Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides

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Aptamers are typically selected from libraries of random DNA (or RNA) sequences through systematic evolution of ligands by exponential enrichment (SELEX), which involves several rounds of alternating steps of partitioning of candidate oligonucleotides and their PCR amplification. Here we describe a protocol for non-SELEX selection of aptamers — a process that involves repetitive steps of partitioning with no amplification between them. Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), which is a highly efficient affinity method, is used for partitioning. NECEEM also facilitates monitoring of bulk affinity of enriched libraries at every step of partitioning and screening of individual clones for their affinity to the target. NECEEM allows all clones to be screened prior to sequencing, so that only clones with suitable binding parameters are sequenced. The entire protocol can be completed in 1 wk, whereas conventional SELEX protocols take several weeks even in a specialized industrial facility.

INTRODUCTION

DNA or RNA aptamers are single-stranded oligonucleotides that can bind proteins, small-molecule compounds and living cells with high affinity and specificity[1,2]. Aptamers are promising affinity ligands with the potential to change the field of affinity probes and replace antibodies as diagnostic, analytical and therapeutic reagents[3–5]. Aptamers have indisputable advantages over antibodies owing to the ease and low cost of production, and the simplicity of chemical modifications and integration into different analytical schemes. The unique properties of aptamers have led to their application in many areas of bioanalytical and biomedical sciences. They have been successfully used in proteomics and development of bioanalytical assays[6], inhibition of enzymes and receptors[7,8], development of artificial enzymes (ribozymes and aptazymes)[9], target validation and screening for drug candidates[10–12], cytometry and imaging of cellular organelles[13], and development of biosensors[14]. Aptamers are gaining a reputation as therapeutic reagents for the treatment of different pathologies[15]. Their potential medical applications also include gene therapy and drug delivery to therapeutic targets[16]. Despite great promise and significant effort in the development of aptamers over a period of 15 yr, they have only been obtained for ~100 protein targets[17]. This slow progress is largely due to the limitations of conventional technologies used for aptamer development.

Aptamers are typically selected from large libraries of random DNA or RNA sequences in a general approach termed systematic evolution of ligands by exponential enrichment (SELEX)[18,19]. In essence, SELEX involves repetitive rounds of two alternating processes: (i) partitioning of aptamers from non-aptamers by separating target-bound DNA from free DNA; and (ii) amplification of aptamers by PCR (**Fig. 1a**).

Non-instrumental methods of partitioning, such as filtration and gel-electrophoresis, were initially used for SELEX, and still dominate the area[20–25]. Because of high background (i.e., the high level of target-non-bound DNA collected along with target-bound DNA), SELEX based on conventional partitioning methods requires numerous rounds of selection (typically >10)[26–30]. As a result, SELEX based on conventional partitioning methods is a lengthy and resource-consuming process, which often leads to DNA structures that bind to the surfaces of the filters or chromatographic supports used in partitioning, rather than to the target[28–30]. Counter selection is successfully employed to eliminate such surface aptamers; however, it introduces additional rounds of selection, making the procedure even longer. Another disadvantage of too many rounds of selection is the limited number of unique aptamer sequences obtained at the output of conventional SELEX[30]. This disadvantage is critical for aptamer-based drug development, which requires as many 'lead molecules' as possible. Finally, if the efficiency of partitioning is too low, SELEX can fail to select aptamers.

Kinetic capillary electrophoresis (KCE) methods[31], which started with the pioneering works of Heegaard and Whitesides on affinity capillary electrophoresis (ACE)[32,33], have established a new methodological platform for the partitioning of aptamers. So far, two distinct KCE methods have been used for the selection of aptamers: non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[34–38] and equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM)[37,39]. Bowser and co-authors were the first to use NECEEM in SELEX; they called the approach CE-SELEX[34,35]. The partitioning efficiency of KCE methods exceeds that of conventional partitioning methods, such as filtration and column chromatography, by at least two orders of magnitude[36].

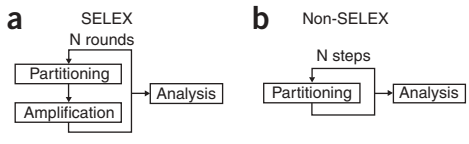

Figure 1 | Schematic representation of SELEX (**a**) and non-SELEX (**b**) selection of aptamers.

As a result, KCE methods decrease the number of rounds of SELEX from ≥ 10 (required by conventional partitioning techniques) to 1–3. In addition, KCE methods can be equally used for selection of aptamers and for measurements of their binding parameters: the equilibrium dissociation constant (K_d), rate constants of complex formation (k_{on}) and dissociation (k_{off}), and the change of enthalpy (ΔH) and entropy (ΔS)[40,41]. KCE methods have been demonstrated to facilitate the selection of 'smart' aptamers — ligands with predefined binding parameters[37,39].

The outstanding partitioning capabilities of KCE methods have recently motivated us to attempt the selection of aptamers in a procedure that does not include intermediate amplification steps; we call this approach non-SELEX[38] (**Fig. 1b**). Excluding repetitive steps of PCR accelerates the procedure of aptamer selection without compromising its efficiency. Omitting repetitive steps of PCR also excludes quantitative errors associated with the exponential nature of PCR amplification, thereby making non-SELEX a useful tool for studies of the properties of DNA libraries with respect to their interaction with targets. For example, non-SELEX can be used to accurately back-calculate the number of aptamer molecules in a naive DNA library. Furthermore, excluding repetitive steps of PCR allows us to avoid the bias related to differences in PCR efficiency with respect to different oligonucleotide sequences. Finally, non-SELEX can potentially provide a viable alternative to SELEX in the commercial development of aptamers[42]. It should be noted that the implementation of non-SELEX with currently available commercial CE instrumentation has a limitation: only a fraction of the collected ligands can be sampled for the next step of non-SELEX. This limitation requires that the fraction of aptamers in the naive library be no lower than 5×10^{-10} for the parameters used in this protocol. Our experience shows that the abundance of aptamers in the naive library is typically $>5 \times 10^{-10}$, thereby making the limitation less important.

In a recent proof-of-principle work, we demonstrated the use of NECEEM-based non-SELEX for the selection of DNA aptamers for h-Ras protein[38]. Here we describe a step-by-step protocol for this method (**Fig. 2**). As the method relies on electrophoretic separation of DNA–target complexes from free DNA, it is applicable to targets with relatively high molecular weights (e.g., proteins and peptides). In the pre-selection steps (Steps 1–10), migration times of the target, naive DNA library and DNA–target complex (if detectable) are measured. These parameters are used to determine the time window for collection of aptamers (we call this the aptamer-collection window). In addition, if the DNA–target complex is detectable, the bulk affinity of the naive library to the target is determined; this is later used to study the progress of aptamer selection. In the selection steps (Steps 11–19), candidate oligonucleotides are partitioned from the rest of the library by multiple steps of NECEEM with no PCR amplification between them. Fractions collected after every step of NECEEM partitioning are kept for subsequent analysis. In the analysis steps (Steps 20–33), all of the collected DNA fractions are concurrently amplified by PCR using the optimized number of PCR cycles, the strands of double-stranded PCR products are separated to obtain single-stranded aptamer pools and, finally, the bulk affinities of aptamer pools to the target are measured. In the cloning and sequencing steps (Steps 34–44), individual sequences from the best pool are amplified by bacterial cloning and asymmetric PCR, and their binding parameters (rate constants and equilibrium constants) to the target are measured. Only aptamers with suitable binding parameters are then sequenced.

