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Original Research

# A cross-contamination-free SELEX platform for a multi-target selection strategy

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**Abstract** Multi-target aptamer selection, known as multiplex systematic evolution of ligands by exponential enrichment (SELEX), is rapidly drawing interest because of its potential to enable high-speed, high-throughput aptamer selection. The parallelization of chemical processes by integrating microfluidic unit operations is a key strategy for developing a multiplex SELEX process. One of the potential problems with on-chip multiplexing chemical processes is cross-contamination. In order to avoid this, we propose a microfluidic network platform that uses pneumatic valves to allow the serial loading and incubation of aptamers with sol-gel entrapped target proteins. After target binding inside the sol-gels, the cross-contamination-free parallel elution of specifically bound aptamers is performed. The platform allows selective binding with five different targets immobilized in sol-gel spots. When eluting bound species, cross-contamination is avoided by sealing the adjacent elution chambers from each other using the pneumatic microvalves. Consequently, we demonstrate specific aptamer binding to the respective protein target and subsequent aptamer elution without any cross-contamination. This proof of concept opens the way to increased automation and microscale parallel processing of the SELEX methodology.

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## Introduction

Aptamers are short oligonucleotide ribonucleic acid (RNA) or single-stranded deoxyribonucleic acid (ssDNA) strands that have great potential as molecular probes that can fold into three-dimensional structures to recognize virtually any molecule[1,2]. The interest in aptamers is driven by their relative ease of development, since the selection process can be performed entirely *in vitro*. In addition, the aptamers can be produced using conventional polymerase chain reaction (PCR), without the need for animal facilities. Aptamer recognition is analogous to that of antibodies, and aptamers can interact tightly and specifically with a wide range of targets, such as small metabolites, toxicants, pollutants, and chemical compounds[3-6]. This affinity is sometimes stronger than antigen-antibody interactions[7], opening the possibility that aptamer capture could be even more specific.

Aptamers can be obtained using an *in vitro* process known as the systematic evolution of ligands by exponential enrichment, or SELEX. In this process, RNA or ssDNA aptamers with high affinity to target proteins can be isolated from large libraries ($10^{15\text{-}16}$) of random sequences through repeated cycles of binding to the target, the elution of bound nucleic acids, and amplification. Performing SELEX against a single target molecule is a well-developed method, such as with the nitrocellulose filter binding method[8-10]. The demand for multiplex aptamer selection has increased, since it would allow the rapid isolation of a large number of selective aptamers against multiple targets. Recently,

several microfluidic platforms have been reported for performing SELEX on a chip[11,12], isolating aptamers against single targets. By integrating multiple targets on a single chip, multiplex aptamer selection can be accomplished[13-15].

One of the potential problems with on-chip multiplexing of chemical processes is cross-contamination. This becomes evident when PCR amplification protocols are used, *i.e.*, in the SELEX process. In our earlier work on multiplex aptamer selection, in which specifically captured aptamers were serially eluted, cross-contamination compromised the specificity of the target molecule aptamer binding[13,14]. In order to avoid this, we propose a pneumatic valving strategy that allows serial loading and incubation for competitive binding of aptamers and the cross-contamination-free parallel elution of specifically bound aptamers. We developed a microfluidic network to realize a full five-plex system by integrating three working components on a single chip (microheaters, microchambers linked with microchannels, and a pneumatically operated microvalve array).

Figure 1 outlines the proposed parallel process for cross-contamination-free multiplex aptamer selection. Target proteins were mixed with sol-gel materials, and the mixtures were spotted on the solid support, supporting the competitive binding of an aptamer library to the different target proteins. Localized heat sources and microvalves were activated to elute the specific aptamers that bound each target protein without any cross-contamination.

