Supporting Information

Beyond epitope binning: directed *in vitro* selection of complementary pairs of binding proteins

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Glossary of Terms

- **Biomarker –** any biological molecule which can be causally linked to a disease state, and which is captured and/or measured in a diagnostic assay. Examples for infectious diseases include molecules from the pathogen (e.g. glycolipids, proteins, DNA) or host-derived molecules (e.g. cytokines, antibodies) produced in response to the pathogen. In the context of this paper, targeted biomarkers are soluble proteins.
- **Affinity reagent** – molecular entities (e.g. antibodies, non-antibody scaffold proteins, aptamers) which participate in bimolecular binding reactions with a target biomarker that are characterized by specificity (K_d of nM-pM and K_d for reactions with non-targets > μM) and reversibility (i.e. noncovalent).
- **Non-specific binding** – a broad term capturing any unintended binding interactions
- **Off-target binding** – undesired binding to any host or pathogen biomolecules
- **Cross-reactive binding** – undesired binding between selected affinity reagents; results in higher limits of detection and the potential for false positive diagnostic results
- **Complementary binding** – simultaneous binding to distinct, non-overlapping epitopes of a biomarker
- **Affinity reagent nomenclature**
    - b signifies that the affinity reagent is labeled with biotin.
    - MBP signifies that the affinity reagent is fused to a maltose-binding protein, which is used as a structured spacer. For our affinity reagents, this spacer is necessary for molecular recognition between biotin and streptavidin.
    - CBD signifies that the affinity reagent is fused to a cellulose-binding domain, which is used to easily functionalize cellulose with a high density of affinity reagents.
    - E1 signifies epitope 1 of a target biomarker.
    - E2 signifies epitope 2 of a target biomarker.
    - SsoRv1656 signifies reduced charge Sso7d that has been engineered to recognize the *Mycobacterium tuberculosis* protein Rv1656.
    - SsoIL6 signifies reduced charge Sso7d that has been engineered to recognize human IL-6.
    - SsoZNS1 signifies reduced charge Sso7d that has been engineered to recognize Zika virus nonstructural protein 1.
    - Examples:
        - bMBP-SsoRv1656.E1
        - SsoRv1656.E2-CBD
        - bMBP-SsoIL6.E1
        - SsoIL6.E2-CBD
        - bMBP-SsoZNS1.E1
        - SsoZNS1.E2-CBD

Materials and Methods

Commercial reagents

Primary detection reagents and dilutions (bold) were: chicken anti-c-Myc (ACMYC; **1:250**) and chicken anti-HA (AHA; **1:1000**) from Exalpha Biologicals, mouse anti-HA.11 (clone 16B12; **1:400**) from Biolegend, and mouse anti-6x-His (clone MA1-21315, HIS.H8; **1:200**) from Thermo Fisher Scientific. Secondary detection reagents were goat anti-mouse AlexaFluor (AF) 647 (A-21235; **1:250**), goat anti-chicken AF488 (A-11039; **1:250**), streptavidin AF647 (S-21374; **1:200**), NeutrAvidin DyLight650 (84607; **1:200**), and streptavidin R-phycoerythrin (S866; **1:100**) from Thermo Fisher Scientific. For later selection processes (i.e. IL-6 and ZNS1), a reduced dilution (**1:1000**) was used for all primary and secondary detection reagents in order to minimize off-target selective pressures which might give rise to binding variants specific to the labeling reagents. Magnetic bead selections were conducted using Dynabeads Biotin Binder (11047) and HisPur NiNTA Magnetic Beads (88831) from Thermo Fisher Scientific. Human IL-6 was purchased commercially with an N-terminal 6x-histidine tag (RayBioTech; 230-00011-10), chemically biotinylated (AcroBio; IL6-H8218), and without any peptide tags (Biolegend: 570808).

Recombinant protein production

rcSso7d-based binding variants were selected against the urine-based tuberculosis (TB) biomarker Rv1656/MT1694(1), human interleukin-6 (IL-6), and Zika virus non-structural 1 protein (ZNS1). Dengue 2 virus non-structural protein 1 (D2NS1) was used for negative selections in the development process for ZNS1-specific affinity reagents.

Human IL-6 was purchased commercially (see above). The bacterial expression plasmid for the TB biomarker Rv1656 was a kind gift from the lab of Antonio Campos-Neto (Forsyth Institute). Plasmids for the ZNS1 and D2NS1 biomarkers were sourced from Sino Biological. TB Rv1656, ZNS1, and D2NS1 were recombinantly produced in BL21(DE3) *E. coli*, as were all soluble variants of the selected rcSso7d clones (either in a fusion protein construct with cellulose-binding domain (CBD) or maltose-binding protein (MBP), or without a fusion partner). All recombinant proteins featured N-terminal 6x-histidine tags for purification and immunostaining. Additional TB biomarkers (except for H4), ZNS1, and D2NS1 were also fused to a C-terminal AviTag biotin acceptor (BA) sequence (MAGGLNDIFEAQKIEWHE) in order to permit *in vivo* biotinylation.(2) The rcSso7d clones in the biotinylated MBP-rcSso7d (bMBP-rcSso7d) fusion format also featured an N-terminal biotin acceptor sequence. For protein expression, all cell cultures were grown at 37°C in Terrific Broth supplemented with kanamycin at a concentration of 50 µg/mL, and induced at an OD_{600} of 0.4 using a final concentration of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacterial cultures featuring AviTag-modified constructs were also supplemented with 10 mM D-biotin (97061-444, VWR) in 10 mM bicine buffer (pH 8.3), to a final concentration of 0.2 mM. Induced cultures were incubated at 20°C for 18-20 hours.

Following recombinant protein expression, cell cultures were lysed via ultrasonication and purified using immobilized metal affinity chromatography (IMAC), as previously described.(3) Purified proteins were buffer exchanged via filter centrifugation using Amicon Ultra Centrifugal Filters with a molecular weight cut-off of 3 kDa (for Rv1656 or rcSso7d) or 10 kDa (for ZNS1, D2NS1, rcSso7d-CBD, or bMBP-rcSso7d). Buffer-exchanged samples were re-suspended in 50 mM HEPES (pH 8.0; for Rv1656), 1x phosphate-buffered saline (PBS; for ZNS1 and D2NS1), or 40 mM sodium acetate (pH 5.5; for rcSso7d clones and fusion proteins). Purified species were quantified using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) and protein purity was assessed using freshly-cast 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel or using 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) with Biorad P/N 161-0374 used as the protein ladder. The gels were visualized using Coomassie blue staining. All bMBP-rcSso7d clones were further purified on a Pierce Monomeric Avidin Agarose Kit

(Thermo Fisher Scientific), following the kit protocol. The rcSso7d clones and rcSso7d fusion proteins were stored at 4°C, and all other proteins were aliquoted and stored at -20°C in 50% (v/v) glycerol.

Magnetic bead sorting

Primary magnetic bead sorting (MBS) was conducted as previously described.(3) In addition to using biotin binder Dynabeads for immobilization of biotinylated protein as detailed previously, HisPur NiNTA beads were also used in primary MBS rounds for immobilization of unbiotinylated biomarker via the 6x-histidine tag. For Rv1656 selections, biotin binder Dynabeads were used. For IL-6, NiNTA beads were used for the first two MBS rounds and biotin binder Dynabeads were used for the third MBS round. For ZNS1, NiNTA beads were used for all MBS rounds. For the third round of MBS for ZNS1, a negative sort was conducted using D2NS1 immobilized on the NiNTA beads, in order to remove clones with off-target binding to D2NS1. All protein incubation steps were conducted with at least 33 picomoles of protein per 10 µL of biotin binder Dynabeads stock solution or per 1 µL of HisPur NiNTA beads stock solution.

Secondary magnetic bead sorting was conducted in a similar fashion. For Rv1656, 60 µL of biotin binder Dynabeads were washed in sterile-filtered 1x PBS/0.1% bovine serum albumin (BSA; this re-suspension solution is termed PBSF). A Dynamag-2 magnetic rack was used to withhold beads while the supernatant was drawn off, and this washing step was repeated twice. In order to ensure surface saturation, all beads were re-suspended with at least 50 picomoles of the bMBP-rcSso7d.E1 species per microliter of stock bead solution – based on protein availability, 60 µL of beads were re-suspended in a volume of 1 mL of PBSF containing 5 nanomoles of protein, amounting to 87 picomoles/µL of Dynabead stock solution. These beads were incubated on a rotary mixer at 4°C for 2 hours, after which they were washed three times in PBSF. One set of beads (equivalent to 45 µL of stock solution) was withheld for negative selection steps, and another set (equivalent to 15 µL of stock solution) was transferred to a 2-mL tube containing 2 nanomoles of Rv1656 (136 picomoles/µL of Dynabead stock solution) in a total volume of 1 mL PBSF. This positive sorting bead preparation was incubated on a rotary mixer at 4°C for 4.5 hours.

During this bead preparation step, the yeast library was prepared for magnetic bead sorting. The appropriate number of yeast cells (the greater of either 20-fold excess of the current library diversity or 10^9 cells) was centrifuged at 2,000 *xg* for 3 minutes and washed in PBSF. Following re-suspension in PBSF, the negative selection beads were spiked into the yeast library (an equivalent of 15 µL Dynabeads stock/selection, to a total volume of 1 mL), and this mixture was incubated on a rotary mixer for at least 1.5 hours at 4°C. Following this period, the yeast library was placed on the magnetic rack for two minutes, and unbound yeast were transferred to a fresh tube. This process was repeated for a total of three negative selection steps.

Following the completion of all three negative selection steps, the positive selection beads (equivalent to 15 µL of the Dynabeads stock) were washed three times in 1 mL of PBSF, and then re-suspended in the negatively sorted yeast library. This mixture was incubated with mixing for at least 2 hours at 4°C, and was then washed with PBSF, discarding any yeast or solution not bound to the magnetic beads. Retained beads and yeast were inoculated into SDCAA medium for outgrowth, and 100 µL of a 200-fold dilution of this solution (ten millionths of the total solution volume) was applied to an SDCAA plate in order to assess the number of retained yeast cells. This plate was grown at 30°C for three days and colonies were counted in order to determine the maximum theoretical diversity within the retained library.

This process was identical for the IL-6 secondary MBS process, except only one negative selection incubation was used, and 660 picomoles of avidin-purified primary affinity reagent bMBP-SsoIL6.E1 was used for a 20 µL volume of Dynabeads stock solution.

For Rv1656, the secondary selection process was started using the yeast population after the third round of MBS, and one additional round of MBS was conducted prior to moving on to FACS. For IL-6, the

secondary selection process was started using the yeast population after the second round of MBS, and one additional round of MBS was conducted prior to moving on to FACS. For ZNS1, the secondary selection process was started using the yeast population after the third round of MBS, and no additional rounds of MBS was conducted prior to FACS to reduce the development process timeline.

Flow cytometry

Yeast populations were prepared for cell sorting as previously described.(3, 4) In short, yeast sub-libraries or clonal populations were outgrown in selective SDCAA media at 30°C for 24 hours, to an OD_{600} of ~4. Yeast cells were then inoculated into SGCAA media (supplemented with 2 g/L of dextrose) at a final OD_{600} of 1.0 (ensuring that the inoculated yeast represented a ten-fold excess of the maximum theoretical library size), and protein expression was induced at 20°C for 24-48 hours. Induced cells were prepared for flow cytometry-based cell sorting by capturing 10^7 yeast cells and re-suspending them at an OD_{600} of 1.0, in 1 mL of PBSF. Cell concentrations and reagent proportions were maintained across all samples, although larger numbers of yeast were prepared as needed to ensure that ten-fold the library size would be screened. Yeast populations to be sorted were centrifuged at 2,000 *xg* for three minutes in order to pellet cells in a gentle manner, preserving viability.

Following harvesting and centrifugation, the yeast pellet was re-suspended in PBSF. Each sample condition was prepared by harvesting the appropriate number of cells for at least 20-fold the library diversity. Generally, a typical expression efficiency of 60% and 50,000 rcSso7d copies per cell was assumed, in order to inform the volumes and concentrations of target biomarker required to ensure a ten-fold molar excess relative to the displayed rcSso7d. Primary incubation steps (with either mouse anti-HA, chicken anti-HA, or chicken anti-cMyc antibodies, and/or the target biomarker) were generally conducted at room temperature over the course of 25-30 minutes (unless the biomarker concentration was sufficiently low to warrant longer incubation times). All following incubation steps (with mouse anti-His IgG, goat anti-mouse IgG AF647, or goat anti-chicken IgG AF488 antibodies, the bMBP-rcSso7d reagent, or streptavidin AF647) were conducted at 4°C over the course of 30 minutes, at the dilutions noted above. In between each incubation step, cells were washed in 1 mL of PBSF and pelleted, and the supernatant was aspirated.

