Flow-Cell-Based Technology for Massively Parallel Characterization of Base-Modified DNA Aptamers

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ABSTRACT: Aptamers incorporating chemically modified bases can achieve superior affinity and specificity compared to natural aptamers, but their characterization remains a labor-intensive, low-throughput task. Here, we describe the “non-natural aptamer array” (N2A2) system, in which a minimally modified Illumina MiSeq instrument is used for the high-throughput generation and characterization of large libraries of base-modified DNA aptamer candidates based on both target binding and specificity. We first demonstrate the capability to screen multiple different base modifications to identify the optimal chemistry for high-affinity target binding. We next use N2A2 to generate aptamers that can maintain excellent specificity even in complex samples, with equally strong target affinity in both buffer and diluted human serum. For both aptamers, affinity was formally calculated with gold-standard binding assays. Given that N2A2 requires only minor mechanical modifications to the MiSeq, we believe that N2A2 offers a broadly accessible tool for generating high-quality affinity reagents for diverse applications.

INTRODUCTION

Base-modified aptamers, in which natural DNA or RNA bases are replaced with non-natural, chemically modified counterparts, can greatly enhance the affinity and specificity of aptamers for protein targets. Gold and co-workers showed that natural aptamers can only target <30% of the human proteome, whereas base-modified aptamers can target more than 80%. Other reports showed that the addition of a non-natural functional group at the 5-position of pyrimidines, such as a cationic amine, can result in aptamers with superior affinity or specificity to natural DNA aptamers. Base-modified aptamers can incorporate a wide range of chemical functional groups beyond those found in nature potentially allowing them to target analytes that would otherwise be difficult or impossible to target. Commercial suppliers such as SomaLogic have been successful in demonstrating the feasibility of re-purposing HTS instruments for parallel aptamer characterization, but there has been no equivalent demonstration of a strategy for characterizing base-modified aptamers in a high-throughput manner.

In this work, we describe a versatile platform that enables the efficient characterization of large numbers of base-modified DNA aptamers in a parallel fashion. Our “non-natural aptamer array” (N2A2) system uses a modified benchtop Illumina MiSeq instrument that can generate up to 10^6 base-modified aptamer sequences in situ within the flow cell, and subsequently measures the binding response and specificity.

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of these sequences in parallel. N2A2 uses a generalizable copper-catalyzed azide–alkyne cycloaddition (CuAAC) “click chemistry”-based approach, initially described by Tolle et al.,\textsuperscript{16} which enables incorporation of virtually any chemical modification into our aptamers with commercially available polymerase enzymes. This simplifies the measurement and comparison of multiple modifications in the same pool of aptamers through consecutive sequencing experiments, each employing a different modification. Additionally, aptamer characterization can be performed directly in complex sample matrices, such that both binding and specificity can be measured simultaneously, enabling the selection of modified aptamers that are optimally suited for a given application.

As an initial demonstration, we used N2A2 to screen enriched libraries containing different modifications in order to identify the best modification for generating aptamers that bind vascular endothelial growth factor (VEGF), a well-characterized protein that offers a useful benchmark for evaluating aptamer screening strategies. We compared the performance of libraries that were modified with either tyrosine or tryptophan, determined that tryptophan-modified libraries exhibit superior performance, and subsequently generated a tryptophan-modified aptamer with substantially better affinity than a previously published natural DNA VEGF aptamer.\textsuperscript{17} We also demonstrate the feasibility of screening for target binding and specificity simultaneously with N2A2 by screening phenylalanine-modified aptamers that bind insulin in diluted serum. This small polypeptide is a challenging target—we are aware of only one insulin aptamer in the literature\textsuperscript{18}—and real-world applications for insulin detection generally require robust affinity in serum samples. Using N2A2, we successfully obtained a base-modified aptamer that exhibits superior affinity to the previously reported aptamer in buffer and outperforms this molecule in serum. For both the VEGF and insulin aptamers, we used gold-standard binding assays to formally measure their affinity and validate the robust target binding observed in our N2A2 assay. We, therefore, conclude that N2A2 has broad potential as a platform for characterizing based-modified aptamers.

