

## Video Article

# Primer-Free Aptamer Selection Using A Random DNA Library

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## Abstract

Aptamers are highly structured oligonucleotides (DNA or RNA) that can bind to targets with affinities comparable to antibodies<sup>1</sup>. They are identified through an in vitro selection process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) to recognize a wide variety of targets, from small molecules to proteins and other macromolecules<sup>2-4</sup>. Aptamers have properties that are well suited for in vivo diagnostic and/or therapeutic applications: Besides good specificity and affinity, they are easily synthesized, survive more rigorous processing conditions, they are poorly immunogenic, and their relatively small size can result in facile penetration of tissues.

Aptamers that are identified through the standard SELEX process usually comprise ~80 nucleotides (nt), since they are typically selected from nucleic acid libraries with ~40 nt long randomized regions plus fixed primer sites of ~20 nt on each side. The fixed primer sequences thus can comprise nearly ~50% of the library sequences, and therefore may positively or negatively compromise identification of aptamers in the selection process<sup>3</sup>, although bioinformatics approaches suggest that the fixed sequences do not contribute significantly to aptamer structure after selection<sup>5</sup>. To address these potential problems, primer sequences have been blocked by complementary oligonucleotides or switched to different sequences midway during the rounds of SELEX<sup>6</sup>, or they have been trimmed to 6-9 nt<sup>7,8</sup>. Wen and Gray<sup>9</sup> designed a primer-free genomic SELEX method, in which the primer sequences were completely removed from the library before selection and were then regenerated to allow amplification of the selected genomic fragments. However, to employ the technique, a unique genomic library has to be constructed, which possesses limited diversity, and regeneration after rounds of selection relies on a linear reamplification step. Alternatively, efforts to circumvent problems caused by fixed primer sequences using high efficiency partitioning are met with problems regarding PCR amplification<sup>10</sup>.

We have developed a primer-free (PF) selection method that significantly simplifies SELEX procedures and effectively eliminates primer-interference problems<sup>11,12</sup>. The protocols work in a straightforward manner. The central random region of the library is purified without extraneous flanking sequences and is bound to a suitable target (for example to a purified protein or complex mixtures such as cell lines). Then the bound sequences are obtained, reunited with flanking sequences, and re-amplified to generate selected sub-libraries. As an example, here we selected aptamers to S100B, a protein marker for melanoma. Binding assays showed  $K_d$ s in the  $10^{-7}$  -  $10^{-8}$  M range after a few rounds of selection, and we demonstrate that the aptamers function effectively in a sandwich binding format.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/2039/>

## Protocol

### 1. Brief Description of the Primer-Free Selection Protocols

A double-stranded DNA library was constructed using PCR with the corresponding oligonucleotides (Figure 1 and 2, Step a), which contain a central random domain of 30 nt, flanked by two primer regions. Two slightly different "primer-free" (PF) protocols were developed. In the dsDNA library, the 5'-region contains an endonuclease "nicking" site for the endonuclease Nt.BstNBI; this enzyme recognizes dsDNA but cleaves only one strand of the DNA substrate. The 3'-region of the dsDNA library contains another "nicking" site, for the endonuclease Nt.BbvCI that also recognizes dsDNA but cleaves only one strand, leaving a CC at the 3'-end (PF<sub>1</sub>), as well as a BspMI endonuclease restriction site, which cleaves both strands leaving no additional 3' nucleotides (PF<sub>2</sub>). We generally employ the PF<sub>1</sub> protocol initially (because it's easier to separate fragments produced during the selection process; see below), and then employ the PF<sub>2</sub> protocol for subsequent rounds of selection.

The 32 nt of 5'-pN30-CC-3' fragment (designated 32+-fragment) and the 30 nt of 5'-pN30-3' fragment (designated 30+-fragment) were respectively generated by Nt.BbvCI/Nt.BstNBI or BspMI/Nt.BstNBI cleavage of the dsDNA library, and the gel-purification. The 32+-fragment contains the 30 nt random domain, with a CC flanking sequence at the 3'-end (PF<sub>1</sub>). The 30+-fragment (PF<sub>2</sub>) consists only the 30 nt random domain sequence. The "self-bridge" 66--fragment (containing the random N30 region with the 5' and 3' flanking sequences) was obtained

by gel purification (Nt.BbvCI or Nt.BstNBI cuts only the upper "+" strand). This self-bridge is obtained directly in the PF<sub>1</sub> protocol, or can be generated and isolated after cutting the library DNA with only NtBbv.CI or Nt.BstNBI for the PF<sub>2</sub> protocol (Figure 1 and 2, Step b). The 32+- or 32+-fragments were incubated with the purified proteins or the cultured melanoma cells to allow the fragments bind to the proteins or the cells, and then the unbound fragments were washed away (Figure 1 and 2, Step c). The bound or selected fragments were used for re-generation of the primer regions.

