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SUPPORTING INFORMATION

Activity-based assessment of an engineered hyperthermophilic protein as a capture agent in paper-based diagnostic tests

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1. MATERIALS

1.1 Reagents

50x Tris-acetate-EDTA buffer and Casamino acids were purchased from Amresco (Solon, OH, USA). Bacto agar, Bacto yeast extract, yeast nitrogen base without amino acids, and Bacto tryptone were purchased from BD Diagnostics (Franklin Lakes, NJ, USA).

Monoclonal mouse anti-HA IgG antibodies were purchased from Biolegend (San Diego, CA, USA). Monoclonal chicken anti-c-Myc IgY antibodies were purchased from Gallus Immunotech Inc. (Cary, NC, USA). Polyclonal rabbit anti-streptavidin IgG antibodies were purchased from Genscript (Piscataway, NJ, USA). Alexa Fluor 488 goat anti-chicken IgG antibodies, Alexa Fluor 647 goat-anti-mouse IgG antibodies, Alexa Fluor 647 streptavidin, and Dynabeads Biotin Binder were purchased from Invitrogen (Carlsbad, CA, USA). Lyophilized streptavidin was purchased from Rockland Antibodies and Assays (Limerick, PA, USA).

Ethidium bromide and 30% acrylamide/Bis Solution were purchased from Bio-Rad (Hercules, CA, USA). Seakem LE agarose was purchased from Lonza (Allendale, NJ, USA). Sodium phosphate monobasic monohydrate and sodium phosphate dibasic were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Eosin 5'-isothiocyanate (EITC) was purchased from Marker Gene Technology (Eugene, OR, USA). Phusion HF polymerase, T4 DNA ligase, NdeI restriction endonuclease, and XhoI restriction endonuclease were purchased from New England Biolabs (Ipswich, MA, USA). Isopropyl β-D-1-thiogalactopyranoside was purchased from Omega Bio-Tek (Norcross, GA, USA). Sodium (meta)-periodate, 10x phosphate-buffered saline (PBS), tricine, D-(+)-galactose, sodium carbonate, N,N,N',N'-tetramethylethylenediamine, sodium dodecyl sulfate (SDS), β-mercaptoethanol, and ammonium persulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) standard (2 mg/mL) and EZ-Link Sulfo-NHS-LC biotin (No-Weigh Format) were purchased from Thermo Scientific (Waltham, MA, USA).

Tris(hydroxymethyl)aminomethane (Tris), sodium acetate (anhydrous), and sodium chloride were purchased via VWR from Avantor Performance Materials (Center Valley, PA, USA). Kanamycin sulfate, ampicillin (sodium salt), LB media, SOC media, glycerol (biotechnology grade), dextrose (anhydrous), Tris-HCl, sodium chloride, imidazole, acetic acid, Molecular Grade Water, 2xYT media, 100x Penicillin-Streptomycin, lyophilized BSA, Coomassie Brilliant Blue G-250 dye, methanol, potassium phosphate dibasic, potassium phosphate monobasic, citric acid monohydrate, and sodium citrate dihydrate were purchased from VWR (Radnor, PA, USA).

1.2 Biological Materials

E. coli derived from the strains DH5α (**F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *gal- pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1**) and BL21(DE3) (**F- *ompT gal dcm lon hsdS*$_B$ (r_B- m_B-) λ(DE3)**) were purchased from Invitrogen. All oligonucleotides were purchased from IDT (San Jose, CA, USA).
Saccharomyces cerevisiae of the EBY100 strain (***GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1 GAL*, Trp- Leu-**), the pCTCON2 plasmid, and the combinatorial library of codon-optimized, charge-reduced Sso7d variants (termed rcSso7d-11) were kind gifts from the lab of Dr. K. Dane Wittrup (Massachusetts Institute of Technology). The charge-reduced scaffold (called rcSso7d) features several mutations at key lysine residues, which serve to reduce the formal charge of the scaffold from +7 to +1. Further modifications made to the rcSso7d scaffold by the Wittrup lab to produce the rcSso7d-11 library will be described in detail in a forthcoming publication.

1.3 Consumables

UltraCruz Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acrodisc PF 0.2 μm Syringe Filters were purchased from the Pall Corporation (Port

Washington, NY, USA). Whatman No. 1 chromatography paper (20 x 20 cm) was purchased from VWR. The Reducing Agent Compatible Pierce Microplate BCA Protein Assay kit and Pierce Biotin Quantitation kit were purchased from Thermo Scientific. All Amicon Ultra Centrifugal Filter Units were purchased from EMD Millipore (Billerica, MA, USA). HisTrap FF Crude 5x1mL columns were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). GenCatch Gel Extraction kits and GenCatch Plasmid Miniprep kits were purchased from Epoch Life Sciences (Missouri City, TX, USA). Corning bottle-top vacuum filters were purchased from Sigma-Aldrich. The Zymo Frozen-EZ Yeast Transformation II and ZymoPrep Yeast Miniprep II kits were purchased from Zymo Research (Irvine, CA, USA).

1.4 Cell Culture Media and Buffers

SDCAA media (pH 4.5) was prepared by dissolving 20 g dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 14.7 g sodium citrate dihydrate, and 4.29 g citric acid monohydrate in DI water, to a final volume of 1 L. This mixture was then sterile-filtered using a Corning bottle-top vacuum filter, and 10 mL of 100x penicillin-streptomycin was added to the solution. Media was stored at 4°C for up to 6 months.

SDCAA agar plates were prepared by dissolving 5.4 g sodium phosphate dibasic, 8.56 g sodium phosphate monobasic monohydrate, 182 g sorbitol, and 15 g agar in DI water to a final volume of 900 mL and autoclaving this solution. A second solution was made by dissolving 20 g dextrose, 6.7 g Difco yeast nitrogen base without amino acids, and 5 g Bacto casamino acids in 100 mL of DI water and sterile-filtering this mixture. These solutions were combined after the autoclaved solution had cooled to ~50°C, and plates were poured in a sterile hood, to a final volume of ~30 mL/plate. Plates were then stored at 4°C for up to 6 months.

SGCAA media was prepared by dissolving 20 g galactose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 5.4 g sodium phosphate dibasic, and 8.56 g sodium phosphate monobasic monohydrate in DI water, to a final volume of 1 L. This mixture was then sterile-filtered.

Yeast freezing solution was prepared by mixing 2% glycerol (v/v) and 0.67% yeast nitrogen base (w/v) in DI water and sterilizing by autoclave. Low-dextrose SDCAA for frozen yeast storage was prepared by dissolving 5 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 5.4 g sodium phosphate dibasic, and 8.56 g sodium phosphate monobasic monohydrate in DI water, to a final volume of 1 L.

Terrific broth media (TB media) was prepared by dissolving 12 g Bacto tryptone, 24 g Bacto yeast extract, and 4 mL biotechnology grade glycerol in 900 mL of DI water. In a separate bottle, 2.31 g potassium phosphate monobasic (0.17 M) and 12.54 g potassium phosphate dibasic (0.72 M) were dissolved in 100 mL of DI water. These solutions were autoclaved separately at 250°C and 10psi for 45 min, and were combined when cooled.

LB media was prepared by adding 31 g of dried LB media to DI water, in a final volume of 1 L, and autoclaving at 250°C and 10psi for 45 min.

LB agar plates were prepared by adding 15 g of Bacto agar to a standard LB media preparation, along with a stir bar. This solution was autoclaved in standard fashion, and was then placed on a magnetic stir plate until the temperature dropped below ~50°C, at which point 1 mL of kanamycin stock solution was added. The mixture was stirred for several minutes more, and then plates were poured in a sterile hood, to a final volume of ~30 mL/plate.

All required antibiotics were added once the solutions had cooled below 50°C. For kanamycin solutions, 1 mL of 1000x kanamycin sulfate stock solution (50 mg/mL in DI water, sterile-filtered and stored at -20°C) was added, to a final concentration of 50 μg/mL. For ampicillin solutions, 1 mL of

1000x ampicillin sodium salt stock solution (100 mg/mL in DI water, sterile-filtered and stored at -20°C) was added, to a final concentration of 100 μg/mL.

Coomassie blue loading buffer for SDS-PAGE gels was prepared by mixing 120 mg of SDS, 1.2 mg of Coomassie Brilliant Blue G-250 dye, 400 μL of glycerol, and 60 mg of β-mercaptoethanol with 600 mM Tris-Cl, pH 6.8, to a final volume of 1 mL.

