Monolith Protocol MO-P-011

Protein A – Herceptin

Protein A is a cell surface receptor found in the cell wall of the bacteria *Staphylococcus aureus*. It's frequent use in biochemical research derives from its ability to bind the Fc region of most immunoglobulins. Herceptin (Trastuzumab) is a monoclonal antibody used to treat breast cancer.

protein – protein interaction | antibody

A1. Target/Fluorescent Molecule

Protein A
uniprot.org/uniprot/P38507

A2. Molecule Class/Organism

Immunoglobulin-binding protein
Staphylococcus aureus

A3. Sequence/Formula

```
AQHDEAQQNA FYQVLNMPNL NADQRNGFIQ SLKDDPSQSA NVLGEAQKLN DSQAPKADAQ QNKFNKDQQS AFYEILNMPN
LNEEQRNGFI QSLKDDPSQS TNVLGEAKKL NESQAPKADN NFNKEQQNAF YEILNMPNLN EEQRNGFIQS LKDDPSQSAN
LLAEAKKLNE SQAPKADNKF NKEQQNAFYE ILHLPNLNEE QRNGFIQSLK DDPSQSANLL AEAKKLNDAQ APKADNKFNK
EQQNAFYEIL HLPNLTEEQR NGFIQSLKDD PSVSKEILAE AKKLNDAQAP KEEDNNKPGK EDGNKPGKED GNKPGKEDNK
KPGKEDGNKP GKEDNKKPGK EDGNKPGKED GNKPGKEDGN KPGKEDGNKP GKEDGNGVHV VKPGDTVNDI AKANGTTADK
IAADNKLADK NMIKPGQELV VDKKQPANHA DANKAQALPE T
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A4. Purification Strategy/Source

Recombinant
anitkoerper-online
ABIN1067599

A5. Stock Concentration/Stock Buffer

5 mg/mL | 122 µM
H_2O

A6. Molecular Weight/Extinction Coefficient

42 kDa
6,930 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

Phosphate-buffered saline (PBS, pH 7.4), 0.05% TWEEN® 20

A8. Labeling Strategy

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

A9. Labeling Procedure

1. Add 45 µL of Labeling Buffer NHS to 5 µL of 122 µM Protein A to obtain 50 µL of a 12 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 3 µL of the 600 µM dye solution with 47 µL of Labeling Buffer NHS to obtain 50 µL of a 36 µM dye solution (3x protein concentration).
4. Mix Protein A and dye in a 1:1 volume ratio (100 µL final volume, 3% final DMSO concentration).
5. Incubate for 30 minutes 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 100 µL of the labeling reaction from step 3 to the center of the column and let sample enter the bed completely.
9. Add 600 µL of dilution buffer after the sample has entered and discard the flow through.
10. Place column in a new collection tube, add 300 µL of dilution buffer and collect the eluate.
11. Keep the labeled protein A (~2 µM) on ice in the dark.

A10. Labeling Efficiency

Measurement of protein concentration and degree of labeling (DOL) using a NanoDrop™:
nanotempertech.com/dol-calculator

Absorbance A_{205}	3.70	Protein concentration	2.77 µM
Absorbance A_{650}	0.499	Degree-of-labeling (DOL)	0.92

B1. Ligand/Non-Fluorescent Binding Partner

Herceptin (Trastuzumab)

B2. Molecule Class/Organism

Monoclonal antibody

B3. Sequence/Formula

Heavy chain

```
EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPTNGYTRY ADSVKGRFTI SADTSKNTAY
LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS
WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPG
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Light chain

```
DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS RFSGSRSGTD FTLTISSLQP
EDFATYYCQQ HYTTPPTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ
ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC
```

B4. Purification Strategy/Source

N/A

B5. Stock Concentration/Stock Buffer

120 mg/mL | 825 µM

B6. Molecular Weight/Extinction Coefficient

145.5 kDa

B7. Serial Dilution Preparation

1. Add 145 µL of dilution buffer to 20 µL of 825 µM Herceptin to obtain 165 µL of a 100 µM solution.
2. Mix 2 µL of 100 µM Herceptin with 198 µL of dilution buffer to obtain 200 µL of a 1 µM Herceptin solution.
3. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 1 µM Herceptin solution into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
4. 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.
5. Mix 2 µL of labeled protein A with 38 µL of dilution buffer to obtain 40 µL of ~100 nM protein A.
6. Mix 4 µL of ~100 nM labeled protein A with 196 µL of dilution buffer to obtain 200 µL of ~2 nM protein A.
7. Add 10 µL of labeled protein A (~2 nM) to each tube from **16** to **1** and mix by pipetting.
8. Incubate for 5 minutes[1] at room temperature in the dark before loading capillaries.