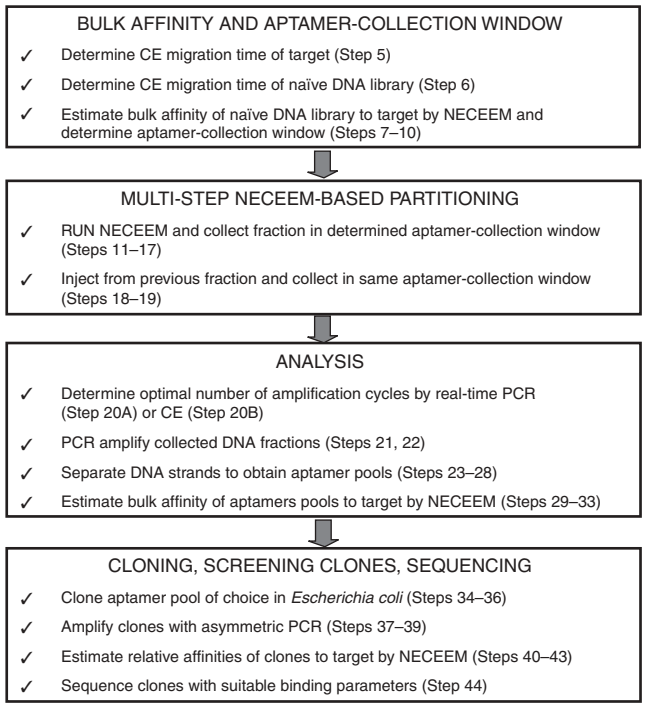

Figure 2 | Flow chart of non-SELEX selection of DNA aptamers using NECEEM for partitioning of aptamers from non-aptamers.

The non-SELEX concept can also be used to develop a similar protocol for selection of RNA aptamers. NECEEM-based partitioning in the protocol can be replaced with partitioning by other KCE methods via minor changes in the procedure. In addition to selection of aptamers, non-SELEX provides the opportunity for selection of affinity ligands from DNA-tagged libraries of small molecules and peptides[43–45]. The DNA tags allow unequivocal identification of the corresponding small molecules when the DNA tags are PCR amplified and sequenced after partitioning. Due to the small size of the molecules with respect to that of the covalently-attached DNA tag, such libraries are expected to have electrophoretic properties identical to those of DNA libraries. SELEX is not applicable to such libraries as small molecules and peptides cannot be amplified by PCR.

MATERIALS

REAGENTS

- Target protein or peptide (see REAGENT SETUP)
- Naive DNA oligonucleotide library (see REAGENT SETUP)
- Oligonucleotide primers: HPLC-purified or PAGE-purified by the supplier (IDT DNA Technologies; see REAGENT SETUP)
- F primer: 5′-CTT CTG CCC GCC TCC TTC C-3′
- R primer: 5′-AGT GTC CGC CTA TCT CGT CTC C-3′
- F-FAM primer: 5′-FAM-CTT CTG CCC GCC TCC TTC C-3′
- R-biotin primer: 5′-biotin-AGT GTC CGC CTA TCT CGT CTC C-3′
- Taq DNA polymerase, 5 units per µl and 10× PCR buffer (Sigma Aldrich, cat. no. D1806)
- dNTP solution (10 mM of each dNTP; Sigma Aldrich, cat. no. D7295)

- 2× real-time PCR master mix with SYBR Green (iQ™ SYBR® Green Supermix; Bio-Rad, cat. no. 170-8882)
- 95% ethanol/3 M sodium acetate, pH 7.0, 33:1 (vol/vol)
- 70% ethanol (vol/vol)
- RNase AWAY solution for removing nucleases and nucleic-acid contamination (Molecular Bio-Products)
- pT7 Blue-3 Perfectly Blunt® cloning kit (Novagene, cat. no. 70182)
- Streptavidin-coated paramagnetic beads (Dynabeads® M-280 Streptavidin; Dynal, cat. no. 112-05D)
- Bead binding/washing buffer: 10 mM Tris-HCl (pH 8.0), 500 mM NaCl and 1 mM EDTA
- DNA storage buffer: 10 mM Tris-HCl, pH 7.4
- Capillary rinsing solutions: 100 mM NaOH, 100 mM HCl and ddH_2O
- RB1: CE run buffer for aptamer selection (50 mM Tris-acetate; pH 8.2)
- RB2: CE run buffer for PCR analysis (25 mM borax; pH 9.3)
- SB1: selection buffer (50 mM Tris-acetate (pH 8.2), 100 mM NaCl and 5 mM $MgCl_2$)
- SB2: 10× selection buffer without NaCl (500 mM Tris-acetate (pH 8.2) and 50 mM $MgCl_2$)
- SB3: 2× selection buffer (100 mM Tris-acetate (pH 8.2), 200 mM NaCl and 10 mM $MgCl_2$)
- ddH_2O purified with a MilliQ water-purification system (Millipore)
- Optional: fluorogenic amine derivatization reagent ATTO-TAG™ FQ (Molecular Probes, cat. no. A-2334) or NanoOrange dye (Molecular Probes, cat. no. N-6666; see TROUBLESHOOTING)

EQUIPMENT

- CE instrument with fraction collection and two detection systems (see EQUIPMENT SETUP)
- Bare-fused silica capillary (Polymicro, cat. no. TSP075375): total length (L_{total}) = 80 cm; length to the detection window ($L_{detection}$) = 70 cm; inner diameter (ID) = 75 μm; outer diameter (OD) = 375 μm
- Real-time thermal cycler or ordinary thermal cycler, programmed with the desired amplification protocols (Bio-Rad or Eppendorf)
- Magnetic bead separator (Magna Rack™ Magnetic Rack for use with ChargeSwitch® and 1.5 ml tubes; Dynal, cat. no. CS15000)
- 0.22 μm pore size filters for filtering of solutions: Millex-GS (0.22 μm), mixed cellulose esters (25 mm), PVC housing, non-sterile (Millipore, cat. no. SLGS025NB)
- PCR primer-design software (see REAGENT SETUP)

REAGENT SETUP

Solutions and buffers All solutions and buffers should be filtered through a 0.22-μm filter before use.