2. BINDER SELECTION AND CHARACTERIZATION

2.1 Combinatorial yeast library revival

Streptavidin-binding variants of rcSso7d were selected from a combinatorial library of EBY100 *Saccharomyces cerevisiae* cells, representing $1.4\ x\ 10^9$ unique, charge-reduced Sso7d clones encoded in the yeast-surface display plasmid pCTCON2. This library, referred to as rcSso7d-11, was received in frozen form, and in order to prepare for flow cytometry, cells representing 20x the library diversity were inoculated into two liters of low-dextrose SDCAA in two baffled shake flasks, and grown over the course of three days. This served to passage and expand the frozen library population for the generation of fresh, additional library aliquots. The revived yeast cultures were centrifuged in a Microfuge X3R from Thermo Scientific at 500 x g and 4°C for ten minutes, and the cell pellet was re-suspended in yeast freezing solution, at a theoretical cell density of $5.4\ x\ 10^9$ cells/mL. These cells were aliquoted into cryogenic vials at approximately 10^{10}cells/vial, and slow-frozen in an isopropanol bath at -70°C.

Sub-aliquots of this population were plated in order to gauge the viability of this passaged population, and to determine the number of vials that must be thawed to capture a 20-fold excess of the theoretical library diversity. SDCAA dilution plates (10^6-fold and 10^7-fold) were prepared and incubated for two days at 30°C, after which the number of visible colonies was counted. The associated viable cell concentration was then compared with the theoretical cell concentration, and the lower of the two ratios was taken, indicating a passaged library viability of 66%.

The theoretical cell concentration was determined by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD_{600}) using a Cary 50 UV–Visible Spectrophotometer from Varian, Inc. (Palo Alto, CA, USA) operated at room temperature. Baseline measurements were taken using bare media, and cell cultures were sufficiently diluted to render the cell solution within the system's linear range of 0.1 to 0.8. An OD_{600} of 1 was taken to be roughly $1\ x\ 10^7$yeast cells.[1]

Given the calculated library viability of 66%, five frozen vials (roughly 1.5-fold more than required using the total cell concentration) were revived in order to capture 20x the library diversity. Note that common practice is to maintain a ratio of at least 10x the library diversity in order to ensure virtually complete library coverage – a ratio of 20x allows for irregularities in cell viability, optical density measurements, etc. SDCAA dilution plates of this revived population indicated a post-freezing viability of 81.2% of the expected viable population, and a final, 17.7-fold over-representation.

2.2 Combinatorial library screening via magnetic bead sorting

This library was first enriched using magnetic bead sorting,[2] in order to capture variants with even marginal affinity to streptavidin, and to reduce the population to a size more amenable to cytometric analysis. Five library aliquots were thawed rapidly at 30°C, and inoculated in 1 L of SDCAA media in a 2 L baffled shake flask. These cultures were incubated overnight at 30°C and 250rpm.

The revived cells were passaged once into fresh SDCAA, and then inoculated once more into 1 L of SDCAA in a 2 L baffled shake flask, and incubated until the culture reached an OD_{600} of approximately 7. Ideally, cells should be induced at an OD_{600} between 2 and 6, in order to induce protein expression while the cells are undergoing log phase growth. However, given the size of the inoculant $(2.8\ x\ 10^{10}\ cells)$, the initial outgrowth was allowed to run long. A 20-fold excess of cells was then centrifuged for 3 minutes at 2000g and 4°C in 250 mL centrifuge bottles, the supernatant was discarded, and the cell pellets were re-suspended in 1 L of SGCAA media, in order to induce expression of the cell surface-displayed combinatorial library. Ordinary induction protocols call for

cells to be induced at an OD_{600} of 1, but due to the size of the library, the cells were induced at a higher cell density of 2.8.

This induction culture was incubated in a 2 L baffled shake flask for 44 hours at 20°C. Following induction, $2.8\,x\,10^{10}$ cells were centrifuged, the supernatant was discarded, and the pellet was re-suspended to a total volume of 20 mL in PBSF (sterile-filtered 1x PBS/0.1% (w/v) BSA), yielding a cell concentration of $1.4\,x\,10^{9}$ cells/mL. This cell suspension was split into twenty 1 mL aliquots in 2 mL microcentrifuge tubes, in order to allow for vigorous mixing and fluid recirculation.

Biotin binder Dynabeads were prepared by washing 50 µL of gently re-suspended bead solution in 1000 µL of PBSF in a 2 mL microcentrifuge tube. After this wash step, the tube was placed on a DynaMag 2 magnetic rack, and given approximately 30 seconds for all beads to be drawn toward the tube wall nearest the rack. The supernatant was carefully drawn off and discarded using a micropipette, and the process was repeated once more. Following the second wash, the beads were re-suspended to a final volume of 1000 µL, and 50 µL of the bead solution was added to each of the twenty tubes containing the cell solution.

These tubes were incubated for 2.5 hours at 4°C on a rotary incubator, in order to properly mix the cells and beads and to allow all potential yeast-surface displayed binders to come into contact with the streptavidin-coated beads. Following this incubation, the tubes were placed on the magnetic rack (taking care to transfer any cell suspension trapped in the top of the microcentrifuge tube back into the tube) and given several minutes in order to allow the beads to navigate the viscous cell suspension and migrate to the tube wall. All unbound yeast cells were carefully drawn off, and the process was repeated, washing the bound beads once in 1 mL of cold PBSF. The captured beads were then re-suspended in 1 mL SDCAA and all aliquots were pooled together and incubated overnight in 1 L of SDCAA media, in order to allow any bound yeast cells to bud off and expand. Serial dilution plates made from the enriched bead solution indicated a post-sort population of $9.6\,x\,10^{5}$ cells.

This magnetic bead sorting process was repeated twice more with increasing wash stringency, in order to apply selective pressure to the population and to select for stronger streptavidin binders. Following outgrowth in SDCAA, $1\,x\,10^{9}$ cells at an OD_{600} of 2-6 were induced in SGCAA at a cell concentration of $1\,x\,10^{7}$ cells/mL, and incubated at 20°C for 40-48 hours. $1\,x\,10^{9}$ induced cells were spun down and re-suspended in 1000 µL PBSF, and 5 µL of pre-washed Dynabeads in 45 µL of PBSF was added to the tube. Following 2.5 hours of co-incubation at 4°C, the beads were once again extracted from the solution and washed in 1000 µL PBSF – two and three times for the second and third magnetic bead sorts, respectively. Following three rounds of magnetic bead sorting, the final enriched population size was found to be $7.4\,x\,10^{5}$. This population was passaged, and prepared for further enrichment via fluorescence-activated cell sorting.

2.3 Combinatorial library screening via fluorescence-activated cell sorting

This yeast library was screened and enriched for streptavidin binders over the course of five sequential rounds of fluorescence activated cell sorting (FACS), following previously described protocols.[1,3,4] Briefly, a twenty-fold excess of the library diversity from the previous sorting round was induced in SGCAA at an OD of 1.0. This culture was grown at 20°C for 40-48 hours, and a twenty-fold library excess of cells were removed and pelleted for 30 seconds at 14,000 x g. The supernatant was aspirated and the cell pellet was re-suspended in 1mL PBSF and centrifuged once more to remove any traces of SGCAA. A 1:250 dilution of chicken anti-c-Myc IgY was prepared, and was conditioned to the desired streptavidin concentration using a 5 µM stock solution of streptavidin Alexa Fluor 647 (SA-AF647). This solution was used to re-suspend the washed yeast pellet to a concentration of $1 \times 10^{7} - 1 \times 10^{8}$ cells/mL, varying the volume to maintain a ten-fold molar excess of

ligand to the yeast-displayed rcSso7d protein (assuming a displayed copy number of 50,000 per cell). Sort-specific details are tabulated in Table S1.

All samples were incubated in the dark in a rotary mixer held at room temperature for at least 30 minutes. Following this primary incubation, the cell solution was centrifuged and the supernatant aspirated, and the resulting cell pellet was washed once in 1 mL of ice-cold PBSF and re-pelleted. This pellet was then re-suspended to a volume of 100 µL in a 1:100 dilution of Alexa Fluor 488 goat anti-chicken IgG, and were incubated on ice in the dark for 15 minutes. The samples were washed once more in 1 mL of ice-cold PBSF, and were kept on ice in pelleted form until the time came to process the library via FACS.

The samples were processed using a BD FACSAria Cell Sorter, running on the FACSDiva software package. Cytometer voltages were set using single-color controls (**488 SCC:** chicken anti-c-Myc IgY followed by Alexa Fluor 488 goat anti-chicken IgG; **647 SCC:** mouse anti-HA IgG followed by Alexa Fluor 647 goat anti-mouse IgG) to fix the binding signal within the dynamic range of the cytometer. Gates were established to select for singlet yeast cells using the forward- and side-scatter signal, and the samples were processed at a rate of approximately 14,000 cells/second. Over the course of the five rounds of FACS, selective pressure was applied to the population by decreasing the ligand concentration from 100 nM to 25 nM, and by increasing the sort gate stringency from taking the top 3% of the sorted population to taking the top 0.2% (Figure S1). Selected cells were sorted into 750 µL of SDCAA, and the captured cells were passaged into 5 mL of SDCAA, thoroughly washing out the sorting receptacle with media. This captured population was expanded for 24-48 hours until saturation was reached, and this culture was then used to inoculate the induction culture for the following sorting round.