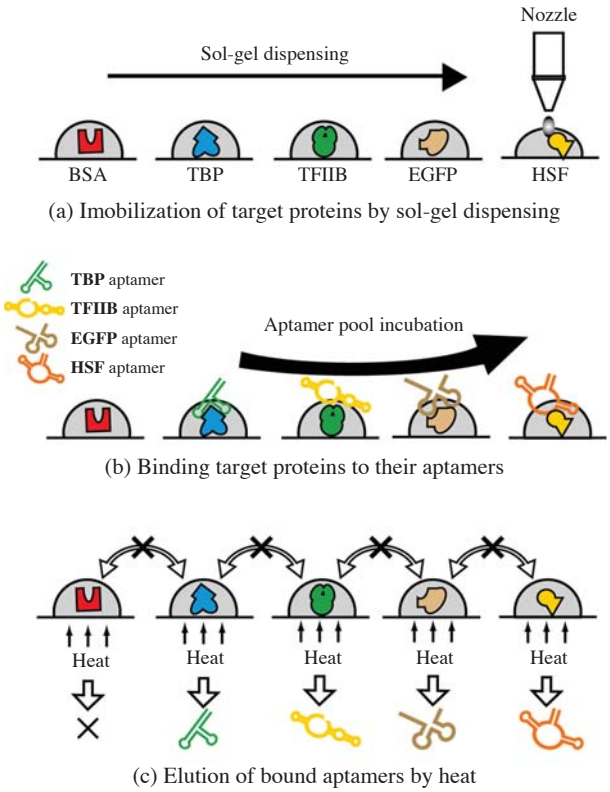



**Figure 1.** (a) The parallel process used for serial aptamer incubation and the cross-contamination-free parallel elution of specifically bound aptamers. Five different targets were immobilized individually in sol-gel spots on the surface of heater electrodes. (b) Subsequently, an RNA mixture (aptamers) was injected into the sol-gel spot array, enabling the aptamers to bind their target proteins. (c) After washing, the bound aptamers were released by applying heat at each sol-gel spot, while the pneumatic valves enabled cross-contamination-free, parallel elution.

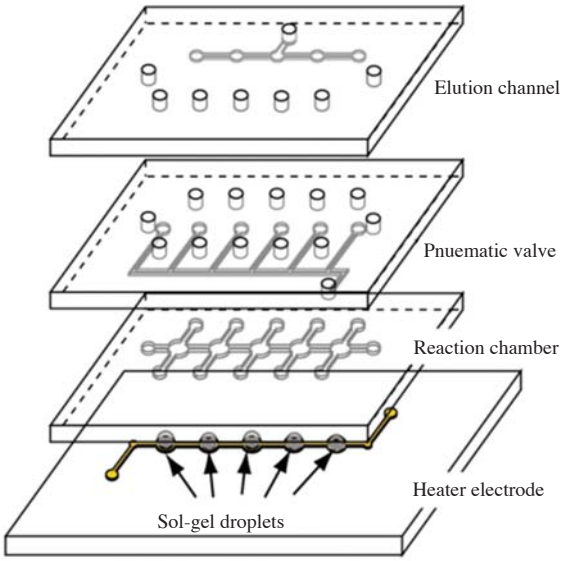
## Results and Discussion

### Pneumatic valve integrated microfluidic platform

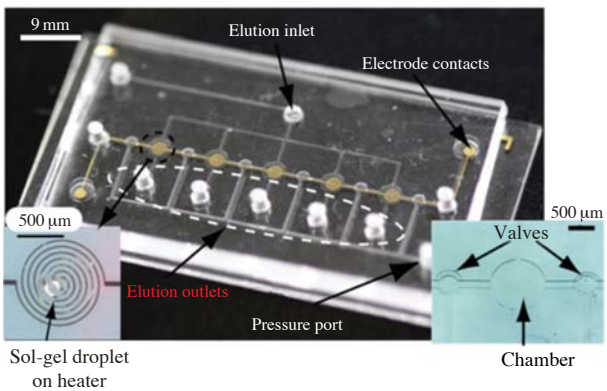
The microfluidic network platform consisted of two chips: a fluidic network chip made from polydimethylsiloxane (PDMS) and a microheater chip fabricated in a glass substrate. Figure 2 shows the assembled microfluidic platform. Three PDMS layers were carefully aligned for the elution channel, pneumatic valve, and reaction chamber layers, and bonded sequentially using oxygen plasma. Then, the sol-gel droplets were spotted on the circular heating electrodes. Finally, the bonded PDMS fluidic network and PDMS-coated glass substrate were bonded together. The microchambers were connected to parallel microchannels with individual elution outlets. The reaction chambers and fluid channels measured 1 mm and 100 μm, respectively. Pneumatic valves[15-17] were located between each microchamber to block mixing of the aptamers eluted from the individual sol gel spots. The fabrication process is explained in detail in the supporting data (Supporting Figure 1). Finally, silicone tubes were inserted into the outlet ports for the fluidic, electrical, and pneumatic connections.

