Monolith Protocol MO-P-012

Herceptin – Protein A

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. In this protocol, Herceptin (Trastuzumab), a monoclonal antibody used to treat breast cancer, is labeled site-specifically after selective reduction of antibody interchain disulfides with a maleimide-dye.

protein – protein interaction | antibody | antibody labeling

A1. Target/Fluorescent Molecule

Herceptin (Trastuzumab)

A2. Molecule Class/Organism

Monoclonal antibody

A3. 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
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A4. Purification Strategy/Source

N/A

A5. Stock Concentration/Stock Buffer

120 mg/mL | 825 µM

A6. Molecular Weight/Extinction Coefficient

145.5 kDa
225,000 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.01% Pluronic® F-127

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – MALEIMIDE 2nd Generation (MO-L014, NanoTemper Technologies GmbH)
1* Dye RED-MALEIMIDE 2nd Generation (10 µg) | 1* B-Column

1* 500 mM TCEP

1* Reduction Buffer (RB): 25 mM H_3BO_3, 25 mM NaCl, 1 mM EDTA, pH 8 (pH adjusted with 50 mM $Na_2B_4O_7$)

A9. Labeling Procedure

1. Add 145 µL of dilution buffer to 20 µL of 825 µM Herceptin to obtain 165 µL of a 100 µM solution. Verify the concentration spectroscopically using an extinction coefficient of 225,000 $M^{-1}cm^{-1}$ (ε_{280}).
2. Mix 16 µL of the 100 µM Herceptin solution with 384 µL of RB buffer to obtain 400 µL of a 4 µM solution.
3. Prepare a fresh 500 mM TCEP stock solution.
4. Mix 2 µL of 500 mM TCEP with 198 µL of RB buffer to obtain 200 µL of 5 mM TCEP.
5. Mix 2.4 µL of 5 mM TCEP with 197.6 µL of RB buffer to obtain 200 µL of 60 µM TCEP.
6. Prepare a PCR-rack with 5 PCR tubes. Transfer 44 µL of RB buffer into tube **1**, 48 µL into tube **2**, 52 µL into tube **3**, 56 µL into tube **4** and 60 µL into tube **5**.
7. Then, add 16 µL of 60 µM TCEP into tube **1**, 12 µL into tube **2**, 8 µL into tube **3** and 4 µL into tube **4**.
8. Add 60 µL of the 4 µM Herceptin solution to tubes **1** – **5** and mix by pipetting to obtain 120 µL of a final Herceptin concentration of 2 µM and TCEP concentrations of 8 µM, 6 µM, 4 µM, 2 µM and no TCEP[1].
9. Incubate tubes for 1 hour at room temperature to selectively reduce disulfide bonds in the hinge region. Afterwards, use Tycho to measure the success of disulfide reduction by measuring one capillary from each of the 5 tubes (see section **C**).
10. Continue with the Herceptin-TCEP mix that shows an ~0.5°C lower first T_i value than unreduced Herceptin (tube **5**). In this protocol: Tube **2** (2 µM Herceptin, 6 µM TCEP).
11. Add 25 µL of DMSO to Dye RED-MALEIMIDE 2nd Generation (10 µg) to obtain a ~530 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
12. Prepare a new PCR tube and mix 1 µL of the ~530 µM dye solution with 19 µM of RB buffer to obtain 20 µL of a ~25 µM dye solution. Then, add 100 µL of the 2 µM reduced Herceptin (tube from step 10)[2].
13. Incubate for 45 minutes at room temperature in the dark.
14. 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.
15. 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.
16. Add 120 µL of the labeling reaction from step 9 to the center of the column and let sample enter the bed completely.
17. Add 400 µL of dilution buffer after the sample has entered and discard the flow through.
18. Place column in a new collection tube, add 480 µL of dilution buffer and collect the eluate.
19. Keep the labeled Herceptin (~0.5 µM) on ice in the dark.

[1] A ~2–3-fold molar excess of active TCEP should be sufficient for disulfide reduction. The 8 µM TCEP concentration may be necessary in case that the TCEP stock has lost some of its activity.
[2] Since unreacted TCEP inactivates the maleimide of the dye and is not removed through an additional purification step, it is essential that the final dye concentration is larger than the final TCEP concentration.

