Electronic Supplementary Material (ESI) for Molecular Systems Design & Engineering.

Supplementary Information

Engineering hyperthermostable rcSso7d as reporter molecule for *in vitro* diagnostic tests

Ki-Joo Sung[1], Eric Miller[1], Hadley D. Sikes[1,2,*]

[1]Department of Chemical Engineering, Massachusetts Institute of Technology,

Cambridge, Massachusetts 02139

[2]Singapore-MIT Alliance for Research and Technology Centre (SMART)

1 CREATE Way, INNOVATION Tower, Singapore 138602

*Corresponding Author:
Professor Hadley Sikes
Telephone: (617) 253-5224
Fax: (617) 253-5224
Email: sikes@mit.edu

Antibody Alternative Scaffolds

Many alternative binding proteins exist and have been studied extensively for the past few decades. Although it is not an extensive list, Table S1 lists a selection of these antibody alternatives and their properties for comparison.

Table S1. Properties of some example antibody alternative scaffolds. K_d: dissociation constant for target binding, T_m: melting temperature. †Produced in the periplasm of *E. coli* to facilitate disulfide bond formation.

	sdAb	*Anticalin*	*DARPin*	*Affibody*	*rcSso7d*
Description	Derived from camelid and cartilaginous fish antibodies	Derived from human lipcalins	Ankyrin repeat motifs	Derived from staphylococcal protein A	Derived from *Sulfolobus solfataricus* DNA-binding protein
Size	12-15 kDa	20 kDa	14-21 kDa	6 kDa	7 kDa
Affinity (K_d)	Down to pM[1]	Down to pM[2]	Down to pM[3]	Down to pM[4,5]	Down to pM[6]
Melting Temperature (T_m)	Highly variable, some 60 to 80°C[1,7]	Parent: 79°C, 53 to 73°C[8–10]	66 to 89°C[11]	Parent: 75°C,[12] 42 to 71°C[5]	Parent: 98°C, 60 to 110°C[13,14]
Structure	Disulfide bonds	Generally non-glycosylated; most have disulfide bonds	No disulfide bonds or glycosylation sites	No disulfide bonds or glycosylation sites	No disulfide bonds or glycosylation sites
Expression system	*S. cerevisiae*[7] *E. coli*†[1]	*E. coli*†[2,8]	*E. coli*[11]	*E. coli*[4]	*E. coli*[6,15]
Production yield	Several mg/L[1]	2 to 20 mg/L[8]	Up to 200 mg/L[11]	100 to 200 mg/L[16]	40 to 130 mg/L[6,15]
Company	Ablynx	Pieris Pharmaceuticals, Inc	Molecular Partners AG	Affibody AB	None

Desirable scaffold properties include strong binding affinity of selected clones, high thermal stability across a genetically diverse range of clones, a lack of disulfide bonds and glycosylation sites for ease of expression in *E. coli*, and high production yields. Based on these criteria, some

potential antibody alternative candidates were eliminated due to highly variable clonal stability and low expression yields in *E. coli*. The remaining scaffolds fulfill our general design criteria, as shown above; however, we chose rcSso7d as our scaffold of interest due to its extremely high inherent thermostability and a binding face which is structurally isolated from its stabilizing, hydrophobic core, increasing the likelihood that mutations in the binding face may have minimal impact on protein stability.

Schematic of rcSso7d Testing Procedure

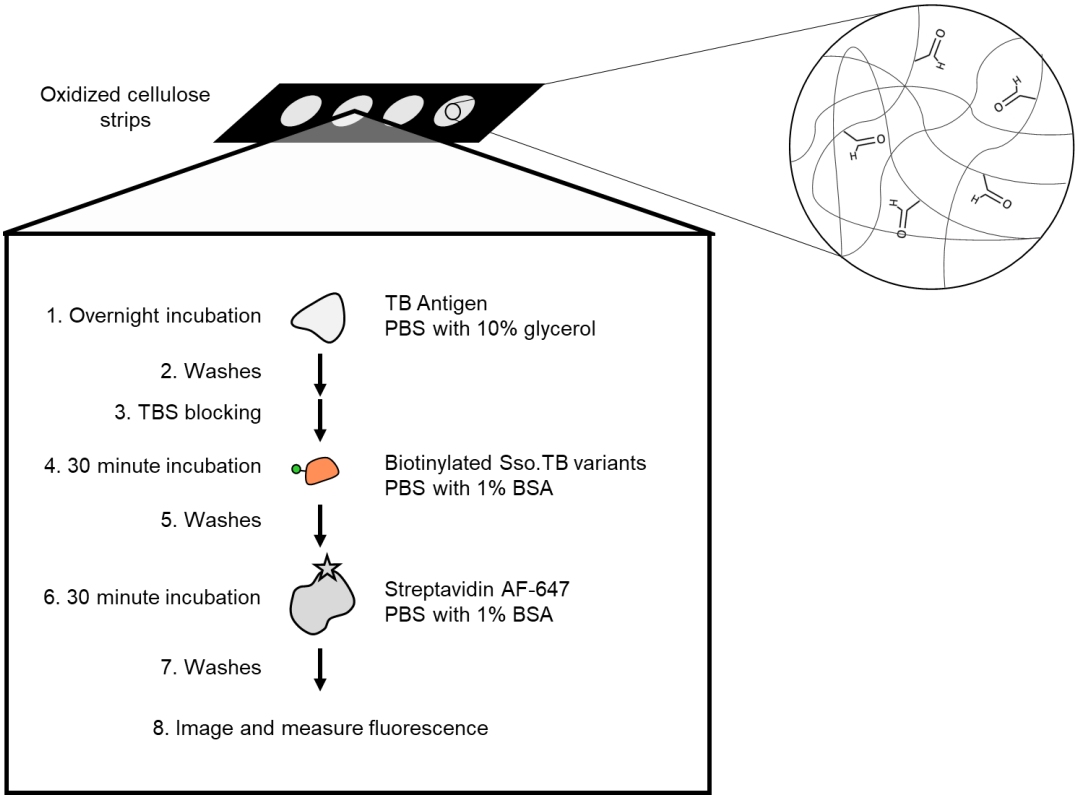

Figure S1. Schematic of rcSso7d testing steps. TB antigen was captured on oxidized cellulose strips (functionalized with aldehydes). After washes and blocking steps, biotinylated Sso.TB was incubated on the strips, followed by incubations with SA AF-647. After washes and drying, the test zones were imaged to measure fluorescence.