### EXPERIMENTAL SECTION

#### Hardware and Software Modifications of MiSeq

Minor modifications were made to an Illumina MiSeq to accommodate the additional reagents introduced to the flow cell during binding experiments. A fluidic inlet line was added to port 23 in the instrument’s internal multiport (Valco Instruments) (Figure S1) and linked to an added external multiport. During an N2A2 run, our software modifications trigger the external multiport to switch ports, so that the appropriate reagent is drawn into the flow cell. Added hardware part numbers can be found in the SI.

Two software modifications were made to the MiSeq to perform N2A2 experiments and control the external hardware. First, we modified the run configuration files to perform a custom set of chemistry steps after sequencing, enabling us to automatically perform custom chemical incorporation and

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Figure 1. Overview of N2A2. (A) N2A2 uses a MiSeq flow cell to generate base-modified aptamer clusters and screen their performance. (B) N2A2 workflow for linking sequence information to binding performance for base-modified aptamers. (C) Schematic of aptamer sequence library as prepared for N2A2 analysis. (D) Schematic of the base-modified aptamer synthesis process. (E) Schematic of the binding assay procedure on the flow-cell surface.
binding assays. The custom chemistry definitions include pumping KOD-XL, EcoRI, or buffer from the additional ports in the MiSeq cartridge; heating the flow cell; incubating reagents on the flow cell; and pumping in externally stored protein dilutions through the external multiport. Detailed changes to the code can be found in the Supplementary Code.

Second, to control our external multiport, we used an automatic file management software (Folder Agent), which monitors the temporary experiment folder and triggers commands upon the creation of .cif files as they are processed. During the second read of the first cycle, the EcoRI site is cleaved. The flow cell is then washed with buffer followed by 50 mM NaOH and 0.25% SDS. Depending on whether we are performing a base-modified or natural DNA run, the flow cell is incubated with click chemistry reagents or selection buffer, respectively, for 40 min, then washed again. Additional details can be found in the SI.

Performing CuAAC Chemistry in N2A2. We utilized the paired-end turnaround procedure of the standard MiSeq workflow to introduce the new nucleobases that allow us to convert natural DNA into base-modified aptamers on the flow cell (Figure 1B). We introduced a commercially available polymerase, KOD-XL, and a new dNTP mix, including C8-alkyne-dUTP, into user-replaceable reagent tubes in the MiSeq reagent cartridge. The new polymerase chain reaction (PCR) mix consists of 804 μL of water, 100 μL of 10X KOD buffer, 20 μL of 10 mM dATP, 20 μL of 10 mM dGTP, 20 μL of 10 mM dCTP, 20 μL of 10 mM C8-alkyne-dUTP, and 16 μL of 2.5 U/μL KOD-XL. During the paired-end turnaround, instead of using the standard amplification master mix, the sequence recipe was edited to use the KOD-XL PCR mix, which was stored in the reagent cartridge. A mixture of 0.1 M CuSO4/0.2 M tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) in H2O and 10 mM azide-modified tryptophan, tyrosine, or phenylalanine was degassed with N2 for at least 15 min. Sodium ascorbate (20 mg) was resuspended in 1 mL of H2O directly before the command. Sodium ascorbate solution (25 μL) was added to the click reaction, and the tube was immediately attached to the external port of the MiSeq. Final concentrations were 1.5 mM CuSO4/THPTA, 0.2 mM azide-conjugate, and 5 mM sodium ascorbate. The alkynes present in the oligos on the flow cell surface were the limiting reagent.

N2A2 VEGF Aptamer Screening Experimental Procedure. The same input pre-enriched DNA libraries and labeled VEGF protein were used for all three experiments, but new sequencing cartridges and flow cells were used for each experiment. For the natural DNA experiment, natural dNTPs were used during the paired-end turnaround. During the conversion process, tyrosine or tryptophan azide was clicked onto the alkyne handle, and then the sequence was cleaved. After collecting the sequence information, we exposed the aptamer libraries to DyLight650-labeled VEGF at concentrations of 1, 10, and 25 μM in both selection buffer and 1% human serum. Before, in between, and after the insulin titrations in buffer and serum, we also performed fiducial mark cycles to ensure that the flow cell was returning to baseline.