Figure 3 shows the system operation during parallel chemical processing. Compressed air was supplied to the pneumatic valves by switching electromagnetic valves. The frequency and duty cycle of the square wave signal for ON/OFF control of the pneumatic valves was programmed using LabVIEW (National Instruments, Austin, TX). The valve control signal output was generated via a National Instruments input/output (I/O) card (DAQ, NI 9264; National Instruments, Austin, TX). The magnitude and frequency of the supplied pressure were monitored using pressure sensors (SDX05D4, Honeywell, USA).

Figure 4 shows the microfluidic operations of the multiplex aptamer selection system: a, b) injection of the aptamer mixture, c) serial washing of all of the




(a) Components of the platform




(b) Picture of the assembled platform

**Figure 2.** The assembled microfluidic platform consisted of three working components (microheaters, microfluidic network, and pneumatic microvalves) for the parallel selection of five target aptamers. (a) Components of the microfluidic network platform, *i.e.*, the fluidic network (reaction chamber and elution channel), pneumatic valve, and heater electrode. (b) Photograph of the assembled platform. The bottom left inset shows a sol-gel droplet over the electrode zone. The shape and size of the PDMS chamber and valve are shown in the bottom right insert. Scale bars indicate the dimensions.

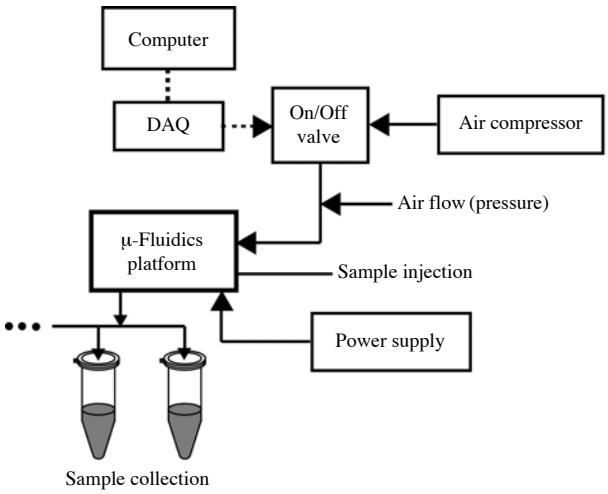



**Figure 3.** Schematic view of the experimental set-up for operating the microfluidic platform.

sol-gel chambers, and d) parallel elution. After priming the microfluidic network with 1 × BB (a), blue ink was introduced to indicate the RNA aptamer mixture (b). To illustrate the washing step, 1 × BB was perfused through the network to confirm that the aptamer mixture (blue ink) was removed (c). Finally, elution buffer (1 × BB) was injected through the elution inlet (lower right inlet) and the eluted samples were collected from the five parallel elution outlets (d), indicated by the red ink. The microvalves were operated during the elution process such that each chamber was isolated to prevent cross-contamination. In the insert of Figure 4d (upper left), the horizontal channels to the left and right were blocked with the pneumatic valves. Consequently, the eluted samples could be transported only to the target outlet, avoiding cross-over to the neighboring outlets.

Sol-gel encapsulation offers a relatively mild route for the immobilization of biomolecules, such as proteins. The proteins are entrapped in the capturing covalent silica gel network, rather than being linked to inorganic materials chemically[18-20]. Our proposed microfluidic platform is based on the sol-gel immobilization of target molecules, *i.e.*, proteins. Sol-gel microdroplets were deposited on a thin PDMS film covering the heating electrode substrate (Figure 2 and Supporting Figure 1). The three-dimensional nanoporous structure of the sol-gel microdroplet allows the diffusion of aptamers into the silica network[13]. Previously, we demonstrated the capture and thermal elution of specific aptamers within sol-gel droplets in a microfluidic format[14]. However, aptamer cross-over from the different sol-gel spots was an evident problem when trying to obtain pure protein target-specific aptamer fractions.

