A High-Throughput Platform for Rapidly Adapting DNA Aptamers to SARS-CoV-2 Evolution

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Abstract
Rapid pathogen evolution, exemplified by SARS-CoV-2 during the COVID-19 pandemic, threatens public health by eroding the effectiveness of vaccines, therapeutics, and diagnostic tools through continuous viral mutation. Although spike protein targeting monoclonal antibodies (mAbs) were developed within 10-12 months of the initial outbreak to serve as key theranostic agents, their redesign has struggled to keep pace with viral evolution, rendering many neutralizing antibodies ineffective. Here we demonstrate a novel platform that integrates a random-rational hybrid library diversification with high-throughput *MiSeq* screening to evolve aptamers as highly versatile recognition elements that can be easily reprogrammed to bind the spike proteins of emerging SARS-CoV-2 strains. Using a repurposed next-generation sequencing (NGS) platform, interactions between 3 different spike proteins and 11,806 unique aptamer variant designs can be effectively screened within a few days. Our starting point is a 40-nt aptamer that binds strongly to the wild-type (WT) spike protein but shows reduced and no affinity toward its Delta and Omicron strains, respectively. With this starting aptamer diversified, our rapid screening method yielded one double mutant that exhibits 4-fold improvement in binding to the Delta spike protein and another double mutant that converts its binding to the Omicron spike protein from no detectable affinity to the k_d of nanomolar range. A selective WT binder was also identified with no binding the two variants of interest. Using this pipeline, we identified bases not previously recognized as part of the motif that contribute critically to spike protein binding. Moreover, our pipeline integrates screening data analysis with molecular dynamics simulations, providing insights into aptamer-protein binding interactions. A sensor was developed based on the identified WT-selective binder, enabling highly specific detection of the WT spike protein with minimal cross-reactivity and robust performance in 40% serum. Together, these results demonstrate that aptamers can be rapidly optimized to bind new variants or selectively recognize a specific strain using the repurposed NGS platform. This work highlights the platform as a highly adaptive technology capable of obtaining aptamers within days to keep pace with rapidly evolving pathogens in future pandemics.

Teaser
A novel high-throughput *MiSeq* screening platform to rapidly evolve spike-protein-binding aptamers to keep pace with viral evolution.

MAIN TEXT

Introduction
By December 2025, the cumulative number of confirmed COVID-19 cases and deaths worldwide had reached 779 million and 7.1 million, respectively (*1*). A major challenge in halting this pandemic arises from the rapid evolution of SARS-CoV-2 virus. Since the outbreak in 2019, the World Health Organization (WHO) has identified five variants of concerns (VOCs), including Alpha, Beta, Gamma,

Delta and Omicron (*2–6*), These variants exhibit markedly higher transmissibility than the original strain (*7*, *8*), leading to extended quarantine, increased hospitalization and vaccine breakthrough infections (*9–15*). To combat the COVID-19 pandemic, at least 17 monoclonal antibodies (mAbs) for both patient treatment and SARS-CoV-2 detection were successfully developed and granted Emergency Use Authorization (EUA) by the U.S. FDA in late 2020 (*16*, *17*). However, these authorizations were later revoked (*18*, *19*) as continuous viral evolution rendered these antibodies ineffective (*20*, *21*). Developing new mAbs at pandemic speed (*7, 8*) and subsequently optimizing them to keep pace with viral mutations (*18*, *20*) remain a formidable challenge. Therefore, a modular, highly adaptable platform that can be rapidly reprogrammed and deployed for both the diagnosis and neutralization of emerging viral variants is urgently needed to support rapid responses for future pandemics (*22*).

Highly adaptable functional nucleic acid technologies can fulfill this need. Analogous to mAbs, DNA aptamers identified through SELEX (Systematic Evolution of Ligands by EXponential enrichment) process (*23–25*) can bind spike proteins with high affinities (k_d values down to the sub nM range (*26–28*)). Compared with mAbs (*19*), aptamers offer comparable binding strength at only a fraction of the cost and production time (*29*, *30*). For instance, a 40-nt DNA aptamer Apt2 developed by Zu and co-workers (*27*) exhibits a k_d of 2 nM when binding spike protein derived from the original wild-type (WT) strain and costs only ~$0.72 per nanomole to synthesize commercially. In the past few years, Dozens of DNA aptamers have been developed to bind different epitopes of the SARS-CoV-2 virus, such as various domains, especially the receptor-binding domain (RBD), of the spike protein (*26*, *28*, *31–33*) and nucleocapsid proteins (*34*). Although some can bind multiple viral strains (*35–37*), most of them faced limited cross-variant robustness. For instance, we found that Apt2 exhibits negligible binding to the spike protein of one of the Omicron subvariants, $\text{Omicron}_{\text{XBB}}$ (**Fig. 1C**), possibly because Apt2 was originally selected against the WT strain, whereas the subvariant harbors 39 amino-acid mutations in its spike protein (21 of them are within the RBD). In other words, similar to mAbs, continuous viral evolution can render SARS-CoV-2-binding aptamers ineffective. Although researchers are adopting machine-learning approaches (*18*) to fine-tune mAbs to keep up with viral evolution, rationally designed mAbs still require production and validation steps that cannot be completed within such a short timeframe. In contrast, aptamers aptamers are inherently more amenable to rapid reengineering to accommodate viral mutations. However, the conventional de novo approach to develop DNA aptamers (i.e., SELEX(*23*)) relies on time-consuming iterative enrichment and counterselection steps to isolate strong and highly specific binders from a highly diverse library (~10^{15} sequences), limiting its ability to generate new aptamers quickly enough to keep pace with viral evolution during a pandemic.

To overcome this limitation, we developed a pipeline based on a repurposed Illumina next-generation sequencing (NGS) platform (*38–52*) that enables massively parallel, quantitative evaluation of aptamer–protein interactions across 10^4-10^7 (depending on the flow cell type, *MiSeq*, *HiSeq* and *NextSeq* and *NovaSeq*) closely related sequences within a single run (*30*, *53–56*). Repurposed NGS flow cells have provided a powerful platform to directly study interactions between target analytes and libraries of DNA (*42*, *55*), RNA (*47*, *48*), modified bases (*57*), and peptide (*44*, *49*). Previously, we employed a repurposed NGS method known as CHAMP (*55*) (Chip-Hybridized Association-Mapping Platform, which eliminates the need to modify Illumina's sequencing protocol) to screen ~40,000 fluorescent nanomaterial species on *MiSeq* flow cells and identify those exhibiting bright emission and distinct colors (*53*). More recently, we have also used CHAMP to optimize and diversify the photophysical properties of fluorogenic DNA (*30*) and RNA (*58*) aptamers. Together with the works from the Soh group (*59–61*, *57*, *51*, *52*), the broad applicability of the repurposed NGS platform in aptamer research has been demonstrated.

While previous work established a high-throughput screening framework for nanomaterial characterization, its ability to rapidly adapt to the emerging viral variants remains limited. To address this, we developed a random–rational hybrid library design (**Fig. 1D**) integrated with CHAMP-based screening. This approach is based on the premise that, for certain virus-binding aptamers, adaptation to new variants does not require complete redesign, but can be achieved through targeted diversification around a functional seeding sequence. By preserving controlled diversity in this local sequence space, the platform enables rapid and directed aptamer reengineering without the need for de novo selection. Using this strategy, we diversified the aforementioned Apt2 through single, double, insertion-deletion (Indel), and doped (i.e., partially randomization, (*62*, *63*)) mutations and screened these 11,806 variants directly on a repurposed NGS flow cell. Apt2 served as an ideal model due to its well-characterized WT RBD binding and early development during the pandemic. By simultaneously measuring binding affinities across all variants without enrichment or loss of sequence diversity, this platform enables systematic exploration of sequence-function relationships and accelerates adaptation to new SARS-CoV-2 variants from months to days. The reusability of the flow cell further supports iterative testing against multiple viral variants on the same platform, facilitating rapid identification of both affinity-enhanced and strain-selective binders. To the best of our knowledge, this study is the first to demonstrate that a repurposed NGS platform can rapidly adapt spike-protein-binding aptamers to keep pace with continuous viral evolution.

In this study, we show that this high-throughput screening strategy enables rapid reengineering of aptamers to adapt to emerging SARS-CoV-2 variants from a single WT binder. Specifically, targeted diversification yielded variants with enhanced binding to Delta, restored affinity toward $\text{Omicron}_{\text{XBB}}$ from undetectable levels to the nM range and enabled the generation of WT-selective binders with minimal cross-reactivity. Integration with molecular dynamics (MD) analysis further revealed that, beyond the loop motif, the upper stem region plays a critical role in spike protein recognition. Finally, a WT-selective aptamer was translated into a strand-displacement fluorescent sensor, achieving highly specific detection with robust performance in complex biological matrices. Together, these results demonstrate a scalable strategy for rapidly adapting molecular recognition elements to evolving pathogens, providing a practical framework for next-generation theranostic development.

Results

Designing an Apt2 Variant Pool for Aptamer Diversification

To demonstrate ability of the platform to rapidly identify new antiviral aptamers capable of adapting to viral evolution, we selected Apt2, a DNA-based SARS-CoV-2 spike-protein-binding aptamer developed by the Zu group to target the WT strain (*27*) as the seeding aptamer, to discover neighboring sequences capable of binding the Delta (B.1.617.2) and $\text{Omicron}_{\text{XBB}}$ (a recombinant of BJ.1 and BM.1.1.1) spike proteins. Apt2 was chosen as a model for its well-characterized WT RBD binding and early development, and these two strains were chosen because both had raised global concern and complicated the COVID-19 pandemic by increasing transmissibility and reducing immune effectiveness. Moreover, these two strains are representative in terms of their mutation magnitudes and appearance timeline (9 vs. 39 amino-acid mutations; identified ~1 vs. ~3 years after the emergence of the original strain, respectively; **Fig. 1A**), making them ideal models for this proof-of-concept study.

The 40-nt Apt2 folds into a hairpin structure, as predicted by mFold (*64*), with the 20-nt loop corresponding to the binding motif identified through the SELEX process (**Fig. 1B**). We first tested the binding affinity of the original Apt2 against the aforementioned three spike proteins using an enzyme-linked oligonucleotide assay (*65*) (ELONA**, fig. S1A**). The results (**Fig. 1C**) were consistent with the structural and sequence divergence of these spike proteins, showing that Apt2 binds

strongly to the wild-type strain (k_d ~2±0.4 nM); however, its binding affinity decreases significantly against the Delta variant (k_d ~89±10 nM) and becomes undetectable with the Omicron$_{XBB}$ strain (no stable k_d determined). When testing a scrambled sequence (Scr), which served as a control, in ELONA assay, no binding affinity to any of the three spike proteins was observed.

