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Beyond Epitope Binning: Directed *in Vitro* Selection of Complementary Pairs of Binding Proteins

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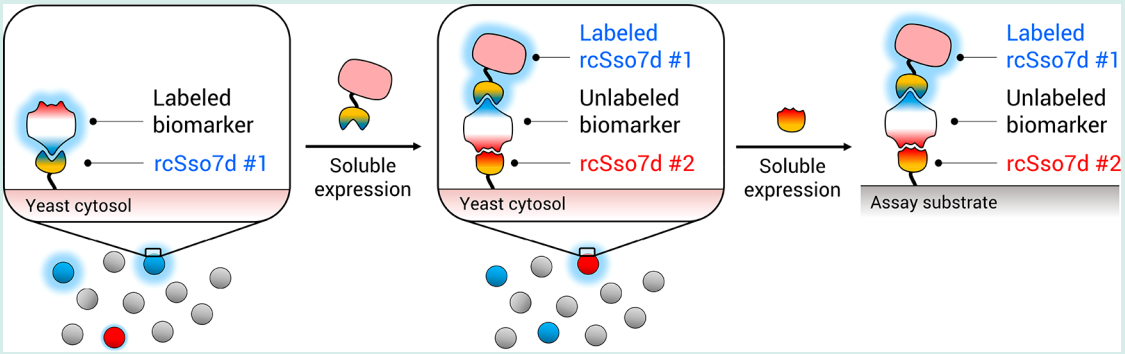
Supporting Information



ABSTRACT: Many biotechnological applications require the simultaneous binding of affinity reagents to nonoverlapping target epitopes, the most prominent example being sandwich immunoassays. Typically, affinity pairs are identified via *post facto* functional analysis of clones that were not selected for complementarity. Here, we developed the Rapid Affinity Pair Identification via Directed Selection (RAPIDS) process, which enables the efficient identification of affinity reagents that function together as complementary pairs, from *in vitro* libraries of ~10^9 variants. We used RAPIDS to develop highly specific affinity pairs against biomarkers of tuberculosis, Zika virus, and sepsis. Without additional trial-and-error screening, these affinity pairs exhibited utility in multiple assay formats. The RAPIDS process applies selective pressure to hundreds of thousands of potential affinity pairs to efficiently identify complementary pairs that bind to separate epitopes without binding to one another or nontargets, yielding diagnostic assays that are sensitive and specific by design.

KEYWORDS: *binding pairs, epitope binning, protein engineering, yeast surface display, sandwich immunoassay*

INTRODUCTION

Affinity reagents are essential to *in vitro* diagnostic tests, permitting the specific detection of protein biomarkers of disease in blood, saliva, urine, and other bodily fluids. These tests commonly employ a "sandwich" assay format, in which one substrate-immobilized affinity reagent captures the targeted biomarker from the bulk fluid, and a second affinity reagent associates a detectable signal with the captured target. This assay format spatially concentrates the target from a complex mixture and enhances specificity by requiring two independent molecular interactions for signal development.[1] However, this approach introduces a degree of complexity to the development of immunoassays. Concurrent binding to the target biomarker requires a complementary pair of affinity reagents, such that each binds with high affinity to sterically distinct parts of the target ("epitopes"). Furthermore, to avoid signal development in the absence of the target, the complementary pair of affinity reagents must not bind to one another.

In order to obtain pairs of complementary affinity reagents for *in vitro* diagnostic applications, binding variants that function in isolation are typically first developed and then later tested for simultaneous binding function in a low-throughput, pairwise fashion. Traditionally, IgG/IgM antibodies have been developed for these applications. This process involves immunizing a host animal (e.g., goat, mouse, chicken) to generate numerous antibodies against the target biomarker and using combinatorial screens to categorize these antibodies based on their epitope specificity ("epitope binning").[2−6] Though this process can yield functional pairs of affinity reagents, it is resource- and time-intensive (requiring 32−44 weeks), assesses a relatively small subset of potential affinity pairs (e.g., 55 potential pairs when assaying ten different clones and testing for self-complementary pairs), and does not impose directed selective pressure for binding pairs.[7−9] *In vivo*

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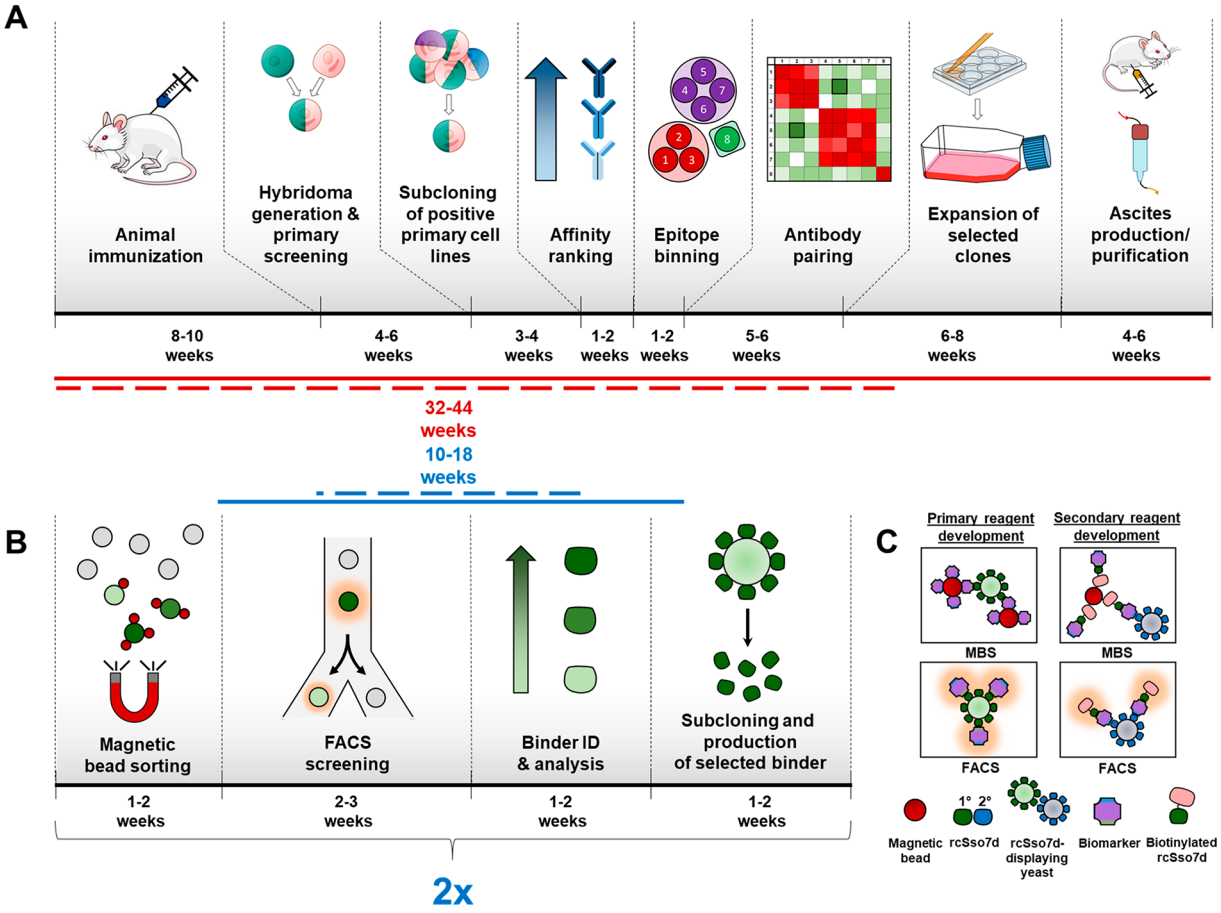