FACS Round	Library Size	Number of Cells	Fold Library Excess	Suspension Volume (µL)	Cell Concentration (cells/mL)	SA-AF647 Concentration (nM)	Sort Gate Stringency
1	7.40E+05	2.00E+07	27.0	200	1.00E+08	100	3.0%
2	1.79E+05	1.00E+07	56.0	100	1.00E+08	100	2.8%
3	2.61E+04	1.00E+07	382.7	100	1.00E+08	100	0.8%
4	6.77E+03	1.00E+07	1477.1	400	2.50E+07	50	1.6%
5	4.90E+03	1.00E+07	2040.0	1000	1.00E+07	25	0.2%

Table S1: Sort conditions for FACS rounds 1-5, including the maximum number of unique library members, the number of cells prepared for each sort, the fold library excess, the volume of PBSF in which the captured cells were re-suspended, the resulting cell concentration, the concentration of streptavidin Alexa Fluor 647 used in each round, and the percentage of the sorted population captured in each round.

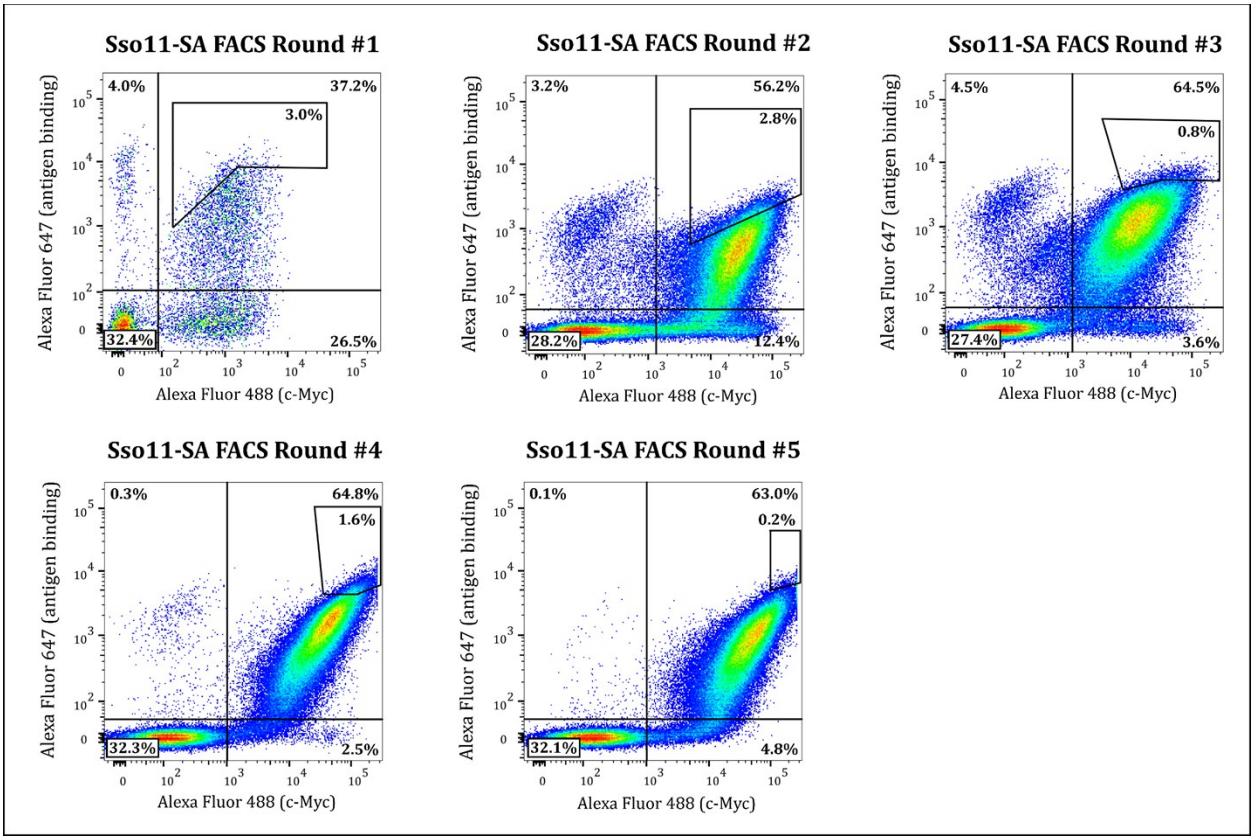

Figure S1: Cytometry plots from Rounds 1-5 of FACS library screening. The corner of each quadrant is labeled with the percentage of the library population found within that quadrant, and the percentage of the population captured is indicated within the sort gate.

2.4 Library sequencing and stable clone generation

Following the fifth FACS library screen, the enriched yeast library consisted of a maximum of 1,270 unique clones. This enriched pool was miniprepped using the ZymoPrep Yeast Miniprep II kit. 1 µL of the harvested plasmid pool at a concentration of ~60 ng/µL was mixed with 40 µL of freshly-thawed electrocompetent DH5α *E. coli*, and this mixture was transferred into a 0.2 cm electroporation cuvette. These samples were subjected to electroporation using a Bio-Rad Micropulser Electroporation Apparatus, applying a voltage of 2.5 kV at a time constant of approximately 5 milliseconds. Immediately following transformation, the cell solution was re-suspended in 1 mL of rich SOC media, and transferred into a rotary incubator at 37°C, where it was incubated for 45-60 minutes. This mixture was then transferred to pre-warmed LB-amp plates and incubated overnight in a stationary incubator held at 37°C.

Transformants of interest were inoculated into 5 mL overnight cultures of LB-amp media and incubated at 37°C for 12-18 hours. These cultures were then mini-prepped using the Epoch Life Sciences GenCatch Plasmid DNA Miniprep Kit. Purified plasmid DNA (pDNA) was eluted in 50 µL of nuclease-free water, and the concentration of this pDNA was quantified using a Nanoquant Plate and an Infinite M200 plate reader from Tecan (Männedorf, Switzerland). Each sequencing sample was prepared in a 10 µL sample at a concentration of 80 ng/µL, and premixed with 5 µL of the pCTCON2 forward sequencing primer at a concentration of 5 µM. These samples were then sent to Quintara Bio for sequencing. Sequence results were analyzed using the online Expasy Translate tool.

The seven samples that were initially sequenced yielded three distinct clones (frequencies are indicated in parentheses and variable residues are underlined in red.):

Clone 1 (2): ATVKFTYQGEEKQVDISKIKIVARDGQYIDFKYDEGGGAYGYGWVSEKDAPKELLQMLEKQ

Clone 2 (4): ATVKFTYQGEEKQVDISKIKIVARDGQWIDFAYDEGGGAKGYGYVSEKDAPKELLQMLEKQ

Clone 3 (1): ATVKFTYQGEEKQVDISKIKIVARDGQWIDFAYDEGGGAIGYGYVSEKDAPKELLQMLEKQ

Unique, sequence-verified clonal *E. coli* stocks were prepared from 5 mL overnight cultures, which were centrifuged and re-suspended in 350 µL of LB-kan media and 500 µL of 50% glycerol. These aliquots were allowed to equilibrate for 15 minutes before being placed in a Mr. Frosty Freezing Container (Thermo Scientific) for a controlled cooling rate of -1°C/minute, and transferred to a -70°C freezer. Miniprepped samples of pCTCON2 plasmid DNA found to encode unique rcSso7d clones were also transformed back into EBY100 yeast using the Frozen-EZ Yeast Transformation II Kit, and plated onto SDCAA agar plates. Following the growth of transformed yeast cells over a 2-4 day incubation at 30°C, these stable clones were expanded in 5 mL overnight SDCAA cultures, and 1 mL of this culture was mixed with 500 µL of 45% (v/v) sterile glycerol, and frozen at -70°C in similar fashion.

2.5 Affinity characterization via yeast surface display and flow cytometry

The model binder displayed in Table S2 (Clone 1) was selected from the sequenced library pool, and was subjected to a titration of streptavidin Alexa Fluor 647 (SA-AF647) in order to determine its affinity in the context of yeast-surface display. This yeast clone was grown overnight in SDCAA at 30°C and inoculated into SGCAA at an OD of 1. This induction culture was then incubated at 20°C for 40-48 hours, and 2×10^7 cells were harvested, centrifuged at 14,000*g* for 30 seconds, washed in PBSF, and centrifuged once more. A 1:250 dilution of chicken anti-c-Myc IgY was prepared, and was used to re-suspend the washed yeast pellet to a concentration of 1×10^7 cells/mL. 50 µL aliquots of this solution were prepared, yielding 5×10^5 cells per sample.

