Quantitative Characterization of Partitioning Stringency in SELEX

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Abstract: Maintaining stringent conditions in SELEX (Systematic Evolution of Ligands by EXponential enrichment) is crucial for obtaining high-affinity aptamers; however, excessive stringency greatly increases the risk of SELEX failure. The control of stringency remains a technical challenge reliant solely on intuition, largely due to the absence of a measure of stringency. Essentially, researchers increase or decrease stringency through its influencers without defining and quantitating it. This study was motivated by our insight that while stringency may not be easily definable via its multiple influencers, it can be delineated by its effect: increasing stringency leads to a decrease in the normalized quantity of binders at the output of partitioning. Building on this insight, we introduce a measure of stringency called the Binder-to-Nonbinder Ratio (BNR) and derive an expression for its experimental determination using a single experimental tool: quantitative PCR. The outcomes of our theoretical analysis and the result of SELEX experiments targeting three distinct protein targets underscore the importance of maintaining a BNR significantly greater than zero to avoid SELEX failure due to excessive stringency – a principle that we term the SELEX non-failure criterion. Utilizing BNR as a measure of stringency alongside this criterion will enable researchers to rationally control SELEX progress.

INTRODUCTION

Every quantitative parameter requires a measure for its meaningful use. Fundamental parameters, such as mass, time, length, and charge, rely on established reference standards for measurement. For nonfundamental parameters, deriving measures necessitates tracing them back to fundamental ones. Take velocity or acceleration, for instance; their measures stem from their definitions, which tie them to their influencing factors. Velocity, for example, is delineated as the ratio of distance traveled to time elapsed. However, some parameters pose challenges in expressing them solely through their influencers. A quintessential example is force, which perplexed scientists for centuries prior to Newton's take on it. His groundbreaking insight was recognizing that force couldn't be generically defined through its influencers but rather should be described through its effect, namely acceleration. This revelation led to the formulation of Newton's second law, $F = ma$, marking the genesis of quantitative science.

In the presented research, we concentrate on the rigorous delineation of partitioning stringency in SELEX (Selection of Ligands by EXponential enrichment), a method employed for selecting oligonucleotide aptamers.[1] Stringency serves as a quantitative parameter — adjustable through changing its quantifiable influencers — yet lacks a definitive measure. Similar to force, stringency of partitioning eludes a straightforward expression through its multiple influencers. Our endeavor aims to define stringency of partitioning through its effect and underscore how establishing a measure for stringency can enhance SELEX methodologies.

Aptamers, oligonucleotides with the ability to bind targets tightly and selectively through multiple non-covalent bonds, find significant utility primarily in diagnostic and therapeutic applications.[2-11] Typically sourced from random-sequence oligonucleotide libraries, aptamers are selection from highly-diverse random-sequence oligonucleotide libraries through SELEX, an iterative process entailing successive rounds of three primary steps (see Figure 1).

In Step 1, the starting library reacts with the target, allowing target-binding oligonucleotides (referred to as binders) to form

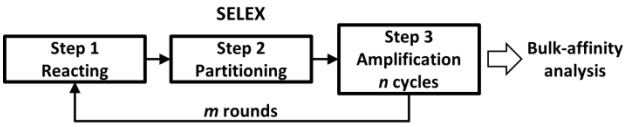

Figure 1. Schematic representation of SELEX. See text for details.

complexes with the target while leaving target-nonbinding oligonucleotides (nonbinders) unbound. It's important to note that the terms "binder" and "nonbinder" merely designate the state of oligonucleotides under specific conditions at the end of Step 1. We acknowledge that strictly speaking all oligonucleotides are binders of different strengths, i.e., capable of forming target–binder complexes,

$$\text{Binder} + \text{Target} \underset{K_d}{\rightleftharpoons} \text{Complex} \qquad (1)$$

with the equilibrium dissociation constants (K_d) spanning from 0 and ∞. Decreasing target concentration will force fewer weak binders than strong binders to form target–binder complexes.

Step 2 is partitioning target−binder complexes from nonbinders. Partitioning is imperfect, *i.e.*, at the output of partitioning, the binders are always contaminated by nonbinders. In addition, partitioning entails duration during which less-stable complexes, characterized by higher dissociation rate constants (k_{off}), dissociate. Consequently, prolonged partitioning disproportionately reduces the population of weak binders compared to strong binders.

Step 3 encompasses amplification, typically via polymerase chain reaction (PCR), of all oligonucleotides (both binders and nonbinders) collected in the second step, generating a binder-enriched library with an increased ratio of strong to weak binders (or binders to nonbinders). This binder-enriched library is then employed in the subsequent round of SELEX.

The binding fitness of the binder-enriched library is assessed through bulk-affinity assays, marking the termination of SELEX when bulk binding ceases to exhibit significant improvement or reaches a desired threshold. The absence of affinity maturation, denoting statistically significant improvement in bulk affinity across rounds, signals SELEX failure. Bulk affinity assays, although resource-intensive and

semi-quantitative, remain the primary means of monitoring SELEX progress in the absence of more refined techniques.

A binder-enriched library with high bulk affinity produced by SELEX can be used "as is" similarly to polyclonal antibodies.[12] In the majority of cases, however, "monoclonal" binders are produced from such a library in several post-SELEX steps, which were not included in the definition of SELEX by its inventors,[1] and are not shown in Figure 1. First, DNA in the binder-enriched library is sequenced, typically using high-throughput sequencing. Second, the sequences are analysed to exclude known nonbinders and rank sequences based on their frequency in the library. Finally, top-ranked sequences are synthesized, and their affinity to the target is assessed in a binding assay. *These post-SELEX steps are not required to characterize binder enrichment and affinity maturation; therefore, they are excluded from our consideration, which focuses solely on the processes of core SELEX shown in Figure 1.*

When performing SELEX, experimentalists typically aim at selecting strong binders, ideally with the lowest achievable values of both K_d and k_{off}. The preference for such binders in SELEX can be achieved by increasing stringency of partitioning.[13-18] As explained above, stringency can be increased by decreasing target concentration in Step 1 and/or increasing the duration of partitioning in Step 2. A means by which the stringency is increased influences the characteristics of the selected binders: decreasing target concentration puts pressure towards the selection of binders with low K_d while increasing the duration of partitioning does this for binders with low k_{off}. Whereas stringency is very attractive as a general descriptor of selective pressure, it has a major limitation. It has no quantitative definition and, accordingly, no measure is assigned to it. Therefore, stringency is only suitable for qualitative/comparative characterization of selective pressure, e.g., "higher stringency" or "lower stringency".

It is intuitive and has never been challenged that tuning SELEX towards the selection of strong binders requires increasing stringency. This relation between stringency and enrichment of strong binders in the library has been demonstrated both theoretically and experimentally.[13-18] Thus, it is desired to have high stringency in SELEX. It is equally intuitive and requires no proof that stringency cannot be increased indefinitely without inducing SELEX failure. Thus, a logical and still unanswered question is how high the plank of stringency can be raised before no binder in the library can get over it. The importance of this question largely motivated our study, which was also inspired by our insight that answering this question is impossible without defining stringency quantitatively.

In this study, we first defined stringency quantitatively through its effect: its influence on the normalized quantity of binders at the output of partitioning. We deduced that the quantity of nonbinders can serve as a normalization parameter and introduced the Binder-to-Nonbinder Ration (BNR) as a measure of stringency. We then used a simple math formalism of partitioning to derive a link between BNR and two parameters easily determinable experimentally with qPCR: the total oligonucleotide quantities at the output of partitioning in the presence and in the absence of the target. Importantly, these quantities are routinely measured by some SELEX practitioners and therefore can be analyzed retrospectively. We used inductive logic to produce a SELEX non-failure criterion: BNR must be significantly greater than zero in each round of SELEX. Using this criterion requires experimental determination of the standard deviation of BNR, which has not been done so far but advantageously can be done retrospectively. Finally, we conducted a comprehensive set of experiments to confirm the validity of the SELEX non-failure criterion. The results of the experiments fully support the theoretical requirement of keeping BNR significantly greater than zero. Thus, our findings suggest that the quantitative measure of stringency can serve as an effective tool for monitoring round-to-round progress of SELEX. In addition to this utilitarian function, the quantitative measure of stringency will advance our fundamental understanding of SELEX.

RESULTS AND DISCUSSION

Deducing a Measure of Stringency. Our first task was to define stringency quantitatively. Stringency depends on many factors including types and concentrations of the target and library, buffers used in Steps 1 and 2, durations of Steps 1 and 2, as well as a method and conditions of partitioning in Step 2 (Figure 1), etc. It may sound counterintuitive at first glance, but incorporating multiple dependencies into a single parameter requires that this parameter is not derived upon any influencers of stringency. Instead, it should be based on the effect of changing stringency as in the Newton's definition of force which we discussed in the introduction. The sole generic effect of increasing stringency is the decrease in the quantity of binders at the output of partitioning. Therefore, we naturally decided to base a measure of stringency on this quantity, which must be normalized to make the quantity independent on the load of the library at the input. A well-established formalism of partitioning shown in Figure 2 will help us to derive a measure of stringency using the normalized quantity of binders.[19-24]

Partitioning is, in essence, a filter rejecting nonbinders (N) and letting binders (B) through. The input and output libraries (L) are comprised of binders and nonbinders and their quantities are linked as:

$$L_{in} = B_{in} + N_{in}$$
$$L_{out} = B_{out} + N_{out} \quad (2)$$

Two major parameters characterizing partitioning are its transmittances for binders (k_N) and nonbinders (k_B) ranging between 0 and 1 and defined as:

$$k_N = N_{out} / N_{in}$$
$$k_B = B_{out} / B_{in} \quad (3)$$

In ideal partitioning, $k_N = 0$ and $k_B = 1$, but in real partitioning, $k_N > 0$ and $k_B < 1$. The value of k_B/k_N is the efficiency of partitioning – the major parameter characterizing the quality of partitioning. Note, k_B/k_N correlates with stringency but can hardly be used as a practical measure of stringency because the experimental determination of k_B is not straightforward.