In the hybridization/ligation reaction, the self-bridge was produced and purified at the same time as the 32+- and 30+-fragment purification; 5'-end primer was same as the library construction and 3'-end primers were replaced with matching primers that also contained an additional Sp6 transcription promoter at the 3'-end (Figure 1 and 2, Step d). The products of the hybridization/ligation reaction were then used for *in vitro* RNA transcription (Figure 1 and 2, Step e). Following the RNA transcription, the ligated DNAs (including the unselected self-bridge DNA fragment) were digested by DNase I to remove interfering background sequences. The reverse transcription (RT)-PCR data have shown that the primer-regenerated products were efficiently reamplified, constituting a "round" of selection (Figure 1 and 2, Step f). The background amplification shown at high cycle numbers in the no-RT control PCR can be completely removed by an additional DNase I digestion, and is not detectable after lower cycle numbers, which we routinely employ.

After 7 rounds of selection, aptamers were characterized for binding properties. Kd's were all in the 10<sup>-7</sup> - 10<sup>-8</sup> M range (Figure 3). In binding assays, various pairs of aptamers showed additive binding, indicating that they target distinct sites on the S100B protein. We therefore tested pairs of aptamers in "sandwich" binding assays, both on glass microarrays (Codelink slides) using fluorescently-tagged second aptamers, and on derivatized gold nanowires with second aptamers coupled to 50 nm gold nanoparticles (AuNPs; Figure 3). In both cases, binding specificity was high: On Codelink microarrays, no sandwich binding was observed with aptamers which did not show additive binding in Kd determinations. With derivatized nanowires we observed virtually no binding to non-target proteins, and individual sandwich complexes can be observed via the aptamer-coupled AuNPs (Figure 3).

## 2. Materials

### 2.1. Generation of PF DNA Library and Reamplification of Bound Fragments

Details are described in Pan and Clawson, 2009; Pan *et al.*, 2008.

### 2.2. Purified Protein Based Selection

1. 20 mM Tris-HCl, pH 7.4
2. 32+-Fragment and 30+-Fragment
3. Selection Buffer (2.5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub> in 1X Phosphate Buffered Saline, pH 7.4, Gibco)
4. Ni-NTA Agarose Beads (Qiagen)
5. Polypropylene Column (Qiagen)
6. Binding Buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, pH7.2, 150 mM NaCl)
7. Expressed, purified S100B protein in Binding Buffer (5 µg/µL)
8. Phenol:ChCl<sub>3</sub>:IAA (pH 7.9, Ambion)
9. 3 M NaAc, pH 5.2
10. 100%, and 70% Ethanol

### 2.3. TOPO Cloning and Sequencing Analysis of the Selected dsDNA Libraries

1. 7<sup>th</sup> round selected PCR products (additional rounds can be performed to continue optimization of aptamer binding)
2. pCR2.1-TOPO Vector (Invitrogen)
3. DH5 Component Cells (Invitrogen)
4. Plasmid Mini Kit (Qiagen)
5. M13 forward primer
6. Informax's Vector NTI (Invitrogen)

### 2.4. Binding Characteristics of the Aptamers

1. Aptamers and control oligos (N<sub>30</sub>CC and N<sub>30</sub>, IDT)
2. T4 Polynucleotide Kinase (New England Biolabs)
3. γ-<sup>32</sup>P-ATP (3000 Ci/mmol; 10 µCi/ml)
4. Purified S100B in Binding Buffer (5 µg/µL)

### 2.5. Sandwich Binding Assays with Purified S100B protein and Selected Aptamers

1. The 1<sup>st</sup> aptamer was coupled to either a.) Codelink microarray slides or b.) Au nanowires.
2. Purified S100B protein, diluted to either 0.125 µM in 70 µl for a) or 1 µM in 50 µl for b).
3. The 2<sup>nd</sup> aptamer was either labeled with a.) Alexafluor 546 for the Codelink array slides, or b.) coupled to 50 nm AuNPs for the studies with derivatized Au nanowires.

