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Article

Quantitative Characterization of Partitioning Stringency in SELEX

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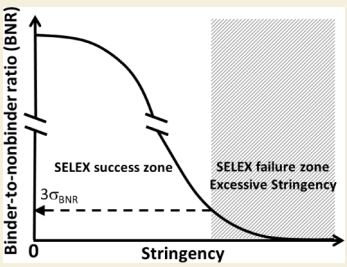
ABSTRACT: Maintaining stringent conditions in SELEX (Systematic Evolution of Ligands by EXponential enrichment) is crucial for obtaining high-affinity aptamers. However, excessive stringency greatly increases the risk of SELEX failure. Controlling stringency has remained a technical challenge, largely dependent on intuition, due to the absence of a clear, quantitative measure of stringency. This study was motivated by our insight that, while stringency is influenced by multiple factors, it can be quantified by its effect: increasing stringency reduces the quantity of binders normalized to that of nonbinders after partitioning. Based on this insight, we propose measuring stringency using the binder-to-nonbinder ratio (BNR), where a lower BNR indicates higher stringency. We derive an experimental method for determining BNR via quantitative PCR. Our theoretical analysis and SELEX experiments using two distinct proteins as selection targets underscore the importance of maintaining a BNR significantly greater than zero to avoid failure, a principle we call the SELEX nonfailure criterion. By employing inverse BNR to quantify stringency and applying this criterion, researchers can more rationally control SELEX progress. The quantitative stringency measure and nonfailure criterion can also be applied to other artificial evolution methods, provided that selected binders are quantifiable.



KEYWORDS: *measure of partitioning stringency in SELEX, round-to-round control of SELEX progress, binder-to-nonbinder ratio at the output of partitioning, SELEX nonfailure criterion*

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 measurements. 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 defined 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 puzzled scientists for centuries before Newton's breakthrough. His key insight was recognizing that force could not be generically defined by its multiple influencers but should instead be described through its effect—acceleration. This led to the formulation of Newton's second law, $F = ma$, marking the beginning of quantitative science.

In the presented research, we focus on the rigorous delineation of partitioning stringency in SELEX (Selection of Ligands by EXponential enrichment),[1,2] a representative approach within the broader class of artificial evolution, also known as in vitro selection. SELEX is employed to select aptamers from random-sequence oligonucleotide libraries. Stringency functions like a quantitative parameter—adjustable by modifying its influencers (the factors it depends on)—yet lacks a definitive measure. Similar to force, partitioning stringency eludes straightforward expression through its multiple influencers. Our aim is to define partitioning stringency by its effect and highlight how establishing a measure for stringency can improve SELEX methodologies and potentially benefit other artificial evolution processes.

Aptamers, oligonucleotides with the ability to bind targets tightly and selectively through multiple noncovalent bonds, are primarily utilized in diagnostic and therapeutic applications.[3−12] Typically sourced from highly diverse random-sequence oligonucleotide libraries, aptamers are selected through SELEX, an iterative process consisting of successive rounds of three primary steps (Figure 1a).

In Step 1, the starting library reacts with the target, allowing target-binding oligonucleotides (referred to as binders) to form complexes with the target while target-nonbinding oligonucleotides (nonbinders) remain unbound. Due to the reversible nature of binding, the process is dynamic and stochastic. It is important to note that in our context, the terms "binder" and "nonbinder" merely designate the state of oligonucleotides under specific conditions at the end of Step 1, just before Step 2 begins. Strictly speaking, all oligonucleotides are binders of different strengths, meaning they are capable of forming target−binder complexes:

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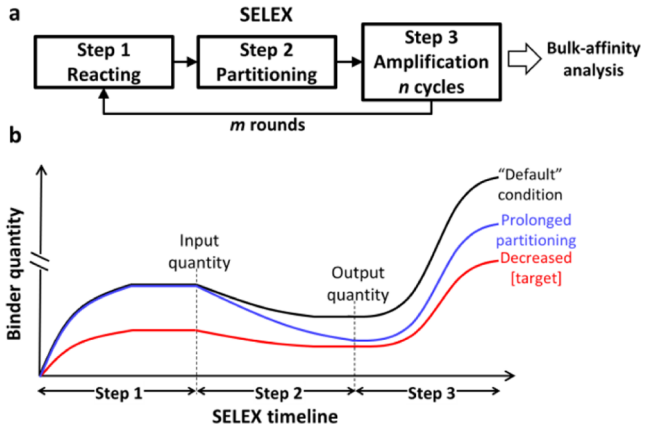

Figure 1. Schematic representation of the SELEX process (a) and the associated changes in binder quantity along the timeline (b). In general, the quantity of binders is equivalent to the quantity of intact target-binder complexes for Steps 1 and 2. See text for details.

$$\text{Binder} + \text{Target} \underset{K_d}{\rightleftharpoons} \text{Complex} \tag{1}$$

with the equilibrium dissociation constants (K_d) ranging from 0 to ∞. Decreasing the target concentration in Step 1 causes fewer weak binders, compared to strong binders, to form target−binder complexes. As a result, a lower input quantity of binders is available at the start of Step 2 (Figure 1b).