Figure 1. Comparison of traditional and RAPIDS processes. (A) Typical timelines and workflow for hybridoma-based development and production of antibody pairs. A host animal is immunized with the target analyte, and host splenocytes are harvested for the production of antibody-producing hybridomas. Target-specific hybridomas are subcloned to yield monoclonal populations, and clones are characterized and ranked by affinity. Selected antibodies are grouped by epitope specificity via sequential binding studies, and pairwise screening is used to identify complementary clones. Selected clones are expressed in a murine host via the generation and chromatographic purification of ascites fluid. (B) Typical timelines and workflow for the rapid affinity pair identification via the direct selection (RAPIDS) process. A naïve yeast surface display (YSD) library is screened *in vitro* via multiple rounds of magnetic bead sorting (MBS) and fluorescence activated cell sorting (FACS). Lead clones are subcloned and recombinantly expressed in a bacterial host. This primary binding variant is employed in secondary reagent development, following the same general selection scheme. (C) Schematic representation of binding complexes during primary and secondary reagent development, for both MBS and FACS.

processes also tend to target the most immunodominant epitope of the biomarker, and thus the likelihood of developing affinity reagents that compete for the same epitope is high. For example, one study found that out of 7,680 hybridomas produced, only 232 clones expressed target-specific antibodies, and 223 of these (96%) targeted the same biomarker epitope.[10] Furthermore, antibodies are prone to nonspecificity, irreproducibility, and lot-to-lot variation;[11,12] therefore, further validation must be conducted on identified antibodies to test for function in the desired diagnostic format.[13] *In vitro* selection processes represent an alternative means of engineering antibody or nonantibody affinity reagents by permitting the display of combinatorial libraries of randomized protein variants on the surface of organisms or carrier particles that harbor the associated coding DNA or RNA (e.g., phage, yeast, ribosomes).[14] After small sublibraries of affinity reagents are selected, pairwise screening can then be conducted to search for potential affinity pairs.[15−17] Both of these epitope-binning approaches require *post facto* classification of selected affinity reagents to ensure function in a chosen diagnostic format.

In recent years, researchers have worked to incorporate selective pressures for dual-epitope binding directly into the *in vitro* development process. One such approach is masked selection, in which a labeled version of the target biomarker is preincubated with a molar excess of a previously selected primary affinity reagent, and this complex is used to rescreen a library. This approach is intended to block the epitope bound by the primary affinity reagent, thus presenting only novel epitopes to potential secondary binding partners.[18,19] However, because masked selection depends upon a reversible, equilibrium binding process to prevent the selection of noncomplementary binding variants, this method does not guarantee development of a secondary affinity reagent that binds to a unique epitope of the biomarker.

Another proposed approach for identifying complementary binding pairs involves the display of randomized pairs of potential binding variants, which are expressed as tandem fusion partners. Stringent wash conditions are used to retain only those variants which are strongly bound to select for dual-epitope binding pairs.[20] However, this process may not always result in the selection of a complementary affinity pair, as a

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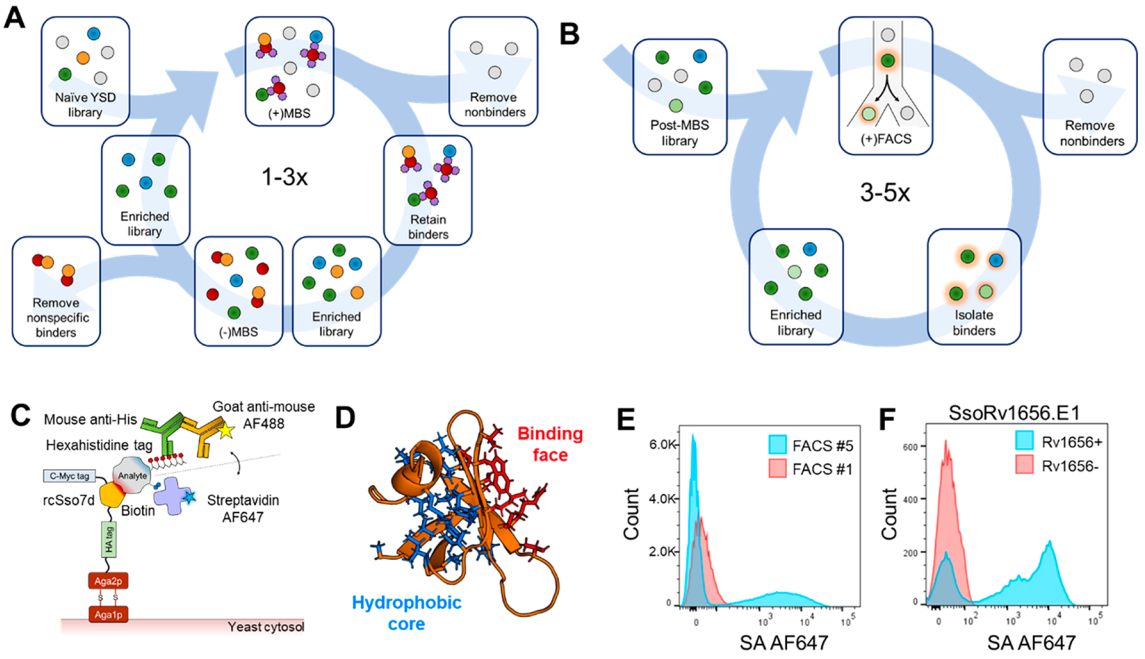

Figure 2. Schematic representation of the standard surface display selection process. (A) MBS schematic for a standard primary affinity reagent development process. Magnetic beads which have been precoated with the diagnostic biomarker are used to retain yeast displaying target-binding rcSso7d variants ("binders"). Negative selection is used to deplete the yeast library of off-target binding variants. (B) FACS schematic for a standard primary affinity reagent development process. Target-binding activity is indicated by the association of a fluorescent signal with rcSso7d-displaying yeast cells. (C) Schematic of the yeast surface display complex. rcSso7d—with flanking HA/c-Myc epitope tags to quantify display efficiency—is expressed as a genetic fusion to the Aga2p protein, which is exported to the exterior of the yeast cell and covalently tethered via a disulfide linkage to the Aga1p species in the cell wall. Target binding is queried via two orthogonal labeling schemes (using the hexahistidine tag or biotin label). (D) Protein ribbon structure of rcSso7d (PDB: 1SSO), highlighting the amino acids of the binding face (red), which is structurally isolated from the hydrophobic core (blue). Mutations are introduced in the red amino acids to generate functional diversity while minimizing potential detrimental impacts to scaffold stability. (E) FACS histograms of yeast sublibraries during the selection of primary affinity reagent SsoRv1656.E1. Histograms depict a significant increase in target-binding activity between selection rounds, with geometric mean intensity values of 6.78 (FACS #1) and 3,255 (FACS #5). (F) FACS histogram of target-specific binding for SsoRv1656.E1 (100 nM Rv1656).

selected pair is more likely to feature a single high affinity binding variant than two variants which bind the same target simultaneously. Desired epitopes can also be directly targeted via library screening against an isolated peptide or a mutated version of the biomarker.[21,22] However, the peptide-based approach may fail to recapitulate the biologically relevant epitope conformation of the native target, and both methods require pre-existing knowledge of the protein structure of the target.