### Thermal retrieval of aptamers

To elute bound RNA aptamers from the target protein, the target-aptamer complex was heated above the melting temperature of the RNA aptamer. Our heater-integrated microfluidic platform consists of five circular coil-shaped zones, linked to each other in series (See

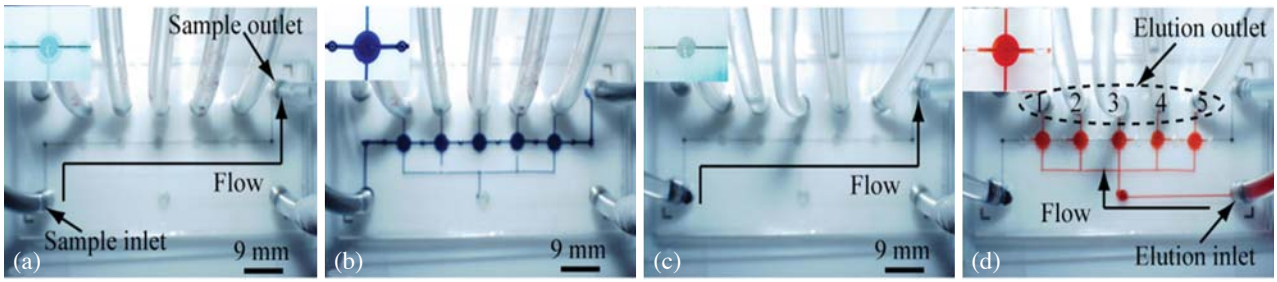



**Figure 4.** Demonstration of fluidic operation during the multiplex aptamer selection process. The introduction of the RNA aptamer mixture (a to b), washing (b to c), and elution process (c to d) are shown. Blue ink (b) indicates that RNA aptamer mixture incubated in the sol-gel chambers. After the washing step, the fluidic networks are clean (c). During the elution step, the pneumatic valves were operated to isolate flow between the chambers. Red ink (elution solution) flows from the elution inlet to five independent outlets. No lateral flow between the chambers is seen. The insert in Figure 4d shows that the pneumatic valves block the lateral channels on each side of the sol-gel chamber.

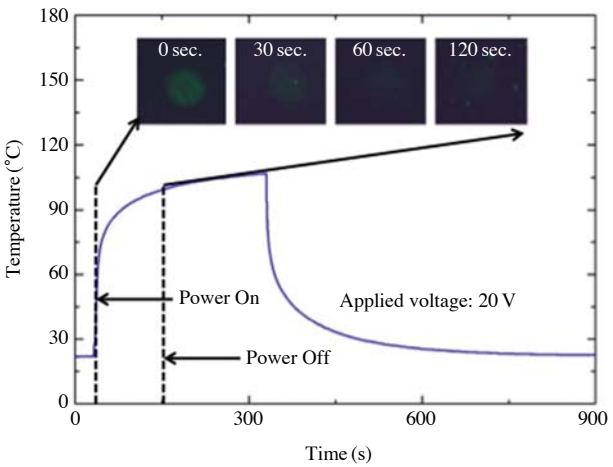



**Figure 5.** Optimal heating condition for denaturing the aptamer in a sol-gel droplet. Inset pictures show the fluorescent signal decay of the DNA aptamer bound to a target protein. Applying 20 V to the heater for at least 30 s is sufficient to reach the melting temperature of the aptamer. The fluorescent signals of the bound DNA decreased with time, and most of the signal disappeared after 30 s.

Supporting Figure 1, lower left). The bound aptamers were released and eluted from the target proteins by thermal denaturation at approximately 90°C. Figure 5 shows the time-resolved release of a fluorescently labeled aptamer from its target protein in a sol-gel droplet with insert images of the fluorescent sol-gel spot at 0, 30, 60, and 120 s.

