Monolith Protocol MO-P-033

Myosin Proximal S2 Subdomain – cMyBP-C

Cardiac myosin binding protein C (cMyBP-C) is specific to heart muscle where it binds to myosin and together with various other proteins provides the contractile motion of the cardiac sarcomers. Mutations in cMyBP-C are a cause of hypertrophic cardiomyopathy, a cardiac muscle disorder which is a leading cause of sudden death in people under age 35.

protein – protein interaction | myosin

A1. Target/Fluorescent Molecule

Human beta cardiac myosin, proximal S2 subdomain (839-968)
uniprot.org/uniprot/P12883

A2. Molecule Class/Organism

Motor protein
Homo sapiens (Human)

A3. Sequence/Formula

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RMVERREAIF CIQYNIRSFM NVKHWPWMKL FFKIKPLLKS AETEKEMATM KEEFQKIKDE LAKSEAKRKE LEEKMVTLLK
EKNDLQLQVQ AEAEGLADAE ERCDQLIKTK IQLEAKIKEV TERAEDEEEI NAELTAKKRK LEDECSELKK DIDDLELTLA
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A4. Purification Strategy/Source

The construct containing an N-terminal His-tag was expressed using the pET 21a expression system in E.coli according to the manufacturer (Qiagen, Germany). Cells were lysed, and protein was purified via a Ni-NTA column (GE) on an FPLC. Fractions were analyzed by SDS-PAGE, fractions with purest protein were pooled and then concentrated and buffer exchanged using Amicon centrifugal filter units.

A5. Stock Concentration/Stock Buffer

10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM TCEP and 100 mM KCl, pH 7.5

A6. Molecular Weight/Extinction Coefficient

N/A

A7. Dilution Buffer

10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 mM KCl, 0.05% TWEEN® 20, pH 7.5

A8. Labeling Strategy

Cysteine labeling with Cy5-maleimide

A9. Labeling Procedure

S2 subdomain of myosin was labeled using a standard cysteine labeling procedure using a Cy5-maleimide dye. A concentrated stock of Cy5 maleimide (2 - 10 mM) was made in DMSO, followed by addition of either 10-fold excess into protein stocks in a DTT-free binding buffer (10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM TCEP and 100 mM KCl, pH 7.5). This mixture was mixed thoroughly, wrapped in aluminum foil and kept on ice overnight. The labeling reaction was terminated by addition of a 10-fold molar excess of DTT. The labeled protein was dialyzed into 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100mM KCl, pH 7.5.

A10. Labeling Efficiency

The labeling efficiency was measured using an extinction coefficient of 250,000 $M^{-1}cm^{-1}$ at 650 nm for Cy5 and by calculating the protein concentration by the Bradford assay. Labeling efficiency for proximal S2 was estimated to be 95%.

B1. Ligand/Non-Fluorescent Binding Partner

Human cardiac myosin binding protein C, full length (cMyBP-C)
uniprot.org/uniprot/Q14896

B2. Molecule Class/Organism

Actin or myosin binding muscle protein
Homo sapiens (Human)

B3. Sequence/Formula

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MPEPGKKPVS AFSKKPRSVE VAAGSPAVFE AETERAGVKV RWQRGGSDIS ASNKYGLATE GTRHTLTVRE VGPADQGSYA
VIAGSSKVKF DLKVIEAEKA EPMLAPAPAP AEATGAPGEA PAPAAELGES APSPKGSSSA ALNGPTPGAP DDPIGLFVMR
PQDGEVTVGG SITFSARVAG ASLLKPPVVK WFKGKWVDLS SKVGQHLQLH DSYDRASKVY LFELHITDAQ PAFTGSYRCE
VSTKDKFDCS NFNLTVHEAM GTGDLDLLSA FRRTSLAGGG RRISDSHEDT GILDFSSLLK KRDSFRTPRD SKLEAPAEED
VWEILRQAPP SEYERIAFQY GVTDLRGMLK RLKGMRRDEK KSTAFQKKLE PAYQVSKGHK IRLTVELADH DAEVKWLKNG
QEIQMSGSKY IFESIGAKRT LTISQCSLAD DAAYQCVVGG EKCSTELFVK EPPVLITRPL EDQLVMVGQR VEFECEVSEE
GAQVKWLKDG VELTREETFK YRFKKDGQRH HLIINEAMLE DAGHYALCTS GGQALAELIV QEKKLEVYQS IADLMVGAKD
QAVFKCEVSD ENVRGVWLKN GKELVPDSRI KVSHIGRVHK LTIDDVTPAD EADYSFVPEG FACNLSAKLH FMEVKIDFVP
RQEPPKIHLD CPGRIPDTIV VVAGNKLRLD VPISGDPAPT VIWQKAITQG NKAPARPAPD APEDTGDSDE WVFDKKLLCE
TEGRVRVETT KDRSIFTVEG AEKEDEGVYT VTVKNPVGED QVNLTVKVID VPDAPAAPKI SNVGEDSCTV QWEPPAYDGG
QPILGYILER KKKKSYRWMR LNFDLIQELS HEARRMIEGV VYEMRVYAVN AIGMSRPSPA SQPFMPIGPP SEPTHLAVED
VSDTTVSLKW RPPERVGAGG LDGYSVEYCP EGCSEWVAAL QGLTEHTSIL VKDLPTGARL LFRVRAHNMA GPGAPVTTTE
PVTVQEILQR PRLQLPRHLR QTIQKKVGEP VNLLIPFQGK PRPQVTWTKE GQPLAGEEVS IRNSPTDTIL FIRAARRVHS
GTYQVTVRIE NMEDKATLVL QVVDKPSPPQ DLRVTDAWGL NVALEWKPPQ DVGNTELWGY TVQKADKKTM EWFTVLEHYR
RTHCVVPELI IGNGYYFRVF SQNMVGFSDR AATTKEPVFI PRPGITYEPP NYKALDFSEA PSFTQPLVNR SVIAGYTAML
CCAVRGSPKP KISWFKNGLD LGEDARFRMF SKQGVLTLEI RKPCPFDGGI YVCRATNLQG EARCECRLEV RVPQ
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B4. Purification Strategy/Source