Rv1656 primary FACS selection was conducted as previously described.(5, 6) Briefly, the sub-library was subjected to five rounds of FACS, and selection stringency was increased by reducing the proportion of captured cells from 1% to 0.1% over the first three rounds, and subsequently reducing the biomarker concentration from 100 nM to 25 nM over rounds 3-5. Throughout this process, the labeling reagents were switched between streptavidin AF647 and the deglycosylated avidin variant NeutrAvidin DyLight 650. Though NeutrAvidin features only 30% sequence homology with streptavidin, subsequent selection processes have employed more orthogonal labeling reagents to prevent the development of off-target binding. Rv1656 secondary selection employed two distinct labeling modes: (**1:** bMBP-SsoRv1656.E1/streptavidin PE; **2:** mouse anti-His IgG/goat anti-mouse IgG AF647). The concentration of the biomarker and the captured proportion were both reduced over five rounds of sorting (100 nM/5%, 100 nM/1%, 20 nM/0.1%, 5 nM/0.1%, and 1 nM/0.1%). Likewise, in order to reduce the risk of enriching the population for binding variants specific to bMBP-SsoRv1656.E1, the concentration of this species also followed the same profile across the selection rounds (100 nM, 100 nM, 20 nM, 5 nM, and 1 nM).

For IL-6 primary selection, we alternated between different labeling reagents (mouse anti-His IgG with goat anti-mouse IgG AF647 and SA AF647) to minimize enrichment of affinity reagents against the labeling reagents and decreased concentration of IL-6 (100 nM, 20 nM, 5 nM, and 1 nM) in the latter sorts to provide selective pressure for higher affinity reagents. For secondary selection, we used bMBP-SsoIL6.E1 (500 nM) with SA AF647 for labeling target binding for positive sorts, decreasing concentration of IL-6 (100 nM, 20 nM, 5 nM, and 1 nM) in latter rounds of FACS. We also incorporated negative

selections against bMBP-SsoIL6.E1 (500 nM) with SA AF647 to remove non-specific or cross-reactive clones.

For ZNS1 primary selection, we conducted all sorts using mouse anti-His IgG with goat anti-mouse IgG AF647. Prior to the positive selections in FACS rounds 2 and 4, we also incorporated negative selections against D2NS1 (200 nM), mouse anti-His IgG, and goat anti-mouse IgG AF647 by collecting the population that does not show binding to these reagents, in order to reduce selection of off-target affinity reagents. The concentration of ZNS1 was decreased over subsequent rounds of FACS to increase selective pressure (100 nM, 20 nM, 5 nM, and 1 nM). For secondary selection, we used 500 nM bMBP-SsoZNS1.E1 with SA AF647 to label for binding. We also incorporated negative selections using biotinylated D2NS1 (200 nM), bMBP-SsoZNS1.E1 (500 nM), and SA AF647 to remove off-target or cross-reactive binding clones.

Fluorescence-activated cell sorting was conducted on a BD FACS Aria running the FACS Diva software package. The forward scatter and side scatter parameters were used in series to identify and select singlet cell populations. Alexa Fluor 647 and DyLight 650 were excited using a laser at 640 nm, and fluorescence was detected using a filter measuring emission at 670 nm, with a 30 nm bandwidth. Alexa Fluor 488 was excited using a laser at 488 nm, and fluorescence was detected using a filter measuring emission at 515 nm, with a 20 nm bandwidth. R-phycoerythrin was excited using a laser at 561 nm, and fluorescence was detected using a filter measuring emission at 582 nm, with a 15 nm bandwidth. Cytometry data was analyzed using the FlowJo software package.

Affinity reagent analysis

After the final FACS library selection for primary or secondary selection, the remaining yeast sub-population was sequenced to determine the population diversity, as described previously.(3) Briefly, the enriched library was miniprepped using the ZymoPrep Yeast Miniprep II kit, and the purified plasmid preparation was transformed into electrocompetent DH5α *E. coli.* Transformation cultures were incubated at 37°C for one hour, and were then plated onto LB-Ampicillin agar plates for overnight stationary incubation at 37°C. Ten to twenty transformed bacterial colonies were grown and miniprepped (Epoch Life Sciences GenCatch Plasmid DNA Miniprep Kit), and the purified plasmids were sent off for sequencing via GeneWiz. Unique clones were identified and stored in *E. coli* cell stocks in -70°C. Miniprepped pCTCON2 plasmids for specific unique rcSso7d clones were transformed back into EBY100 *S. cerevisiae* using the Frozen-EZ Yeast Transformation II Kit (Zymo Research) and stored in *S. cerevisiae* cell stocks in -70°C.

Identified rcSso7d clones in EBY100 were then analyzed using yeast surface display via FACS analysis. Yeast cells prepared for FACS analysis following a similar protocol listed under the previous section. Affinity characterization for selected rcSso7d clones was conducted following a similar protocol as outlined previously.(3) If multiple clones were identified after sequencing, the highest-affinity clone with lowest non-specific binding was selected for further processing.

Selected rcSso7d variants were cloned from the pCTCON2 yeast surface display plasmid into pET28b(+) bacterial expression plasmids, following a protocol outlined previously.(3, 5, 6) Three different plasmid constructs were used: 1) N-terminal AviTag biotin acceptor sequence (b) with maltose-binding protein (MBP) (bMBP-rcSso7d), 2) C-terminal cellulose-binding domain (CBD) (rcSso7d-CBD), and 3) no fusion partners (rcSso7d). Each of the plasmid constructs contain an N-terminal hexahistidine tag for purification purposes. The primers used for cloning (**Supplementary Table 1**) amplify the rcSso7d variant to insert the selected clone into the previously constructed pET28b(+)-bMBP-rcSso7d, pET28b(+)-rcSso7d-CBD, and pET28b(+)-rcSso7d plasmids.(3, 5, 6) Successfully cloned plasmids were stored in DH5α *E. coli* cell stocks and also transformed into BL21(DE3) *E. coli* for protein expression (see above). Complete DNA and protein sequences are compiled in **Supplementary Table 2**.

Bio-layer interferometry

Kinetic analysis was conducted with ForteBio's Octet® RED96 Bio-Layer Interferometry platform, using Streptavidin (SA) sensor tips. Sample concentrations were assessed using a BCA assay and adjusted to the desired concentrations using kinetics buffer (1x PBS, 0.1% w/v BSA, and 0.02% v/v Tween-20 for Rv1656 experiments; 1x PBS, 0.1% w/v BSA, and 0.05% v/v Tween-20, pH 5 for IL-6 and ZNS1 experiments). Streptavidin-coated BLI sensors were hydrated in kinetics buffer for ten minutes at room temperature. All wells were filled with a 200 µL solution volume and all steps were performed at 30°C with mixing at 1000 rpm.

Evaluation of binding kinetic parameters was initiated by incubating the pre-hydrated streptavidin tip first in kinetics buffer to obtain a signal baseline (60 seconds). Next the tips were contacted with the ligand solution (containing the bMBP-rcSso7d variant for desired characterization) for various periods of time ranging from 60 seconds to 160 seconds, depending on the desired loading thickness outputted in the sensorgram. After another baseline incubation in the kinetics buffer (60 seconds), the tip was contacted with the biomarker solution (Rv1656, IL-6, or ZNS1) for the association step (300 seconds) before incubation in kinetics buffer for the dissociation step (600 seconds). Multiple concentrations of the biomarker were tested in order to measure multiple binding curves for more reliable kinetic constants.

For SsoRv1656.E1, 5 nM bMBP-SsoRv1656.E1 was used for ligand loading at 60 seconds, followed by various concentrations of Rv1656 for the association step (1.25, 0.625, and 0.3125 nM). For SsoRv1656.E2, 50 nM bMBP-SsoRv1656.E2 was used for ligand loading at 160 seconds, followed by various concentrations of Rv1656 for the association step (9, 3, and 1 nM). For SsoIL6.E1, SsoIL6.E2, SsoZNS1.E1, and SsoZNS1.E2 kinetics analysis, the associated bMBP-rcSso7d affinity reagent (10 nM) was used in the ligand loading step, and various concentrations of IL-6 and ZNS1 was used for the association steps (81, 27, 9, 3, and 1 nM target biomarker) . For SsoIL6.E1, SsoZNS1.E1, and SsoZNS1.E2, the ligand was loaded for 60 seconds. For SsoIL6.E2, the ligand was loaded for 90 seconds.

In addition to the above samples, a reference sensor was also tested during each experiment. This entailed the same steps as above, with the exception of incubating in kinetics buffer without the biomarker for the association step. A baseline sensor was also conducted, which consisted of just kinetics buffer during each incubation in order to assess potential baseline drift over the course of the experiment.

Kinetics data sensorgrams were analyzed using ForteBio's Data Analysis 8.2 software. Single reference subtraction was used to subtract out the reference sensor values (with bMBP-rcSso7d loaded onto the sensor and subsequent association and dissociation steps occurring in just the kinetics buffer) from the sample curves. Kinetic parameters were obtained by applying a global fit to the curves using a 1:1 binding fit model.

For Rv1656 sequential binding assays, pre-hydrated tips were dipped into fresh kinetics buffer for 60 seconds in order to establish a signal baseline. Five controls were developed along with a single experimental sample, to create the following immunocomplexes:

1. bMBP-SsoRv1656.E1/Rv1656/SsoRv1656.E2-CBD;
2. bMBP-SsoRv1656.E1/Rv1656/(blank);
3. bMBP-SsoRv1656.E1 /(blank)/SsoRv1656.E2-CBD;
4. (blank)/(blank)/SsoRv1656.E2-CBD;
5. (blank)/Rv1656/SsoRv1656.E2-CBD;
6. (blank)/(blank)/(blank)

Appropriate soluble concentrations and association times were established for samples containing bMBP-SsoRv1656.E1 (5 nM), Rv1656 (10 nM), and SsoRv1656.E2-CBD (500 nM). All association steps were conducted for 300 seconds, and the final dissociation step lasted for 600 seconds.

For IL-6 sequential binding assays, bMBP-SsoIL6.E2 (94 nM) was loaded onto the sensor tip for 300 seconds. Subsequently, the tip was incubated with 1 μM IL-6 (Biolegend) for 300 seconds and 1 μM SsoIL6.E1 for another 300 seconds to complete the full kinetic assay. Control assays were conducted using a similar protocol with 1 μM IL-8 (Biolegend) in the secondary binding step instead of IL-6.

For ZNS1 sequential binding assays, bMBP-SsoZNS1.E2 (10 nM) was loaded onto the sensor tip for 180 seconds, followed by a wash step in kinetics buffer for 60 seconds. Then, the tip was incubated in the biomarker solution (100 nM ZNS1) for 300 seconds, followed by another incubation in the complementary affinity reagent solution (5 μM SsoZNS1.E1-CBD) for 300 seconds. A control was assessed alongside the experimental sample, with the same parameters and samples as the sample, with the exception of kinetics buffer used in the place of the biomarker solution during the biomarker incubation step.

Paper-based assays

Whatman No. 1 chromatography paper was patterned with hydrophilic test zones by printing hydrophobic ink as previously described.(7) Paper-based immunoassays were developed using sequential protein addition and wicking of the flow-through from the transverse side of the paper, and the test zones were washed twice with 20 μL of 1x PBS following each incubation step. Protein immobilization on the hydrophilic cellulose test zones was conducted using rcSso7d-CBD (cellulose-binding domain) fusion proteins, which binds in high density to the surface of cellulose.(5) rcSso7d-CBD was diluted in 40 mM of sodium acetate buffer (pH 5.5) to 30-60 μM, and 6 μL was applied to each test zone for a 30 second incubation time. Test zones were then contacted for 30 minutes with 10 μL of the relevant biomarker (Rv1656, IL-6, or ZNS1) in 1% PBSA (1x PBS with 1% w/v BSA)—or just 1% PBSA in the absence of target biomarker for negative controls.

For Rv1656 paper-based assays, sample wells were subsequently contacted for 30 minutes with 10 μL of the complementary bMBP-rcSso7d variant (256 nM) in a McIlvaine buffer system designed to minimize non-specific binding (pH 5; 103 mM Na_2HPO_4/48.5 mM citric acid), supplemented with 1% w/v BSA. These samples were subsequently washed with this buffer at pH 5 (without BSA), and finally the samples were contacted in the dark for 30 minutes with 10 μL of SA AF647 (256 nM). Samples were allowed to air-dry in the dark prior to imaging.