BA-Sso.TB as a Detection Reagent

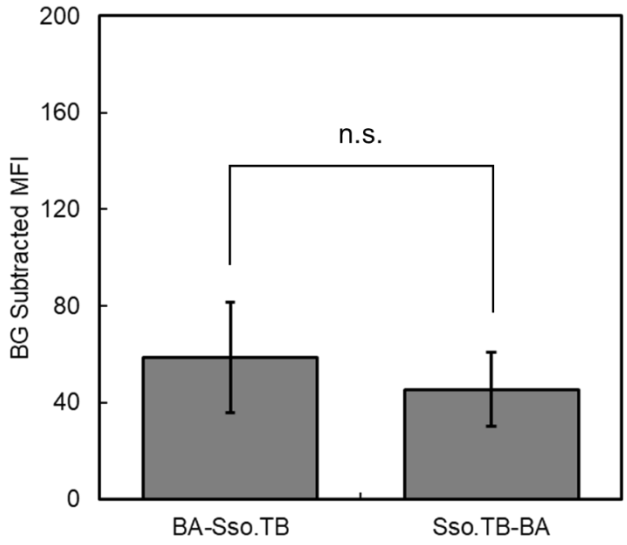

Figure S2. Background subtracted fluorescence signal (MFI: mean fluorescence intensity) of BA-Sso.TB variants (BA-Sso.TB with N-terminal BA modification and Sso.TB-BA with C-terminal BA modification) in the presence of TB antigen immobilized on oxidized cellulose strips and its respective negative control lacking TB antigen. The background subtracted MFI is low, indicating a need for improved signal output by investigating different biotinylation methods and protein variants. No statistical difference was observed between the two variants using $P < 0.05$, indicating minimal difference with orientation of BA. Each data point consists of an average of least eight replicates over multiple days, and error bars indicate standard deviations. n.s.: Not significant.

Sso.TB-b_x as a Detection Reagent

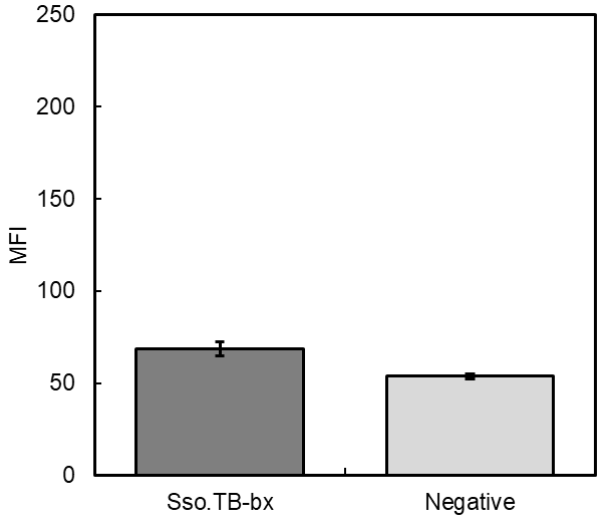

Figure S3. Fluorescence signal (MFI: mean fluorescence intensity) of Sso.TB-b_x in the presence of TB antigen immobilized on oxidized cellulose strips and its respective negative control lacking TB antigen. The background subtracted MFI was lower than for BA-Sso.TB and Sso.TB-BA (Figure S2). Each data point consists of an average of four replicates, and error bars indicate standard deviations.

BA-Link-Sso.TB as a Detection Reagent

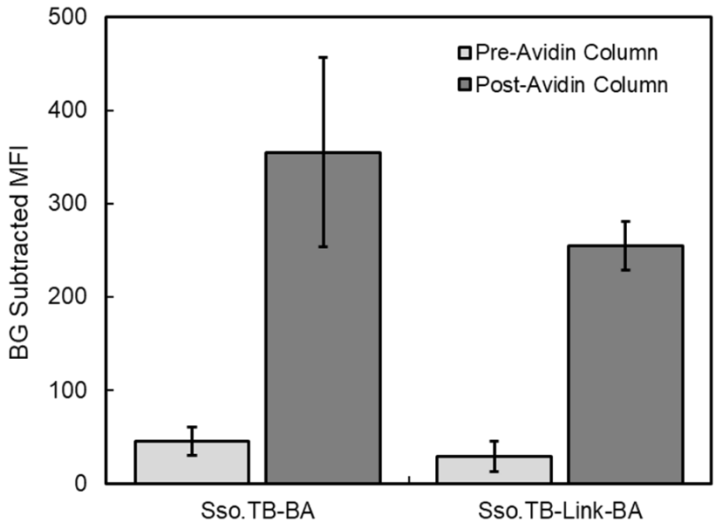

Figure S4. Background subtracted MFI (mean fluorescence intensity) of Sso.TB-BA and Sso.TB-Link-BA (both with C-terminal modifications to ensure no orientation-dependent effects) for both sub-populations (pre-avidin column and post-avidin column purified). The MFI from the background signal (negative control lacking TB antigen) was subtracted from the MFI in the presence of TB antigen immobilized on oxidized cellulose strips. The addition of the extra $(G_4S)_2$ linker sequence did not improve signal read-out and therefore was not pursued for further studies. However, comparing the variance between post-avidin purified Sso.TB-BA and Sso.TB-Link-BA suggests lower variance with Sso.TB-Link-BA, which may be useful for assays that require high reproducibility, even at the expense of reduced signal output. Each data point consists of an average of least eight replicates over multiple days, and error bars indicate standard deviations.

