High-Throughput Discovery of Aptamers for Sandwich Assays

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ABSTRACT: Sandwich assays are among the most powerful tools in molecular detection. These assays use "pairs" of affinity reagents so that the detection signal is generated only when both reagents bind simultaneously to different sites on the target molecule, enabling highly sensitive and specific measurements in complex samples. Thus, the capability to efficiently screen affinity reagent pairs at a high throughput is critical. In this work, we describe an experimental strategy for screening "aptamer pairs" at a throughput of 10^6 aptamer pairs per hour—which is many orders of magnitude higher than the current state of the art. The key step in our process is the conversion of solution-phase aptamers into "aptamer particles" such that we can directly measure the simultaneous binding of multiple aptamers to a target protein based on fluorescence signals and sort individual particles harboring aptamer pairs via the fluorescence-activated cell-sorter instrument. As proof of principle, we successfully isolated a high-quality DNA aptamer pair for plasminogen activator inhibitor 1 (PAI-1). Within only two rounds of screening, we discovered DNA aptamer pairs with low-nanomolar sensitivity in dilute serum and excellent specificity with minimal off-target binding even to closely related proteins such as PAI-2.

Sandwich assays such as the enzyme-linked immunosorbent assay (ELISA) are widely used in biomedical research and clinical diagnostics because they enable highly sensitive and specific detection of target molecules even in complex and heterogeneous samples. The sensitivity and specificity of the ELISA relies on "antibody pairs" that bind to two different binding sites on the target molecule, such that the detection signal is generated only when both antibodies bind simultaneously. Finding suitable reagent pairs is the most important step in developing sandwich assays; however, it is extremely challenging to generate monoclonal antibody pairs using conventional antibody discovery techniques (such as the hybridoma process). This is because antibodies are usually generated toward the most immunogenic site on the target protein (often called the "hot spot"), while other antibodies toward other sites, which are necessary to create the antibody pairs, are far more rare. Thus, researchers must generate and screen a large number of clones in the hopes of finding suitable monoclonal antibody pairs; this process is time-consuming, expensive, and prone to failure. For these reasons, polyclonal antibodies are often used and monoclonal antibody pairs are currently available for a very small fraction of the human proteome. Thus, there is considerable interest in using alternative reagents to replace antibody pairs.

Aptamers offer an appealing alternative to antibodies because they are chemically synthesized, renewable, and can be manufactured in an economical and highly reproducible manner. Furthermore, aptamers are discovered via in vitro selection, which provides avenues for circumventing the "hot spot" problem that plagues the discovery of monoclonal antibody pairs. Assays such as the enzyme-linked aptasorbent assay (ELASA) and the enzyme-linked oligonucleotide assay (ELONA) prove that aptamers can be successfully incorporated into standard assay formats, and a number of innovative methods have been reported for generating novel aptamer pairs. For example, Shi et al. reported a two-step process in which one aptamer is used to mask a domain of the target protein, allowing the selection of aptamers that bind to other sites on this protein. More recently, Ochsner et al. developed a method to generate aptamer pairs where the capture and detection aptamers were selected with distinct modified nucleotide chemistries in order to target different epitopes of the same target. Additionally, a Luminex platform was used to screen sandwich assays by simultaneously testing multiple bead-conjugated capture aptamers, with each aptamer identifiable through the use of a different bead color. In another approach, our group identified aptamer candidates from a selection against angiopoietin-2 by using high-throughput sequencing. These sequences were then used to synthesize custom aptamer arrays; using a known target-binding aptamer, we were able to discover DNA—aptamer pairs for the angiopoietin-2 protein. While these works show that aptamers can rival the specificity and sensitivity of antibodies for sandwich assays, the discovery
of aptamer pairs remains fairly low throughput, which ultimately limits their utility as affinity reagents.

The key to successfully implementing aptamer pairs as useful affinity reagent pairs is the ability to screen a large number of candidate pairs at a high throughput. In this work, we report a methodology that can screen more than $10^6$ aptamer pairs per hour—many orders of magnitude higher than the current state of the art (Figure 1). Our method (called “particle display of aptamer pairs” or PDAP) allows us to directly measure the simultaneous binding of individual aptamers as pairs (based on fluorescence signals) and sort them using a fluorescence-activated cell sorter instrument (FACS). As proof of principle, we performed a PDAP screen for the plasminogen activator inhibitor-1 (PAI-1), an important biomarker of thrombosis and atherosclerosis. In only two rounds of PDAP screening we discovered the first DNA aptamer pair for PAI-1 that enables highly sensitive detection in diluted serum in a sandwich assay format. This aptamer pair is highly specific with minimal off-target binding, even to closely related proteins such as PAI-2. PDAP offers many advantages over previous methods because it does not require labeling of the target protein, which makes it generalizable for a wide range of targets. In addition, the screening process is performed in complex samples (such as diluted serum) so that the resulting aptamer pairs can be used directly in a sandwich assay format without further modifications. Finally, although a FACS is not standard lab equipment, it is becoming more widely available, making our method accessible to many laboratories.