Step 2 involves partitioning the target−binder complexes from nonbinders. Partitioning is imperfect; the binders are always contaminated by nonbinders at the output of this step. Additionally, partitioning takes time during which less-stable complexes, characterized by higher dissociation rate constants (k_{off}), may dissociate. As a result, prolonged partitioning disproportionately reduces the population of weak binders compared to strong binders, leading to lower output quantity of binders at the end of Step 2 (Figure 1b).

Step 3 involves amplification, typically via polymerase chain reaction (PCR), of all oligonucleotides (both binders and nonbinders) collected during the second step. This amplification generates a binder-enriched library with an increased ratio of strong to weak binders (or binders to nonbinders). The 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, which denotes a statistically significant improvement in bulk affinity across rounds, signals SELEX failure. Although resource-intensive and semiquantitative, bulk affinity assays 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", similar to polyclonal antibodies.[13] However, in most cases, "monoclonal" binders are produced from such a library in several post-SELEX steps, which are not included in the original definition of SELEX by its inventors,[1] and are not shown in Figure 1a. First, DNA from the binder-enriched library is sequenced, typically using high-throughput sequencing. Second, the sequences are analyzed to exclude known nonbinders and rank sequences based on their frequency in the library. Finally, the 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 core SELEX processes shown in Figure 1a.

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.[14−19] As explained above, stringency can be increased by decreasing the target concentration in Step 1 and/or increasing the duration of partitioning in Step 2. The method by which stringency is increased influences the characteristics of the selected binders: decreasing the target concentration puts pressure toward selecting binders with low K_d, while increasing the partitioning duration favors binders with low k_{off}. While stringency is attractive as a general descriptor of selective pressure, it has a major limitation: it has no quantitative definition and, accordingly, no quantitative measure is assigned to it. Thus, stringency is suitable only for qualitative or comparative characterization of selective pressure, e.g., "higher stringency" or "lower stringency".

It is intuitive, and has never been challenged, that tuning SELEX toward the selection of strong binders requires increasing stringency. This relationship between stringency and the enrichment of strong binders in the library has been demonstrated both theoretically and experimentally.[14−20] As a result, high stringency is desirable in SELEX. However, while it is equally intuitive and requires no proof that stringency cannot be increased indefinitely without inducing SELEX failure, experimental evidence now confirms this notion.[21] Thus, a logical yet unanswered question is how high can the plank of stringency be raised before no binder in the library can clear the bar? The importance of this question largely motivated our study, which was also inspired by our insight that answering it is impossible without defining stringency quantitatively.

In this study, we first defined stringency quantitatively through its effect—its influence on the quantity of binders normalized to that of nonbinders at the output of partitioning. We focused on the normalized quantity of binders because increasing stringency always reduces the output quantity of binders. This occurs either by reducing the input quantity of binders (in the case of lower target concentration) or directly reducing the output quantity (in the case of extended partitioning duration) (Figure 1b). We further introduced the inverse Binder-to-Nonbinder Ratio (BNR) as a quantitative measure of stringency. Using a simple mathematical formalism of partitioning—which, however, grasps its kinetic nature—we derived a link between BNR and two parameters that are 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, needed to calculate BNR, are routinely measured by some SELEX practitioners to get insights into SELEX progress. Thus, they can be analyzed retrospectively to compute BNR and quantitatively assess stringency. Using inductive logic, we established a SELEX nonfailure criterion: BNR must be significantly greater than zero in each round of SELEX. This criterion is novel and nontrivial; to the best of our knowledge, it has not been previously reported or applied. Without this criterion, BNR cannot be productively used to guide SELEX. Applying this criterion requires experimental determination of the standard

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deviation of BNR, which, although not yet done, can also be completed retrospectively. Finally, we conducted a comprehensive set of experiments to confirm the validity of the SELEX nonfailure criterion. The results fully support the theoretical requirement of keeping BNR significantly greater than zero. Our findings suggest that this quantitative measure of stringency can serve as an effective tool for monitoring round-to-round progress of SELEX.