Flow Cytometry Binding Assays. We generated non-natural aptamer particles, without flow-cell primer sequences, via previously established methods. Details can be found in the SI. Positive control beads with aptamer SL2B (for VEGF) or aptamer IGA3 (for insulin) were generated by ordering the biotinylated aptamer (Table S1) and immobilizing onto MyOne SA Dynabeads according to manufacturer's instructions. Beads (1 μL) and VEGF (0.39–50 nM) or insulin (0.39–50 μM) in 100 μL of selection buffer were incubated at room temperature for 40 min. The tube was placed on a magnetic rack for 30 s, washed twice with 100 μL of cold selection buffer, and resuspended in 100 μL of cold selection buffer. The beads' fluorescence was measured by flow cytometry.

RESULTS AND DISCUSSION

Overview of the N2A2 Platform. N2A2 is designed to efficiently characterize the target binding and specificity of many base-modified aptamers from an enriched pool. The N2A2 is fabricated by converting the Illumina MiSeq instrument such that base-modified aptamer clusters can be synthesized and characterized directly on the sequencing flow cell (Figure 1A). The workflow entails three main steps: sequencing, conversion, and in situ binding measurements (Figure 1B). It begins with the sequencing of a DNA library during read 1 of a paired-end sequencing run. During the paired-end turnaround and read 2, the DNA clusters are converted into base-modified aptamer clusters. This is achieved via a CuAAC-based click chemistry reaction with alkylene-modified nucleobases, which can be incorporated into aptamer sequences with a standard commercially available polymerase. Finally, during the rest of read 2, the flow cell is incubated with fluorescently labeled protein to screen for target binding and specificity, with intensity information collected for each cluster. These screening and sequencing data are processed to generate a phenotype-genotype linked map of all the clusters. The initial libraries that we employed comprise a randomized region flanked by forward primer (FP) and reverse primer complementary (RPc) sequences.

We first enrich our initial library for binding to the target using conventional enrichment methods (e.g., SELEX or particle display) to reduce the pool's diversity to a scale that can effectively be screened on the N2A2 (up to ~10^6 sequences). The enriched aptamer pool is then amplified with a pair of adaptor sequences, producing a library of aptamers that feature an EcoRI recognition sequence adjacent to the RPc sequence (Figure 1C). In a separate amplification step, Nextera XT indices that include the Illumina-defined flow-cell primers are added onto the 5' and 3' ends of each library molecule. Once the library is prepared, we perform paired-end sequencing of the aptamer pool with a V3 MiSeq kit. During this process, the aptamer pool is displayed on the flow cell as antisense strands, with a 5' reverse primer sequence and 3' FP complementary sequence; sequencing produces a FASTQ file that represents these sequences as sense strands.

The conversion of DNA into base-modified aptamers is achieved through a three-step process (Figure 1D). The first read of sequencing generates the natural DNA template and FASTQ data (step 1). In the paired-end turnaround step, C8-
alkyne-dUTP is substituted for native dTTP during bridge PCR using the KOD-XL polymerase, which can incorporate the modification with high fidelity (step 2).

This produces aptamer clusters with alkyne handles that are compatible with post-synthesis modification via click chemistry. This enables us to replace every T with virtually any chemical functional group that we wish to incorporate. Next, during natural DNA aptamer analysis only, we remove the adaptor and flow-cell primer sequences that were added to the 3′ end of the aptamers to prevent potential steric hindrance between the aptamer and the target. Our library incorporates an EcoRI recognition sequence between the aptamer and the adaptor primer; by hybridizing a complementary strand to the primer and recognition sequence, we form a double-stranded cut-site that enables enzymatic excision of the 3′ sequencing primer, index, and flow-cell primer sequences. During post-N2A2 affinity validation, the natural and non-natural aptamer candidates are tested without the indices and flow-cell primer sequences. Finally, during the second read of the paired-end sequencing process, the desired chemical modifications are conjugated to the aptamer through click chemistry (step 3).

The entire process is performed directly by the MiSeq on the flow cell. This is achieved by editing the XML files to control the MiSeq instrument to incorporate the necessary reagents (i.e., non-natural dNTPs, enzymes, and buffer) stored in the custom tubes in the Illumina sequencing cartridge. Only modest hardware modifications are required, as shown in Figure S1. Control experiments for modified cluster generation and click reactions are shown in Figures S2 and S3.