Target protein or peptide The target protein or peptide should be dissolved in SB1 selection buffer at a concentration of 10–100 μM. Storage conditions and duration will vary depending on the target. ▲ CRITICAL The target should have high purity to ensure that aptamers are selected only for the target and not the impurities.

Oligonucleotide library design The DNA library has a universal design with two constant regions (for PCR amplification) at the ends, a fluorescent label (FAM) at the 5′-end and a randomized region in the center. The length of the randomized region can be between 30 and 60 nt. A typical length of each constant region is between 18 and 20 nt. A typical library might be 5′-FAM-CTT CTG CCC GCC TCC TTC C-(39N)-GGA GAC GAG ATA GGC GGA CAC T-3′. Resuspend the naive library in DNA storage buffer to a concentration of 100 μM. The library can be stored at –20 °C for several years. ▲ CRITICAL Choose manual mixing when ordering the library to ensure a 1:1:1:1 ratio of the four nucleotides in the randomized region of the library.

PCR primer design PCR primers should be designed using appropriate software (e.g., http://scitools.idtdna.com/Primerquest) to eliminate self-dimers and hetero-dimers, according to standard primer-design considerations. Resuspend the primers in DNA storage buffer to a concentration of 10 μM. The primers can be stored at –20 °C for several years.

EQUIPMENT SETUP

CE instrument with fraction collection and two detection systems The two types of detection system are as follows: (i) laser-induced fluorescence (LIF) detection with excitation of fluorescence at 488 nm and detection of fluorescence at 520 nm; and (ii) light-absorbance detection at 280 nm. Technically suitable CE instruments are produced by Beckman-Coulter (PA 800 and PACE MDQ) and Agilent Technologies. We have only used the Beckman-Coulter instruments but plan to test the Agilent Technologies instruments in the near future.

PROCEDURE

Library preparation ● TIMING 0.5 h

1| Mix 5 μl of 100 μM naive DNA library in DNA storage buffer with 5 μl SB3, heat the mixture in a thermal cycler to 94 °C and cool down to 20 °C at a rate of 0.5 °C per s. This should take ≤5 min.

▲ CRITICAL STEP The temperature treatment is critical for proper folding of single-stranded (ss) DNA oligonucleotides. Choose the selection buffer to maintain the native structure of the target. The buffer should preferably have <200 mM total salts, and its viscosity should be similar to that of water. The selection buffer can also contain DNase inhibitors to prevent degradation of DNA. A single step of negative selection for DNA inhibitors can be performed before the selection for the target to ensure aptamers for DNase inhibitors are not selected.

■ PAUSE POINT Store the mixture at room temperature (20–25 °C) for ≤6 h.

2| Prepare a 100-nM solution of the folded DNA library (from Step 1) in 100 μl SB1.

Capillary preparation ● TIMING 0.5 h

3| Install a new capillary. All commercial CE instruments with fraction collection have detectors shifted from the end of the capillary. To calculate the migration time of any species to the end of the capillary, the migration time to the detector has to be multiplied by the following conversion factor (f):

$$f = L_{total}/L_{detector}. \quad (1)$$

Here L_{total} and $L_{detector}$ are lengths from the capillary inlet to its outlet and detector, respectively. In our case, f = 80 cm/70 cm = 1.14.

4| Rinse the capillary with approximately four capillary volumes (15 μl) of each of the following capillary rinising solutions: 100 mM HCl, 100 mM NaOH, ddH_2O and RB1. For L_{total} = 80 cm and capillary ID = 75 μM, rinsing is carried out at a pressure of 137 kPa (20 psi) for 2 min for each solution. Set the temperature of the capillary and sample storage at 15 °C.

▲ CRITICAL STEP The CE run buffer is chosen to facilitate the best quality separation of a target from DNA. The buffer should preferably have <200 mM total salts to prevent excessive Joule heating and interference with the subsequent PCR reaction. If the target is a protein, use Tris-based or borate-based CE run buffers with a pH between 7 and 9.

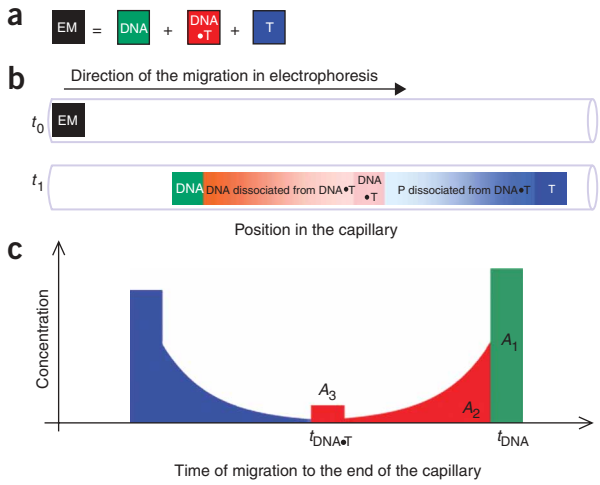

Figure 3 | Schematic representation of NECEEM-based determination of DNA affinity to the target (T). (**a**) Components of the equilibrium mixture (EM): DNA, DNA–target complex (DNA●T) and T. (**b**) NECEEM-based separation of DNA, T and DNA●T. A short plug of the EM is injected into a capillary at time t_0. High voltage is then applied. It is assumed that T migrates faster than DNA; DNA●T typically has an intermediate mobility. Equilibrium fractions of free DNA and T migrate as individual zones (green and blue rectangles), which do not change in time. The equilibrium fraction of DNA●T continuously dissociates during separation, leaving smears of DNA (red) and T (blue). By time t_1, only a fraction of DNA●T remains intact (red rectangle). (**c**) The parameters obtained form a single NECEEM electropherogram for the determination of K_d and k_{off}, areas A_1–A_3 (defined in Step 9), and migration times of the complex ($t_{DNA•T}$) and free DNA (t_{DNA}).

Determination of library bulk affinity and aptamer-collection window ● TIMING 3–4 h

5| Inject 150 nl (13.7 kPa or 2 psi for 13 s) of the target solution (10–100 μM) into the capillary and run CE in RB1 at 375 V cm^{-1} (normal polarity and positive electrode at the injection end) with light-absorbance detection (at 280 nm for protein targets). Determine the migration time of the target to the detector and multiply by $f = 1.14$ to calculate the migration time to the end of the capillary. Usually, 40 min is enough to observe a peak of the target.

▲ CRITICAL STEP Targets typically do not bear fluorophores; therefore, target concentrations suitable for light-absorbance detection are used to identify the target in this step. The remaining steps of the procedure involve fluorescently labeled DNA, so LIF detection is used to identify DNA and DNA–target complexes.

? TROUBLESHOOTING

6| Inject 150 nl of 100 nM folded naive DNA library from Step 2 into the capillary and run CE in RB1 at 375 V cm^{-1} with LIF detection. Determine the migration time of the naive DNA library to the detector and multiply it by f (see Step 3) to calculate the migration time to the end of the capillary.