**MATERIALS AND METHODS**

**PDAP Screening Process.** We obtained our initial “pre-enriched” pool as previously described. Briefly, this process consisted of one round of conventional selection with the target on magnetic beads followed by two rounds of particle display to obtain the pre-enriched pool used here.

For our PDAP screens, we employed a known, 5′-end Quasar 670 fluorophore-labeled high-affinity PAI-1 aptamer as a detection aptamer. Round 1 of PDAP was performed with the pre-enriched pool in PBSMCT (PBS at a final MgCl$_2$ concentration of 1.5 mM) at a concentration of approximately 20 000 beads/μL. The beads were sonicated to avoid aggregation, then incubated with 50 nM His-tagged PAI-1 (Millipore) containing 0.1% fetal bovine serum (FBS) and 5 μM free His-tag peptide (GenScript). The free His-tag peptide was added to eliminate aptamers recognizing the His tag on the PAI-1 protein. After the initial target incubation for 1.5 h, the pre-enriched pool on beads was separated from the unbound target by using a magnetic rack. The target-bound pre-enriched aptamer pool was then incubated with 50 nM of Q670-labeled detection aptamer for 30 min. The aptamer candidate pool was then resuspended in PBSMCT buffer.

Following target incubation then labeling with the detection aptamer, we sorted particles that contained high fluorescence. We established a reference gate by running unlabeled aptamer particles (in the absence of target and the detection aptamer) through the cell sorter. We sorted in the aptamer pairs gate, which was a gate established to collect all particles with fluorescence greater than that shown in the reference gate. We collected 7400 events for the next round using a BD FACS Aria I cell sorting instrument. We then eluted the detection aptamer from the isolated magnetic beads by heating the bead solution to 95 °C for 5 min followed by sonication of the beads. The beads were separated from the solution using a magnetic rack, and the supernatant was carefully removed. Aptamer candidates were resuspended in water and then amplified by polymerase chain reaction (PCR) under the following conditions: 95 °C for 2 min, followed by 20 cycles of 95 °C for 15 s, 59 °C for 30 s,
and 72 °C for 45 s. Following PCR, the DNA was purified and quantified with a NanoDrop spectrophotometer. Emulsion PCR was used to generate monoclonal particles for an additional round of PDAP screening, in which the concentration of PAI-1 was lowered to 25 nM and the amount of FBS was increased to 0.2%. Under these conditions, we collected 10^300 events. The aptamer candidates isolated in round 2 were then amplified as previously described for 21 cycles in preparation for cloning and sequencing. Negative control reactions with 0 nM PAI-1 were carried out for each round of screening to evaluate nonspecific interactions between the detection aptamer and the aptamer particles. An Accuri flow cytometer was used to evaluate the progress of each round of screening.

The round 2 pool was cloned using a TOPO TA cloning kit (Life Technologies) into a plasmid vector. The vectors were then transformed into chemically competent Escherichia coli cells. The cells were grown overnight on kanamycin-treated plates at 37 °C, after which individual colonies were picked and sent for Sanger sequencing by Genewiz.

### RESULTS AND DISCUSSION

**PDAP Overview.** The key step in the PDAP screen is the conversion of solution-phase aptamers into “aptamer particles” through the emulsion PCR process as previously reported by our group (Figure 1, step 1). Using this process, each aptamer particle displays ∼10^5 copies of a single aptamer sequence on the surface of a polymer bead. We designed an aptamer library with 60 randomized nucleotides flanked by a pair of 20-nucleotide primer-binding sites (see Supporting Information for sequences). After incubating these aptamer particles with the target protein (step 2), we add a fluorescently labeled detection aptamer that is known to possess high affinity for the target (step 3). In this work, we used an aptamer previously reported by our group that binds to PAI-1 with an equilibrium dissociation constant (K_D) of 339 pM.