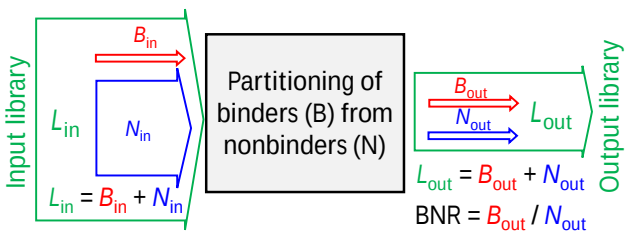

Figure 2. Schematic representation of partitioning of binders (B) from nonbinders (N). See text for details.

In the decisive initial rounds of SELEX, binders always constitute only a minute fraction of the input library: $B_{in} << N_{in}$. This inequality constitutes the sole assumption in our derivation of the measure of stringency.

N_{out} represents a background caused by the leakage on oligonucleotides through partitioning even in the absence of the target. If $B_{in} << N_{in}$ (our assumption), then N_{out} is a constant value that can be used to normalize B_{out} through constructing a unitless parameter naturally termed Binder-to-Nonbinder Ratio (BNR):

$$\mathrm{BNR} = B_{out} / N_{out} \tag{4}$$

Note, we drop the subscript "out" for BNR as the binder-to-nonbinder ratio of the input library is not considered here.

Being a ratio of quantities of DNA, BNR is a unitless parameter. Increasing stringency leads to decreasing BNR down to zero. Because N_{out} (background) cannot be zero, decreasing stringency will always lead to a finite upper limit of BNR. This limit depends only on the background: the lower the background, the higher the upper limit of BNR. To conclude this consideration, BNR is clearly and logically linked with stringency and can be used as a quantitative descriptor of stringency.

Practical Means of BNR Determination. To be a practical measure of stringency, that can be easily adopted and used by aptamer developers, BNR must be determinable with the tools of the core SELEX loop shown in Figure 1, i.e., partitioning and PCR. Based on BNR definition (eq. (4)), it can be calculated if both B_{out} and N_{out} are known. N_{out} can be determined simply by sampling the input library to the partitioning in the absence of the target. Under the assumption of $B_{in} << N_{in}$, the output library does not contain binders in the absence of target, and according to the second equation in eq. (2) we get:

$$N_{out} = L_{out,-T} \tag{5}$$

where "–T" designates the absence of the target. When the target is present in partitioning (+T), the output library may contain binders, and their quantity can be expressed by rearranging the second equation in eq. (2) and using eq. (5) as follows:

$$B_{out} = L_{out,+T} - N_{out} = L_{out,+T} - L_{out,-T} \tag{6}$$

By substituting eqs. (5) and (6), into eq. (4) we can express BNR through two experimentally determinable parameters, $L_{out,+T}$ and $L_{out,-T}$:

$$\mathrm{BNR} = \frac{L_{out,+T}}{L_{out,-T}} - 1 \geq 0 \tag{7}$$

The values of $L_{out,+T}$ and $L_{out,-T}$ can be easily determined by quantitating DNA with qPCR in the output libraries when the input library is sampled for partitioning in the presence and the absence of the target, respectively.

$L_{out,+T}$ and $L_{out,-T}$ are likely measured regularly by aptamer selectors. For example, the authors were informed that Somalogic has always measured $L_{out,+T}$ and $L_{out,-T}$. Thus, BNR can be calculated retrospectively to help researchers understand the predictive power and limitations of BNR as a risk-management tool in SELEX (see below).

Eq. (7) for BNR was derived from the mass balance (eq. (2)) and the definition of BNR (eq. (4)) using a single simplifying assumption: $B_{in} << N_{in}$. Accordingly, eq. (7) does not require any validation and is applicable to all methods of partitioning and all types of libraries and targets in the early rounds of SELEX when nonbinders dominate the library.

BNR as a "Risk-Management Tool" in SELEX. Let's employ BNR to address the question regarding the highest stringency in SELEX that does not result in its failure. The progression of SELEX necessitates that the output library contains binders. Theoretically, this necessity translates into a requirement of BNR > 0. However, in practice, BNR is always determined with some experimental uncertainly. Thus, for BNR to indicate the presence of binders in the output library, BNR must be statistically significantly greater than zero:

$$\mathrm{BNR} > n\sigma_{\mathrm{BNR}} \tag{8}$$

where σ is the standard deviation of BNR and n is the confidence level (1, 2, 3, …). The value of σ_{BNR} is defined by rules of error propagation from eq. (7) as follows (see Section S1 for details):

$$\sigma_{\mathrm{BNR}} = \sqrt{\left(\frac{\sigma_{L_{out,+T}}}{L_{out,-T}}\right)^2 + \left(\sigma_{L_{out,-T}}\frac{L_{out,+T}}{L_{out,-T}^2}\right)^2} \tag{9}$$

where σ with subscripts $L_{out,-T}$ and $L_{out,+T}$ are standard deviations of $L_{out,-T}$ and $L_{out,+T}$, respectively. To minimize the amount of experimentation required for the determination of σ_{BNR}, we can make a reasonable assumption that the standard deviation is proportional to the square root of L_{out}, which is common for random noise (see Section S1 for details):

$$\sigma_{L_{out,+T}} = \sigma_{L_{out,-T}}\sqrt{\frac{L_{out,+T}}{L_{out,-T}}} \tag{10}$$

By substituting eq. (10) into eq. (9) we obtain the following formula for the assessment of σ_{BNR}:

$$\sigma_{\mathrm{BNR}} \approx \sigma_{L_{out,-T}}\frac{L_{out,+T}}{\left(L_{out,-T}\right)^2}\sqrt{1+\frac{L_{out,-T}}{L_{out,+T}}} \tag{11}$$

which only requires the determination of the standard deviation of $L_{out,-T}$. The latter can be found by conducting repetitive sampling for partitioning of a defaults amount of the library without the target and quantitating DNA with qPCR. This value is expected not to change significantly for the same method and identical conditions of partitioning and thus can be determined only once provided that the method and conditions are not changed.

Eq. (7) is the necessary condition for SELEX to progress, defining the highest stringency as the one corresponding to BNR = $n\sigma_{\mathrm{BNR}}$. We can choose the confidence level n based on the specifics of selection. As a default value, we currently consider n = 3, although coming to a consensus value of n will likely require the analysis of a large amount of experimental data of SELEX accompanied by the values of BNR and σ_{BNR}. It is also an open question whether n will depend on a method of partitioning used in SELEX. Machine learning tools may aid in the analyses required for answering this and other questions, which are beyond the scope of this work. The next step in this proof-of-concept work was to confirm the conclusions of the theoretical framework experimentally.

Experimental Design. Our experiments did not aim to select individual aptamers, that would necessarily require using post-SELEX steps — these were purposefully excluded from our consideration. Instead, our experimental study aimed to test how varying BNR (via varying target concentration) influences the SELEX progress assessed through affinity maturation via a bulk-affinity assay of the output library. This assay relies on optimized measurements of the fraction of unbound library

(R),[25] and is deemed to provide accurate comparison of SELEX carried out at different target concentrations.

To investigate how target aptagenicity affects BNR, we conducted SELEX for two target proteins: His-tagged MutS (93 kDa) and non-tagged thrombin (35 kDa), for both of which successful aptamer selections were performed several times.[26-29] A single DNA library with 40 randomly positioned nucleotides was used in all selections. We chose four target concentrations covering two orders of magnitude: 500, 100, 10, and 1 nM. Capillary electrophoresis (CE)-based SELEX is typically conducted without round-to-round increase of stringency; therefore, we planned to conduct four selections with constant target concentrations (500, 100, 10, and 1 nM) through the rounds as our main set of experiments. However, considering that a graduate increase of stringency is often used for surface-based partitioning, we also included a similar experiment in our study.

We opted for partitioning by CE, which reliably supports partitioning efficiency (k_B/k_N) of 10^4–10^9 (orders of magnitude higher than that of surface-based partitioning).[21, 24, 28, 30-39] Partitioning by CE typically facilitates SELEX completion in 3 to 4 rounds. It was demonstrated multiple times that additional rounds of CE-based partitioning are not productive or can even be counterproductive, leading to worsening bulk affinity.[30-33, 40, 41] Therefore, we limited our SELEX to three rounds, which was deemed sufficient for conclusions drawn in this work. Due to the pronounced adhesion of both target proteins to the bare inner wall of the fused-silica capillary, we used capillaries with a polyvinyl alcohol (PVA) coating, which diminished the adhesion along with suppressing the electroosmotic flow (EOF).[42, 43] The suppressed EOF necessitated the use of a CE mode termed complex-last NECEEM, in which the unbound DNA (nonbinders) moves inside the capillary faster that the protein–DNA (target−binder) complexes.[24] In the next paragraph, we provide some essentials of our SELEX.

In Step 1, the target was mixed with the library, and the mixture was incubated for 1 hour to allow the formation of target−binder complexes, serving as a positive control. As a negative control, we used a mixture of the library with a target matrix void of the target. In Step 2, a small volume of the mixture was injected into the capillary (the resulting sample plug's length was approximately 5% of the capillary length), and target−binder complexes were separated from the unbound library by electrophoresis. A fraction was collected in a pre-determined time window, where binders should elute (see Section S2 for the determination of the binder-elution window). In Step 3, the collected fraction underwent a two-stage PCR amplification — quantitative PCR (qPCR) followed by asymmetric PCR (aPCR) — to produce the binder-enriched library for the next round of SELEX as well as obtain $L_{out,+T}$. For consistency across selections, we used a constant 10-µM concentration of the starting library in Round 1. Subsequently, we employed a 0.33-µM output library as input for later rounds. To confirm the robustness and the reproducibility of the selection results, we repeated two of the four selections for thrombin, particularly those for 10-nM and 500-nM target concentrations.