Protein species	**Primary structure (N → C)** (**Variable AA residues** and **library-wide scaffold modifications**)
WT Sso7d	ATVKFKYKGEEKQVDISKIK**K**V**W**R**V**GK**M**I**S**F**T**YDEGGGK**T**G**R**G**A**VSEKDAPKELLQMLEKQKK
rcSso7d-SA	ATVKF**T**Y**Q**GEEKQVDISKIK**I**V**A**R**D**G**Q**Y**I**D**F**K**Y**DEGGG**A**Y**G**Y**G**W**V**SEKDAPKELLQMLEKQ

Table S2: Primary protein structure of wild-type Sso7d and the selected streptavidin-binder, rcSso7d-SA. Residues highlighted in blue represent mutations that were made to the scaffold prior to combinatorial mutagenesis and library creation, and which should be present in all selected binders.

Stock solutions of SA-AF647 were prepared at concentrations of 1, 0.2, and 0.04 µM, and these stock solutions were used to prepare samples at SA-AF647 concentrations ranging from 50 nM to 10 pM. The volume required at each concentration was calculated such that streptavidin would be available in ten-fold molar excess of the displayed binder. The yeast aliquots were pipetted into these pre-mixed samples, and all samples were incubated in the dark in a rotary mixer held at room temperature for at least 820 minutes. This is the period of time required for the most dilute sample to reach 95% of equilibrium binding, assuming a K_D of 0.6 nM, and employing the relation:

$$t_{95\%} \approx \frac{3}{k_{on}([SA] + K_d)}$$

Solution volumes and minimum required incubation times are shown in Table S3.

SA-AF647 Concentration (nM)	Time for 95% EQ (minutes) Presumed Kd = 0.6nM	$\frac{\#\ SA-AF647}{\#\ Sso7d}$	Cell Volume (µL)	Stock SA-AF647 Concentration (µM)	Stock Volume to Add (µL)	Buffer Volume to Add (µL)
50	9.88	422.6	50	1	10.53	150
25	19.53	205.9	50	1	5.13	150
10	47.17	81.1	50	1	2.02	150
8	58.14	64.8	50	1	1.61	150
6	75.76	48.5	50	1	1.21	150
4	108.70	88.7	50	1	2.21	500
2	192.31	44.2	50	1	1.10	500
1	312.50	22.2	50	0.2	2.76	500
0.8	357.14	17.7	50	0.2	2.21	500
0.6	416.67	13.3	50	0.2	1.65	500
0.4	500.00	16.9	50	0.2	2.10	1000
0.2	625.00	16.5	50	0.2	2.05	2000
0.1	714.29	16.3	50	0.04	10.15	4000
0.05	769.23	16.2	50	0.04	10.08	8000
0.025	800.00	16.1	50	0.04	10.04	16000
0.01	819.67	12.9	50	0.04	8.01	32000

Table S3: Experimental conditions for the determination of the K_D of yeast-surface displayed rcSso7d-SA, via the titration of streptavidin Alexa Fluor 647. This setup was used for each of three technical replicates.

Following this primary incubation, the cell solutions were centrifuged and the supernatant was aspirated, and the resulting cell pellets were washed in 1 mL of ice-cold PBSF. These pellets were then re-suspended to a volume of 50 µL in a 1:100 dilution of Alexa Fluor 488 goat anti-chicken IgG, and were incubated on ice in the dark for 15 minutes. The samples were then washed once more in 1 mL of ice-cold PBSF, and were kept on ice in pelleted form until flow cytometric analysis.

Cytometric analysis was conducted using a BD FACS LSR II flow cytometer running on the FACSDiva software package. Samples were re-suspended in 500 µL of PBSF and transferred to a polystyrene round-bottomed tube. Cytometer voltages were set using single-color controls to fix the binding signal within the dynamic range of the cytometer. Gates were established to select for singlet yeast cells using the forward- and side-scatter signal, and the samples were analyzed on low speed, capturing 20,000 events per run.

Single color controls (SCCs) were prepared in order to set cytometer voltages and a secondary binding control was prepared in order to ensure that the selected binder underwent no non-specific binding to the secondary labeling reagents. The AF 647 SCC was prepared by incubating a control pellet in a 1:100 dilution of mouse anti-HA IgG, followed by a 1:100 dilution of Alexa Fluor 647 goat anti-mouse IgG. The AF 488 SCC was prepared by incubating a control pellet in a 1:250 dilution of chicken anti-c-Myc IgY, followed by a 1:100 dilution of Alexa Fluor 488 goat anti-chicken IgG. The

secondary binding control was prepared by incubating a control pellet in a 1:100 dilution of Alexa Fluor 488 goat anti-chicken IgG and a 1:100 dilution of Alexa Fluor 647 goat anti-mouse IgG. Representative control plots are shown below, in Figure S2.

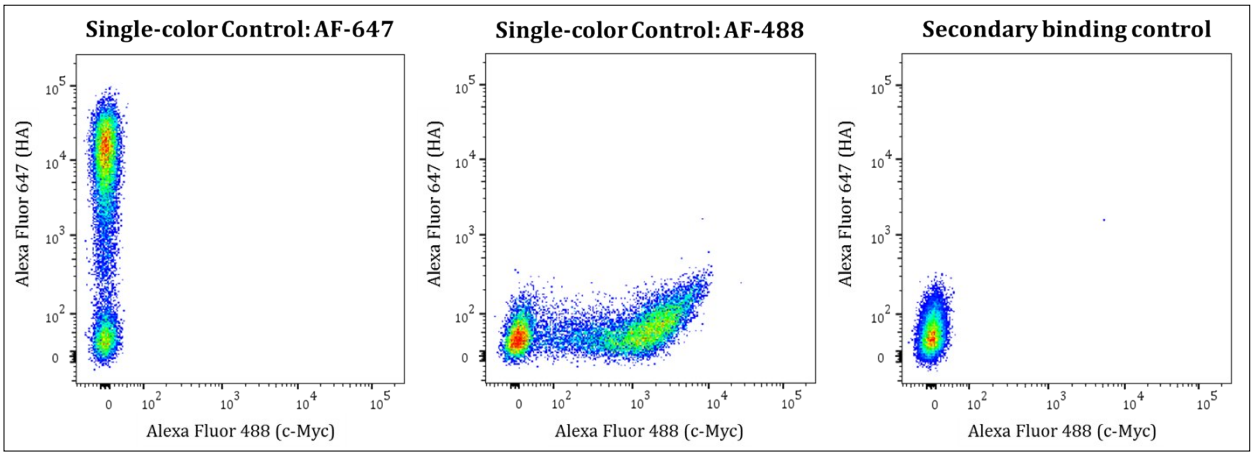

Figure S2: Representative cytometry plots from the single-color controls (AF-647 and AF-488, respectively), and the secondary binding control. Binding signal is confined to the expected fluorescence ranges for both single-color controls, and virtually no binding is observed for the secondary binding control, indicating no non-specific binding to secondary labeling reagents.

Analysis files (.fcs) were processed using the FlowJo software package. Singlet populations were gated using the signal from plots of FSC-A vs. SSC-A, FSC-W vs. FSC-H, and SSC-W vs. SSC-H. The non-induced fraction of the resulting population was determined by using the Alexa Fluor 488 single-color control. The total geometric mean fluorescence (MFU_{tot}) from the Alexa Fluor 647 channel was calculated for each experimental sample. This value was used in conjunction with the associated antigen concentration to determine the additional parameter values $(MFU_{min}, MFU_{range}, and\ K_d)$ in the following relation:

$$MFU_{tot} = MFU_{min} + \frac{MFU_{range} \times [SA - AF647]}{[SA - AF647] + K_d}$$

The Excel Solver function was used to minimize the sum of the squared differences between the calculated and experimentally determined values of MFU_{tot}. Three biological replicates were completed on separate days following the same protocol, and the averaged K_d from these three independent experiments is the reported affinity of 556 ±136 pM.

The plot of the yeast-surface display titration data was generated by averaging the individual data points from the three technical replicates and fitting the resulting curve to the above functional form. A Matlab script employing the command *fminsearch* was created in order to determine optimal parameter values such that the squared error between the line of best fit and the data

points was minimized. The parameters and associated r^2 value (calculated via the formula $r^2 = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2}$, which accounts for the presence of a constant term) are tabulated in Table S7.