Taking the previously identified interaction profile between the seeding aptamer Apt2 and the WT spike protein into consideration, a mutant pool was designed using a random-rational hybrid algorithm to ensure a controlled diversity in this local sequence space, which included four subgroups: single mutations (120 sequences), double mutations (6,508 sequences), indels (single insertions or deletions, 152 sequences), and doped (i.e., partially randomized) mutants (4,500 sequences, generated with a mutation rate of 10% within the 20-nt loop motif and filtered to remove duplicates with the other subgroups). Based on the calculation (**Methods** and **fig. S2**), most doped mutants contain either triple (59%) or quadruple (28%) mutations (**Fig. 1D**), representing a moderate yet meaningful degree of diversification. The simple mutants (single, double mutants and indels) were expected to improve binding to the Delta variant, whereas the doped mutants, with their more disrupted motifs, held greater potential to strongly bind to Omicron$_{XBB}$.

A

Spike protein variant	Identified time	Number of mutated a.a.	Virus features
WildType (WT)	Dec 2019	-	Baseline strain
Delta (B.1.617.2)	May 2021	9	Increased transmissibility
Omicron$_{XBB}$	Sept 2022	39	Highly immune evasive

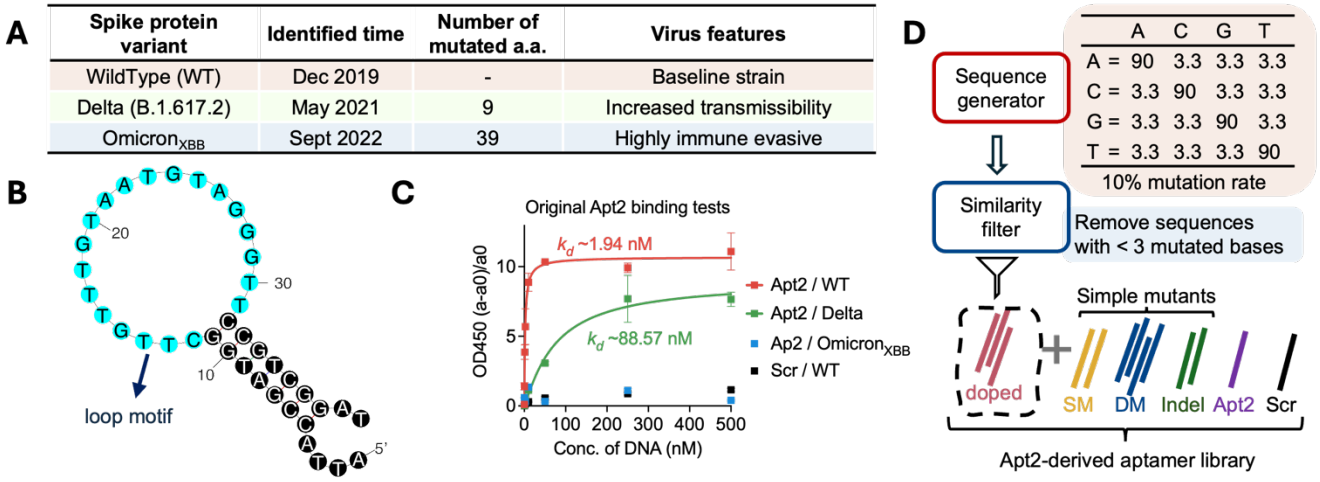

Fig. 1. Apt2 chosen as the seeding strand for SARS-CoV-2 spike protein aptamer diversification and screening. (**A**) The selected SARS-CoV-2 spike protein (trimer) variants in the experiment. Both Delta and Omicron$_{XBB}$ drove major global waves during the COVID-19 pandemic through their mutations, with the more recent one Omicron$_{XBB}$ being distinctly different than the original strain. a.a.: amino acid(s). (**B**) The secondary structure of Apt2 as predicted by mFold, with the 20-nt loop motif region identified by the previous SELEX, highlighted in light blue. (**C**) Characterization of the seeding aptamers and the negative control sequence with ELONA. k_d(Apt2/WT) ~2±0.4 nM; k_d(Apt2/Delta) ~89±10 nM. (**D**) Aptamer library design for chip screening. SM: single mutants; Indel: insertion and deletion mutants; DM: double mutants; Scr: scrambled. percentage of each design category: doped: 37.6%; SM/Indel: 2.06%; DM: 58.7%; Apt2: 0.83%; Scr: 0.83%.

High-throughput Screening of Apt2-derived Pool on an Illumina NGS Flow Cell

To screen the mutant pool in a high-throughput manner, we synthesized the library, added sequencing adapters and loaded it onto an Illumina *MiSeq* flow cell (**Fig. 2A**). Before screening, we first confirmed that the sequencing adapters and primers did not disrupt aptamer binding (**figs. S3-S4**). After sequencing, the strands were denatured, and the flow cell was thoroughly rinsed to remove any excess dyes. The fiducial marker PhiX was then labeled with an Atto488-tagged PhiX probe and imaged (**Fig. 2B**) for mapping purpose (*30*, *53*). To perform a binding test, the His-tagged spike protein was labeled via an Alexa647-conjugated anti-His-tag antibody and injected into the flow cell. The fiducial markers and bound proteins were imaged under fluorescence microscopy using two different filter cubes (**Methods**). The spike protein binding capacity of each sequence was estimated by the mean fluorescence intensity among its duplicates on the chip (**Methods**). By digesting the bound proteins and denaturing the DNA sequences, the same flow cell could be reset to its initial state for sequential screening of binders to another protein target (**Fig. 2C**). This approach allows the binding characterization of the entire pool against multiple SARS-CoV-2 spike protein variants to be performed on a single chip, thereby reducing experimental cost and material usage.

As a proof-of-concept, we performed the first screening against WT spike protein. The microscope images showed clear signals from fiducial markers under the FITC channel and from captured spike proteins under the Cy5 channel (**Fig. 2D, upper**). The positions of the bright FITC puncta matched the coordinates recorded in the FASTQ file (**Fig. 2D, left**), confirming successful image alignment. Within the field of view, regions corresponding to the Apt2 sequence co-localized with several bright Alexa647 puncta, whereas those corresponding to the Scr sequence remained dark (**Fig. 2D, right**). These results demonstrated that binding was visualized as intended. Next, we cleaned the chip surface and performed another round of screening against $\text{Omicron}_{\text{XBB}}$. We compared the acquired fluorescence intensities with the original Apt2 and Scr signals. The results showed that Apt2 sequences exhibited significantly higher fluorescence intensity than Scr when introducing WT (**Fig. 2E, left**), while the brightness of them was similar when the protein target switched to the $\text{Omicron}_{\text{XBB}}$ (**Fig. 2E, middle**), which agreed with our ELONA results that Apt2 is a strong binder for the WT while a non-binder for the $\text{Omicron}_{\text{XBB}}$ (**Fig. 1C**). A third round of screening against Delta was then performed following the chip regeneration (**Fig. 2E, right**).

