Electronic Supplementary Material (ESI) for Sensors & Diagnostics.
This journal is © The Royal Society of Chemistry 2023

Engineering thermostable affinity proteins for use in high-throughput immunoassay formats

Huan Jia[1,a], Nazirulmubin Abdul Moomen[1,a], Jeanette Leong[1,a], Patthara Kongsuphol[1,a], Zhi Feng Sherman Lim[a], Carmen Sze Min Pui[a], Yuxuan Tan[a], Ki-Joo Sung[b], Hadley D. Sikes[a,b,*]

[a]Antimicrobial Resistance Interdisciplinary Research Group (AMR-IRG), Singapore-MIT Alliance in Research and Technology (SMART), #03-10/11 Innovation Wing, 1 CREATE way, Singapore 138602

[b]Department of Chemical Engineering, Massachusetts Institute of Technology (MIT), 77 Massachusetts Ave. E19-502C Cambridge, MA 02139, USA

[1]HJ, NAM, JL and PK contributed equally as co-first authors.

*Corresponding author email: sikes@mit.edu

1. Supplementary Results

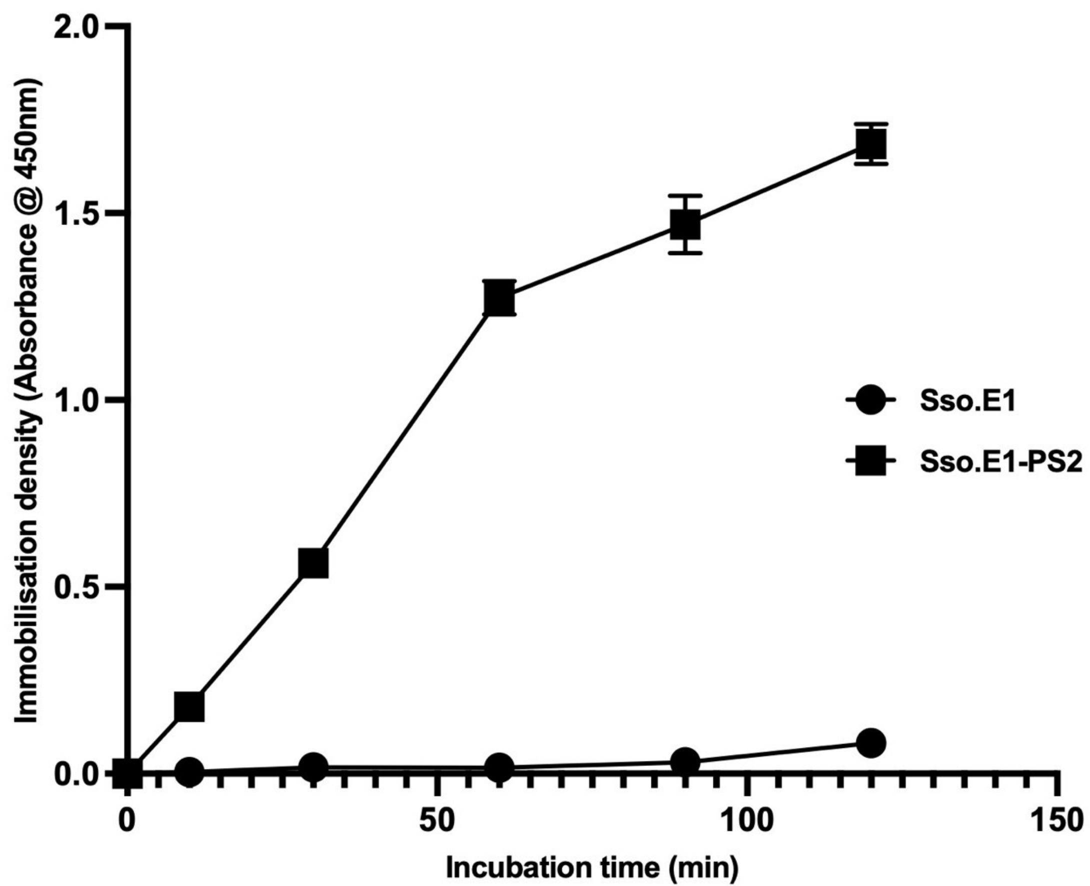

Figure S1. Changes in immobilisation density over time for Sso.E1 and Sso.E1-PS2 constructs. Data are mean ± s.d. with n = 3 independent replicates.