YSD titration			
$\mathbf{MFU_{min}}$	$\mathbf{MFU_{range}}$	$\boldsymbol{K_d}$	$\boldsymbol{r^2}$
0.037	0.995	0.518	0.980

Table S4: Parameter values for the calculated line of best fit for the combined yeast-surface display titration data.

3. rcSso7d-SA PRODUCTION AND CHARACTERIZATION

3.1 Plasmid cloning of streptavidin-binding rcSso7d variant

In order to express the identified streptavidin-binding rcSso7d variant in soluble form, the gene encoding this protein species (rcSso7d-SA) was cloned from a pCTCON2 yeast-surface display plasmid into a pET28b(+) bacterial expression plasmid. This plasmid construct includes an N-terminal hexahistidine fusion tag, which allows for facile purification using immobilized metal affinity chromatography (IMAC). Forward and reverse primers were designed and characterized using the IDT Oligoanalyzer tool, and the oligonucleotide sequences are reported in Table S4.

Oligonucleotides	**DNA Sequence** (***NdeI*** and ***XhoI*** sites)
pCTCON2 forward sequencing primer	5'-GTTCCAGACTACGCTCTGCAGG-3'
pCTCON2 reverse sequencing primer	5'-GATTTTGTTACATCTACACTGTTG-3'
pET28b+ T7-forward sequencing primer	5'-TAATACGACTCACTATAGGG-3'
pET28b+ T7-reverse sequencing primer	5'-GCTAGTTATTGCTCAGCGG-3'
pCTCON2 to pET28b+ forward cloning primer	5'-AGGCAGTCT**CATATG**GCAACCGTGAAATTCAC-3'
pCTCON2 to pET28b+ reverse cloning primer	5'–ACCCCT**CTCGAG**TTATTGCTTTTCCAGCATCTG-3'

Table S5: Oligonucleotide sequences of primers used in sequencing reactions and plasmid cloning of selected binder rcSso7d-SA.

This primer pair was designed to append flanking *NdeI* and *XhoI* restriction sites to the 5' and 3' ends of the gene of interest, and used in a polymerase chain reaction (PCR) to produce the desired amplicon. The PCR mix contained 10 µL of 5x Phusion HF polymerase buffer, 1 µL of dNTP mix (10 mM of each base), 1 µL each of the forward and reverse primers at a concentration of 10 µM, 100 ng of template DNA (freshly miniprepped pCTCON2 vector containing the rcSso7d-SA gene), 1 µL of Phusion HF polymerase, and PCR-grade water to a final volume of 50 µL. The thermocycling profile featured an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of (i) denaturation at 95°C for 30 seconds, (ii) primer annealing at 58.3°C for 30 seconds, and (iii) extension at 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes.

Following production and amplification of the 273-bp target gene product, the DNA samples were electrophoretically separated in 1% (w/v) Seakem LE agarose in TAE buffer and viewed by ethidium bromide staining. The 50 µL aliquot of PCR product was mixed with 10 µL of 6x loading buffer, and

following a transfer of this mixture into the wells, electrophoresis was conducted at 100 V for 50 minutes. The visualization of bands was performed using an Electrophoresis Chemi Imager Cabinet from Alpha Innotech (now Cell Biosciences; Santa Clara, CA, USA) operating in UV Transillumination mode, and images were processed using ImageJ software (version 1.49, NIH; Bethesda, MD, USA). The product band was excised using a clean razor blade, extracted from the agarose gel and purified using a GenCatch Gel Extraction kit, and quantified using a Tecan plate reader.

The PCR amplicon and a freshly-miniprepped pET28b(+) vector were then digested using the *NdeI* and *XhoI* restriction enzymes. Both reactions were run as double digests, and contained 1 μg (or the maximum available) of the DNA construct to be digested. The reactions were conditioned in 5 μL of 10x CutSmart buffer, and 1 μL of each of the *NdeI* and *XhoI* enzymes. PCR-grade water was added to a total volume of 50 μL, and these reactions were incubated at 37°C for 3 hours. Following this period, the restriction enzymes were inactivated via a 10-minute incubation at 65°C. These digested products were once again separated and visualized via an agarose gel (using a lane containing uncut plasmid as a negative control), and the bands corresponding to the desired products were excised and purified.

These products were quantified, and a 20 μL ligation reaction was prepared, containing 0.06 picomoles of the digested rcSso7d-SA insert and 0.02 picomoles of the digested pET-28b(+) vector backbone. These products were mixed with 2 μL of 10x T4 DNA ligase buffer and 1 μL of T4 DNA ligase, and filled to the final volume with PCR-grade water. A negative ligation control lacking the digested insert was also prepared. These ligation reactions were incubated overnight at 16°C, and inactivated via a 10-minute incubation at 65°C. Completed ligation reactions were stored at -20°C until future use.

3.2 rcSso7d-SA plasmid transformation and heterologous expression

The prepared ligation reactions (1 μL) were then transformed into electrocompetent DH5-α *E. coli* cells and these 1 mL transformation mixtures were plated in their entirety on pre-warmed LB-kan plates. Following overnight stationary incubation at 37°C, no colonies were observed on the ligation negative control plate, and five colonies were picked from the experimental ligation plate and transferred into liquid medium. These were grown as overnight cultures, miniprepped, and sequenced in order to determine which of the colonies contained the successfully cloned gene of interest.

Following sequencing and identification of the colonies containing the rcSso7d-SA construct in a pET-28b(+) vector, the corresponding miniprepped plasmid was transformed into electrocompetent BL21 (DE3) *E. coli* (Invitrogen) cells. A single transformant was picked from this reaction, and grown in an overnight culture of LB-kan media at 37°C and 250 rpm. This culture was then inoculated directly into 1 liter of TB-kan media to yield an initial OD of 0.01. This culture (contained in a 2-liter baffled shake flask for improved aeration) was allowed to expand, and was induced at an OD of 0.4 with a final concentration of 500 μM isopropyl β-D-1-thiogalactopyranoside. Following induction, the cell culture was incubated at 20°C and 250 rpm for 18-20 hours, and was subsequently spun down at 4000*g* and frozen at -20°C until ready for harvest.

3.3 rcSso7d-SA purification

Induced cells were lysed on ice in an IMAC loading buffer (50 mM Tris buffer, 300 mM NaCl, 10 mM imidazole, pH 7.6 at 4°C) by ultrasonication in a Branson Sonifier from Branson Ultrasonics (Danbury, CT, USA), via fifteen cycles of 30 seconds at 50% duty and 50% power output, followed by 30 seconds cool-down. This crude lysate was clarified using Acrodisc PF 0.2 μm Syringe Filters, and loaded onto a pre-equilibrated HisTrap FF Crude 1 mL column at a volumetric flowrate of 0.6 mL/min and a temperature of 4°C. All fluid handling was conducted using an Äkta Purifier from GE Healthcare Life Sciences. The loaded column was then washed with 10 column volumes (CVs) of loading buffer,

and the bound protein was eluted over a linear imidazole gradient at 1 mL/min. The gradient ran from 0-100% elution buffer (50 mM Tris buffer, 300 mM NaCl, 500 mM imidazole, pH 7.6 at 4°C) over the course of 20 CVs. Protein elution was tracked using a UV_{280} trace (Figure S3). Purified protein fractions were captured during the elution phase when the UV_{280} trace rose above 750 mAU, from approximately 47-60 CVs.

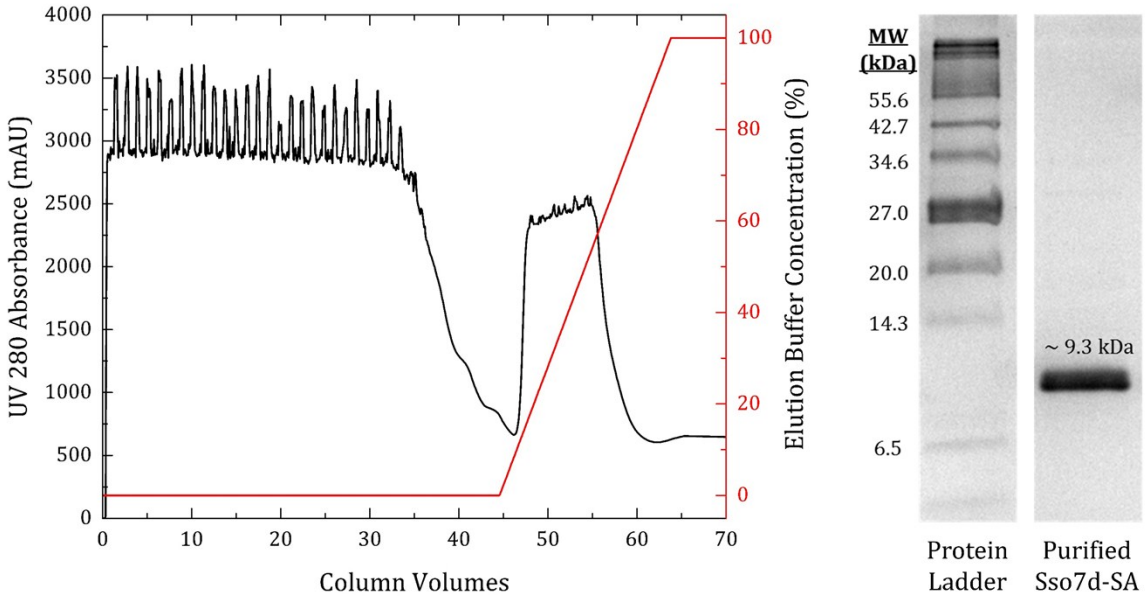

Figure S3: Representative chromatogram from the IMAC purification of rcSso7d-SA, and SDS-PAGE gel of purified fraction. The large UV_{280} peak from CVs 0-47 represents the loading phase, wherein unbound host cell protein flows through to waste and His-tagged rcSso7d-SA is retained on the nickel column. The large peak coinciding with the linear gradient of elution buffer represents the elution of bound rcSso7d -SA. The SDS-PAGE image indicates that the captured elution peak is monomeric and of high purity.

