Monolith Protocol MO-P-067

Bovine Serum Albumin – Zinc

Bovine serum albumin (also known as BSA) is a serum albumin protein derived from cows, which is often used as a protein concentration standard in lab experiments. Among its 583 amino acid residues, it contains 17 intrachain disulfide bonds as well as one free thiol group, which can be used for site-specific labeling with a thiol-reactive (maleimide) fluorescent dye. Apart from fatty acids and a variety of small molecules in the blood, BSA is also associated with the transport of metal ions such as zinc (Zn^{2+}).

protein – ion interaction | Zn

A1. Target/Fluorescent Molecule

Bovine serum albumin (BSA)
uniprot.org/uniprot/P02769

A2. Molecule Class/Organism

Serum protein
Bos taurus (Bovine)

A3. Sequence/Formula

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DTHKSEIAHR FKDLGEEHFK GLVLIAFSQY LQQCPFDEHV KLVNELTEFA KTCVADESHA GCEKSLHTLF GDELCKVASL
RETYGDMADC CEKQEPERNE CFLSHKDDSP DLPKLKPDPN TLCDEFKADE KKFWGKYLYE IARRHPYFYA PELLYYANKY
NGVFQECCQA EDKGACLLPK IETMREKVLA SSARQRLRCA SIQKFGERAL KAWSVARLSQ KFPKAEFVEV TKLVTDLTKV
HKECCHGDLL ECADDRADLA KYICDNQDTI SSKLKECCDK PLLEKSHCIA EVEKDAIPEN LPPLTADFAE DKDVCKNYQE
AKDAFLGSFL YEYSRRHPEY AVSVLLRLAK EYEATLEECC AKDDPHACYS TVFDKLKHLV DEPQNLIKQN CDQFEKLGEY
GFQNALIVRY TRKVPQVSTP TLVEVSRSLG KVGTRCCTKP ESERMPCTED YLSLILNRLC VLHEKTPVSE KVTKCCTESL
VNRRPCFSAL TPDETYVPKA FDEKLFTFHA DICTLPDTEK QIKKQTALVE LLKHKPKATE EQLKTVMENF VAFVDKCCAA
DDKEACFAVE GPKLVVSTQT ALA
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A4. Purification Strategy/Source

Carl Roth GmbH
8076.2

A5. Stock Concentration/Stock Buffer

Powdered

A6. Molecular Weight/Extinction Coefficient

66.5 kDa
43,800 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

Phosphate buffered saline (PBS, pH 7.4)[1]

A8. Labeling Strategy

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

A9. Labeling Procedure

1. Add 3 mL of dilution buffer to 20 mg of BSA to obtain a 100 µM solution. Mix carefully with a pipette to dissolve all protein and avoid creating air bubbles.
2. Mix 20 µL of 100 µM BSA with 180 µL of Labeling Buffer MALEIMIDE to obtain 200 µL of a 10 µM BSA solution.
3. Mix 2 µL of 500 mM TCEP with 198 µL of Labeling Buffer MALEIMIDE to obtain 200 µL of 5 mM TCEP.
4. Mix 3.2 µL of 5 mM TCEP with 196.8 µL of Labeling Buffer MALEIMIDE to obtain 200 µL of 80 µM TCEP.
5. Mix 50 µL of the 80 µM TCEP solution with 100 µL of the 10 µM BSA solution to obtain 150 µL of a 6.7 µM BSA, 26.7 µM TCEP solution (4x protein concentration).
6. Incubate for 30 minutes at room temperature.
7. Add 13.2 µL of DMSO to Dye RED-MALEIMIDE 2nd Generation (10 µg) to obtain a ~1 mM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
8. Mix 10 µL of the 1 mM dye solution with 40 µL of Labeling Buffer MALEIMIDE to obtain 50 µL of a 200 µM dye solution.
9. Add the 150 µL of the BSA-TCEP mix (step 5) to the 200 µM dye solution to obtain 200 µL of a 5 µM BSA, 20 µM TCEP, 50 µM dye solution (10x protein concentration, 2.5x TCEP concentration[2], 5% DMSO).
10. Incubate for 30 minutes at room temperature in the dark.
11. 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.
12. 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.
13. Add 200 µL of the labeling reaction from step 9 to the center of the column and let sample enter the bed completely.
14. Add 400 µL of dilution buffer after the sample has entered and discard the flow through.
15. Place column in a new collection tube, add 500 µL of dilution buffer and collect the eluate.
16. Keep the labeled BSA (~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_{280}	0.082	Protein concentration	1.76 µM
Absorbance A_{650}	0.125	Degree-of-labeling (DOL)	0.36