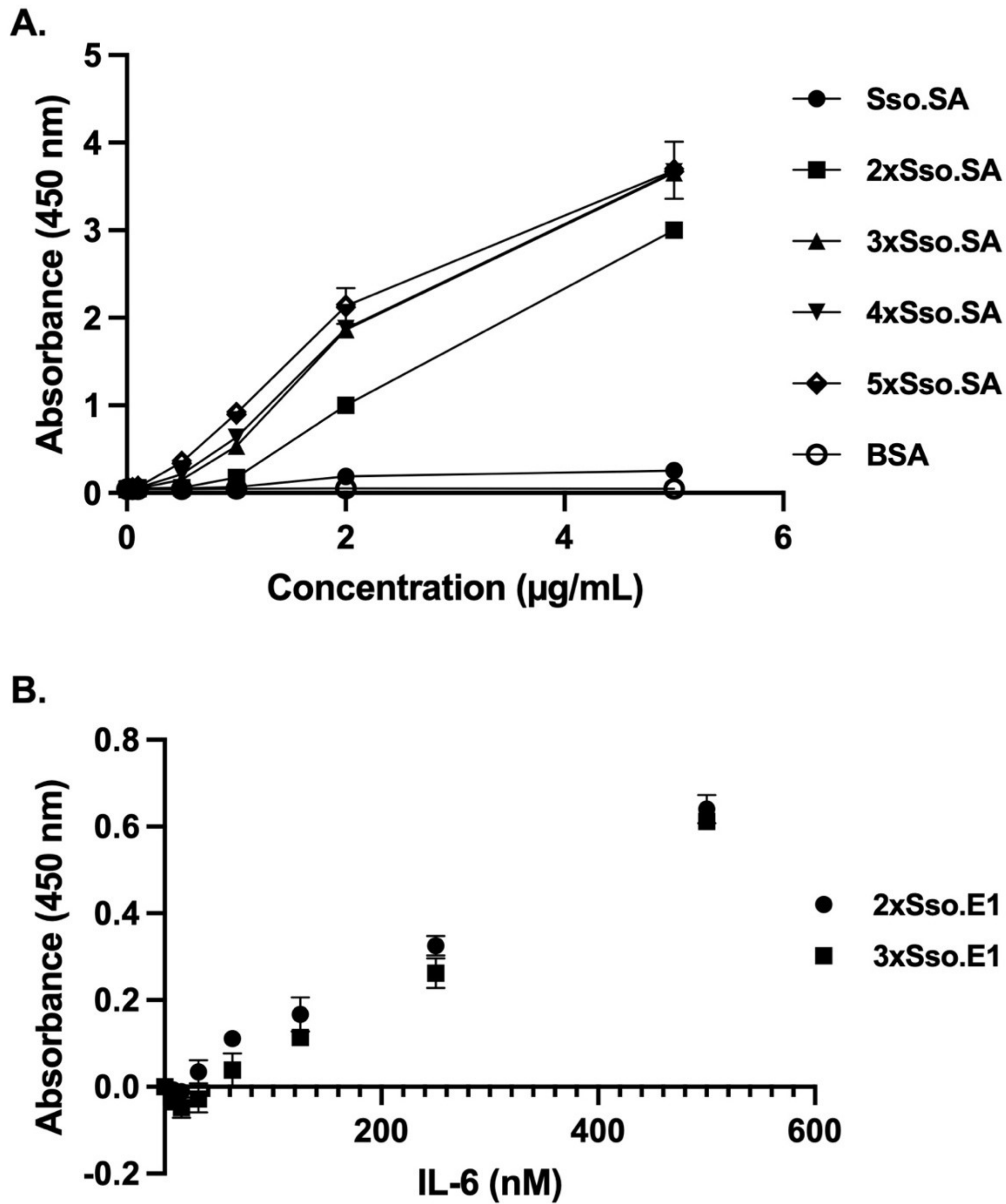

Figure S2. ELISA results on repetitive units constructs. A. Detection of streptavidin by different repetitive units of Sso.SA constructs; B. IL-6 detection ELISA for 2xSso.E1 and 3xSso.E1 constructs. Data in **A, B** are mean ± s.d. with n = 2 (**A**) or n = 3 (**B**) independent replicates.

2. Materials and methods

2.1. Plasmid Construction

Figure 1A shows a schematic of the various gene constructs employed in this work. Figure S3 and Table S1 give information about the gene structures and primers used for molecular cloning. All constructs were cloned into the pET28b vector, which encodes an N-terminal 6xHis tag and a Kanamycin-resistant gene.