After every round of SELEX, qPCR was used to determine $L_{out,+T}$ and $L_{out,-T} \equiv N_{out}$; we also knew the values of $N_{in} \equiv L_{in}$. Cumulatively this data allowed us to calculate k_N with eq. (3) and BNR with eq. (7) for each round. The bulk-affinity assay was performed for the starting library and outcome libraries after each round of SELEX, and its results were used to judge the progress of SELEX. In our bulk-affinity assay, R — representing the fraction of unbound library obtained in the bulk affinity test — serves as an indicator of affinity maturation: decreasing values of R indicate improving affinity of the library to the target. $R > 0.5$ is expected for Round 0, while affinity maturation progressively reduces R for subsequent rounds. To mitigate the poor accuracy associated with R measurement close to its limits (0 and 1), we systematically adjusted the protein concentration in the bulk affinity test in a stepwise fashion to maintain R within the desired range of 0.3 to 0.7. Note that having bulk affinity assays after every round is excessive and done here only for correlation of BNR with affinity maturation. The determination of BNR is much easier than assessing affinity maturation.

To judge whether the condition of eq. (7) is satisfied, we determined the value of σ_{BNR} with eq. (11) as explained above. To confirm that this approach of σ_{BNR} determination is acceptable, we also determined σ_{BNR} in a straightforward way, i.e., by conducing multiple sets of positive-control and negative-control experiments for one constant target concentration (500 nM thrombin). The values of $L_{out,+T}$ and $L_{out,-T}$ were determined and used to calculate BNR values. The mean value of BNR and its standard deviation were then estimated (Section S1). Details of all the above-described experimental procedures can be found in Section S6.

Determination of BNR in SELEX with Constant Round-to-Round Target Concentration. Following our plan, we completed three-round SELEX for both MutS and thrombin with four constant round-to-round target concentrations. A detailed summary of k_N and BNR values can be found in Section S3. Notably, the k_N values were found to be in the range of 10^{-4} to 10^{-3} and 10^{-6} to 10^{-5} for our NECEEM-based SELEX for thrombin and MutS, respectively. The difference in k_N values between the two targets was attributed to different resolutions of the proteins-DNA complexes from the DNA nonbinders between the two protein targets of different sizes (thrombin molecule is smaller than MutS). Essentially, partitioning for thrombin was performed with an approximately 100 times higher nonbinder background (N_{out}) than partitioning for MutS. Considering that BNR is inversely dependent on N_{out} (eq. (4)), it is expected that the theoretical range of BNR values in SELEX for MutS would be approximately two orders of magnitude higher than in SELEX for thrombin, given the same target concentration and quantity of the input library. Indeed, the experimental values of BNR in SELEX for MutS were consistently within 1 to 2 orders of magnitude higher than in SELEX for thrombin at the same target concentration (Figure 3a).

There are a couple of other reasons for variations in experimental BNR values that need to be mentioned. First, the uncertainty associated with the qPCR-determined $L_{out,+T}$ and $L_{out,-T}$ values can lead to potential variations of ~10% under the same experimental conditions (see error calculations in Section S1). Second, the nature of the target, specifically its aptagenicity, defines the binder abundance in the starting library for a specific target and the upper limit of $L_{out,+T}$. Intuitively, an ideal SELEX would have a high binder abundance in the starting library (high $L_{out,+T}$) together with a low nonbinder background (low k_N or N_{out}), leading to a high BNR value much greater than zero.

A consistent trend observed in the BNR values for both targets was a decrease in BNR with decreasing target concentration, ultimately reaching BNR ≈ 0 (Figure 3a). This

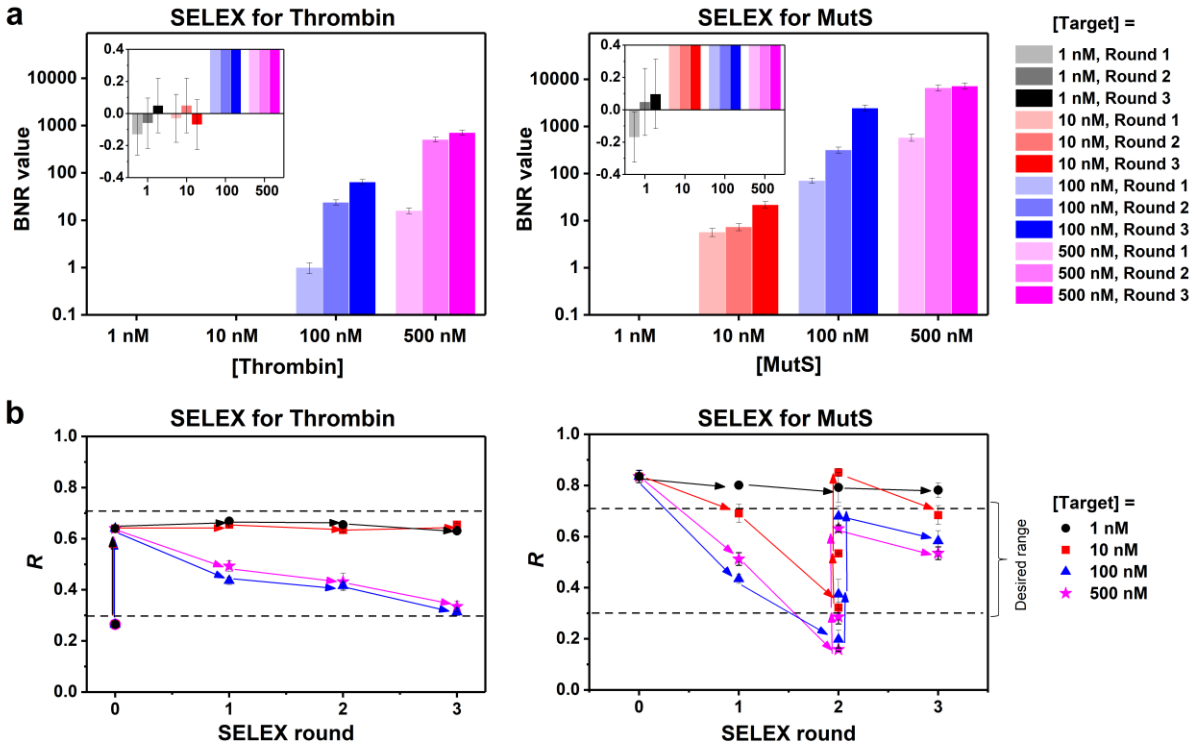

Figure 3. Comparison of BNR values **(a)** and bulk affinities represented by *R* values **(b)** to evaluate the selection outcomes for MutS and thrombin under four different constant (throughout the rounds of selection) target concentrations. In **(a)**, the inset displays the same data but with a linear ordinate, focusing on the lower BNR range. In **(b)**, the measurements of *R* in each selection followed a previously established workflow for assessment of bulk affinity, starting with a protein concentration of 1 μM. To ensure that *R* remained within the desired range, we systematically adjusted the target concentration in the bulk affinity workflow in a stepwise manner. The vertical arrow connecting points on the graph indicates a 10-fold decrease in target concentration for the same selection round, which was implemented to maintain *R* within the desired range.

trend was expected; at higher target concentration, more target molecules are available to bind oligonucleotides in the library, increasing $L_{out,+T}$ and BNR. In contrast, when the target is deficient, only the most tightly bound binders remain bound to the target and are collected at the output of partitioning, resulting in lower $L_{out,+T}$ and BNR values. In SELEX for thrombin, the BNR value decreased to below $3\sigma_{BNR}$ when the target concentration reached (on the way down) 10 nM. Since partitioning for MutS had a lower nonbinder background (lower k_N), the BNR value decreased to below $3\sigma_{BNR}$ at a lower target concentration of 1 nM.

Another important observation from Figure 3a was that when the BNR value in Round 1 was below $3\sigma_{BNR}$ (as seen in SELEX for 1-nM and 10-nM thrombin as well as 1-nM MutS), there was no detectable increase in the BNR value in subsequent rounds for a given target concentration. However, when BNR value in Round 1 was much greater than $3\sigma_{BNR}$, BNR consistently increased from round to round, peaking in Round 3 (e.g., SELEX for 100-nM and 500-nM thrombin; and SELEX for 10-nM, 100-nM, and 500-nM MutS). This increase in BNR between rounds for such target concentrations indicates that the fraction of binders was increasing with progress of SELEX, possibly indicating its outcome (which will be discussed in detail below).

Correlation between BNR with Affinity Maturation for Constant Round-to-Rounds Target Concentration. To validate BNR as a tool for assessing SELEX progress, we compared BNR values with affinity maturation monitored using the bulk-affinity assay.[25] The results of the bulk affinity assay are summarized in Figure 3b, where *R* is plotted against the SELEX round number for every target concentration, with Round 0 being the starting library prior to the first partitioning (see Section S4 for the electropherograms and calculations of *R* values).

We found that BNR values correlated with affinity maturation. In SELEX for thrombin, round-to-round affinity maturation (progressive decrease of *R*) was observed only for 100-nM and 500-nM target concentrations for which BNR was greater than 3σ. The *R* value remained unchanged from round to round for 10 nM and 1 nM target concentrations, for which BNR was below 3σ. A similar trend was seen in SELEX for MutS, with the only difference being that the target concentration resulting in no affinity maturation and BNR < 3σ was 1 nM. For higher target concentrations rounds-to-round affinity maturation was observed and BNR values were greater than 3σ. The comparison of BNR to affinity maturation confirms that BNR can serve as an unambiguous real-time indicator of SELEX progress. To mitigate the risk of SELEX failure, one needs to maintain BNR > 3σ for all rounds of SELEX, including the critical Round 1. It is important to emphasize that our experimental results lead to conclusions identical to the conclusions derived from the analysis of our mathematical framework in Figure 2.