[1] Longer incubation can lead to aggregation due to the multiple IgG-binding sites of Protein A.

B8. SD-Test

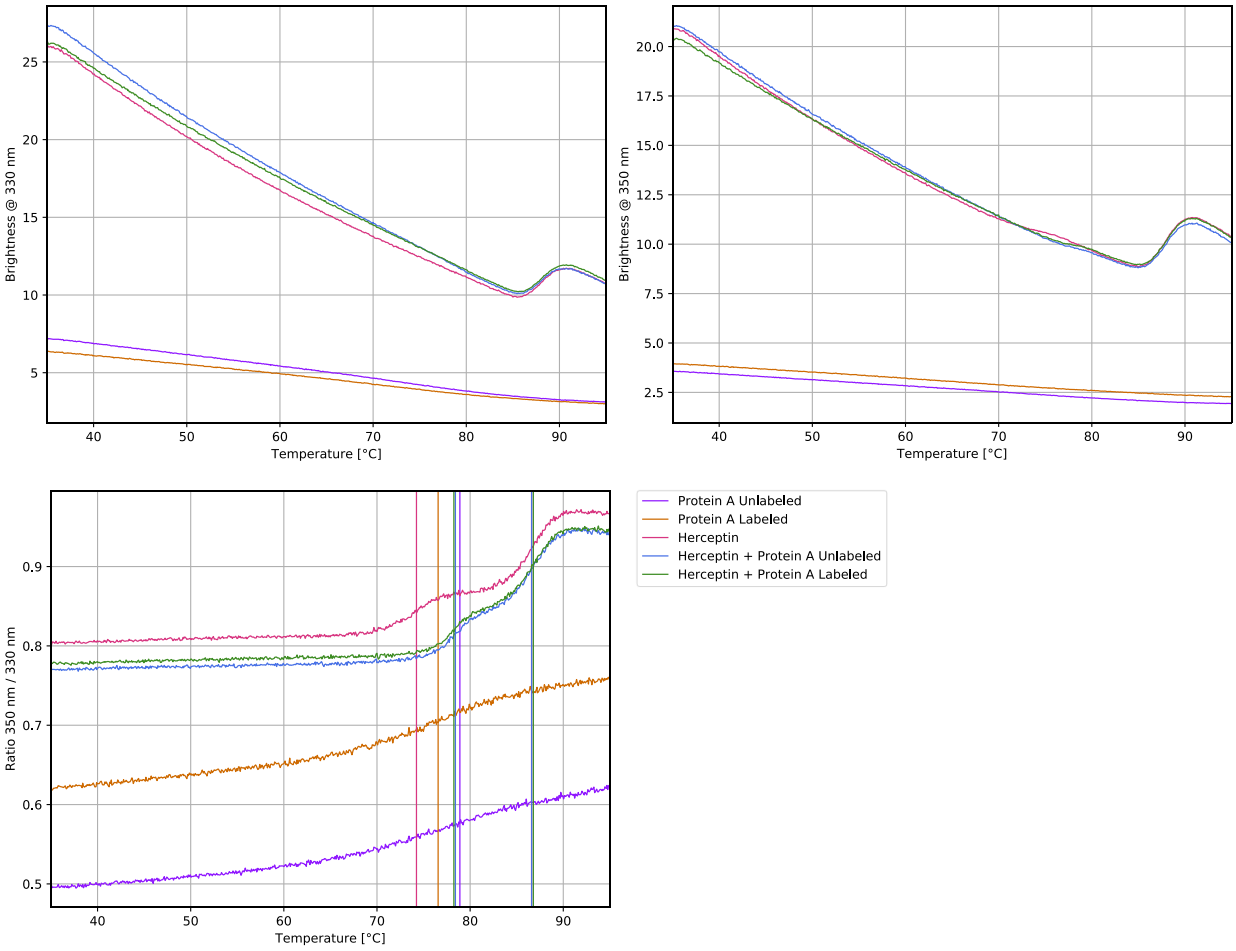
1. When checking for specificity of ligand-induced changes in initial fluorescence with the SD-Test, do **not** centrifuge[2] the remainder of tubes **1** to **3** and **14** to **16**.
2. Prepare the SD-mix: Dilute 400 µL of 10% SDS and 40 µL of 1 M DTT in 560 µL water to obtain a solution containing 4% SDS and 40 mM DTT.
3. Transfer 7 µL of the SD-mix to six PCR tubes.
4. Add 7 µL from the remainder of tubes **1** to **3** and **14** to **16** to the tubes containing 7 µL SD-mix. Mix well by pipetting.
5. Place samples on a heat block set to 95°C for 5 minutes to denature the protein, then allow to cool at 25°C for 10 minutes before loading into capillaries.

