

Ideal-Filter Capillary Electrophoresis (IFCE) Facilitates the One-Step Selection of Aptamers

An T. H. Le, Svetlana M. Krylova, Mirzo Kanoatov, Shrey Desai and Sergey N. Krylov*

Abstract: Selection of aptamers from oligonucleotide libraries currently requires multiple rounds of alternating steps of partitioning of binders from nonbinders and enzymatic amplification of all collected oligonucleotides. Here we report a highly-practical solution for reliable one-step selection of aptamers. We introduce partitioning by Ideal-Filter Capillary Electrophoresis (IFCE) in which binders and nonbinders move in the opposite directions. The efficiency of IFCE-based partitioning reaches 10^9 , which is ten million times higher than that of typical solid-phase partitioning methods. One step of IFCE-based partitioning is sufficient for selection of a high-affinity aptamer pool for a protein target. Partitioning by IFCE promises to become an indispensable tool for fast and robust selection of binders from different types of oligonucleotide libraries.

Aptamers are oligonucleotides that can bind target molecules with high affinity and selectivity;^[1] they find a variety of practical applications.^[2] Aptamers are typically selected from random-sequence oligonucleotide libraries in a process termed SELEX.^[1-3] SELEX involves iterated rounds of (i) incubation of the library with the target followed by partitioning of target-binding oligonucleotides (binders) from target-non-binding oligonucleotides (nonbinders) and (ii) polymerase-chain-reaction (PCR) amplification of all collected oligonucleotides until the binder-to-nonbinder ratio (B/N) reaches a desired value, preferably greater than unity. Remarkably, SELEX fails to select binders in 70% of attempts.^[4] This multi-round procedure is prone to failure inherently because PCR preferentially amplifies nonbinders, which are less structured oligonucleotides than binders and are, hence, more easily accessible to polymerases.^[5] As a result, SELEX enriches readily amplifiable nonbinders instead of binders if the efficiency of enriching binders in partitioning is lower than the efficiency of enriching these nonbinders in PCR amplification.^[6] An obvious solution to this taunting problem is increasing the efficiency of partitioning to the level at which its single step becomes sufficient for reaching the desired B/N . There have been several reports claiming one-step selection of aptamers.^[7] However, neither of the suggested methods has been independently confirmed since their introduction in 2005-2012, thus, questioning at least their transferability and practicality and likely their reliability. Here we report on a quantitatively-validated, highly-practical, and easily-adoptable approach for one-step selection of aptamers. We hope that the new approach will be adopted and successfully used by many in the large and diverse *in-vitro* selection community.

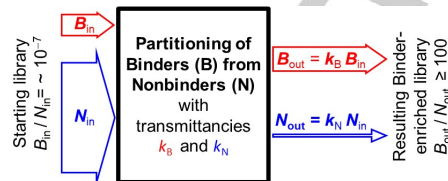


Figure 1. Schematic depiction of one-step selection of binders from an oligonucleotide library. See text for details.

This work was inspired by our understanding that there are two major reasons for the lack of a robust and practical way of one-step selection of aptamers. The first reason is methodological: while high efficiencies of partitioning are the implied goal, they are typically not measured and not used to guide developments or substantiate claims of one-step selection. The second reason is technological: it is extremely difficult to achieve high efficiencies of partitioning due to a relatively high nonbinder background. This background is caused by (i) adsorption of nonbinders to surfaces in solid-phase methods,^[8] and (ii) non-uniform migration of nonbinders in homogeneous electrophoresis-based methods.^[9] Here we address both the methodological and technological issues *via* a rational approach in which: (i) the efficiency of partitioning required for one-step selection is theoretically estimated, (ii) a new partitioning method is developed to reach the required efficiency, and (iii) one-step selection of aptamers from a random-sequence oligonucleotide library is finally demonstrated.

First, we theoretically estimated the efficiency of partitioning which should guarantee one-step binder selection; the selection scheme is depicted in Figure 1. The quantities of binders and nonbinders at the input of partitioning are B_{in} and N_{in} and at the output are B_{out} and N_{out} , respectively. The output values are related to the input ones *via* transmittances of partitioning for binder and nonbinders, k_B and k_N , respectively: $B_{out} = k_B B_{in}$ and $N_{out} = k_N N_{in}$. The value of k_N is a fraction of nonbinders that penetrates through partitioning, contaminates binders, and, as a result, creates nonbinder background. If we chose $B_{out}/N_{out} \geq 100$ as a criterion of completed selection (a criterion of $B_{out}/N_{out} \geq 1$ is typically considered acceptable), then one step of partitioning is sufficient for completing selection when the efficiency of partitioning (defined as k_B/k_N) relates to the starting binder abundance (B_{in}/N_{in}) as:

$$k_B/k_N \geq 100 / (B_{in}/N_{in}) \quad (1)$$

Values of B_{in}/N_{in} are hard to estimate in SELEX. Our estimate *via* binder selection from a random-sequence DNA library in three consecutive steps of partitioning without PCR amplification between them gave $B_{in}/N_{in} \sim 10^{-7}$.^[10] According to eq. (1), this estimate suggests $k_B/k_N \sim 10^9$ as an efficiency of partitioning which should suffice binder selection in one step. Assuming that $k_B \approx 1$ (which is typically satisfied), we can conclude that reaching $k_N \sim 10^{-9}$ is sufficient for one-step selection. Further, we use this value as a guide in our development of a partitioning method suitable for one-step selection of binders from oligonucleotide libraries.

