton Research Catalog #HR4-729**

Note

The MPIB Crystallization Facility offers in-house users about 25 commercial and in-house crystallization screens which allow to test a wide variety of initial precipitant conditions, providing more than 2000 different mixtures of buffers, precipitating agents and additives. All of the screen reservoir solutions are formatted into MRC 2 lens crystallization 96-well plates (8 x 12 array, SWISSCI UVXPO-2LENS). The protein samples are dispensed in  0.1 µL -  0.5 µL sitting drops with a nanoliter Phoenix crystallization pipetting robot (Art Robbins Instruments). The plate is sealed with Polyolefin transparent adhesive film (hj-bioanalytik). The plates can be stored at 3 different temperatures ( 4 °C ,  12 °C and  19 °C). Crystallization drops are automatically imaged over four weeks at regular time intervals. Two in-house designed visualization systems are operated in temperature-controlled rooms (at  4 °C and  19 °C). These systems are based on the combination of an automated Leica optical stereomicroscope with a storage cabinet assisted by a robotic arm. All crystallization plates are barcoded. A dedicated web interface allows in-house users to analyze their imaged crystallization experiments remotely.

Buffers and reagents:

- Clusterin mutant TL1 with oligo-mannose N-glycans (see protocol "Clusterin purification from HEK293E cells", dx.doi.org/10.17504/protocols.io.bvvkn64w). Add α-mannosidase-I inhibitor kifunensine (MedChemExpress) dissolved in water to the HEK293E medium ( 16 micromolar (µM) final concentration) during protein expression in order to obtain TL1 with oligo-mannose N-glycans.
- The protein should be used for crystallization immediately after size exclusion chromatography and concentration by ultrafiltration. The particular stock of TL1 in  20 millimolar (mM) Na-acetate  5.0 ,



[M] 100 millimolar (mM) NaCl and [M] 1 millimolar (mM) EDTA yielding crystals for diffraction experiments had a concentration of 10.4 mg ml^{-1} ([M] 211 micromolar (μM)).

Note

The protein should be concentrated close to the solubility limit, typically $5\text{--}50 \text{ mg ml}^{-1}$ in a low-concentration buffer (~ [M] 20 millimolar (mM) buffering agent and [M] 50 millimolar (mM) - [M] 200 millimolar (mM) salt). This is to screen the influence on crystallization of pH and low ionic strength of the precipitant solution. If the protein needs some substance for stability such as a ligand or reducing agent, this should be included into the buffer.

- Nextal Classics crystallization screen solutions (Qiagen, Hilden, Germany).
- Index crystallization screen solutions (Hampton Research, Aliso Viejo, CA)
- JCSG-plus crystallization screen solutions (Molecular Dimensions, Sheffield, UK)
- glycerol
-  3 inch wide Crystal Clear Sealing Tape **Hampton Research Catalog #HR4-506**



Preparation of the Crystal Screening Plates

- 1 Reformat commercial screening solution into 2 ml deep well blocks using a Tecan EVO 200.
- 2 Transfer  70 μL of the respective crystallization solution into the reservoirs of a SWISSCI UVXPO-2LENS crystallization plate. 
- 3 Seal the plate with clear tape for intermediate storage.

Note

Incomplete factorial screens were initially introduced in the 1990ies to sparsely screen the crystallization condition space, i.e. precipitants (high concentrations of polymers, non-volatile alcohols and salts) are varied against co-precipitants (salts, polymers and non-volatile alcohols) and against buffers (pH) (Jancarik and Kim 1991). Some screens were derived from actual crystallization conditions, some were de-novo developed.

Crystallization Experiments

- 4 Remove the tape from the screening plates with dispensed reservoir solutions.
- 5 Clarify the protein solution by centrifugation at  16900 x g, 4°C, 00:10:00 . 
- 6 Fill Phoenix crystallization pipetting robot with  90 μL clarified supernatant. 
- 7 Insert first plate.
- 8 Dispense 200 nl protein solution and 200 nl reservoir per well. 
- 9 Seal wells with transparent adhesive film.

- 10 Repeat steps 7 to 9 for the remaining screens.
- 11 Incubate duplicate crystallization plates at 🌡️ 4 °C and 🌡️ 19 °C .



Note

Clusterin mutant TL1 crystals were observed after 25 days at 🌡️ 4 °C in Classics condition H3 and Index conditions E9 and G3.



Fig. 1 Microscopic image of “ugly” Clusterin mutant TL1 crystals in Classics condition H3. The overall image dimensions are approximate 0.75 x 1 mm.

Note

Crystals may have regular three-dimensional, thin plate or needle-shaped appearance with straight edges. Depending on the crystal lattice type, crystal may show optical birefringence that can be detected with two rotatable polarization filters above and below the sample in a stereo light microscope. Sometimes dust particles and textile fiber fragments can have the appearance of crystals. Often microcrystals and so-called spherulites are found which can be optimized by grid-screening around the crystallization condition, e.g. precipitant concentration vs. co precipitant concentration, and/or by micro-seeding with crushed crystals. Amorphous brownish precipitates and liquid-liquid phase separation (oil droplets) are negative outcomes. Less than 50% of the experiments should remain clear after equilibration, otherwise the protein sample concentration should be increased in subsequent crystallization screens. If crystallization still cannot be achieved, the protein should be further engineered to remove flexible parts. A sequence homolog of the protein of interest with different surface residues might allow better crystal packing.