Comparing N-terminal and C-terminal Modifications

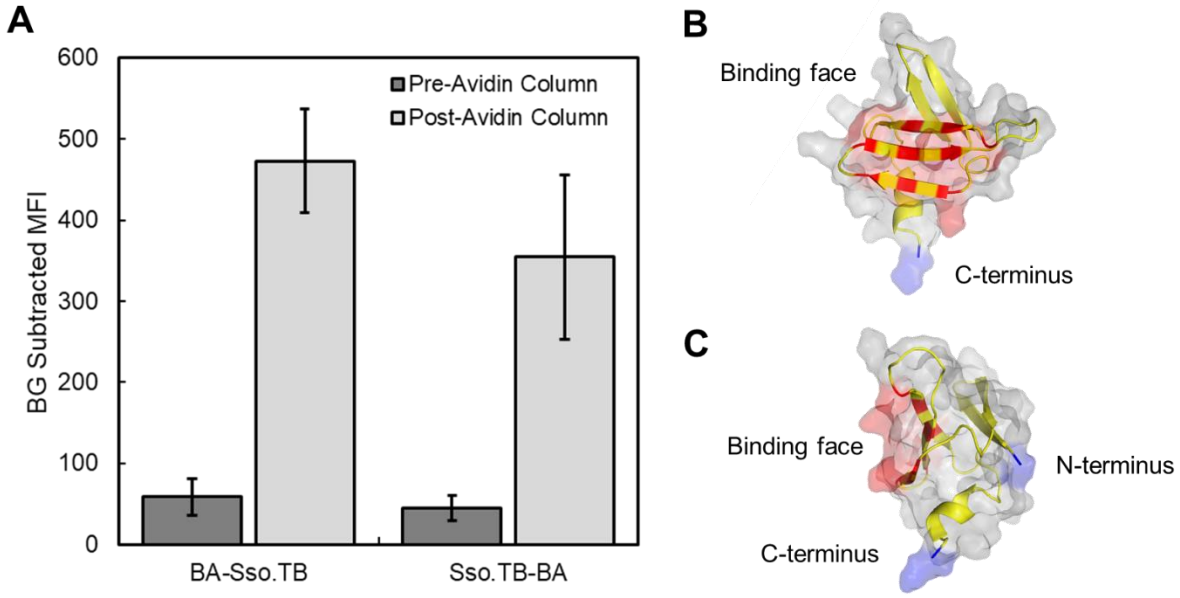

Figure S5. Comparison of N-terminal and C-terminal BA modifications on Sso.TB. (A) Background subtracted MFI (mean fluorescence intensity) of BA-Sso.TB and Sso.TB-BA for both sub-populations (pre-avidin column and post-avidin column purified). The MFI from the background signal (negative control lacking TB antigen) was subtracted from the MFI in the presence of TB antigen immobilized on oxidized cellulose strips. Data for pre-avidin column purified is identical to data shown in Figure S2, which showed no significant difference in signal. Post-avidin column purification, the N-terminal BA variant showed moderately higher signal than the C-terminal BA variant. Each data point consists of an average of least eight replicates over multiple days, and error bars indicate standard deviations. (B) Front view of the rcSso7d protein, showing the C-terminus adjacent to the binding face. (C) Side view of the rcSso7d protein, showing the N-terminus on the opposite side of the binding face. The orientation of the termini may result in steric hindrance effects leading to a lower signal for Sso.TB-BA.

Additional Controls for Specificity of Binding

We conducted two additional control experiments to test for nonspecific binding of the biotinylated MBP-fusion construct and to test for specificity of the Sso.TB against the TB antigen. Both experiments used TB antigen immobilized on oxidized cellulose followed by 330 nM of the biotinylated protein and using 3.3 μM SA AF 647 to generate a signal. Figure S6 tests for nonspecific binding of the biotinylated MBP-rcSso7d. A different rcSso7d binder that does not demonstrate binding affinity to TB antigen (Sso.Other) was cloned into the MBP format, expressed and purified, and chemically biotinylated to generate MBP-Sso.Other-b_x, following the same procedure outlined in the Materials and Methods. Quant*Tag assay was used on MBP-Sso.Other-b_x to quantify approximately 20 biotins per protein post-chemical conjugation. By testing a different clone of rcSso7d in the biotinylated MBP-rcSso7d format, we verified that the binding signal generated from MBP-Sso.TB-b_x is from binding of the Sso.TB to the TB antigen, not from nonspecific interactions of any MBP-rcSso7d-b_x to the TB antigen or cellulose.

Amino acid sequence of Sso.Other:

MATVKFTYQGEEKQVDISKIK**W**V**R**R**D**GQ**I**I**Y**F**N**YDEGGGA**W**G**W**G**D**VSEKDAPKELLQMLEKQ

Figure S7 shows a variation of a competitive binding assay, in which TB antigen is immobilized on oxidized cellulose and followed by either 1) MBP-Sso.TB-b_x or 2) MBP-Sso.TB-b_x with 10 μM additional soluble TB antigen. With additional soluble TB antigen applied to the test zones with the binding protein, the binding signal is lower, indicating specific binding of the Sso.TB to the TB antigen.