Solid-phase methods are most widely used for partitioning aptamers; practical solid-phase methods have $k_N \geq 10^{-2}$.^[8]

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Supporting information including the Experimental Section and the ORCID identification numbers of the authors can be found under:
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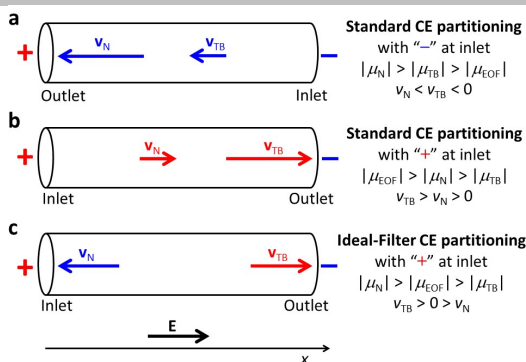


Figure 2. Schematics of CE-based partitioning of target-binder complexes (TB) from nonbinders (N). Panels (a) and (b) show standard CE-based partitioning with velocity vectors of complexes and nonbinders directed towards the capillary outlet biased at “+” and “-”, respectively. Panel (c) shows IFCE-based partitioning with the velocity vector of complexes directed towards the capillary outlet at “-” and with a counter-directed velocity vector of nonbinders.

Homogeneous partitioning by capillary electrophoresis (CE) is more instrumentation-intensive but proven to bring k_N down to 10^{-5} .^[7] Therefore, we used CE as an instrumental platform for development of a partitioning method with the required $k_N \sim 10^{-9}$.

Partitioning by CE is based on free-flow separation of target-binder complexes (TB) from nonbinders in an electric field. The main reason for nonbinder background in CE-based partitioning is nonuniform migration of oligonucleotides:^[9] there is always a small part of nonbinders that tails towards binders and creates nonbinder background in the binder-collection time window.^[7] Two known practical modes of CE-based partitioning differ by polarity (Figure 2a,b), but in both of them nonbinders and target-binder complexes move in the same direction.^[7, 10–11] These modes do not operate as a physical filter which is supposed to let binders through but reject nonbinders. We hypothesized that the k_N in CE-based partitioning could be decreased if the target-binder complexes and nonbinders moved in the opposite directions (Figure 2c) making CE function as a physical filter (but without the issue of non-specific adsorption inherent to real filters) and giving this mode of CE a name of Ideal-Filter CE (IFCE).

IFCE is equivalent to the following relation between the velocity of nonbinders, v_N , and that of binders, v_B : $v_N < 0 < v_{TB}$. This relation can be achieved if electrophoretic mobilities of the target-binder complex, μ_{TB} , and nonbinders, μ_N , relate to the mobility of electroosmotic flow (EOF), μ_{EOF} , as:

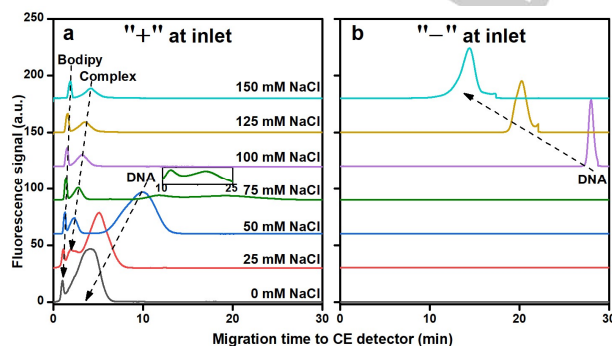


Figure 3. The effect of NaCl added to RB on migration pattern of protein-DNA complexes and unbound DNA in CE with “+” (a) and “-” (b) at the inlet, respectively. The equilibrium mixture contained 100 nM MutS protein, 100 nM fluorescently-labeled DNA aptamer of MutS, and 150 nM Bodipy (EOF marker) and was incubated for 30 min at room temperature. No peaks were observed after 30 min; therefore, only the first 30 min of 50-min runs are shown.

$|\mu_N| > |\mu_{EOF}| > |\mu_{TB}|$. The latter relation can be achieved, in turn, by decreasing $|\mu_{EOF}|$ via increasing the ionic strength of the running buffer, I_{RB} . We increased the value of I_{RB} by introducing NaCl in concentrations ranging from 25 to 150 mM to the RB (50 mM Tris-HCl pH 7.0); the corresponding values of I_{RB} ranged from approximately 50 to 200 mM (see Section S1). The electric field strength used in this study was 200 V/cm; it was chosen as the highest value that caused no overheating of RB inside the capillary (see Section S2). MutS protein (MW \approx 90 kDa, $pI \approx$ 5.2) was used as a target in this study. MutS is a part of the cellular DNA repair machinery. DNA aptamers for MutS have been previously selected by a 3-round SELEX process based on standard CE partitioning.^[11d] One of such aptamers was used in this study to model binders (in the presence of MutS) and nonbinders (in the absence of MutS). All equilibrium mixtures of MutS and DNA were incubated for 30 min at room temperature. Experimental details can be found in Section S6.

In a set of experiments testing whether $v_{TB} > 0 > v_N$ could be achieved in CE by increasing I_{RB} , the sample was an equilibrium mixture of MutS with its previously selected and characterized fluorescently-labeled DNA aptamer. The equilibrium mixture contained the MutS-aptamer (target-binder) complex and an unbound aptamer (nonbinder). To be able to detect nonbinders moving with very small velocities, we conducted these experiments with a short separation distance of $l = 4.5$ cm and with a long run time of $t = 50$ min. Every equilibrium mixture was run with two CE polarities: “+” at the inlet (Figure 3a) and “-” at the inlet (Figure 3b); the second was required to detect nonbinders when $v_N < 0$. The magnitude of the minimum “detectable” velocity was $|v_{min}| = l/t = 0.9$ mm/min. The peak of the complex was detected only with “+” at the inlet and for all concentrations of NaCl suggesting that $v_{TB} > 0$ for all I_{RB} values tested. The peak of nonbinders was detected with “+” at the inlet for $[NaCl] \leq 50$ mM, and with “-” at the inlet for $[NaCl] \geq 100$ mM, suggesting that $v_N > |v_{min}|$ for $[NaCl] \leq 50$ mM and $v_N < -|v_{min}|$ for $[NaCl] \geq 100$ mM. The peak of nonbinders was not detected with either polarity for $[NaCl] = 75$ mM suggesting $|v_N| < |v_{min}|$. These results prove that $v_{TB} > 0 > v_N$ can be satisfied by increasing I_{RB} (to $I_{RB} \geq 150$ mM in our case, which corresponds to $[NaCl] \geq 100$ mM).