Purified protein fractions were 1000x buffer exchanged into 50 mM sodium acetate resuspension buffer, pH 5 in order to remove imidazole. Captured protein fractions were combined into an Amicon Ultra-15 Centrifugal Filter Unit with a 3kDa membrane, and spun at 4,000 x *g* for 1 hour. The concentrated protein solution (~1.5 mL) was then diluted in sodium acetate buffer to 15 mL, and the process was repeated 3 times more, to yield an approximate 1000x dilution. This buffer-exchanged protein solution was then diluted once more up to 10 mL, and the protein concentration was quantified using a Reducing Agent Compatible Microplate BCA Protein Assay Kit. This process determined the concentration of purified protein to be ~4.22 mg/mL, indicating a total yield of 42.2 g/L of bacterial culture.

3.4 SDS-PAGE characterization

Protein samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). rcSso7d-SA samples were characterized using 16% gels cast in-lab on the same day, for resolving proteins within the molecular weight range of 5-60 kDa. The gel separating buffer was

prepared by combining 300 mL Tris-Cl/SDS buffer (3 M Tris-Cl, 3 g/L SDS, pH 8.45), 55 mL DI water, and 95 mL glycerol. The gel stacking buffer was prepared by combining 129 mL Tris-Cl/SDS buffer and 321 mL DI water.

The separating gel was prepared by combining 2.66 mL of 30% acrylamide/Bis Solution, 2.29 mL of separating buffer, 50 µL of ammonium persulfate (AMPS) solution (1 g AMPS in 10 mL DI water, frozen at -80°C), and 4 µL of N,N,N',N'-tetramethylethylenediamine (TEMED). This solution was mixed briefly, and pipetted in between glass plates held in a casting frame, until the fluid level was within 1 cm of the well comb. This gel layer was allowed to solidify over the course of 45 minutes, using an overlay of DI water to level the fluid surface. The stacking gel was prepared by combining 500 µL of 30% acrylamide/Bis Solution, 2 mL of stacking buffer, 25 µL of AMPS, and 4 µL of TEMED. This solution was mixed briefly, pipetted into the assembled gel sandwich until overflow, and allowed to solidify over the course of 45 minutes a well comb had been inserted into the top of the assembly.

Following the gelation of the stacking gel, the gel assembly was re-seated in the buffer chamber of a Mini-Protean Tetra Cell electrophoresis unit, and the inner chamber was filled with fresh cathode buffer (60.55 g Tris base, 89.6 g Tricine, and 5 g SDS in 1 L of DI water, diluted 5x). The outer chamber was filled to the first line with anode buffer (1 M Tris-Cl, pH 8.9, diluted 5x).

The protein sample of interest was diluted to 0.15 mg/mL in 10 µL, and mixed with 2 µL of 6x Coomassie blue loading buffer in a 200 µL PCR tube. This protein sample was subjected to heating at 95°C for 5 minutes, and loaded into the freshly-prepared gel. Electrophoresis was conducted over the course of 45 minutes at 200V using a PowerPac HC HighCurrent Power Supply, and the gel was removed from the assembled cassette. The gel was rinsed in DI water, and then fixed and stained in 100 mL of hot fixing solution (50% v/v methanol, 10% v/v glacial acetic acid, 40% v/v DI water, microwaved for 45 seconds) and 5 mL of Coomassie stain (50% v/v methanol, 10% v/v glacial acetic acid, 40% v/v DI water, 3 g Coomassie Brilliant Blue G-250 dye), and incubated on a VWR digital rocker for 45 minutes. The stained gel was then rinsed in DI water, and de-stained in 100 mL of hot de-staining solution (30% v/v methanol, 10% v/v glacial acetic acid, 60% DI water, microwaved for 45 seconds), and incubated on a shaking rocker for 60 minutes. The de-staining solution was then removed and replaced with DI water, and the gel was incubated overnight on the digital rocker.

Sample images were then captured using the Electrophoresis Chemi Imager Cabinet operating in Reflective White Light mode, and processed using ImageJ (Figure S3).

3.5 Production cost analysis

Cost analysis was conducted for the production of rcSso7d-SA, and compared to the purchase cost of a comparable rabbit anti-streptavidin polyclonal antibody. The individual reagents were selected for the minimum price available via the vendor sites, and are tabulated below. The final molar yield is found to be ~68-fold greater for rcSso7d-SA than for the polyclonal antibodies, and at a final price point that is ~100-fold cheaper. Other analyses have found diagnostic primary antibodies to be even more expensive, [56] with a liberal estimated cost/test of $0.11-$0.13. When compared with reagent costs on this scale, the rcSso7d scaffold is at least 380-fold more cost-effective than antibodies as a diagnostic capture agent.

Chemical	Supplier	Supplier Code	Stock Size (g/mL/count)	Batch Usage (g/mL/count)	Stock Cost	Batch Cost
Yeast Extract	VWR	90000-726	500	24.000	$96.29	$4.62
Tryptone	Fisher-Scientific	DF0123-17-3	500	12.000	$58.74	$1.41
Glycerol	VWR	97062-452	1000	4.000	$25.07	$0.10
Potassium diphosphate monobasic	Macron Fine Chemicals	7100-12	500	2.310	$44.71	$1.12
Potassium phosphate dibasic	VWR	BDH9266-2.5KG	2500	12.540	$142.63	$0.72
Sodium chloride	VWR	97061-274	500	8.766	$26.45	$0.46
Imidazole	Amresco	0527-1KG	1000	8.680	$243.69	$2.12
Tris base	VWR	97063-888	1000	1.975	$74.60	$0.15
Tris HCl	VWR	IC10313001	1000	0.806	$193.84	$0.16
Sodium acetate	Macron Fine Chemicals	MK-7800-500	500	0.658	$58.21	$0.08
Glacial acetic acid	VWR	97065-042	2625	0.269	$66.50	$0.01
HisTrap FF Crude Column	GE Healthcare	11-0004-58	5	1	$173.50	$34.70
Amicon Ultra 0.5 Centrifugal Filter Units (3 kDa)	EMD Millipore	UFC500324	24	1	$86.73	$3.61

		rcSso7d -SA	Commercial pAb-SA
	Total Cost	$49.25	$69.00
	Total Yield (μg)	42,200	5,000
Conservative, 6 μL/test	**Mass Usage per Test (μg)**	0.738	6.000
	Number of Tests	57182	833
	Cost/Test	$0.00086	$0.08280
Liberal, 2 μL/test	**Mass Usage per Test (μg)**	0.246	2.000
	Number of Tests	171545	2500
	Cost/Test	$0.00029	$0.02760
	Fold Improvement, # Tests	68.62	
	Fold Improvement, Cost/Test	96.14	

Table S6: Cost analysis of the production and purification of rcSso7d-SA, as compared with a commercial polyclonal blend of anti-SA IgG antibodies. Reagents used in the expression and purification process are listed above, as well as their unit prices and the cost incurred in the production of a single batch of rcSso7d-SA. The total cost basis is compared below, for both the conservative case, wherein 6 μL of solution are required for each test, and the liberal case, where only 2 μL of solution are required per test. Fold improvement in terms of the molar yield and cost per test are tabulated below.