The DNA coding full-length human cMyBP-C protein was cloned from a vector obtained from Open Biosystems into pFastBac1 transfer plasmid (Invitrogen). The resulting vector encoding full-length MyBP-C was used for the site-specific transposition of an expression cassette into a bacmid. Isolation of recombinant bacmid DNA, transfection of Sf9 cells (ThermoFisher) and isolation of high titer viral stocks were carried out according to manufacturer's instructions. Infected cells were lysed, and the protein was purified via an N-terminal FLAG tag epitope. Protein quality was checked by SDS-PAGE. Purified fractions of the protein were dialyzed in binding buffer. The dialyzed protein was dephosphorylated and subsequently phosphorylated at the M domain.

Phosphorylated MyBP-C was then centrifuged at 350,000 × g for 15 min to get rid of any aggregates before using for Microscale Thermophoresis. Protein concentrations were quantified by the Bradford assay. Fresh reparations of MyBP-C were used (less than 3 days old), and all binding experiments were performed at 23°C.

B5. Stock Concentration/Stock Buffer

10 mM Imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100 mM KCl, pH 7.5

B6. Molecular Weight/Extinction Coefficient

N/A

B7. Serial Dilution Preparation

A 16-step 2:1 1:1 serial dilution of cMyBP-C was prepared in dilution buffer. The starting concentration was 150 µM. 5 µL of the highest concentration were added to tube 1. 5µl of dilution buffer were pipetted to tubes 2-16 of the 1:1 serial dilution. Starting from tube 2, 10 µL (from the concentrated protein stock) were transferred from one tube to the next dilution step and mixed. From the last tube, 10 µL were discarded to have a final volume of 5 µL in all tubes of the 1:1 serial dilution. Care was taken to maintain 0.05% tween-20 in all dilutions starting from tube 1. After the 1:1 serial dilution was prepared, 1uL from a 250 nM target stock solution (Cy5-labeled proximal S2) was added to each dilution step leading to a final target concentration of 50 nM target and a highest ligand concentration of 150 µM. The tubes were incubated for 30 – 60 min before loading of the 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

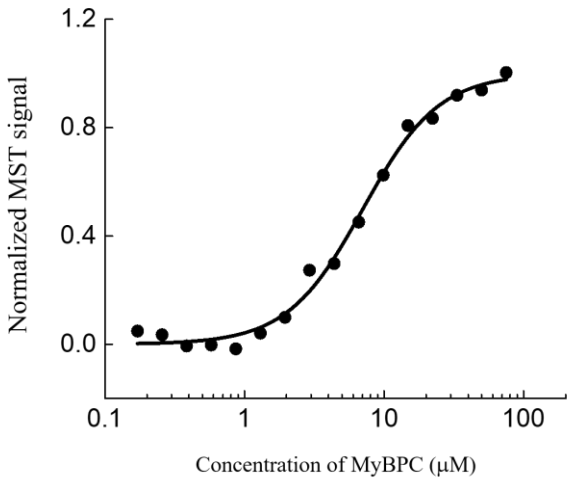
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100 mM KCl, pH 7.5
50 nM myosin S2 subunit | 150 µM – 340 nM cMyBP-C | 23°C | high MST power | 30% excitation power

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

K_d = 7 µM Nag and Trivedi, Nat. Struct. Mol. Biol. 24 (2017) 525–533



D5. Reference Results/Supporting Results

K_d = 5 µM Isothermal Titration Calorimetry (ITC)
Gruen and Gautel, J Mol Biol 286 (1999) 933-949

E. Contributors

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