Paper-based assay development proceeded similarly for ZNS1 and IL-6 assays, except the bMBP-rcSso7d and SA AF647 species were prepared at a concentration of 512 nM, and all protein incubation steps and wash steps were conducted in the same solution (1% PBSA and 1x PBS, respectively).

Fluorescence microscopy was used to measure the amount of target biomarker captured, as described previously.(3) Samples were exposed for 150 ms using a Cy5 filter and imaged using Metamorph software (Molecular Devices, Sunnyvale, CA). Captured fluorescent images were processed as previously detailed using the ImageJ software package (US National Institutes of Health) to determine the mean fluorescence intensity (MFI). Values represent an average of at least four replicates, and error bars indicate standard deviations of the calculated mean fluorescence intensity.

Bead-based assays

SsoIL6.E1 was conjugated on carboxylated polystyrene bead (Biorad; 171506011) by activating the carboxyl group with a solution containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ThermoFisher Scientific) and N-hydroxysulfosuccinimide (Sigma Aldrich) (EDC/NHS). Prior to carboxyl group activation, beads were vortexed for at least 30 seconds, and 1.25 x 10^6 beads were aliquoted into a

1.5 mL tube. Beads were centrifuged for 14,000 *xg* for 4 minutes, and the supernatant was carefully discarded. This centrifugation parameter was used throughout the conjugation protocol. Beads were washed twice with 50 mM MES buffer (pH 5.5), and the carboxyl groups were activated with 30 µL of 25 mg/mL EDC and 25 mg/mL NHS mixture for 30 minutes at room temperature on a rotator. To conjugate SsoIL6.E1 on the beads, 100 µM of 94 nM SsoIL6.E1 in 1x PBS pH 7.4 was added to the activated beads and incubated on a rotator for 14-18 hours at 4 °C. After incubation, the beads were washed twice with PBST (1x PBS with 1% Tween20) and blocked with 5% human serum (in 1x PBS) for 1 hour at room temperature.

For the IL-6 bead-based assay, 5,000 beads were used for each sample. Beads were incubated with 150 µL of various concentrations of IL-6 (ranging from 12.5 to 400 nM) for 1 hour at room temperature. A sample without IL-6 (0 nM) was also conducted. Afterwards, the beads were washed with PBST twice and incubated with 1 µM of bMBP-SsoIL6.E2 for 1 hour at room temperature. The beads were washed with PBST twice, and fluorescent signal was generated by incubating the beads with 1 µg/mL of streptavidin R-phycoerythrin (SA-PE; Roche; 05065925103) for 30 minutes at room temperature in the dark. A similar protocol was used for the control experiment, in which a non-target protein (IL-8; 400 nM) was used during the biomarker incubation instead of IL-6. Flow cytometry (Attune NxT, ThermoFisher Scientific) was used to measure the resulting fluorescent signal (excitation: 561 nm; emission: 585 nm; bandwidth: 16 nm). The forward-scatter parameter was used to identify and analyze singlet bead populations.

Well-plate ELISA

SsoIL6.E1 was immobilized on flat-bottom 96 well polystyrene plates (Greiner Bio-One) by incubating SsoIL6.E1 (50 µg/mL) in each well for 14 to 18 hours at 4 °C. The plates were washed three times with PBST (1x PBS with 1% Tween20), blocked with 5% fetal bovine serum for 1 hour at room temperature and washed for another three times with PBST to remove unbound serum proteins. Different concentrations of (carrier-free) human IL-6 or TNF-α biomarkers (Biolegend), ranging from 0-200 nM, were added to each well and incubated for 2 hours at room temperature. The plates were washed three times with PBST, incubated with 1000 nM bMBP-SsoIL6.E2 for 1 hour at room temperature, and washed for another three times with PBST to remove unbound bMBP-SsoIL6.E2. To generate colorimetric signal, plates were washed three times with PBST and incubated with 1:2000 streptavidin HRP (Biolegend) for 30 minutes at room temperature. Plates were then washed four times with PBST and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma Aldrich/Merck, Singapore) for 3-10 minutes until the reactions turned blue. Reactions were stopped by adding equal volume of 1N H_2SO_4 to TMB to each well. Optical signals were read at 450 nm using Biotek Synergy 4 Multi Mode Microplate Reader (Biotek Instrument, Inc., USA). For all incubation steps, 100 µL solution volumes were used for each well. For all washing steps, 200 µL of PBST was used for each well. Experiments were conducted in duplicate.

Statistical analysis

For paper-based assay data sets, the mean fluorescence intensity (MFI) was reported. The error bars represent the standard deviation from the mean of four independent replicates. ELISA experiments were conducted in duplicate and the mean of these results is reported. The error bars in the ELISA experiments represent the standard deviation from the mean. For bead-based assays, mean fluorescence intensity (MFI) was reported. The error bars represent the standard deviation within a fluorescent bead population from one experiment. Where fluorescence intensity values are reported for flow cytometry plots, these represent the geometric mean fluorescence intensity of 10,000 cells, unless otherwise stated.

Supplementary Tables:

Supplementary Table 1: Oligonucleotide sequences of primers used in plasmid cloning.

#	Oligo Name	DNA Sequence (*NdeI*, *XhoI*, *BamHI*, and *SpeI* 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-BamHI-App-rev	5′-ACCCCT**CTCGAG**TTATTA**GGATCC**TTGCTTTTCCAGCATCTG-3′	66.3
4	rcSso-SpeI-for	5′-TCGTGTCT**ACTAGT**GCAACCGTGAAATTCACATACC-3′	63.1

Supplementary Table 2: DNA and primary amino acid sequences of representative protein constructs

Species	cDNA (5'-3')	Protein Sequence (N->C)
bMBP-SsoRv1656.E1	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGATGGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACTCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTGCAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTGTGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGTAAAGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFEAQKIEWHELKGGGGSGGGGSEFKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNSGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTGSGGGGSGGGGSTSATVKFTYQGEEKQVDISKIKSVWRRGQRIWFRYDEGGGAWGAGKVSEKDAPKELLQMLEKQ*
SsoRv1656.E2-CBD	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTGGGTGCGTCGTTACGGCCAGTACATTGGTTTTTCTTATGATGAAGGTGGTGGTGCCTGGGGTAAAGGTTATGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGATCCGGAGGTGGAGGTTCTGGTGGAGGAGGATCTGGAGGTGGTGGTTCTCCGGTATCAGGCAATTTGAAGGTTGAATTCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAATACCGGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACAGAAAGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATTACTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTTGAAATAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCACATACAAGGTAGATTTGCAAAGAATGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTGCTTCACAGTTTGTTGAATGGGATCAGGTAACACCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCTAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIKWVRRYGQYIGFSYDEGGGAWGKGYVSEKDAPKELLQMLEKQGSGGGGSGGGGSGGGGSPVSGNLKVEFYNSNPSDTTNSINPQFKVTNTGSSAIDLSKLTLRYYYTVDGQKDQTFWCDHAAIIGSNGSYNGITSNVKGTFVKMSSSTNNADTYLEISFTGGTLEPGAHVHIQGRFAKNDWSNYTQSNDYSFKSASQFVEWDQVTPYLNGVLVWGKEP*

bMBP-SsoRv1656.E2	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAT GGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTG GTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAC GGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAA AGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATG GCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTA CAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAA GGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGA CGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACT CTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCC GTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCA AGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAA TAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCAC GTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGA AGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTG CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTGGGTG CGTCGTTACGGCCAGTACATTGGTTTTTCTTATGATGAAGGTGGTGGTGCCTGGGGTAAAGGTTA TGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFE AQKIEWHELKGGGGSGGGGSEFKIEEGKLVI WINGDKGYNGLAEVGKKFEKDTGIKVTVEHP DKLEEKFPQVAATGDGPDIIFWAHDRFGGYA QSGLLAEITPDKAFQDKLYPFTWDAVRYNGK LIAYPIAVEALSLIYNKDLLPNPPKTWEEIP ALDKELKAKGKSALMFNLQEPYFTWPLIAAD GGYAFKYENGKYDIKDVGVDNSGAKAGLTFL VDLIKNKHMNADTDYSIAEAAFNKGETAMTI NGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP FVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAA TMENAQKGEIMPNIPQMSAFWYAVRTAVINA ASGRQTVDEALKDAQTGSGGGGSGGGGSTSA TVKFTYQGEEKQVDISKIKWVRRYGQYIGFS YDEGGGAWGKGYVSEKDAPKELLQMLEKQ*
SsoIL6.E1	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTGT GGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGTAAA GTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKIKIVGRHGQWIYFWYDEGGGADG NGWVSEKDAPKELLQMLEKQ*

bMBP-SsoIL6.E1	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAT GGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTG GTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAC GGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAA AGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATG GCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTA CAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAA GGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGA CGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACT CTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCC GTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCA AGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAA TAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCAC GTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGA AGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTG CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTG TGGCGTCGTGGCCAGCGTATTTGGTTTCGTTATGATGAAGGTGGTGGTGCCTGGGGTGCAGGTAA AGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFE AQKIEWHELKGGGGSGGGGSEFKIEEGKLVI WINGDKGYNGLAEVGKKFEKDTGIKVTVEHP DKLEEKFPQVAATGDGPDIIFWAHDRFGGYA QSGLLAEITPDKAFQDKLYPFTWDAVRYNGK LIAYPIAVEALSLIYNKDLLPNPPKTWEEIP ALDKELKAKGKSALMFNLQEPYFTWPLIAAD GGYAFKYENGKYDIKDVGVDNSGAKAGLTFL VDLIKNKHMNADTDYSIAEAAFNKGETAMTI NGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP FVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAA TMENAQKGEIMPNIPQMSAFWYAVRTAVINA ASGRQTVDEALKDAQTGSGGGGSGGGGSTSA TVKFTYQGEEKQVDISKIK**I**V**G**R**H**GQ**W**I**Y**F**W** YDEGGGA**D**G**N**G**W**VSEKDAPKELLQMLEKQ*
SsoIL6.E1-CBD	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGATCGTGG GTCGTCATGGCCAGTGGATTTACTTTTGGTATGATGAAGGTGGTGGTGCCGATGGTAACGGTTGG GTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGATCCGGAGGTGG AGGTTCTGGTGGAGGAGGATCTGGAGGTGGTGGTTCTCCGGTATCAGGCAATTTGAAGGTTGAAT TCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAATACC GGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACAGAA AGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATTA CTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTT GAAATAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCACATACAAGGTAGATTTGC AAAGAATGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTGCTTCACAGTTTG TTGAATGGGATCAGGTAACACCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCTAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKIK**I**V**G**R**H**GQ**W**I**Y**F**W**YDEGGGA**D**G **N**G**W**VSEKDAPKELLQMLEKQGSGGGGSGGGG SGGGGSPVSGNLKVEFYNSNPSDTTNSINPQ FKVTNTGSSAIDLSKLTLRYYYTVDGQKDQT FWCDHAAIIGSNGSYNGITSNVKGTFVKMSS STNNADTYLEISFTGGTLEPGAHVHIQGRFA KNDWSNYTQSNDYSFKSASQFVEWDQVTPYL NGVLVWGKEP*
SsoIL6.E2	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAACCAAGAACGTGT ACCGTTGGGGCCAGCATATTTGGTTTGACTATGATGAAGGTGGTGGTGCCGCAGGTTATGGTAAA GTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKTK**N**V**Y**R**W**GQ**H**I**W**F**D**YDEGGGA**A**G **Y**G**K**VSEKDAPKELLQMLEKQ*