[1] Detergents (e.g. TWEEN® 20) cannot be used as they bind to a similar binding site as Zn^{2+} (Coverdale et al., Metallomics, 2019, 11, 1805).
[2] As maleimide can also react with TCEP, it is important to have an excess of maleimide over TCEP in the labeling reaction.

B1. Ligand/Non-Fluorescent Binding Partner

Zn^{2+}

B2. Molecule Class/Organism

Ion

B3. Sequence/Formula

$ZnCl_2$

B4. Purification Strategy/Source

Carl Roth GmbH
T887.1

B5. Stock Concentration/Stock Buffer

Powdered

B6. Molecular Weight/Extinction Coefficient

136.28 Da

B7. Serial Dilution Preparation

1. Dissolve 0.34 g of $ZnCl_2$ in 10 mL of ddH_2O to obtain a 250 mM $ZnCl_2$ stock solution. Add 4 drops of HCl (1.2 M) to acidify the solution and completely dissolve all $ZnCl_2$.
2. Mix 2 µL of the 250 mM $ZnCl_2$ stock with 998 µL of ddH_2O to obtain a 1 mL of a 500 µM $ZnCl_2$ solution[3].
3. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 500 µM $ZnCl_2$ solution into tube **1**. Then, add 10 µL of ddH_2O to tubes **1** 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 8 µL of labeled BSA (~2 µM) and 192 µL of dilution buffer containing 0.005% Pluronic® F-127 to obtain 200 µL of ~80 nM labeled BSA solution.
6. Add 10 µL of this solution to each tube from **16** to **1** and mix by pipetting.
7. Load capillaries immediately[4].

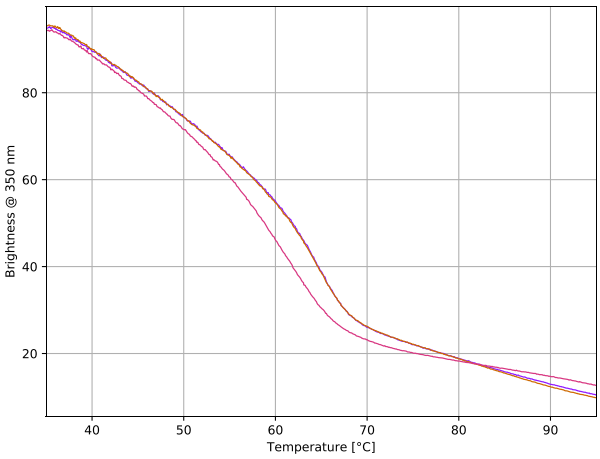
[3] Due to the low solubility of $ZnCl_2$ at non-acidic pH and higher ionic strength, as well as its tendency to form insoluble $Zn(OH)_2$ at pH > 7, the dilution series is prepared in ddH_2O (see also Krezel et al., Archives of Biochemistry and Biophysics 611 (2016) 3-19).