Once the conversion process is complete, we screen aptamer−target binding in situ within the flow cell. A single fiducial mark sequence is included in the sequenced DNA library, and we incubate the flow cell with a fluorescently labeled oligonucleotide complementary to this sequence, which serves as a positive binding control in all steps of the experiment (Figure 1E, step 1). Next, we incubate the aptamer clusters on the flow cell with fluorescently labeled target proteins (step 2), wash with buffer, and use the MiSeq to capture images of the flow cell. These images provide the fluorescence intensity of all clusters simultaneously, which is representative of the amount of target captured by each aptamer cluster. Next, we increase the concentration of the target protein, wash, and image, repeating this process at multiple concentrations. Seven different concentrations were tested, with additional cycles included to confirm return to baseline. These intensities provide binding information for all
Among the published DNA aptamers for VEGF described to date, the SL2B aptamer discovered by the Yung group exhibits the highest affinity.\textsuperscript{17} We used this aptamer as a positive control for our natural DNA N2A2 screen (Table S1). We aligned the aptamer sequences from our screen to the intensity data as described above and removed sequences of incorrect lengths or containing other errors (SI Methods and Table S2).

Both base-modified aptamer populations showed increased binding to VEGF relative to the natural DNA library, but the W-modified aptamers exhibited considerably higher affinity compared to both natural DNA and Y-modified aptamers. (Figure 2A) We also noted that the SL2B aptamer (dotted red line and top histogram) performs slightly below the average of our natural DNA pool. Many strong signal outliers can be seen for both the W- and Y-modified pools in the boxplots. Based on these findings, we determined that the W modification is a superior choice for the selection of high-affinity VEGF aptamers, and focused our subsequent efforts on the characterization of W-modified aptamers.

Interestingly, we found that the binding signal of the aptamers was not linearly proportional to the number of W modifications (Figure 2B). The average fluorescent signal of aptamers increased along with the number of modifications, indicating that the W-modifications positively affect aptamer affinity, but the aptamers with the strongest signal were not the ones with the most modifications. Instead, several of our top binders had only 2–4 W modifications. Additionally, we observed that in the top 1 and 0.1% of binders with the strongest signal, even aptamers with higher numbers of W modifications generally tend not to contain long contiguous stretches of modified bases (Figure S4).

Since N2A2 allows us to characterize many copies of the same sequence, we examined the relationship between copy number and binding performance, as aptamer studies often rely on a high copy number in the final round of enrichment to choose potential hits.\textsuperscript{23} We selected a trio of aptamers from the flow cell: VEGF-3 represented the top of the 3rd quartile of performs, VEGF-4 had a low copy number and high binding signal, and VEGF-5 had a high copy number and high binding signal (Figure 2A,C). We see that VEGF-4 exhibits the best performance of the three aptamers, demonstrating how N2A2 allows us to identify promising low-copy-number hits that would otherwise have been overlooked with purely sequence-count-based SELEX analysis.

We used a bead-based flow cytometry assay\textsuperscript{32} to characterize eight W-modified aptamers (including VEGF-3, -4, and -5) that exhibited target-binding signal in the top quartile of the natural, Y, or W N2A2 experiments at 1 nM VEGF. We found that all eight aptamers selected with N2A2 consistently performed better than SL2B (Figure S5 and Table S3). The best aptamer, VEGF-4, showed a $K_d$ of 3.3 nM in flow cytometry assays. In comparison, the positive control SL2B aptamer exhibited a $K_d$ of 16.9 nM using the same assay. Importantly, the affinity of SL2B as measured on the N2A2 was consistent with measurements obtained with the bead-based flow cytometry assay (Figure S6). We also validated the performance of aptamer VEGF-4 by biolayer interferometry (BLI) and again confirmed that this non-natural aptamer outperforms SL2B in terms of VEGF affinity (Figure S7). These results demonstrate how N2A2 experiments can guide the selection of an aptamer with optimal chemical modifications for a given target and facilitate the rapid characterization of many base-modified aptamers in parallel.
Figure 3. Assessing F-modified insulin aptamer binding in buffer and serum. (A) N2A2 fluorescence images of aptamer clusters. A non-specific aptamer (top row) shows a strong signal from insulin binding in the buffer (left), but this signal disappears in serum (right). In contrast, a high-specificity aptamer (bottom row) shows strong insulin binding in both buffer and serum. (B) Z-scores from four representative families: 6 and 96, which retain binding to 10 μM insulin in both buffer and 1% serum, and 181 and 2120, which show reduced binding in 1% serum. Individual aptamer Z-scores are plotted in both buffer and serum conditions, with gray lines connecting datapoints for each sequence. A Z score of 1 is 1 standard deviation above the mean. (C) Flow cytometry-based bead-binding assay for ins24 and natural DNA aptamer IGA3 in buffer (blue) and 1% human serum (orange).