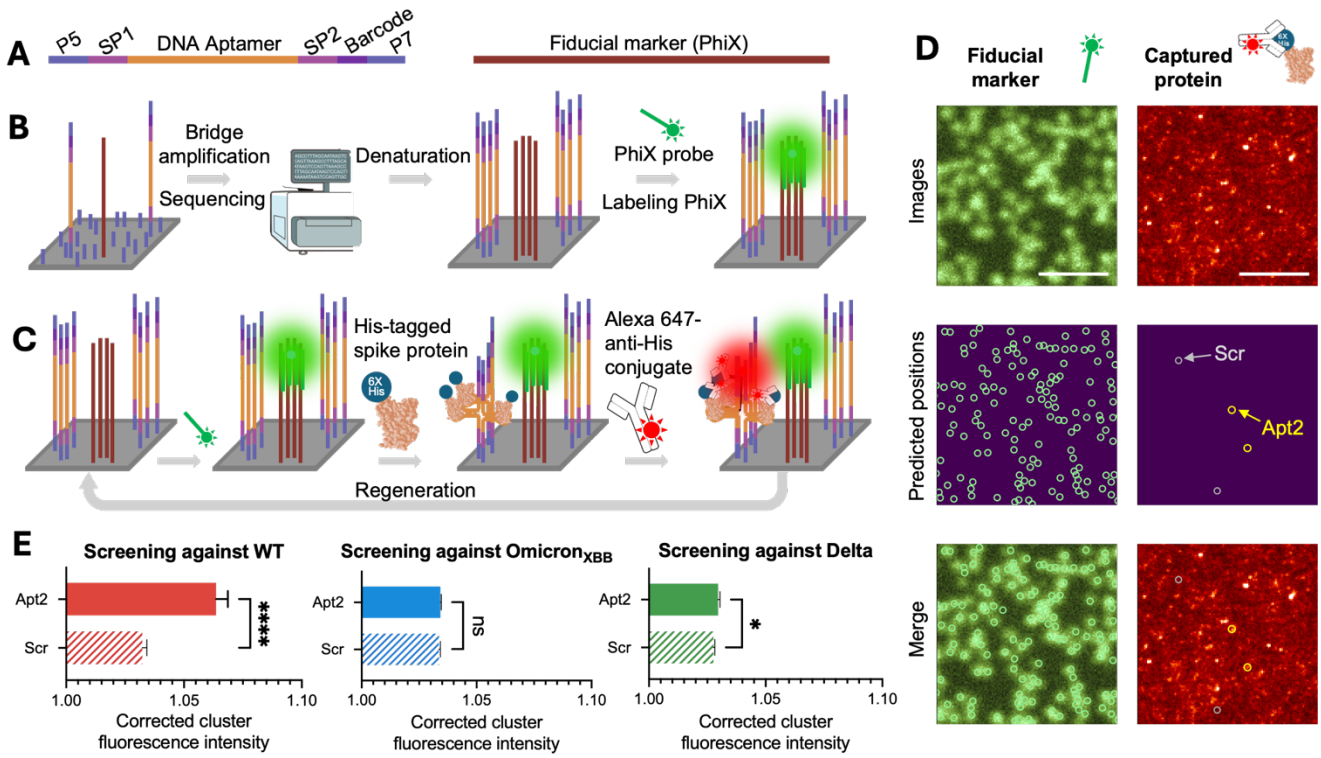

Fig. 2. Overview of SARS-CoV-2 spike protein variants specific aptamer identification on Illumina NGS chips. (**A**) The DNA pool described in **Fig. 1** are sandwiched in between Illumina sequencing adapters and synthesized on the flow cell surface. (**B**) NGS library was sequenced on a *MiSeq* chip through standard PE75 protocol and was denatured to expose ssDNA on the chip surface. Control sequences (i.e., PhiX) were labeled with an Atto488-tagged probe (fiducial marker). (**C**) To image SARS-CoV-2 spike protein binding on the chip, we first incubated the flow cell with 100 nM His-tagged spike protein trimer, which was then stained by the Alexa647 His-tag antibody conjugate. After imaging, the chip was regenerated by 0.1 N NaOH treatment, with another spike protein variant added in the following round. (**D**) A representative field of view (FOV) of PhiX labeled with Atto488-tagged probes (left, top) for mapping purpose, and WT spike protein labeled with Alexa647-His-tag antibody conjugate (right, top). The polonies representing the PhiX sequences, the seeding aptamer Apt2 and the negative control sequence (Scr) are highlighted in green (left, middle), yellow and light gray (right, middle) circles, respectively, which were identified after the CHAMP alignment. The merged figures (bottom) show the successful mapping of sequence positions and the chip images. Scale bars are 10 µm. (**E**) Corrected fluorescence intensities of the polonies correlating the seeding aptamers and the negative control sequence (Scr) on the chip when co-incubated with WT, Omicron$_{XBB}$, and Delta spike proteins, respectively. Error bars reflect the SEM (standard error of the mean) of each group. Number of duplicates of each trial can be found in **Supplementary Table S3**. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, $p > 0.05$.

Enhancing the Affinity for Delta by Modifying the Aptamer Stem

Here we sought to identify mutants with improved binding to the Delta strain, as a proxy for adaptation to an earlier stage of SARS-CoV-2 evolution. To evaluate whether Delta binding affinity could be enhanced by variants in the pool, we quantified the percentage change in fluorescence signal for each sequence relative to original Apt2 (hereafter referred to as percentage change) across three independent screening trials, each performed against a different strain. Analysis of the combined WT and Delta screening data (**fig. S5**) showed that the sequences were approximately evenly split between those exhibiting higher and lower fluorescence compared with Apt2 in the Delta screen. Nevertheless, because the fluorescence signals of Apt2 and Scr were less clearly separated in the Delta screen than in the WT screen (**Fig. 2E**), the calculated percentage changes for the Delta could be less reliable. This limitation was further supported by streptavidin-mediated surface binding assays mimicking the condition on the chip (**fig. S1B**), in which several high-fluorescence candidates failed to show consistently enhanced binding in ELONA (e.g., T3C-A39C; **Fig. 3C** and **fig. S6**).

To overcome the challenge, we changed the strategy by examining binding patterns at the sequence level using the screening data. The effects of individual sequences on a given viral strain were summarized as percentage changes and visualized across a series of heatmaps. Examination of the single mutations (**Fig. 3A**) and indels (**fig. S7**) datasets for Apt2 against the Delta strain revealed that the 20-nt loop motif (residues C12 to T31), although originally identified from SELEX, was also conserved for Delta binding, since any point mutation or insertion within this region disrupted interaction. Double-mutation heatmap on the stem further showed that the bases close to the loop are also intolerant with mutations (**fig. S8**). Based on this observation, we restricted subsequent analysis to mutants with alterations confined to the stem region to preserve Delta binding. These candidates were ranked according to the difference in percentage change between WT and Delta, with the goal of enhancing selectivity while minimizing cross-reactivity. The top five sequences with the most negative differences were selected for further evaluation (**Fig. 3B**). The binding properties of them toward WT and Delta were evaluated using ELONA (**Fig. 3C**). Most variants exhibited binding profiles similar to that of the original Apt2. Among them, A8G-A39T showed a modest improvement in binding affinity for Delta relative to Apt2 (k_d ~20±4 nM vs. 89±10 nM). Notably, this variant retained very strong binding to WT (k_d ~0.8±0.2 nM) while exhibiting negligible affinity for Omicron$_{XBB}$ (**Fig. 3D**).

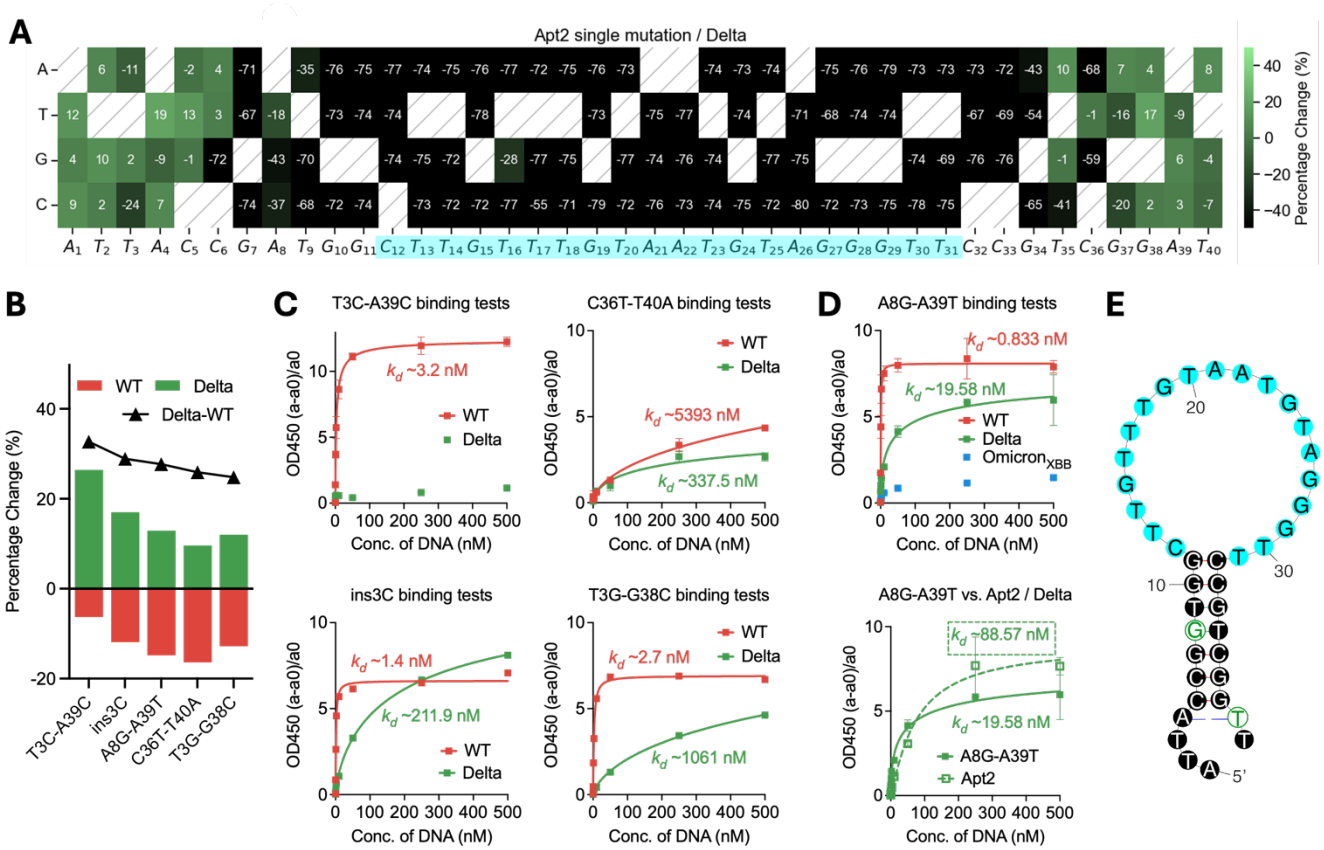

Fig. 3. Identifying Apt2-A8G-A39T as an improved Delta binder. (**A**) Apt2 single-mutation/Delta heatmap. The color of each cell represents the percentage change compared to the original Apt2/Delta with the color bar showing on the right. Cells representing the unmutated sequences are shown as diagonally hatched. The heatmap implied the immutability of the loop motif (highlighted in cyan). (**B**) Top five stem-altered candidates ranked by the Delta-WT percentage-change difference. Percentage changes for WT and Delta are shown as red and green bars, respectively. The black line indicates the Delta-WT percentage-change difference. (**C**)-(**D**) ELONA revealed that among all 5 candidates, A8G-A39T can bind slightly stronger than the seeding aptamer Apt2 (k_d ~20±4 nM vs. k_d ~89±10 nM, right). Further investigation showed that A8G-A39T has a similar binding pattern to the original Apt2, which is a strong WT binder (k_d ~0.8±0.2 nM), medium Delta binder, and does not bind Omicron$_{XBB}$. (**E**) A8G and A39T substitutions (highlighted by the green circles) were highlighted in the mFold-predicted secondary structure of Apt2. The motif was highlighted in cyan.

Identification of Omicron$_{XBB}$ Binders among the Doped Mutants

To assess whether our platform can identify aptamers targeting newly evolved viral variants, we screened the designed pool for Omicron$_{XBB}$ binders, focusing on the doped subgroup to maximize sequence diversity. Most fluorescence signals were comparable to those of Apt2 and Scr (**Fig. 4A**), indicating that the majority exhibit weak or no binding to Omicron$_{XBB}$. Notably, a small subset displayed a pronounced increase in fluorescence intensity and was selected for further investigation. To improve binding specificity and reduce background from multi-target screening, we narrowed the selection to five candidates with negligible fluorescence in both the WT and Delta screens (**Fig. 4B**, highlighted in blue). Among these, two were double mutants in the 20-nt loop motif, while three originated from the doped mutation subgroup (**Fig. 4B**, zoom-in), suggesting that, unlike WT and Delta, the loop motif alone is insufficient to support Omicron$_{XBB}$ binding (**figs. S11-12**).