We then studied how increasing I_{RB} affected the nonbinder background. The sample was DNA without any target. Two-minute fractions were collected and DNA quantities in them were determined via quantitative PCR (qPCR). Such experiments were

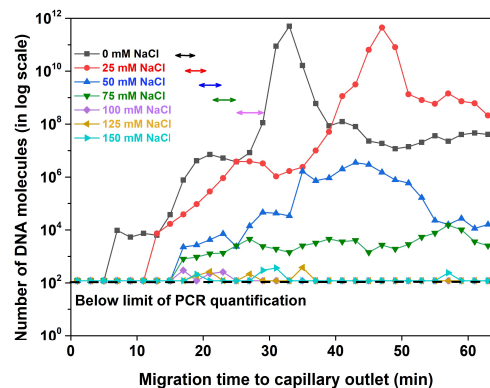


Figure 4. The effect of NaCl added to RB on the migration pattern of DNA in CE with “+” at the inlet. The sample contained 10 μ M DNA and 150 nM Bodipy (EOF marker and a reference for correct start time of collecting the first fraction). Separation distance was 34 cm. Fractions were collected every 2 min and concentrations of DNA in them were determined with qPCR and used to calculate DNA quantities in these fractions. These quantities are shown on the y-axis in the graph. The double-headed arrows indicate estimated elution windows of the aptamer (see Section S3).

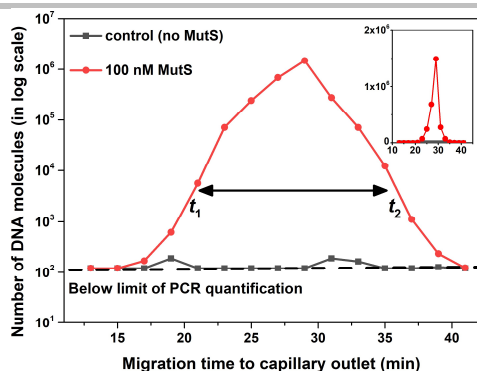


Figure 5. IFCE-based partitioning of DNA binders of MutS protein from the unbound library. The sampled equilibrium mixture was 47 nL in volume and contained 10 μ M random-sequence DNA library and 100 nM MutS protein. Separation distance was 34 cm. The inset shows the same data but with a linear ordinate.

carried out with different I_{RB} , and the results were presented as "DNA quantity in a corresponding fraction vs migration time of this fraction to the capillary outlet" (Figure 4). In agreement with our hypothesis, increasing I_{RB} led to decreasing the DNA (nonbinder) background. Remarkably, the background decreased down to and below the limit of quantitation (LOQ) of qPCR at $[NaCl] \geq 100$ mM. The values of k_N were calculated as integrals under DNA curves within the binder-collection time windows (see Section S3) divided by the total quantity of DNA sampled into the capillary. The latter was calculated as an integral under the DNA curve for $[NaCl] = 0$ within a 0 to 50 min time window. For different concentrations of NaCl in RB we obtained the following values of k_N : 8×10^{-6} (0 mM NaCl), 6×10^{-7} (25 mM NaCl), 2×10^{-8} (50 mM NaCl), 9×10^{-9} (75 mM NaCl), and 6×10^{-10} (100 mM NaCl). Adding 100 mM NaCl to RB resulted in 1.3×10^4 times (!) decrease in k_N in comparison to no NaCl in RB. We did not calculate k_N for $[NaCl] \geq 125$ mM as the quantity of DNA was well below the LOQ of qPCR. These experiments prove that IFCE (*i.e.* $v_{TB} > 0 > v_N$) can drastically decrease the nonbinder background and reach $k_N \sim 10^{-9}$. In the rest of the study, IFCE was conducted with RB containing 100 mM NaCl ($I_{RB} \approx 150$ mM).

So far we assumed that $k_B \approx 1$, and, thus, k_B/k_N was anticipated to be predominantly defined by k_N . In principle, k_B can be much lower than unity due to binder loss in partitioning. In solid-phase partitioning, the "best" aptamers can be lost due to the inability to soft-dissociate them from the surface-immobilized target. In CE-based partitioning, aptamers can be lost due to an incorrectly-determined binder-collection time window. Here, we proved our assumption of $k_B \approx 1$ by (i) determining the quantity of MutS-aptamer (target-binder) complex sampled, $B_{in} = (8.9 \pm 0.9) \times 10^8$, (ii) determining the quantity of aptamers (binders) collected in the binder-collection time window corresponding to the elution time window of the MutS-aptamer complex, $B_{out} = (7.3 \pm 0.5) \times 10^8$, and (iii) calculating $k_B = B_{out}/B_{in} = 0.8 \pm 0.3 \approx 1$. See Section S4 for details.

To test if the achieved in IFCE efficiency of partitioning of $k_B/k_N > 1.7 \times 10^9$ could facilitate one-step selection of aptamers we conducted partitioning of MutS binders from a random-sequence DNA library. To exclude the effect of potential contamination of solutions with traces of the aptamer used to measure k_N and k_B , the library was designed with PCR-priming regions different from those of the aptamer. A sample of the equilibrium mixture containing the library ($B_{in} + N_{in} \approx 2.8 \times 10^{11}$)