N2A2 Enables Screening for Specificity. N2A2 can also be utilized to simultaneously screen base-modified aptamers for both target binding and specificity. As an example, we isolated a base-modified aptamer that can specifically bind to insulin in diluted human serum. For this experiment, we chose a phenylalanine (F) modification based on the crystal structure of the insulin receptor, which reveals that F plays an important role in the hydrophobic pocket of the insulin-binding site.29 This residue also tends to be over-represented in protein-binding sites and enhances the binding of base-modified aptamers. Insulin is a polypeptide hormone made up of 51 amino acids (molecular weight 5.8 kDa),33,34 and generating high-affinity aptamers to polypeptides is much more challenging than for larger proteins. To our knowledge, only one aptamer has been described in the literature for insulin to date (by Yoshida et al.), which exhibits a K_{d} of ~10 μM.18

To generate F-modified insulin aptamers, we performed two rounds each of positive and negative SELEX and two rounds of particle display (SI Methods), and the resulting pool was characterized on N2A2 (Table S4). Briefly, we measured the fluorescence intensity of F-modified aptamer clusters when incubated with 1, 10, and 25 μM insulin in buffer or 1% human serum (Figures S8 and S9). By comparing the cluster intensities in buffer and serum, we can readily discriminate between “non-specific” aptamers, which exhibit fluorescence under buffer conditions but no longer generate a signal in serum, from “specific” aptamers that retain their fluorescence intensity in both conditions (Figure 3A). For each aptamer cluster, we examined the Z-score at each insulin concentration in both buffer and 1% serum to reduce cycle-to-cycle variance and make the populations more directly comparable (SI Methods). Z-score is defined as \((x - \mu)/\sigma\), where \(x\) is the aptamer fluorescence, \(\mu\) is the mean fluorescence of the entire population, and \(\sigma\) is the standard deviation of the entire population. Since we have many aptamers and aptamer families and want to describe the standard deviations from the population mean, the Z-score is an appropriate metric to compare each aptamer to the overall population in a given cycle.

Finally, N2A2 allows us to examine both individual aptamers and aptamer family performance. Family clustering has previously been used to choose SELEX aptamer candidates after HTS,35 however, N2A2 allows us to visualize how an entire family performs and which are the best candidates within the family. We performed family clustering with a Levenshtein edit distance of ≤5 (SI Methods and Figure S10) and identified two distinct types of aptamer families. One group of families generally showed a reduced Z-score in 1% serum, such as families 181 and 2120, while the other mostly retained their fluorescent signal in 1% serum, such as families 6 and 96 (Figure 3B).

We tested many aptamer candidates from family clusters that retained binding in serum, as well as aptamers that did not cluster into a family but had high binding performance, at various insulin concentrations by flow cytometry (Tables S5 and S11). Family 6 stood out because its mean fluorescence was the highest among all families, and we further investigated sequence ins24—a high copy-number representative. We used the same bead-based flow cytometry method mentioned above to compare the insulin affinities of ins24 and IGA3, the Yoshida et al. aptamer,18 and obtained K_{d}s of 2.6 ± 0.6 and 12.7 ± 1.1 μM, respectively, in buffer (Figure 3C). Critically, only ins24 retained equally strong affinity when we performed the same assay in 1% serum, with a K_{d} of 4.8 ± 0.7 μM. In contrast, IGA3 exhibited dramatically lower affinity, and we were unable to obtain a meaningful K_{d} measurement. A follow-up microscale thermophoresis experiment with fluorophore-labeled ins24 and unlabeled insulin confirmed that our aptamer has a K_{d} of 2.4 ± 0.4 and 2.6 ± 0.6 μM in buffer and serum conditions, respectively (Figure S12). Although the affinity of the selected aptamer is modest, these results demonstrate that N2A2 can rapidly identify base-modified aptamer sequences that outperform natural DNA in terms of binding to challenging small polypeptide targets, and which maintain consistent binding performance even in high-background serum samples.