A streptavidin-mediated surface binding assay was used to evaluate the binding profiles of the five selected sequences (**Fig. 4C**). In this assay, aptamer candidates were immobilized on streptavidin-coated microwell plates via a biotin moiety. His-tagged spike proteins and an anti-His-fluorophore conjugate were sequentially introduced, with washing steps between incubations (**Methods**; **fig. S1B**). Fluorescence intensities were measured using a plate reader and converted to percentage changes relative to the Apt2/WT positive baseline. Four of the five candidates suppressed binding to WT and Delta but did not exhibit improved binding to Omicron$_{XBB}$. In contrast, doped854, a triple mutant (T17G-T23G-T25G; **Fig. 4D**), showed a modest enhancement in binding to Omicron$_{XBB}$. This result was further supported by ELONA (**Fig. 4E**), which confirmed that doped854 binds Omicron$_{XBB}$ with higher affinity than the original Apt2 (k_d ~7±0.9 nM vs. undetectable).

To investigate structural features that might underlie the unique binding behavior of doped854, we examined its ability to activate thioflavin T (ThT), a fluorogenic dye sensitive to G-quadruplex (G4) structures (*66*–*70*). ThT fluorescence was measured in the presence of Apt2, doped854, a rationally designed G4-forming mutant (G4Apt2: C12G-T16G-T17G-T23G-T25G; **Fig. 4F**), and control sequences known to strongly activate (D-22AG-40 (*67*, *70*)) or not respond to ThT (cTBA (*71*, *72*)). G4Apt2 was designed based on the canonical G-quadruplex motif ($G_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}$) (*73*). All three aptamer variants enhanced ThT fluorescence, with doped854 producing a 53% stronger signal than Apt2 in the screening buffer and G4Apt2 yielding an even larger increase (~90%). As expected, all signals were lower than that of D-22AG-40, a known G4 dimer (**Fig. 4G**). These results suggest that Apt2 does not form, or only weakly stabilizes, a G-quadruplex structure. Conversely, doped854 can form or stabilize such a topology (*74*), contributing to its ability to bind the evolved Omicron$_{XBB}$ strain.

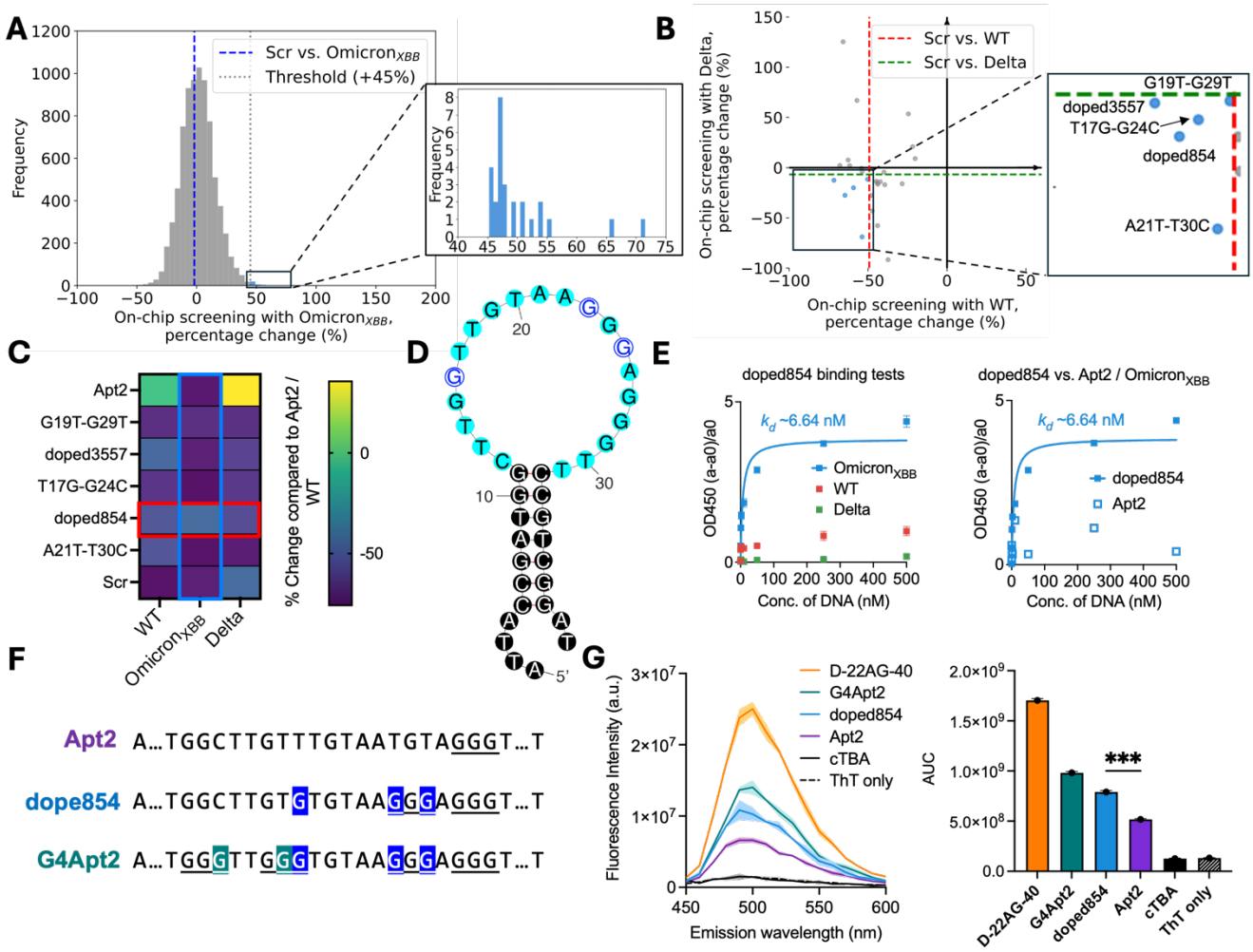

Fig. 4. Identifying Apt2-doped854 as an OmicronXBB-selective binder. (**A**) Sequences with at least +45% higher intensity than that of Apt2/Omicron$_{XBB}$ were selected out from the whole pool (bars in blue). (**B**) These selected candidates' WT and Delta binding data were retrieved and summarized in a scatterplot, with those with neglect binding affinity for WT (to the left of the red dotted line) and Delta (below the green dotted line) highlighted as blue puncta. (**C**) Streptavidin-mediated surface binding assay was used to test the bindings of Omicron$_{XBB}$ candidates selected from (**A**)-(**B**). Promising candidate doped854 was highlighted in a red box. Binding affinity for Omicron$_{XBB}$ comparison among the candidates are highlighted in a blue box. The color scale is shown on the right. (**D**) T17G, T23G and T25G substitutions (highlighted by the blue circles) were indicated in the mFold-predicted secondary structure of Apt2. The loop motif was highlighted in cyan. (**E**) ELONA revealed that Apt2-doped854 can bind Omicron$_{XBB}$, developing a new binding feature compared to the original Apt2 (k_d ~7±0.9 nM vs. undetectable). (**F**) Sequences of Apt2, doped854 and G4Apt2. Residues T2 to A8, T31 to A39 were omitted. Mutated bases in doped854 were highlighted in blue and further altered bases in G4Apt2 were highlighted in teal. G-tracts (G3s) were underscored. (**G**) Fluorescence activation of thioflavin T by DNA strands. D-22AG-40 is a G4 dimer known for strongly enhancing ThT's fluorescence. cTBA is the reverse complementary sequence of thrombin's aptamer TBA, which has no guanine in the sequence. Fluorescence was measured using 1 μM DNA and 1 μM thioflavin T. Shadings represent the standard deviations. Area under curve (AUC) was used to generate the overall intensities shown in the bar chart. Two-tailed un-paired *t* test was performed to compare the fluorescence intensities between doped854 and Apt2. P-value = 0.0001.

Enhancement of the Binding Specificity for the Wild-Type Strain by Mutation

Although Apt2 was originally selected against the WT spike protein, it also binds the Delta strain (**Fig. 2B**). Beyond improving binding affinity, we attempted to optimize specificity toward WT, with the goal of identifying aptamers that preferentially recognize WT while minimizing cross-reactivity with Delta. By isolating WT-specific binders, we aimed to improve the precision of diagnostic tools. Highly specific detection of WT provides unambiguous strain assignment, which is essential for generating strain-resolved data to support studies of viral evolutionary pathways(*75*).

To evaluate whether the designed pool contains candidates with improved WT specificity, we revisited the WT–Delta combinatorial dataset, with particular emphasis on the x-axis (**fig. S5A**). Most sequences clustered near the Scr/WT baseline (red dashed line), consistent with Apt2 being a strong WT binder that outperforms most of its mutants; overall, WT measurements exhibited greater consistency than those obtained for Delta. WT-specific candidates were defined as sequences located to the right of the y-axis (brighter than Apt2/WT) while remaining below the green dotted line (darker than Scr/Delta), and were highlighted in red. This distribution indicated the feasibility of identifying WT-specific mutants within the current pool. We next examined the binding profiles of these candidates against $\text{Omicron}_{\text{XBB}}$ (**fig. S5B**), where their signals clustered around zero, suggesting minimal binding to $\text{Omicron}_{\text{XBB}}$ and, consequently, high binding specificity for WT.

To identify sequence features associated with WT specificity, we compared the single-mutation heatmaps of Apt2 for WT and Delta. This analysis revealed that the G11 position, located at the junction between the loop and stem, tolerates a G to A substitution when binding WT, but this same mutation markedly disrupts binding to Delta (**Fig. 5A**). Consistent with this observation, the G11A single mutant showed minimal changes in binding $\text{Omicron}_{\text{XBB}}$ (**Fig. 5B**; +18%, +7%, and -75% for WT, $\text{Omicron}_{\text{XBB}}$, and Delta, respectively). Based on these results, we hypothesized that the G11A mutation could enhance the binding specificity of Apt2 toward the WT spike protein. Supporting this hypothesis, the WT double-mutation heatmap displayed a reddish horizontal stripe and a corresponding vertical stripe within the predominantly negative (dark) region (**fig. S10**). These stripes correspond to G11A-containing double mutants, in which G11A is paired with a second mutation either upstream or downstream of position 11. Notably, these cells with less negative values did not extend broadly across the heatmap, suggesting that retention of WT binding requires the second mutation to be positioned away from the loop motif. Taken together, these observations led us to refine our hypothesis: Apt2 double mutants carrying G11A, combined with a second substitution outside the loop motif, might serve as selective WT binders. Chip-screening results for these double mutants are summarized in a series of bar plots (**Fig. 4C**). As expected, Delta binding was disrupted for all such double mutants, while WT binding varied depending on the type and position of the second mutation.