and MutS was subjected to IFCE. Two-minute fractions were collected and analyzed by qPCR to build a "DNA quantity vs migration time to the capillary outlet" electropherogram; the control experiment was similar, but MutS in the equilibrium mixture was replaced with RB (Figure 5). B_{out} and N_{out} were calculated as integrals under the curves within the target-binder complex collection time window, $t_1 - t_2$ (13 to 31 min), in IFCE and control experiment, respectively. They were found to be $B_{out} \approx 2.9 \times 10^6$ and $N_{out} \approx 1.1 \times 10^3$, and, accordingly, $B_{out}/N_{out} \approx 2.6 \times 10^3$. Thus, IFCE could support $B_{out}/N_{out} \gg 100$, which confirmed completed selection using the chosen very strong criterion of selection completion. This experiment independently confirmed that $k_N = N_{out}/N_{in} \sim 10^{-9}$ can be reached in a real selection (from a random-sequence DNA library and in the presence of a protein target). The knowledge of the quantity of the sampled nonbinders $N_{in} \approx B_{in} + N_{in}$ and the quantity of sampled binders $B_{in} \approx B_{out}$ (as $k_B \approx 1$) uniquely allowed estimation of the initial binder abundance: $B_{in}/N_{in} \approx 1.0 \times 10^{-5}$. In other words, approximately 0.001% of the random-sequence library were bound to MutS in the equilibrium mixture containing 100 nM MutS and stayed bound for the duration of the 1-h long IFCE partitioning run. The initial abundance is obviously not an invariant; it depends on the natures of target and library, their concentrations, incubation time, *etc.* IFCE can uniquely facilitate studies which are needed to understand how B_{in}/N_{in} depends on these parameters.

As a final step, we amplified a fraction containing the highest quantity of complexes by PCR using a fluorescently-labeled primer. After amplifying DNA in this fraction by PCR, we performed a pressure-aided IFCE-based binding test with fluorescence detection (see Section S5).^[12] This test revealed an apparent equilibrium dissociation constant of the enriched library of $K_{d,app} \approx 40$ nM and confirmed successful selection of a high-affinity aptamer pool in one step of IFCE partitioning. For comparison, selecting a pool with similar $K_{d,app}$ by classical CE-based partitioning required three rounds of SELEX.^[11d] This successful one-step selection, in turn, confirmed correctness of our estimate that $k_N \sim 10^{-9}$ was sufficient for one-step selection. Cumulatively, this study proves that our approach of rationally developing a partitioning method for one-step selection of binders from oligonucleotide libraries does work.

To summarize, the condition of IFCE, *i.e.*, the migration of target-binder complexes and nonbinders in the opposite directions, was achieved by raising I_{RB} to a physiological value at physiological pH. The higher I_{RB} is also expected to suppress non-specific interactions, *e.g.* of the protein target with nonbinders and the inner capillary wall (the latter is arguably the main limitation of CE-based partitioning). The value of I_{RB} may be increased above 150 mM used in our study to further suppress the non-specific interactions if needed. On the other hand, if only a minimal increase in I_{RB} is desired (*e.g.* to minimize heat generation), an equivalent decrease in $|\mu_{EOF}|$ can be achieved by employing lower concentrations of salts with larger cations (*e.g.* K^+ , Rb^+ , Cs^+).^[13] Addition of passivating agents, such as bovine serum albumin and non-ionic surfactants, to RB may also potentially aid the suppression of non-specific interactions. IFCE allowed reaching uniquely low nonbinder background with $k_N \sim 10^{-9}$. This value is $\sim 10^7$ lower than k_N values of practical solid-phase partitioning methods. Importantly, such extremely low k_N was reached without sacrificing k_B which was near unity. The resulting k_B/k_N was sufficient for selection of a potent aptamer pool for MutS protein in one step of partitioning. Note, that in IFCE-based binder selection, oligonucleotide amplification by PCR or another process is needed only for binder identification; therefore, high-fidelity amplifying enzymes (*e.g.* polymerases) must be used. While the proof of IFCE

was done here with a random-sequence DNA library, we foresee that IFCE will be directly applicable to selection of binders from other anionic libraries with a uniform charge. For example, IFCE should greatly benefit selection of binders from DNA-encoded libraries to which SELEX is not applicable due to inability to PCR-amplify such libraries.^[14]

Conflict of interest

The authors declare no conflict of interest.

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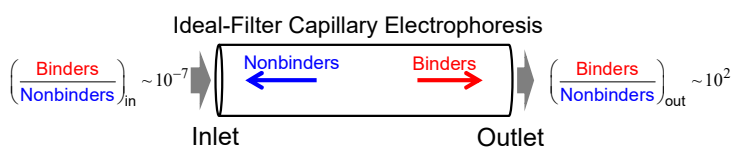
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Entry for the Table of Contents

Layout 2:

COMMUNICATION



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Ideal-Filter Capillary Electrophoresis

We introduce *Ideal-Filter Capillary Electrophoresis (IFCE)*, which facilitates reliable and practical one-step selection of oligonucleotide aptamers for protein targets. IFCE will also be applicable to selection of binders from DNA-encoded libraries of small-molecules.

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Section S1: Values of I_{RB} for RBs used in this study

Table S1. RBs used in this study and their corresponding values of I_{RB}

RB	I_{RB} (mM)
50 mM Tris HCl pH 7.0	46
50 mM Tris HCl pH 7.0, 25 mM NaCl	71
50 mM Tris HCl pH 7.0, 50 mM NaCl	96
50 mM Tris HCl pH 7.0, 75 mM NaCl	121
50 mM Tris HCl pH 7.0, 100 mM NaCl	146
50 mM Tris HCl pH 7.0, 125 mM NaCl	171
50 mM Tris HCl pH 7.0, 150 mM NaCl	196

Note, that the I_{RB} value for 1×PBS buffer (138 mM NaCl, 2.7 mM KCl, 1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4) is 162 mM.

Section S2: Determination of electric field strength (E) that guarantees no overheating

The value of I_{RB} was changed by introducing NaCl in concentration ranging from 25 to 150 mM to RB. A CE instrument utilized in our study allowed capillary thermo-stabilization *via* its contact with a liquid coolant stabilized at T_{coolant} . The central longest part of the capillary was washed by the coolant and had temperature $T_{\text{cooled}} \geq T_{\text{coolant}}$; while the two short flanking parts of the capillary were not in contact with the coolant and their temperature $T_{\text{non-cooled}}$ was higher than T_{cooled} . The goal of this part of the study was to find E which guaranteed $T_{\text{cooled}} \leq 20^\circ\text{C}$ and $T_{\text{non-cooled}} \leq 42^\circ\text{C}$ at $T_{\text{coolant}} = 15^\circ\text{C}$.