? TROUBLESHOOTING

7| Prepare 10 μl equilibrium mixture in SB1, consisting of 100 nM folded naive DNA library from Step 2 and 10–100 μM of the target. Incubate the mixture at room temperature for 15 min. The incubation temperature might vary depending on the target and the desirable thermodynamic parameters of the aptamer–target interaction[41].

8| Inject 150 nl of the equilibrium mixture from Step 7 into the capillary and run NECEEM in RB1 at 375 V cm^{-1} with LIF detection. This procedure is represented schematically in **Figure 3**.

? TROUBLESHOOTING

9| Calculate the bulk K_d value of the DNA library to the target using the following formula:

$$K_d = \frac{[T]_{tot}\{1+A_1/(A_2+A_3)\} - [DNA]_{tot}}{1+(A_2+A_3)/A_1}. \quad (2)$$

Here $[T]_{tot}$ and $[DNA]_{tot}$ are the total concentrations of the target and DNA, respectively, A_1 is the area of the peak of free DNA divided by the migration time of free DNA, A_2 is the area of the peak of DNA that dissociated from the complex during NECEEM divided by the migration time of free DNA and A_3 is the area of the peak of the intact complex that reached the detector divided by the migration time of the complex. Strictly speaking, K_d cannot be measured for the interaction of the target with a highly heterogeneous DNA library. The value of bulk K_d for the target–library interaction gives no indication of the K_d values of individual aptamers in the library. It is only a measure of an effective abundance of aptamers in the library, which is used for qualitatively monitoring the progress of selection — a decrease of this value throughout the steps of partitioning indicates that the selection is progressing. Adsorption of species to capillary walls can cause tailing of electrophoretic peaks. The division of areas by corresponding migration times is performed to ensure that peak areas are proportional to the amounts of the species. This procedure is necessary for CE instruments with the on-column detection mode only (http://www.beckman.com/literature/Bioresearch/a1774a.pdf). For CE instruments with past-column detection, normalization is not required. The electropherogram in **Figure 3** can also be used to calculate the rate constant of complex dissociation as follows[36,37]:

$$k_{off} = \frac{1}{t_{DNA•T}} \ln\left(\frac{A_2+A_3}{A_3}\right). \quad (3)$$

? TROUBLESHOOTING

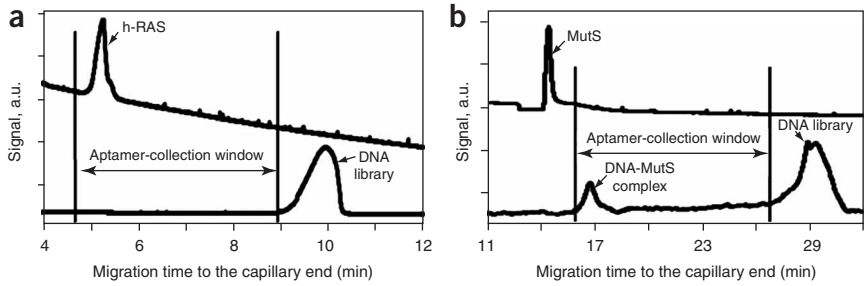

Figure 4 | Choosing an aptamer-collection window. (**a**) A complex of DNA with the target (h-Ras protein) is not detectable in the bulk-affinity assay. The top and bottom traces are electropherograms of h-Ras protein (UV detection at 280 nm), and the equilibrium mixture of h-Ras and naive DNA library (fluorescence detection), respectively. (**b**) A complex of DNA with the target (MutS protein) is detectable in a bulk-affinity assay. The top and bottom traces are electropherograms of MutS protein (UV detection at 280 nm), and the equilibrium mixture of MutS and DNA library (fluorescence detection), respectively.

10| Determine the aptamer-collection window. If the peak of the DNA–target complex is not detectable in the bulk-affinity assay (see example in **Fig. 4a**), the aptamer-collection window should be between the left boundaries of the peaks of the target and the naive DNA library. If the peak of the DNA–target complex is detectable (see example in **Fig. 4b**), the aptamer-collection window starts from the left boundary of the peak of the complex and ends before the left boundary of the peak of the naive DNA library. By choosing a proper aptamer-collection window, 'smart' aptamers (that is, aptamers with the desired kinetic and thermodynamic parameters) can be developed[36,37,39].

▲ **CRITICAL STEP** The width and the position of the aptamer-collection window define the quality of partitioning of the target-bound DNA molecules from the excess of the unbound DNA; the better the aptamer-collection window is separated from the peak of the naive DNA library, the higher the efficiency of aptamer selection.

? TROUBLESHOOTING

Multi-step NECEEM-based partitioning ● **TIMING 4–5 h**

11| Mix 5 µl of 50 µM naive DNA library from Step 1 with 5 µl of 1–1,000 nM target in SB1. Incubate the mixture at room temperature for 15 min. Steps 12–15 can be carried out during this incubation time.

▲ **CRITICAL STEP** We suggest that the selection should start with a target concentration two orders of magnitude below the bulk K_d value of the naive DNA library determined in Step 9. Most of the selected aptamers will have a K_d value below the target concentration.

12| Replace capillary rinsing solutions and RB1 with fresh aliquots.

▲ **CRITICAL STEP** Precautions should be taken during CE separation and fraction collection not to contaminate the buffers and workplace with the naive DNA library. New vials of solutions and buffers should be used for every CE separation.

13| Prepare an outlet vial containing 5 µl of the target solution in SB1; this will be used to collect the aptamer fraction in Step 17. The concentration of the target is the same as in Step 11, although lower target concentrations can be used to increase the stringency of selection.

14| Rinse the capillary with RNase AWAY solution (137 kPa or 20 psi for 5 min) keeping the capillary coolant at 25 °C.

15| Rinse the capillary as described in Step 4.

16| Inject 150 nl of the reaction mixture from Step 11 into the capillary; the mixture contains 25 µM naive DNA library (a total of 2×10^{12} molecules) and 0.5–500 nM target.

17| Run NECEEM in RB1 and collect a fraction in the aptamer-collection window determined in Step 10. The fraction is collected in the outlet vial prepared in Step 13. The total volume in the outlet vial (including the 5 µL from Step 13) varies between ~5 and 10 µl depending on the aptamer-collection window.

18| Incubate the collected fraction for 15 min at room temperature.

19| Repeat Steps 12–18 twice more; in Step 16, inject 150 nl of the mixture from the outlet vial from Step 17. This is the end of partitioning in non-SELEX. The number of partitioning steps is usually two or three, and is no more than four (**Fig. 5**). Fractions collected from each step are used in Steps 20 and 21 to study the progress of selection.

■ **PAUSE POINT** The fractions need to be stored at –20 °C. DNA in the fractions is stable for several months.