Finally, library screening methods in the sandwich immunoassay format have been demonstrated in the context of phage-based biopanning. In this "sandwich panning" approach, surfaces (e.g., 96-well plates) are coated with previously selected affinity reagents, used to capture the desired target, and libraries of phages are screened for binding to available secondary epitopes.[23−25] However, these heterogeneous panning methods often lead to the identification of false-positive secondary binding candidates. In one sandwich panning study, only 3 of the 200 clones suspected to be secondary binding reagents were actually found to interact with a novel target epitope.[24]

Here, we present a novel approach called Rapid Affinity Pair Identification via Directed Selection (RAPIDS). The RAPIDS process consists of a flow cytometry-based *in vitro* method for 1) identifying a high-quality primary affinity reagent and 2) using this primary affinity reagent in the soluble phase to directly select for secondary affinity reagents that simultaneously bind to distinct epitopes of the target biomarker. By directly identifying pairs that function together during the selection process, the RAPIDS approach removes the trial-and-error aspect of traditional screening methods and allows for immediate validation of the affinity pair in an *in vitro* diagnostic format. Counterselection can also be incorporated to prevent the selection of cross-reactive or nonspecific affinity reagents.

In this study, we demonstrate the use of the RAPIDS approach for the directed development of three affinity pairs based on the rcSso7d scaffold protein.[26−29] We developed these pairs for the specific detection of 1) a urine-based tuberculosis antigen (Rv1656),[30] 2) a human cytokine (interleukin-6, IL-6),[31] and 3) a Zika virus antigen (non-structural protein 1, NS1) which shares significant protein sequence homology (51−53%) with a Dengue virus NS1 variant.[32] We validated the specificity and function of these affinity pairs in several *in vitro* contexts (flow cytometry, bead-based assays, well-plate ELISAs, and paper-based assays). With the RAPIDS process, we demonstrate efficiency, versatility, and increased control over the development of functional affinity pairs for use in diagnostic assays.

RESULTS AND DISCUSSION

The RAPIDS Method. To address the limitations of the most common process for developing complementary affinity pairs (e.g., animal immunization and epitope-binning) (Figure 1A), we developed the RAPIDS method. In contrast to the traditional development process for antibody pairs, the RAPIDS method requires only 10−18 weeks (Figure 1B)

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Table 1. Binding Face Amino Acid Sequences and Biophysical Parameters[a]

protein	binding face shorthand tag	k_{on} (1/(M s))	k_{off} (1/s)	K_d (M)
SsoRv1656.E1	SWRRWRWAK	2.3×10^5	7.6×10^{-5}	3.3×10^{-10}
SsoRv1656.E2	WRYYGSWKY	1.1×10^5	4.7×10^{-4}	4.2×10^{-9}
SsoIL6.E1	IGHWYWDNW	1.5×10^5	6.3×10^{-4}	4.2×10^{-9}
SsoIL6.E2	NYWHWDAYK	1.4×10^5	5.2×10^{-4}	3.7×10^{-9}
SsoZNS1.E1	SIKHWAWSK	1.5×10^5	1.6×10^{-4}	1.1×10^{-9}
SsoZNS1.E2	RYWIDGISWS	1.6×10^5	2.7×10^{-4}	1.7×10^{-9}

[a]Binding face shorthand tags show the variable binding face amino acid residues. Biophysical parameters (k_{on}, k_{off}, and K_d) for the selected affinity reagents were measured using biolayer interferometry (BLI).