The following SUMET equations were utilized for determination of T_{cooled} and $T_{\text{non-cooled}}$ (Patel, K. H.; Evenhuis, C. J.; Cherney, L. T.; Krylov, S. N. Simplified universal method for determining electrolyte temperatures in a capillary electrophoresis instrument with forced-air cooling. *Electrophoresis* 2012, 33, 1079-1085):

$$T_{\text{cooled}} = T_{\text{coolant}} + \Delta T_{\text{cooled}} = T_{\text{coolant}} + \frac{c \left(\frac{V \times I}{L} \right)^n}{g + \left(\frac{V \times I}{L} \right)^n} \times \frac{V \times I}{L} \quad (\text{Eq. S1})$$

$$T_{\text{non-cooled}} = T_{\text{coolant}} + \Delta T_{\text{non-cooled}} = T_{\text{coolant}} + ka \left(\frac{V \times I}{L} \right) \times \frac{V \times I}{L} \quad (\text{Eq. S2})$$

Here, T_{coolant} is the set temperature of coolant in $^\circ\text{K}$; V is the set voltage in kV; I is the obtained current in μA ; L is the total length of the capillary in mm; c , g , n , k and a are instrument-specific and capillary-dependent empirical parameters. For a capillary with inner diameter of 75 μm , these values are as follows: $c = 1.437$; $g = 0.881$; $n = 0.250$; $a = 0.95$.

Since the highest I_{RB} corresponds to the greatest Joule heat, it was sufficient to determine E which satisfied these conditions for RB containing 150 mM NaCl; RBs with lower I_{RB} would then automatically satisfy the two conditions. Accordingly, the electric current was measured for RB containing 150 mM NaCl during 1 min for each of six values of the applied voltage and for six corresponding values of E : 20, 100, 200, 300, 400 and 500 V/cm. The collected current-voltage data and the SUMET program were used to determine T_{cooled} and $T_{\text{non-cooled}}$ for each E ; they are shown in Table S2.

Table S2. Values of T_{cooled} and $T_{\text{non-cooled}}$ for RBs with the highest I_{RB} (50 mM Tris-HCl pH 7.0, 150 mM NaCl) and for different values of E

E (V/cm)	T_{cooled} (°C)	$T_{\text{non-cooled}}$ (°C)
20	15.0	25.2
100	15.5	29.4
200	17.5	41.5
300	21.7	57.6
400*	26.7	67.5
500*	29.9	70.6

Capillary with a total length of 50 cm and inner diameter of 75 μm was used. Temperature of coolant was 15 °C. Electrical current measurements were obtained by applying 1, 5, 10, 15, 20 and 25 kV for a period of 1 min at each voltage.

*Current exceeded 300 μA , which is the current limit in the CE instrument used.

The highest E satisfying both $T_{\text{cooled}} \leq 20$ °C and $T_{\text{non-cooled}} \leq 42$ °C was 200 V/cm; the sought temperatures were $T_{\text{cooled}} = 17.5$ °C and $T_{\text{non-cooled}} = 41.5$ °C. We then conducted current-voltage measurements at $E = 200$ V/cm for RBs with lower ionic strengths and determined T_{cooled} and $T_{\text{non-cooled}}$; they are shown in Table S2.

Table S3. Values of T_{cooled} and $T_{\text{non-cooled}}$ for different RBs (based on Eq. S1 and Eq. S2)

RB	T_{cooled} (°C)	$T_{\text{non-cooled}}$ (°C)
50 mM Tris HCl pH 7.0	15.5	29.0
50 mM Tris HCl pH 7.0, 25 mM NaCl	15.7	31.0
50 mM Tris HCl pH 7.0, 50 mM NaCl	16.2	34.0
50 mM Tris HCl pH 7.0, 75 mM NaCl	16.6	36.6
50 mM Tris HCl pH 7.0, 100 mM NaCl	17.0	38.7
50 mM Tris HCl pH 7.0, 125 mM NaCl	17.3	40.4
50 mM Tris HCl pH 7.0, 150 mM NaCl	17.5	41.5

Capillary with a total length of 50 cm and inner diameter of 75 μm was used. The voltage was set at 10 kV and temperature of coolant was 15 °C. Electrical current measurements were obtained for different RBs by applying 10 kV for a period of 1 min.

As expected all values of T_{cooled} were in a range of 15.0–17.5 °C and all values of $T_{\text{non-cooled}}$ were higher than 17.5 °C and lower than 41.5 °C. Thus, an electric field of 200 V/cm guaranteed $T_{\text{cooled}} \leq 20$ °C and $T_{\text{non-cooled}} \leq 42$ °C and was used in the rest of this study.

To avoid sample exposure to higher temperatures in the inlet non-cooled part of the capillary, the sample was propagated through this part by pressure at $E = 0$, and the voltage was applied only when the sample was 2 cm inside the cooled part of the capillary. The intact complexes were still exposed to the elevated $T_{\text{non-cooled}} \leq 41.5$ °C while passing through the non-cooled part at the capillary outlet.

Section S3: Determination of elution windows of the MutS-aptamer complex

Aptamer-collection time windows were determined as widths of the bases of peaks of MutS-aptamer complexes in CE experiments performed with NaCl-free RB and fluorescence detection (Figure S1).

