Monolith Protocol MO-P-065

MCU – MCUR1

Mitochondrial calcium homeostasis plays a key role in cellular physiology and regulates cell bioenergetics, cytoplasmic calcium signals and activation of cell death pathways. The mitochondrial inner membrane calcium uniporter (MCU) which mediates calcium uptake into mitochondria constitutes the pore-forming and calcium-conducting subunit of the uniporter complex (uniplex). The key regulator of mitochondrial calcium uniporter (MCUR1) which is required for calcium entry into mitochondrion, plays a direct role in uniporter-mediated calcium uptake via a direct interaction with MCU.

protein – protein interaction | membrane-bound coiled-coil protein | calcium | mitochondria

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A1. Target/Fluorescent Molecule

N-terminal domain of calcium uniporter protein, mitochondrial (MCU_{75-233})
uniprot.org/uniprot/Q8NE86

A2. Molecule Class/Organism

Transmembrane pore-forming and calcium-conducting subunit of mitochondrial calcium uniporter
Homo sapiens (Human)

A3. Sequence/Formula

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MVTVVYQNGL PVISVRLPSR RERCQFTLKP ISDSVGVFLR QLQEEDRGID RVAIYSPDGV RVAASTGIDL LLLDDFKLVI
NDLTYHVRPP KRDLLSHENA ATLNDVKTLV QQLYTTLCIE QHQLNKEREL IERLEDLKEQ LAPLEKVRIE GSW
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A4. Purification Strategy/Source

E. coli cells were grown to an optical density of OD_{600} = 0.5 and expression was induced with 1 mM isopropyl b-D-thiogalactoside (IPTG). Following incubation for 4 h at 37°C for *E. coli* C41 and 24 h at 12°C for ArcticExpress (DE3), cells were harvested by centrifugation. The cell pellet was resuspended in lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 4 mM $MgCl_2$, DNaseI, 1 mM phenylmethylsulfonyl fluoride (PMSF) and cOmplete EDTA-free Protease Inhibitor Cocktail (Roche), and subsequently lysed by sonication. Following centrifugation of the cell lysate to remove cell debris, the supernatant was loaded on a Ni-NTA Agarose column pre-equilibrated with buffer A (20 mM Tris, pH 7.6, 150 mM NaCl). Bound proteins were eluted with a two-step gradient including a step of 5% buffer B (20 mM Tris, pH 7.6, 500 mM NaCl, 0.5 M imidazole) followed by linear gradient of 5-100% buffer (B). Protein-containing fractions were dialyzed against buffer A and incubated with TEV protease for His-tag cleavage. Cleaved protein was separated from histidine-tagged TEV protease and proteolytic fragments, by reloading the sample on Ni-NTA. Fractions containing cleaved protein were pooled and purified to homogeneity by gel filtration on Superdex 75.

MCU_{75-233} and $MCUR1_{160-230}$ were purified under denaturing conditions by stirring the cell lysate in 6 M guanidine hydrochloride (Gua-HCl) at room temperature for 1 h. Following centrifugation, the supernatant was loaded on Ni-NTA Agarose column equilibrated with 20 mM Tris, pH 8.0, 300 mM NaCl, 6 M Gua-HCl. Bound proteins were eluted with a linear gradient of 0-0.5 M imidazole in the same buffer and refolded by dialysis against buffer containing 20 mM Tris, pH 7.6, 150 mM NaCl and 1 mM DTT. Following cleavage by TEV protease, the refolded protein was purified to homogeneity by applying a second Ni-NTA column followed by gel filtration on Superdex 75 as described above.

A5. Stock Concentration/Stock Buffer

2.2 mg/mL | 125 µM
50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% TWEEN® 20, 1 mM TCEP

A6. Molecular Weight/Extinction Coefficient

17.57 kDa
11,460 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% TWEEN® 20

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – NHS 2nd Generation (MO-L011, NanoTemper Technologies GmbH)
1* Dye RED-NHS 2nd Generation (10 µg) | 1* B-Column