BNR and Affinity Maturation in SELEX with Decreasing Round-to-Round Target Concentration. Purposely decreasing the target concentration with the progression of SELEX is a common strategy, particularly when low-efficiency partitioning methods are employed, such as

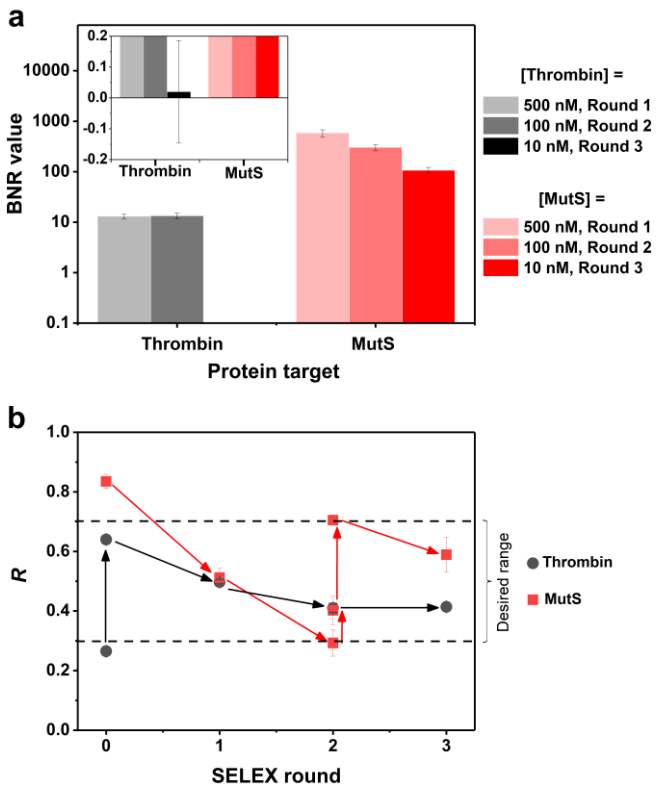

Figure 4. Comparison of BNR values **(a)** and bulk affinities represented by *R* values **(b)** to evaluate the outcomes of SELEX for MutS and thrombin under decreasing round-to-round target concentration. In **(a)**, the inset displays the same data but with a linear ordinate scale, focusing on the lower BNR range. In **(b)**, the measurements of *R* in each SELEX followed a published workflow for assessment of bulk affinity in a similar manner to the prior SELEX procedures for constant round-to-round target concentration (refer to Figure 3).

separation on magnetic beads and nitrocellulose filters. SELEX based on such methods typically involves more than 10 rounds with target concentration decreasing with in successive rounds.[44, 45] This strategy aims to steer SELEX towards selecting binders with higher affinity (lower K_d values). There is no indication to suggest that the requirement of BNR being statistically significantly greater than zero would not apply to this SELEX strategy. Even is experimentalists increase the stringency with progressive rounds of SELEX, it is still crucial to ensure that the resulting BNR value remains above 3σ for each and every round. If BNR falls below 3σ, one should return to the conditions of the previous round to minimize the chance of SELEX failure.

To confirm this experimentally, we conducted SELEX for both thrombin and MutS with target concentrations decreasing from round to round: 500 nM in Round 1, 100 nM in Round 2, and 10 nM in Round 3 (Figure 4). Our data revealed that for thrombin, affinity maturation ceased when the target concentration decreased down to 10 nM with BNR < 3σ in Round 3. In contrast, the bulk affinity improved throughout SELEX for MutS, as the determined BNR values were > 3σ for all three concentrations (refer to Section S5 for a summary of BNR and *R* values obtained in this series of decreasing target concentration).

CONCLUDING REMARKS

In summary, we employed deductive logic to univocally establish the relationship between SELEX stringency and BNR. Increasing stringency leads to a decrease in BNR, which theoretically ranges between 0 and ∞ practically has a finite upper limit due to a nonzero nonbinder background of partitioning. BNR offers the advantage of being a unitless measure fundamentally independent of the specifics of partitioning methods used in SELEX. Through a known formalism of partitioning (Figure 2) and simple mathematics, we obtained eq. (7), $\mathrm{BNR} = (L_{\mathrm{out},+\mathrm{T}}/L_{\mathrm{out},-\mathrm{T}}) - 1$, providing a practical method of determining BNR. BNR, a ratio between the output library's amount in the presence and absence of the target reduced by unity, is straightforward to determine, requiring only the tools of SELEX itself, namely partitioning and PCR. Moreover, using inductive logic, we concluded that every round of SELEX must have a BNR value statistically significantly greater than zero ($\mathrm{BNR} > n\sigma_{\mathrm{BNR}}$) to maximize the probability of success. To illustrate the use of BNR, we conducted a comprehensive set of experiments involving CE-based SELEX for two protein targets at varying stringency levels, employing both constant and decreasing round-to-round target concentration. We determined BNR and conducted a bulk-affinity assay after every round of SELEX. A perfect correlation was observed between the BNR values and affinity maturation status: when BNR was not statistically significantly greater than zero, affinity maturation was not detectable. Thus, both the theory and experiments strongly advocate for conducting SELEX so that BNR of every round is statistically significantly greater than zero. Below, we place some common aspects of SELEX in the context of BNR and the SELEX non-failure criterion.

Stringency in SELEX is typically controlled by changing protein concentration or partitioning duration. However, the definition of BNR suggests another means of controlling stringency: by altering the nonbinder background of partition, $L_{\mathrm{out},-\mathrm{T}}$, equivalent to decreasing k_N. If CE is uses for partitioning, the nonbinder background can be changed by choosing a different mode of CE-based partitioning as different modes have different k_N values.[24] Another means of increasing BNR can be using a more superior starting library, such as a chemically modified DNA library with a greater bulk affinity to the target.[46-48]

The probability of SELEX non-failure is intricately tied to the confidence level (n) of BNR exceeding zero, with $n = 3$ representing a default value corresponding to a 99.5% probability that BNR > 0. This statistical aspect is crucial for dispelling misconceptions in SELEX. Currently, practitioners may persist with SELEX even if no detectable affinity maturation occurs after 10 or 20 rounds. However, statistical analysis suggests that if BNR equals zero (within experimental error) in any round, it indicates excessive stringency, rendering SELEX success improbable. Monitoring BNR emerges as an efficient tool for risk management in SELEX, being both less expensive and more robust than traditional bulk affinity assays for tracking affinity maturation.

We advocate for the adoption of BNR as a measure of stringency in SELEX, determined by practitioners and reported along with its standard deviation routinely as an objective indicator of SELEX progress. Furthermore, the collation of BNR data from the SELEX community will serve as a valuable resource for validating our proposed stringency control model across various partitioning methods. Our ongoing efforts include monitoring BNR and confirming the efficacy of our

proposed monitoring model for stringency through recent SELEX campaigns, such as the one targeting carbonic anhydrase II using a different CE-based partitioning method (complex-first NECEEM). The BNR values for each SELEX round, alongside affinity maturation progress, are available in the attached Excel spreadsheet in the Supporting Information. We strongly encourage SELEX practitioners to contribute retrospective or ongoing project data to this dataset, which will undergo regular updates.

This collaborative endeavor not only validates our proposed stringency control model but also establishes a comprehensive data repository to address critical questions regarding its applicability. One such question pertains to the relationship between the confidence level (n) in eq (8) and the probability of SELEX success or failure, a relationship likely dependent on SELEX specifics such as partitioning method. We anticipate that answering this question will necessitate a significant amount of empirical data, with analysis potentially benefiting from machine learning tools. This communal effort underscores the importance of participation from the aptamer community to advance SELEX research and its applications.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the Publication's website:

Determination of the standard deviation of BNR (σ_{BNR}) (Section S1); Determination of the binder-elution windows (Section S2); Summary of nonbinder background (k_N) and BNR values obtained in SELEX for MutS and thrombin (Section S3); Data analysis for the bulk affinity assays (Section S4); Summary of BNR values and bulk affinity analysis obtained in decreasing target concentration series (Section S5); Experimental details (Section S6) (PDF)

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Notes

The authors declare no competing financial interest.

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Graphics for the Table of Contents Only

Stringency control in SELEX is currently subjective while excessive stringency leads to SELEX failure. We introduce “Binder-to-Nonbinder Ratio (BNR)” measured via qPCR after partitioning, a parameter that characterizes stringency quantitatively. BNR should be statistically greater than zero to prevent excessive stringency and highly-probable SELEX failure. This approach provides practical guidance for rationalizing stringency levels in SELEX experiments.