4. PAPER SAMPLE PRODUCTION AND PROCESSING

4.1 Eosin 5'-isothiocyanate coupling reaction

Lyophilized streptavidin was reconstituted to a concentration of 1 mg/mL in sterile molecular grade water, and was desalted using 10kDa Amicon Ultra-0.5 mL Centrifugal Filter Units. The streptavidin solution (1 mL) was spun down to a volume of ~25 µL in a tabletop centrifuge operating at 4°C and 14,000g. This solution was then re-suspended in 0.1 M sodium bicarbonate buffer (pH 9.0), to yield a final volume of 100 µL at a concentration of 10 mg/mL. EITC (1 mg) was dissolved in DMSO to a final concentration of 10 mg/mL, and 10 µL of this solution was added to the 100 µL streptavidin solution. This reaction mixture was protected from the light and stored overnight at 4°C. Over the course of the reaction, the isothiocyanate moiety of EITC reacts non-preferentially with free amine groups within the protein (the N-terminus and lysine side chains), to form a covalent thiourea bond.

Following reaction, the mixture was diluted with 890 µL of PBS to a final streptavidin concentration of 1 mg/mL. The mixture was then loaded onto UltraCruz Micro G-25 Spin Columns and centrifuged according to manufacturer protocols, in order to remove unreacted EITC via size exclusion chromatography.

The average EITC conjugation efficiency of this reaction was determined via UV-Vis absorbance spectroscopy, using a Nanoquant Plate and an Infinite M200 plate reader to measure the background-subtracted absorbance of the solution at 280nm and 525nm. The conjugation efficiency and final protein concentration was then determined using Beer's law, in the form of the following two equations:

$$\frac{n_{EITC}}{n_{SA}} = \frac{\left({Abs_{525}}/{\varepsilon_{EITC,525}}\right)}{\left(Abs_{280} - \left({Abs_{525}\varepsilon_{EITC,280}}/{\varepsilon_{EITC,525}\varepsilon_{SA,280}}\right)\right)}$$

$$C_{SA} = \left(Abs_{280} - \left({Abs_{525}\varepsilon_{EITC,280}}/{\varepsilon_{EITC,525}\varepsilon_{SA,280}}\right)\right)b$$

where $\frac{n_{EITC}}{n_{SA}}$ is the number of EITC molecules conjugated per streptavidin molecule, C_{SA} is the molar concentration of streptavidin, Abs_{280} & Abs_{525} are the background-corrected absorbance at 280 nm and 525 nm, respectively, the experimentally-determined extinction coefficients are $\varepsilon_{EITC,280} = 26{,}800\ M^{-1}cm^{-1}$, $\varepsilon_{EITC,525} = 90{,}200\ M^{-1}cm^{-1}$, $\varepsilon_{SA,280} = 173{,}000\ M^{-1}cm^{-1}$, and the optical path-length is $b = 0.05\ cm$.

These calculations determined a conjugation efficiency of 2.8 moles of EITC/mole of streptavidin, and that the concentration of the streptavidin-eosin conjugate was 8.6 µM. This SA-EITC solution was diluted 1:1 with glycerol, aliquoted, and stored at -20°C.

4.2 Aldehyde-functionalized paper preparation

Aldehyde-functionalized paper was prepared by submerging 3" x 8" sheets of Whatman No. 1 chromatography paper in a 30 mM $NaIO_4$ solution held at 65°C for 2 hours. The activated paper was then washed three times, by dipping the sheets in fresh deionized water for one minute. Excess water was allowed to run off, and after the final wash each sheet was blotted with paper towels. The sheets were allowed to dry in a fume hood overnight, and were then placed in an envelope and stored in a desiccator for at least twelve hours.

Following the drying process, a 5 x 7 array of test strips, each containing four independent test zones 3 millimeters in diameter, was printed onto each sheet using a Xerox ColorQube 3570 solid ink printer set to the default parameters for photo-quality printing. The printed sheets were then heated in an oven at 150°C for 150 seconds to allow the wax to melt all the way through to the back of the paper and expand slightly across the paper. As a result, each sheet of paper had a number of circular, hydrophilic, aldehyde-functionalized test zones (with a final diameter of 2 millimeters) separated by the hydrophobic wax. The presence of aldehyde groups in the test zones was confirmed by adding 2 µL of 2,4-dinitrophenylhydrazine (Brady's reagent) and observing a color change from yellow to dark orange. The printed sheets were stored in a desiccator until use.

4.3 Paper-based assay development

This aldehyde-functionalized paper was used to assay the activity of the streptavidin-binding species, as detailed in the protocol by Lathwal et al.[7] Briefly, the protein species of interest were mixed with a final concentration of 10% v/v glycerol.

A commercially-available, polyclonal blend of anti-SA rabbit IgG antibodies was purchased in lyophilized form from Genscript and reconstituted in PCR-grade water to a concentration of 5 mg/mL. This solution was aliquoted and frozen at -20°C according to manufacturer recommendations, and each aliquot was only allowed to go through a single freeze-thaw cycle. In all paper-based assays, the polyclonal antibodies (pAbs) were diluted to 1 mg/mL (6.67 µM) in its optimal resuspension buffer - phosphate-buffered saline (PBS), pH 7.4 - and glycerol at a final concentration of 10% (v/v). For all assays, rcSso7d-SA was diluted in its optimal resuspension buffer (50 mM sodium acetate, pH 5) to 123.44 µg/mL (13.33 µM) – twice the molar concentration of the bivalent pAbs, such that an equal number of binding faces would be represented on the test surface for each species.

For each experimental condition, 6 µL of the relevant solution was pipetted onto each of the four test zones in a single test strip, in order to prepare technical replicates. This test strip, measuring 2.8 cm x 1.5 cm, was suspended upon supports within an empty pipette box, such that the fluid wicking through the circular test zones would not contact the surface below. This pipette box was partially filled with distilled water in order to render it a humid chamber, and a weight was placed on top of the closed box in order to maintain a humid atmosphere and prevent evaporation.

These test strips were incubated for 16 hours at room temperature, and were then blotted on a clean Kimwipe in order to remove excess solution. The test zones were then washed twice with 20 µL of 1x PBS, blotting away the flow-through each time, and any unreacted free aldehydes were inactivated via a one hour incubation with 10 µL of 1x TBS. Following this incubation, the test zones were washed twice more with 20 µL of 1x PBS, and the samples were contacted with 10 µL of streptavidin-eosin conjugate, diluted to a concentration of 330 nM in 1x PBS containing 1% w/v bovine serum albumin (BSA). The test strips were incubated for 30 minutes in the dark to prevent photo-bleaching of the conjugated eosin, and were then washed twice with 20 µL of ice-cold PBS and allowed to air-dry in the dark.

The negative control for these tests was prepared with BSA, diluted in PBS and 10% v/v glycerol to a concentration of 1 mg/mL. This served to passivate the reactive aldehyde groups, and mimic the presence of immobilized protein species on the surface of the paper. The positive control was prepared using biotinylated BSA. This species was produced using the EZ-Link Sulfo-NHS-LC Biotinylation kits in the No-Weigh format, and biotinylation efficiency was quantified using a Pierce Biotin Quantitation kit. The 1 mg/mL stock solution of biotinylated BSA was diluted to 0.9 mg/mL via the addition of glycerol, to a final volumetric concentration of 10%.

At the concentrations stated above, the commercial polyclonal antibody blend and rcSso7d-SA preparation exhibit similar binding activities (Figure S4). Though the polyclonal antibodies may have slightly higher surface-immobilized activity, the rcSso7d species yields comparable values, and thus its surface-bound activity is sufficient for paper-based diagnostic applications. Additionally, rcSso7d-SA can be affinity matured using standard protein engineering approaches (e.g. error-prone PCR), whereas polyclonal antibody blends are less amenable to manipulation.