In addition to its utilitarian function, the quantitative measure of stringency will advance our fundamental understanding of SELEX. Moreover, our method of quantitatively assessing stringency through BNR can be broadly applied to other artificial evolution methodologies, such as display techniques and selection of binders from DNA-encoded libraries, provided there is a means to quantify the output after partitioning with and without the target. While no formal framework existed until now for quantitatively monitoring evolutionary progress and adjusting selection conditions, we have learned that companies like GlaxoSmithKline and Somalogic routinely use qPCR to measure these quantities during selections involving DNA-encoded libraries and xenonucleic acids (XNAs), respectively, as part of their quality control processes. Additionally, several academic laboratories have implemented workflows to quantify output quantities in order to track selection progress for DNA aptamers,[19,22,23] and to optimize selection conditions for DNA,[20] XNA,[24] and DNA-encoded libraries.[25]

While these efforts were previously conducted without a formal model, they were all aimed at gaining insights into the selection process and adjusting stringency accordingly for better outcomes. We anticipate that the BNR model will serve as a universal basis for measuring stringency and predicting selection progress across various artificial evolution processes.

MATERIALS AND METHODS

Reagents and Materials

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.[26] The poly(vinyl alcohol) (PVA)-coated capillaries were prepared as described elsewhere.[27] 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 consisted of the annealed starting library at 10 μM and the protein target at the chosen concentration. For Rounds 2 and 3, a binder-enriched library at 330 nM was used instead of the 10 μM starting library. Unless otherwise stated, the target concentration in the equilibrium mixture was kept constant across all three SELEX rounds. The equilibrium mixtures were incubated at room temperature (21 °C) for 1 to allow the binding reaction to approach chemical equilibrium. The mixture was then 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. This 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

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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 labeled 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 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-long 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.

Reproducibility Assessment of Background Binding in Magnetic-Bead Partitioning

An N40 DNA library (0.3 nmol) was incubated in 30 μL of bead selection buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) along with 1 mg/mL of sheared salmon sperm DNA (Fisher Scientific, Mississauga, ON, Canada). A suspension of 20 μL of MagnaBind streptavidin beads (Fisher Scientific, Mississauga, ON, Canada) was washed three times and then resuspended in 20 μL of the same buffer. This bead suspension was mixed with the DNA library solution, resulting in a total volume of 50 μL, and incubated for 30 min on a rotator. Postincubation, the beads were separated and washed three times with 100 μL of the bead selection buffer. To elute any background DNA library molecules (or $L_{out,-T}$), the beads were resuspended in 50 μL of bead selection buffer and heated at 72 °C for 5 min. The eluted solution was then separated and subjected to one round of qPCR to measure the DNA concentration, as described in the earlier section "PCR procedures and generation of binder-enriched library". For this experiment, the same N40 DNA library and unlabeled primer pair used in the MutS selection were employed. The experiment was repeated seven times to assess the reproducibility of $L_{out,-T}$ measurements in magnetic-bead partitioning.

RESULTS AND DISCUSSION

Quantitative 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 these steps, as well as the method and conditions of partitioning in Step 2 (Figure 1). It might seem counterintuitive, but to incorporate these multiple dependencies into a single parameter, this parameter must not be directly derived from any individual influencers of stringency. Instead, it should be based on the overall effect of changes in stringency, similar to Newton's definition of force, as discussed in the introduction. The universal effect of increasing stringency is the reduction in the number of binders after partitioning. Therefore, we decided to base our quantitative measure of stringency on this quantity, which needs to be normalized to make it independent of the library load at the input. A well-established partitioning formalism (Figure 2) allows us to derive a measure of stringency using the normalized quantity of binders.[28−33]

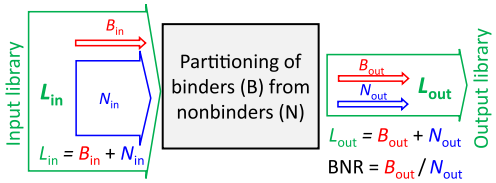

Figure 2. Schematic representation of partitioning of binders (B) from nonbinders (N). See text for details.

Partitioning essentially acts as a filter, removing nonbinders (N) and allowing binders (B) to pass through. The input and output libraries (L) are comprised of binders and nonbinders (defined in the introduction), linked by the following equations:

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

Two key parameters describe partitioning: the transmittances for binders (k_B) and nonbinders (k_N), both ranging between 0 and 1, defined as

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

In ideal partitioning, $k_N = 0$ and $k_B = 1$, however, in real partitioning, $k_N > 0$ and $k_B < 1$. The value of k_B/k_N represents the efficiency of partitioning, the main parameter characterizing the overall quality of partitioning.