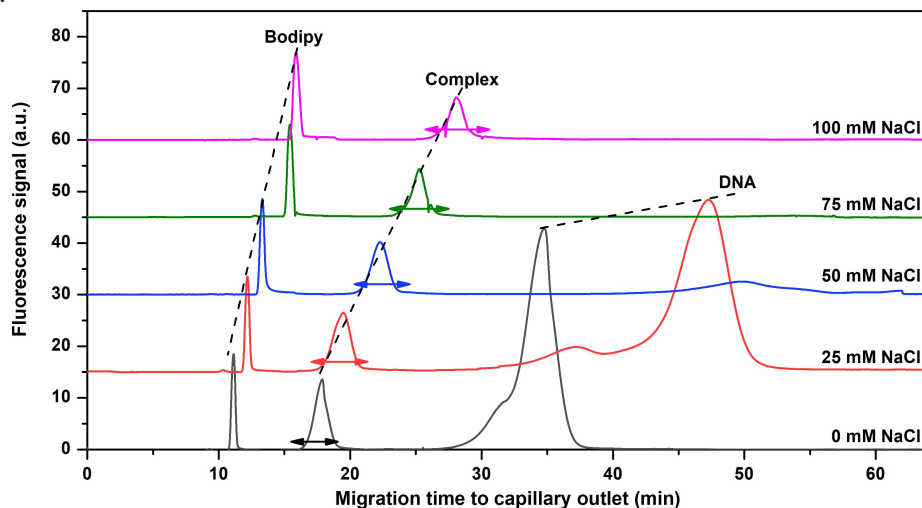


Figure S1. Determination of time windows for MutS-aptamer complex collection for RBs based on 50 mM Tris-HCl pH 7.0 and containing concentrations of NaCl varying from 0 to 100 mM. The equilibrium mixture contained 100 nM MutS, 100 nM fluorescently-labeled aptamer, and 150 nM Bodipy (EOF marker). Electrophoresis was carried out with an electric field of 200 V/cm, “+” at the capillary inlet, and a separation distance of 34 cm. The double-headed arrows indicate estimated elution windows of the MutS-aptamer complex.

Section S4: Details on the determination of efficiency of binder collection

We found k_B experimentally as $k_B = B_{out} / B_{in}$, where B_{in} is the number of target-binder complexes sampled and B_{out} is the number of binders collected in the binder-collection time window corresponding to the elution time window of the target-binder complexes. We first used NaCl-free RB in which the target-binder complexes and nonbinders migrate in the same direction (see Figure 2b in the main text) allowing for very accurate determination of B_{in} by using fluorescence detection (Figure S2). A known volume of the equilibrium mixture containing MutS and its DNA aptamer was sampled for a CE run, in which both fluorescence and qPCR detections were used leading to two electropherograms for each CE run (Figure S2a,b).

The value of $B_{in} = (8.9 \pm 0.9) \times 10^8$ was found from an electropherogram with fluorescence detection shown in Figure S2a as the multiplication product of (i) the total concentration of aptamer in the equilibrium mixture, (ii) the volume of the sampled equilibrium mixture, and (iii) a relative amount of aptamer that was bound to MutS in the equilibrium mixture (relative to the total sampled amount of aptamer). We made no correction for complex dissociation during CE as less than 1% of the MutS-aptamer complex dissociated during complex migration to the capillary exit. The degree of complex dissociation during CE-based partitioning of MutS-aptamer complex from the unbound aptamer was assessed *via* determination of the value of the rate constant of complex dissociation (k_{off}):

$$k_{off} = \frac{\ln((A_C + A_D) / A_C)}{t_{detection}} = 0.003 \text{ min}^{-1}$$

where A_D and A_C are areas under the electropherograms segments (Figure 5a in the main text) corresponding to complex decay and the intact complex at the time of its detection, $t_{detection}$, respectively. The quantity of intact complex at the capillary outlet (B_{out}) was calculated as:

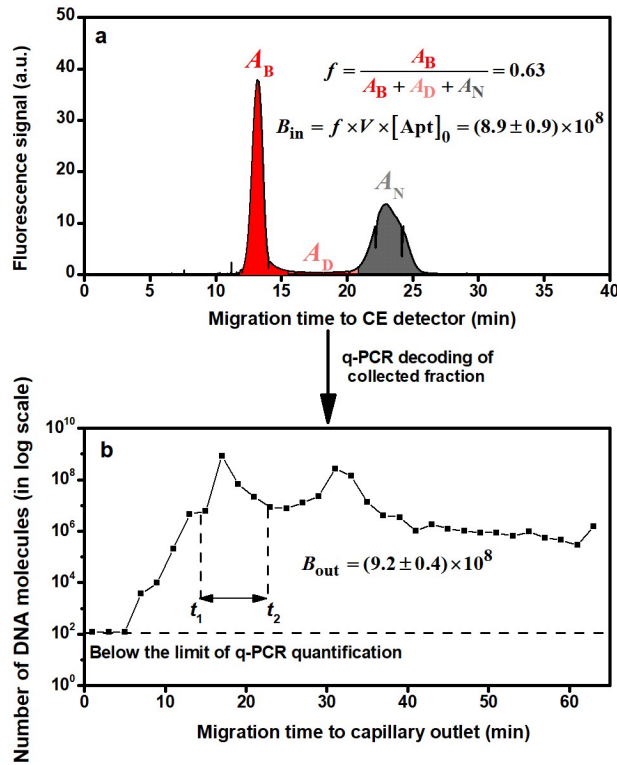


Figure S2. Determination of B_{in} (a) and B_{out} (b) for IFCE-based partitioning of MutS-bound aptamer and the unbound aptamer. A known volume, $V = 47$ nL, of the equilibrium mixture containing 200 nM MutS and 50 nM fluorescently-labeled aptamer ($[Apt]_0 = 50$ nM) was injected and CE was carried out with an electric field of 200 V/cm with a separation distance of 34 cm. Fluorescence detection was used to obtain an ordinary electropherogram shown in Panel a. Two-min fractions were collected and the number of aptamer copies was determined in each fraction by qPCR to produce an electropherogram in Panel b. A relative amount of MutS-bound aptamer (f) was found from the shaded areas in Panel a, and B_{in} was determined with formulas shown within the panel. B_{out} was calculated as an integral under the curve in Panel b within the binder-collection time window $t_1 - t_2$.

$$B_{out} = B_{detected} \exp(-k_{off}(t_{out} - t_{detection})) = 8.8 \times 10^8$$

Here, $B_{detected}$ is the amount of complex that was intact at the time of its passing the detector ($B_{detected} = 8.9 \times 10^8$) and t_{out} is the time when the complex reached the capillary outlet. The value of $(t_{out} - t_{detection})$ was found to be 4 min. Thus, less than 1% of the complex dissociated during complex migration to the outlet from the detector.