To verify the aptamer release while heating, we chose TBP protein and the known affinity ligand TATA-dsDNA as a model system. For the binding assay, 50 μL of Cy-3-labeled TATA DNA (200 pmol) were introduced to TBP immobilized sol-gel droplet chambers in the microfluidic platform. After a 1-h incubation, the microchannel and chambers were washed with 1 × BB with 0.01% Tween 20 at 90 μL/min for 30 min. While applying 20 V to the electrode, we monitored the decaying fluorescent signal over 120 s. The melting temperature of the aptamer (> 90°C) was reached within 30 s, and most of the fluorescent signal disappeared in 120 s (Figure 5). Since the elution process was carried out for 4 min, we believe that all of the bound aptamers were released from the target protein.

### Carry-over-free multiplex aptamer elution

Five sol-gel droplets containing BSA, TBP, TFIIB, eGFP, and HSF proteins were spotted on the centers of separate microheaters. Each sol-gel droplet could entrap approximately 30 fmole of protein[14]. An equivalent amount of each RNA aptamer was dissolved in 50 μL 1 × BB[13] (1X binding buffer with 12 mM HEPES pH7.9, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT), and perfused through the microchannel and chambers for aptamer target binding. After washing, the bound aptamers were eluted thermally at 20 V.

Each fraction eluted from the five chambers was expected to hold the aptamer specific to its sol-gel-entrapped target protein. Figure 6 shows the resulting PCR data, demonstrating the interaction between the target proteins and their specific aptamers using our microfluidic platform.

The fraction eluted from the negative control chamber (Chamber 1) correctly showed no signal as neither of the aptamers had affinity to BSA, also indicating that the pneumatic sealing of the chambers alleviated the problem of cross-contamination during the thermal elution step[13]. All of the primer sets were included in the PCR experiment, and the results matched the corresponding immobilized target protein in each microfluidic chamber. As shown in Figure 6, the gel electrophoresis shows aptamer-specific binding and