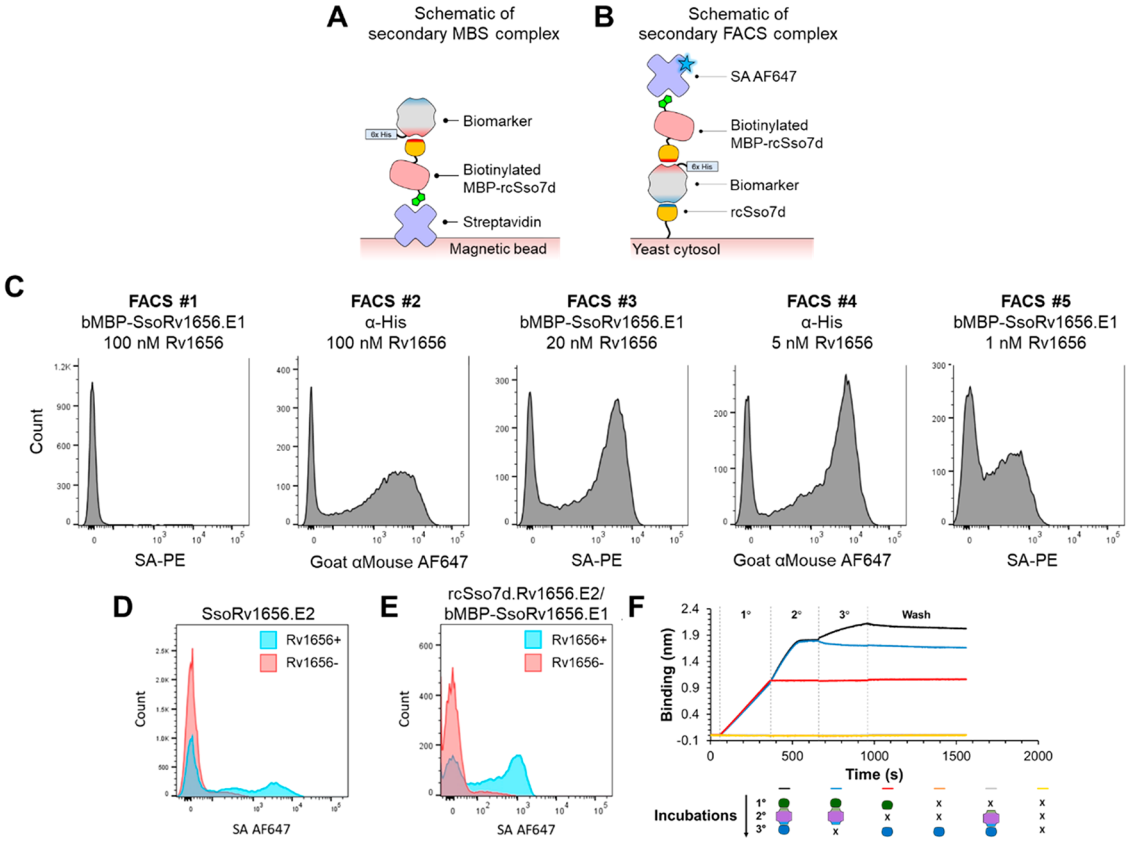

Figure 3. Overview of the secondary selection process: Identification of an affinity pair against TB Rv1656. (A) Schematic of the protein complex for secondary MBS. Oriented target display mediated by binding to the primary epitope (red) presents nonoverlapping epitopes (blue) for yeast sorting. (B) Schematic of the protein complex for secondary FACS. Fluorescent labeling of yeast cells is mediated by the simultaneous binding of the biotinylated primary rcSso7d clone to the primary target epitope (red) and the binding of the displayed rcSso7d variant to a novel epitope (blue). (C) FACS histograms for selection of the secondary affinity reagent SsoRv1656.E2. Target-specific binding activity increases throughout the process despite the decreasing target concentration. (D) FACS histogram of target-specific binding for SsoRv1656.E2 (100 nM Rv1656). (E) FACS histogram of target-specific binding for SsoRv1656.E2/bMBP-SsoRv1656.E1 in the full-sandwich immunocomplex (100 nM Rv1656, 100 nM bMBP-SsoRv1656.E1). (F) Sequential binding of bMBP-SsoRv1656.E1 (1°, 5 nM), Rv1656 (2°, 10 nM), and SsoRv1656-CBD (3°, 500 nM) assessed via biolayer interferometry (BLI). The latter three curves (orange, gray, and yellow) overlap with each other along the *x*-axis of the plot.

and permits the simultaneous screening of a large combinatorial library of potential binding pairs. This process consists of two distinct phases (Figure 1C). The primary development phase selects affinity reagents from a large combinatorial library, strictly based on target binding activity. In the secondary development phase, the selected primary rcSso7d clone (green) is used to apply an epitope-specific selective pressure to ensure selection of a secondary affinity reagent that targets a sterically distinct epitope on the target biomarker (blue). To enrich the population for cells bearing target-binding rcSso7d variants, the RAPIDS process employs magnetic bead sorting[33] (MBS; Figures 2A and S1) and fluorescence-activated cell sorting (FACS; Figures 2B and S2) to screen through a combinatorial yeast surface display library featuring randomized variants of a binding protein.[34]

Affinity Pair Development against the TB Biomarker Using RAPIDS. To demonstrate the RAPIDS process, we used the technique to select rcSso7d-based affinity reagents against the urine-based tuberculosis (TB) biomarker, Rv1656. We identified a high-affinity primary binding variant via standard yeast surface display (Figure 2C), screening through a combinatorial protein library as previously described.[35] In this process, we screened through a population of over 10^9 distinct clones with mutations in the structurally isolated binding face

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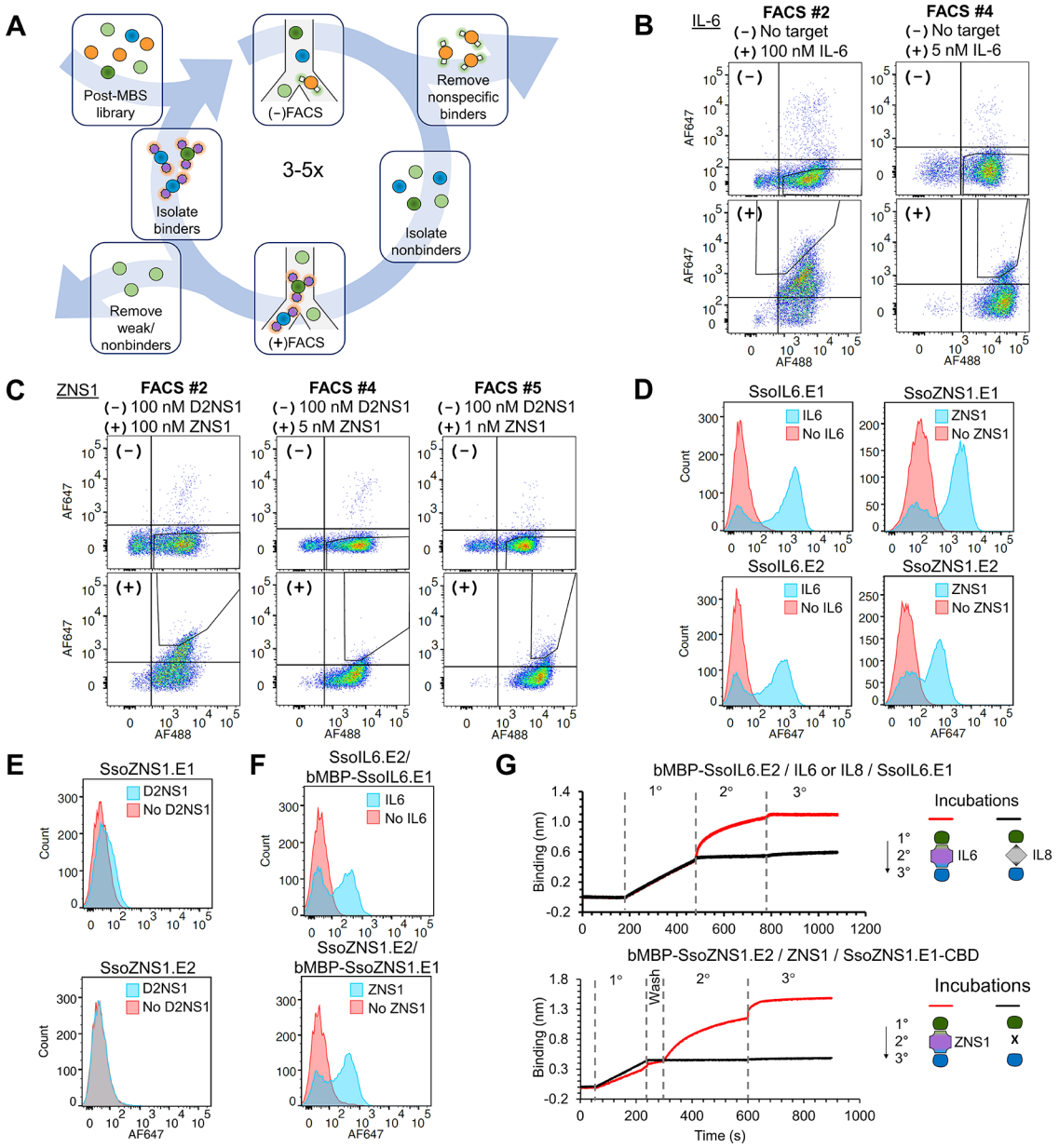