A10. Labeling Efficiency

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

Absorbance A_{280}	0.147	Protein concentration	0.64 µM
Absorbance A_{650}	0.083	Degree-of-labeling (DOL)	0.67

B1. Ligand/Non-Fluorescent Binding Partner

Protein A
uniprot.org/uniprot/P38507

B2. Molecule Class/Organism

Immunoglobulin-binding protein
Staphylococcus aureus

B3. 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

B4. Purification Strategy/Source

Recombinant
anitkoerper-online
ABIN1067599

B5. Stock Concentration/Stock Buffer

53.7 mg/mL | 1.28 mM
H_2O

B6. Molecular Weight/Extinction Coefficient

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

B7. Serial Dilution Preparation

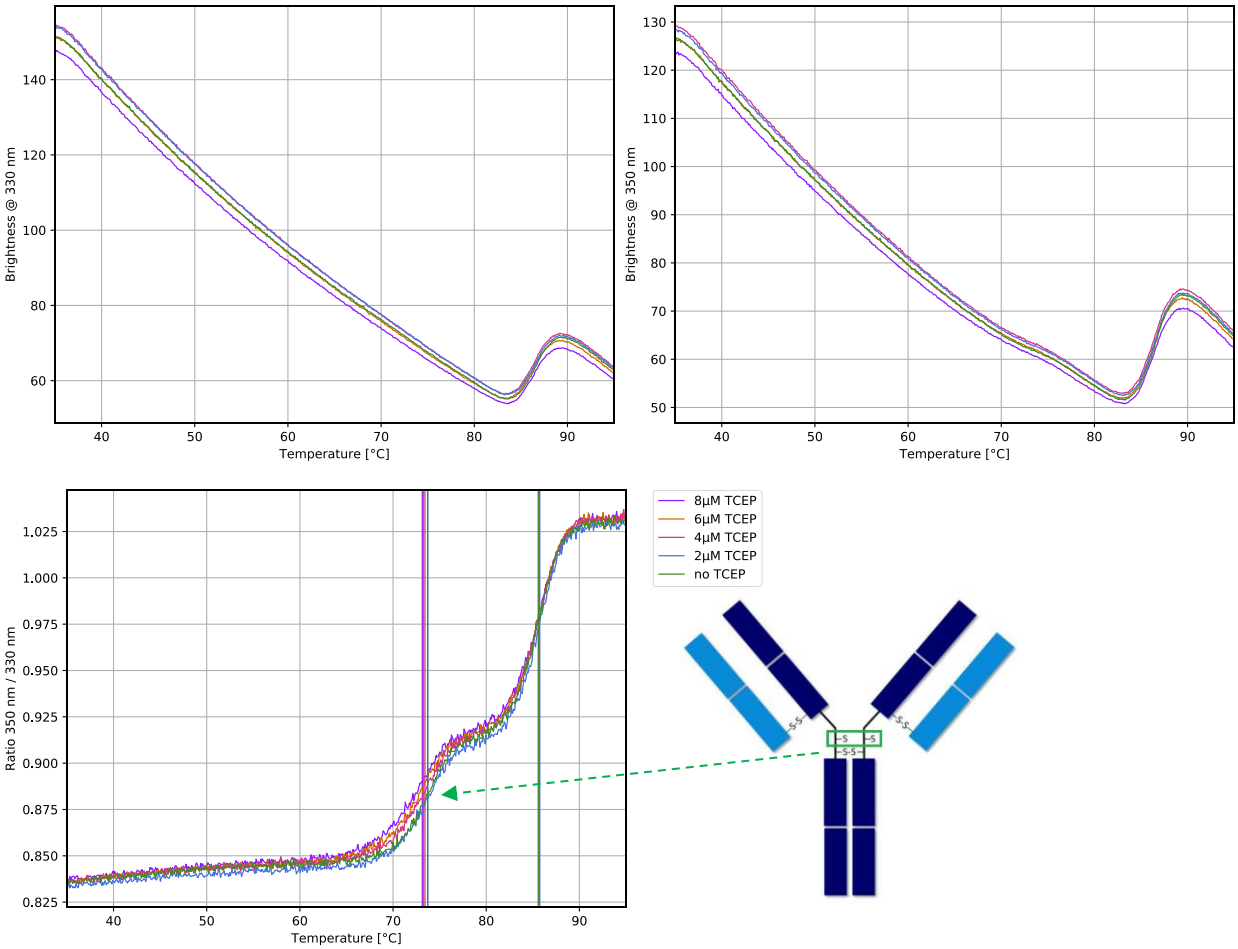
1. Mix 3.3 µL of 1.28 mM protein A with 196.7 µL of dilution buffer to obtain 200 µL of a 20 µM solution.
2. Mix 2 µL of the 10 µM protein A solution with 398 µL of dilution buffer to obtain 400 µL of a 100 nM solution.
3. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 100 nM protein A solution 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 Herceptin (~0.5 µL) with 498 µL of dilution buffer to obtain 500 µL of ~2 nM Herceptin.
6. Add 10 µL of labeled Herceptin (~2 nM) to each tube from **16** to **1** and mix by pipetting.
7. Load capillaries immediately[3].