To further narrow down candidates for WT-specific binders, we applied the streptavidin-mediated surface binding assay to a subset of G11A-containing double mutants to characterize their interactions with three SARS-CoV-2 spike protein strains. Mutants carrying substitutions at A1 or T40 were excluded, as changes in on-chip fluorescence at these positions are more likely attributable to interactions with chip primers rather than genuine effects on aptamer-protein binding (**fig. S4**). All remaining candidates showed disrupted binding to Delta relative to the parental Apt2. Among them, the point mutant G11A and the double mutants C5A-G11A, C5T-G11A, and G11A-C38G also exhibited noticeable reductions in WT binding (**Fig. 4D**). In comparison, the double mutants A4T-G11A and A4G-G11A displayed a WT-specific binding feature, with A4T-G11A (**Fig. 4E**) retaining the strongest interaction with WT. Despite a previously observed but unexplained elevation in the Delta signal in this assay, A4T-G11A showed substantially higher fluorescence for WT than for Delta,

408 demonstrating robust WT specificity.

409

410 We characterized the selected mutant A4T-G11A's binding kinetics toward the indicated strains (**Fig.**
411 **4F**). A4T-G11A retained strong binding affinity for WT, comparable to that of the parental Apt2 (k_d
412 ~1±0.04 nM vs. 2±0.4 nM), while binding to Delta was markedly suppressed (k_d ~89±10 nM vs.
413 undetectable). These results confirmed A4T-G11A as a strong and WT-specific binder for the SARS-
414 CoV-2 spike protein trimer, demonstrating that sequence-level screening can simultaneously enable
415 affinity optimization and effective counterselection.

416

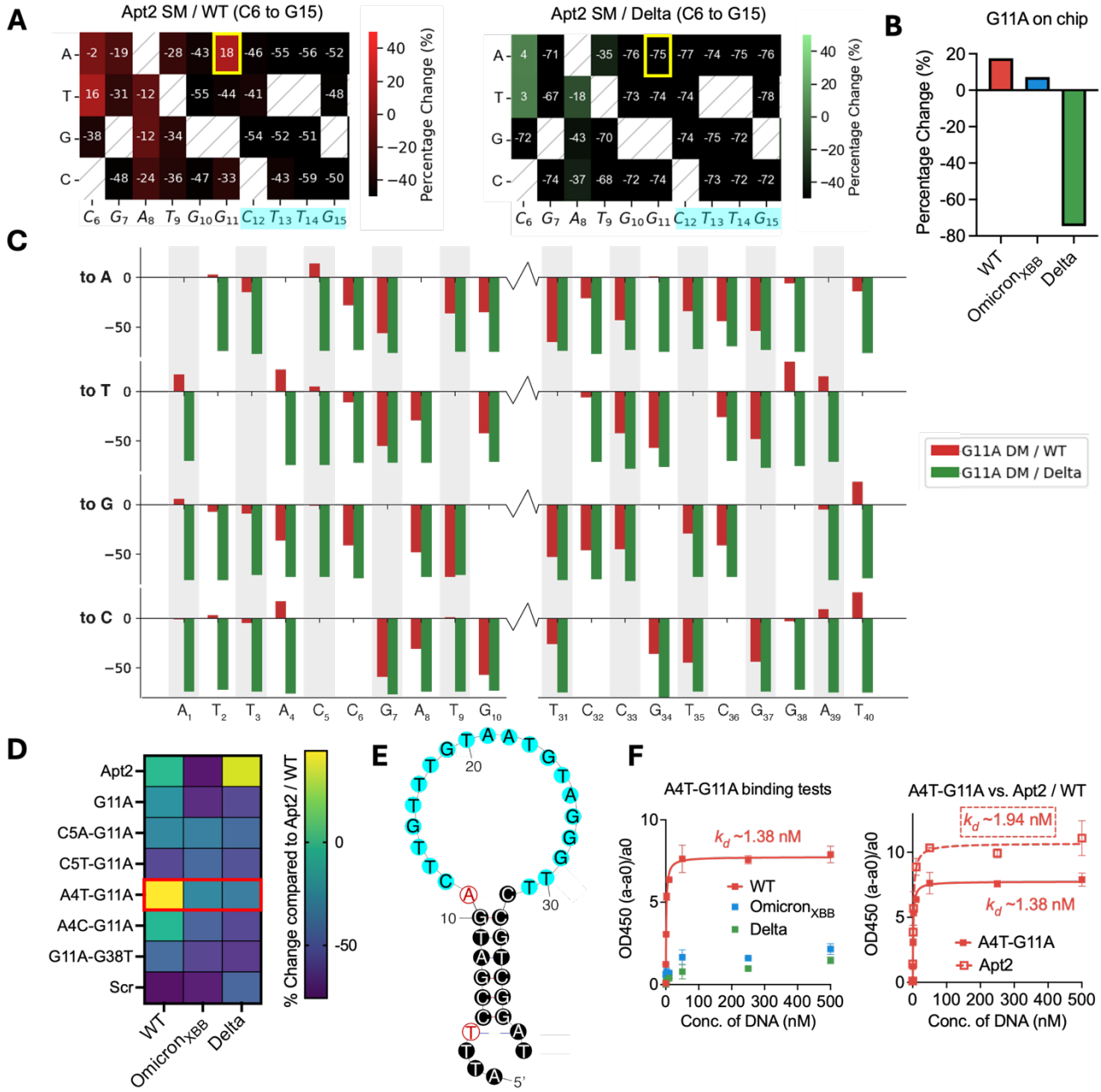

Fig. 5. Identifying Apt2-A4T-G11A as a more selective WT binder. (**A**) Part (residues C6 to G15) of the Apt2 single-mutation/WT (left, red) and Apt2 single-mutation/Delta (right, green) heatmaps. Bases within the loop motif were highlighted in cyan. G11A point mutation cells were highlighted in yellow boxes, which exhibited that G11A single mutants (SM) sustained the binding to WT but repressed the binding to Delta. Color bars are on the right of the heatmaps. Full heatmaps can be found in **fig. S6A** and **Fig. 3A**, respectively. (**B**) According to *MiSeq* screening, G11A's bindings to WT and Omicron$_{XBB}$ are similar to those of the original Apt2 (+18% and +7%), but G11A has a much lower binding to Delta (-75%). (**C**) Bar charts showed selected G11A-containing DMs' binding characteristics for WT (red) and Delta (green). The x axis represented the second mutation position; y axis showed the percentage change of the corresponding DM. (**D**) Streptavidin-mediated surface binding assay was used to test the bindings of WT-specific candidates selected from (**C**). The color scale is shown on the right. (**E**) A4T and G11A substitutions (highlighted by the red circles) were indicated in the mFold-predicted secondary structure of Apt2. The motif was highlighted in cyan. (**F**) ELONA showed that A4T-G11A remains to bind WT strongly (k_d ~1±0.04 nM vs. k_d ~2±0.4 nM, right), while it efficiently suppresses the binding to Delta and showing no detectable binding to Omicron$_{XBB}$ (left).

Analyze the binding pattern of Apt2 with *MiSeq* screening data and molecular dynamics simulations

To demonstrate that our platform can do more than screening and can also elucidate binding patterns useful for rational design, we examined the WT binding data. We initially assumed that the loop motif of Apt2 (residues C12 to T31) was solely responsible for binding WT, with the stem serving only as a structural scaffold or linker. However, this assumption was challenged by several observations. First, the previously identified Delta-enhanced binder A8G-A39T, which also showed improved WT binding, converted the A8·T35 Watson-Crick base pair into a G8·T35 wobble pair, a change expected to destabilize the structure. Second, the single mutant T9C, which should stabilize the stem by converting a T9·G34 wobble pair into a C9·G34 WC pair, instead completely abolished binding to all SARS-CoV-2 spike protein variants, both on chip (**fig. S9A**) and in ELONA (**Fig. 6A**). Further analysis revealed a functional asymmetry within the stem region. The upper three base pairs (T9·G34 to G11·C32) were highly intolerant to substitution by alternative WC or wobble pairs, suggesting that their role in binding extends beyond simple structural stabilization (**Fig. 6C**, above the dashed line). In contrast, the lower four base pairs (C5·G38 to A8·T35) exhibited substantially greater tolerance to base-pair alterations (**Fig. 6C**, below the dashed line), indicating a more limited contribution to protein binding.

To provide a molecular-level insight into the possible aptamer binding pattern, we further applied molecular dynamics (MD) simulations to investigate the structural behavior of the aptamer strands (**fig. S13-14**) (*76*). Structural superposition of two most populated cluster representatives (**Fig. 6B**), RMSF (Root-Mean-Square Fluctuation) (*77*) results (**Fig. 6D**) and the simulation trajectories (step = 100, **Supplementary Movies 1-3**) showed that Apt2, G11A and doped854 had very flexible tail (residues A1 to A4, A39 to T40, green) parts and equally rigid stem (C5·G38 to G11·C32, magenta) through the 10-ns simulation, suggesting that (i) truncating the 4-nt tail might not affect the binding; (ii) The upper stem (T9·G34 to G11·C32) remains paired as predicted by mFold, despite their additional role beyond structural stabilization; (iii) the alteration of the binding profiles could ascribe to the different sub-structures formed within the loop. Within the loop region, residues T16 to T20 in the original Apt2 showed greater stability during the 10-ns simulation compared to the Delta non-binders G11A and doped854. For the $\text{Omicron}_{\text{XBB}}$, residues A21 to T25 displayed distinct stabilization, coinciding with two of the three mutations that form a consecutive G tract and lead to an alternative folded conformation, which aligns with the observations from the ThT experiments (**Fig. 4G**). The loop regions exhibited sharp bending towards the stems, suggesting that the role of the upper stem might be to turn the aptamer into a preferred orientation to facilitate the binding, rather than to directly contact the proteins.

In addition, according to the RMSF plot, the G11A single mutant did not pose too much effect on the position of itself or neighboring bases, instead, it probably altered the selectivity of the binding by inducing the structure change in the loop. To investigate the G11A single mutant's binding mechanism, we performed two more simulations on G11C and G11T for comparison (**figs. S15-16**). On one hand, G11A and G11T share relatively similar conformations; however, with the latter one's structure much more dynamic and unstable according to the RMSF analysis (**Fig. 6F**). On the other hand, G11C held a distinct 3D structure compared to G11A, G11T and the original Apt2: G11C exhibits a uniquely inward-collapsed loop architecture centered along the short stem axis, whereas Apt2, G11A and G11T adopt a more open and outward-projecting loop conformation, resulting in highly similar overall topologies which could enable the WT binding (**Fig. 6E**).