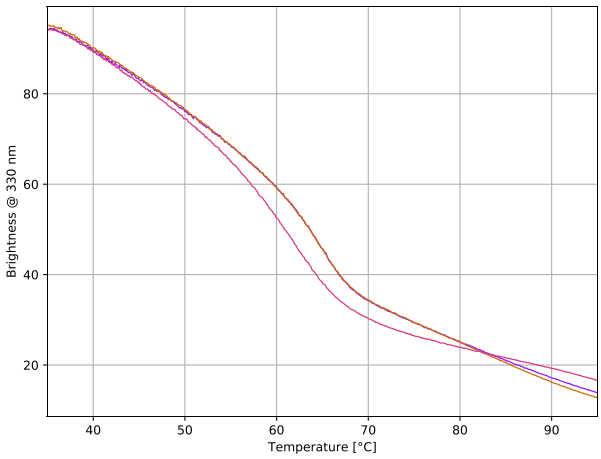
[4] Longer incubation times can lead to BSA aggregation at high $ZnCl_2$ concentrations, as well as precipitation of Zn_{2+} in the phosphate buffer.

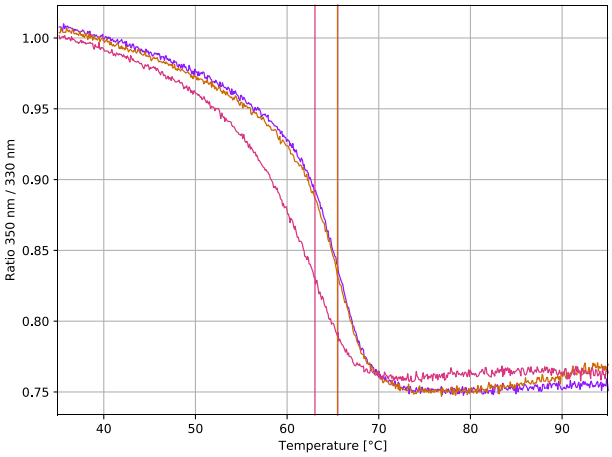
Validation of TCEP activity using Tycho NT.6:

nanotempertech.com/tycho

no TCEP	10 µL of 10 µM BSA + 5 µL of Labeling Buffer MALEIMIDE	T_i = 65.5°C
26.7µM TCEP	10 µL of 10 µM BSA + 5 µL of 80 µM TCEP	T_i = 65.5°C
1.67mM TCEP	10 µL of 10 µM BSA + 5 µL of 5 mM TCEP	T_i = 63.0°C[5]