Figure 4. Generality of the RAPIDS approach: identification of affinity pairs against IL-6 and ZNS1. (A) FACS schematic including negative selections. The induced yeast sublibrary is first incubated with nontarget proteins and labeled with fluorescent reagents in order to collect cells with positive rcSso7d display but no off-target binding. The sorted cells are then immediately relabeled with the target and corresponding fluorescent reagents before a second sort to collect cells with a positive, target-specific binding signal. The isolated cell population is expanded through outgrowth, and the process is repeated. (B, C) Representative FACS plots for secondary affinity reagent selection against IL-6 from FACS #2 and #4 (B) and ZNS1 from FACS #2, #4, and #5 (C). The plots shown consist of those rounds of sorts using a negative sort (against bMBP-SsoIL6.E1/SA AF647 for IL-6 or D2NS1/bMBP-SsoZNS1.E1/SA AF647 for ZNS1) prior to a positive sort. Gates drawn indicate the gates used for sorting. (D) FACS histograms of target-specific binding with the target-positive sample (blue) and the target-negative sample (red). Histograms represent binding of the selected primary affinity reagents (SsoIL6.E1 and SsoZNS1.E1; top) or secondary affinity reagents (SsoIL6.E2 and SsoZNS1.E2; bottom) to their respective biomarkers (16 nM). (E) FACS histograms demonstrate minimal off-target binding of SsoZNS1.E1 and SsoZNS1.E2 to D2NS1 (100 nM). (F) FACS histograms of full-sandwich, target-specific binding for IL-6 (top) and ZNS1 (bottom) with rcSso7d.E2/bMBP-rcSso7d.E1 in a yeast surface display format. The target-positive sample (blue) was incubated with 16 nM of the biomarker, and the target-negative sample (red) indicates the absence of the target biomarker. (G) Sequential binding demonstration in a full-sandwich format using biolayer interferometry (BLI).

of the rcSso7d scaffold (red, Figure 2D).[27] rcSso7d-displaying yeast cells are incubated with a soluble, purified target biomarker, and target-specific binding activity can be assessed using fluorophore-conjugated labeling reagents. In order to avoid isolating clones that bind to the labeling reagents, two orthogonal sets of labeling reagents can be iteratively switched between sorting rounds (**1:** mouse anti-His IgG antibody/goat anti-mouse IgG antibody AF647; **2:** streptavidin (SA) AF647) (Figure 2C).

We observed significant enrichment of Rv1656-specific binding variants over the course of five rounds of FACS, noting a 480-fold increase in the target-specific binding signal

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within the rcSso7d-displaying yeast population (Figure 2E).[35] We sequenced a subset of the yeast sublibrary and observed multiple instances of a single clone within the enriched population (SsoRv1656.E1; E1: epitope targeted by the primary affinity reagent; Table 1; Figure S3). We validated the Rv1656-specific binding activity of this primary binding species in the yeast-surface display format (Figure 2F) and integrated this selected primary rcSso7d clone into a fusion construct (Figure S4) which enables its straightforward labeling and facile production in bacteria (via *in vivo* biotinylation of a fusion construct incorporating the maltose-binding protein (MBP) and the rcSso7d clone; referred to as bMBP-SsoRv1656.E1).[36,37]

To apply an orientation-specific selection pressure for the identification of a secondary rcSso7d clone, we coated magnetic beads with the primary rcSso7d clone, followed by the Rv1656 biomarker. This ensures preferential target orientation, such that only nonoverlapping epitopes are available for binding by a secondary affinity reagent. In this secondary MBS process, we sorted the Rv1656 yeast sublibrary resulting from the final round of primary MBS (Figure 3A; library size ~10^6).

Having used secondary MBS to enrich the library for binding variants specific to a secondary epitope, we employed secondary FACS to apply additional selective pressure for high-quality, complementary secondary affinity reagents (Figure 3B). The biotinylated primary rcSso7d clone bound to fluorophore-conjugated streptavidin was used as the labeling reagent in secondary FACS. Given that the signal is only observed when simultaneous binding occurs between 1) the rcSso7d clone displayed on the surface of the yeast cell, 2) the unlabeled target biomarker, and 3) the biotinylated primary rcSso7d variant, this scheme ensures that a fluorescent signal indicates binding to nonoverlapping epitopes on the biomarker. The secondary yeast library was sorted via five rounds of FACS, alternating between different labeling methods (**1:** bMBP-SsoRv1656.E1/streptavidin PE; **2:** mouse anti-His IgG antibody/goat anti-mouse IgG antibody AF647) (Figure 3C) to minimize enrichment of affinity reagents that cross-react with labeling reagents or the primary rcSso7d clone. In order to select those variants with the greatest affinity, the concentration of Rv1656 was also reduced over the course of this process, to a final concentration of 1 nM.

Upon sequencing a subset of the yeast sublibrary after the final round of FACS, we observed multiple instances of a single unique Rv1656-specific clone (SsoRv1656.E2; E2: epitope targeted by the secondary affinity reagent; Table 1; Figure S3). This rcSso7d variant demonstrated target-specific binding activity when challenged with the labeled biomarker in the yeast surface display format (Figure 3D). In order to demonstrate the target-specific binding signal in the "full-sandwich" immunocomplex format, yeast cells displaying SsoRv1656.E2 were sequentially incubated with the unlabeled Rv1656 biomarker, followed by bMBP-SsoRv1656.E1 and SA AF647 (Figure 3E). Full-sandwich complex formation was also confirmed via biolayer interferometry (BLI), via conjugation of biotinylated bMBP-SsoRv1656.E1 to streptavidin-coated fiber-optic tips, followed by incubation with Rv1656 and the secondary rcSso7d clone fused to a cellulose-binding domain (SsoRv1656.E2-CBD; Figure 3F). The identified affinity reagents demonstrated highly preferential binding to Rv1656 relative to other recombinant TB biomarkers (Figure S5).[38] We also determined the biophysical binding constants (k_{on}, k_{off}, and K_d) for the Rv1656-specific complementary affinity pair (Table 1; Figure S6).

Generality of the RAPIDS Approach: Affinity Pair Selection against Zika Virus NS1 and IL-6. We next sought to assess the generality of the RAPIDS process by developing pairs of affinity reagents against two additional soluble biomarkers: human interleukin-6 (IL-6) and Zika virus nonstructural protein 1 (ZNS1). IL-6 is a proinflammatory cytokine and an anti-inflammatory myokine, and it is often used as an indicator of infection, tissue injury, and other inflammatory responses.[31] ZNS1 is secreted into the blood-stream by cells infected by the Zika virus and has been targeted as the diagnostic biomarker.[32]

In order to ensure binding specificity of the selected affinity reagents and to demonstrate flexibility of the RAPIDS process to meet relevant design criteria for different affinity applications, we incorporated additional negative selection steps into the selection process for these two targets. These counterselection steps were particularly important when developing affinity reagents against Zika NS1, given its sequence and structural homology to other flavivirus NS1 variants (e.g., Dengue NS1). To remove binding variants that demonstrate off-target binding activity, we introduced a two-step counterselection process into the RAPIDS scheme (Figure 4A). First, the yeast sublibrary is labeled with nontarget proteins, and cells that bind to these proteins are discarded. The retained yeast population is then immediately relabeled with the target protein and is re-sorted via FACS to collect the population fraction which demonstrates analyte-specific binding activity. This two-step process can be interspersed between traditional FACS rounds as needed, based on the observed level of nonspecific binding activity within the sublibrary. We chose Dengue virus type 2 nonstructural protein 1 (D2NS1) to use for counterselections in the affinity reagent development process for ZNS1. To ensure enrichment of secondary rcSso7d clones that do not cross-react with the selected primary rcSso7d affinity reagents, we also included negative selections against these primary rcSso7d clones during the RAPIDS process for both IL-6 and ZNS1.

