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Crystallization of TMEM55B 80-166 2CysMUT alone and in Complex with a RILPL1 391-403 Peptide

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We use this protocol and it's working

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Abstract

This protocol describes the set up and harvesting of Crystals from a 24-well hanging drop setup. Preceding to this larger scale setup, crystallography screens were conducted to determine a condition in which TMEM55B 80-166 2CysMUT alone and in complex with a RILPL1 391-403 peptide forms crystals. This conditions were then repeated in a larger volume, varying the pH, precipitant, and/or salt concentration to further optimize crystal formation to obtain optimal results at the Synchrotron. The best crystals were then harvested for X-ray crystallography at the synchrotron and subsequent structure determination.



Materials

Consumables

- 24 well plates (Greiner, 662150)
- MRC 2 well crystallization plates (Jena Bioscience, CPL-135)
- Siliconized glass cover slips 22 mm diameter (Jena Bioscience, CSL-107)
- Silicone grease medium viscosity (Jena Bioscience CGR-101)
- 3kDa MWCO concentrator tubes for microcentrifuge (Amicon Ultra 0.5ml, Ultracel, 3kDa MWCO)

Reagents

- Several commercial 96 well screens, i.e. PACT premierTM HT-96, MD1-36; JCSG-plusTM HT-96, MD1-40; Clear StrategyTM Screen I HT-96, MD1-31; Clear StrategyTM Screen II HT-96, MD1-32; Rigaku Wizard Classic 1 & 2 HT-96, MD15-W12-B; ProPlexTM HT-96, MD1-42; Structure Screen 1 + 2 HT-96, MD1-30
- Purified TMEM55B 80-166 2CysMUT
- RILPL1 391-403 peptide, custom made from Genscript, > 98% purity, reconstituted in Gel filtration buffer (20 mM Tris pH8.0 at 4°C, 150 mM NaCl, 1 mM DTT) to a concentration of 2 mg/ml. Note: The same buffer as TMEM55B 80-166 2CysMUT is in after final SEC purification step.
- Stock solutions of buffers, precipitants, and salts
- Glycerol
- Liquid nitrogen

Equipment

- Tabletop microcentrifuge (Ohaus or VWR MicroStar 12)
- Vibration-free fridge/incubator (RuMed, Rubart Apparate GmbH)
- Crystallography loops of various sizes (DT MicroLoopsTM, MiTiGen)
- Magnetic Cryovials (Jena Bioscience)
- Unipucks (Jena Bioscience, X-CC-110)
- Magnetic loop base pen
- Cryogenic storage dewar
- CX100 Cryo Express dry shipper (Jena Bioscience)
- Mosquito nanolitre pipetting robot (SPT Labtech)

Method

1 Screening for crystal formation

1.1 Pre-concentrate TMEM55B to a concentration between 1-2 mg/ml (this should be around 0.5-2 ml volume).

1.2 Determine its molar concentration and add a 2 x molar excess of the RILPL1 peptide to the TMEM55B construct.

1.3 Screening for our TMEM55B construct alone was performed with protein concentrated up to 10 mg/ml

Note

At this concentration, the construct will precipitate over time, so setting up the screen in a short amount of time is crucial.

1.4 Prepare MRC 2 96-well plates by transferring 70 µl of the screening solutions from the commercial 96-well stock to the plate, row by row using an 8-channel pipette.

1.5 Load the protein samples to the loading strip of the Mosquito nanolitre pipetting robot.

1.6 Place the MRC-02 plate in the pipetting robot.

1.7 Run program that mixes 100 nl reservoir with 100 nl of protein. Alternative 150 nl at a 1:1 ratio was used on occasion.

1.8 Incubate at 290K in a low vibration refrigerator.

Note

Set up a suitable control. This can be the concentrator flow through or a different protein. If crystals appear in the control drop, there is a great chance the crystal is not the desired protein/peptide complex.

Note

Unlike most other proteins, TMEM55B crystals took up to 21 days to occur and grow to maturity.

- 2 Setting up crystallography in 24 well hanging drop format
 - 2.1 Pre-concentrate TMEM55B to a concentration between 1-2 mg/ml (this should be around 0.5-2 ml volume).
 - 2.2 Determine its molar concentration and add a 2x molar excess of the RILPL1 peptide to the TMEM55B construct.
 - 2.3 Concentrate complex to a concentration of approximately 20-30 mg/ml in a tabletop centrifugation concentrator. Final volume is about 50-100 μ l.
 - 2.4 Prepare 1 ml of the different conditions in the individual wells of the 24 well plate, see Table 1 as example.