? TROUBLESHOOTING

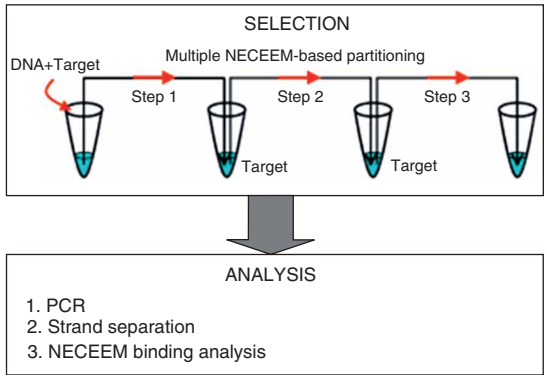

Figure 5 | Partitioning of aptamers from non-aptamers in non-SELEX by multiple steps of NECEEM with no PCR amplification between the steps.

TABLE 1 | Touchdown PCR program for Step 20A(ii).

Cycle number	Denaturation	Annealing	Polymerization
1	30 s at 94 °C	–	–
2–19	10 s at 94 °C	10 s at 72–55 °C	10 s at 72 °C
	–	(–1 °C per cycle)	–
20–33	10 s at 94 °C	10 s at 55 °C	10 s at 72 °C
Hold at 4 °C	–	–	–

Determination of optimum number of cycles for preparative PCR ● TIMING **2–5 h**

20| The optimum number of cycles for amplification of the aptamer pools for subsequent analysis can be determined by either real-time PCR (option A) or CE (option B). Option A is the preferred method.

(A) Analytical real-time PCR ● TIMING **2 h**

(i) Set up 20 µl amplification reactions for each fraction collected in Steps 17 and 19 according to the conditions in the following table:

Reagents	Reaction mixture
2× real-time PCR master mix with SYBR Green	10 µl
F primer (10 µM)	0.6 µl (300 nM final)
R primer (10 µM)	0.6 µl (300 nM final)
Template	1 µl
ddH_2O	7.8 µl

▲ CRITICAL STEP Unlabeled primers are used here because the emission wavelength of FAM is the same as that of SYBR Green; therefore, FAM can interfere with the SYBR Green-based detection in real-time PCR.

(ii) Amplify the reactions using the touchdown PCR program detailed in **Table 1**. Touchdown PCR does not require additional calibration of the annealing temperature of the primer[46].

(iii) Real-time PCR produces an S-shaped amplification curve (product yield versus number of cycles); it is necessary to determine how many cycles are required for the reaction mixture to generate the product at a level of 50–60% of the maximum[47].

▲ CRITICAL STEP This is the number of cycles required to generate the optimal amount of amplified library product in the preparative PCR (Steps 21 and 22).

(B) Analysis of PCR products with CE (if real-time PCR is not available) ● TIMING **5 h**

(i) Set up four identical 10 µl PCR amplification reactions for each fraction collected in Steps 17 and 19. This can be achieved by setting up a master mix, as shown in the following table, and pipetting 10 µl into four individual tubes:

Reagents	Reaction mixture
10× PCR buffer	4 µl
10 µM F-FAM primer	1.2 µl (300 nM final)
10 µM R-biotin primer	1.2 µl (300 nM final)
10 mM dNTPs	0.8 µl (200 µM final)
5 U μl^{-1} Taq DNA polymerase	0.4 µl (0.05 U μl^{-1} final)
Template	2 µl
ddH_2O	30.4 µl

▲ CRITICAL STEP The FAM-labeled primer is used in this PCR reaction so that the reaction product can be detected in LIF-based CE.

(ii) Amplify the reactions using the touchdown PCR program detailed in **Table 2**. For each fraction, remove one of the four vials after 15, 20, 25 and 30 PCR cycles immediately after the polymerization step at 72 °C.

(iii) Rinse the capillary as described in Step 4, but use RB2 instead of RB1.

TABLE 2 | Touchdown PCR program for Step 20B(ii).

Cycle number	Denaturation	Annealing	Polymerization
1	30 s at 94 °C	–	–
2–19	10 s at 94 °C	10 s at 72–55 °C (–1 °C per cycle)	10 s at 72 °C
20–30	10 s at 94 °C	10 s at 55 °C	10 s at 72 °C
Hold at 4 °C	–	–	–

(iv) For each PCR sample from Step 20B(ii), inject 50 nl of the PCR sample into the capillary and run CE in RB2 at 375 V cm^{-1} with LIF detection. Observe CE separation of the PCR product from the F-FAM primer (**Fig. 6**).
▲ CRITICAL STEP Choose the number of PCR cycles for which the area of the CE peak corresponding to the PCR product is 10–20% of the total area of all peaks. This is the optimum number of cycles for preparative PCR (Steps 21 and 22).

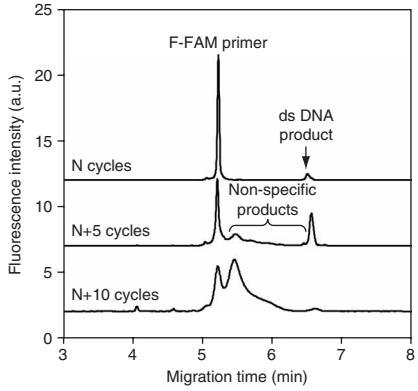

Figure 6 | Gel-free CE analysis of PCR products. N is the optimum number of PCR cycles where the PCR yield is 10–20%.

Preparative PCR of collected fractions for affinity analysis ● TIMING **2 h**

21| Set up 100 µl PCR amplification reactions for each collected fraction from Steps 17 and 19. Use the remainder of the collected fractions as template. The recipe for the PCR reaction is shown in the following table:

Reagents	Reaction mixture
10× PCR buffer	10 µl
10 µM F-FAM primer	3 µl (300 nM final)
10 µM R-biotin primer	3 µl (300 nM final)
10 mM dNTPs	2 µl (200 µM final)
5 U μl^{-1} Taq DNA polymerase	1 µl (0.05 U μl^{-1} final)
Template	5 µl
ddH_2O	76 µl

▲ CRITICAL STEP The volume of a template might vary so the total volume of the PCR reaction mixture should be adjusted to ensure that the template constitutes 5% of the total volume of the PCR reaction. A pair of FAM-labeled and biotin-labeled primers is used in this PCR reaction to produce a FAM-labeled aptamer and a biotin-labeled complementary strand. Biotin facilitates strand separation, whereas FAM allows LIF detection in CE.

22| Amplify the reactions using the touchdown PCR program described in Step 20A(ii). Use the optimum number of cycles determined in Steps 20A(iii) or 20B(iv); this number might vary from fraction to fraction.

Separation of DNA strands ● TIMING **1 h**

23| For each PCR sample from Step 22, aliquot 20 µl streptavidin beads into an empty microfuge tube. Wash the beads twice with 200 µl bead binding/washing buffer. After each wash, remove the beads from the solution with a magnetic bead separator.