We used RAPIDS to develop complementary pairs of rcSso7d-based affinity reagents against IL-6 (SsoIL6.E1/E2) and ZNS1 (SsoZNS1.E1/E2) (Table 1; Figures S3, S7, and S8), incorporating negative selections as outlined above. Consistent with previous studies, we observed significant enrichment of aromatic residues within the binding faces of these selected variants.[27] Representative FACS plots from the secondary negative selection sorts (Figure 4B,C) demonstrate reduction of off-target or cross-reactive binding in subsequent sublibraries. Following the primary selection process, we identified seven unique IL-6-specific clones within two groups of similar sequences varying by only one or two amino acid differences ("subfamilies") and one ZNS1-specific subfamily (Figure S9). Initial analysis of the IL-6 clones within the different subfamilies did not result in the identification of complementary affinity pairs (Figure S10). The rcSso7d clone with the highest binding affinity was selected as the primary affinity reagent for each biomarker (SsoIL6.E1 and SsoZNS1.E1). During the secondary selection process, we identified one unique clone against IL-6 (SsoIL6.E2) and one unique clone against ZNS1 (SsoZNS1.E2) (Table 1; Figure S3). Though ZNS1 is a hexameric protein, neither SsoZNS1.E1 nor SsoZNS1.E2 demonstrated appreciable binding to a multivalent epitope of the NS1 biomarker (Figure

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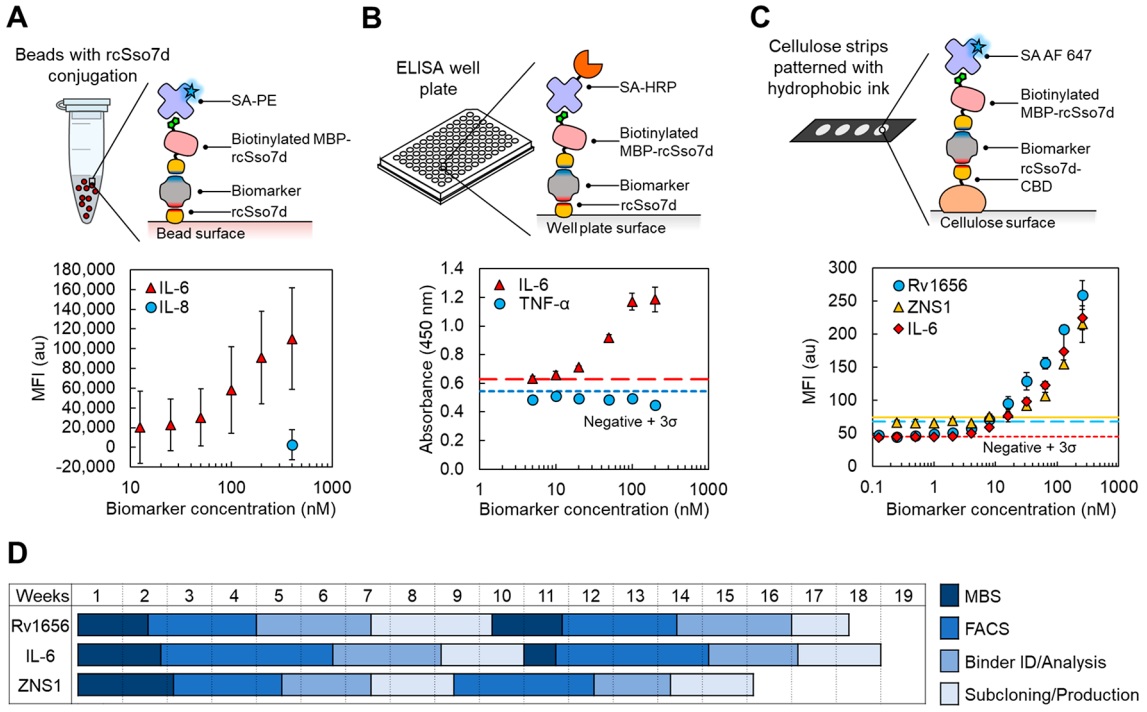

Figure 5. Functionality of affinity pairs in *in vitro* assay formats. (A) Schematic of bead-based assay using beads conjugated with SsoIL6.E1 (above). Beads were incubated with the target (IL-6; red triangles) or the nontarget protein (IL-8; blue circles), followed by bMBP-SsoIL6.E2 and SA-PE (R-phycoerythrin). Flow cytometry was used to measure the resulting fluorescence (below). Each data point represents the mean fluorescence intensity (MFI) from one experiment, and error bars signify the standard deviation of the beads within each sample. (B) Schematic of well-plate ELISA (above). SsoIL6.E1 was coated onto polystyrene wells, followed by incubations of IL-6 (red triangles) or the nontarget protein TNF-α (blue circles). A signal was generated using bMBP-SsoIL6.E2 and SA-HRP (horseradish peroxidase). Absorbance measurements are shown at 450 nm (below). Dotted lines represent the background signal in the absence of the biomarker. Each data point consists of an average of two replicates, and error bars signify standard deviation. (C) Schematic of paper-based assay using cellulose strips patterned with hydrophobic ink (above). An rcSso7d clone in the rcSso7d-CBD fusion construct is immobilized on the cellulose surface, followed by the target biomarker (Rv1656, blue circles; ZNS1, yellow triangles; and IL-6, red diamonds), and labeled with its complementary rcSso7d clone in the bMBP-rcSso7d fusion construct and SA-AF647. MFI is shown for each affinity pair (below). Dotted lines represent the background signal in the absence of the biomarker. Each data point consists of an average of four replicates, and error bars signify standard deviation. (D) Developmental timelines for all three pairs of binding proteins using the RAPIDS process.

S11), indicating the need for a pair of distinct affinity reagents for ZNS1.

In order to confirm target-specific binding for the selected affinity reagents, we challenged the clonal E1 and E2 yeast cultures with their respective target biomarkers (IL-6 or ZNS1). A strong binding signal was observed following incubation with the respective targets (Figure 4D). SsoZNS1.E1 and SsoZNS1.E2 also showed minimal off-target binding to D2NS1, demonstrating specificity of the selected affinity reagents (Figure 4E). Additionally, SsoIL6.E1 and SsoIL6.E2 both demonstrated specificity to IL-6 over another cytokine, IL-8 (Figure S12). To confirm separate epitope binding of the identified affinity reagents, we conducted full-sandwich binding experiments. Yeast cells displaying the E2 variant were sequentially incubated with the biomarker and the bMBP-rcSso7d.E1 fusion construct, followed by fluorophore-conjugated streptavidin for labeling. The binding pairs demonstrated strong target-specific binding activity and also showed minimal cross-reactivity to one another in the absence of the target (Figure 4F). To further validate separate epitope binding, we used BLI to assess the sequential binding activity of the soluble protein species in the full-sandwich format (Figure 4G). We also measured the biophysical binding parameters (k_{on}, k_{off}, and K_d) for the selected complementary affinity pairs for IL-6 and ZNS1 (Table 1; Figure S13).