Construction of repetitive units of Sso.E1

In a prior work, employing the RAPIDS method, a complementary pair of rcSso7d proteins specific to IL-6 were identified and published.[1,2] The rcSso7d proteins were assigned as Sso.E1 and Sso.E2 respectively as capture and reporting reagents, where Sso.E2 was fused with biotin acceptor (BA) and maltose binding protein (MBP) as BA-MBP-Sso.E2.[1,2] Additionally, BA-MBP-Sso.E1 construct was generated. The repeating units of 2xSso.E1. and 3xSso.E1. were constructed using Sso.E1 and BA-MBP-Sso.E1 as templates. **Table S1** provides the primers and DNA templates utilized for cloning each construct.

pET28b plasmid encoding BA-MBP-Sso.E1 was utilized as a template for polymerase chain reaction (PCR) to construct an insert comprising a 5'-(G4S)2 linker sequence and a 1xSso.E1 sequence with a *BsaI* restriction enzyme site (**Figure S3A and S3B**). As a vector, the pET28b plasmid carrying the Sso.E1 gene was amplified by PCR along with the introduction of a BsaI restriction enzyme site. Both insert and vector products were cleaved by the BsaI restriction enzyme and ligated to generate the 2xSso.E1 construct using a scarless Golden Gate assembly kit (NEB, Singapore). Every PCR reaction was performed with KOD Hot Start DNA polymerase (Sigma-Aldrich, Singapore). The PCR product of the insert fragment was purified utilizing the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Asia Pacific, Singapore), whilst the PCR product of the vector fragment was purified utilizing the DNA Gel Extraction Minipreps Kit (Bio Basic Asia Pacific, Singapore).

To generate 3xSso.E1, 3 fragments were ligated together, (i). Sso.E1 carrying 5'-$(G_4S)_2$ linker fragment produced by PCR from BA-MBP-Sso.E1, (ii). 2xSso.E1 fragment produced by PCR from previous described pET28b-2xSso.E1 construction and (iii). backbone pET28b as vector by PCR using BA-MBP-Sso.E1 plasmid. Golden Gate Assembly kit was used for ligation (**Figure S3C**).

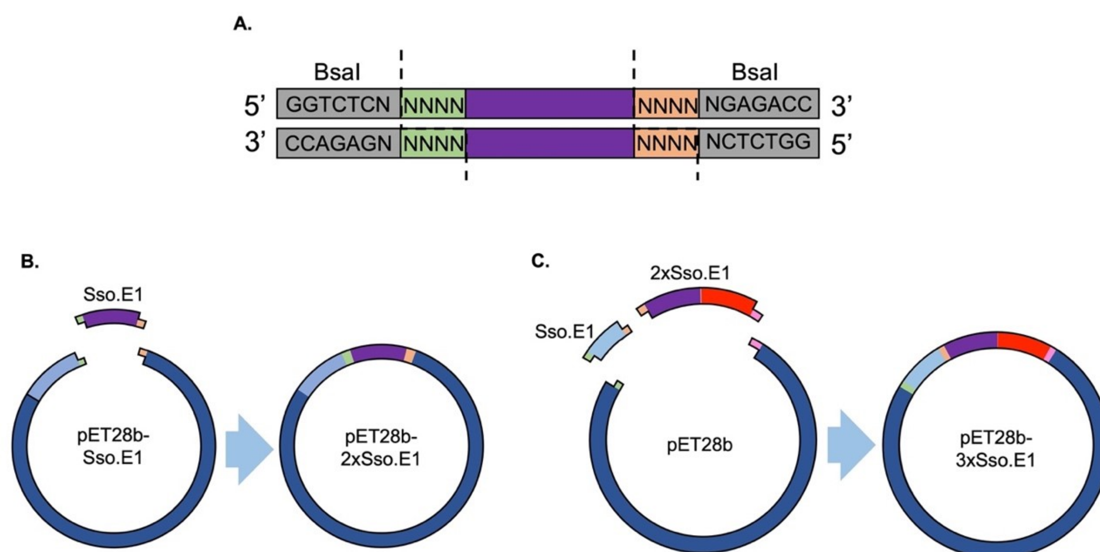

Figure S3. Construction of 2x.SsoE1 and 3x.SsoE1 using Golden Gate Assembly technique. **(A)** Principle of Golden Gate Assembly. Enzyme digestion site is downstream of the *BsaI* enzyme recognition site. Following the digestion, 4 overhang sequences are exposed to guide the assembly. **(B)** Insert and vector used for construction of 2xSso.E1 and **(C)** Inserts and vector used for construction of 3xSso.E1.