Importantly, the efficiency of partitioning (k_B/k_N) inversely correlates with stringency: as stringency increases, k_B/k_N decreases. However, measuring stringency based on k_B/k_N is impractical because quantifying k_B requires knowing B_{in} (the number of binders at the input), which is challenging.[26] Therefore, we use the fact that the ratio k_B/k_N correlates with the Binder-to-Nonbinder Ratio (BNR), defined as B_{out}/N_{out}. Since B_{out} and N_{out} can be quantified experimentally (as

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demonstrated below), we propose using BNR to define a practical measure of stringency (S):

$$S \equiv \frac{1}{\mathrm{BNR}} \equiv \frac{1}{B_{\mathrm{out}}/N_{\mathrm{out}}} \tag{4}$$

Since both BNR and S are ratios of oligonucleotide quantities, they are unitless parameters. Theoretically, S ranges from 0 to 1, however, the lower limit of zero is unattainable because N_{out} (background) cannot be zero, and B_{out} cannot be infinite.

Practical Means of Stringency Determination

For stringency (S) to be a useful quantitative parameter, it must be measurable using the core SELEX loop tools (Figure 1), namely partitioning and PCR. Based on the definition of BNR (eq 4), S can be calculated directly if both B_{out} and N_{out} are known. N_{out} can be determined by partitioning the input library without the target:

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

where "−T" denotes the absence of the target.

When the target is present (+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:

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

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

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

$$S \equiv \frac{1}{\mathrm{BNR}} = \frac{L_{\mathrm{out},-\mathrm{T}}}{L_{\mathrm{out},+\mathrm{T}} - L_{\mathrm{out},-\mathrm{T}}} \leq \infty \tag{7}$$

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

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

Equations 7 were derived from the mass balance (eq 2) and the definitions of BNR and S (eq 4). Accordingly, eq 7 do not require any validation and are applicable to all methods of partitioning. While eq 7 are equivalent to each other, it is more practical to operate with BNR to simplify the mathematics. Therefore, all further derivations are performed for BNR rather than S.

BNR as a "Risk-Management Tool" in SELEX

Let us use BNR to address the question of the highest stringency in SELEX that does not result in failure. For SELEX to progress, the output library must contain binders. Theoretically, this means that BNR must be greater than zero. However, in practice, BNR is always determined with some degree of experimental uncertainty. Therefore, for BNR to reliably indicate the presence of binders in the output library, it 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 represents the confidence level (1, 2, 3, ...). The value of σ_{BNR} is defined by error propagation rules from eq 7 as follows (see Section S1 for details):

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

where σ with subscripts $L_{\mathrm{out},-\mathrm{T}}$ and $L_{\mathrm{out},+\mathrm{T}}$ are standard deviations of $L_{\mathrm{out},-\mathrm{T}}$ and $L_{\mathrm{out},+\mathrm{T}}$, respectively. To minimize the amount of experimentation required to determine σ_{BNR}, we assume that the standard deviation is proportional to the square root of L_{out}, which is typical for random noise (see Section S1 for details):

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

By substituting this into eq 9, we obtain the following formula for the assessment of σ_{BNR}:

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

This approach requires determining the standard deviation of $L_{\mathrm{out},-\mathrm{T}}$, which can be done once by repeatedly sampling a fixed quantity of the library for partitioning without the target. This value is expected to remain constant as long as the partitioning method and conditions do not change.

Equation 7 sets the condition for SELEX to progress, with the highest allowable stringency (S) corresponding to lowest allowable BNR, equal to $n\sigma_{\mathrm{BNR}}$. The confidence level n can be selected based on the specific experiment. A default value of n = 3 is used here, though establishing a consensus may require analyzing large SELEX data sets. It is also unclear if n will vary depending on the partitioning method. Machine learning tools could help address this and other related questions, though these are beyond the scope of the current study. The next step in this proof-of-concept work was to confirm the conclusions of the theoretical framework experimentally. However, it is important to note that since the BNR framework was developed from "first-principles", it does not inherently require experimental validation. Instead, the primary purpose of the experimental component is to provide a practical demonstration of BNR application to the SELEX process, offering practitioners an instructive and actionable example.

Experimental Design

Our experiments did not aim to select individual aptamers, which would require post-SELEX steps—these were purposefully excluded from our study. Instead, our goal was to test how varying BNR influences SELEX progress. BNR can be adjusted by either changing the target concentration or the duration of partitioning. In this study, we varied target concentrations, assessing SELEX progress using bulk-affinity assays, which measure the fraction of unbound library (R).[34]

To investigate how target aptagenicity affects BNR, we conducted SELEX for two target proteins: His-tagged MutS (93 kDa) and nontagged thrombin (35 kDa), both of which have been successfully used in previous aptamer selections.[35−38] A single DNA library with 40 random nucleotides was used, and we examined four target concentrations: 500,