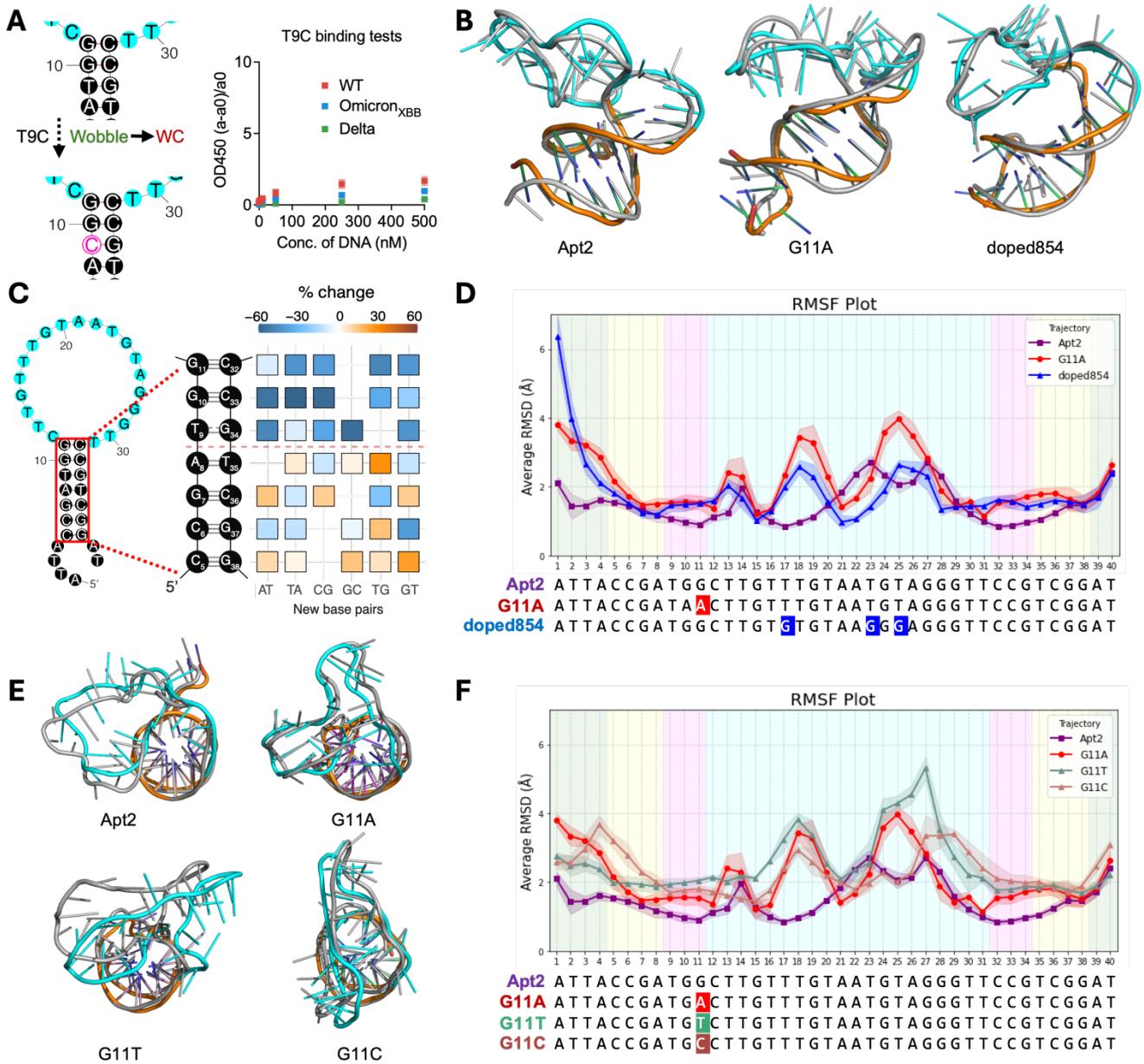

Fig. 6. Structure analysis with on-chip data and MD simulation. (**A**) ELONA for Apt2-T9C, indicating nearly no-binding to all spike protein variants. (**B**) Representative structures in each of the top two cluster superpositions within the latter 5 ns during the 10-ns simulation time of Apt2 (left; 33.6% and 33%), G11A (middle; 28.8% and 25%), and doped854 (right; 29.8% and 29.6%). The loop motifs were highlighted in cyan. The second top structures were highlighted in grey. (**C**) Base-pair-substitution heatmap for WT screening in the stem section. The x-axis indicates the new base pair for substitution; y-axis refers to the base pair being replaced. The pink dotted line separated base pairs with higher (below) and much lower (beyond) tolerance towards base-pair substitution. (**D**) RMSF (root-mean-square fluctuation) of Apt2, G11A and doped854. (**E**) Representative structures (top views) in each of the top two cluster superpositions within the latter 5 ns during the 10-ns simulation time of Apt2 (top left; 33.6% and 33%), G11A (top right; 28.8% and 25%), G11T (bottom left; 33.2% and 22.6%) and G11C (bottom right; 37% and 30.2%). The loop motifs were highlighted in cyan. The second top structures were highlighted in grey. (**F**) RMSF of Apt2, G11A, G11T and G11C. Color code for (**D**) and (**F**): tails: green; lower stem: yellow; upper stem: magenta; loop (motif): cyan.

500 **Selective diagnostics for one of the three variant strains with strand displacement reaction**

501 Here we demonstrate an application of the optimized aptamer through our platform, by converting
502 the highly WT-selective aptamer A4T-G11A (**Fig. 5**) into a strand-displacement fluorescent aptasen-
503 sor (*78*, *79*) specifically for SARS-CoV-2 WT spike protein detection. This design aimed to enhance
504 diagnostic precision, as highly specific detection of WT enables unambiguous strain assignment,
505 providing a foundation for generating strain-resolved data to inform studies of viral evolutionary
506 pathways.

507

508 We have proven that the lower stem of the aptamer can be replaced by other base pairs (**Fig. 6C**),
509 however, they cannot be truncated directly, based on the on-chip profiling results (**fig. S9C**). ELONA
510 validation confirmed that truncation at C5·G38 yielded the shortest construct that retained strong
511 WT binding (**fig. S19**). A4T-G11A was truncated, had the lower stem scrambled, and was extended
512 on the 5' end (*80–83*) and labeled with FAM (denoted as wt sensor), allowing hybridization with an
513 11-nt Iowa Black quencher-labeled complementary strand (Q-cDNA, **Fig. 7A**). Quenching efficiency
514 and baseline stability were evaluated prior to sensing measurements, with ~86% fluorescence
515 quenching achieved at a 1:2 aptamer: Q-cDNA ratio (**fig. S20A**).

516

517 Upon addition of 200 nM WT spike protein, fluorescence was enhanced by ~3 fold, significantly
518 exceeding the responses observed for Delta and $\text{Omicron}_{\text{XBB}}$ at the same concentration (**fig. S20B**).
519 A calibration curve was constructed from the fluorescence enhancement upon WT addition with a
520 four-parameter logistic (4PL) model (**Fig. 7B, left**) (*84*). The response exhibited a linear relation-
521 ship above 6.25 nM (**Fig. 7B, right**), from which a limit of detection (LOD) of 5.5 nM was estimated
522 using 3σ/slope. In contrast, the responses to Delta and $\text{Omicron}_{\text{XBB}}$ remained near baseline, demon-
523 strating strong specificity for the WT strain. We further evaluated sensor selectivity against a panel
524 of common proteins (**Fig. 7C**). All non-target proteins produced negligible fluorescence changes,
525 even at 5-fold higher concentrations (500 nM). Finally, the aptasensor maintained robust perfor-
526 mance in complex biological matrices, retaining ~2-fold signal enhancement in 40% human serum
527 (**Fig. 7D**), supporting its potential applicability in clinical samples (*85–87*).