	1	2	3	4	5	6
A	0.15M KSCN 0.1M Tris pH8.7 18% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.6 18% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.5 18% PEG5000 / MME Orig. H11 Clear Strategy II	0.15M KSCN 0.1M Tris pH8.4 18% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.2 18% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.0 18% PEG5000 / MME
B	0.1M KSCN 0.1M Tris pH8.7 18% PEG5000 / MME	0.1M KSCN 0.1M Tris pH8.6 18% PEG5000 / MME	0.1M KSCN 0.1M Tris pH8.5 18% PEG5000 / MME	0.1M KSCN 0.1M Tris pH8.4 18% PEG5000 / MME	0.1M KSCN 0.1M Tris pH8.2 18% PEG5000 / MME	0.1M KSCN 0.1M Tris pH8.0 18% PEG5000 / MME
C	0.05M KSCN 0.1M Tris pH8.7 18% PEG5000 / MME	0.05M KSCN 0.1M Tris pH8.6 18% PEG5000 / MME	0.05M KSCN 0.1M Tris pH8.5 18% PEG5000 / MME	0.05M KSCN 0.1M Tris pH8.4 18% PEG5000 / MME	0.05M KSCN 0.1M Tris pH8.2 18% PEG5000 / MME	0.10M KSCN 0.1M Tris pH8.0 18% PEG5000 / MME
D	0.15M KSCN 0.1M Tris pH8.7 15% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.6 15% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.5 15% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.4 15% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.2 15% PEG5000 / MME	0.15M KSCN 0.1M Tris pH8.0 15% PEG5000 / MME

Table 1: Table of 24well hanging drop optimization of crystallography. The deposited structure of the complex was harvested from condition C3, a variation of the original condition from the 96 well plate.



2.5 Apply silicone grease on the rim of the 24 wells to allow air-tight sealing of the well with a glass cover slip.

2.6 Place a drop of 1 μ l of the reservoir on a siliconized glass cover slip.

2.7 Mix with 1 μ l of the concentrated protein/peptide complex.

Note

Set up a suitable control. This can be the concentrator flow through or a different protein. If crystals appear in the control drop, there is a great chance the crystal is not the desired protein/peptide complex.

2.8 Place glass cover slip inverted on the 24 well to close it, with the drop facing the inside of the reservoir. The silicone grease seals the well and prevents evaporation.

2.9 Incubate at 290K in a low-vibration fridge.

2.10 Crystals appear after a relatively long period of incubation, approximately 2 weeks after setup.

3 Harvesting of the crystals

Note

The crystals can be either harvested into magnetic cryovials that retain a small reservoir of liquid nitrogen or directly into the Unipuck. It is important that the crystal condition harvested is clearly identifiable to match the condition but also the purification with the respective crystal.

3.1 Prepare a cryoprotectant solution of the reservoir of a condition harvested from with 30% glycerol.

3.2 Prepare magnetic cryovials or the Unipucks by submerging them into a vessel filled with liquid nitrogen. Maintain nitrogen levels throughout the harvesting to avoid damaging the crystals by thawing.

3.3 Harvest the crystal with a crystal loop on the tip of the magnetic pen. Dip the crystal briefly into the cryoprotectant and transfer into the liquid nitrogen with the magnetic

cryovials or Unipucks.

Note

Cryoprotection of crystals can be done by various dipping times, or by adding a much larger volume of the cryoprotectant to the drop with the crystals. Small variations can influence the quality of a dataset obtained at the synchrotron.

- 3.4 Store the magnetic cryovials in a cryogenic dewar until transfer to a Unipuck.
- 3.5 If required, transfer the crystals from the magnetic cryovials to Unipucks for shipping.
- 3.6 Transfer the Unipucks with the samples to a pre-cooled dry shipper. Maintain liquid nitrogen until before shipping to the Synchrotron. Pour out all liquid nitrogen from the dry shipper for shipping.
- 3.7 Shipping dewars with crystals housed in pucks under cold conditions are shipped to synchrotrons using FEDEX. Typically the synchrotrons used are NSLS2 at Brookhaven, New York; and the Soleil synchrotron in Saclay, France.
- 4 Crystal handling and data collection at the Synchrotron
 - 4.1 Crystals are mounted at the beamline remotely by our local contacts at the synchrotron.
 - 4.2 We typically do not go the synchrotron for data collection, it is collected remotely using software specific for each beamline at the various synchrotron facilities.

Note

Data collection parameters will be found from the raw images that we have deposited along with the refined crystal structure in data repositories.