24| Mix 100 µl of PCR-amplified samples from Step 22 with 100 µl bead binding/washing buffer.

25| Resuspend the beads from Step 23 in the sample solutions from Step 24, and gently pipette up and down to mix them. Incubate at room temperature for 20 min, with periodic gentle mixing to facilitate faster binding of DNA to the beads.

26| Remove the beads from the solution using a magnetic separator and discard the solution. Wash the beads three times with 200 µl streptavidin bead binding/washing buffer at room temperature for 2 min each time.

27| Add 40 µl of 100 mM NaOH to the beads to facilitate the dissociation of aptamers from their complementary strands, which are attached to the streptavidin beads. Gently pipette the mixture up and down, and incubate for 5 min at room temperature. Remove the beads from the solution with the magnetic separator.

28| Transfer the solution, which contains the aptamer pool, to a new 0.2 ml vial. Add 4 µl of 1 M HCl solution followed by 5 µl SB2. If the DNA needs to be concentrated or the buffer needs to be changed, standard ethanol precipitation can be carried out, as described in **Box 1**. The aptamer pools are used for further analysis in Steps 29 and 34.
■ PAUSE POINT The aptamer pools can be stored at –20 °C for several years.

NECEEM-based affinity analysis of aptamer pools ● TIMING **3–4 h**

29| Treat 5 µl of each aptamer pool from Step 28 as described in Step 1.

30| Mix 5 µl of the aptamer pool from Step 29 and 5 µl of the target in SB1. Incubate the mixture at room temperature for 15 min. The concentration of the target should be equal to or higher than that in Step 11.

BOX 1 | DNA PRECIPITATION ● TIMING 1 h

1. Add 150 µl of 95% ethanol/3 M sodium acetate, pH 7.0, 33:1 (vol/vol; the solution should be at –20 °C) to each sample.
2. Centrifuge each sample for 20 min at 10,000–20,000*g* and 10 °C.
3. Carefully remove the supernatant and add 500 µl of 70% ethanol (vol/vol) at –20 °C to the pellet.
4. Centrifuge for 3 min at 10,000–20,000*g* and 10 °C and remove the supernatant.
5. Dry DNA under vacuum to remove ethanol traces and re-dissolve each dry sample in 15 µl SB1. The resulting solutions contain pools of ssDNA aptamers for subsequent affinity analysis.

31| Rinse the capillary as described in Step 4.

32| Inject 50 nl of the equilibrium mixture from Step 30 into the capillary and run NECEEM in RB1 at 375 V cm^{-1}.

33| Calculate the bulk K_d value of each aptamer pool to the target using equation (2). As alternatives to NECEEM, the affinity of the DNA pool or individual clones to the target can be estimated by a filter-binding assay. We previously showed that both methods give comparable results[36].
? TROUBLESHOOTING

Cloning and screening clones ● TIMING 24 h

34| Select the aptamer pool that shows suitable (typically, the highest) binding affinity; calibrate the PCR as described in Step 20 and perform preparative PCR of that pool (use the sample from Step 28) as described in Steps 21 and 22, but using unlabeled primers.
▲ **CRITICAL STEP** Labels on the PCR product would interfere with ligation in the subsequent cloning procedure.

35| Clone the resulting PCR products as described in the manual for the pT7 Blue-3 Perfectly Blunt cloning kit (http://www.emdbiosciences.com/docs/docs/PROT/TB183.pdf). Plate the transformed bacteria on selective media and incubate at 37 °C overnight.

36| Number the colonies on the bottom of the plate for future sampling for sequencing, remove the colonies, resuspend each in an individual vial with 50 µl ddH_2O and incubate in a boiling water bath for 5 min. Return the plate to 37 °C for a few hours to re-grow the colonies; these will be required for plasmid isolation for sequence analysis (Step 44).

37| Pellet the bacterial debris by centrifugation for 10 min at 10,000*g* and 20 °C. Use 5 µl supernatant as a template for the subsequent PCR reaction.

38| Set up asymmetric PCR as described in the following table:

Reagents	Reaction mixture
10× PCR buffer	10 µl
10 µM F-FAM primer	3 µl (300 nM final)
1 µM R primer	3 µl (30 nM final)
10 mM dNTPs	2 µl (200 µM final)
5 U $µl^{-1}$ Taq DNA polymerase	1 µl (0.05 U $µl^{-1}$ final)
Template	5 µl
ddH_2O	76 µl

39| Amplify the reactions using the touchdown PCR program detailed in **Table 3**.
▲ **CRITICAL STEP** A FAM-labeled primer is used in the asymmetric PCR reaction to produce FAM-labeled single-stranded aptamers. Asymmetric PCR is performed with an excess of the primer that is used to synthesize the aptamer strand; the complementary strand is thereby synthesized in much smaller amounts. This avoids the need for DNA strand separation when screening clones for binding affinity. Note that asymmetric PCR cannot be used to amplify random DNA libraries (Steps 21 and 22).

TABLE 3 | Touchdown PCR program for Step 39.

Cycle number	Denaturation	Annealing	Polymerization
1	30 s at 94 °C	–	–
2–19	10 s at 94 °C	10 s at 72–55 °C (–1 °C per cycle)	10 s at 72 °C
20–50	10 s at 94 °C	10 s at 55 °C	10 s at 72 °C
Hold at 4 °C	–	–	–

40| Mix 5 µl of each PCR sample from Step 39 individually with 5 µl of the target in SB1 (at the concentration given in Step 30). Incubate the mixture at room temperature for 15 min.

41| Rinse the capillary as described in Step 4.

42| Inject 50 nl of the mixture from Step 40 into the capillary and run NECEEM in RB1 at 375 V cm^{-1} (**Fig. 7**).

43| Estimate the K_d values for clones using equation (2).
? TROUBLESHOOTING

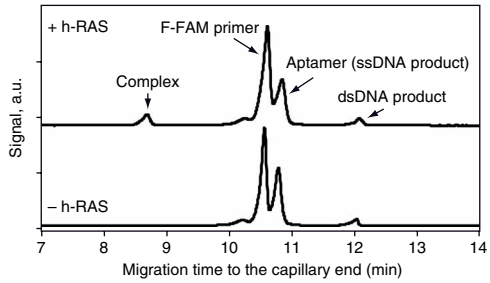

Figure 7 | NECEEM-based screening of clones for binding affinity after asymmetric PCR. In the top, a complex of a clone with a target (h-Ras) is detected. The bottom shows a control CE run of the clone after asymmetric PCR without the target.

44| Choose aptamers with desirable K_d values. Place the appropriate colonies from the plates in Step 36 in selective liquid medium, grow overnight at 37 °C and isolate plasmid DNA using any standard method or kit. Sequence the cloned aptamer using a dideoxy-DNA sequencing method[48] as described in the GenomeLab™ sequencing chemistry protocol (Beckman Coulter).