[2] Centrifugation will cause larger complexes of Herceptin and Protein A to precipitate out of the aqueous solution.

C. Applied Quality Checks

Validation of structural integrity and functionality of labeled protein A and Herceptin using Tycho NT.6:
nanotempertech.com/tycho

Protein A (UL)	1 µL of 122 µM protein A + 40 µL of dilution buffer	T_i = 78.9°C
Protein A (L)	10 µL of B-Column eluate (~2.9 µM)	T_i = 76.7°C
Herceptin	5 µL of 1 µM Herceptin + 5 µL of dilution buffer	$T_{i,1}$ = 74.2°C | $T_{i,2}$ = 86.6°C
Herceptin + protein A (UL)	5 µL of 1 µM Herceptin + 5 µL of 2.9 µM protein A	$T_{i,1}$ = 78.4°C | $T_{i,2}$ = 86.6°C
Herceptin + protein A (L)	5 µL of 1 µM Herceptin + 5 µL of B-Column eluate (~2.9 µM)	$T_{i,1}$ = 78.3°C | $T_{i,2}$ = 86.8°C



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

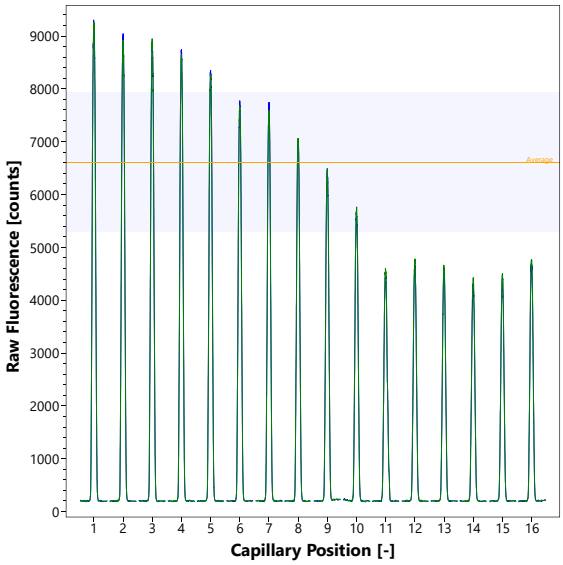
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

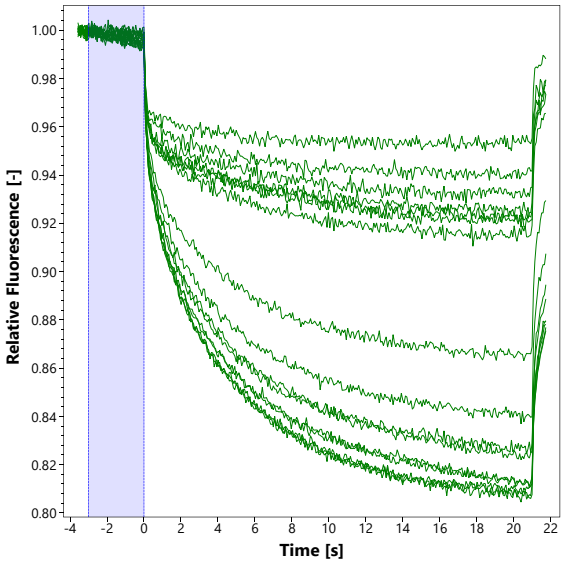
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