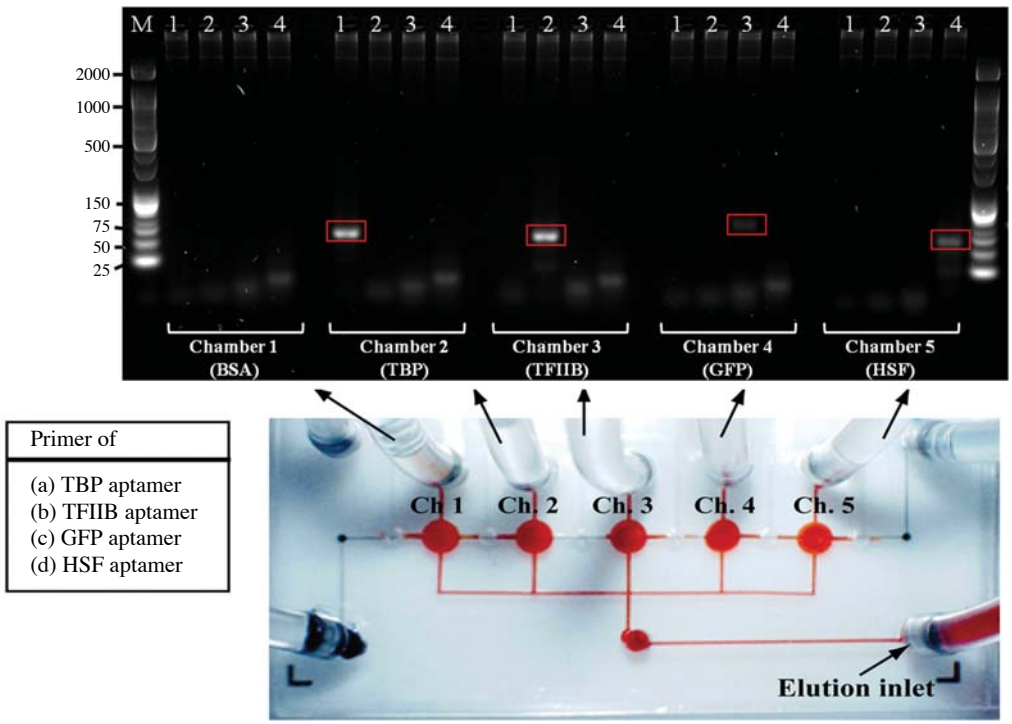



**Figure 6.** Aptamer recovery from each chamber. Elution solution (red ink) flowed from the elution inlet to the five independent outlets. During the elution step, the pneumatic valves were operated to isolate the chambers from each other, and the heater was activated at 20 V for 4 min to release the bound aptamers. Eluted samples were reverse transcribed (Invitrogen, CA) into cDNAs using 1 μM specific primers (reverse primers shown in Table 1) The reverse transcription was performed as following protocol 60 min at 42˚C, 5 min at 85˚C. cDNAs were amplified (NEB Taq DNA polymerase, MA) using 1 μM F, R primers (shown in Table 1. with red arrows) and 1 mM dNTPs with 18 cycles amplification (95˚C 30 s, 54˚C 1 min, and 72˚C 1 min)[13,14].

no cross-contamination of the aptamers eluted from the adjacent chambers, demonstrating the absolute pneumatic sealing of the individual chambers (chambers 2-5 are in lanes 1-4, respectively). This confirms the successful implementation of the microfluidic-pneumatic-valve-controlled multiplex aptamer selection and elution strategy.

## Conclusions

We report a microfluidic network platform for the parallelization of a five-plex aptamer selection process that avoids cross-contamination between specifically bound aptamers in the elution step using a new pneumatic valve solution. The platform was realized with the integration of three functional components-the fluidic network, pneumatic valves, and microheaters. In addition to the benefit of being cross-contamination free, the parallel elution allows a faster process turnover compared with earlier work.

Although the platform was designed to elute aptamers without cross contamination in single round selection process, it should be developed allowing repeated rounds to obtain high affinity aptamer, i.e multiplatform integration.

## Methods

### Protein/aptamer/DNA preparations

Yeast TBP, yeast TFIIB, human HSF1, and enhanced GFP (eGFP) were chosen as target proteins. Bovine serum albumin (BSA) was used as a negative control. Full-length His-tagged versions of TBP and TFIIB were purified from BL21-DE3 cells following a standard His-tagged protein purification protocol. Purified BSA, HSF1, and eGFP proteins were purchased from Stressgen (Stressgen, UK).

To prepare the four aptamer sets, we used an *in-vitro* transcription system, according to the manufacturer's protocol (MEGAshortscript™ Kit, Ambion). The sequences of the aptamers were as follows:

TBP-specific aptamer: 5′-GGG AGA AUU CAA CUG CCA UCU AGG CAG CCA AGG UAA ACA AUU CAG UUA GUG GAA UGA AAC UGC CCA ACA CCA GAA GUA CUA CAA GCU UCU GGA

CUC GGU-3′[21],

TFIIB-specific aptamer: 5′-GGG AGA AUU CAA CUG CCA UCU AGG CAA AGA GCU AAU GUA GGA UGC UGG GGU AGU CCA GCC CUA GAA UAA GCG CUA GUA CUA CAA GCU UCU GGA CUC GG-3′[22],

GFP-specific aptamer: 5′-GGA CUG CGA GGG AGC ACG AAA CGU CGU GGC GCA AUU GGG UGG GGA AAG UCC UUA AAA GAG GGC CAC CAC AGA AGC AAU GGG CUU CUG GAC UCG GU-3′[23].

As the aptamer against eGFP, we used a known GFP aptamer, which also selectively binds eGFP, as determined using an electrophoretic mobility shift assay (EMSA)[22,23]:

HSF-specific aptamer: 5′-GGG AGA AUU CAA CUG CCA UCU GGC AUC GCG AUA CAA AAU UAA GUU GAA CGC GAG UUC UCC AUC AGU CUA CAA GCU UCU GGA CUC GAU-3′[24].

After amplifying the aptamer DNA construct, each PCR product was purified with a QIAquick Purification Kit (QIAGEN, Germany). Then, 1 μg of the DNA template was transcribed into RNA *in vitro* for 4 h using a MEGAshortscript kit and precipitated directly with two volumes of ethanol at −20°C for 2 h. For incubation, 10 ng of each RNA species were mixed in 50 μL 1 × BB and introduced into the microfluidic device.

To evaluate the micro-heating, Cy3-labeled complementary dsTATA-DNA was synthesized by IDT, USA (5′-Cy3-GGG AAT TCG GGC TAT AAA AGG GGG ATC CGG-3′ and 5′-CCG GAT CCC CCT TTT ATA GCC CGA ATT CCC-3′).