bMBP-SsoIL6.E2	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAT GGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTG GTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAC GGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAA AGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATG GCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTA CAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAA GGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGA CGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACT CTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCC GTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCA AGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAA TAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCAC GTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGA AGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTG CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAACCAAGAACGTG TACCGTTGGGGCCAGCATATTTGGTTTGACTATGATGAAGGTGGTGGTGCCGCAGGTTATGGTAA AGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFE AQKIEWHELKGGGGSGGGGSEFKIEEGKLVI WINGDKGYNGLAEVGKKFEKDTGIKVTVEHP DKLEEKFPQVAATGDGPDIIFWAHDRFGGYA QSGLLAEITPDKAFQDKLYPFTWDAVRYNGK LIAYPIAVEALSLIYNKDLLPNPPKTWEEIP ALDKELKAKGKSALMFNLQEPYFTWPLIAAD GGYAFKYENGKYDIKDVGVDNSGAKAGLTFL VDLIKNKHMNADTDYSIAEAAFNKGETAMTI NGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP FVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAA TMENAQKGEIMPNIPQMSAFWYAVRTAVINA ASGRQTVDEALKDAQTGSGGGGSGGGGSTSA TVKFTYQGEEKQVDISKTKNVYRWGQHIWFD YDEGGGAAGYGKVSEKDAPKELLQMLEKQ*
SsoIL6.E2-CBD	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAACCAAGAACGTGT ACCGTTGGGGCCAGCATATTTGGTTTGACTATGATGAAGGTGGTGGTGCCGCAGGTTATGGTAAA GTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGATCCGGAGGTGG AGGTTCTGGTGGAGGAGGATCTGGAGGTGGTGGTTCTCCGGTATCAGGCAATTTGAAGGTTGAAT TCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAATACC GGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACAGAA AGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATTA CTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTT GAAATAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCACATACAAGGTAGATTTGC AAAGAATGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTGCTTCACAGTTTG TTGAATGGGATCAGGTAACACCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCTAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKTKNVYRWGQHIWFDYDEGGGAAG YGKVSEKDAPKELLQMLEKQGSGGGGSGGGG SGGGGSPVSGNLKVEFYNSNPSDTTNSINPQ FKVTNTGSSAIDLSKLTLRYYYTVDGQKDQT FWCDHAAIIGSNGSYNGITSNVKGTFVKMSS STNNADTYLEISFTGGTLEPGAHVHIQGRFA KNDWSNYTQSNDYSFKSASQFVEWDQVTPYL NGVLVWGKEP*

bMBP-SsoZNS1.E1	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAT GGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTG GTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAC GGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAA AGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATG GCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTA CAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAA GGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGA CGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACT CTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCC GTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCA AGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAA TAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCAC GTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGA AGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTG CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTG ATCCGTAAAGGCCAGCATATTTGGTTTGCTTATGATGAAGGTGGTGGTGCCTGGGGTAGCGGTAA AGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFE AQKIEWHELKGGGGSGGGGSEFKIEEGKLVI WINGDKGYNGLAEVGKKFEKDTGIKVTVEHP DKLEEKFPQVAATGDGPDIIFWAHDRFGGYA QSGLLAEITPDKAFQDKLYPFTWDAVRYNGK LIAYPIAVEALSLIYNKDLLPNPPKTWEEIP ALDKELKAKGKSALMFNLQEPYFTWPLIAAD GGYAFKYENGKYDIKDVGVDNSGAKAGLTFL VDLIKNKHMNADTDYSIAEAAFNKGETAMTI NGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP FVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAA TMENAQKGEIMPNIPQMSAFWYAVRTAVINA ASGRQTVDEALKDAQTGSGGGGSGGGGSTSA TVKFTYQGEEKQVDISKIK**S**V**I**R**K**GQ**H**I**W**F**A** YDEGGGA**W**G**S**G**K**VSEKDAPKELLQMLEKQ*
SsoZNS1.E1-CBD	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGTCTGTGA TCCGTAAAGGCCAGCATATTTGGTTTGCTTATGATGAAGGTGGTGGTGCCTGGGGTAGCGGTAAA GTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGATCCGGAGGTGG AGGTTCTGGTGGAGGAGGATCTGGAGGTGGTGGTTCTCCGGTATCAGGCAATTTGAAGGTTGAAT TCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAATACC GGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACAGAA AGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATTA CTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTT GAAATAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCACATACAAGGTAGATTTGC AAAGAATGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTGCTTCACAGTTTG TTGAATGGGATCAGGTAACACCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCTAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKIK**S**V**I**R**K**GQ**H**I**W**F**A**YDEGGGA**W**G **S**G**K**VSEKDAPKELLQMLEKQGSGGGGSGGGG SGGGGSPVSGNLKVEFYNSNPSDTTNSINPQ FKVTNTGSSAIDLSKLTLRYYYTVDGQKDQT FWCDHAAIIGSNGSYNGITSNVKGTFVKMSS STNNADTYLEISFTGGTLEPGAHVHIQGRFA KNDWSNYTQSNDYSFKSASQFVEWDQVTPYL NGVLVWGKEP*

bMBP-SsoZNS1.E2	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAT GGCGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAACTTAAGGGTGGTG GTGGTAGCGGTGGTGGCGGTTCAGAATTCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAAC GGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAA AGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATG GCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTA CAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATC TGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAA GGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGA CGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACT CTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCC GTGGGCATGGTCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCA AGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAA TAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCAC GTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGA AGCCCTGAAAGACGCGCAGACTGGATCCGGTGGTGGTGGTAGCGGTGGTGGCGGTTCAACTAGTG CAACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGCGTGTG TACCGTTGGATCGGCCAGGACATTGGTTTTATCTATGATGAAGGTGGTGGTGCCAGCGGTTGGGG TAGCGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAATAA	MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFE AQKIEWHELKGGGGSGGGGSEFKIEEGKLVI WINGDKGYNGLAEVGKKFEKDTGIKVTVEHP DKLEEKFPQVAATGDGPDIIFWAHDRFGGYA QSGLLAEITPDKAFQDKLYPFTWDAVRYNGK LIAYPIAVEALSLIYNKDLLPNPPKTWEEIP ALDKELKAKGKSALMFNLQEPYFTWPLIAAD GGYAFKYENGKYDIKDVGVDNSGAKAGLTFL VDLIKNKHMNADTDYSIAEAAFNKGETAMTI NGPWAWSNIDTSKVNYGVTVLPTFKGQPSKP FVGVLSAGINAASPNKELAKEFLENYLLTDE GLEAVNKDKPLGAVALKSYEEELAKDPRIAA TMENAQKGEIMPNIPQMSAFWYAVRTAVINA ASGRQTVDEALKDAQTGSGGGGSGGGGSTSA TVKFTYQGEEKQVDISKIK**R**V**Y**R**WI**GQ**D**I**G**F **I**YDEGGGA**S**G**W**G**S**VSEKDAPKELLQMLEKQ*
SsoZNS1.E2-CBD	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGC AACCGTGAAATTCACATACCAAGGCGAAGAAAAACAGGTGGATATTAGCAAAATCAAGCGTGTGT ACCGTTGGATCGGCCAGGACATTGGTTTTATCTATGATGAAGGTGGTGGTGCCAGCGGTTGGGGT AGCGTGAGCGAAAAAGATGCACCGAAAGAACTGCTGCAGATGCTGGAAAAGCAAGGATCCGGAGG TGGAGGTTCTGGTGGAGGAGGATCTGGAGGTGGTGGTTCTCCGGTATCAGGCAATTTGAAGGTTG AATTCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAAT ACCGGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACA GAAAGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAA TTACTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTAC CTTGAAATAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCACATACAAGGTAGATT TGCAAAGAATGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTGCTTCACAGT TTGTTGAATGGGATCAGGTAACACCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCTAA	MGSSHHHHHHSSGLVPRGSHMATVKFTYQGE EKQVDISKIK**R**V**Y**R**WI**GQ**D**I**G**F**I**YDEGGGA**S** G**W**G**S**VSEKDAPKELLQMLEKQGSGGGGSGGG GSGGGGSPVSGNLKVEFYNSNPSDTTNSINP QFKVTNTGSSAIDLSKLTLRYYYTVDGQKDQ TFWCDHAAIIGSNGSYNGITSNVKGTFVKMS SSTNNADTYLEISFTGGTLEPGAHVHIQGRF AKNDWSNYTQSNDYSFKSASQFVEWDQVTPY LNGVLVWGKEP*

Supplementary Figures:

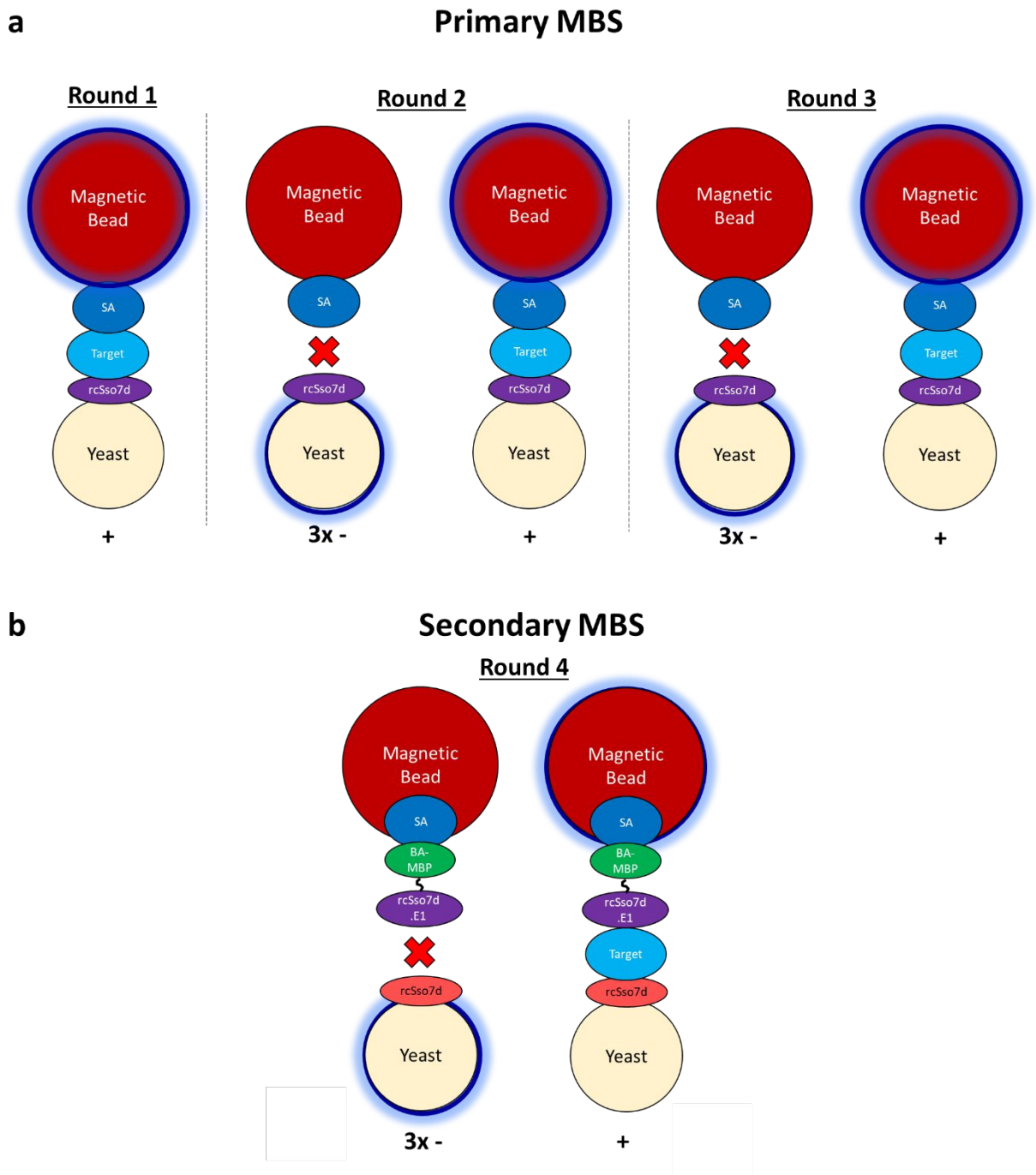

Figure S1: Detailed schematic of magnetic bead sorting (MBS). a) Schematic for representative primary MBS, demonstrating three rounds of sorting. rcSso7d proteins are expressed on the surface of yeast cells. Biotinylated target biomarker is immobilized onto the streptavidin (SA)-coated magnetic beads, although other immobilization methods can be used as well. The first round will typically consist of a positive sort, collecting any yeast cells that bind to the target-immobilized magnetic bead (highlighted in blue). In the subsequent sorting rounds, up to three rounds of negative sorts are conducted consecutively by collecting the yeast cells that do not bind to the magnetic beads (highlighted in blue) in the absence of target biomarker. Immediately after the negative sorts, a positive sort is conducted with the unbound

yeast population. b) Schematic for representative secondary MBS, demonstrating an additional round of MBS using the yeast population after the third round of primary MBS. The primary rcSso7d clone in the biotinylated format (bMBP-rcSso7d.E1) is immobilized onto the SA-coated magnetic beads. Negative sorts are conducted in the absence of target biomarker to collect the yeast cells (highlighted in blue) that do not bind to the beads. A positive sort is conducted immediately after, using magnetic beads coated with bMBP-rcSso7d.E1, which binds to the target. Therefore, only the yeast cells that bind to a non-overlapping epitope on the target will be collected.