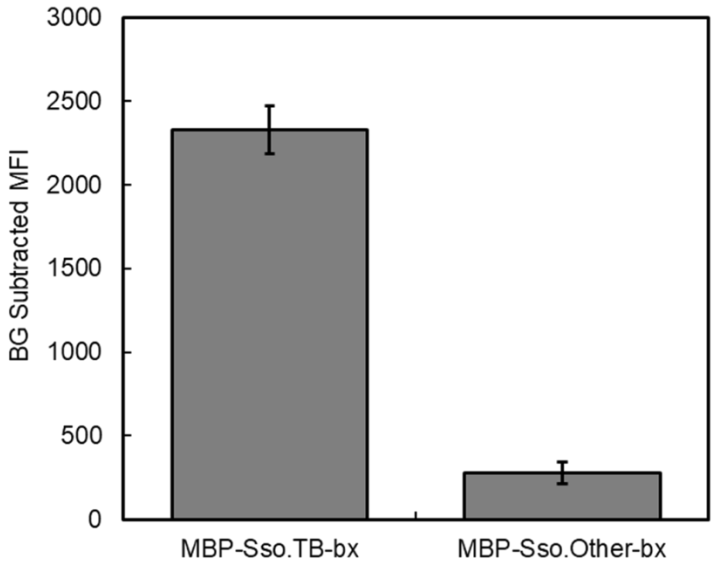

Figure S6. Control experiment testing for nonspecific binding of the biotinylated MBP-Sso.TB. Both MBP-Sso.TB-b_x and MBP-Sso.Other-b_x were tested against immobilized TB antigen. Background subtracted MFI (mean fluorescence intensity) of MBP-Sso.TB-b_x and MBP-Sso.Other-b_x is shown. The MFI from the background signal (negative control lacking TB antigen) was subtracted from the MFI in the presence of TB antigen immobilized on oxidized cellulose strips. The low signal of MBP-Sso.Other-b_x against TB antigen in comparison to the high signal of MBP-Sso.TB-b_x against TB antigen demonstrates that the binding signal from the Sso.TB variants is not a result of nonspecific interactions. Each data point consists of an average of four replicates, and error bars indicate standard deviations.

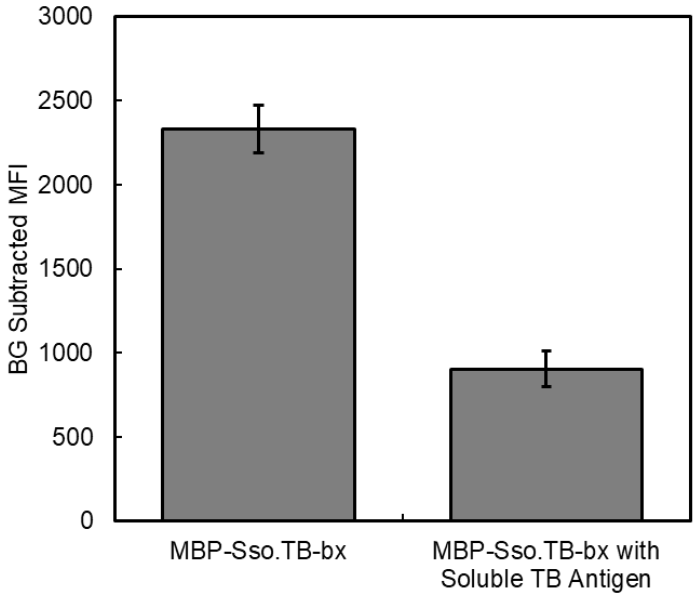

Figure S7. Control experiment testing for specificity of Sso.TB to the TB antigen. MBP-Sso.TB-b_x was tested in a competitive assay via the application of MBP-Sso.TB-b_x with additional soluble TB antigen to the test zones with immobilized TB antigen. Background subtracted MFI (mean fluorescence intensity) of MBP-Sso.TB-b_x without and with the addition of soluble TB antigen is shown. The MFI from the background signal (negative control lacking TB antigen) was subtracted from the MFI in the presence of TB antigen immobilized on oxidized cellulose strips. The reduction in signal with the presence of additional soluble TB antigen indicates specific binding of Sso.TB to the TB antigen. Each data point consists of an average of three replicates, and error bars indicate standard deviations.

SDS-PAGE

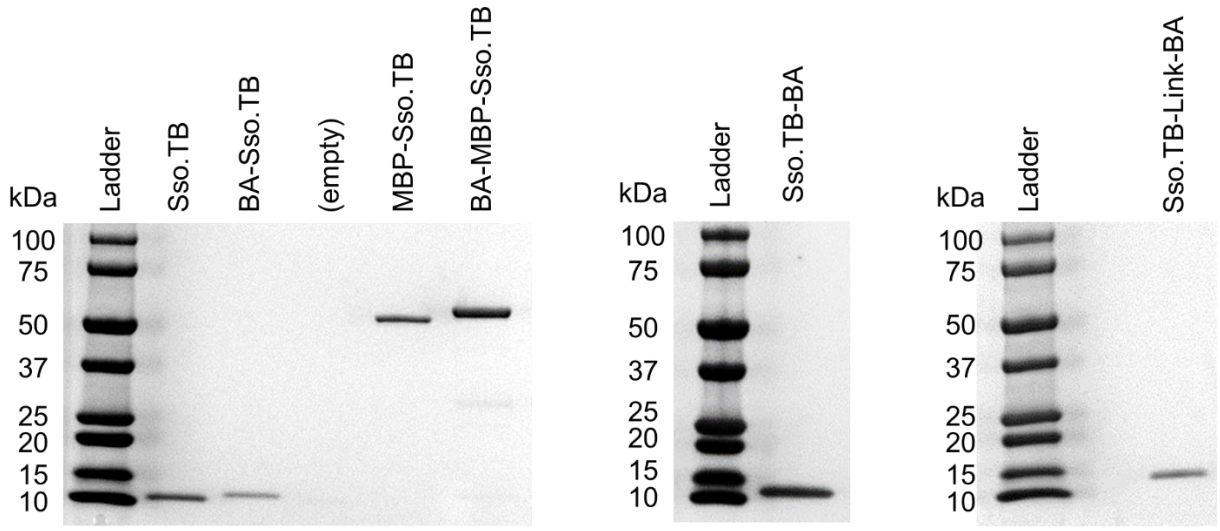

Figure S8. Original SDS-PAGE gel images for the Sso.TB variants to indicate protein purity.

Figure 2 is a combination of the three gels for fluidity.

Biotin Quantitation

We used a Quant*Tag kit (Vector Labs) rather than the more common HABA (4'-hydroxyazobenzene-2-carboxylic acid) assay due to unreliable results for some of the protein variants. HABA assay requires the biotin to displace HABA by binding to avidin, resulting in a decreased absorbance at 500 nm wavelength. Table S2 depicts some of the absorbance values collected when attempting to quantify biotinylation efficiency for two variants, BA-Sso.TB and MBP-Sso.TB-b_x. Values for chemically biotinylated MBP-Sso.TB-b_x appeared reasonable with an expected drop in absorbance with the presence of biotinylated protein.

