Monolith Protocol MO-P-050

Affibody – IgM-Fc (stoichiometry)

Affibodies are small (~6 kDa) recombinant proteins which bind in a monovalent fashion to their target with high specificity and affinity. The affibody used here binds to membrane-associated IgM-BCRs (mIgM-BCR). The B cell antigen receptor (BCR) consists of a plasma membrane–bound antibody that is associated with a pair of signaling proteins. Antigen binding to the BCR stimulates B cells to differentiate into antibody-secreting cells. Since mIgM-BCRs are arranged as homodimers, it is expected that two affibodies bind to a single IgM molecule.

protein – protein interaction | affibody | IgM | stoichiometry

---

A1. Target/Fluorescent Molecule

Anti-IgM Affibody[1] Molecule

A2. Molecule Class/Organism

Anti-IgM affibody
Escherichia coli

A3. Sequence/Formula

N/A

A4. Purification Strategy/Source

Recombinant protein, produced in *E. coli* according to the manufacturer (Affibody, Bromma, Sweden)
Abcam Germany
ab36088

A5. Stock Concentration/Stock Buffer

5 µM
PBS, pH 7.4

[1] Affibody® affinity ligands are unique research reagents, produced using innovative protein-engineering technologies. They are small, simple proteins composed of a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A. Protein A is a surface expressed protein of *Staphylococcus aureus*. This scaffold has excellent features as affinity ligands and can be designed to bind with high affinity to any given target protein in a monovalent manner. The domain consists of 58 amino acids, 13 of which are randomized to generate Affibody® libraries with a large number of ligand variants. Thus, the libraries consist of a multitude of protein ligands with an identical backbone and variable surface-binding properties. In function, Affibody® Molecules are like monoclonal antibodies in the sense that they recognize a single epitope. Compared to conventional antibodies, the most striking dissimilarity of Affibody® molecules is their small size and monovalent binding. Affibody® molecules have a molecular weight of 6 kDa, compared to the molecular weight of antibodies, which is ~150 kDa.

A6. Molecular Weight/Extinction Coefficient

7 kDa

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$

A8. Labeling Strategy

Maleimide-labeling[2] with AberriorStar635P (Aberrior GmbH, Göttingen, Germany)

A9. Labeling Procedure

1. Resuspend the AberriorStar635P dye in DMSO to a final concentration of 10 mg/mL.
2. Incubate 70 nmol of the affibody for 60 min at room temperature (RT) in 500 µL reducing buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10 mM EDTA, 100 mM TCEP).
3. Do a buffer exchange using NAP5 desalting columns (GE Healthcare) to an argon-degassed buffer containing 50 mM HEPES pH 7.6, 150 mM NaCl, 10 mM EDTA.
4. Mix the affibody with a ~5-10 molar excess of the maleimide fluorophore and incubate at RT for 60 min.
5. To quench the remaining reactive fluorophores, add 50 mM of cysteamine chloride and incubate for 15 min at RT.
6. Remove free dye using a size exclusion column or a self-packed desalting column with G-25 superfine media (GE Healthcare, USA).
7. Measure the degree of labeling (DOL).

A10. Labeling Efficiency

DOL = 0.95 - 0.99

B1. Ligand/Non-Fluorescent Binding Partner

Recombinant Human LMW IgM-Fc domain (monomeric)
uniprot.org/uniprot/P01871

B2. Molecule Class/Organism

IgM Fc molecule
Homo sapiens (Human)

[2] The anti-IgM affibody contains a single C-terminal cysteine.

B3. Sequence/Formula

```
VIAELPPKVS VFVPPRDGFF GNPRKSKLIC QATGFSPRQI QVSWLREGKQ VGSGVTTDQV QAEAKESGPT TYKVTSTLTI
KESDWLGQSM FTCRVDHRGL TFQQNASSMC VPDQDTAIRV FAIPPSFASI FLTKSTKLTC LVTDLTTYDS VTISWTRQNG
EAVKTHTNIS ESHPNATFSA VGEASICEDD WNSGERFTCT VTHTDLPSPL KQTISRPKGV ALHRPDVYLL PPAREQLNLR
ESATITCLVT GFSPADVFVQ WMQRGQPLSP EKYVTSAPMP EPQAPGRYFA HSILTVSEEE WNTGETYTCV VAHEALPNRV
TERTVDKSTG AHHHHHH
```

B4. Purification Strategy/Source

Purified by Immobilized Metal Affinity Chromatography (IMAC) according to the manufacturer's protocol
Absolute antibody, Oxford, UK
Pr00108-15.5

B5. Stock Concentration/Stock Buffer

18.8 µM
PBS with preservative (0.02% Proclin 300)

B6. Molecular Weight/Extinction Coefficient

74.5 kDa
85,080 $M^{-1}cm^{-1}$ (ε_{280})

B7. Serial Dilution Preparation

1. Prepare a PCR-rack with 16 PCR tubes. Mix 3.7 µL of 18.8 µM IgM-Fc with 16.3 µL of dilution buffer in tube **1** to obtain 20 µL of a 3.5 µM solution. 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. Add 10 µL of labeled affibody (10 nM) to each tube from **16** to **1** and mix by pipetting.
4. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

Dilution series over small concentration range to precisely determine stoichiometry

1. Prepare a PCR-rack with 16 new PCR tubes. Mix 6 µL of 18.8 µM IgM-Fc with 34 µL of dilution buffer in tube **1** to obtain 40 µL of a 2.8 µM solution. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
2. Prepare a 3:1 serial dilution by transferring 30 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 30 µL from tube **16** to get an equal volume of 10 µL for all samples.
3. Mix 100 µL of labeled affibody (10 nM) and 100 µL of unlabeled affibody (790 nM) to obtain 200 µL of a 400 nM affibody solution.[3]
4. Add 10 µL of labeled affibody (800 nM) to each tube from **16** to **1** and mix by pipetting.
5. Centrifuge samples for 10 min at RT before loading capillaries.