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

C. Applied Quality Checks

Validation of structural integrity of Herceptin and efficiency of TCEP reduction using Tycho NT.6:
nanotempertech.com/tycho

8 µM TCEP	44 µL of RB buffer + 16 µL of 60 µM TCEP + 60 µL of 4 µM Herceptin	T_i^1 = 73.1°C	T_i^2 = 85.7°C
6 µM TCEP	48 µL of RB buffer + 12 µL of 60 µM TCEP + 60 µL of 4 µM Herceptin	T_i^1 = 73.3°C	T_i^2 = 85.7°C
4 µM TCEP	52 µL of RB buffer + 8 µL of 60 µM TCEP + 60 µL of 4 µM Herceptin	T_i^1 = 73.4°C	T_i^2 = 85.6°C
2 µM TCEP	56 µL of RB buffer + 4 µL of 60 µM TCEP + 60 µL of 4 µM Herceptin	T_i^1 = 73.7°C	T_i^2 = 85.7°C
0 µM TCEP	60 µL of RB buffer + 60 µL of 4 µM Herceptin	T_i^1 = 73.7°C	T_i^2 = 85.6°C

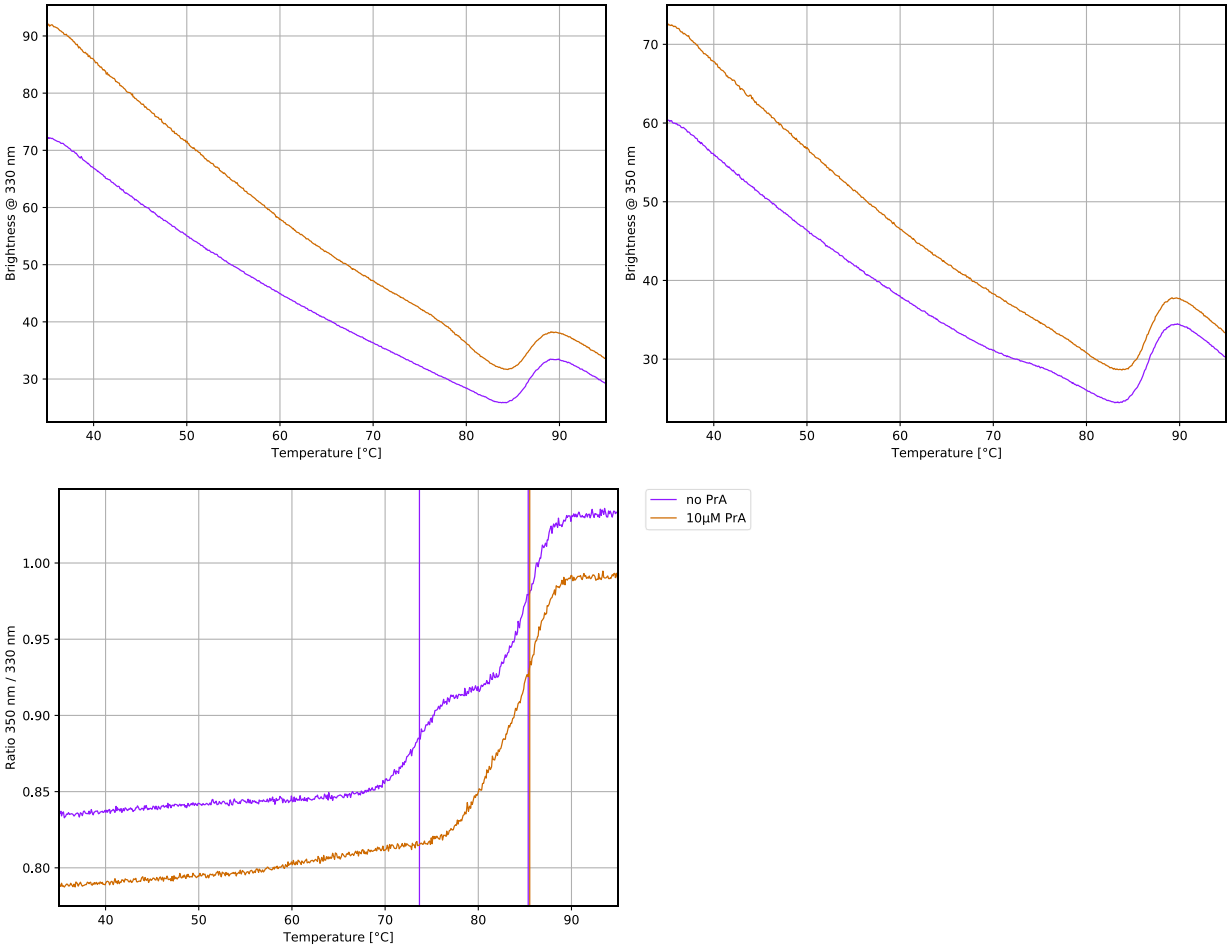


Successful TCEP reduction at concentrations > 4 µM is visible as a slightly destabilized T_i^1 while T_i^2 is not affected.