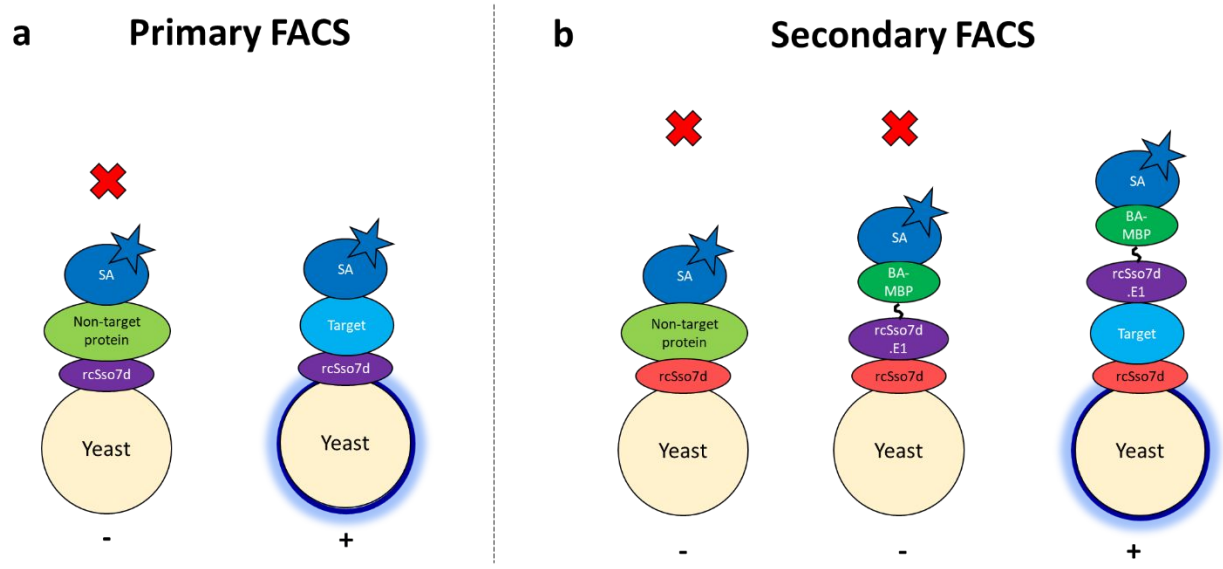

Figure S2: Detailed schematic of FACS. a) Schematic for representative primary FACS. rcSso7d proteins are expressed on the surface of yeast cells. Positive sorts can be conducted by collecting the yeast cells (highlighted in blue) that bind to the target based on fluorescence labeling of the target. Alternatively, a negative sort can be conducted first to collect yeast cells that do not demonstrate off-target binding, before immediately relabeling the collected cells with the target biomarker for a positive sort. b) Schematic for representative secondary FACS. The primary rcSso7d clone in the biotinylated format (bMBP-rcSso7d.E1) is used to label bound target biomarker to collect only the yeast cells (highlighted in blue) that bind to a non-overlapping epitope on the target. Negative sorts may similarly be used to collect cells that do not bind to non-target proteins or the primary affinity reagent. This population may then be immediately relabeled for a positive sort.

SsoRv1656.E1	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AATCAAGTCTGTGTGGCGTCGTGGCCAG CGTATTTGGTTTCGTTATGATGAAGGTGG TGGTGCCTGGGGTGCAGGTAAAGTGAGC GAAAAAGATGCACCGAAAGAACTGCTGC AGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKIKSVWRRGQRIWFRY DEGGGAWGAGKVSEKD APKELLQMLEKQ
SsoRv1656.E2	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AATCAAGTGGGTGCGTCGTTACGGCCAG TACATTGGTTTTTCTTATGATGAAGGTGG TGGTGCCTGGGGTAAAGGTTATGTGAGC GAAAAAGATGCACCGAAAGAACTGCTGC AGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKIKWVRRYGQYIGFSYD EGGGAWGKGYVSEKDA PKELLQMLEKQ
SsoIL6.E1	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AATCAAGATCGTGGGTCGTCATGGCCAG TGGATTTACTTTTGGTATGATGAAGGTGG TGGTGCCGATGGTAACGGTTGGGTGAGC GAAAAAGATGCACCGAAAGAACTGCTGC AGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKIKIVGRHGQWIYFWYD EGGGADGNGWVSEKDA PKELLQMLEKQ
SsoIL6.E2	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AACCAAGAACGTGTACCGTTGGGGCCAG CATATTTGGTTTGACTATGATGAAGGTGG TGGTGCCGCAGGTTATGGTAAAGTGAGC GAAAAAGATGCACCGAAAGAACTGCTGC AGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKTKNVYRWGQHIWFDY DEGGGAAGYGKVSEKD APKELLQMLEKQ
SsoZNS1.E1	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AATCAAGTCTGTGATCCGTAAAGGCCAG CATATTTGGTTTGCTTATGATGAAGGTGG TGGTGCCTGGGGTAGCGGTAAAGTGAGC GAAAAAGATGCACCGAAAGAACTGCTGC AGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKIKSVIRKGQHIWFAYD EGGGAWGSGKVSEKDA PKELLQMLEKQ
SsoZNS1.E2	ATGGCAACCGTGAAATTCACATACCAAG GCGAAGAAAAACAGGTGGATATTAGCAA AATCAAGCGTGTGTACCGTTGGATCGGC CAGGACATTGGTTTTATCTATGATGAAGG TGGTGGTGCCAGCGGTTGGGGTAGCGT GAGCGAAAAAGATGCACCGAAAGAACTG CTGCAGATGCTGGAAAAGCAA	MATVKFTYQGEEKQVDI SKIKRVYRWIGQDIGFIYD EGGGASGWGSVSEKDA PKELLQMLEKQ

Figure S3: Full sequences of selected rcSso7d affinity reagents against TB Rv1656, IL-6, and ZNS1. Nucleic acid and amino acid sequences for the selected rcSso7d affinity reagents. Red indicates the amino acid sequences in the binding face of the rcSso7d clone. Blue indicates an additional mutation detected that was not in the binding face.

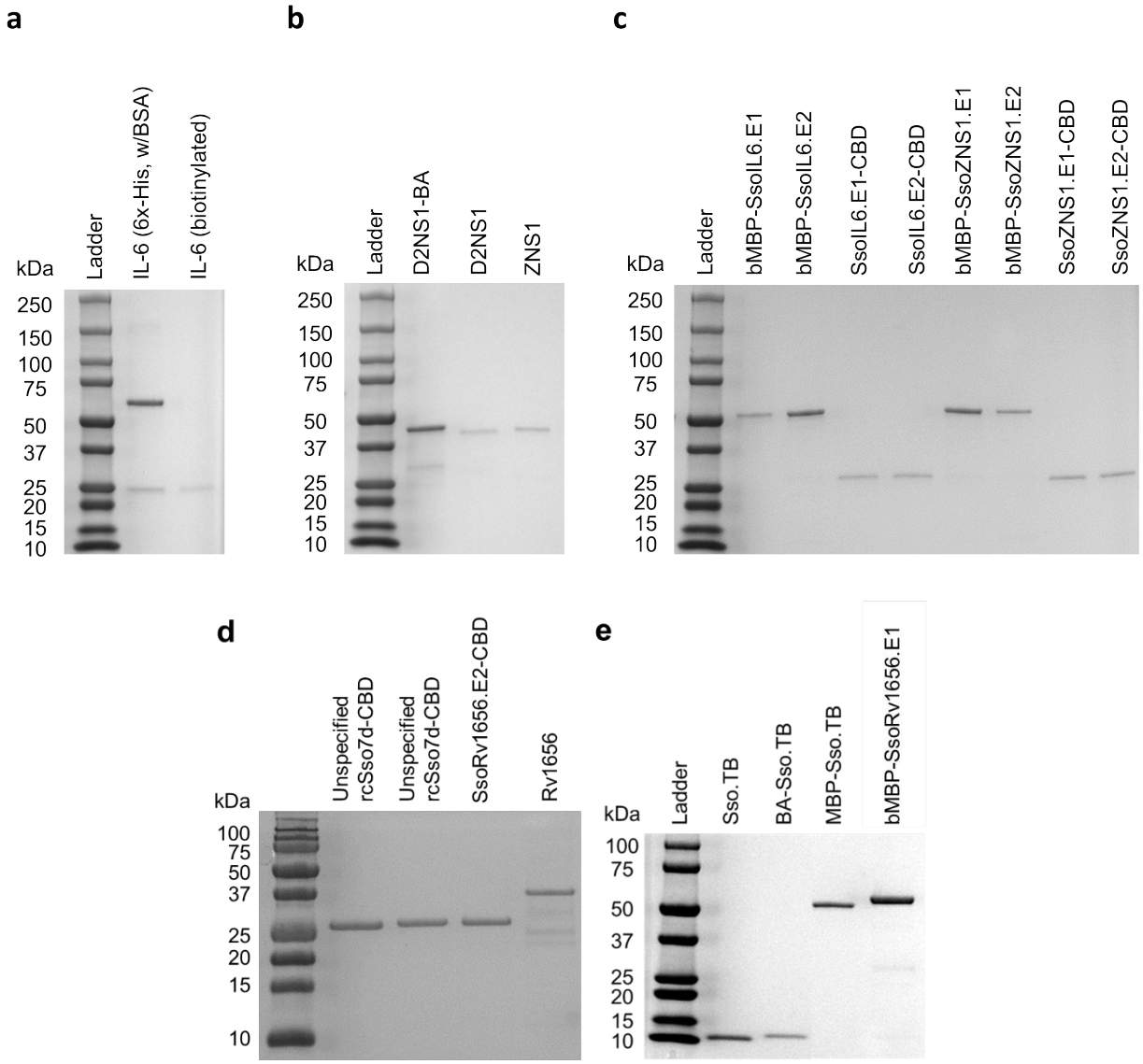

Figure S4: SDS-PAGE gels for protein preparations used in selection processes and for all soluble rcSso7d variants. a) SDS-PAGE for the commercially purchased human IL-6 (MW: 23 kDa): 6x-His IL-6 (RayBioTech) and biotinylated IL-6 (Acro Biosystems). The larger band present is from the addition of bovine serum albumin (BSA; MW: 66 kDa), which was recommended as a carrier protein for long-term storage by the supplier. b) SDS-PAGE for the recombinantly produced Dengue 2 NS1 with C-terminal biotin acceptor sequence (D2NS1-BA; MW: 48 kDa), Dengue 2 NS1 (D2NS1; MW: 44 kDa), and Zika NS1 (ZNS1; MW: 43 kDa). c) SDS-PAGE for the recombinantly produced rcSso7d variants for IL-6 and ZNS1: bMBP-SsoIL6.E1 (MW: 54 kDa), bMBP-SsoIL6.E2 (MW: 54 kDa), SsoIL6.E1-CBD (MW: 28 kDa), SsoIL6.E2-CBD (MW: 28 kDa), bMBP-Sso7d.ZNS1.E1 (MW: 54 kDa), bMBP-SsoZNS1.E2 (MW: 54 kDa), SsoZNS1.E1-CBD (MW: 28 kDa), and SsoZNS1.E2-CBD (MW: 28 kDa). d) SDS-PAGE (15%, hand-cast) for SsoRv1656.E2-CBD (MW: 28kDa) and Rv1656 (MW: 35 kDa). Additional lanes correspond to unspecified rcSso7d-CBD variants, but are included for completeness. e) SDS-PAGE for bMBP-SsoRv1656.E1. Adapted from Sung et al. (2018). Additional lanes correspond to various SsoRv1656.E1 fusion constructs not considered here, but are included for completeness.