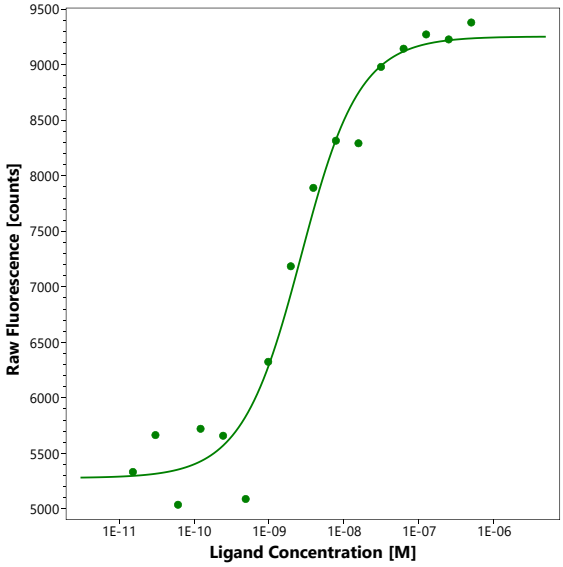
Phosphate-buffered saline (PBS, pH 7.4), 0.05% TWEEN® 20
1 nM protein A | 500 nM – 15 pM Herceptin | 25°C | medium MST power | 20% excitation power

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

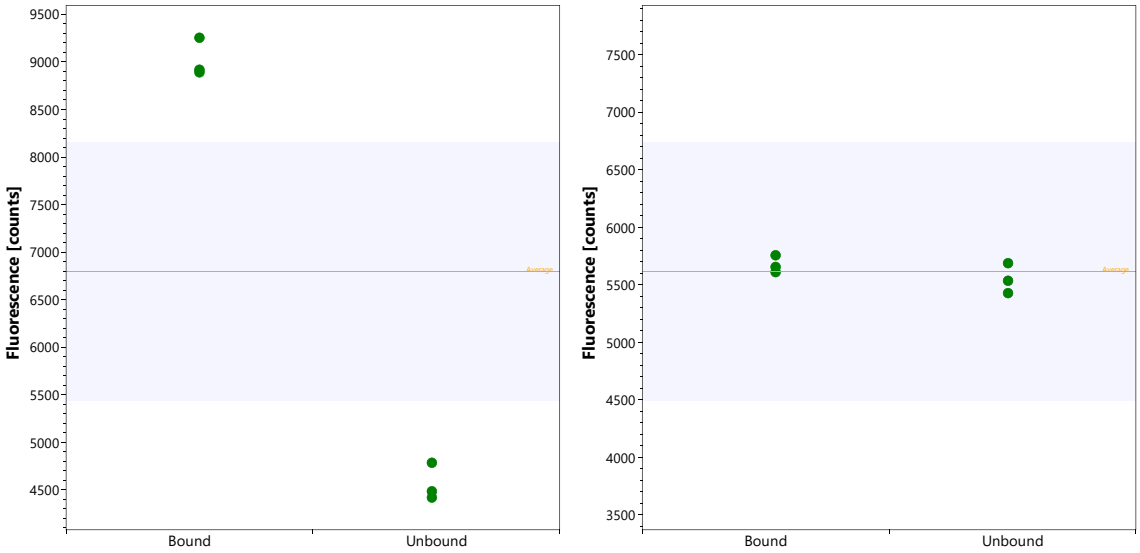
K_d = 2.15 nM Initial Fluorescence Analysis







SD-Test[3]:



D5. Reference Results/Supporting Results

K_d = 7.1 nM Surface Plasmon Resonance (SPR)
Svensson et al., Eur J Biochem 258 (2001) 890-896

E. Contributors

Andreas Langer[4]

[3] Due to the ligand-dependent fluorescence changes, an SD-test was performed to confirm binding-dependent change. As the changes in initial fluorescence were concluded to be binding-specific, the initial fluorescence data was used for binding curve fit and K_d determination.
[4] NanoTemper Technologies GmbH, München, Germany | nanotempertech.com