## 3. Methods

### 3.1. Generation of PF DNA Library

Detailed Methods and Protocols for aptamer selection can be found in Pan and Clawson, 2009; Pan *et al.*, 2008.

### 3.2. Aptamer Selection using Purified S100B Protein

Human S100 calcium binding protein B (S100B, Gene ID#6285) was used as a target. The His<sub>6</sub>-tagged S100B protein (98 amino acids in length) was expressed and purified by using the QIAexpressionist system. (QIAGEN). If desired or indicated, the His<sub>6</sub>-tag can be removed enzymatically. During the rounds of selection, 1  $\mu$ L sample from each step was saved for liquid scintillation counting to determine the overall binding efficiency.

#### 3.2.1. Preparation of PF Library-DNA Fragments

1. Re-suspend the 32<sup>+</sup> and 30<sup>+</sup> fragments in 40  $\mu$ L of 20 mM Tris-HCl, pH 7.4.
2. Heat 3 min at 85°C, and cool down to 37°C for 3 min in an incubator of 37°C.
3. Add 760  $\mu$ L of selection buffer (2.5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub> in 1X Phosphate Buffered Saline, pH 7.4, GIBCO) and incubate for 3 min at 37°C, then keep at room temperature (RT) for 10 min.
4. Pass through a column contained Ni-NTA agarose-beads (QIAGEN) pre-washed with selection buffer, then keep at RT until use.

#### 3.2.2. Preparation of Ni-NTA Agarose-Bead Bound S100B at RT

1. Spin down 400  $\mu$ L of Ni-NTA agarose beads for 3 sec and discard the supernatant.
2. Wash the beads with 400  $\mu$ L of binding buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, pH7.2, 150 mM NaCl) by gently pipetting 5 times, then spin down and discard the supernatant.
3. Repeat step 2 twice.
4. Add 400  $\mu$ L of purified S100B (5  $\mu$ g/ $\mu$ L, suspended in binding buffer) and gently pipette 5 times every 3 min for total of 15 min.
5. Spin down and discard the supernatant.
6. Wash the bead-bound S100B with 400  $\mu$ L of binding buffer by gently pipetting 3 times, then spin down and discard the supernatant.
7. Repeat step 6 twice.

#### 3.2.3. Selection of S100B-bound Aptamers

1. Transfer the 32<sup>+</sup>- and 30<sup>+</sup>-fragments from the 3.2.1 to the bead-bound S100B.
2. Incubate for 15 min and mix gently every 3 min by gently pipetting.
3. Wash the S100B-DNA complex with 800  $\mu$ L of binding buffer by gently pipetting, then spin down and discard the supernatant.
4. Repeat step 3 twice.

#### 3.2.4. Recovery of the S100B-Selected Aptamers

1. Add 200  $\mu$ L 20 mM Tris-HCl (pH7.4), heat 3 min at 85°C, vortex 1 min and spin down, then transfer the supernatant to a fresh tube.
2. Repeat step 1 once, and combine the supernates.
3. Purify the selected DNA-fragments as per steps 9-12 from previous publications (11, 12).

#### 3.2.5. TOPO Cloning and Sequencing Analysis for Identification of Consensus Aptamer Sequences

1. Clone the 10<sup>th</sup> round selected PCR products into pCR2.1-TOPO vector (from Invitrogen). Additional cloning can be done as further selections are performed.
2. Sequence 40-50 single colonies with M13 forward primer (we use the Molecular Genetics Core Facility at the Hershey Medical Center).
3. Align the selected sequences using Informax's Vector NTI (Invitrogen).
4. Determine the consensus sequences based on the alignments.
5. Consensus aptamers were then purchased from Integrated DNA Technologies.