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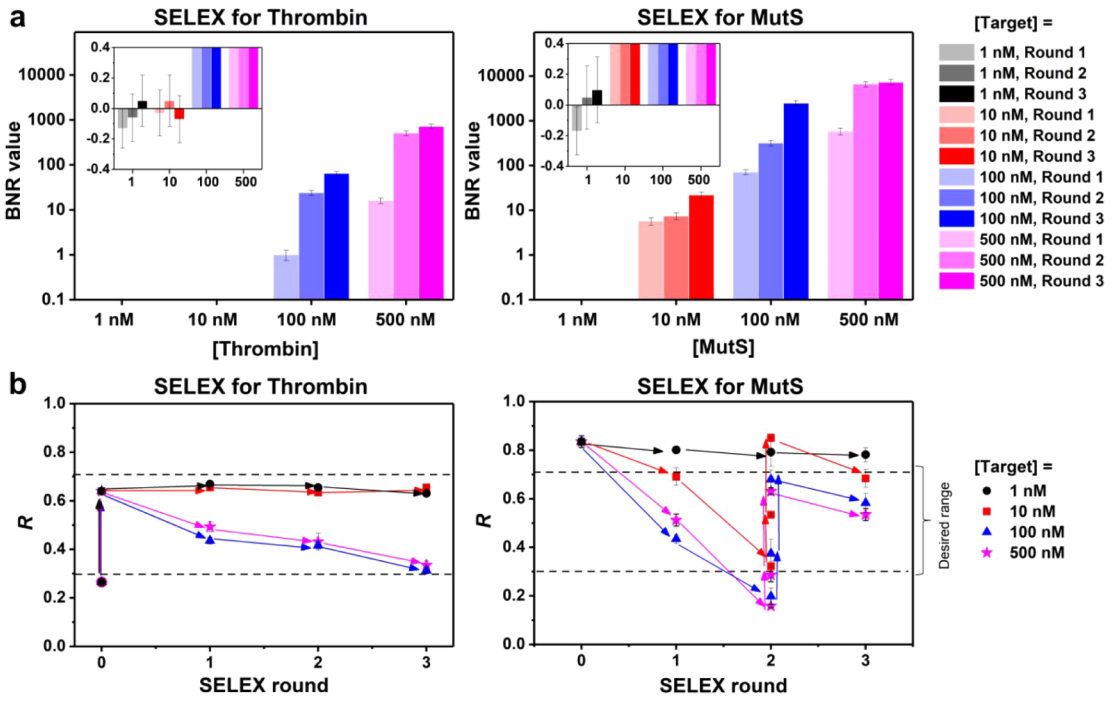

Figure 3. Comparison of BNR values (a) and bulk affinities represented by R values (b) to evaluate the selection outcomes for MutS and thrombin under four different constant (throughout the rounds of selection) target concentrations. In (a), the inset displays the same data but with a linear ordinate, 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.

100, 10, and 1 nM. Capillary electrophoresis (CE) was used for partitioning, as it reliably supports high partitioning efficiency (k_B/k_N) of 10^4–10^9 (orders of magnitude higher than that of surface-based partitioning).[30,33,37,39−48] Typically, CE-based SELEX is completed in 3 to 4 rounds without increasing stringency between rounds. Thus, we conducted four selections with constant target concentrations. To compare, we also included experiments with gradually increasing stringency, which is often used in surface-based partitioning.

Since multiple studies have shown that additional rounds of CE-based partitioning are either unproductive or counterproductive, we limited our SELEX to three rounds, which were sufficient for drawing conclusions.[39−42,49,50] To address strong adhesion of target proteins to the capillary walls, we used poly(vinyl alcohol) (PVA)-coated capillaries, which also suppress electroosmotic flow (EOF).[27,51] The suppressed EOF necessitated the use of a CE mode called complex-last NECEEM, where nonbinders move faster through the capillary than target−binder complexes.[33] In the next paragraph, we provide some essentials of our NECEEM-based SELEX.

In Step 1, the target was mixed with the library and incubated for 1 h to form target−binder complexes, serving as the positive control. The negative control was a mixture of the library with a target matrix void of the target. In Step 2, a small volume of the mixture (about 5% of the capillary length) was injected into the capillary and target−binder complexes were separated from the unbound library by electrophoresis. A fraction was collected in a predetermined 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 amplificationquantitative 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, and a 0.33-μM input library for subsequent rounds. To ensure robustness and reproducibility, we repeated two of the four thrombin selections, specifically for 10-nM and 500-nM target concentrations.

After every SELEX round, qPCR was used to determine $L_{out,+T}$ and $L_{out,-T} \equiv N_{out}$; we also knew the values of $N_{in} \equiv L_{in}$. These data allowed us to calculate k_N with eq 3 and BNR with eq 7 for each round. Bulk-affinity assays were performed on the starting library and outcome libraries after each round to track the SELEX progress. In this assay, R represents the fraction of unbound library and serves as an indicator of affinity maturation. Higher R values (>0.5) are expected for Round 0, while lower R values in subsequent rounds signal improved affinity. To mitigate the poor accuracy associated with R measurements close to its limits (0 and 1), we adjusted protein concentration stepwise to keep R between 0.3 to 0.7.[34] Although performing bulk-affinity assays after every round is excessive, it was done here to correlate BNR with affinity maturation.