Incorporation of Affinity Pairs into Test Formats. In order to assess the utility of the identified affinity pairs for *in vitro* applications, we sought to incorporate the selected affinity reagents into three common binding assay formats: bead-based assays, well-plate enzyme-linked immunosorbent assays (ELISA), and paper-based immunoassays. To assess rcSso7d functionality in a bead-based assay format, we conjugated SsoIL6.E1 to carboxyl polystyrene beads, challenged these beads with various concentrations of human IL-6 or a representative nontarget cytokine (human IL-8), and labeled the beads using bMBP-SsoIL6.E2 in complex with streptavidin R-phycoerythrin (SA-PE). Bead fluorescence was quantified using flow cytometry. The results indicate a clear increase in the fluorescence signal with increasing concentration of IL-6 and demonstrate a lack of signal in the presence of nontarget IL-8. (Figure 5A) These findings demonstrate the specificity and functionality of the identified binding pair in a bead-based assay format. Bead-based performance was also assessed using a plate reader to quantify fluorescence for suspensions of magnetic beads (Figure S14).

Next, the IL-6 assay was implemented in a polystyrene 96-well plate to demonstrate applicability for traditional well-plate ELISA. We coated the polystyrene surfaces of the wells with SsoIL6.E1, followed by various concentrations of human IL-6 or another representative nontarget cytokine (TNF-α; tumor

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necrosis factor α) to challenge the affinity pair against another off-target protein. We then used bMBP-SsoIL6.E2 to label the captured biomarker, followed by streptavidin-HRP (horseradish peroxidase). The SsoIL6 affinity pair detected the presence of IL-6 with no discernible positive signal when challenged with tumor necrosis factor alpha (TNF-α) (Figure 5B), further demonstrating specificity and applicability of the selected rcSso7d pair in an ELISA format.

Lastly, the target-specific binding activity of the affinity pairs was assessed in a paper-based assay format, using hydrophobic ink to delineate circular hydrophilic wells on cellulose strips. For each pair of affinity reagents, one clone was incorporated into a cellulose-binding domain (CBD) fusion construct for high-density protein immobilization on cellulose.[35] After applying various concentrations of antigen to the paper wells, we used the other rcSso7d clone in the bMBP-rcSso7d format to label the captured biomarker. All three pairs showed an increased signal with increasing biomarker concentration (Figures 5C and S15), demonstrating utility in a paper-based assay format.

Assessment of Development Timeline. The detailed development timelines for all three pairs of binding proteins indicate that the RAPIDS process was consistently completed within 18 weeks for each target biomarker (Figure 5D). In order to shorten this development process, we eliminated the additional MBS round(s) for ZNS1 secondary affinity reagent selection and instead started immediately with secondary FACS using the post-primary MBS yeast sublibrary. It may be possible to further shorten the RAPIDS process timeline, as discussed below (Figure S16).

DISCUSSION

Traditional methods for the development of complementary affinity reagents rely on time-intensive, low-throughput pairwise screening approaches. This process typically involves selection of affinity reagents solely on the basis of target-specific binding activity. However, diagnostic applications feature stringent design criteria (e.g., minimal cross-reactivity or off-target binding, and function in the desired assay format), and thus additional screening of selected affinity reagents must often be conducted *post facto*. Here, we developed a novel process for the directed selection of affinity pairs in a fully *in vitro* platform. This RAPIDS process employs a stringent, pair-specific selection pressure, which ensures that binding variants are selected solely on the basis of complementary binding to nonoverlapping biomarker epitopes. This process also enables the selection of affinity reagents specific to less immunodominant epitopes which might not be targeted by *in vivo* development methods.

With this *in vitro* platform, we can screen the full diversity of a combinatorial library—or a sublibrary previously enriched for target-specific binding clones—to explore all potential candidate pairs, rather than the limited subset typically tested with traditional methods like epitope binning. We demonstrated the applicability and generality of the RAPIDS method by generating complementary affinity pairs against three different biomarkers (tuberculosis Rv1656, human IL-6, and Zika virus NS1), each within an 18-week development timeline. The RAPIDS process can be leveraged to identify affinity reagents that function together as a pair in a relevant diagnostic format without extensive screening through sublibraries dominated by false-positive secondary binding candidates. These selected reagents can be designed for limited cross-reactivity with their complementary affinity reagent (enabled via counterselections using the primary affinity reagent) and can be designed to discriminate between highly homologous target biomarkers (enabled via counterselections using off-target proteins, e.g., Dengue 2 NS1). Compatibility of the RAPIDS process with counterselection methods is particularly critical for the production of multiplexed assays, in which cross-reactivity would yield significant background noise and assay cross-talk.[39] We also demonstrated that the functionality of the selected affinity pairs is not assay-dependent and is generalizable to a variety of different formats, including yeast surface display, bead-based assays, well-plate ELISAs, and paper-based assays.

The RAPIDS process may be further optimized to reduce the timeline required to develop affinity pairs. For instance, the yeast sublibrary used to initiate the secondary selection process can be modified, while still screening the library diversity required for the identification of high-quality affinity reagents. During magnetic bead sorting (MBS), avid binding of the multivalent yeast cells to the target-coated beads ensures retention of all target-specific binding variants, regardless of binding affinity or epitope specificity.[33] By conducting an additional, orientation-specific magnetic bead sort at the start of the secondary selection process, an early selective pressure is applied to enrich the population for affinity reagents specific to a novel, secondary epitope. However, this additional MBS round can be omitted to instead start the secondary selection process with secondary FACS (as demonstrated during the ZNS1 development process). The timeline can potentially be further compressed by starting the secondary selection process using a more enriched sublibrary from the primary selection process (e.g., after primary FACS #1). However, care must be taken to ensure that the selected starting sublibrary has not been overenriched for binding variants specific to the primary, immunodominant target epitope, in which case secondary affinity reagents would be scarce or absent.

The development timeline can also be shortened using methods which enable the production of soluble affinity reagents directly from the yeast surface display format.[40,41] This would permit the omission of primary binding variant subcloning, bacterial expression, and protein purification steps. Process throughput can be significantly enhanced by shortening the affinity pair development timeline, allowing protein engineers to rapidly address novel protein biomarkers with lower capital requirements and reduced trial-and-error.

The RAPIDS process is generalizable, as it can be applied in additional display platforms (e.g., phage, ribosome)[14] and with other scaffold proteins (e.g., nanobodies, DARPins, anticalins, affibodies, fibronectins).[42−46] The development scheme can also be adapted for the selection of a hybrid affinity pair, employing a well-validated antibody as the primary affinity reagent in order to select for a complementary affinity reagent. In addition, since the RAPIDS approach uses *in vitro* selection processes, the selections can be conducted in relevant fluid samples (e.g., serum, urine) to ensure that the identified affinity reagents recognize the target biomarker in its native format. Furthermore, the RAPIDS process has the potential to be used in nondiagnostic application contexts that require multiepitope binding affinity reagents, such as therapeutic bispecific antibodies[47,48] and high-affinity, heterodimeric binding reagents which avidly bind a single target.[49−51] The RAPIDS scheme enables the facile identification of complementary affinity reagents via the application of a directed

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selective pressure, and thus this technique may support many different affinity reagent development processes within the biotechnology industry.