### Preparation of the sol-gel microarray and protein immobilization

Five sol-gel microdroplets containing individual target proteins were spotted over the heater zones (Chamber 1, BSA; Chamber 2, TBP; Chamber 3, TFIIB; Chamber 4, eGFP; Chamber 5, HSF). The sol-gel microdroplets were dispensed using a piezo-driven, non-contact dispenser (DW-SolB, PCL, Korea) with a nanoliter volume range (approximately 50 nL/droplet).

Four proteins (TBP, TFIIB, eGFP, and HSF) were chosen as targets, and BSA was used as a negative control. The target proteins were encapsulated in sol-gel matrix, according to the manufacturer's recommendations (SolB™-chip preparation protocol, PCL, Korea, www.pclchip.com). Using the formulation for 30-100 kDa encapsulation[14,25,26], approximately 30 fmole of each protein were mixed with SolB reagents and spotted onto the central region of the microheater using a non-contact microdispensing instrument (DW-SolB, PCL, Korea), and the single spot volume was calculated automatically using auto-drop volume detection software (around 50 nL per solgel droplet).

### Microfluidic and valve operation sequence

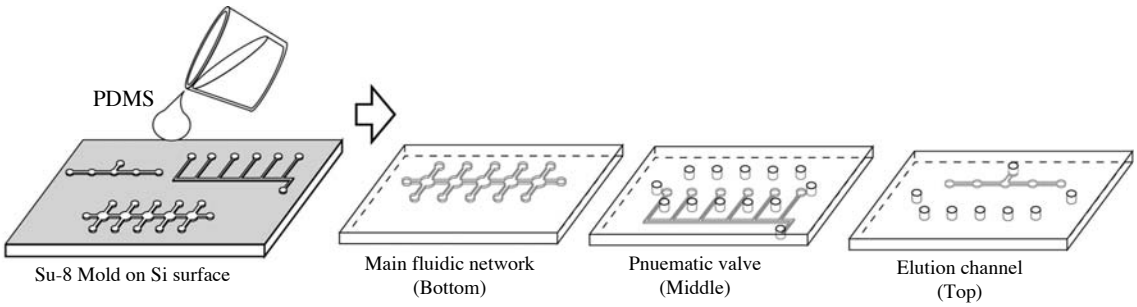
After immobilizing the target proteins on the platform, an aptamer mixture was introduced through the sample inlet port in a 50-μL total volume using a syringe pump (10 μL/min for 5 min) and retained for incubation for 1 h. By incubating the aptamer solution with all of the target protein sol-gel spots simultaneously, a competitive binding assay was performed, which reduced interference from unspecific binding. Subsequently, washing was performed at 90 μL/min for 30 min. Elution buffer (1 × BB) was injected through the elution inlet (Figure 4d) at 50 μL/min for 4 min: 2 min for heating plus 2 min to collect the aptamers. While heating, the pneumatic valves were operated to block the serial channel linking the chambers, ensuring that each chamber was eluted without any cross-contamination.

### Gel electrophoresis of the PCR products

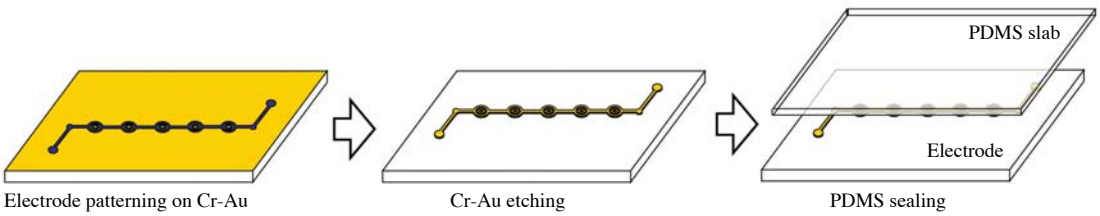
To evaluate the specific aptamer interaction, the apta-