At this point the aptamer-coated beads can be interrogated individually for fluorescence intensity. Particles displaying a “capture aptamer” that can pair with the detection aptamer will bind to the target at a site other than that bound by the detection aptamer. This leaves the detection aptamer’s binding site open and results in a highly fluorescent aptamer particle (step 4 inset, right). In contrast, if the detection aptamer and the aptamer particle do not form a pair (either because the aptamer particle does not recognize the target or recognizes the same site as the detection aptamer), the aptamer particle will show low fluorescence and will be discarded (step 4 inset, left). Importantly, we perform PDAP in a background of diluted FBS so that the resulting aptamer pairs can recognize PAI-1 with high specificity and could be used in sandwich assays without further optimization.

**PDAP Shows Rapid Enrichment of Aptamer Pairs.** We started the PDAP process with a random library containing 1 nmol of oligonucleotides (∼6 × 10^14 molecules). Due to the inherent throughput limitations of FACS (∼4 × 10^7 particles/h), it is impractical to directly perform PDAP on a library of this size. In order to reduce the size of the pool without sacrificing diversity, we performed a pre-enrichment step in which we conducted one round of conventional, bead-based...
selection for aptamers that bind to PAI-1, followed by two rounds of PDAP screening (see Materials and Methods).

In order to maximize the specificity of the aptamer pairs, we performed our PDAP screen in diluted FBS. In round 1, we incubated the pre-enriched pool with 50 nM PAI-1 in 0.1% FBS, and added 5 μM of free His-tag to avoid enriching aptamers that bind to the His-tag on the PAI-1 protein. To distinguish aptamer particles that form pairs from those that do not, we defined the “reference” and “aptamer pairs” gates before performing the sorting (Figure 2a). We defined the reference gate by running unlabeled aptamer particles through the flow cytometer. The aptamer pairs gate was defined to isolate the population of aptamer particles that show higher fluorescence than the reference gate. We tuned the stringency by reducing the target concentration and increasing the FBS concentration between the rounds of screening. We collected these aptamer particles as templates to synthesize aptamer particles for round 2. In round 2 we increased the screening stringency by decreasing the concentration of PAI-1 to 25 nM and increasing the FBS concentration to 0.2%, while keeping the concentration of His-tag at 5 μM.

We observed considerable enrichment of aptamer pairs after only two rounds of PDAP. We evaluated the results of the PDAP screening process by measuring aptamer pair formation in the pre-enriched, round 1, and round 2 pools under the same experimental conditions (Figure 2a). We observed that 9.8% of the round 2 pool resides within the aptamer pairs gate relative to 1.6% in round 1 and only 1.0% in the pre-enriched pool. When we performed the same measurement without PAI-1, we observed negligible signal, indicating that the fluorescence signals detected by FACS were indeed the result of aptamers binding to PAI-1 in a sandwich format, and not a product of parasitic aptamer–aptamer interactions (Supporting Information, Figure S1).

To obtain individual sequences of capture aptamers isolated in round 2, we cloned the pool into the pCR4-TOPO vector, transformed these plasmids into chemically competent bacterial cells, and sequenced 32 of the clones (Supporting Information, Table S1). We obtained 24 unique aptamer sequences and used them to synthesize a set of 24 aptamer particles by carrying out PCR with forward-primer coated magnetic beads. We measured the target binding of each of these aptamer particles in a sandwich format assay in both buffer and FBS. After incubating these aptamer particles with PAI-1 and the detection aptamer, we performed flow cytometry and plotted the fluorescence intensity of each capture aptamer to determine its relative binding affinity (Supporting Information, Figure S2).

**CA-1 Forms an Aptamer Pair with High Sensitivity.** Capture aptamer 1 (CA-1) generated the strongest signal in both buffer and FBS, and we therefore selected this aptamer for further evaluation in a series of control experiments. First, we confirmed that CA-1 only binds to a single site on the target protein, observing negligible binding when CA-1 was used as both the capture and detection aptamer in a PAI-1 sandwich assay (Supporting Information, Figure S3a). Second, we verified that CA-1 does not bind to the polyhistidine tag of PAI-1 by performing a sandwich assay with PAI-1 in the presence of a 100-fold molar excess of His-tag (Supporting Information, Figure S3b).