### 3.3. Sandwich Binding Assays with Pairs of Selected Aptamers

#### 3.3.1. Microarray format using fluorescently labeled 2<sup>nd</sup> aptamers.

1. Consensus 1<sup>st</sup> Aptamers were synthesized with a 5-amineC6 moiety. They were suspended in printing buffer to a final concentration of 15  $\mu$ M and spotted onto Codelink Activated Slides (GE Healthcare/Amersham Biosciences) using an Apogee Discoveries MicroGrid Arrayer in the DNA Microarray facility PSU, University Park, following Codelink recommended protocols. Each slide was printed with 12 arrays.
2. Hybridization was carried out in a 2 x 8 format Microarray Hybridization Cassette (Array It, TeleChem International). The Microarray Cassette was sealed using nuclease-free adhesive sealing foil (AlumaSeal II, Research Products International) to prevent evaporation.
3. S100B protein was diluted in PBS to the appropriate concentration in 70  $\mu$ L and applied to the wells of the microarray cassette. Binding to the aptamers printed on the Codelink slide was for 1 hour at room temperature. The wells of the cassette were then individually washed 3X with PBS + 5 mM MgCl<sub>2</sub> (PBBSM), and excess wash buffer was blotted. The fluorescently labeled aptamers were diluted in PBBSM to 0.125  $\mu$ M in 70  $\mu$ L, and then heated to 85 °C for 3 minutes followed by 3 minutes on ice. Aptamers were applied to the wells of the microarray cassette, and incubated for one hour at room temperature. Wells were again individually washed 3X with PBBSM. The cassette was then taken apart, and the whole slide was rinsed in PBBSM. The slide was then thoroughly dried by centrifugation, and scanned using a Scanning Packard Biosciences ScanArray 4000XL (Perkin Elmer).

#### 3.3.2. Derivatized Nanowire format using 2<sup>nd</sup> aptamers coupled to 50 nm AuNPs

Gold NWs (~5  $\mu$ M in length, 320 nm in diameter) were synthesized by galvanostatic electrodeposition within porous alumina membranes following previously published protocols (13, 14). Upon dissolution of the porous membrane, the nanowires were resuspended in 1 mL ethanol.

As noted, DNA was purchased from Integrated DNA Technologies. Gold nanoparticles (50 nm) were purchased from Ted Pella. Thiolated DNA was cleaved with 100 mM DTT in 0.1 M sodium phosphate pH 8.3 for one hour and then purified in a Centri-spin 10 column.

### Aptamer attachment to Gold NWs and NPs

A 50  $\mu$ L aliquot of Au nanowires was placed into a 0.5 mL non-stick centrifuge tube and rinsed into 10 mM phosphate buffer, 300 mM NaCl, pH 7.4. DNA thiolated at the 5' end (with a 10 T spacer at 5' end) was added at a final concentration of 0.4  $\mu$ M to the wires. The sample was vortexed for 30 min and then rinsed by centrifugation (8100 g) three times with the 10 mM phosphate buffer, 300 mM NaCl, pH 7.4 and three times with 50 mM phosphate buffer, 5 mM MgCl<sub>2</sub>, pH 7.2. The DNA coated wires were resuspended in 100  $\mu$ L buffer to dilute by half.

DNA-derivatized AuNPs were prepared by adding 50  $\mu$ L 100 mM 2<sup>nd</sup> aptamer (with a spacer of 10 T's at the 5'-end) to 1 mL 50 nm AuNPs and heated at 37°C for one hour. Following heating, 10 mM sodium phosphate buffer, 1M NaCl, pH 7.4 was added (25  $\mu$ L two times, followed by 100  $\mu$ L, 150  $\mu$ L, and 128  $\mu$ L) at 0.5 hour intervals. The conjugates were left on a 37°C heat block overnight before use. Samples were rinsed 3 times with 50 mM phosphate buffer, 5 mM MgCl<sub>2</sub>, pH 7.2. Conjugates were resuspended in 120  $\mu$ L buffer.