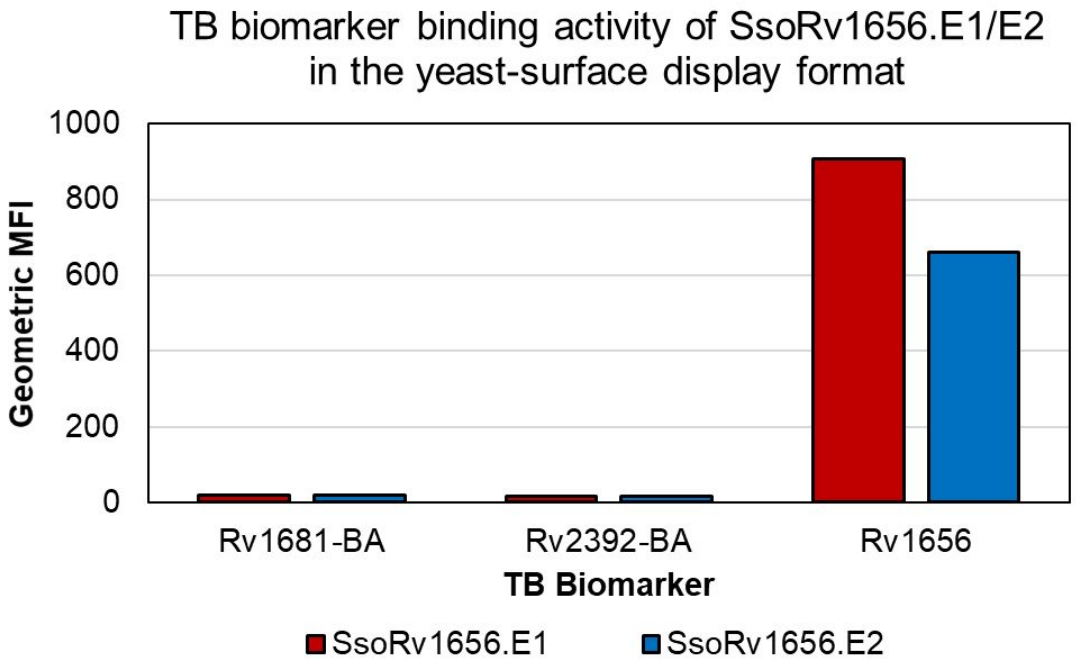

Figure S5: Specificity of SsoRv1656.E1 and SsoRv1656.E2 when challenged with other TB biomarkers. FACS histograms demonstrate minimal off-target binding of SsoRv1656.E1 and SsoRv1656.E2 to other tuberculosis biomarkers (Rv1681-BA and Rv2392-BA, both at a concentration of 100 nM; BA: *in vivo* biotin acceptor sequence; Kashino et al., 2008; Pollock et al., 2013). Target/off-target binding was visualized using mouse anti-His IgG/goat anti-mouse IgG AF647 labeling reagents. Only Rv1656 demonstrates appreciable target-binding – the low-signal population in that histogram corresponds to non-displaying yeast cells. Histograms represent ~10,000 cells.

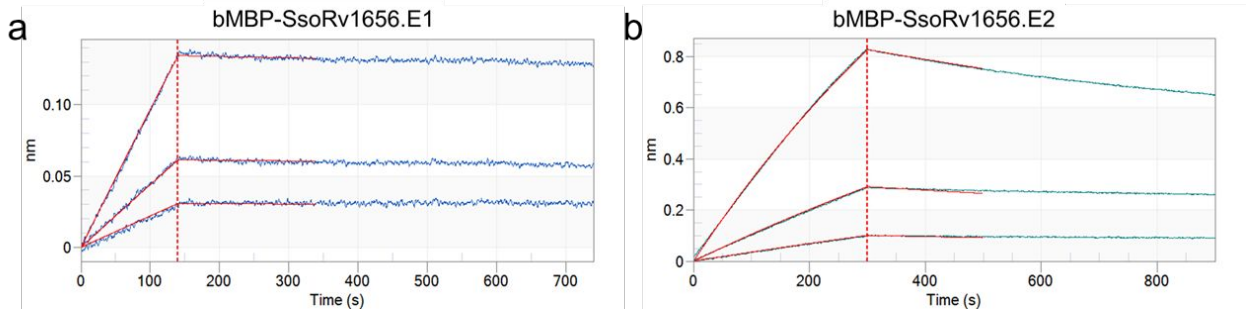

Figure S6: Bio-layer interferometry curves for kinetics measurements for Rv1656 affinity reagents. a) BLI traces for bMBP-SsoRv1656.E1 immobilized on streptavidin-coated tips (60 seconds in a 5 nM solution to form a 0.3 nm layer) and contacted for 140 seconds with Rv1656 at concentrations ranging by factors of 2 from 10 nM to 0.3125 nM. The dissociation step was conducted over the course of 600 seconds. b) BLI traces for bMBP-SsoRv1656.E2 immobilized on streptavidin-coated tips (160 seconds in a 50 nM solution to form a 2.5 nm layer) and contacted for 300 seconds with Rv1656 at concentrations ranging by factors of 3 from 243 nM to 1 nM. The dissociation step was conducted over the course of 600 seconds. Profiles were fit to heterogeneous binding models and the bottom four traces exhibiting limited biphasic behavior were used to globally fit the profiles and identify appropriate kinetic parameters.

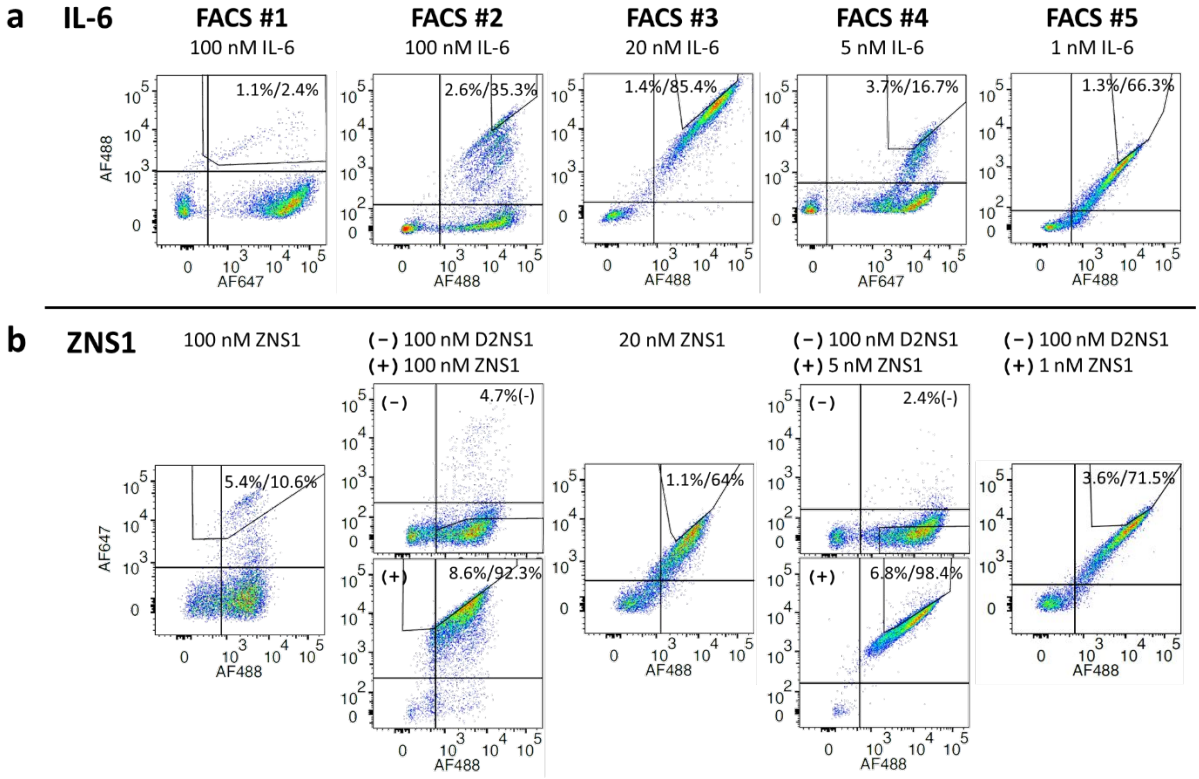

Figure S7: FACS dot plots for primary selection of rcSso7d against IL-6 and ZNS1. a) Primary FACS plots for the selection of affinity reagents against human IL-6. Concentrations of IL-6 used are listed above the plots, with a reduction in concentration from 100 nM to 1 nM for increased stringency. Numbers on the top right of each plot correspond to the percentage of cells collected / the percentage of cells displaying binding signal. b) Primary FACS plots for the selection of affinity reagents against Zika virus NS1. Negative sorts using Dengue 2 NS1 (D2NS1) were conducted prior to the positive sorts for FACS rounds 2 and 4. Concentrations of ZNS1 and D2NS1 used are listed above the plots. Numbers on the top right of the positive sort plots correspond to the percentage of cells collected / the percentage of cells displaying binding signal. The number on the top right of the negative sort plots correspond to the percentage of cells displaying non-target binding signal.

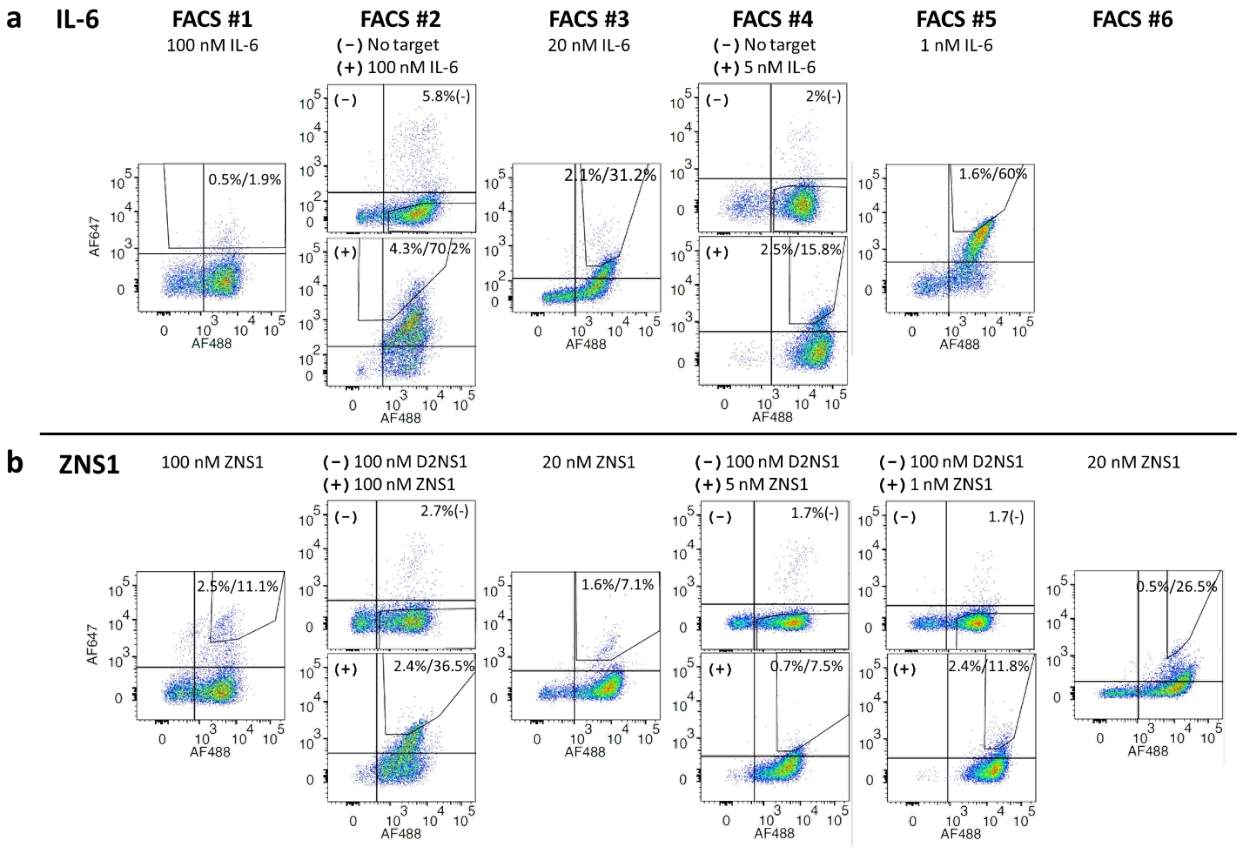

Figure S8: FACS dot plots for secondary selection of rcSso7d against IL-6 and ZNS1. a) Secondary FACS plots for the selection of affinity reagents against human IL-6. Negative sorts using the labeling reagents (bMBP-SsoIL6.E1 and SA AF647) were conducted prior to the positive sorts for FACS rounds 2 and 4. Concentrations of IL-6 used are listed above the plots, with a reduction in concentration from 100 nM to 1 nM for increased stringency. Numbers on the top right of the positive sort plots correspond to the percentage of cells collected / the percentage of cells displaying binding signal. The number on the top right of the negative sort plots correspond to the percentage of cells displaying non-target binding signal. b) Secondary FACS plots for the selection of affinity reagents against Zika virus NS1. Negative sorts using Dengue 2 NS1 (D2NS1) and the labeling reagents (bMBP-SsoZNS1.E1 and SA AF647) were conducted prior to the positive sorts for FACS rounds 2 and 4. Concentrations of ZNS1 and D2NS1 used are listed above the plots. Numbers on the top right of the positive sort plots correspond to the percentage of cells collected / the percentage of cells displaying binding signal. The number on the top right of the negative sort plots correspond to the percentage of cells displaying non-target binding signal.