Table S2. Absorbance values before addition of biotinylated protein (HABA/Avidin) and after addition of protein (HABA/Avidin/Protein) for three separate attempts. The calculated number of biotins per protein are shown based on the change in absorbance. MBP-Sso.TB-b_x showed reasonable values for biotinylation, but BA-Sso.TB did not show a noticeable change in absorbance; in some cases, the absorbance increased minutely. †High concentration of protein added, resulting in large change in absorbance but still small number of biotins per protein.

	BA-Sso.TB			MBP-Sso.TB-b_x		
	Run 1	Run 2	Run 3†	Run 1	Run 2	Run 3
Absorbance (HABA/Avidin)	0.739	0.768	0.741	0.754	0.798	0.741
Absorbance (HABA/Avidin/Protein)	0.742	0.777	0.571	0.737	0.777	0.622
Calculated # of Biotins per Protein	-0.157	-0.061	0.111	11.1	10.4	11.7

Since testing the BA-Sso.TB variant on cellulose strips did show binding of streptavidin, we determined that the HABA assay was unable to accurately quantify biotinylation efficiency for some of the protein variants. Based on the results from the avidin column purification and testing

on cellulose strips, the lack of accessible biotins for BA-Sso.TB and similarly *in vivo* biotinylated variants may explain the inaccurate results obtained using the HABA assay, since it does require that the biotin displace HABA by binding to avidin. If the biotin is inaccessible and cannot bind to avidin, that would explain the absence of color development.

The Quant*Tag results for MBP-Sso.TB-b_x matched the results obtained from HABA assay, verifying that the unreliable results for some of the variants was not a result of issues with the assay itself. The Quant*Tag reagent reacts to the presence of all biotins, even those that may not be accessible to bind to avidin like the HABA assay requires.[17] Since the reagent in the Quant*Tag kits also reacts with amino groups, the values obtained for the biotinylated proteins were adjusted based on the values from their respective unbiotinylated proteins (Sso.TB for BA-Sso.TB, Sso.TB-BA, and Sso.TB-Link-BA; MBP-Sso.TB for BA-MBP-Sso.TB). We also verified that the post-avidin column populations had approximately 100% biotinylation efficiency using the Quant*Tag assay on BA-MBP-Sso.TB (results not shown).

Materials and Methods

Selection of rcSso7d Clone Against TB Antigen Rv1656

The DNA and primary amino acid sequence of Sso.TB are included below. Bolded amino acids represent the antigen-specific residues in the variable binding face. This Rv1656-binding variant was selected via yeast surface display, and was isolated from the same combinatorial library as that outlined in Miller et al.[15]

cDNA Sequence:

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGG
CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGT
GTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGT
AAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA

Primary amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIK**S**V**W**R**R**GQ**R**I**W**F**R**YDEGGGA**W**G**A**G
KVSEKDAPKELLQMLEKQ

Production of Gene Constructs

Sso.TB

Sso.TB was cloned from the pCTcon2 yeast display plasmid into the pET28b(+) bacterial expression plasmid as previously described.[6] Briefly, polymerase chain reaction (PCR) amplification of the desired gene was conducted using the primers **rcSso7d-for** and **rcSso7d-rev** (Table S3), at an annealing temperature of 58.3 °C. This PCR amplicon was subjected to an *NdeI/XhoI* double digest at 37 °C for three hours (adding the *NdeI* enzyme after two hours to prevent aberrant cleavage). Following gel purification, this cleaved product was ligated into the digested pET-28b(+) plasmid backbone for ten minutes at room temperature in order to generate the stable Sso.TB construct. All ligation mixtures were purified using the DNA Clean and Concentrator-5 Kit from Zymo Research (Irvine, CA, USA), and eluted in 12 μL of PCR-grade

water. 4 μL of this ligation product was transformed into DH5α E. coli (**F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) gal- phoA supE44 λ- thi-1 gyrA96 relA1**) via electroporation. The entirety of this transformation mixture was plated on LB-kan plates and incubated overnight at 37 °C. Positive clones were verified via both 5' and 3' sequencing, using the T7 promoter and T7 terminator sequencing primers.

N-terminal MBP-Sso.TB

The maltose-binding protein-rcSso7d (MBP-Sso.TB) construct was prepared via stepwise restriction cloning projects. The MBP fusion partner was isolated from the pBADM41-MBP plasmid sourced from the European Molecular Biology Laboratory (EMBL). PCR was conducted using the **MBP-NdeI-for** and **MBP-BamHI-rev** primers, at an annealing temperature of 56 °C. This amplicon was subjected to an *NdeI/BamHI* double digest at 37 °C for one hour, and the gel-purified product was ligated into the digested pET28b-rcSso7d.SA-CBD plasmid (previously described), in the place of rcSso7d.SA.

This sequence-verified plasmid product was then used as the vector backbone for the second step of the process. Sso.TB was amplified using the **rcSso-BamHI-for** and **rcSso7d-rev** primers, at an annealing temperature of 59 °C. This step served to append the appropriate restriction sites to the rcSso7d gene, as well as a 5'-linker sequence encoding the $(G_4S)_2$ linker. This product was subjected to a *BamHI/XhoI* double digest at 37°C for three hours, and the gel-purified product was ligated into the digested pET28b-MBP-CBD vector backbone.