SUPPORTING INFORMATION

Quantitative Characterization of Stringency in SELEX

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Additional supplementary files
The following supplementary files can be found on Figshare:
Link: https://doi.org/10.6084/m9.figshare.24992766

File name	Description/Experiment
BNR and *R* values.xlsx	Calculation of BNR and *R* values in SELEX experiments for MutS and thrombin
Figures.zip	Source files for Figures used in the main text and SI (Origin and png files)
BNR data from SELEX community.xlsx	Compilation of BNR data gathered from SELEX experiments for various protein targets, sourced from the SELEX community

Section S1: Determination of the standard deviation of BNR (σ_{BNR}) through error propagation

Finding σ_{BNR} by determining BNR multiple times and then calculating σ_{BNR} as

$$\sigma_{BNR} = \sqrt{\frac{\sum_{i=1}^{N}\left(BNR_i - \frac{1}{N}\sum_{i=1}^{N} BNR_i\right)^2}{N-1}} \tag{S1}$$

requires multiple experiments to find $L_{out,+T}$, which is too resource-intensive. Therefore, we opted to use rules of error propagation that can greatly simplify finding σ_{BNR}. BNR is defined as:

$$BNR = \frac{L_{out,+T}}{L_{out,-T}} - 1 \tag{S2}$$

If standard deviations of $L_{out,+T}$ and $L_{out,-T}$ weakly correlate with each other, then the standard deviation of σ_{BNR} can be approximated by the following rule of error propagation:

$$\sigma_{BNR} = \sqrt{\left(\frac{\partial BNR}{\partial L_{out,+T_0}}\right)^2 {\sigma_{L_{out,+T_0}}}^2 + \left(\frac{\partial BNR}{\partial L_{out,-T_0}}\right)^2 {\sigma_{L_{out,-T_0}}}^2} = \sqrt{\left(\frac{\sigma_{L_{out,+T}}}{L_{out,-T}}\right)^2 + \left(\sigma_{L_{out,-T}} \frac{L_{out,+T}}{{L_{out,-T}}^2}\right)^2} \tag{S3}$$

This expression can be simplified to avoid the need to determine $L_{out,+T}$. The standard deviation of L_{out} depends on the value of L_{out} itself. The exact link between them is case-dependent and difficult to decipher, but we can approximate this dependence by the rules of, e.g., shot noise which call for the standard deviation of L_{out} to be proportional to the square root of L_{out}:

$$\begin{aligned} \sigma_{L_{out,+T}} &= a\sqrt{L_{out,+T}} \\ \sigma_{L_{out,-T}} &= a\sqrt{L_{out,-T}} \end{aligned} \tag{S4}$$

where a is a constant. By dividing these equations, we can obtain:

$$\frac{\sigma_{L_{out,+T}}}{\sigma_{L_{out,-T}}} = \sqrt{\frac{L_{out,+T}}{L_{out,-T}}} \tag{S5}$$

We can rewrite this expression as:

$$\sigma_{L_{out,+T}} = \sigma_{L_{out,-T}} \sqrt{\frac{L_{out,+T}}{L_{out,-T}}} \tag{S6}$$

By substituting eq. (S6) into eq. (S3), we derive the following formula for evaluating σ_{BNR}:

$$\sigma_{BNR} \approx \sigma_{L_{out,-T}} \frac{L_{out,+T}}{\left(L_{out,-T}\right)^2} \sqrt{1 + \frac{L_{out,-T}}{L_{out,+T}}} \tag{S7}$$

To confirm that all assumptions used in this derivation are valid, we compared σ_{BNR} obtained with eq. (S1) and eq. (S7). This comparison involved conducing multiple sets of positive-control (with target) and negative-control (without target) experiments at a constant target concentration (500 nM thrombin). The values of $L_{out,+T}$ and $L_{out,-T}$ were determined by qPCR and used to calculate the corresponding BNR values with eq. (S1). Then, the values of $L_{out,-T}$ were used to compute the standard deviation of $L_{out,-T}$, which was used to calculate σ_{BNR} with eq. (S7). The results of calculations are summarized in Table S1 below.

Table S1. Comparison of σ_{BNR} obtained± via eq. (S1) and eq. (S7) in this supporting document. Five sets of positive and negative controls were conducted using 10 μM DNA library and 500 nM thrombin to find $L_{out,+T}$ and $L_{out,-T}$, respectively.

Parameter	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Mean	σ
$L_{out,+T}$	7.1×10^9	5.9×10^9	7.5×10^9	6.9×10^9	6.9×10^9	6.9×10^9	0.6×10^9
$L_{out,-T}$	4.3×10^8	4.0×10^8	5.4×10^8	4.6×10^8	4.7×10^8	4.6×10^8	0.5×10^8
BNR *via* eq. (S2)	15.75	13.91	12.98	14.04	13.69	**14.1**	**1.0***
σ_{BNR} *via* eq. (S7)	2.15	2.06	1.43	1.79	1.71	**1.8****	**0.3*****

*This is σ_{BNR} calculated with eq. (S1)
**This is mean σ_{BNR} calculated by averaging σ_{BNR} values determined with eq. (S7) for 5 experiments
***This is the standard deviation of σ_{BNR} determined with eq. (S7)

To summarize, we obtained:
BNR = 14.1 ± 1.0
Eq. (S1) σ_{BNR} = 1.0
Eq. (S7) σ_{BNR} = 1.8 ± 0.3

As we can see, σ_{BNR} values obtained with eq. (S7) overestimate σ_{BNR} obtained with eq. (S1) by approximately a factor of 2, which is acceptable as this overestimation can be compensated by choosing a smaller value of n in the criterion BNR > $n\sigma_{BNR}$. Therefore, we use eq. (S7) to assess σ_{BNR} values of the obtained BNR data in all our experiments.

Section S2: Determination of the binder-elution window

To determine the binder-elution window, NECEEM-based partitioning was conducted using a mixture of the starting DNA library (100 nM) and a relatively high concentration of the target (1 μM). Peaks of protein−DNA complexes were detected for both protein targets at such high concentrations, allowing us to identify binder-elution windows to be used in SELEX. It is noted that in the case of thrombin, the resolution between target−binder complexes and DNA nonbinders was poorer than in the MutS case, primarily due to the smaller size of thrombin molecule. As such, the partitioning in SELEX for thrombin experienced a much higher nonbinder background (k_N is in a range of 10^{-4}–10^{-3} for thrombin *vs* 10^{-6}–10^{-5} for MutS).

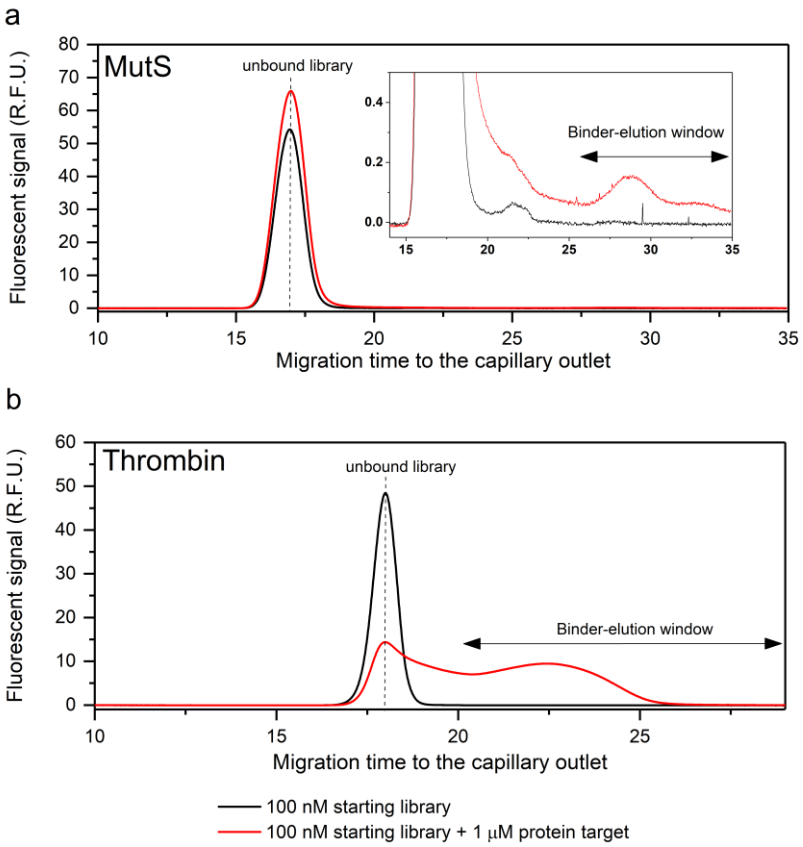

Figure S2-1. Determination of binder-elution window for NECEEM-based SELEX for MutS **(a)** and thrombin **(b)**. Based on the migration profile of DNA nonbinders and target−binder complexes, elution of target−binder complexes was conducted using pressure after NECEEM-based partitioning for 26 min and 20 min in SELEX procedures for MutS and thrombin, respectively. In this complex-last NECEEM mode, the first peak (from the left) corresponds to the unbound library while the second peak corresponds to the target−binder complex.

Section S3: Summary of nonbinder background (k_N) and BNR values obtained in SELEX for MutS and thrombin

Note S3-1: Summary of k_N and BNR values obtained in SELEX for MutS

We conducted three-round SELEX for His-tagged MutS with four constant round-to-round target concentrations: 1, 10, 100 and 500 nM. Each round involved a set of positive controls (in the presence of target) and negative controls (in the absence of target) to determine $L_{out,+T}$ and $L_{out,-T}$, respectively. After every round, DNA was quantitated with qPCR and k_N and BNR values were determined. The results are shown in **Table S3-1** below.

Table S3-1. Summary of k_N and BNR values obtained in SELEX for MutS. The three values in each cell correspond to Round 1, Round 2, and Round 3 of SELEX, respectively.

[MutS] in SELEX	k_N (= N_{out}/N_{in})	BNR (= $L_{out,+T}/L_{out,-T} - 1$)	σ_{BNR}
1 nM	(6.94×10^{-5}, 8.14×10^{-5}, 3.94×10^{-5})	(−0.17, 0.05, 0.1)	(0.16, 0.21, 0.21)
10 nM	(5.65×10^{-5}, 2.15×10^{-5}, 8.44×10^{-5})	(5.7, 7.4, 22)	(1.1, 1.3, 3.3)
100 nM	(6.94×10^{-5}, 2.65×10^{-5}, 4.36×10^{-5})	(71, 318, 2479)	(9.1, 45, 350)
500 nM	(5.26×10^{-5}, 2.35×10^{-5}, 5.67×10^{-5})	(582, 6609, 7299)	(97, 934, 1032)

Note S3-2: Summary of k_N and q values obtained in SELEX for thrombin

Similar to SELEX procedures for MutS, we completed three-round SELEX for thrombin with four constant round-to-round target concentrations and estimated k_N and BNR values for every round after DNA quantitation with qPCR. We repeated two of the four SELEX rounds for thrombin to ensure quantitative reproducibility of the results (10-nM and 500-nM SELEX experiments). The data are shown in **Table S3-2** below.