528

529

530

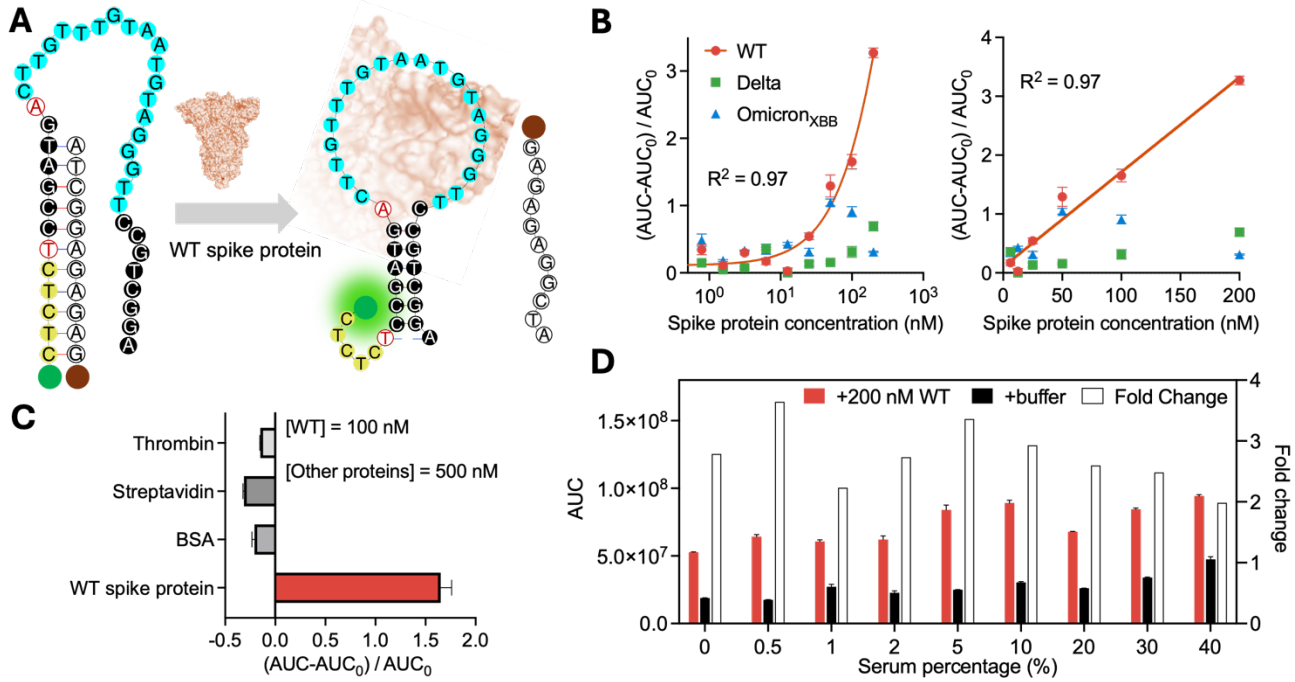

Figure 7. Precise diagnostics for SARS-CoV-2 WT with highly selective Apt2 mutant identified from the chip. (**A**) Schematic of the strand-displacement fluorescent sensor for rapid detection of SARS-CoV-2 spike proteins. The aptamer is modified with FAM (wt sensor), and the complementary DNA (cDNA) strand is modified with an Iowa Black quencher. Color code: loop: cyan; mutated bases: red circles; 5' overhang: yellow. (**B**) Calibration curves for WT spike protein detection. Data were fitted using a four-parameter logistic (4PL) model over the full concentration range (0.4-200 nM, left) and a linear model over the higher concentration range (6.25-200 nM, right). The limit of detection (LOD) was determined from the linear fit. (**C**) Selectivity analysis of the aptasensor against a panel of proteins. Non-target proteins were tested at 5× higher concentrations than the WT spike protein. (**D**) Stability and reactivity of the WT selective aptasensor in response to 200 nM WT spike protein across varying serum percentages (0-40%). For (**B**)-(**D**), each condition was measured in duplicate.

Discussion

Here we demonstrate a massively parallel strategy for rapidly diversifying and screening spike-protein-binding aptamers within days, enabling either retention of binding to newly emerged viral strains or enhancement of specificity toward a particular strain. Starting from an early WT-spike protein aptamer Apt2, we were able to identify a mutant with improved binding to the Delta strain, a nM-level binder for $\text{Omicron}_{\text{XBB}}$, and a WT-specific mutant with the binding selectivity significantly enhanced. The platform also provided systematic exploration of the sequence-function relationship, revealing the binding pattern structurally incorporated with molecular dynamics simulations. Finally, we turned the selected WT-specific aptamer into a strand-displacement fluorescence sensor, enabling the precise diagnostics for SARS-CoV-2 WT.

Although virus-targeting aptamers are unlikely to replace monoclonal antibodies (mAbs) in the foreseeable future because of intrinsic limitations, including rapid renal clearance, susceptibility to nuclease degradation, and limited ability to engage immune effector functions (*88–90*), nevertheless, aptamers remain attractive for biosensing and certain therapeutic applications because of their low production cost, high stability, small size, and low immunogenicity (*91–93*). Our results highlight an additional advantage of aptamers: adaptability. Once an initial antiviral aptamer has been identified, it can be rapidly reengineered to recognize newly emerging variants without requiring de novo selection each time. In addition to improving binding to evolved strains, this study also demonstrates that aptamers can be tuned for strain-selective recognition, which may be particularly valuable for diagnostic applications requiring precise variant discrimination and for generating strain-resolved datasets to support studies of viral evolution (*75*, *94–96*). We also demonstrated that aptamers offer functionality beyond binding (*97*) by converting a WT-selective variant into a strand-displacement fluorescent sensor. Their ability to be readily engineered into structure-switching aptasensors (*78*, *87*) provides a functional advantage over mAbs in certain sensing and diagnostic applications.

Repurposed NGS platforms offer a unique advantage for aptamer engineering by enabling massively parallel and quantitative interrogation of large, closely related sequence spaces in a single experiment. Prior studies (*38*, *42*, *45–47*, *49*, *50*, *55*, *59*), including our own (*30*, *53*, *58*, *98*), have demonstrated the versatility of this approach across diverse molecular systems, establishing repurposed NGS flow cells as a broadly useful platform for functional screening and sequence-property mapping. In this context, the key strength of NGS-based screening is not only throughput, but also the ability to retain and evaluate sequence diversity directly, rather than relying on iterative enrichment steps. This work extends this capability to a distinct challenge of rapid adaptation of antiviral aptamers to continuous viral evolution. Rather than treating each novel viral variant as a de novo discovery problem, we show that a repurposed NGS platform can be used to reengineer an existing functional aptamer through a random-rational hybrid diversification around a validated seeding aptamer. This strategy preserves the efficiency of high-throughput screening while introducing a more directed and adaptable framework for molecular redesign. As a result, the platform supports both rapid recovery of binding toward newly emerged variants and deliberate tuning of strain specificity, highlighting a specific advantage of our approach over existing repurposed NGS applications: it is not limited to characterizing or optimizing static molecular functions, but can instead be used to keep pace with an evolving biological target. The ability to iteratively screen related sequence libraries on the same platform further strengthens its practical value for emerging-pathogen surveillance and response.

Beyond the *MiSeq* screening, molecular dynamics simulations provide a structural framework for interpreting the variant-dependent binding behaviors observed experimentally (**Fig. 6**). These

results suggest that the upper stem region, rather than serving solely as a passive scaffold, contributes directly to target recognition. Within the loop, distinct segments appear to govern variant-specific interactions, indicating that binding specificity is distributed across multiple localized motifs rather than confined to a single region. Notably, substitutions at position G11 exert a pronounced effect on global conformation. While the adenine substitution preserves a loop orientation compatible with binding, alternative substitutions lead to structural rearrangements or destabilization that disrupt target interaction. This provides a structural explanation for the experimentally observed selectivity at this position. Importantly, this suggests that aptamer optimization need not rely solely on empirical screening, but can be guided by structural considerations, providing a foundation for rational aptamer design in response to evolving viral targets.

More broadly, this work addresses a challenge highlighted by the COVID-19 pandemic: the lack of a modular molecular-recognition platform that can be readily reprogrammed to keep pace with viral evolution. Although the demonstration here was SARS-CoV-2 spike protein, this strategy should be extendable to other viral targets. In a future outbreak scenario, this could enable a practical workflow in which libraries diversified around an existing antiviral aptamer are prepared on repurposed NGS flow cells and screened immediately against newly emerging variants. The library size can also be scaled up by utilizing other types of NGS flow cells (*MiSeq*, *HiSeq* and *NextSeq* and *NovaSeq*) or leveraging multiple chips. Because these chips can remain functional for years under appropriate storage (when soaked in an appropriate buffer and stored at 4 °C) and can be reused for sequential assays, the same platform could support iterative screening as new strains arise, without requiring complete library redesign unless full target escape occurs.

In summary, we developed a new method to rapidly evolve aptamers against newly emerging viral strains, providing a potential tool for responding to current and future pandemics. We demonstrated its effectiveness by identifying sequences that bind the 9-amino-acid-mutated Delta variant and the 39-amino-acid-mutated $\text{Omicron}_{\text{XBB}}$ variant within a diversified pool based on Apt2, which was originally selected against the baseline strain WT. This streamlined workflow can be readily adapted to a broad range of biomedical applications in the future.

Materials and Methods

Doped pool mutation probability calculation

Let the number of the doped bases be L and the mutation rate be m. The theoretical number of the mutated bases n follows the binomial distribution.

$$n \sim binomial(L, m)$$

$$Pr_0(n|m, L) = \binom{L}{n} m^n (1-m)^{L-n}$$

With the n>2 filtration, the ultimate probability of n given m and L can be calculated as follows.

$$Pr(n|m, L) = \begin{cases} 0, & n < 3 \\ \dfrac{Pr_0(n|m, L)}{1 - \sum_0^2 Pr_0(i|m,\ L)}, & n \geq 3 \end{cases}$$

In this study, we set L = 20 (only dope the loop region) and m = 0.1. The theoretical and actual frequency of each mutation number can be found in **fig. S2**.

638 **Apt2 library construction for NGS sequencing**
639 Apt2 variants library with PCR primers was synthesized by Twist Biosciences. Forward and reverse PCR
640 primers with Illumina sequencing adapters were synthesized by IDT. The overlap extension PCR process
641 was performed using Q5 High-Fidelity 2× Master Mix from NEB (#M0492) following protocol provided
642 by the manufacturer using a thermal cycler (Applied Biosystems). After PCR amplification, the library
643 amplicons were purified with the PCR cleanup kit (#T1030, Monarch). The length of the amplicons was
644 checked with a 2% agarose gel (**fig. S21**). The concentration and quality of the purified library amplicons
645 was quantified using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). After PCR pro-
646 cess, PhiX control V3 sequences (#FC-110-3001, Illumina) were diluted and mixed with Apt2 amplicons
647 to prepare a 20 µl of 2 nM solution according to the requirement of our sequencing facility. The coverage
648 for both PhiX and the library sequences were targeted to be 15% ~ 20% of overall sequencing space. The
649 mixture was then subjected to *MiSeq* PE75 sequencing and the final loading concentration was at 8 pM.
650
651 **Illumina *MiSeq* chip preparation**
652 After sequencing, *MiSeq* chips were stored at 4°C in 20 mM sodium phosphate buffer (SPB, pH 6.6).
653 Before experiments, the *MiSeq* chip was incubated with 20 µl 0.1 N NaOH solution for 5 min at room
654 temperature and rinsed with 20 µl 20 mM SPB (pH 6.8) 3 times to remove excess NaOH. This step was
655 to denature DNA strands which might contain residual fluorescent dyes from the sequencing process. The
656 chip was further photobleached by sequentially exposing each FOV (5 rows, 65 FOVs per row, 2 layers,
657 220 × 220 μm^2 each FOV) under an 80% excitation power for 1 min. The PhiX sequences were then
658 labeled using an Atto488-tagged probe (1 µM and 20 µl) at 37°C for 40 min. The flow cell was rinsed 3
659 times by the screening buffer (40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 6 mM $MgCl_2$, 2.5 mM $CaCl_2$,
660 2.5 mM KCl, 0.1% Tween 20, 0.2 mg/mL BSA) to remove excess fluorophores. 20 µL 100 nM of His-
661 tagged SARS-CoV-2 spike protein variants (#SPN-C52H3, #SPN-C52He, #SPN-C5248; Acro Biosys-
662 tems) were then introduced to the chip surface by pipetting and incubated at 37°C for 1 hour. The protein
663 was stored in nuclease-free water at 1 µM and further diluted in screening buffer at room temperature for
664 30 min. The flow cell was rinsed 3 times by the screening buffer to remove unbound proteins. 20 µL of
665 6×-His-tag mAb-Alexa647 conjugate (#MA1-21315-A647, Invitrogen) were then introduced to the chip
666 surface by pipetting and incubated at room temperature for 15 min. The mAb-dye conjugate was diluted
667 at 1:500 ratio with the screening buffer. Excess conjugate was washed away with screening buffer.
668
669 **Fluorescence microscopy and chip image acquisition**
670 An open-source software, Micro-Manager (*99*), was used to control an sCMOS camera (ORCA-Flash 4.0,
671 Hamamatsu), an xyz translation stage (ProScan III, Prior Scientific), and an auto-shutter (Lambda SC,
672 Shutter Instrument) on an Olympus IX71 fluorescence microscope for all our *MiSeq* screening experi-
673 ments. An LED illuminator and a 60× water-immersion objective (UPLSAPO60XW, Olympus) were
674 used in the IX71 system. The detailed setup of images acquisition could be found in our previous reports.
675 (*30*, *53*) In brief, for each round of experiments, we acquired 3 rows of TIFF images with 60 field of views
676 (FOVs, 220×220 μm^2) per row on both sides of the chip (totally 360 images). This covers 5.81 mm^2
677 surface area. For each row of images, we first recorded fiducial marker images (1 second exposure time,
678 green channel, Ex/Em: 480/40 nm, 535/50 nm), and then acquired spike protein images with red channels
679 (Ex/Em: 620/60 nm, 700/75 nm, cat. no. 49006, Chroma) under the same imaging settings. The scanning
680 step size (220 µm per step) and exposure time at each position were all precisely controlled by our auto-
681 scan algorithm and shutter control.
682
683 **Illumina *MiSeq* chip regeneration**
684 After loading the samples to the chip and acquiring the images, the flow cell was regenerated by digesting
685 the proteins and denaturing the anchored DNA sequences. 20 µL proteinase K mix (#P8107, NEB) was
686 added to the flow cell by pipetting and incubated at 40°C for 2 hours. Excess proteinase and protein
687 fragments were washed away by 20 mM SPB buffer. This was followed by a 0.1N NaOH denaturation.
688 Excess base was removed by 20 mM SPB buffer.