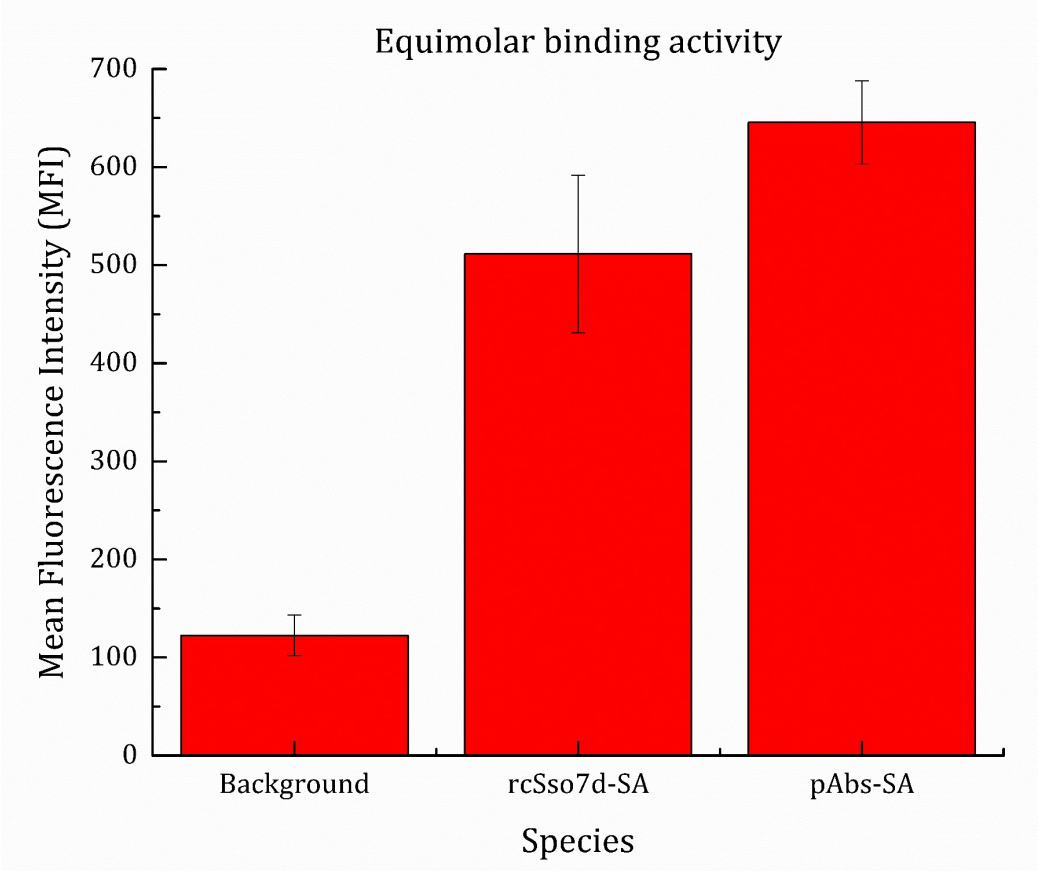

Figure S4: Binding activity of BSA, pAbs-SA, and rcSso7d-SA under equimolar conditions. Four technical replicates were prepared for each of three independent experiments.

4.4 Fluorescence microscopy and sample processing

Fluorescence microscopy was used to detect the presence of eosin on the surface of the paper following the development of the immunoassay. Each test zone was imaged using an IX81 microscope purchased from Olympus (Center Valley, PA, USA), with a 4X objective lens and a 10X eyepiece lens. The samples were exposed for 1000ms (unless otherwise noted) using a TxRed-4040C filter set purchased from Semrock (Rochester, NY, USA). The excitation range for this filter set is 540-580nm, and the emission range is 600-640nm. This sufficiently matches the excitation/emission maxima of EITC (λ_{ex} = 521nm, λ_{em} = 544nm). Samples were imaged using the Metamorph software package from Molecular Devices (Sunnyvale, CA, USA). A Lumen 200 with a Prior Lumen Bulb (Item #P-LM200BI) was purchased from Prior Scientific (Rockland, MA, USA) and used as the light source.

The mean fluorescence intensity of each test zone image was calculated by averaging the constituent pixel intensities using ImageJ. The Auto Threshold function was used to create a mask capturing all relevant areas within the test zone and excluding particulate matter and heterogeneities, and the mean pixel intensity was measured within the bounds of this mask. Data were concatenated and processed using a script written in Matlab (Mathworks, Natick, MA, USA).

5. ACTIVITY-BASED CHARACTERIZATION

5.1 Surface-bound activity assay

A serial dilution of streptavidin-eosin conjugate was prepared, and incubated on the hydrophilic test zones for at least 500 minutes, a period of time sufficient for the most dilute sample to reach 95% of equilibrium binding (using the known affinity of approximately 500 pM). Solution volumes and minimum required incubation times are tabulated in Table S6. These samples were imaged via fluorescence microscopy, following the previously-outlined protocols. This experiment was conducted in duplicate on two separate days, and the signal from the eight technical replicates was averaged to yield the given data points.

SA-EITC Concentration (nM)	Time for 95% EQ (minutes) Presumed K_d = 0.5nM	Test Zone Volume (µL)	Stock SA-EITC Concentration (nM)	Stock Volume to Add (µL)	Buffer Volume to Add (µL)
200	2.49	10	4300	9.860	202.140
100	4.98	10	200	72	72
50	9.90	10	100	94	94
30	16.39	10	50	138	92
20	24.39	10	30	180	90
18	27.03	10	20	45	5
16	30.30	10	20	40	10
14	34.48	10	20	35	15
12	40.00	10	20	30	20
10	47.62	10	20	70	70
8	58.82	10	10	40	10
6	76.92	10	10	30	20
4	111.11	10	10	20	30
2	200.00	10	10	8	32
1	333.33	11	10	4	36
0.5	500.00	21	10	2	38

Table S7: Experimental conditions for the determination of the apparent K_D of paper-immobilized rcSso7d-SA, via the titration of streptavidin-eosin conjugate. This setup was used for each of the eight technical replicates.

5.2 Thermal challenge antigen-binding assay

Both rcSso7d-SA and a commercially-available, polyclonal blend of anti-SA rabbit IgG antibodies were subjected to a thermal challenge antigen-binding assay in order to investigate their relative thermal denaturation kinetics under conditions of accelerated degradation. Both species were prepared according to the protocols detailed previously, in sufficient volume for all necessary experimental samples.

These bulk preparations were then split into 30 µL aliquots in separate PCR tubes and weighed, and the samples were incubated in a thermocycler held at 95°C for various periods of time. Following this incubation period, the samples were massed again, and the volume of diluent lost through evaporation was replaced in order to prevent artefactual results due to differences in protein concentration between samples. 10 µL of each sample was then added to aldehyde-functionalized

chromatography paper test zones and incubated overnight for 16-18 hours. The test zones were then washed and neutralized according to established protocols, and were contacted with a 330 nM SA-EITC solution for 30 minutes.

These samples were then imaged via fluorescence microscopy, following the previously-outlined protocols. In order to produce binding curves which naturally range from 1 to 0, the background-corrected relative activity was scaled by the background-subtracted activity observed for the species prior to thermal exposure. Here, I_{BG} is the mean fluorescence intensity of the negative control, in which a 1 mg/mL solution of bovine serum albumin is immobilized and similarly contacted with the streptavidin-eosin conjugate. This corrects for non-specific binding of the SA-EITC to the substrate, or to non-functional regions of the immobilized proteins. The dimensionless quantity employed is as follows:

$$\square = \frac{I - I_{BG}}{I_0 - I_{BG}}$$

This experiment was conducted in triplicate on three separate days (four sample wells each day), for a total of twelve technical replicates. Each set of four replicates was rendered dimensionless relative to its respective negative control, in order to account for inter-day variations in absolute signal. These relative activities were then averaged to yield the given data points.

5.3 Curve fitting and parameter determination

The plots of surface-immobilized activity showed strong agreement with a quadratic line of best fit, following the relation below:

$$y = Ax^2 + Bx + C$$

The same Matlab script used to fit the yeast surface display titration data was used to determine optimal parameter values for this new functional form. These parameters, and the corresponding r^2 values, are tabulated in Table S8.

	Surface-immobilized activity			
	A	**B**	**C**	r^2
rcSso7d-SA	-0.018	16.271	258.370	0.9998
Baseline	-0.0045	3.417	225.394	0.9976

Supplementary Table S8: Parameter values for the calculated line of best fit for the combined surface-immobilized activity data.

The accelerated degradation activity assay data was fit to a sigmoidal curve using a five-parameter functional form:

$$y = \frac{A - B}{\left(1 + \left({}^{t}/_{C}\right)^{D}\right)^{E}} + B$$

Given that the dimensionless activity ranges between an upper asymptote at 1 and a lower asymptote at 0, the values of the parameters A and B were pinned at 1 and 0, respectively. This reduces the equation to a three-parameter sigmoid:

$$y = \frac{1}{\left(1 + \left({}^{t}/_{C}\right)^{D}\right)^{E}}$$

Here, C is the time at the inflection point, D is the slope at the inflection point, and E allows for asymmetrical slopes of asymptotic approach. The same Matlab script was used to determine optimal parameter values for this new functional form. These parameter values are tabulated in Table S9, as are the corresponding r^2 values (calculated via the formula $r^2 = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i y_i^2}$, which accounts for the lack of a constant term).

	95°C $t_{1/2}$						
	A	**B**	**C**	**D**	**E**	**r^2**	**$t_{1/2}$**
rcSso7d-SA	1	0	107.406	1.481	1.157	0.9992	94.00
pAb-SA	1	0	0.155	18.083	0.048	0.9874	0.34

Supplementary Table S9: Parameter values for the calculated line of best fit for the combined $t_{1/2}$ activity data following incubation at 95°C.

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