To ensure eq 7 was met, we calculated σ_{BNR} using eq 11. Prior to this, we validated this approach by determining σ_{BNR} directly, i.e., by running 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 used to calculate BNR, and the mean BNR and its standard deviation were estimated (Section S1).

It is important to emphasize that while the experimental portion of this study primarily serves a demonstrative purpose,

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it is both rigorous and comprehensive in its scope. Specifically, it includes data from 10 independent SELEX campaigns, encompassing a variety of conditions to instructively show how the BNR framework can be used in practice and confirm experimentally the theoretical expectations. This extensive experimental data set not only reinforces the theoretical findings but also provides practitioners with actionable insights and a reliable reference for implementing the BNR framework in their own SELEX workflows.

Determination of BNR in SELEX with Constant Round-To-Round Target Concentration

In line with our experimental plan, we completed three rounds of SELEX for both MutS and thrombin, maintaining constant target concentration throughout. These experiments were conducted at four target concentrations for each protein, with detailed k_N and BNR values provided in Section S3. Notably, the k_N values for our NECEEM-based SELEX experiments ranged from 10^{-4} to 10^{-3} for thrombin and from 10^{-6} to 10^{-5} for MutS. The variation in k_N values between the two targets is attributed to differences in the resolution of the protein−DNA complexes from nonbinding DNA, influenced by the size disparity between the protein targets—thrombin being smaller than MutS. This size-dependent increase in k_N has been extensively studied elsewhere.[29,33]

Since N_{out} is proportional to k_N, as shown in eq 3, smaller proteins lead to higher N_{out} values. Consequently, partitioning for thrombin was performed with an approximately 100-fold greater nonbinder background (N_{out}) than for MutS. Given that BNR is inversely dependent on N_{out} (eq 4), the theoretical range of BNR values in SELEX for MutS is expected to be about 2 orders of magnitude higher than for thrombin. Indeed, our experimental results showed that BNR values for MutS were consistently one to 2 orders of magnitude higher than those for thrombin at the same target concentration (Figure 3a).

Variations in BNR can be attributed to two main factors. First, the uncertainty in qPCR measurements of $L_{out,+T}$ and $L_{out,-T}$ values, which can cause up to 10% variation (see error calculations in Section S1). Second, the nature of the target (i.e., its aptagenicity) defines the binder abundance in the starting library and the upper limit of $L_{out,+T}$. An ideal SELEX would have a high binder abundance in the starting library (high $L_{out,+T}$) and 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 nearly zero (Figure 3a). This was expected—higher target concentrations allow more binding, increasing $L_{out,+T}$ and BNR, while lower target concentrations result in only the strongest binders being collected, reducing both $L_{out,+T}$ and BNR values. In SELEX for thrombin, BNR dropped below $3\sigma_{BNR}$ when the target concentration reached (on the way down) 10 nM, whereas for MutS, this occurred at lower target concentration of 1 nM due to its lower nonbinder background.

Another important observation from Figure 3a was that when BNR in Round 1 was below $3\sigma_{BNR}$ (as seen in SELEX for 1-nM and 10-nM thrombin and 1-nM MutS), no detectable increase in BNR was seen in subsequent rounds. However, when BNR in Round 1 was much greater than $3\sigma_{BNR}$, BNR consistently increased with each round, peaking in Round 3. This was seen in SELEX for 100-nM and 500-nM thrombin; and 10-nM, 100-nM, and 500-nM MutS, suggesting an increasing fraction of binders as SELEX progressed, potentially indicating its outcome (to be discussed in detail below).

Correlation Between BNR and Affinity Maturation for Constant Round-To-Round Target Concentration

To validate BNR as a tool for tracking SELEX progress, we compared BNR values with affinity maturation, which was monitored using bulk-affinity assays (Figure 3b).[34] In these assays, the fraction of unbound library (R) was plotted against the SELEX round number for each target concentration, with Round 0 representing the starting library prior to the first partitioning (see Section S4 for the electropherograms and R value calculation).

We found that BNR values correlated well with affinity maturation. For thrombin, a progressive decrease in R (indicating affinity maturation) was observed only at 100-nM and 500-nM target concentrations, where BNR was greater than 3σ. No change in R was observed at 10 and 1 nM, where BNR was below 3σ. A similar trend was observed for MutS, with no affinity maturation at 1 nM, where BNR was below 3σ. For higher concentrations, affinity maturation was consistent, with BNR values above 3σ.