● **TIMING**

Day 1: Preparation of equipment and solutions (1 h), and determination of library bulk affinity and aptamer-collection window (3–4 h).
Day 2: Multi-step NECEEM-based partitioning (4–5 h).
Day 3: Determination of optimum number of PCR cycles (2–5 h), preparative PCR of collected fractions (2 h) and separation of DNA strands (1 h).
Day 4: NECEEM-based affinity analysis of aptamer pools (3–4 h), cloning (≥5 h–overnight bacterial growth).
Day 5: Asymmetric PCR (2 h) and NECEEM-based screening of clones for binding affinity (1 h per clone).

? TROUBLESHOOTING

See **Table 4** for troubleshooting advice.

TABLE 4 | Troubleshooting table.

Step	Problem	Explanation and solution
5	Target is not detectable by UV	The concentration of the target is too low. Label the target with a fluorogenic amine derivatization reagent ATTO-TAG™ FQ or NanoOrange dye to identify it with LIF detection.
		Target adheres to silica walls of the capillary. Use a coated capillary and change the polarity of the electric field (negative electrode at the injection end of the capillary).
6	DNA library gives many peaks	The naive DNA library contains impurities. Purify DNA with PAGE under denaturing conditions.
8	No peak of complex observed	Bulk affinity of target to the naive DNA library is too low. Choose one or more of the following options: increase the concentration of the target; decrease the concentration of NaCl in the selection buffer; decrease the incubation temperature of the target with DNA; incubate the target with the DNA library overnight; decrease the temperature of the capillary.
	No free DNA is detectable	Bulk affinity of the target to the naive DNA library is too high. Choose one or more of the following options: decrease the concentration of the target; increase the concentration of sodium chloride in the selection buffer; increase the incubation temperature of the target with the DNA library; increase the temperature of the capillary.
9	No dissociation of complex is detectable	The complex is highly stable. The area of dissociation is negligible. Assume that $A_2 = 0$ in equations (2) and (3).
	Two or more peaks of complexes are observed	The target interacts with DNA with several stoichiometries. Summarize peak areas of all complexes to calculate bulk affinity.
10	Narrow window between DNA–target complex and naive DNA library	Poor separation between DNA and DNA–target complex. Choose one or more of the following options: increase the length of the capillary; change the pH and concentration of the CE run buffer; evaluate a CE run buffer of a different nature.
19	Current is unstable or drops	Air bubbles inside the capillary or loose electrode contact. Choose one or more of the following options: de-gas and filter the CE run buffer; decrease the voltage that drives electrophoresis; ensure that both electrodes and both ends of the capillary reach the solutions in the vials.

TABLE 4 | Troubleshooting table (continued).

Step	Problem	Explanation and solution
33	No improvement in affinity of pools compared with bulk affinity of naive DNA library	No aptamers were selected. Choose one or more of the following options: increase the concentration of the target; increase the concentration of the DNA library; decrease the concentration of sodium chloride in the selection buffer; use a capillary with a larger inner diameter; increase the injection volume.
	Affinities of aptamer pools are only slightly higher than bulk affinity of naive DNA library	Aptamers with high K_d are selected. Increase the stringency parameters of aptamer selection: decrease the concentration of the target; increase the concentration of sodium chloride in the selection buffer; increase the temperature during incubation of the equilibrium mixture and its NECEEM separation; narrow down the aptamer-collection window; collect only the complex; decrease the incubation time of the equilibrium mixture.
43	No peak of complex is detected	No aptamers were produced in asymmetric PCR. Check the insertion in the vector with PCR and agarose gel or try another clone.

ANTICIPATED RESULTS

The described protocol results in dozens of unique DNA (RNA) sequences capable of binding a target with prominent affinity and selectivity. The sequences are analyzed to find homologous structures with common regions. Next, one or two aptamers from each homologous group are synthesized chemically and analyzed with KCE to find kinetic and equilibrium binding parameters[40,41]. **Figure 8** shows an example for NECEEM-based affinity analysis of a chemically synthesized aptamer for h-Ras protein. To improve binding and nuclease stability of aptamers they can be further truncated and chemically modified[5].

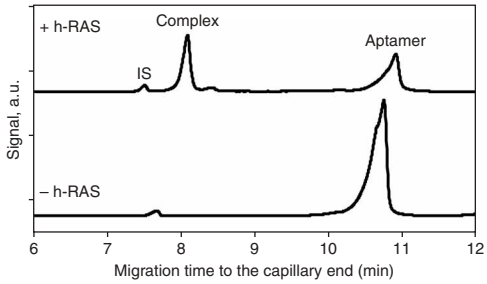

Figure 8 | NECEEM-based affinity analysis of a chemically synthesized aptamer for h-Ras protein. In the top, a complex of an aptamer with a target (h-Ras) is detected. The bottom shows a control of an aptamer without the target. Fluorescein is used as an internal standard (IS). The separation conditions are as described in Step 42.