We then determined the equilibrium dissociation constant ($K_D$) of CA-1 using two different analytical methods, in order to independently verify the accuracy of our affinity measurements. First, we used a flow-cytometry-based fluorescence method, in which we labeled PAI-1 bound to CA-1 aptamer particles with a FITC-conjugated monoclonal antibody targeting the His-tag. We evaluated binding over a range of PAI-1 concentrations, fitting the data as previously described,$^7,28$ and obtained a $K_D$ of 34.3 nM (Figure 2b). We also measured the $K_D$ of CA-1 by using microscale thermophoresis (MST),$^29$ a solution-based approach that does not require conjugation to a solid support. This entailed maintaining a constant concentration of fluorophore-labeled CA-1 while incubating with varying concentrations of PAI-1. These reaction mixtures were scanned in capillaries, and normalized fluorescence data were analyzed with a model that accounts for high receptor concentrations.$^{27,28}$ The binding assay results yielded a solution-phase $K_D$ of 15.8 nM. Although we observed a 2-fold difference between the measurements obtained via flow cytometry and MST, this result is consistent with past reports directly comparing $K_D$ determinations from solution-phase and solid support-based methods.$^{30}$

**Aptamer Pairs Achieve Nanomolar Target Sensitivity.** We next determined the performance of CA-1 in a sandwich assay format by performing a series of flow-cytometry assays to determine the sensitivity of our aptamer pair in both buffer and serum. Briefly, we incubated CA-1 aptamer particles with PAI-1 at concentrations ranging from 0 to 300 nM in either buffer or dilute FBS. We then washed the beads and incubated with fluorescently labeled detection aptamer. Following a second washing step, we measured fluorescence with a flow cytometer.
We subsequently determined the minimum PAI-1 concentration that could be reliably detected above the background. We calculated the limit of detection as described previously for sandwich assays, based on the mean fluorescence signal of control reactions with no protein target plus 3 times the standard deviation of these reactions (NC + 3SD). Details are provided in the Supporting Information. We found that our aptamer pair assay could effectively detect less than 10 nM concentrations in both buffer and diluted serum, with a limit of detection of 4.6 and 5.8 nM, respectively. These results demonstrate that aptamer pairs generated by PDAP can achieve highly sensitive and specific detection of target analytes even in complex, interference-rich samples.

**Aptamer Pairs Show Excellent Specificity.** To assess the specificity of our aptamer pair in a sandwich assay, we measured its binding to 10 “nontarget” proteins spanning a wide range of sizes and isoelectric points. For example, interleukin-4 (IL-4) is a 15 kDa cytokine involved in allergic inflammation with a pI of 9.26, while complement 3 (C3) is a much larger (185 kDa) protein with a pI of 5.7 that plays a prominent role in the innate immune response. Each protein was tested in a sandwich assay, using aptamer CA-1 on magnetic beads for protein capture and the Qasuar 670-labeled detection aptamer as a signaling reagent. After carrying out the assay at a fixed protein concentration, we performed signal measurement using a flow cytometer (see Materials and Methods).

Our aptamer pair exhibited exquisite specificity for PAI-1 (Figure 3b). For example, the binding signal for PAI-1 was 70-fold greater than that obtained with IgG, which generated the largest response among the nontarget proteins. Importantly, our aptamer pair showed negligible affinity for PAI-2, which exhibits 41% homology to PAI-1. These results indicate that our aptamer pair is not merely capable of discriminating against unrelated proteins, but also offers a valuable tool for distinguishing PAI-1 from related proteins with considerable homology. This combination of target selectivity and sensitivity could make these aptamers a useful tool for clinical diagnostics and highlights the strong performance that can be achieved by PDAP-generated aptamer pairs under rigorous testing conditions.

**CONCLUSION**

In this work, we demonstrate a rapid and efficient strategy for isolating high-quality aptamer pairs that can be directly used in sandwich assays. Our screening methodology enables direct screening of aptamer pairs at a throughput of >10^8 aptamer pairs per hour—many orders of magnitude higher than the current state of the art. Major advantages of our method include the fact that it does not require labeling of the target protein and the screen can be performed in heterogeneous samples such as diluted serum. Within only two rounds of PDAP screening, we were able to discover DNA aptamer pairs for PAI-1 with low-nanomolar affinity and excellent specificity with minimal off-target binding even to closely related proteins such as PAI-2. Although FACS is not a standard laboratory instrument currently, it is becoming widely accessible to the general research community. Given this, we believe our screening methodology offers a useful strategy for the discovery of affinity reagent pairs for sensitive and specific molecular detection.