Construction of Sso.E1 with PS peptide tags

All constructs with PS peptide tags, including Sso.E1-PS1, Sso.E1-PS2 and Sso.E1-PS3, were synthesized in pUC57 vector while PS2-Sso.E1 was synthesized in pET28b vector by Bio Basic Asia Pacific (Singapore). All constructs in pUC57 plasmids were amplified using PCR and cloned into pET28b vector.

To fuse PS2 to the N terminus of 2xSso.E1, PS2-Sso.E1 was used as a PCR template to amplify pET28b vector that carries the PS2 tag. 2xSso.E1 insert was amplified from pET28-2xSso.E1 plasmid. Inserts and vector were fused together at *NheI* (NEB, Singapore) and *XhoI* (NEB, Singapore) restriction enzyme sites using T4 DNA ligase (NEB, Singapore).

Table S1. Primer information used in generation of repetitive Sso.E1 and PS peptide tagged Sso.E1.

Primer Name	DNA Sequence, 5'-3' (*BsaI*, *XhoI*, and *NheI* sites)	Annealing Temperature (°C)	Number of PCR cycles	Template	PCR product	Final Construct
Rep IL6.2 vec-F	**GGTCTC**ACTCGAGCACCACCACCACCA	59	30	pET28b(+) Sso.E1	pET28b(+) Sso.E1 without stop codon	pET28b(+) 2xSso.E1
Rep IL6.2 vec-R	**GGTCTC**ATTGCTTTTCCAGCATCTGCAGCAGTT	59	30			
Rep IL6.2 ins-F	**GGTCTC**TCGAGTTATTGCTTTTCCAGCATCTGCAG	59	30	pET28b(+) BA-MBP-Sso.E1	GS Linker + Sso.E1 insert	

	CAGTTCTTT					
Rep IL6.2 ins-R	GGTCTCTGCAAGGTGGTGGTGGTAGCGGTGG	59	30			
Rep Vec-F	AAGGGTCTCAGTTGGCTGCTGCCACCGC	67.2 72	5 30	pET28b(+) 2xSso.E1	pET28b(+) backbone	pET28b(+) 3xSso.E1
Rep Vec-R	ACAGGTCTCATCCGCTCACAATTCCCCTATAGTGAGTCGTATTA	67.2 72	5 30			
Ins1 2x-F	AACGGTCTCGCGGATAACAATTCCCCTCTAGAAATAATTTTGTT	55.4 62.3	5 30	pET28b(+) 2xSso.E1	2xSso.E1 with GS Linker in between each unit	
Ins1 2x-R	ACAGGTCTCACACCTTGCTTTTCCAGCATCTG	55.4 62.3	5 30			
Ins2 1x-F	AACGGTCTCCGGTGGTGGTGGTAGC	55.4 62.3	5 30	pET28b(+) BA-MBP-Sso.E1	GS Linker + Sso.E1 insert	
Ins2 1x-R	ACAGGTCTCCCAACTCAGCTTCCTTTCG	55.4 62.3	5 30			
XhoI-F-PS2N	GCAATAACTCGAGCACCACC	47 50.1	5 30	pET28b(+) PS2-Sso.E1	pET28b(+) backbone + PS2 + GS linker	pET28b(+) PS2-(**n**)xSso.E1
NheI-R-PS2N	AATGCTAGCACTAGTTGAACCG	47 50.1	5 30			
Sso NheI-F	TACGCTAGCGCAACCGTGAAATTC	45 53.3	5 30	pET28b(+) (**n**)xSso.E1	(**n**)xSso.E1 insert	
Sso XhoI-R	CGCCTCGAGTTATTGCTTTTCCAG	45 53.3	5 30			

'n' refers to the number of Sso.E1 units.

2.2. Protein expression and purification

Plasmids were transformed into BL21(DE3) E. coli cells and cultivated at 37°C shaker until the optical density (OD600) reached between 0.6 and 0.8. 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) induced the expression of proteins. The culture was further cultivated in an incubator at 16°C and 160 rpm for 16 to 20 hours. The production of BA-MBP-Sso.E2 required the addition of 0.1 mM biotin during the induction step. The 10 mM biotin stock solution was prepared by dissolving 24 mg d-biotin (Sigma-Aldrich, Singapore) in 10 mL of warm 10 mM bicine buffer (pH 8.3) and stirring until the biotin was dissolved completely. Afterward, the solution was filtered and sterilized with a 0.22 m filter (Sartorius, Germany).