**Table 1.** Primer set for PCR amplification of target aptamers.

| Name | Sequence |
|---|---|
| TBP aptamer | GGGAGAATTCAACTGCCATCTAGGCAGCCAAGGTAAACAATTCAGTTAGTGGAATGAAACTGCCCAACACCAGAAGTACTACAAGCTTCTGGACTCGGT |
| Primer | TBPF → ← TBPR |
| TFIIB aptamer | GGGAGAATTCAACTGCCATCTAGGCAAAGAGCTAATGTAGGATGCTGGGGTAGTCCAGCCCTAGAATAAGCGCTAGTACTACAAGCTTCTGGACTCGGT |
| Primer | TFIIBF → ← TFIIBR |
| GFP Aptamer | GGACTGCGATGGGAGCACGAAACGTCGTGGCGCAATTGGGTGGGGAAAGTCCTTAAAAGAGGGCCACCACAGAAGCAATGGGCTTCTGGACTCGGT |
| Primer | GFPF → ← GFPR |
| HSF aptamer | GGGAGAATTCAACTGCCATCTGGCATCGCGATACAAAATTAAGTTGAACGCGAGTTCTCCATCTAGTACTACAAGCTTCTGGACTCGAT |
| Primer | HSFF → ← HSFR |




(a) Fabrication of PDMS fluidic network




(b) Fabrication of heater electrode

**Supplementary Figure 1.** Fabrication of the fluidic network and heater electrode. (a) The PDMS fluidic network consists of three components (top left): 1) a network in which five chambers are linked by a main channel for incubation and parallel channels for elution, 2) a pneumatic valve array on the main channel sealing each sol-gel spot from the others, and 3) an elution channel for eluting the sol-gel bound aptamers. (b) A Au/Cr microheater was fabricated on the glass substrate. After depositing chromium and gold layers, the microheater structure was patterned using standard photolithography and etched to obtain spiral-shaped circular electrodes. Finally, a thin ($>100$ μm) PDMS layer was spin-coated on the electrode chip to prevent fluid interaction and oxidation of the resistive heater, and to provide a surface enabling enhanced adhesion of the sol-gel droplets.

mers eluted from each target chamber were confirmed by PCR analysis. The elute from each target chamber in the microfluidic platform was reverse transcribed and used as template with amplification for 18 cycles of 95°C 30 s, 54°C 1 min, and 72°C 1 min. A specific primer set against each aptamer species was designed (red arrows) and synthesized (IDT, USA), as shown in Table 1. The amplified PCR products were analyzed using 2% agarose gel electrophoresis.

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## Supporting Information

Supplementary Figure 1 illustrates the fabrication of the micro-platform integrated with micro-resistive Cr-Au heaters and the fluidic network with the reaction chambers, pneumatic valves, and elution channels for the five-plex-SELEX process on a chip. The microchambers were linked in parallel with microchannels. The rubber-seal pneumatic valves ensured no cross-contamination between adjacent sol-gel spots during the elution process.

As shown in Figure 1a, a negative photoresist (SU-8 2075, MicroChem) was spin-coated and patterned. The mold master was cleaned thoroughly with isopropyl alcohol and de-ionized (DI) water. A prepolymer of PDMS (Silopt 184, Dow Corning Toray) was mixed with a curing reagent in a 10 : 1 weight ratio, and poured over the mold master. The mixture was kept at around 0.02 MPa for 30 min in a vacuum chamber to remove the trapped air bubbles from the liquid PDMS. After curing at 75°C for 2 h, the PDMS structure was peeled off the SU-8 mold, and the essential through-holes for interconnections, *i.e.*, the electric, fluidic,

and pneumatic ports, were punched out. The height of the patterning mold master was 100 μm. The reaction chambers measured 1 mm and the fluid channels measured 100 μm.

A circular microheater (see Figure 1b) was fabricated from Cr/Au layers deposited on a glass substrate and patterned via the sequential etching of the chrome and gold layers. The size of the microheaters matched the reaction chamber (1 mm diameter) to provide homogenous heating of the sol-gel spot. The Cr-Au heater was 50 μm wide, and the total resistance of the heater was 287Ω at room temperature. After fabricating the microheater, a thin PDMS layer (~100 μm) was coated on the glass surface as an electric and fluid insulation layer to prevent electrode oxidation during the resistive heating process, yet allowing rapid heat transfer to the sol-gel spot.

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