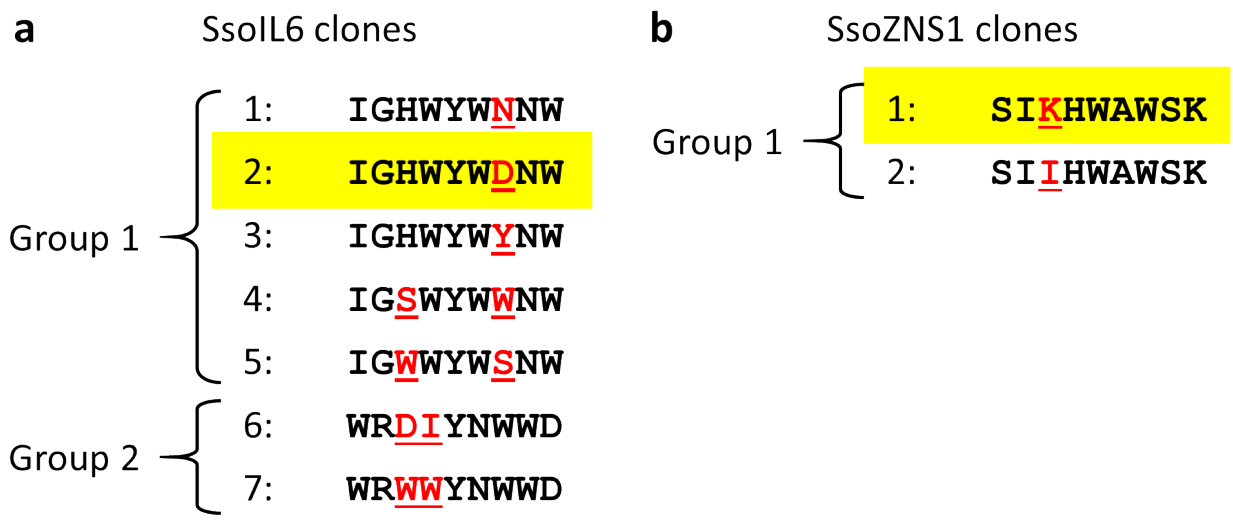

Figure S9: Shorthand tag for the amino acid binding face sequences of all identified rcSso7d affinity reagents against IL-6 and ZNS1 from primary selection process. a) Binding face sequences of the seven identified unique clones from the IL-6 primary selection process. Sequences have been sorted into two groups based on sequence similarity ("sub-family"). Red and underlined amino acids highlight the sequences that differ in each sub-family. b) Binding face sequences of the two identified unique clones from the ZNS1 primary selection process. Sequences show sequence similarity ("sub-family"). Red and underlined amino acids highlight the sequences that differ in the sub-family. Yellow highlighted clones are the ones selected as SsoIL6.E1 and SsoZNS1.E1 and used for secondary selection.

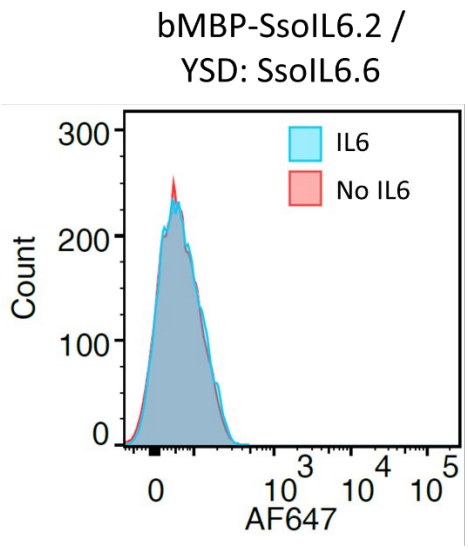

Figure S10: Representative FACS histogram demonstrating overlapping epitope binding of SsoIL6 clones identified from primary selection process. One clone was chosen from each sub-family of the identified rcSso7d clones after primary selection against IL-6: clone 2 and clone 6. These variants were cloned into the bMBP-rcSso7d format and tested using yeast-surface display against all of the other SsoIL6 clones for potential complementarity. None of the tested pairs showed non-overlapping epitope binding. A sample FACS histogram of one of the pairs tested (clones 2 and 6) demonstrates the lack of binding signal in the presence of IL-6, even though each bind separately to IL-6, suggesting that they are competing for the same or similar epitope.

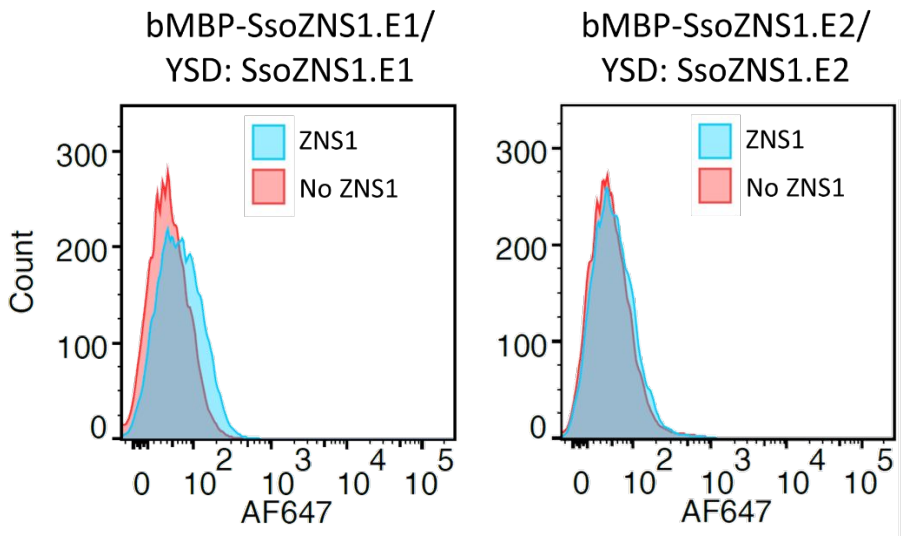

Figure S11: FACS histograms demonstrating SsoZNS1.E1 and SsoZNS1.E2 bind to unique epitopes of ZNS1. Since NS1 is a hexameric protein, it may be possible to use the same affinity reagent as both capture and reporter in a sandwich assay; therefore, the two clones selected against ZNS1 (SsoZNS1.E1 and SsoZNS1.E2) were tested for binding to a multivalent epitope. SsoZNS1.E1 was displayed on the surface of yeast cells, followed by 100 nM ZNS1, and labeled with bMBP-SsoZNS1.E1 and SA AF647 (left), and SsoZNS1.E2 was displayed on the surface of yeast cells, followed by 100 nM ZNS1, and labeled with bMBP-SsoZNS1.E2 and SA AF647 (right). The absence of positive binding signal in both cases indicate that the SsoZNS1.E1 and SsoZNS1.E2 clones demonstrate binding to unique, non-repetitive or multivalent epitopes of ZNS1; therefore, the use of distinct affinity pairs is necessary.

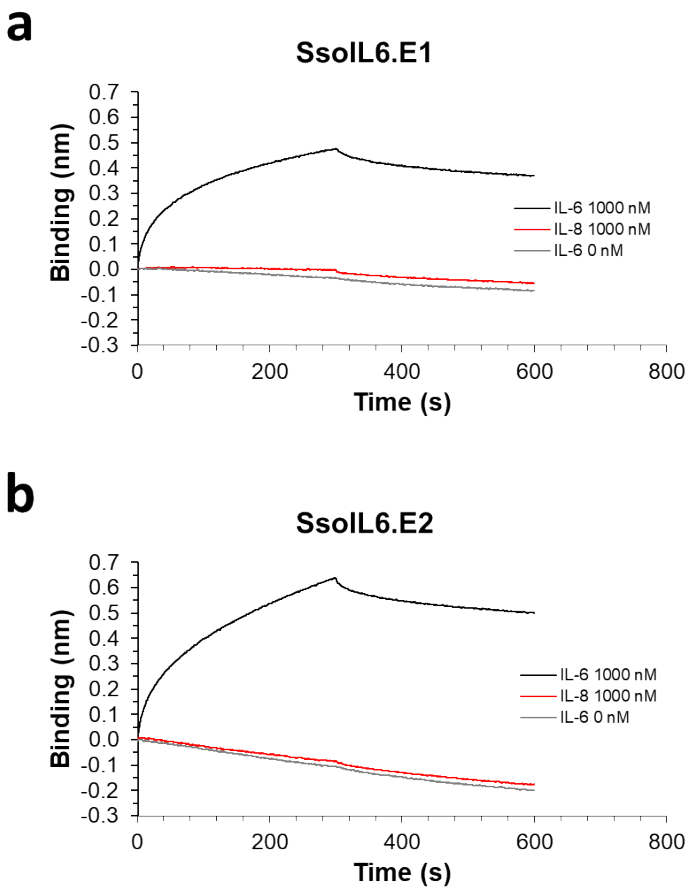

Figure S12: Specificity of SsoIL6.E1 and SsoIL6.E2 when challenged with IL-8 in bio-layer interferometry. Bio-layer interferometry plots with either SsoIL6.E1 (a) or SsoIL6.E2 (b) immobilized on the sensor tip, followed by immersion a solution either containing 1000 nM IL-6, 1000 nM IL-8, or just buffer (0 nM IL-6) for 300 seconds. Following association, the tips were immersed in just buffer for 300 seconds of dissociation. Curves indicate specific binding to IL-6 (association of signal) as compared to the non-target protein IL-8 or in the absence of IL-6.

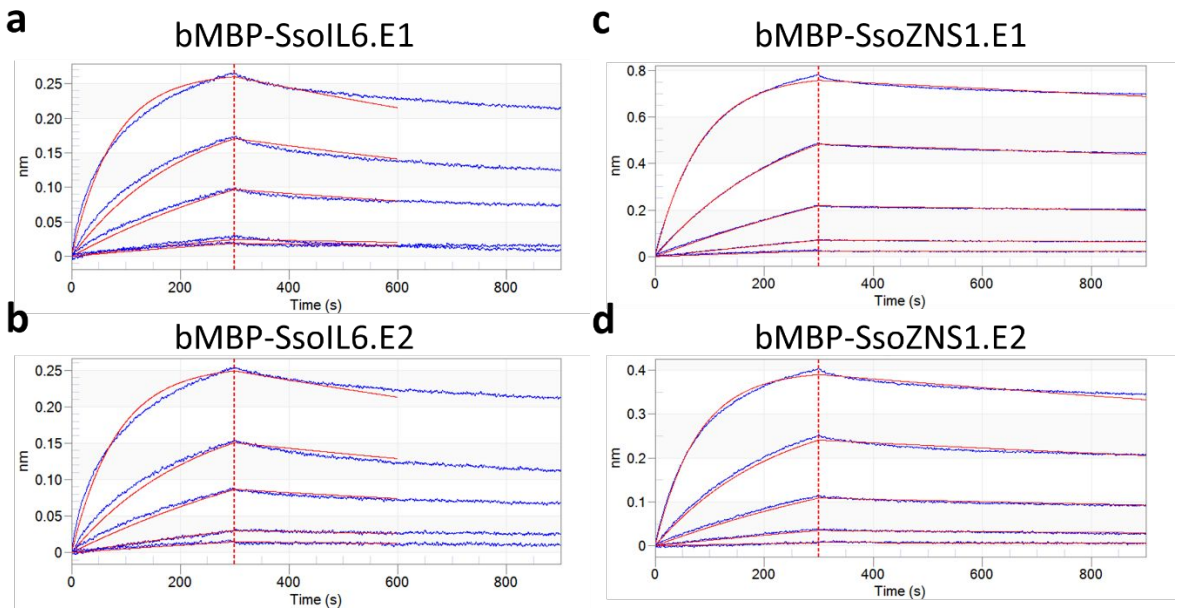

Figure S13: Bio-layer interferometry curves for kinetics measurements for IL-6 and ZNS1 affinity reagents. Bio-layer interferometry plots with either bMBP-SsoIL6.E1 (a), bMBP-SsoIL6.E2 (b), bMBP-SsoZNS1.E1 (c), or bMBP-SsoZNS1.E2 (d) immobilized on the SA sensor tip, followed by immersion a solution either containing various concentrations of IL-6 or ZNS1 (81, 27, 9, 3, and 1 nM) for 300 seconds of association. Following association, the tips were immersed in just buffer for 600 seconds of dissociation. The curves were analyzed and fitted using ForteBio's analysis software with a 1:1 binding fit model to estimate kinetics parameters (k_{on}, k_{off}, and K_d).