The value of $B_{out} = (9.2 \pm 0.4) \times 10^8$ was then determined from the electropherogram with qPCR detection shown in Figure S2b in the main text by calculating an integral under the DNA curve within the binder-collection time window of 15 to 23 min. Knowing B_{out} and B_{in} allowed us to calculate $k_B \equiv B_{out} / B_{in} = 1.0 \pm 0.1$.

We then conducted an IFCE experiment (with RB containing 100 mM NaCl) and found $B_{out} = (7.3 \pm 0.5) \times 10^8$, which is slightly lower than the value of $(9.2 \pm 0.4) \times 10^8$ obtained for NaCl-free RB. The difference was presumably due to additional ions' in IFCE affecting the strength of ionic bonds in protein-DNA complexes. These experiments demonstrated that by choosing a proper binder-collection time window we are able to satisfy our assumption of $K_b \approx 1$.

Section S5: Evaluation of binding affinity of aptamer pool selected for Mutts

The equilibrium mixture of the enriched DNA library with 100 nM MutS was subjected to pressure-aided IFCE with fluorescent detection. A distinct peak corresponding to MutS-DNA complexes was observed in a time window of 7 to 9 min followed by a smaller peak of unbound DNA with a maximum at 11 min. The apparent equilibrium dissociation constant of $K_{d,app} \approx 40$ nM for the interaction of the enriched library with MutS protein was found from Figure S3.

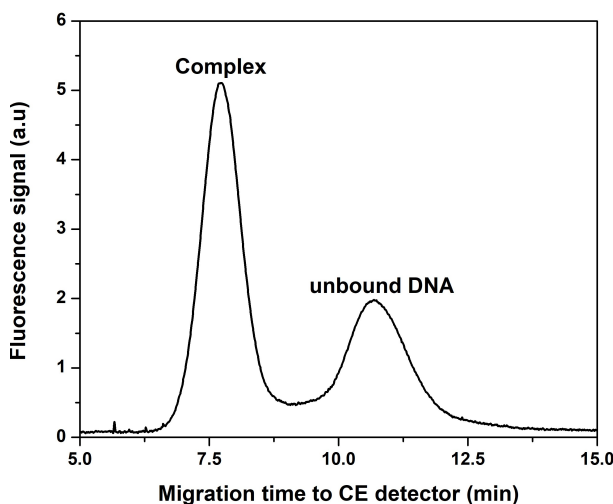


Figure S3. Pressure-aided IFCE separation of the components of equilibrium mixture containing 20 nM fluorescently labeled enriched DNA library and 100 nM MutS. The running buffer was 50 mM TrisHCl pH 7.0, 100 mM NaCl. CE was carried out with an electric field of 200 V/cm (“+” at the inlet) with a pressure supplement of 0.2 psi. The separation distance was 34 cm.

Section S6: Experimental section

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 *Thermus aquaticus* MutS protein (MW \approx 90 kDa, pI 6.0) was expressed and purified as described previously (Beloborodov, S. S.; Bao, J.; Krylova, S. M.; Shala-Lawrence, A.; Johnson, P. E.; Krylov, S. N., Aptamer facilitated purification of functional proteins. *J. Chromatogr. B.* 2018, 1073, 201-206). All DNA molecules were custom synthesized by Integrated DNA Technologies (Coralville, IA, USA). Bodipy (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) was purchased from Life Technologies Inc. (Burlington, ON, Canada). The RB was 50 mM Tris-HCl with NaCl ranging from 0 to 150 mM at pH 7.0. The sample buffer was always identical to RB to prevent adverse effects of buffer mismatch. Accordingly, all dilutions of sample components used in CE experiments were done by adding the corresponding RB.

DNA sequences

The DNA aptamer with affinity toward MutS protein was previously selected in our laboratory (clone 2-06) (Drabovich, A. P.; Berezovski, M.; Okhonin, V.; Krylov, S. N., Selection of smart aptamers by methods of kinetic capillary electrophoresis. *Anal. Chem.* 2006, 78, 3171-3178), and its

fluorescein amidite (FAM)-labeled version was used here: 5'-FAM-CTT CTG CCC GCC TCC TTC CTG GTA AAG TCA TTA ATA GGT GTG GGG TGC CGG GCA TTT CGG AGA CGA GAT AGG CGG ACA CT-3'. For aptamer-selection study, a synthetic FAM-labeled DNA library (N40) with a 40-nt random region was used: 5'-FAM-AGC CTA ACG CAG AAC AAT GG-(N40)-CGA TGC CAG GTT AAA GCA CT-3'. The following primers were used for PCR amplification of the MutS aptamer: forward primer (MutS_uF), 5'-CTT CTG CCC GCC TCC TTC C-3'; reverse primer (MutS_uR), 5'-AGT GTC CGC CTA TCT CGT CTC C-3'. Two sets of primers were used to amplify binders selected from the naïve library. The first set of primers was an unlabelled forward primer (N40_uF), 5'-AGC CTA ACG CAG AAC AAT GG-3', and an unlabeled reverse primer (N40_uR), 5'-AGT GCT TTA ACC TGG CAT CG-3'. The second set contained a FAM-labeled forward primer (N40-famF), 5'-FAM-AGC CTA ACG CAG AAC AAT GG-3', and a biotin-labeled reverse primer (N40-biotinR), 5'-Biotin-TEG-AGT GCT TTA ACC TGG CAT CG-3'.