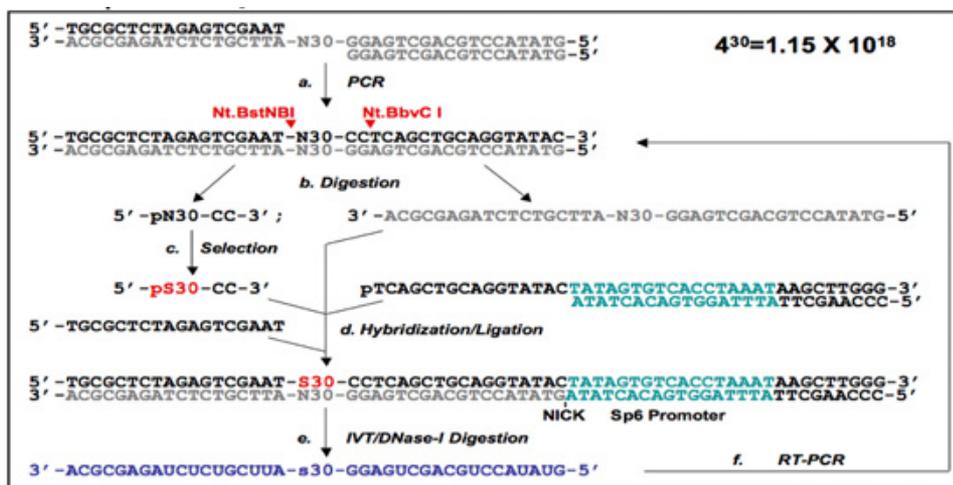
### Sandwich Hybridization on NWs

DNA coated wires, 1  $\mu$ L diluted, were added to buffer in PCR tubes. S100B protein or HtrA1 control protein (1  $\mu$ g/ $\mu$ L) was added for a final concentration of 1  $\mu$ M in 50  $\mu$ L buffer (~ 10-12 protein molecules added per square nanometer of Au nanowire surface). Samples were vortexed for ~ 2 hrs. Wires were rinsed by centrifugation (8100 g) three times with 50 mM phosphate buffer, 5 mM MgCl<sub>2</sub>, pH 7.2 and were each resuspended in 20  $\mu$ L AuNP/DNA conjugates. The wires were vortexed in the conjugates for 2 hr, and then were rinsed 5x by centrifugation (1300 g) with 50 mM phosphate buffer, 5 mM MgCl<sub>2</sub>, pH 7.2 to remove the excess nanoparticles. The samples were resuspended in 20  $\mu$ L buffer and dried on Au coated Si wafers for FE-SEM analysis.

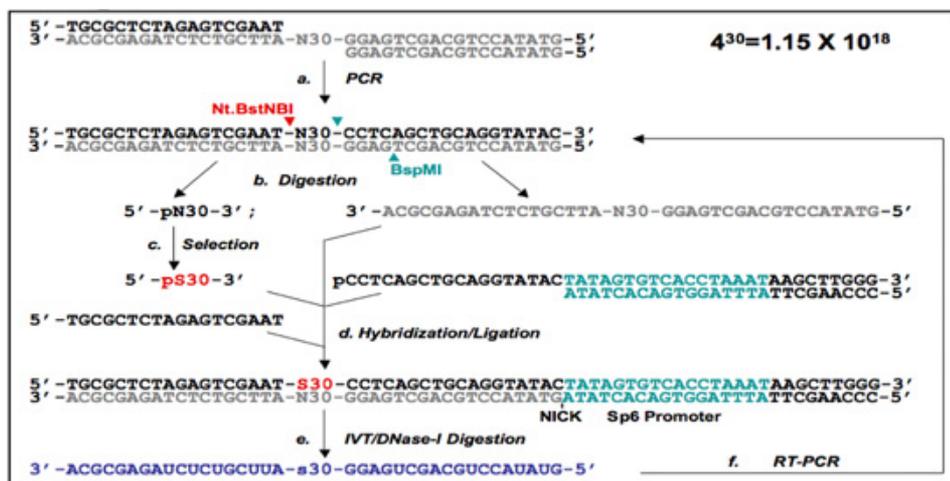
FE-SEM images of the nanowires were obtained using a Leo 1530 Field Emission Scanning Electron Microscope using a Schottky field-emission electron source at a 5.00 kV operating voltage.

## 4. Representative Results

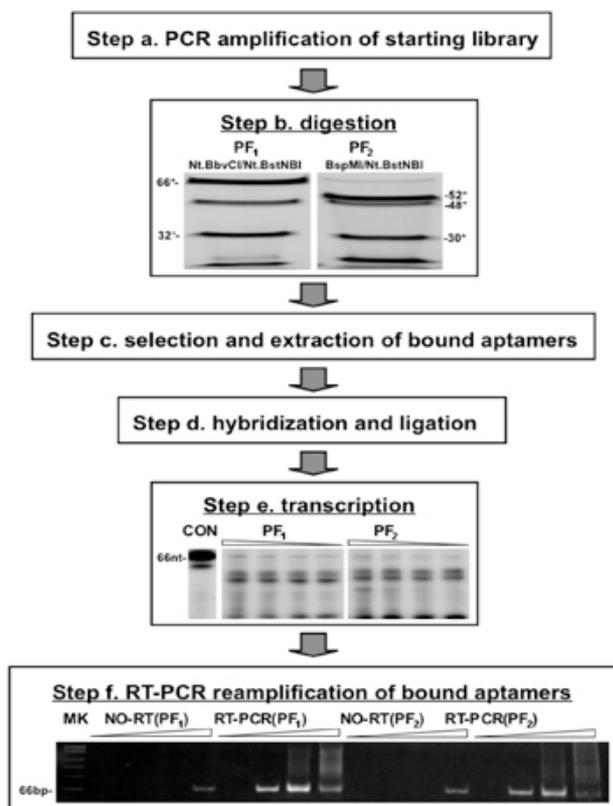
Figure 1. The 5'-Primer-Free (PF<sub>1</sub>) and Primer-Free (PF<sub>2</sub>) Protocols.