Fluorescence image alignment algorithm

To identify the sequences behind polonies, we applied a revised imaging alignment algorithm from **C**hip-**H**ybridized **A**ssociated **M**apping **P**latform, or CHAMP (*55*), which was developed by Jung *et al.* The detailed description of implementing CHAMP algorithm on epi-fluorescence-microscope-acquired images could be found in our previous report (*30*, *53*). In brief, FASTQ coordinates were first Gaussian blurred to build a synthetic image and then transformed into Fourier space. TIFF images were also Fourier transformed and performed cross-correlation with transformed FASTQ images to register the rough position of each TIFF image. If the maximum cross-correlation value is larger than that of control position (other positions with higher read density), this TIFF image will perform precision alignment using least-squares constellation mapping to the potential target region to find detailed parameters (e.g., scaling factor, rotation angle). The output files included the x-y coordinates and the corresponding sequence ID of each cluster within the FOV and were saved individually according to different image positions. To analyze our Apt2 images, we corrected the uneven illumination using flat-field correction and bootstrapped to compute the median intensity of each activator (baseline corrected) in order to rank the Apt2 mutants' brightness.

Flat-field correction

To correct the uneven illumination across each FOV. We implemented flat-field correction. A Gaussian-blur filter ($\sigma = 100$) was applied on each FOV itself to generate the flat-field reference image for each FOV. The corrected images used for the following analysis were computed as follows:

$$I_{corrected} = I_{aptamer}\text{-}I_{dark} \,/\, (I_{flat\text{-}field\ image}\text{-}I_{dark})$$

Where $I_{corrected}$ is the flat-field-corrected images, $I_{aptamer}$ is the original TIFF images acquired under red channel, $I_{flat\text{-}field\ image}$ is the TIFF image after Gaussian blurring, and I_{dark} is the dark image acquired using 1 second exposure time when illumination source is turned off. When calculating the percentage changes, the intensities were subtracted by 1 to remove the average background from the protein and dye's non-specific binding to the chip surface.

ELONA binding assay

The binding affinities quantified in this study were all produced with ELONA (*65*) (enzyme-linked oligonucleotide assay). Briefly, 96-well high-binding microplates (#3690, Corning) were coated with the SARS-CoV-2 spike protein variant at a concentration of 1.33 µg/mL in PBS (pH 7.4, Invitrogen #AM9624) and incubated at 4 °C overnight. After washing with PBST (PBS containing 0.05% Tween-20), wells were blocked with blocking buffer (5% w/v BSA in PBST) for 1 hour at room temperature. Biotin-labeled aptamers (IDT) at various concentrations (3 duplicates for each concentration) were then added and incubated for 90 min at room temperature to allow binding. After washing, bound aptamers were detected using streptavidin-HRP conjugate (#RABHRP3-600UL, Millipore Sigma), followed by addition of TMB substrate (#555214, BD Bioscience). Washing was introduced between the steps with the screening buffer. The reaction was stopped with stop solution (10% H_2SO_4), and absorbance was measured at 450 nm using a microplate reader (SpectraMax i3, Molecular Devices). Control with scrambled Apt2 (Scr) were included. OD450 values were normalized with the lowest value on the whole plate.

$$OD450_{normalized} = (OD450_{raw} - OD450_{min})/OD450_{min}$$

The dissociation constant (k_d) was generated by fitting the concentration (c)-absorbance (A) curve to a Hill equation with a dynamic Hill slope (h), given that the signal produced by an enzymatic reaction might not be in a strictly linear relation with the binding. Each of the 3 duplicates was fitted to the model separately. The reported k_ds are composed of the mean k_d of the duplicates with the standard deviation:

$$\frac{A - A_0}{A_0} = \frac{B_{max} c^h}{k_d + c^h}$$

Streptavidin-mediated surface binding assay

In addition to ELONA, streptavidin-mediated surface binding assay was also used to estimate and compare the bindings for the DNA sequences and spike protein variants. Biotin-labeled aptamers (IDT) at 2.5 μM (3 duplicates) were then added to the 96-well streptavidin-coated pre-blocked black microplates (#15503, Thermo Fisher). and incubated for 2 hours at room temperature to be anchored onto the plate surface. After washing, 33 nM spike protein variants were added and incubated for 1 hour to allow binding, followed by addition of the 0.002× 6×-His-tag mAb-Alexa488 conjugate (#MA1-21315-A488, Invitrogen) for 30 min at room temperature. After washing away excess conjugate, 100 μL screening buffer was added to each well to enable the fluorescence measurement. Fluorescence was measured at Ex/Em 485/535 nm using the microplate reader. Control with scrambled Apt2 (Scr) were included.

3D structures prediction for Apt2 and its mutants of interests

Starting from the predicted secondary structure of Apt2 by mfold (*64*), we replaced all Ts with Us to generate its equivalent ssRNA rApt2. The corresponding 3D structures rApt2 was then modeled and visualized in RNAComposer (*100*, *101*). The preliminary 3D structure of Apt2 was then obtained by substituting bases T back for U. To relax the aptamer system, a refinement process was carried out based on molecular dynamics simulation to produce the input structure for the MD simulation. The input structures of G11A, doped854, G11C, G11T was generated by performing mutagenesis to the Apt2's 3D structure by PyMol (*102*).

Molecular dynamics simulation of selected aptamers

The 3D structures of Apt2 and mutants of interests were obtained as described in the previous section. The structures were then soaked into a box of water with a size of 65Å × 65Å × 65Å. Counter ions such as Na^+ were added to neutralize the system. Additional NaCl, KCl, $MgCl_2$ and $CaCl_2$ were added to obtain salt concentrations of 150, 6, 2.5 and 2.5 mmol/L, respectively, to mimic the correct buffer condition. The systems underwent restrained minimization and restrained gradual heating processes to reach equilibration before 10-ns production simulation for analysis (*103*, *104*). All simulations in this study were performed with the AMOEBA polarizable force field (*105–107*).

Simulated frame clustering

A pairwise RMSD (Root mean square deviation) matrix was computed from the aligned frames to serve as the distance metric. We applied agglomerative hierarchical clustering to this matrix, iteratively merging the closest clusters based on a complete linkage method. The resulting dendrogram was cut at a threshold of RMSD = 4 to partition the trajectory into distinct conformational states. and then the centroid is taken as representative structure. The central frames were collected from each cluster and superposed to visualize for each mutant of interests.

RMSF calculations

Root mean square fluctuation (RMSF) analysis was conducted to evaluate the flexibility of individual residues within the backbones of aptamer structures during MD simulations . The RMSF values were calculated over the equilibrated trajectory using PyLOOS, according to the equation:

$$(i) = \sqrt{\langle (r_i(t) - \langle r_i \rangle)^2 \rangle_t}$$

where $r_i(t)$ represents the position of atom i at time t, and $\langle r_i \rangle$ denotes its time-averaged position after aligning each frame to the first one. Only the equilibrated portion of the trajectory (last 5 ns) was used for the calculation.

784 **Strand-displacement fluorescent sensor test**

785 The fluorescence aptasensor experiments were performed in a half-area black 96-well microplate
786 (#675076, Greiner Bio-One) using the microplate reader mentioned in the previous section. The excitation
787 wavelengths were set to 480 nm; the emission spectra were collected from 510-700 nm. 2.5 μL of 1 μM
788 wt sensor, 40.5 μL of screening buffer and 2 μL of proteins at different concentrations were premixed in
789 the wells and incubated at RT for 30 min. 5 μL of 1 μM Q-cDNA was then added the wells before meas-
790 uring the fluorescence. The fluorescence intensity for each sample was calculated through the area under
791 curve (AUC) of the emission spectrum. For the spike-in serum test, 50% human serum was used to yield
792 buffer with 0-40% serum. The mixture was heat-inactivated at 85°C for 10 min. The supernatant was
793 collected as the new buffer. The data were represented as $(AUC-AUC_0) / AUC_0$, where AUC_0 denotes the
794 fluorescence with the mixture of 2 μL of screening buffer, and AUC was the fluorescence with 2 μL of
795 protein addition.
796

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Supplementary Materials

Tables S1 to S3, Notes, figs. S1 to S21, Movies S1 to S3