C-terminal Sso.TB-BA

rcSso7d variants bearing an C-terminal AviTag (Sso.TB-BA) were prepared via splice-overlap extension. PCR amplification of the Sso.TB variant was conducted using the **rcSso7d-for** and

rcSso-BA-Bridge-rev primers, at an annealing temperature of 58.3°C. PCR amplification of the biotin acceptor sequence (taken from pET30b-MBD2-h4-eGFP-BA plasmid outlined in Tam et al[18]) was performed using the **rcSso-BA-Bridge-for** and **BA-rev** primers, at an annealing temperature of 50.6 °C. The resulting amplicons were gel-purified, and 100 ng of each product was added to a second amplification reaction, along with 1 µL each of the **rcSso7d-for** and **BA-rev** primers. This PCR step was run using the standard PCR protocol, at an annealing temperature of 67.5 °C (the annealing temperature specific to the overlap region).

The resulting amplicon was digested with the *NdeI/XhoI* enzymes at 37°C, as was the pET28b-Sso.TB plasmid. The digested amplicon was ligated into the vector backbone and transformed into DH5α *E. coli* as previously described, and a subset of the resulting colonies were sequenced in order to identify the desired variant. Positive clones were verified using 5' and 3' sequencing, using the T7 promoter and T7 terminator sequencing primers.

For subsequent cloning processes, an internal *BamHI* site was inserted between the rcSso7d and biotin acceptor sequences. This was done via a stepwise-process, first introducing a 3' *BamHI* site to the rcSso7d sequence and integrating it into the rcSso7d-CBD format, and then introducing a 5' *BamHI* site to the biotin acceptor sequence and integrating it in the place of the CBD fusion partner. This permits modular cloning of additional rcSso7d variants into the BA-Sso construct.

<u>C-terminal Sso.TB-Link-BA</u>

The construct featuring a $(G_4S)_2$ linker sequence between the Sso.TB and C-terminal BA was prepared via traditional restriction cloning. PCR was used to modify the Sso.TB gene with a 3' linker sequence, using the **rcSso7d-for** and **rcSso-3PL-rev** primers at an annealing temperature of 58.3 °C. This product was subjected to an *NdeI/BamHI* double digest at 37 °C for one hour, and the gel-purified product was ligated into the digested pET28b-Sso.TB-BA plasmid (featuring an

internal BamHI site). The binding ability of this construct was compared against the C-terminal BA-Sso.TB construct to ensure no orientation-dependent effects.

N-terminal BA-Sso.TB

rcSso7d variants bearing an N-terminal AviTag (BA-Sso.TB) were prepared via non-directional restriction cloning. PCR amplification of the biotin acceptor sequence was performed using the **NdeI-BA-for** and **NdeI-BA-rev** primers, at an annealing temperature of 55.1 °C. This served to append *NdeI* sites at both the 5' and 3' ends of the biotin acceptor sequence. The resulting amplicon was digested with the *NdeI* enzyme at 37 °C, as was the pET28b-Sso.TB plasmid. The digested amplicon was ligated into the vector backbone and transformed into DH5α *E. coli* as previously described, and a subset of the resulting colonies were sequenced in order to identify variants containing the correctly oriented N-terminal biotin acceptor sequence.

N-terminal BA-MBP-rcSso7d

The construct for the MBP-rcSso7d fusion protein with an N-terminal AviTag on the MBP was prepared via stepwise restriction cloning projects, along with splice-overlap extension to develop modular cloning of other variants into the BA-MBP-rcSso7d construct. PCR was conducted on the previously constructed MBP-rcSso7d plasmid using **MBP-NdeI-EcoRI-for** and **MBP-BamHI-rev2** primers, at an annealing temperature of 59.6 °C. The additional *EcoRI* restriction site was added such that the AviTag sequence could be inserted in between the *NdeI* and *EcoRI* sites at the N-terminus. This PCR product was used for splice-overlap extension to add a $(G_4S)_2$ linker sequence at the 3' end of the MBP sequence, using **MBP-NdeI-EcoRI-for** and **LinkerMBP-SpeI-rev** primers, along with **MBP-Linker-SpeI-BamHI-oligo** to add the linker sequence, at an annealing temperature of 64.8 °C. PCR was then conducted on the rcSso7d sequence using MBP-

rcSso7d plasmid as a template, using **rcSso-SpeI-for** and **rcSso7d-rev** primers, in order to append a *SpeI* restriction site at the 5' end of the sequence. The MBP-Linker amplicon was subjected to an *NdeI/SpeI* double digest, rcSso7d amplicon was subjected to an *SpeI/XhoI* double digest, and the pET28b(+) backbone was subjected to an *NdeI/XhoI* double digest, all at 37 °C for one hour. The gel-purified products were ligated into the digested plasmid, transformed into DH5α *E. coli* as previously described, and subsequent colonies were sequenced to form a MBP-rcSso7d construct with extra restriction sites at the 5' end.

To add in the AviTag sequence into the 5' end of the new MBP-rcSso7d construct, PCR was conducted on the AviTag sequence using **AviTag-NdeI-for** and **AviTag-Linker-AflII-rev** primers, at an annealing temperature of 60.6 °C. The PCR product was used for splice-overlap extension to add a $(G_4S)_2$ linker sequence at the 3' end of the AviTag sequence, using **AviTag-NdeI-for** and **LinkerAviTag-EcoRI-rev** primers, along with **AviTag-Linker-EcoRI-AflII-oligo** to add the linker sequence, at an annealing temperature of 60.5 °C. The *AflII* site was added for extra modularity, in case of future cloning projects. The resulting amplicon and the new MBP-rcSso7d construct were subjected to an *NdeI/EcoRI* double digest at 37 °C for one hour. The gel-purified products were ligated together to form the final construct. The binding ability of this construct was compared against the N-terminal BA-Sso.TB construct to ensure no orientation-dependent effects.

Table S3. Oligonucleotide sequences of primers used in plasmid cloning of the rcSso7d.TB variants.