Default conditions for CE and fraction collection

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). Uncoated fused-silica capillaries, with a total length of 50 cm and a 10.2-cm distance from one of the ends to the detection zone were used. The two capillary ends were used as inlets interchangeably in experiments requiring different separation distances. The separation distance was defined as the distance in the capillary from the sample position at the time of electric field application to the detection point. Prior to every run, the capillary was rinsed successively with 0.1 M HCl, 0.1 M NaOH, deionized H₂O, and a run buffer for 3 min each. The sample contained 10 μ M annealed oligonucleotides (melted at 90 °C for 2 min and gradually cooled down to 20 °C at a rate of 0.5 °C/s) and 150 nM Bodipy. When specified, the sample also contained 100 nM MutS protein. The sample mixture was incubated for 30 min at a room temperature (22–24 °C) and then injected with a pressure pulse of 0.5 psi \times 10 s to yield a 10 mm long sample plug. The injected sample plug was propagated through the uncooled part of the capillary at the inlet by injecting a 5.7 cm long plug of the RB with a pressure pulse of 0.3 psi \times 90 s. CE was carried out at an electric field of 200 V/cm (10 kV over 50 cm). The duration of electrophoretic runs without fraction collection was 50 min. The duration of electrophoretic runs with fraction collection was 64 min. Collection vials contained 20 μ L of the RB each and were switched every 2 min; 32 fractions were collected for each run.

Quantitative PCR

DNA in the collected fractions was amplified and quantitated by qPCR using a CFX ConnectTM instrument from Bio-rad. q-PCR reagent mixture was prepared by combining IQ SYBR Green Supermix from Bio-Rad (Mississauga, ON, Canada) with unlabeled DNA primers at final concentrations of 1 \times SYBR Green Supermix, 100 nM MutS_uF, and 100 nM MutS_uR. qPCR reaction mixture was prepared by adding 18 μ L of the qPCR reagent mixture to a 2- μ L aliquot of each fraction immediately before thermocycling. The thermocycling protocol was: 95 °C for 3 min, 95 °C for 10 s (denaturation), 56 °C for 10 s (annealing), 72 °C for 10 s (extension), followed by a plate read at 72 °C and a return to the denaturation step (bypassing the 95 °C \times 3 min step) for a total of 43 cycles. All reactions were performed in duplicates.

Specifics of single-round aptamer selection

Fraction collection and qPCR detection were similar to the procedures described in the previous two sections with a few modifications specified below. The equilibrium mixture contained: 10 μ M N40 library, 100 nM MutS protein, and 150 nM Bodipy. For qPCR, 1 \times SYBR Green Supermix and the unlabeled primers for N40 library (100 nM of each N40_uF and N40_uR) constituted the qPCR reagent mixture. The fraction which eluted at minute 29 and contained the highest amount of MutS-DNA complexes was subjected to preparative PCR. The procedure of preparative PCR involved two rounds of amplification. In the first round, the fraction was amplified by qPCR in quintuplicates as previously described. An S-shaped amplification curve was plotted, and the PCR product was removed two cycles into the exponential phase of the curve. After qPCR, 100 μ L of the five combined PCR reactions was purified using MinElute[®] PCR purification kit (QIAGEN, Mississauga, ON, Canada) as per manufacturer's instructions. DNA was then eluted using 20 μ L of 50 mM TrisHCl, pH 7.0. Once product's purity was verified by native PAGE, it was subjected to asymmetric PCR. Five μ L of DNA was added to 45 μ L of asymmetric PCR reagent mixture from New England Biolabs Inc. (MA, USA). Final concentrations of PCR reagents in the reaction mixture were: 1 \times Standard *Taq* Reaction Buffer, 1 μ M N40-famF, 50 nM N40-biotinR, 2.5 units/ μ L *Taq* DNA Polymerase, and 200 μ M dNTPs mix. The reaction was performed in duplicates with the following temperature protocol: 94 $^{\circ}$ C for 30 s (initial denaturation, performed once), 94 $^{\circ}$ C for 10 s (denaturation), 56 $^{\circ}$ C for 10 s (annealing), and 72 $^{\circ}$ C for 10 s (extension). Seventeen cycles of asymmetric PCR were run. Ten μ L of MagnaBind[™] streptavidin beads suspension (Thermo Scientific, IL, USA) 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 room temperature. The beads were magnetized, discarded, and the PCR product was then purified using the MinElute[®] PCR purification kit. The final product was eluted using 20 μ L of 50 mM TrisHCl, pH 7.0, and 2 μ L of 1 M NaCl was added to bring NaCl concentration to 100 mM.

To determine DNA concentration in the enriched library pool, serial dilutions of N40-famF (2 μ M, 1 μ M, 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 (Thermo Scientific, IL, USA). DNA concentration in the enriched library pool was found to be 1.2 μ M. For a pressure-aided IFCE-based binding test of the enriched pool (used to determine $K_{d,app}$), a 47 nL plug of the equilibrium mixture containing 20 nM enriched library and 100 nM MutS in 50 mM Tris-HCl, 100 mM NaCl, pH 7.0, (default running buffer for IFCE) was injected into 50-cm-long capillary by a 0.5 psi \times 10 s pressure pulse. The sample mixture was propagated through the non-cooled portion of the capillary by injecting a 5.7-cm-long plug of RB with a pressure pulse of 0.3 psi \times 90 s. CE was carried out at an electric field of 200 V/cm with "+" at the inlet. In addition to applying voltage, a pressure of 0.20 psi was applied to the capillary inlet to supplement the electric field and ensure that the nonbinders reach the detector. The pressure-aided IFCE allowed detection of target-binder complexes and nonbinders, which is required for determination of $K_{d,app}$ (Kanoatov, M.; Krylov, S. N., Analysis of DNA in phosphate buffered saline using kinetic capillary electrophoresis. *Anal. Chem.* 2016, 88, 7421-7428).