This comparison confirms that BNR is an effective "real-time" indicator of SELEX progress. To mitigate the risk of SELEX failure, it is important to maintain BNR > 3σ in all rounds, especially in the critical Round 1. Our experimental results aligned with the conclusions of our mathematical framework in Figure 2.

BNR and Affinity Maturation in SELEX with Decreasing Round-To-Round Target Concentration

Decreasing the target concentration during SELEX is a common strategy, particularly when low-efficiency partitioning methods are employed, such as separation on magnetic beads or nitrocellulose filters. SELEX based on such methods typically involves more than 10 rounds, with target concentration decreasing in successive rounds.[52,53] This strategy aims to steer SELEX toward selecting binders with higher affinity (lower K_d values). The requirement for BNR to be greater than 3σ remains critical in this strategy. If BNR falls below 3σ, reverting to the conditions of the previous round minimizes the risk of SELEX failure.

To confirm this experimentally, we conducted SELEX for thrombin and MutS with decreasing target concentrations: 500, 100, and 10 nM in Rounds 1, 2, and 3, respectively (Figure 4). For thrombin, affinity maturation ceased when the target concentration dropped to 10 nM, as BNR fell below 3σ in Round 3. In contrast, for MutS, affinity maturation continued, with BNR values remaining above 3σ at all three concentrations (refer to Section S5 for a summary of BNR and R values obtained in this series of decreasing target concentration).

Practical Aspects of Application of BNR and the SELEX Nonfailure 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 via improving the efficiency of partitioning (k_B/k_N): by altering the nonbinder background of partition, $L_{out,-T}$, equivalent to decreasing k_N. If CE is used for partitioning, the nonbinder background can be changed by choosing a different mode of CE-based partitioning as different modes have

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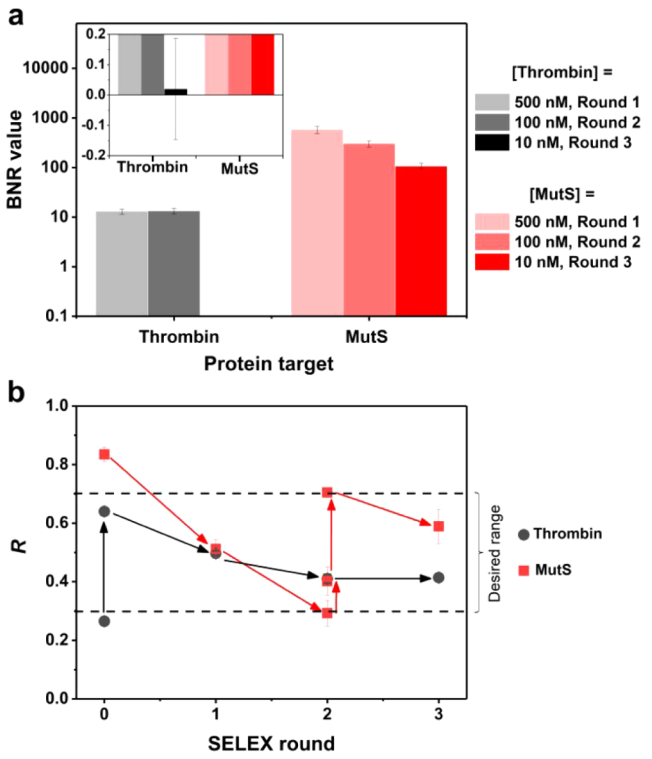

Figure 4. Comparison of BNR values (a) and bulk affinities represented by R values (b) to evaluate the outcomes of SELEX for MutS and thrombin under decreasing round-to-round target concentration. In (a), the inset displays the same data but with a linear ordinate scale, focusing on the lower BNR range. 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).

different k_N values.[33] Another means of increasing BNR can be using a more superior starting library, such as a chemically modified DNA library with greater bulk affinity for the target, thereby raising k_B.[54−56]

Counter-selection, although not part of the three core SELEX steps, is commonly performed as a standalone selection round to eliminate nonspecific binders by using a nontarget molecule.[57] The BNR framework can be readily adapted to counter selection rounds, where its purpose shifts to evaluating the removal of weakly or nonspecifically binding sequences. In such cases, counter-selection often involves conditions of high target concentration or lower stringency. Monitoring BNR during counter-selection ensures the value remains significantly greater than zero, thereby confirming the effectiveness of this step in refining the aptamer pool. Further details on BNR's application to counter-selection are provided in Section S6.

The probability of SELEX nonfailure 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, drastically reducing the probability of successful SELEX. 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.