Table S3-2. Summary of k_N and BNR values obtained in SELEX for thrombin. The three values in each cell correspond to Round 1, Round 2, and Round 3 of SELEX, respectively.

[Thrombin] in SELEX	k_N (= N_{out}/N_{in})	BNR (= $L_{out,+T}/L_{out,-T} - 1$)	σ_{BNR}
1 nM	(5.20×10^{-4}, 6.45×10^{-4}, 6.83×10^{-4})	(−0.13, −0.06, 0.05)	(0.13, 0.16, 0.17)
10 nM	(4.89×10^{-4}, 2.93×10^{-4}, 4.14×10^{-4})	(−0.03, 0.05, −0.07)	(0.15, 0.17. 0.16)
10 nM (repetition)	(4.64×10^{-4}, 3.73×10^{-4}, 6.44×10^{-4})	(−0.08, 0.10, −0.10)	(0.15, 0.18, 0.15)
100 nM	(5.05×10^{-4}, 5.15×10^{-4}, 3.44×10^{-4})	(0.99, 24, 65)	(0.26, 2.9, 7.7)
500 nM	(4.27×10^{-4}, 4.78×10^{-4}, 6.61×10^{-4})	(16, 508, 719)	(2.2, 59, 83)
500 nM (repetition)	(3.98×10^{-4}, 4.87×10^{-4}, 5.63×10^{-4})	(14, 430, 800)	(2.1, 50, 93)

Section S4: Data analysis for bulk affinity assays

Note S4-1: Electropherograms and calculation of *R* in SELEX for MutS

We used a previously published bulk affinity workflow (Teclemichael, E.; Le, A. T. H.; Krylova, S. M.; Wang, T. Y.; Krylov, S. N. Bulk Affinity Assays in Aptamer Selection: Challenges, Theory, and Workflow. *Anal. Chem.* 2022, *94*, 15183−15188) to evaluate the progress of SELEX for MutS at four different target concentrations. The bulk affinity assay was conducted using a constant DNA concentration of 1 nM and a starting target concentration of 1 µM (**Figure S4-1**). The target concentration in the bulk affinity assay was subsequently decreased in a stepwise fashion (i.e., 1 µM → 100 nM → 10 nM) to ensure that *R* value (fraction of unbound library) stays within its desired range of 0.3–0.7.

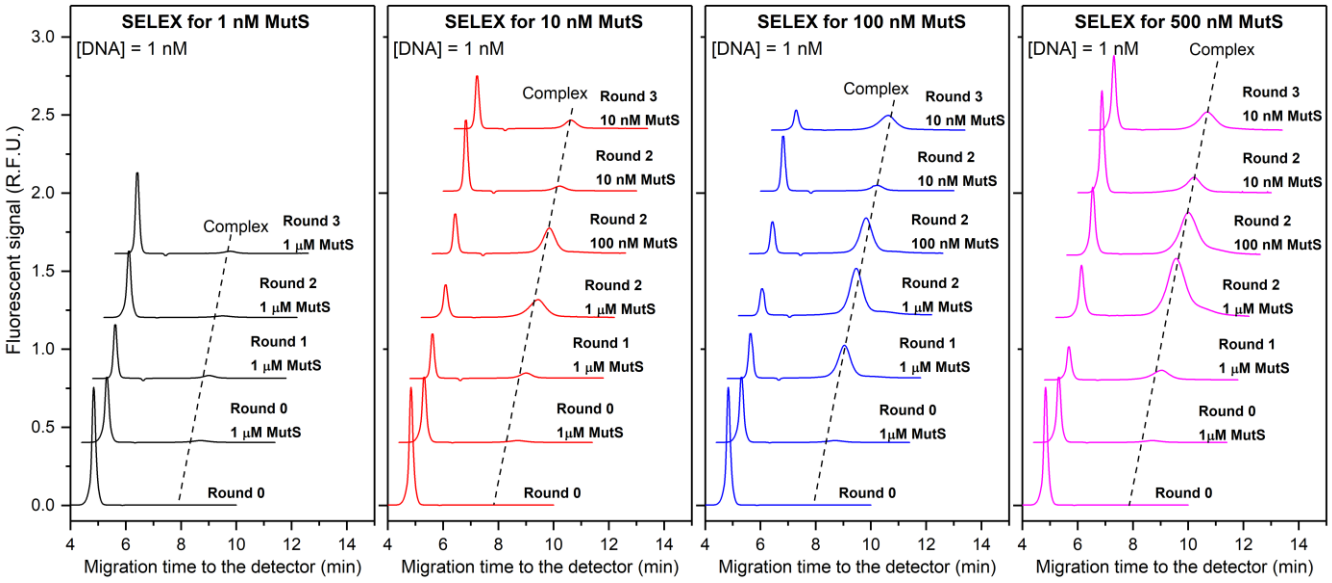

Figure S4-1. Bulk affinity tests of the starting library and the binder-enriched libraries obtained in SELEX for MutS at four different target concentrations using the published bulk affinity workflow. Black, red, blue, and magenta traces represent SELEX procedures using 1, 10, 100, and 500 nM MutS, respectively. The experiments were conducted in triplicates and only the representative electropherograms are shown here. The dashed line indicates the position of the target−binder complex in each electropherogram while the leftmost peak corresponds to the unbound DNA library.

The *R* value in the bulk affinity test was then estimated from the ratio of the peak area of unbound DNA library to the total peak area of unbound library and target−binder complex in the corresponding electropherograms using the NAAP program (Kanoatov, M.; Galievsky, V. A.; Krylova, S. M.; Cherney, L. T.; Jankowski, H. K.; Krylov, S. N. Using Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) for Simultaneous Determination of Concentration and Equilibrium Constant. *Anal. Chem.* 2015, *87*, 3099−3106). The results are shown in **Table S4-1** below.

Table S4-1. Summary of *R* values for the starting library (Round 0) and binder-enriched libraries (Round 1–3) obtained in SELEX for MutS at 4 different target concentration schemes: 1, 10, 100, and 500 nM MutS. Note that *R* values for Round 0 were the same for all SELEX procedures.

[Mut] in SELEX	**Round**	[Target] used in the affinity test	*R* values for triplicate runs	**Mean *R* ± standard deviation**
	0	1 µM	0.802, 0.845, 0.857	**0.835 ± 0.024**
1 nM	**1**	1 µM	0.822, 0.791, 0.791	**0.801 ± 0.015**
	2	1 µM	0.790, 0.865, 0.721	**0.792 ± 0.059**
	3	1 µM	0.763, 0.759, 0.823	**0.782 ± 0.029**
10 nM	**1**	1µM	0.643, 0.700, 0.731	**0.691 ± 0.036**
	2	1 µM	0.324, 0.296, 0.346	**0.322 ± 0.021**
	2	100 nM	0.531, 0.537, 0.535	**0.534 ± 0.003**
	2	10 nM	0.850, 0.871, 0.833	**0.851 ± 0.016**
	3	10 nM	0.634, 0.700, 0.719	**0.684 ± 0.037**
100 nM	**1**	1 µM	0.411, 0.443, 0.451	**0.435 ± 0.018**
	2	1 µM	0.214, 0.231, 0.151	**0.198 ± 0.035**
	2	100 nM	0.376, 0.445, 0.304	**0.375 ± 0.058**
	2	10 nM	0.625, 0.709, 0.707	**0.680 ± 0.039**
	3	10 nM	0.637, 0.570, 0.542	**0.583 ± 0.400**
500 nM	**1**	1µM	0.511, 0.482, 0.544	**0.512 ± 0.025**
	2	1 µM	0.164, 0.159, 0.151	**0.158 ± 0.006**
	2	100 nM	0.325, 0.260, 0.274	**0.286 ± 0.028**
	2	10 nM	0.646, 0.633, 0.613	**0.631 ±0.014**
	3	10 nM	0.508, 0.569, 0.528	**0.535 ± 0.025**

Note S4-2: Electropherograms and calculation of *R* in SELEX for thrombin

Similar to our treatment of SELEX results for MutS, we applied the bulk affinity workflow to assess the progress of SELEX for thrombin across four different target concentrations. The workflow maintained a constant DNA concentration of 20 nM and began with a target concentration of 1 µM (**Figure S4-2**). At 1 µM target concentration, significant binding of the starting library to thrombin was observed as the corresponding *R* value was below 0.3. Consequently, the target concentration was reduced by 10 folds from 1 µM to 100 nM. This adjustment was made to elevate the *R* value of the starting library (Round 0) to a level within the desired range (0.3–0.7); this target concentration (100 nM) remained fixed for later rounds.