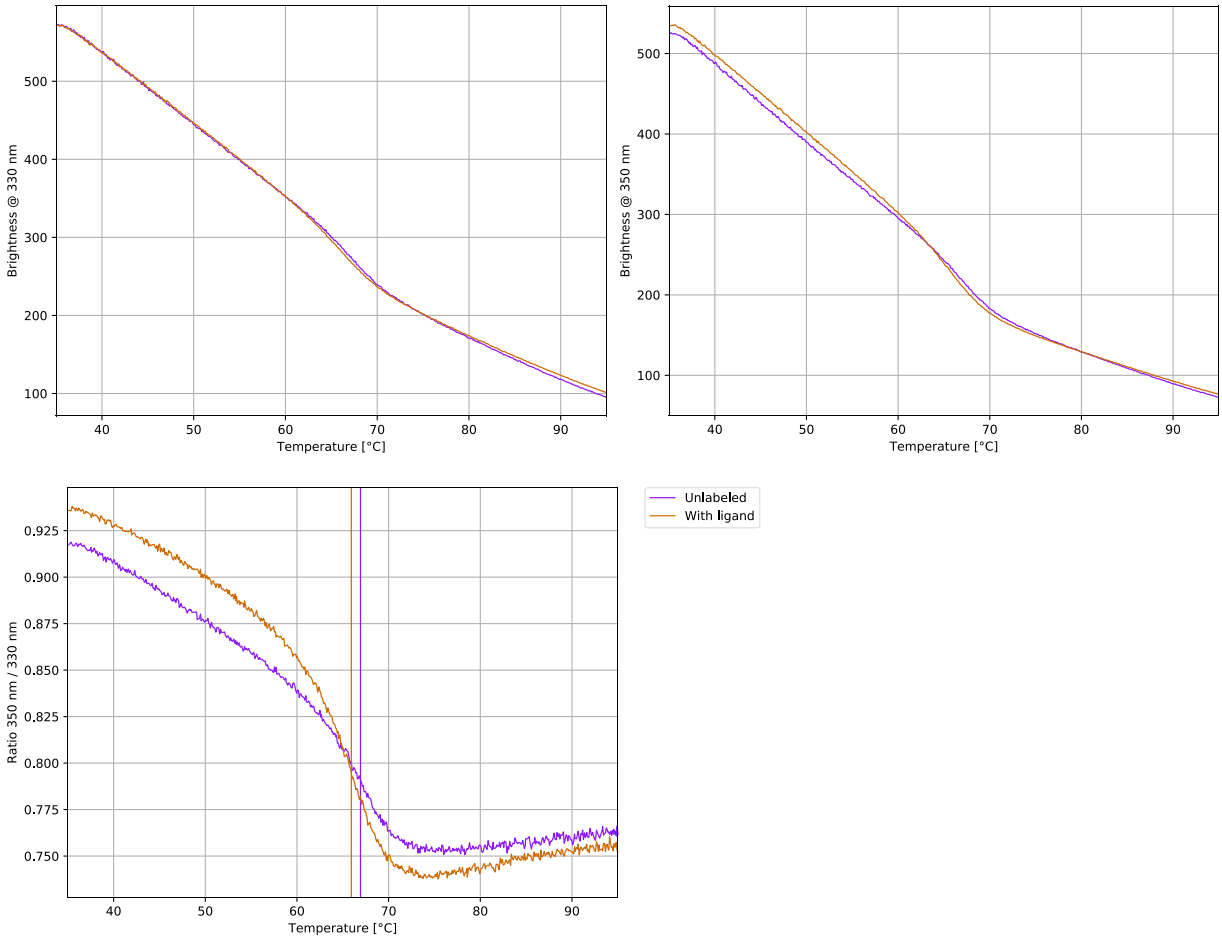




[5] While 26.7 µM TCEP has no effect on the structural integrity of BSA, 1.67 mM TCEP significantly destabilizes the protein, presumably by the reduction of intrachain disulfide bonds.

Validation of structural integrity and functionality of BSA using Tycho NT.6:
nanotempertech.com/tycho

Unlabeled	10 µL of ddH_2O + 10 µL of 100 µM BSA	T_i = 66.9°C
With ligand	8 µL of ddH_2O + 2 µL of 500 µM $ZnCl_2$ + 10 µL of 100 µM BSA	T_i = 65.9°C



D1. MST System/Capillaries

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

D2. MST Software

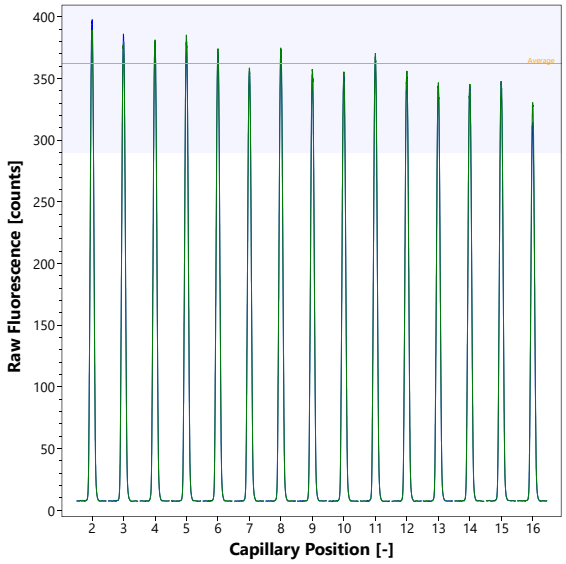
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