Crystal Harvesting and Cryoprotection

- 12 Under a stereo light microscope at the appropriate temperature (here  4 °C), cut open the respective well by cutting the adhesive film with a scalpel. Cover the well with a cover slip between operations to prevent evaporation. 

Note

If the crystals form clusters so that crystals would overlap in the beam path during data collection at certain rotation angles, try to break the cluster apart by crushing the center with a sharp glass needle. Then use large monocrystalline fragments. Small satellite crystals are not a problem.

- 13 Manually fish the crystals with a cryoloop attached to the magnetic CrystalWand tool as a handle. Handling is easiest when the cryoloop lumen is slightly bigger than the crystal.

Note

If you find the drop covered with a transparent skin, try to first move the skin aside with a sharp glass needle. Otherwise, the skin tends to wrap around crystals. If the crystal sticks to the skin, try to rip the skin around the crystal. If the crystal sticks to the plastic bottom, try to break it loose with a sharp glass needle. For transfer, try to sling the loop around the crystal and move it to the surface. Turn the loop vertical so that the crystal rides on the nylon loop. Then pull the loop with the crystal out of the drop.



- 14 Transfer the crystal to a  0.5 μL droplet of the respective cryobuffer followed by 10-
 00:20:00 equilibration. This droplet can be pipetted into the neighboring lens in the same well.

20m

**Note**

1. Crystallization buffers containing 30-35% or more organic compounds usually work as cryobuffers, i.e. snap-frozen buffer shows clear optical appearance and no ice rings in the diffraction pattern. if the concentration is lower, prepare a cryobuffer with the precipitants at the same concentrations and additional cryoprotectant (glycerol, ethylene glycol, MPD, DMSO or sucrose) to reach 35% organics content. high salt conditions can be augmented with 15-20% cryoprotectant (LiCl and Li_2SO_4 also work as cryoprotectant). if you only have pre-formulated crystallization buffer from a commercial crystal screen, reduce the volume of a 100 μL aliquot by 15% under vacuum (e.g. in a speedvac device) and add  15 μL cryoprotectant to prepare a cryobuffer. this was done in the case of TL1 using glycerol as cryoprotectant. cryoprotectants tend to stick to pipette tips. check how you can achieve complete transfer.

2. If crystals dissolve in the cryobuffer, use a cryobuffer with slightly higher precipitant concentration, so-called "mother liquor". Reduce incubation time and droplet size.

3. If the concentration of the additional cryoprotectant is above 10%, prepare cryobuffers with intermediate cryoprotectant concentration by mixing cryobuffer with crystallization buffer and transfer stepwise to prevent cracking of crystals. A 50% intermediate buffer was used for TL1 crystals.

- 15 Fish the crystals as above and quickly dip the mounted crystal into liquid nitrogen. Keep submerged.

Note

There is enough time to check for the presence and correct orientation of the crystal (long axis along the spindle) in the loop before snap-cooling.

- 16 Under liquid nitrogen, transfer the mounted crystal into a basket sample holder/transport container.

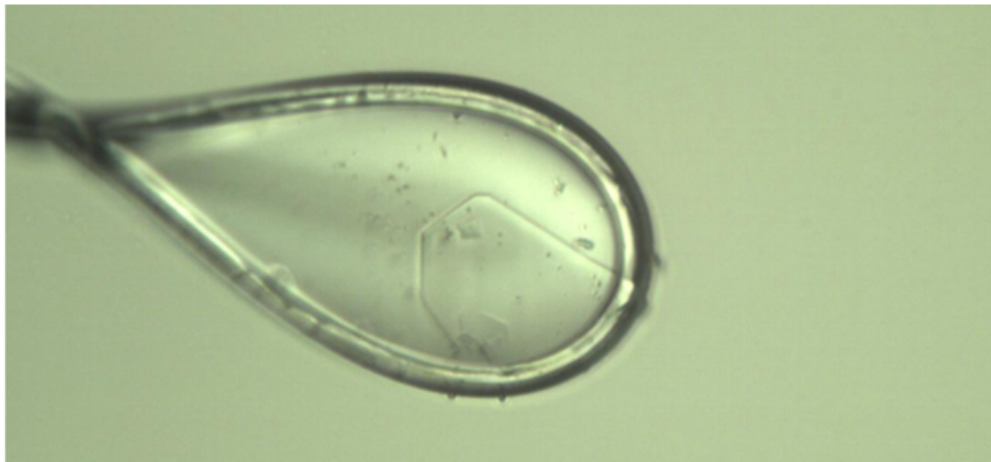


Fig. 2 Microscopic image of a plate-shaped crystal of Clusterin mutant TL1 from Classics condition H3 mounted in a nylon cryoloop (top view). This picture was acquired immediately before diffraction data collection. The crystal is surrounded by a thin film of clear cryobuffer. The spindle (not shown) is oriented horizontally. The diameter of the nylon loop is 10 μm . This crystal grew in addition to the needles in Fig. 1.

Protocol references

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