#	Oligo Name	DNA Sequence (*NdeI*, *XhoI*, *BamHI*, *EcoRI*, *SpeI*, and *AflII* sites)	Annealing Temp. (°C)
1	rcSso7d-for	5'-AGGCAGTCT**CATATG**GCAACCGTGAAAT-3'	63.3
2	rcSso7d-rev	5'-ACCCCT**CTCGAG**TTATTGCTTTTCCAGCATCTG-3'	64

3	rcSso-BA Bridge-rev	5’-TCGTTCAGGCCGCCCGCCATTTGCTTTTCCAGCATCTGCA-3’	72.5
4	rcSso-BA Bridge-for	5’-TGCAGATGCTGGAAAAGCAAATGGCGGGCGGCCTGAACGA-3’	72.5
5	BA-rev	5’-TGGTG**CTCGAG**TTTATTCATGC-3’	55.6
6	NdeI-BA-for	5’-CTACGC**CATATG**GCGGGCGGCCTG-3’	68
7	NdeI-BA-rev	5’-GCAAGG**CATATG**GTCATGCCATTCAATT-3’	60.1
8	MBP-NdeI-for	5’-GCGGCG**CATATG**AAAATCGAAGAAGGTAAA-3’	61
9	MBP-BamHI-rev	5’-GATACG**GGATCC**AGTCTGCGCGTCTTT-3’	63.7
10	rcSso-BamHI-for	5’-GCATACATATG**GGATCC**GGTGGTGGTGGTAGCGGTGGTGGCG GTTCAATGGCAACCGTGA-3’	74.1
11	rcSso-3PL-rev	5’-TTTAA**GGATCC**TGAACCGCCACCACCGCTACCACCACCACCTT GCTTTTCCAGCA-3’	73
12	MBP-NdeI-EcoRI-for	5’-CGGCGG**CATATGGAATTC**AAAATCGAAGAAGGTAAACTGG-3’	64.8
13	MBP-BamHI-rev2	5’-CTTATT**GGATCC**AGTCTGCGCGTCTTTCAGGG-3’	64.6
14	MBP-Linker-SpeI-BamHI-oligo	5’-TTATT**ACTAGT**TGAACCGCCACCACCGCTACCACCACCACC**GG ATCC**AGTCTGCGCGTCT-3’	73.4
15	LinkerMBP-SpeI-rev	5’-TTATT**ACTAGT**TGAACCGCCACCACCGCTACCAC-3’	64.8
16	rcSso-SpeI-for	5’-TCGTGTCT**ACTAGT**GCAACCGTGAAATTCACATACC-3’	63.1
17	AviTag-NdeI-for	5’-CTAAA**CATATG**ATGGCGGGCGGCCTGAACG-3’	65.6
18	AviTag-Linker-AflII-rev	5’-CCACCACC**CTTAAG**TTCATGCCATTCAATTTTCTGCGC-3’	65.8
19	AviTag-Linker-EcoRI-AflII-oligo	5’-TTATT**GAATTC**TGAACCGCCACCACCGCTACCACCACCACC**CT TAAG**TTCATGC-3’	70.4
20	LinkerAviTag-EcoRI-rev	5’-TTATT**GAATTC**TGAACCGCCACCACCGCTACCACC-3’	65.5

Recombinant protein expression, purification, and characterization

Expressions of all protein constructs were conducted in a BL21(DE3) strain of *E. coli* and induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The BA variants were supplemented with free biotin during expression via the addition of 0.1 mM D-biotin to assist with higher biotinylation efficiency. After overnight expression, the cells were cultivated and lysed via sonication. The recombinant rcSso7d protein products were then purified from the clarified lysate through IMAC using HisTrap FF crude columns (GE Healthcare) and buffer exchanged into 40 mM sodium acetate (pH 5.5) using Amicon Ultra Centrifugal Filters. The TB antigen was expressed and purified as described previously.[15]

All purified proteins were quantified using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). To verify purity, the proteins were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) with Biorad P/N 161-0374 used as the protein ladder. The gel was stained with Coomassie Brilliant Blue G-250.

After IMAC purification and buffer exchange for BA-Sso.TB, Sso.TB-BA, Sso.TB-Link-BA, and BA-MBP-Sso.TB, a portion of the purified protein was further purified on a Pierce Monomeric Avidin Agarose Kit (Thermo Fisher Scientific) to purify the proteins with accessible biotins. The kit protocol was followed, and the provided Regeneration buffer was used for elution. The elution fractions were pooled together and concentrated down using Amicon Ultra Centrifugal Filters. The yield of protein bound and eluted from the column was determined by quantifying the amount of protein eluted using a BCA assay and dividing the amount of protein in elution fractions by the total amount of protein applied to the column.

Sso.TB and MBP-Sso.TB were chemically biotinylated using EZ-Link Sulfo-NHS-LC-Biotin No-Weight format (Thermo Fisher Scientific), following the protocol provided by the manufacturer. For example, for MBP-Sso.TB, 1.5 mL of 43 μM protein (3.27 mg total) was reacted with 40 molar excess of Sulfo-NHS-LC-Biotin. The conjugated proteins were then desalted using Micro G-25 Spin Columns (Santa Cruz Biotech) to remove free biotins. To quantify the amount of biotins per protein for the chemically biotinylated and the *in vivo* biotinylated proteins, Quant*Tag (Vector Labs) was used following the manufacturer's protocol. Unbiotinylated Sso.TB and MBP-Sso.TB were used to subtract out any background absorbance for BA-Sso.TB, Sso.TB-BA, Sso.TB-Link-BA, BA-MBP-Sso.TB, and MBP-Sso.TB-b_x.

Table S4. Number of primary amines for Sso.TB and MBP-Sso.TB (lysine groups and the N-terminus of the protein). Primary amines are sites that can react with the Sulfo-NHS-LC-Biotin.

	MW (Da)	Number of primary amines
Sso.TB	9353.7	9
MBP-Sso.TB	50467.35	45

Fabrication and testing on oxidized cellulose assay test strips

Whatman No. 1 chromatography paper was oxidized for aldehyde functionalization and test zones were printed using hydrophobic ink as described previously.[19] Protein immobilization on the oxidized test zones occurred via overnight incubation of 6 uL of 20 uM of the antigen in 1x PBS and 10% glycerol. After blocking any unreacted aldehyde sites with 10 uL of 1x TBS for one hour, 10 uL of 330 nM of the biotinylated rcSso7d variant in 1% PBSA (1x PBS with 1% w/v bovine serum albumin) was applied to each test zone for 30 minutes followed by 10 uL of 330 nM SA AF-647 (Thermo Fisher Scientific) for 30 minutes in the dark. After each incubation steps, the test

zones were washed twice with 20 uL of 1x PBS. Samples were allowed to air-dry in the dark before imaging. Schematic of rcSso7d testing can be found in Figure S1.