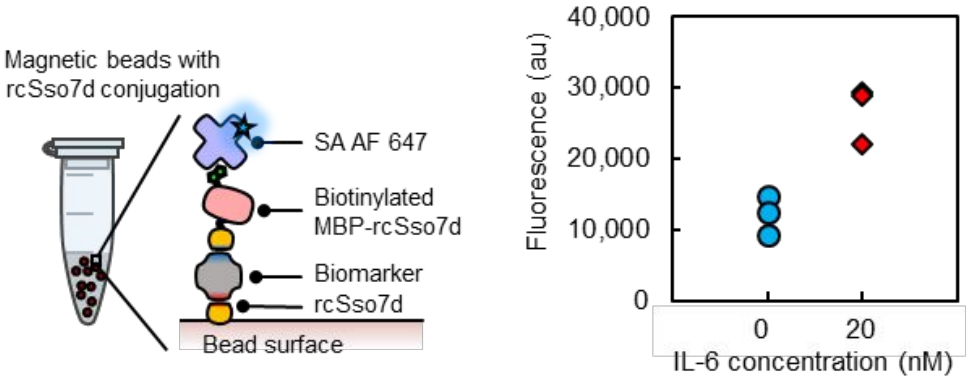

Figure S14: Magnetic bead-based assay format with SsoIL6 affinity pair. SsoIL6.E1 was chemically conjugated to LodeStars 2.7 Carboxyl magnetic beads (Agilent). $7x10^6$ beads were washed with 1 mL of 1% PBSA (1x PBS with 1% w/v BSA) and then placed on a Dynamag-2 magnetic rack to collect the beads and remove the supernatant. The beads were resuspended in 300 µL of 20 nM of IL-6 in 1% PBSA (or just 1% PBSA in the absence of target biomarker for the negative controls) and incubated for 30 minutes at room temperature on a rotator. After washing the beads with 1 mL of PBST (1x PBS with 1% Tween20) then twice with 1 mL 1% PBSA, the beads were incubated with 300 µL of 50 nM of bMBP-SsoIL6.8 for 30 minutes at room temperature on a rotator. After washing the beads with 1 mL of PBST (1x PBS with 1% Tween20) then twice with 1 mL 1% PBSA, they were incubated with 300 µL of 200 nM SA AF647 for 15 minutes at room temperature on a rotator, covered in foil. After a final wash of the beads with 1 mL of PBST (1x PBS with 1% Tween20) then twice with 1 mL 1% PBSA, the beads were resuspended in 175 µL of PBSA, and then 150 µL of the beads was transferred to a Corning black flat 96-well plate. A plate reader was used to measure fluorescence intensity with excitation at 640 nm and emission at 670 nm. Three replicates were developed for each sample and negative control. The results indicated a clear increase in fluorescence signal in the presence of IL-6, signifying specificity and functionality of the identified binding pair in a bead-based assay format

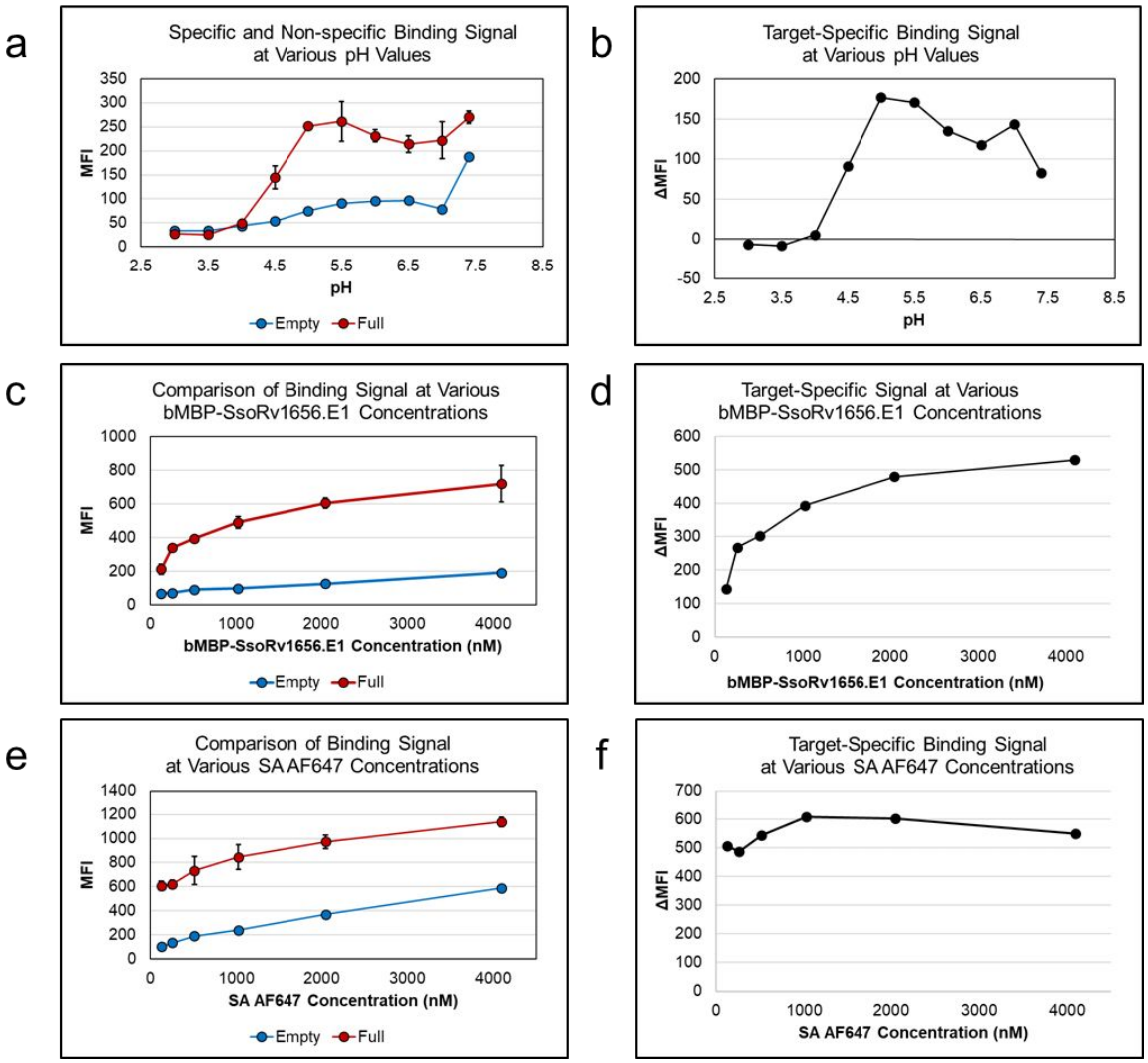

Figure S15: Parameter screens for Rv1656 paper-based assays. a) Scan of buffer pH used in bMBP-SsoRv1656.E1 incubation and wash steps. Various pH solutions were formulated using McIlvaine buffer. 30 μM SsoRv1656.E2-CBD; 256 nM Rv1656/bMBP-SsoRv1656.E1/SA AF647. Empty denotes assays contacted with 1x PBS rather than an Rv1656 solution. b) Difference between empty and full sandwiches, indicating target-specific binding signal. c) Scan of bMBP-SsoRv1656.E1 concentration using a pH 5 solution for incubation and wash steps. 30 μM SsoRv1656.E2-CBD; 256 nM Rv1656/SA AF647. Empty denotes assays contacted with 1x PBS rather than an Rv1656 solution. d) Difference between empty and full sandwiches, indicating target-specific binding signal. e) Scan of SA AF647 concentration using a bMBP-SsoRv1656.E1 concentration of 2.048 μM and a pH 5 solution for incubation and wash steps. 30 μM SsoRv1656.E2-CBD; 256 nM Rv1656, 2.048 μM bMBP-SsoRv1656.E1. Empty denotes assays contacted with 1x PBS rather than an Rv1656 solution. f) Difference between empty and full sandwiches, indicating target-specific binding signal. All samples were imaged in the Cy5 channel and exposed for 80 ms.

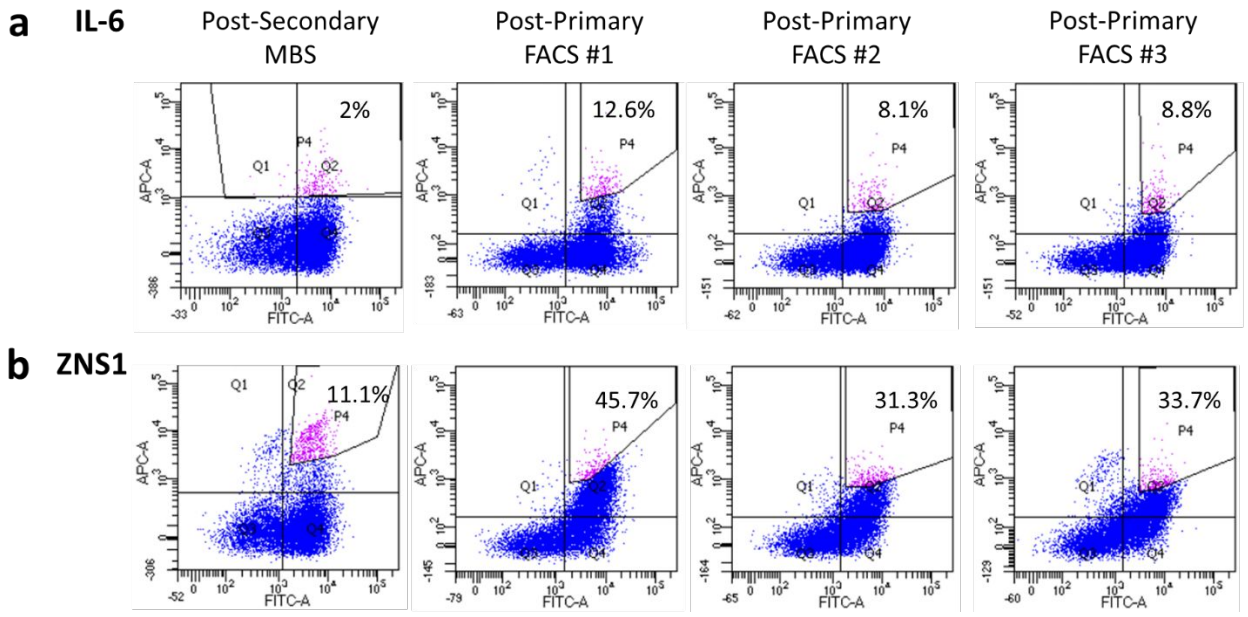

Figure S16: FACS plots analyzing various primary selection populations for potential secondary binding signal. a) The post-FACS populations during primary selection for SsoIL6.E1 after round #1, #2, and #3, which have been labeled with 100 nM IL-6 and 200 nM bMBP-SsoIL6.E1 to assess potential secondary binding signal. These plots are compared to the post-secondary MBS population (secondary FACS #1), which was used to identify the SsoIL6.E2 affinity reagent. Percentage in corner signifies percentage of cells with positive binding signal (top two quadrants). b) The post-FACS populations during primary selection for SsoZNS1.E1 after round #1, #2, and #3, which have been labeled with 100 nM ZNS1 and 200 nM bMBP-SsoZNS1.E1 to assess potential secondary binding signal. These plots are compared to the post-primary MBS population (secondary FACS #1), which was used to identify the SsoZNS1.E2 affinity reagent. Percentage in corner signifies percentage of cells with positive binding signal (top two quadrants). In both (a) and (b), a decently strong positive signal for secondary binding is still present even when using a more-enriched population from primary selection, signifying that it may be possible to identify secondary affinity reagents using this more enriched population in order to further reduce developmental time.