(A) The PF<sub>1</sub> DNA-aptamer selection protocol. The library oligonucleotides are annealed together, and subjected to PCR amplification to yield a double-stranded DNA library (a). The 5'-end primer-free (PF<sub>1</sub>) random region from the PF single-stranded DNA libraries is prepared by Nt.BstNBI /Nt.BbvCI digestions (cleavage sites are marked with red arrowheads) and gel-purifications, and the self-bridge (black arrow) is isolated by gel purification (b). After selection (c), the selected sequences (designated pS30-CC) are hybridized with their corresponding oligomers and the self-bridge and ligated to re-generate the previously removed primer regions. At this stage, primers are introduced that contain an Sp6 promoter at the 3'-end (d). This is then used to transcribe RNA containing the selected regions (e). Finally, the RNA is then exclusively re-amplified (the template DNA is digested) using RT-PCR (f), and the selected sub-library is ready for the next round of selection.

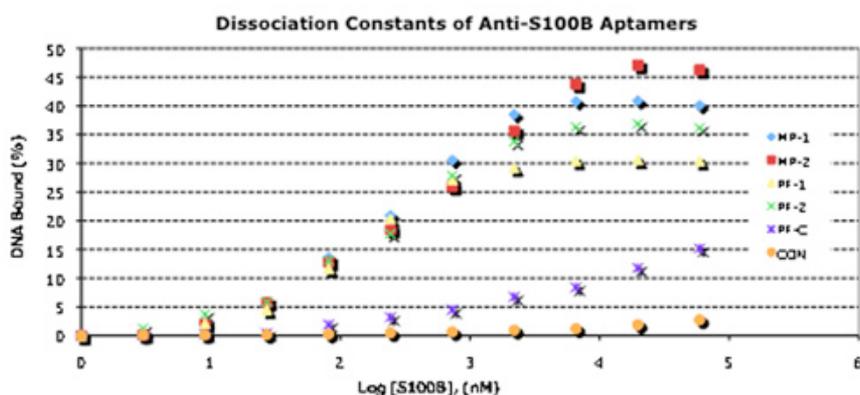


(B) The PF<sub>2</sub> DNA-aptamer selection protocol. The library oligonucleotides are annealed together and subjected to PCR amplification to yield a double-stranded DNA library (a). The primer-free (PF<sub>2</sub>) random region from the PF single-stranded DNA library is prepared by Nt.BstNBI/BspMI digestions (cut sites are denoted with arrowheads) and gel purification. The self-bridge is isolated either from the PF<sub>1</sub> protocol and/or by single-digestion of the starting library with Nt.BstNBI (b), coupled with gel purification. After selection (c), the selected sequences (designated pS30) are hybridized with their corresponding oligomers and the self-bridge, and ligated to regenerate the previously removed primer regions. As in PF<sub>1</sub>, primers are introduced that contain an Sp6 promoter at 3'-end (d). This is then used to transcribe RNA containing the selected regions (e). The RNA is then exclusively reamplified using RT-PCR (f), and the selected sub-library is ready for the next round of selection.