As a measure of stringency, BNR is associated with SELEX's ability to select for stronger or weaker binders. However, this association is not explicit, and we do not believe that BNR can be directly correlated with specific binding constants, such as K_d or k_{off}, of the selected aptamers. At present, we view BNR primarily as a tool to guide SELEX by helping to prevent failure, rather than as a direct predictor of binding affinity.

The effectiveness of BNR as a predictive model for the robustness of SELEX decreases with decreasing precision of L_{out} measurements. In CE-based partitioning, the relative standard deviation (RSD) of L_{out} was small, approximately 10% (Table S1). However, CE is not the predominant method of partitioning in SELEX, being limited to laboratories with significant expertise in instrumental analytical chemistry. The dominant means of partitioning in SELEX is via "pool-down" using targets immobilized on magnetic beads.[52] Therefore, we examined the prospect of using the BNR predictive model in aptamer selection using the pool-down partitioning approach. Advantageously, such evaluation could be performed by assessing only the RSD of $L_{out,-T}$ as eq 11 contains only its standard deviation.

Given the nature of bead-based methods, potential issues with reproducibility may arise from multiple factors, including variation in bead-suspension-handling steps and washing steps. We perceive the nonuniformity of bead suspension to be a major contributor. Consequently, we expected lower reproducibility in $L_{out,-T}$ for bead-based partitioning compared to CE-based partitioning. To address the nonuniformity concern, we rigorously vortexed the bead suspension before and/or after each pipetting step. Details of these experiments can be found in Materials and Methods section.

Our results showed that the RSD of $L_{out,-T}$ for bead-based partitioning was around 35% (Table S7). Addressing other sources of irreproducibility could most likely lower this value. Although the magnetic-bead method exhibits higher uncertainty in L_{out} values, which may impact the predictive power of the BNR model, we deem this uncertainty acceptable. Further refinements to improve quantitative reproducibility in this partitioning method will enhance the predictive capability of the BNR model when used for this method. Additionally, given the multiround nature of bead-based SELEX, we anticipate that the BNR model can also be utilized to detect PCR bias, which may arise after numerous selection rounds.[58] In such cases, a decrease in BNR values, despite unchanged stringency, would signal the presence of bias.

We advocate for the adoption of BNR as the basis for a quantitative measure of stringency in SELEX, to be determined by practitioners and routinely reported along with its standard deviation as an objective indicator of SELEX progress. BNR can be universally applied to retrospective or ongoing SELEX projects, provided that consistent conditions are maintained across both target-present and target-absent experiments. Crucially, proper determination of the standard deviation of $L_{out,-T}$ is required when partitioning methods or conditions change. This ensures reproducibility and broad applicability across various SELEX methods. Additionally, 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 stringency monitoring model through recent SELEX

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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 data set, which will undergo regular updates.

Lastly, while this work establishes BNR as the basis for the quantitative measure of stringency in SELEX and a predictor of SELEX progress, the principles outlined here extend to other artificial evolution processes, including display techniques such as phage, ribosome, yeast surface, and mRNA display, as well as directed evolution.[59,60] The concept of defining stringency and employing BNR as the foundation for its quantitative measure is universally applicable across these methodologies. By carefully adjusting selection pressures and ensuring good reproducibility of L_{out} measurements in the selection process, the BNR predictive model can be reliably applied to enhance selection outcomes across various artificial evolution methods. This demonstrates its utility as the basis for a universal measure of stringency and as a predictor of selection progress in artificial evolution processes.

CONCLUDING REMARKS

This study quantitatively established the relationship between SELEX stringency and the Binder-to-Nonbinder Ratio (BNR), offering a novel method for measuring and controlling stringency. Increasing stringency decreases BNR, with a theoretical range of the latter from zero to infinity, though in practice, BNR has a finite upper limit due to nonbinder background in partitioning.

BNR, a unitless parameter independent of specific partitioning methods, serves as a practical, effective, and universal measure of stringency. Building on this framework, we introduced a new SELEX nonfailure criterion, ensuring that BNR remains statistically greater than zero to minimize the risk of SELEX failure.

To validate this approach, we conducted a series of experiments using CE-based SELEX for two protein targets at varying concentrations, employing both constant and decreasing round-to-round target concentrations. The strong correlation between BNR values and affinity maturation observed in these experiments supports the theoretical requirement for maintaining a positive BNR throughout all rounds.

By adopting inverse BNR as a quantitative stringency measure, this method provides a systematic framework for optimizing SELEX progress. Moreover, the approach holds promise for broader applications in other artificial evolution techniques, where quantifiable binders are involved, enabling more efficient and successful selection outcomes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00890.

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); utilization of the BNR framework in counter-selection (Section S6); reproducibility assessment of $L_{out,-T}$ measurements for magnetic-bead partitioning (Section S7) (PDF)

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Notes

The authors declare no competing financial interest.

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