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1. Wilson, D.S. & Szostak, J.W. *In vitro* selection of functional nucleic acids. *Annu. Rev. Biochem.* **68**, 611–647 (1999).
2. Famulok, M., Mayer, G. & Blind, M. Nucleic acid aptamers — from selection *in vitro* to applications *in vivo*. *Acc. Chem. Res.* **33**, 591–599 (2000).
3. Rimmele, M. Nucleic acid aptamers as tools and drugs: recent developments. *Chembiochem* **4**, 963–971 (2003).
4. Jayasena, S.D. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin. Chem.* **45**, 1628–1650 (1999).
5. Lee, J.F., Stovall, G.M. & Ellington, A.D. Aptamer therapeutics advance. *Curr. Opin. Chem. Biol.* **10**, 282–289 (2006).
6. Bock, C. *et al.* Photoaptamer arrays applied to multiplexed proteomic analysis. *Proteomics* **4**, 609–618 (2004).
7. Surugiu-Warnmark, I., Warnmark, A., Toresson, G., Gustafsson, J.A. & Bulow, L. Selection of DNA aptamers against rat liver X receptors. *Biochem. Biophys. Res. Commun.* **332**, 512–517 (2005).
8. Pestourie, C., Tavitian, B. & Duconge, F. Aptamers against extracellular targets for *in vivo* applications. *Biochimie* **87**, 921–930 (2005).
9. Davidson, E.A. & Ellington, A.D. Engineering regulatory RNAs. *Trends Biotechnol.* **23**, 109–112 (2005).
10. Mayer, G. & Jenne, A. Aptamers in research and drug development. *BioDrugs* **18**, 351–359 (2004).
11. Nutiu, R., Yu, J.M. & Li, Y. Signaling aptamers for monitoring enzymatic activity and for inhibitor screening. *Chembiochem* **5**, 1139–1144 (2004).
12. Famulok, M. & Mayer, G. Intramers and aptamers: applications in protein–function analyses and potential for drug screening. *Chembiochem* **6**, 19–26 (2005).
13. Ulrich, H., Martins, A.H. & Pesquero, J.B. RNA and DNA aptamers in cytomics analysis. *Cytometry A* **59**, 220–231 (2004).
14. Stojanovic, M.N. & Kolpashchikov, D.M. Modular aptameric sensors. *J. Am. Chem. Soc.* **126**, 9266–9270 (2004).
15. Nimjee, S.M., Rusconi, C.P. & Sullenger, B.A. Aptamers: an emerging class of therapeutics. *Annu. Rev. Med.* **56**, 555–583 (2005).
16. Patil, S.D., Rhodes, D.G. & Burgess, D.J. DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS J.* **7**, 61–77 (2005).
17. Lee, J.F., Hesselberth, J.R., Meyers, L.A. & Ellington, A.D. Aptamer database. *Nucleic Acids Res.* **32**, D95–D100 (2004).
18. Ellington, A. & Szostak, J. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818–822 (1990).
19. Tuerk, C. & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505–510 (1990).
20. O'Connell, D. *et al.* Calcium-dependent oligonucleotide antagonists against L-selectin. *Proc. Natl. Acad. Sci. USA* **93**, 5883–5887 (1996).
21. Ulrich, H. *et al. In vitro* selection of RNA molecules that displace cocaine from the membrane-bound nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **95**, 14051–14056 (1998).
22. Burke, D.H., Scates, L., Andrews, K. & Gold, L. Bent pseudoknots and novel RNA inhibitors of type 1 human immunodeficiency virus (HIV-1) reverse transcriptase. *J. Mol. Biol.* **264**, 650–666 (1996).
23. Drolet, D.W., Jenison, R.D., Smith, D.E., Pratt, D. & Hicke, B. A high throughput platform for systematic evolution of ligands by exponential enrichment (SELEX). *Comb. Chem. High Throughput Screen.* **2**, 271–278 (1999).
24. Beutel, B.A. & Gold, L. *In vitro* evolution of intrinsically bent DNA. *J. Mol. Biol.* **228**, 803–812 (1992).
25. Ciesiolka, J. *et al.* Affinity selection-amplification from randomized ribooligonucleotide pools. *Methods Enzymol.* **267**, 315–335 (1996).

26. Irvine, D., Tuerk, C. & Gold, L. SELEXION. Systematic evolution of ligands by exponential enrichment with integrated optimization by non-linear analysis. *J. Mol. Biol.* **222**, 739–761 (1991).
27. Vant-Hull, B., Antonio, P.-B., Davis, R.H. & Gold, L. The mathematics of SELEX against complex targets. *J. Mol. Biol.* **278**, 579–597 (1998).
28. Schneider, D., Gold, L. & Platt, T. Selective enrichment of RNA species for tight binding to *Escherichia coli* rho factor. *FASEB J.* **7**, 201–207 (1993).
29. Chen, H. & Gold, L. Selection of high-affinity RNA ligands to reverse transcriptase: inhibition of cDNA synthesis and RNase H activity. *Biochemistry* **33**, 8746–8756 (1994).
30. Tian, Y. *et al.* Dissecting protein:protein interactions between transcription factors with an RNA aptamer. *RNA* **1**, 317–326 (1995).
31. Petrov, A., Okhonin, V., Berezovski, M. & Krylov, S.N. Kinetic capillary electrophoresis (KCE): a conceptual platform for kinetic homogeneous affinity methods. *J. Am. Chem. Soc.* **127**, 17104–17110 (2005).
32. Heegaard, N.H.H. & Robey, F.A. Use of capillary zone electrophoresis to evaluate the binding of anionic carbohydrates to synthetic peptides derived from human serum amyloid P component. *Anal. Chem.* **64**, 2479–2482 (1992).
33. Chu, Y.H., Avila, L.Z., Biebuyck, H.A. & Whitesides, G.M. Use of affinity capillary electrophoresis to measure binding constants of ligands to proteins. *J. Med. Chem.* **35**, 2915–2917 (1992).
34. Mendonsa, S.D. & Bowser, M.T. *In vitro* evolution of functional DNA using capillary electrophoresis. *J. Am. Chem. Soc.* **126**, 20–21 (2004).
35. Mosing, R.K., Mendonsa, S.D. & Bowser, M.T. Capillary electrophoresis-SELEX selection of aptamers with affinity for HIV-1 reverse transcriptase. *Anal. Chem.* **77**, 6107–6112 (2005).
36. Berezovski, M. *et al.* Nonequilibrium capillary electrophoresis of equilibrium mixtures: a universal tool for development of aptamers. *J. Am. Chem. Soc.* **127**, 3165–3171 (2005).
37. Drabovich, A.P., Berezovski, M., Okhonin, V. & Krylov, S.N. Selection of smart aptamers by methods of kinetic capillary electrophoresis. *Anal. Chem.* **78**, 3171–3178 (2006).
38. Berezovski, M., Musheev, M., Drabovich, A. & Krylov, S.N. Non-SELEX selection of aptamers. *J. Am. Chem. Soc.* **128**, 1410–1411 (2006).
39. Drabovich, A., Berezovski, M. & Krylov, S.N. Selection of smart aptamers by equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM). *J. Am. Chem. Soc.* **127**, 11224–11225 (2005).
40. Berezovski, M. & Krylov, S.N. Nonequilibrium capillary electrophoresis of equilibrium mixtures — a single experiment reveals equilibrium and kinetic parameters of protein–DNA interactions. *J. Am. Chem. Soc.* **124**, 13764–13765 (2002).
41. Berezovski, M. & Krylov, S.N. Thermochemistry of protein–DNA interaction studied with temperature-controlled nonequilibrium capillary electrophoresis of equilibrium mixtures. *Anal. Chem.* **77**, 1526–1529 (2005).
42. Thiel, K. Oligo oligarchy — the surprisingly small world of aptamers. *Nat. Biotechnol.* **22**, 649–651 (2004).
43. Kanna, M.W., Rozenman, M.M., Sakurai, K., Snyder, T.M. & Liu, D.R. Reaction discovery enabled by DNA-templated synthesis and *in vitro* selection. *Nature* **431**, 545–549 (2004).
44. Garner, Z.J. *et al.* DNA-templated synthesis and selection of a library of macrocycles. *Science* **305**, 1601–1605 (2004).
45. Takahashi, T.T., Austin, R.J. & Roberts, R.W. mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.* **28**, 159–165 (2003).
46. Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. & Mattick, J.S. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**, 4008 (1991).
47. Musheev, M.U. & Krylov, S.N. Selection of aptamers by systematic evolution of ligands by exponential enrichment: addressing the polymerase chain reaction issue. *Anal. Chim. Acta* **564**, 91–96 (2006).
48. Sanger, F., Nicklen, S. & Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5471 (1977).