[3] To achieve saturating conditions, but not to saturate the detector of the Monolith, labeled affibody needs to be mixed with unlabeled affibody.

D1. MST System/Capillaries

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

D2. MST Software

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

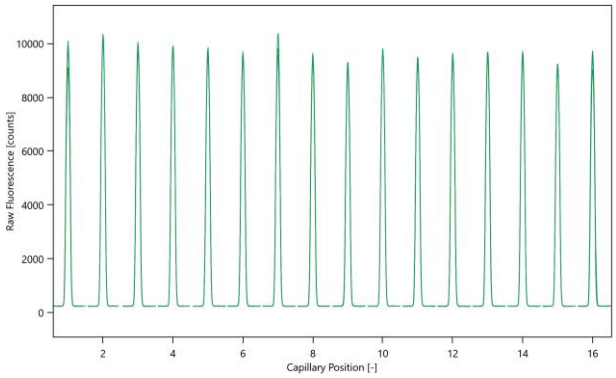
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

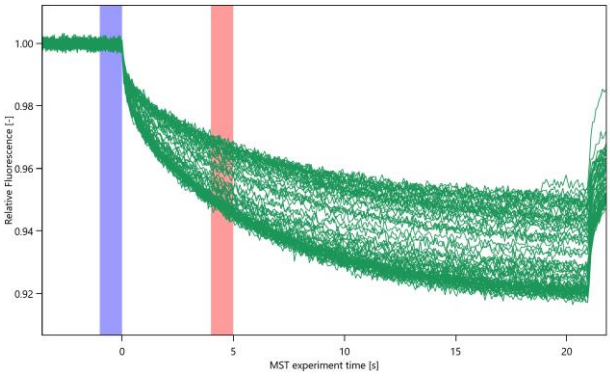
50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$
5 nM affibody-Star635P | 1750 nM – 0.05 nM IgM-Fc | medium MST power | 20% excitation power

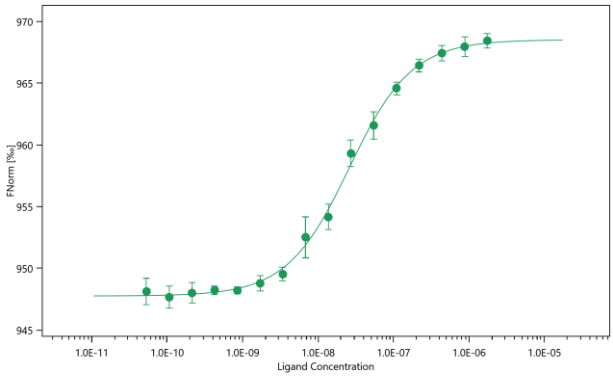
50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$
5 nM (labeled) + 395 nM (unlabeled) affibody-Star635P | 1400 nM – 18 nM IgM-Fc | medium MST power | 20% excitation power

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

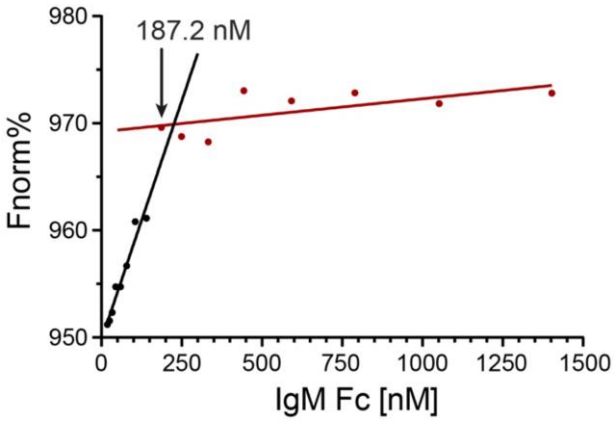
K_d = 28 ± 0.4 nM Gomes de Castro et al., Nature Communications 10, 820 (2019)







Intersection of linear fits[4] at ~187.2 nM of IgM-Fc (400 nM of affibody) → 1 : 2.1 stoichiometry



D5. Reference Results/Supporting Results

N/A

E. Contributors

Shama Sograte Idrissi[5], Felipe Opazo[5], Teresia Hallström[6]

[4] Linear regression lines of the saturated and non-saturated data points were set manually.

[5] Center for Biostructural Imaging of Neurodegeneration, University Medical Center Göttingen, Göttingen, Germany
[6] NanoTemper Technologies GmbH, München, Germany | nanotempertech.com