Fluorescent microscopy was used to measure level of binding, as described previously.[6] Samples were exposed for 250 ms using a Cy5 filter and imaged using Metamorph software (Molecular Devices, Sunnyvale, CA). Captured fluorescent images were processed on ImageJ (US National Institutes of Health) to determine the mean fluorescence intensity (MFI), as described previously.[6] The background (fluorescence without presence of antigen) was subtracted from the sample to obtain the background subtracted MFI. Error bars indicate standard deviations. Statistical analysis was performed using JMP (SAS Institute). Significant differences between samples were evaluated by the Student's *t*-test, using $P < 0.05$ as significant.

Polymerization-based amplification

Eosin 5'-isothiocynate was conjugated to streptavidin (SA-eosin) as described previously.[20] The colorimetric results were generated following the same protocol listed above until after application of the reporter protein, BA-MBP-Sso.TB. Afterwards, 10 uL of 330 nM of SA-eosin in 1% PBSA was applied to both positive and negative test zones for 30 minutes. After wash steps, we applied an aqueous solution of 200 mM poly(ethylene glycol) diacrylate, 100 mM 1-vinyl-2-pyrrolidinone, 150 mM triethanolamine, 0.4 μM eosin Y, 1.6 mM phenolphthalein, and 0.02 N hydrochloric acid to each test surface and illuminated the surfaces with green light for 100 seconds. After rinsing with diH_2O, hydrogel generation was visualized by the addition of 2 μL of 0.5 M NaOH and imaged immediately using an iPhone 6s camera.

Enzymatic amplification

HRP-conjugated streptavidin (SA-HRP) was purchased from Thermo Fisher Scientific. After incubation of the test strips with BA-MBP-Sso.TB, 10 uL of 10 μg/mL of SA-HRP in 1% PBSA was applied to both positive and negative surfaces for 30 minutes. An aqueous solution of 5 mg/mL 3,3-diaminobenzidine (DAB) with 0.1% v/v hydrogen peroxide (H_2O_2) was prepared immediately before use, using VWR Life Science DAB Substrate Tablets (5 mg per tablet). After wash steps of the test zones, the DAB solution was applied. After 8 minutes, the surfaces were washed with diH_2O and imaged using an iPhone 6s camera.

Sequences and primary amino acid sequences of rcSso7d.TB constructs

rcSso7d.TB

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGG
CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGT
GTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGT
AAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIKSVWRRGQRIWFRYDEGGGAWGAG
KVSEKDAPKELLQMLEKQ

C-terminal rcSso7d.TB-BA

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGG
CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGT
GTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGT
AAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAATGGCGGGCG
GCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIKSVWRRGQRIWFRYDEGGGAWGAG
KVSEKDAPKELLQMLEKQMAGGLNDIFEAQKIEWHE

N-terminal BA-rcSso7d.TB

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGG
CGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGACCATATGGCAACCGT
GAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTGTGGCGT
CGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGTAAAGTGA
GCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMAGGLNDIFEAQKIEWHDHMATVKFTYQGEEKQVDISKIKSVWR
RGQRIWFRYDEGGGAWGAGKVSEKDAPKELLQMLEKQ

rcSso7d.TBLink-BA

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGG
CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGT
GTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGT
AAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGTGGTGGTG
GTAGCGGTGGTGGCGGTTCAGGATCCATGGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAA
AATTGAATGGCATGAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIKSVWRRGQRIWFRYDEGGGAWGAG
KVSEKDAPKELLQMLEKQGGGGSGGGGSGSMAGGLNDIFEAQKIEWHE

MBP-rcSso7d.TB

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGA
AAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGA
AGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTG
GAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACG
ACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCA
GGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCG
ATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCT
GGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAA
CCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTAT
GAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACTCTGGCGCGAAAGCGGGTCTGA
CCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGA
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GACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAAC
CGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGA
GTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAATAAAGACAAACCGCTG
GGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACTA
TGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTTCTGGTATGC
CGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTGAAAGAC
GCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAATGGCAACCGTGAAATTCA
CATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTGTGGCGTCGTGGCCA
GCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGTAAAGTGAGCGAAAAA
GATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKL
EEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYP
IAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKY
ENGKYDIKDVGVDNSGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNI
DTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPL
GAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKD
AQTGSGGGGSGGGGSMATVKFTYQGEEKQVDISKIKSVWRRGQRIWFRYDEGGGAWGAGKVSEK
DAPKELLQMLEKQ

BA-MBP-rcSso7d.TB

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGA
TGGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGG
TGGTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATT
AACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAA
TTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGG
CGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTG
TTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCG
TACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAA
CAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTG
AAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGA
TTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGG
CGTGGATAACTCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACAC
ATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGA
CCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGT
ACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAAC
GCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAG
GTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGA
GTTGGCGAAAGATCCACGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCG
AACATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCG
GTCGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGG
TGGTGGCGGTTCAACTAGTGCAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGAT
ATTAGCAAAATCAAGTCTGTGTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTG
GTGGTGCCTGGGGTGCAGGTAAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCT
GGAAAAGCAATAA

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFEAQKIEWHELKGGGGSGGGGSEFKIEEGKLVIWI
NGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGL
LAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKEL
KAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNSGAKAGLTFLVDLIKNKH
MNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGIN
AASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMP
NIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTGSGGGGSGGGGSTSATVKFTYQGEEKQVD
ISKIKSVWRRGQRIWFRYDEGGGAWGAGKVSEKDAPKELLQMLEKQ

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