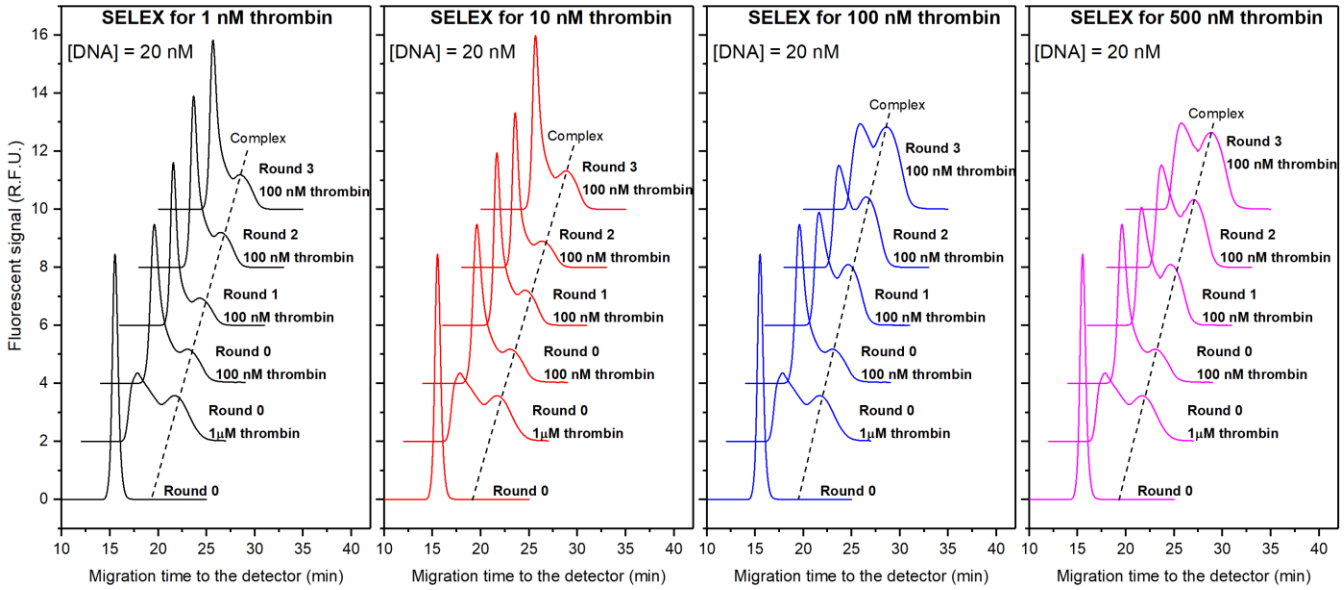

Figure S4-2. Bulk affinity tests of the starting library and binder-enriched libraries obtained in SELEX for thrombin at four different target concentrations using the published bulk affinity workflow. Black, red, blue, and magenta traces represent SELEX procedures with 1, 10, 100, and 500 nM thrombin, respectively. The affinity test for every round was conducted in triplicates and only the representative electropherograms are shown here. The dashed lines indicate positions of the target−binder complexes while the leftmost peak corresponds to the unbound DNA library.

In SELEX for thrombin, the resolution between the unbound library and the target−binder complex was relatively poor (**Figure S4-2**), leading to challenges in calculating *R* based on distinct peak areas of the unbound library and target−binder complex. Therefore, for the thrombin case, we determined *R* value by utilizing the peak height ratio of unbound library in the presence of target to that in its absence. The peak heights and migration times were obtained with 32 Karat Software. The results can be found in **Table S4-2** below.

Table S4-2. Summary of *R* values for the starting library (Round 0) and binder-enriched libraries (Round 1–3) obtained in SELEX for thrombin at four different target concentrations: 1, 10, 100 and 500 nM thrombin. Note, *R* values for Round 0 were the same for all SELEX experiments.

[Thrombin] in SELEX	**Round**	[Target] used in the affinity test	*R* values for triplicate runs	**Mean *R* ± standard deviation**
–	**0**	1 µM	0.261, 0.266, 0.268	**0.265 ± 0.004**
	0	100 nM	0.640, 0.636, 0.644	**0.640 ± 0.004**
1 nM	**1**	100 nM	0.660, 0.663, 0.682	**0.669 ± 0.012**
	2	100 nM	0.644, 0.656, 0.665	**0.655 ± 0.011**
	3	100 nM	0.635, 0.627, 0.630	**0.631 ± 0.004**
10 nM	**1**	100 nM	0.649, 0.654, 0.662	**0.655 ± 0.007**
	2	100 nM	0.627, 0.635, 0.640	**0.634 ± 0.007**
	3	100 nM	0.653, 0.660, 0.651	**0.655 ± 0.005**
10 nM (repetition)	**1**	100 nM	0.683, 0.685, 0.682	**0.684 ± 0.002**
	2	100 nM	0.671, 0.671, 0.674	**0.672 ± 0.002**
	3	100 nM	0.640, 0.651, 0.653	**0.648 ± 0.007**
100 nM	**1**	100 nM	0.453, 0.429, 0.427	**0.436 ± 0.015**
	2	100 nM	0.403, 0.411, 0.435	**0.416 ± 0.016**
	3	100 nM	0.322, 0.320, 0.296	**0.313 ± 0.015**
500 nM	**1**	100 nM	0.474, 0.490, 0.515	**0.493 ± 0.021**
	2	100 nM	0.403, 0.470, 0.420	**0.431 ± 0.035**
	3	100 nM	0.341, 0.350, 0.313	**0.335 ± 0.020**
500 nM (repetition)	**1**	100 nM	0.489, 0.502, 0.501	**0.497 ± 0.008**
	2	100 nM	0.402, 0.427, 0.443	**0.424 ± 0.020**
	3	100 nM	0.349, 0.337, 0.338	**0.341 ± 0.007**

Section S5: Summary of BNR values and bulk affinity analysis obtained in decreasing target concentration series

We conducted a three-round SELEX for His-tagged MutS and thrombin, implementing decreasing target concentrations from 500 nM (Round 1) to 100 nM (Round 2) and further down to 10 nM (Round 3). Similar to the prior SELEX procedures for constant target concentration scheme (refer to **Section S3** and **S4**), each round involved both positive control (in the presence of the target) and negative control (in the absence of the target) to determine the BNR values.
Furthermore, we also applied a similar procedure of the bulk affinity workflow to assess the SELEX progress with decreasing round-to-round target concentrations for both protein targets, aiming to obtain comparable *R* values for each round of SELEX. The obtained BNR and *R* values in every SELEX round are presented in **Table S5-1**, while the electropherograms from the bulk affinity workflow are shown in **Figure S5-1**.

Table S5-1. Summary of BNR and *R* values for the starting library (Round 0) and binder-enriched libraries (Round 1–3) obtained in SELEX for His-tagged MutS and thrombin with decreasing round-to-round target concentrations. Note, *R* values for Round 0 were the same for all SELEX experiments.

Target	Round	[Target] used in the affinity test	*R* values for triplicate runs	Mean *R* ± standard deviation	BNR ± standard deviation
MutS	**0**	1 µM	0.802, 0.845, 0.857	**0.835 ± 0.024**	**N/A**
	1	1 µM	0.511, 0.482, 0.544	**0.512 ± 0.031**	**582 ± 97**
	2	1 µM	0.340, 0.284, 0.255	**0.293 ± 0.044**	**302 ± 43**
	2	100 nM	0.365,0.456, 0.387	**0.402 ± 0.048**	
	2	10 nM	0.699,0.712,0.704	**0.705 ± 0.007**	
	3	10 nM	0.647, 0.591, 0.529	**0.589 ± 0.059**	**107 ± 15**
Thrombin	**0**	1 µM	0.261, 0.266, 0.268	**0.265 ± 0.004**	**N/A**
	0	100 nM	0.640, 0.636, 0.644	**0.640 ± 0.004**	**N/A**
	1	100 nM	0.493, 0.491, 0.508	**0.497 ± 0.009**	**13 ± 1.4**
	2	100 nM	0.395, 0.409, 0.427	**0.410 ± 0.015**	**13 ± 1.7**
	3	100 nM	0.408, 0.413, 0.421	**0.414 ± 0.007**	**0.02 ± 0.17**

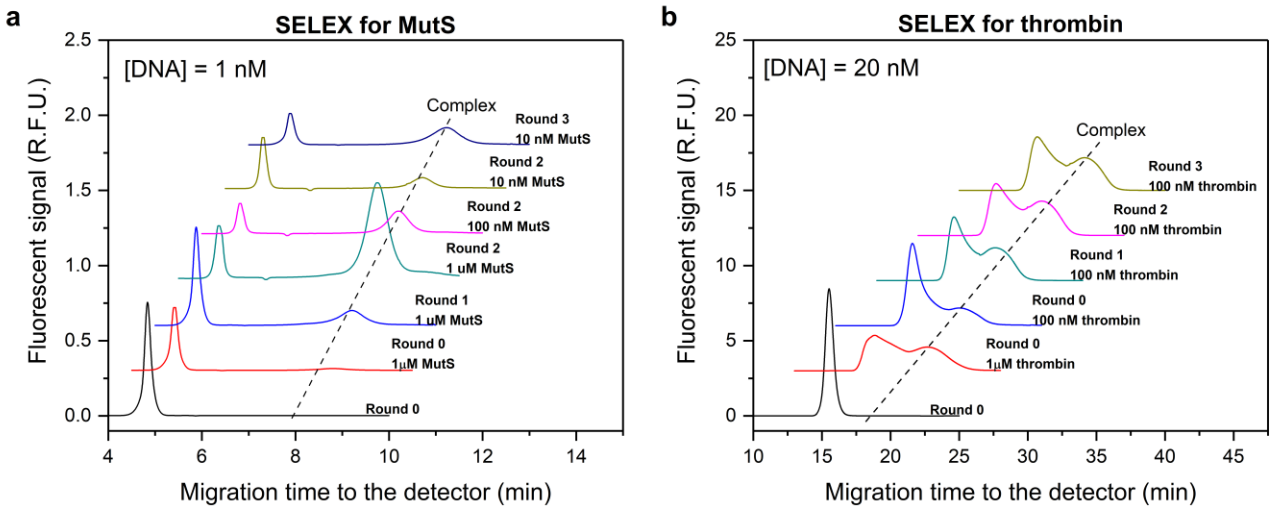

Figure S5-1. Bulk affinity tests of the starting library and binder-enriched libraries obtained in SELEX for MutS **(a)** and thrombin **(b)** with decreasing round-to-round target concentrations using the published bulk affinity workflow. The affinity test for every round was conducted in triplicates and only the representative electropherograms are shown here. The dashed lines indicate positions of the target−binder complexes while the leftmost peak corresponds to the unbound DNA library.