The identification of complementary pairs of affinity reagents is a critical capability for immunoassay development, and the RAPIDS technique enables the efficient screening of hundreds of thousands of pairwise interactions to yield clones that function together with improved functionality and performance, relative to those isolated via traditional methods. We envision that this rapid development platform will accelerate affinity pair discovery efforts throughout the biomedical field, leading to the development of highly sensitive and specific immunoassays which address unmet medical needs.

MATERIALS AND METHODS

A detailed description of all materials and methods can be found in the Supporting Information. A brief version is included below.

Recombinant Protein Production. All recombinant proteins were expressed in BL21(DE3) *E. coli* (IPTG induction at 20 °C for 18−20 h) and purified using Ni-NTA immobilized metal affinity chromatography (IMAC), as previously described and outlined in the Supporting Information.[29,37]

Magnetic Bead Sorting. The combinatorial yeast surface display library was prepared as previously described.[27] Primary magnetic bead sorting (MBS) was conducted as previously described and detailed in the Supporting Information.[29] For the ZNS1 selection process, negative MBS was conducted by immobilizing the off-target protein (i.e., D2NS1 for ZNS1 selection processes) on the magnetic beads, incubating the beads with the yeast library, and disposing of any cells bound to the beads.

Secondary MBS was conducted in a similar fashion and was initiated using the yeast sublibrary resulting from either the second or third round of primary MBS. Prior to a round of positive secondary MBS, one to three rounds of negative MBS were conducted. Negative MBS was conducted by incubating the bMBP-rcSso7d.E1 species with biotin binder Dynabeads on a rotary mixer at 4 °C for at least 2 h. Negative selection beads were washed and then incubated with the induced yeast library at 4 °C for at least 2 h, after which any bead-bound cells were discarded. For positive secondary MBS sorts, the bMBP-rcSso7d.E1 species was immobilized on the magnetic beads as outlined above. Following a wash step, the beads were incubated with the unbiotinylated biomarker on a rotary mixer at 4 °C for at least 2 h. After additional washes, the beads were incubated with the induced yeast cells on a rotary mixer at 4 °C for at least 2 h. Finally, unbound yeast cells were discarded, and the retained beads and yeast were inoculated into fresh SDCAA medium for library outgrowth.

No additional rounds of MBS were conducted prior to FACS for the ZNS1 secondary selection process.

Flow Cytometry. Yeast populations were prepared for flow cytometry as previously described.[29,52] The soluble biomarker concentration was decreased over subsequent sorting rounds to apply increasing selective pressure for higher affinity reagents. Rv1656 primary FACS selection was conducted by alternating between different labeling reagents to prevent off-target binding, as previously described and outlined in the Supporting Information.[35,37] Rv1656 secondary FACS selection also alternated between two different labeling modes (**1:** bMBP-SsoRv1656.E1/SA-PE to select for affinity reagents that bind to nonoverlapping epitopes; **2:** mouse anti-His IgG/goat anti-mouse IgG AF647 to reduce off-target binding).

IL-6 primary selection also employed alternating labeling reagents. For secondary selection, all sorts were conducted with bMBP-SsoIL6.E1/SA AF647 for labeling to ensure selection of complementary affinity reagents. To remove nonspecific or cross-reactive clones, negative selections were conducted immediately prior to the second and fourth rounds of FACS by labeling the induced yeast population with only bMBP-SsoIL6.E1/SA AF647. Cells that did not display a binding signal were collected and were then relabeled with IL-6 and screened again for secondary binding activity.

ZNS1 primary selection involved negative selections against D2NS1 and the labeling reagents to reduce selection of off-target affinity reagents. For secondary selection, all sorts were conducted with bMBP-SsoZNS1.E1/SA AF647 for labeling, with negative selections using biotinylated D2NS1, bMBP-SsoZNS1.E1, and SA AF647 to remove off-target or cross-reactive clones.

Affinity Reagent Characterization and Validation. After the final FACS library selection, the remaining yeast subpopulation was sequenced to determine the population diversity, and selected clones were characterized, as described previously.[29] Selected variants were cloned into pET28b(+) bacterial expression plasmids, following protocols identified previously.[29,35,37]

Kinetic analysis was conducted using the ForteBio's Octet RED96 Bio-Layer Interferometry platform, using Streptavidin (SA) sensor tips. Additional details, including information on the sequential binding assays, are outlined in the Supporting Information.

Paper-based assays were developed following a similar protocol as previously described.[35,53] The complementary bMBP-rcSso7d variant and SA AF647 were used to label the captured biomarker in full-sandwich assays. Fluorescence microscopy was used to quantify biomarker binding, as described previously.[29]

Bead-based assays were conducted by conjugating SsoIL6.E1 to carboxylated polystyrene beads. SsoIL6.E1-conjugated beads were incubated with various concentrations of IL-6 or IL-8 for 1 h, followed by sequential incubations with bMBP-SsoIL6.E1 for 1 h and SA-PE for 30 min. Flow cytometry was used to quantify the resulting binding signal.

Well-plate ELISAs were conducted by immobilizing SsoIL6.E1 on flat-bottom 96-well polystyrene plates overnight. After blocking, wells were contacted with different concentrations of IL-6 or TNF-α for 2 h, followed by sequential incubations with bMBP-SsoIL6.E2 and SA-HRP. A signal was generated by contacting developed wells with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, and this signal was measured using a plate reader.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.9b00176.

Glossary of terms; materials and methods for RAPIDS process, affinity reagent characterization, and assay development; gene and protein sequences; schematics of binding events; SDS PAGE analysis of recombinant

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proteins; supplementary flow cytometry, biolayer interferometry, and interfacial binding assay data (PDF)

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Author Contributions

▽E.A.M. and K.-J.S. contributed equally and are listed alphabetically. E.A.M., K.-J.S., and H.D.S. conceived the study. E.A.M. and K.-J.S. conceptualized, executed, and optimized the RAPIDS process. E.A.M. and K.-J.S. performed experiments to select affinity reagents, characterize clones, implement paper-based assays, and analyze the resulting data. P.K. designed bead and well plate-based assays. With direction from P.K., H.Q.A.-Y. conducted BLI experiments with IL-6, V.T. conducted well-plate ELISA, Y.T. conducted bead-based experiments, and each analyzed the resulting data. K.P.-Y. expressed and purified IL-6 affinity reagents. S.B. and F.M.K. assisted with the affinity reagent selection process. I.G.K. contributed to production of a biomarker. E.A.M. and K.-J.S. wrote the manuscript with input from H.D.S.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

RAPIDS, rapid affinity pair identification via directed selection; ZNS1, Zika nonstructural protein 1; D2NS1, Dengue 2 nonstructural protein 1; IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; MBS, magnetic bead sorting; FACS, fluorescence activated cell sorting; BLI, biolayer interferometry; AF488, Alexa Fluor 488; AF647, Alexa Fluor 647; SA-PE, streptavidin R-phycoerythrin; MBP, maltose-binding protein; CBD, cellulose-binding domain; bMBP, biotinylated maltose-binding protein; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal affinity chromatography

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