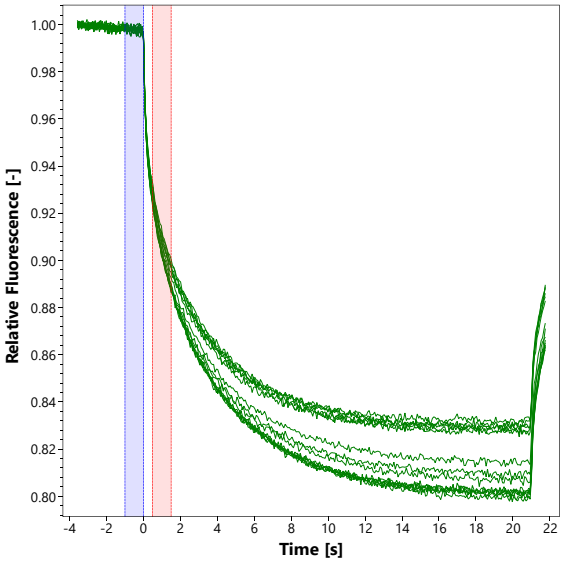
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

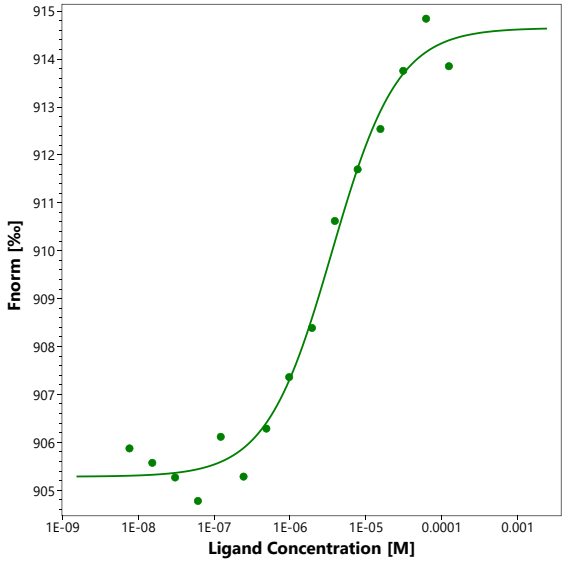
0.5X Phosphate buffered saline (PBS, pH 7.4), 0.0025% Pluronic® F-127[6]
40 nM BSA | 250 µM – 7.6 nM $ZnCl_2$ | 23°C | high MST power | 40% excitation power

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

K_d = 3.5 µM

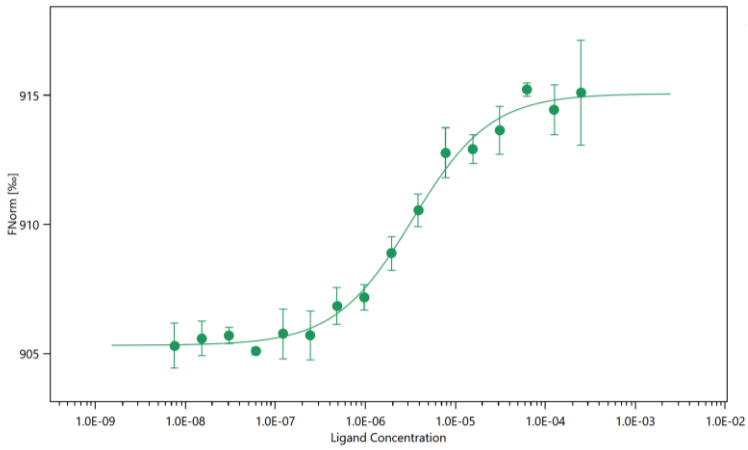






[6] Higher concentrations of detergent interfere with Zn^{2+} binding due to the shared binding sites.

K_d = 3.3 ± 0.4 µM (n = 3)



D5. Reference Results/Supporting Results

K_d = 0.3 µM Competitive spectrophotometry
Oyoshi et al., Journal of Inorganic Biochemistry 75 (1999) 213–218

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