Section S6: Experimental details

Materials and solutions

All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Fused-silica capillaries with inner and outer diameters of 75 and 360 μm, respectively, were purchased from Molex Polymicro (Phoenix, AZ, USA). Recombinant His-tagged MutS protein (MW ≈ 90 kDa, p*I* 6.0) was purchased from Prospec Protein Specialist (Ness Ziona, Israel). Recombinant human alpha-thrombin protein (MW ≈ 36.7 kDa, p*I* 6.4–7.6) was purchased from Fisher Scientific (Mississauga, ON, Canada). All DNA molecules were custom synthesized by Integrated DNA Technologies (Coralville, IA, USA). CE running buffers were 50 mM Tris-HCl pH 8.0 and 50 mM Tris-acetate pH 8.2 for SELEX for MutS and thrombin, respectively. The sample buffer was always identical to the running buffer to avoid the adverse effects of buffer mismatch. Accordingly, all dilutions of sample components in CE experiments were done by adding the corresponding running buffer.

DNA sequences

All DNA stock solutions were subjected to annealing by incubation at 90 °C for 2 min before being cooled to 20 °C at a rate of 0.5 °C/s, prior to the dilution and preparation of the equilibrium mixtures. To avoid cross-contamination between the SELEX procedures for two different protein targets, distinct synthetic fluorescein amidite (FAM)-labeled, 40-nt random DNA libraries (referred to as N40) with unique primer regions were used as follows: (*i*) for MutS: 5′-FAM-CTC CTC TGA CTG TAA CCA CG-N40-GC ATA GGT AGT CCA GAA GCC-3′, and (*ii*) for thrombin: 5′-FAM-CTA CGG TAA ATC GGC AGT CA-(N40)-AT CTG AAG CAT AGT CCA GGC-3′.
Two sets of primers were used to amplify binders selected from the starting library. The primers in the first set were unlabeled and employed for quantitative PCR (qPCR). These primers had the following sequences: (*i*) for MutS: 5′-CTC CTC TGA CTG TAA CCA CG-3′(forward) and 5′-GGC TTC TGG ACT ACC TAT GC-3′(reverse), and (*ii*) for thrombin: 5′-CTA CGG TAA ATC GGC AGT CA-3′ (forward) and 5′-GCC TGG ACT ATG CTT CAG AT-3′(reverse). For asymmetric PCR (aPCR), the second set of primers included a fluorescently labeled version of the forward primer and a biotin-labeled version of the reverse primer: (*i*) for MutS: 5′-Alexa Fluor488-CTC CTC TGA CTG TAA CCA CG-3′(forward) and 5′-Biotin-TEG-GGC TTC TGG ACT ACC TAT GC (reverse), and (*ii*) for thrombin: 5′-Alexa Fluor488-CTA CGG TAA ATC GGC AGT CA-3′ (forward) and 5′-Biotin-TEG-GCC TGG ACT ATG CTT CAG AT-3′(reverse).

CE Instrumentations

All CE experiments were performed with a P/ACE MDQ apparatus (SCIEX, Concord, ON, Canada) equipped with a laser-induced fluorescence (LIF) detection system. Fluorescence was excited with a blue line (488 nm) of a solid-state laser and detected at 520 nm using a spectrally-optimized emission filter system (Galievsky, V. A.; Stasheuski, A. S.; Krylov, S. N. Improvement of LOD in fluorescence detection with spectrally nonuniform background by optimization of emission filtering. *Anal. Chem.* 2017, *89*, 11122-11128). The poly(vinyl alcohol) (PVA)-coated capillaries were prepared as described elsewhere (de Jong, S.; Krylov, S. N. Pressure-based approach for the analysis of protein adsorption in capillary electrophoresis. *Anal. Chem.* 2012, *84*, 453−458). The total length of the capillary was 80 cm for most of the experiments, except for the bulk affinity tests conducted in SELEX for MutS, where the capillary length was 50 cm. In all cases, the detection window was positioned 10 cm away from the outlet of the capillary. Prior to every run, the PVA-coated capillary was rinsed with the running buffer at 20 psi (138 kPa) for 8 min. The coolant temperature was set at 15 °C.

Specifics of CE-based fraction collection

In Round 1, the equilibrium mixture contained the annealed starting library of 10 μM and the protein target of chosen concentration; 330 nM binder-enriched library was used for Rounds 2 and 3 instead of 10 μM. The target

concentration in the equilibrium mixture was kept constant throughout the three rounds of SELEX. The equilibrium mixtures were incubated at room temperature (21 °C) for 1 h to approach chemical equilibrium in the binding reaction. The equilibrium mixture was injected into the capillary by a pressure pulse of 1 psi (6.9 kPa) × 28 s, resulting in a sample plug of 3.7 cm in length. The sample plug was propagated by a pressure pulse of 0.9 psi (6.2 kPa) × 45 s (to yield a 5.4 cm-long buffer plug) to pass the uncooled region of the capillary before applying the electric field. Partitioning was carried out using reversed polarity (anode at the outlet) at 25 kV for 26 and 20 min in SELEX procedures for MutS and thrombin, respectively. After CE-based partitioning, elution of the target−binder complex was facilitated by pressure at 5 psi (34.5 kPa) for 1 min into a fraction-collection vial containing 20 μL of the running buffer.

PCR procedures and generation of binder-enriched library

The eluted binder-enriched library was amplified and quantitated by two rounds of qPCR using CFX Connect instrument (Bio-Rad, ON, Canada). The qPCR reagent mixture was prepared to obtain final concentrations of 1×Q5 High-Fidelity 2×Master Mix (New England BioLabs, Whitby, ON, Canada), 1×SYBR Green (Fisher Scientific, Mississauga, ON, Canada), 500 nM unlabeled forward primer, and 500 nM unlabeled reverse primer. Before thermocycling, the qPCR reaction mixture was prepared by adding a 2 μL aliquot of the eluted fraction to 18 μL of the qPCR reagent mixture. The PCR thermocycling protocol was as follows: 98 °C for 30 s (initialization, performed once), 98 °C for 10 s (denaturation), 65 °C for 20 s (annealing), and 72 °C for 20 s (extension), followed by a plate read at 72 °C and a return to the denaturation step for a total of 40 cycles. All qPCR reactions were performed in duplicate. In the first round of qPCR, the eluted fraction was quantitated using an eight-point calibration curve. An S-shaped amplification curve was then plotted for the eluted fraction. In the second round of the qPCR, the qPCR product of the eluted fraction was removed when it was two cycles into the exponential phase of the previously plotted amplification curve. After qPCR, 100 μL of the qPCR product was later purified using the MinElute® PCR purification kit (QIAGEN, Missisauga, ON, Canada) as per manufacturer's instructions. Once product's purity was verified by native PAGE, it was subjected to aPCR. Five μL of DNA was added to 45 μL of aPCR reagent mixture from New England Biolabs Inc. (Whitby, ON, Canada). Final concentrations of PCR reagents in the reaction mixture were: 1×Q5® Reaction Buffer, 1 μM fluorescently labeled forward primer, 50 nM biotin-labeled reverse primer, 0.02 units/μL Q5® High-Fidelity DNA Polymerase, and 200 μM dNTPs mix. The reaction was performed in duplicates with the following temperature protocol: 98 °C for 30 s (initial denaturation, performed once), 98 °C for 10 s (denaturation), 65 °C for 20 s (annealing), and 72 °C for 20 s (extension). Eighteen cycles of aPCR were run. Ten μL of MagnaBind™ streptavidin beads suspension (Fisher Scientific, Mississauga, ON, Canada) was washed three times and resuspended in bead washing/binding buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA pH 8.0). Once amplified, the duplicate PCR reactions were combined and incubated with streptavidin magnetic beads for 30 min at a room temperature (23 ± 1 °C). The beads were magnetized, discarded, and the PCR product was then purified using the MinElute® PCR purification kit as per manufacturer's instructions.
To quantitate the DNA concentration in the binder-enriched library, serial dilutions of the fluorescently labelled forward primer (2 μM, 1 μM, 500 nM, 250 nM, 125 nM, 62.5 nM, and 31.25 nM) were prepared to build a standard curve by measuring fluorescence intensity at 519 nm with NanoDrop 3300 Fluorospectrometer (Fisher Scientific, Mississauga, ON, Canada). The purified binder-enriched library was then ready for the next round of SELEX.

Specifics of bulk affinity test

Equilibrium mixtures of either the starting library or the binder-enriched library and varying target concentrations were prepared and incubated at room temperature for 1 h prior to injection into the capillary. Throughout all the bulk affinity tests, the concentrations of the starting library or the binder-enriched library remained constant (i.e., 1 nM in SELEX for MutS and 20 nM for SELEX for thrombin). In the case of MutS bulk affinity tests, a 50-cm capillary was used to shorten the separation time while still ensuring the desired resolution between the unbound library and the target−binder complex. As such, the conditions for MutS bulk affinity tests were readjusted as follows: (*i*) sample injection at 0.5 psi (3.4 kPa) × 20 s to create a 2.1 cm-long sample plug, (*ii*) buffer propagation

at 0.9 psi (6.2 kPa) × 30 s to yield a 5.8 cm-long buffer plug and pass the uncooled capillary region and (*iii*) separation at 25 kV with reversed polarity (anode at the capillary outlet) for a duration of 15 min. Due to the poorer resolution in SELEX for thrombin, the bulk affinity tests were continued to be conducted using an 80-cm capillary. The conditions for thrombin bulk affinity tests were the same as conditions used in the SELEX procedure with the total separation time of 25 min.