A9. Labeling Procedure

1. Add 84 µL of Labeling Buffer (20 mM HEPES, pH 7.6, 150 mM NaCl) to 16 µL of 125 µM MCU_{75-233} to obtain 100 µL of a 20 µM solution.
2. Add 25 µL of DMSO to Dye RED-NHS 2nd Generation (10 µg) to obtain a ~600 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
3. Mix 10 µL of the 600 µM dye solution with 90 µL of Labeling Buffer to obtain 100 µL of a 60 µM dye solution (3x protein concentration).
4. Mix MCU_{75-233} and dye in a 1:1 volume ratio (200 µL final volume, 5% final DMSO concentration).
5. Incubate for 1 hour at room temperature in the dark.
6. In the meantime, remove the top cap of the B-Column and pour off the storage solution. Remove the bottom cap and place with adapter in a 15 mL tube.
7. Fill the column with dilution buffer and allow it to enter the packed resin bed completely by gravity flow. Discard the flow through collected. Repeat this step 3 more times.
8. Add 200 µL of the labeling reaction from step 4 to the center of the column and let sample enter the bed completely.
9. Add 300 µL of dilution buffer after the sample has entered and discard the flow through.
10. Place column in a new collection tube, add 600 µL of dilution buffer and collect the eluate.
11. Prepare 10 µL aliquots of labeled MCU_{75-233} (~2 µM) and store at -80°C.

A10. Labeling Efficiency

N/A

B1. Ligand/Non-Fluorescent Binding Partner

Mitochondrial calcium uniporter regulator 1 ($MCUR1_{160-230}$)
uniprot.org/uniprot/Q96AQ8

B2. Molecule Class/Organism

Membrane-bound coiled-coil protein
Homo sapiens (Human)

B3. Sequence/Formula

GAMGSRKLYF DTHALVCLLE DNGFATQQAE IIVSALVKIL EANMDIVYKD MVTKMQQEIT FQQVMSQIAN VKKD

B4. Purification Strategy/Source

See A4.

B5. Stock Concentration/Stock Buffer

3.63 mg/mL | 436 µM
50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% TWEEN® 20, 1 mM TCEP

B6. Molecular Weight/Extinction Coefficient

8.32 kDa
2,980 $M^{-1}cm^{-1}$ (ε_{280})

B7. Serial Dilution Preparation

1. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 436 µM $MCUR1_{160-230}$ solution into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
2. Prepare a 1:1 serial dilution by transferring 10 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 10 µL from tube **16** to get an equal volume of 10 µL for all samples.
3. Mix 4 µL of labeled MCU_{75-233} (~2 µM) with 196 µL of dilution buffer to obtain 200 µL of ~40 nM MCU_{75-233}.
4. Add 10 µL of labeled MCU_{75-233} (~40 nM) to each tube from **16** to **1** and mix by pipetting.
5. Incubate for 15 minutes at room temperature in the dark and then centrifuge at 13,000 rpm for 10 minutes before loading capillaries.

D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

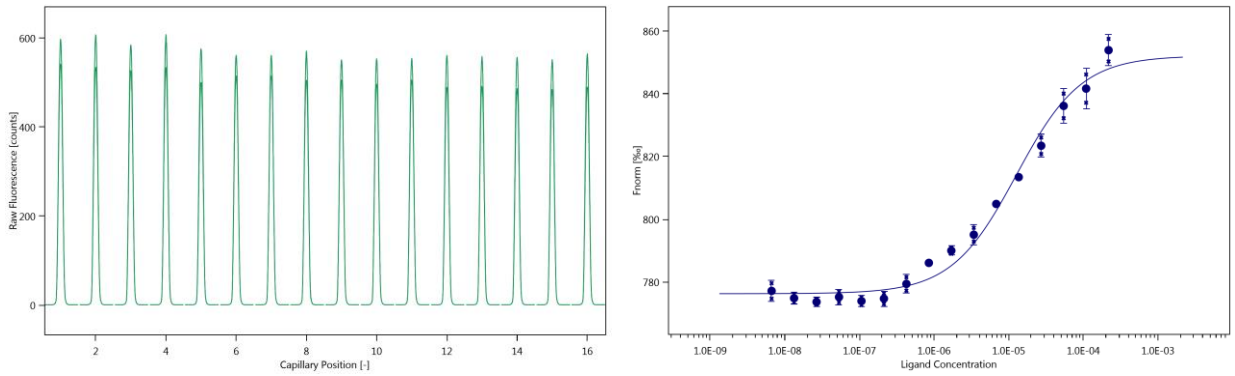
MO.Control v1.6 | MO.AffinityAnalysis v2.3 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% TWEEN® 20
20 nM MCU_{75-233} | 218 µM – 6.6 nM $MCUR1_{160-230}$ | 25°C | high MST power | 100% excitation power

D4. MST Results (Capillary Scan/Time Traces/Dose Response)

K_d = 12.7 ± 3.5 µM Adlaka et al., Structure 27, 464–475 (2019)



D5. Reference Results/Supporting Results

N/A

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