Confirmation of the binding of protein A to Herceptin using Tycho NT.6:

nanotempertech.com/tycho

no PrA	5 µL of 2 µM Herceptin + 5 µL of dilution buffer	$T_{i,1}$ = 73.7°C | $T_{i,2}$ = 85.4°C
10 µM PrA	5 µL of 2 µM Herceptin + 5 µL of 20 µM protein A	$T_{i,1}$ = N/A | $T_{i,2}$ = 85.5°C



Protein A binds to the Fc domain of Herceptin and stabilizes it so much that the first T_i disappears.

D1. MST System/Capillaries

Monolith NT.115^{Pico} Red (NanoTemper Technologies GmbH)
Capillaries Monolith NT.115 (MO-K022, NanoTemper Technologies GmbH)

D2. MST Software

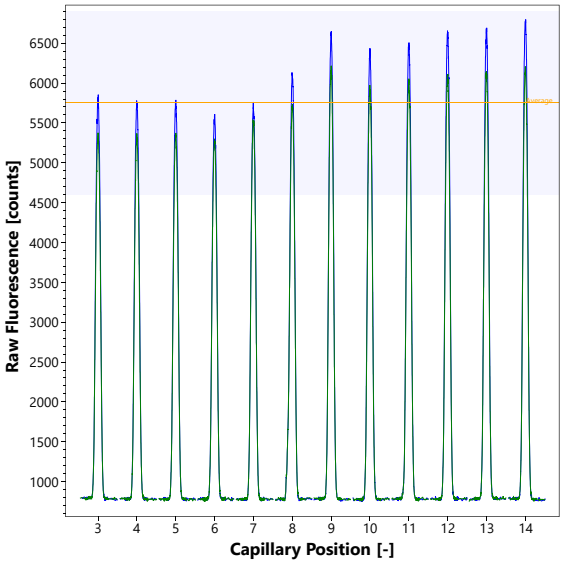
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

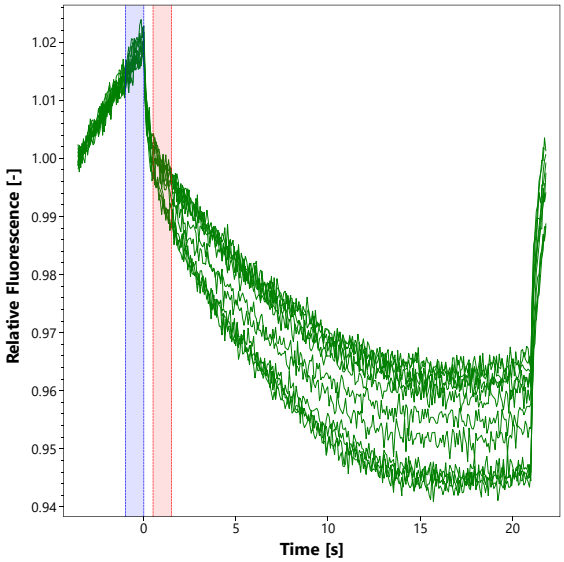
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

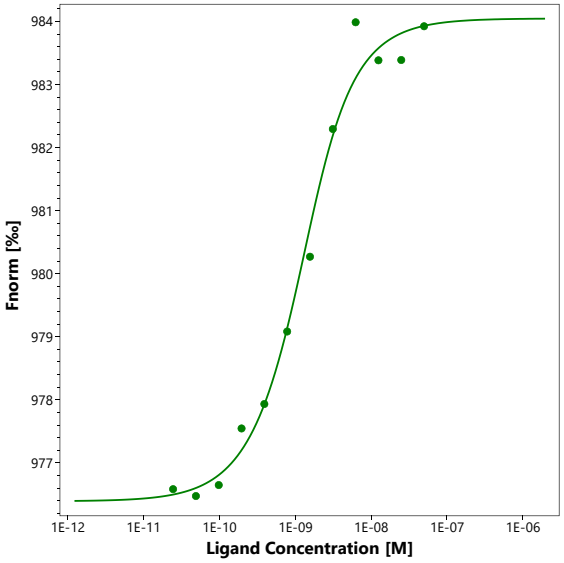
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.01% Pluronic® F-127
1 nM Herceptin | 200 nM – 6.1 pM protein A (binding sites)[4] | 25°C | medium MST power | 80% excitation power

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

K_d = 758 pM







D5. Reference Results/Supporting Results

N/A

[4] As protein A has 4 binding sites for IgGs, the ligand concentration was multiplied with a factor of 4.

E. Contributors

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