CONCLUSIONS

We have demonstrated a workflow for screening and characterization of up to millions of base-modified aptamers. Our N2A2 platform is a modified Illumina MiSeq instrument and exploits that technology's sophisticated flow cell and
imaging apparatus to analyze vast arrays of base-modified aptamer candidates in situ, in a manner that directly couples binding data to defined sequences based on their position within the flow cell. Since our approach uses a click chemistry-based modification strategy, one can employ commercially available polymerase enzymes to incorporate virtually any base modification without meaningfully changing the experimental workflow.

We have shown that N2A2 can be used to compare the binding performance of DNA aptamer libraries incorporating different base modifications and identified that the tryptophan modification improves affinity for the target protein VEGF. We isolated a novel W-modified aptamer with improved affinity to the best natural DNA aptamer in the literature. N2A2 can also be used to simultaneously screen base-modified aptamers for specificity and target binding. We screened a library of F-modified aptamers for insulin in both buffer and diluted serum and obtained an aptamer that retained its affinity in both conditions, an improvement over the previously published aptamer.

We emphasize that N2A2 is not a replacement for the entire SELEX process; rather it allows for the simultaneous characterization of an enriched library of up to \( \sim 10^6 \) sequences. This represents a multiple-log increase in the number of natural or base-modified aptamers that can be analyzed in a single experiment. Identification of optimal base modifications and sequence variants can be accomplished within 2−3 N2A2 runs. Additionally, due to the versatility of the platform, one can readily test a wide range of experimental conditions—including diverse buffers, complex backgrounds, and pH levels—in rapid succession. The binding measurements derived from N2A2 are intended to assist in the selection of promising aptamers from vast libraries of candidates, but the use of gold-standard binding assays is necessary for the formal measurement of affinity for those sequences, as we have done in this work.

This is also a platform with considerable potential for extension in the future—for example, studying single and double mutant libraries of an individual sequence to create sequence fitness landscapes. The scale of this assay is well suited for creating sequence fitness landscapes, as Knight and coworkers did, and for predicting novel aptamers with machine learning. Such gain- and loss-of-function mutational landscapes could be deeply informative for the introduction of further modifications to base-modified aptamers and yield aptamers with further optimized binding properties. Libraries featuring multiple modifications in parallel could be screened using the selection techniques developed by Gawande et al. The platform could also be extended to RNA aptamers—previous work from the Lis' and Greenleaf labs has demonstrated the profiling of RNA−protein interactions on a sequencing flow cell, where RNA strands were synthesized after the flow cell was removed from the sequencer. Alternatively, the range of functions screened—such as structure switching—could be expanded by modifying the scripting of reagents delivered during read 2. Additional modifications to the XML and driving scripts could also facilitate stepwise sequencing, natural aptamer screening, click modification, and base-modified aptamer screening within a single experiment.

Given that our system is built upon a widely available sequencing instrument and uses a straightforward and versatile click chemistry approach to achieve aptamer modifications, we believe that N2A2 offers a broadly accessible solution to researchers looking to develop specialized aptamers for challenging applications.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c04777.

Materials, hardware modification to Miseq, software modification to Miseq, validating conversion to base-modified aptamers, protein labeling, SELEX and particle display for VEGF and insulin pre-enrichment, DNA preparation for high throughput sequencing, sequencing data alignment to images, sequence preprocessing and sequence-intensity alignment, aptamer family clustering, Z score calculation, BLI binding assays, MST measurements, and flow cytometry binding assays (PDF)

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**Notes**

The authors declare no competing financial interest.

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