At 4°C, cells were harvested by ultracentrifuging at 4,000 g for 15 minutes. The cell pellets were subsequently resuspended in Lysis Buffer (50 mM Tris-HCl pH7.6, 300 mM NaCl, and 10 mM Imidazole) with a protease inhibitor cocktail (Nacalai Tesque Inc., USA). Before undergoing ultracentrifugation at 20,000 g for 35 minutes at 4°C, the cell

suspension was sonicated for 1-3 minutes at 50-70% amplitude. Before loading a gravity flow column, supernatant was incubated with NuviaTM (BioRad, USA) immobilized metal affinity chromatography (IMAC) Ni-Charged Resin at 4°C for three hours. 10 column volumes (CV) of buffer containing 50 mM and 100 mM Imidazole were used to wash the resin. Six cycles of elution with 1 CV of buffer containing 300 mM Imidazole were followed by two cycles of elution with 1 CV of the Elution Buffer (50 mM Tris-HCL pH 7.6, 300 mM NaCl, 500 mM Imidazole).

Concentrated fractions containing the protein of interest were subjected to buffer exchange with 1x PBS, pH 7.6 using an Amicon centrifugal filter (Millipore, USA) with a molecular weight cut off (MWCO) no greater than half the size of the protein. All proteins fused with PS tag were purified using size exclusion chromatography with a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) in 1x PBS, pH 7.6.

Proteins were separated by SDS-PAGE and stained with Coomassie blue. Gel pictures were taken utilizing Gel Doc Imaging Systems (Bio-Rad, USA).

2.3. Half ELISA for checking immobilisation density

96-well polystyrene plates (Cellstar, Greiner bio-one) were coated with 100 µL of 125 nM Sso.E1 constructs in each well. On a 300 rpm shaker, the proteins were incubated for various durations ranging from 10 to 120 minutes at room temperature. Each well was washed three times with 200 µL of washing buffer (1x PBS with 0.1% v/v Tween-20) and blocked for 1 hour at room temperature with 200 µL of blocking reagent (5% v/v FBS diluted in 1x PBS). The plate was washed with 200 µL of the washing buffer three times. Each well was added with 100 µL of anti-His antibody conjugated to HRP at dilution factor of 2500 (Sigma-Aldrich Pte. Ltd.) and incubated for 1 hour at room temperature in the absence of light. Each well was washed with the washing buffer three times. Each well was then filled with 100 µL of TMB and incubated for 2 to 4 minutes. Reactions were stopped using 100 µL of 2 M sulfuric acid. Using a Synergy HTX Multi-Mode Reader, the absorbance was measured at 450 nm (BioTek, USA).

2.4. Full sandwich assay

Each well of the 96-well plate was coated with 100 µL of 500 nM capture proteins and incubated for 10 minutes at room temperature on a plate shaker at 300rpm. Each well

was washed three times with 200 µL of washing buffer and blocked for one hour at room temperature with 200 µL of blocking reagent on a 300 rpm shaker. Each well was washed with the washing buffer three times. Human IL-6 recombinant protein (Biolegend, USA) was prepared by two-fold serial dilution in PBS to yield samples with final concentrations ranging from 7.81 to 500 nM. In each well, 100 µL of IL-6 sample with various concentrations were incubated for 2 hours at room temperature on a 300 rpm shaker. Each well was washed with the washing buffer three times. After adding 100 µL of 500 nM Sso.E2 to each well, they were incubated for one hour at room temperature with shaking at 300 rpm. For signal generation, 100 µL of 1:2000 diluted SA-HRP (Sigma-Aldrich, Singapore) in PBS was applied to each well and incubated at room temperature for 30 minutes. Each well was washed three times with the washing buffer and incubated for two to four minutes with 100 µL of TMB substrate (Sigma-Aldrich, Singapore). Reaction was stopped with 100 µL of 2 M sulfuric acid. Absorbance at 450 nm was measured with a Synergy HTX Multi-Mode Reader.

2.5. Statistical analysis

GraphPad Prism 9 (9.5.1) was utilized for all statistical analysis. Triplicate data were obtained for each group of experiments and error bars in Figures represent standard deviation about the mean. Ordinary one-way ANOVA was carried out for multiple comparisons of fold changes in immobilisation as shown in **Figure 2B**.

References

1. Miller, E. A. *et al.* Beyond epitope binning: directed in vitro selection of complementary pairs of binding proteins. *ACS Combinatorial Science* **22**, 49-60 (2019).
2. Sung, K.-J., Miller, E. A. & Sikes, H. D. Engineering hyperthermostable rcSso7d as reporter molecule for in vitro diagnostic tests. *Molecular Systems Design & Engineering* **3**, 877-882 (2018).