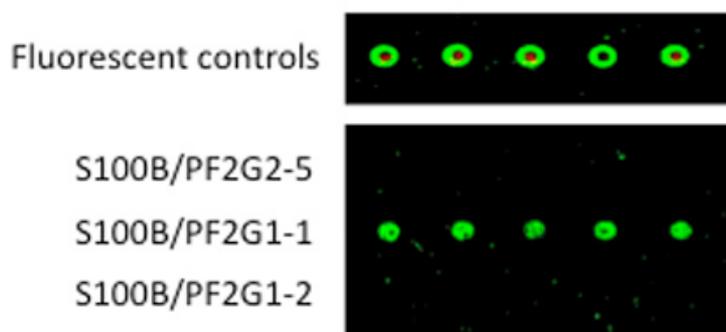
**Figure 2. Schematic representation and experimental results/reduction to practice of the PF selection protocol.** Designated steps correspond to those depicted in Figure 1. After digestion of the PF library constructed by PCR (Step a) with restriction endonucleases (as indicated), the digested products were separated by PAGE on a 10% gel under denaturing conditions. The corresponding fragments obtained with the PF<sub>1</sub> and PF<sub>2</sub> selection protocols are shown (Step b). After selection (Step c), the bound aptamers are hybridized with their corresponding oligomers and the self-bridge, and ligated to regenerate the previously removed primer regions. At 3'-end, primers are introduced that contain an Sp6 promoter at 3'-end (Step d). <sup>32</sup>P-labeled RNAs were transcribed using ligated products of 1, 0.5, 0.25, 0.125 μl from 5 μl reactions, and were separated by PAGE on 6% gels under denaturing conditions (Step e). The transcripts were then treated with DNase to removed the unselected random DNA regions, reverse transcribed into cDNA, and then reamplified for 7, 14, 21 or 28 PCR cycles. Products were separate by PAGE on 8% gels under non-denaturing conditions. The 66 nt fragment represents the full-length product containing the selected pS30-CC and pS30 sequences as re-embedded within the 5' and 3' flanking sequences. Migration of PhiX174/HinI markers is shown at

left (Step f). Controls included omission of the reverse transcription (RT) step. A small amount of product in such samples could be seen at high cycle numbers; this presumably can be completely eliminated with a second (or prolonged) DNase digestion step.

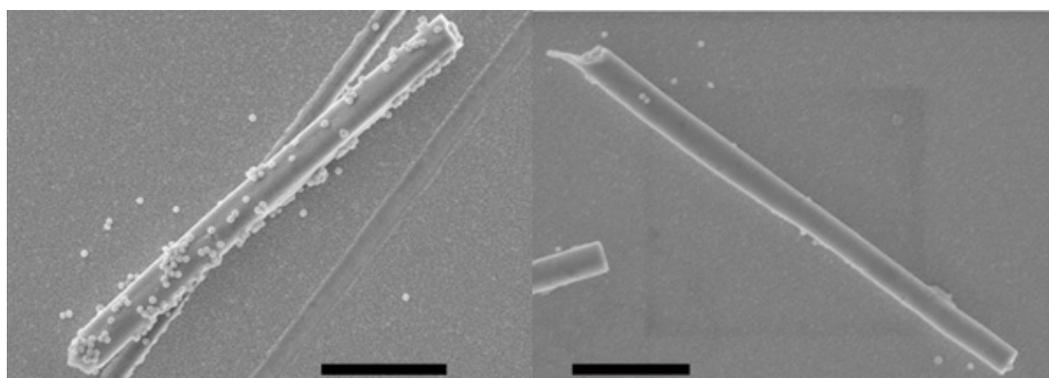


**Figure 3. Binding Characteristics of Selected Aptamers and Their Paired Use in a Sandwich Format.**

**A.** Concentration-Dependent <sup>32</sup>P-Aptamer Binding for K<sub>d</sub> Determination. Aptamers were 5'-end labeled using γ-<sup>32</sup>P-ATP (3000Ci/mmol, ~ 10 mCi) and T4 Polynucleotide Kinase (New England Biolabs), and binding was determined as described.



**B.** 5'-amine-derivatized 1<sup>st</sup> aptamers were coupled to Codelink microarrays. Purified S100B protein was bound to the 1<sup>st</sup> aptamer, the slides were rinsed thoroughly, and AlexaFluor546-labeled 2<sup>nd</sup> aptamer was bound to the 1<sup>st</sup> aptamer:S100B complexes. After thorough rinsing, Fluorescence was quantified using a ScanArray scanner.



**C.** 5'-thiol derivatized 1<sup>st</sup> aptamers were coupled to Au nanowires using standard thiol chemistry. 2<sup>nd</sup> aptamers were coupled to 50 nm AuNPs in the same manner. Purified S100B protein (left) or purified HtrA1 control protein (right) was then bound first to the derivatized nanowires. After thorough rinsing, 2<sup>nd</sup> aptamer-50 nm AuNPs were subsequently bound to the 1<sup>st</sup>-aptamer-nanowire:S100B complexes. After thorough rinsing, bound sandwich complexes were visualized using field-emission scanning electron microscopy. Scale bars = 1 μm.

## Disclosures

No conflicts of interest declared.

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