STAR Protocols

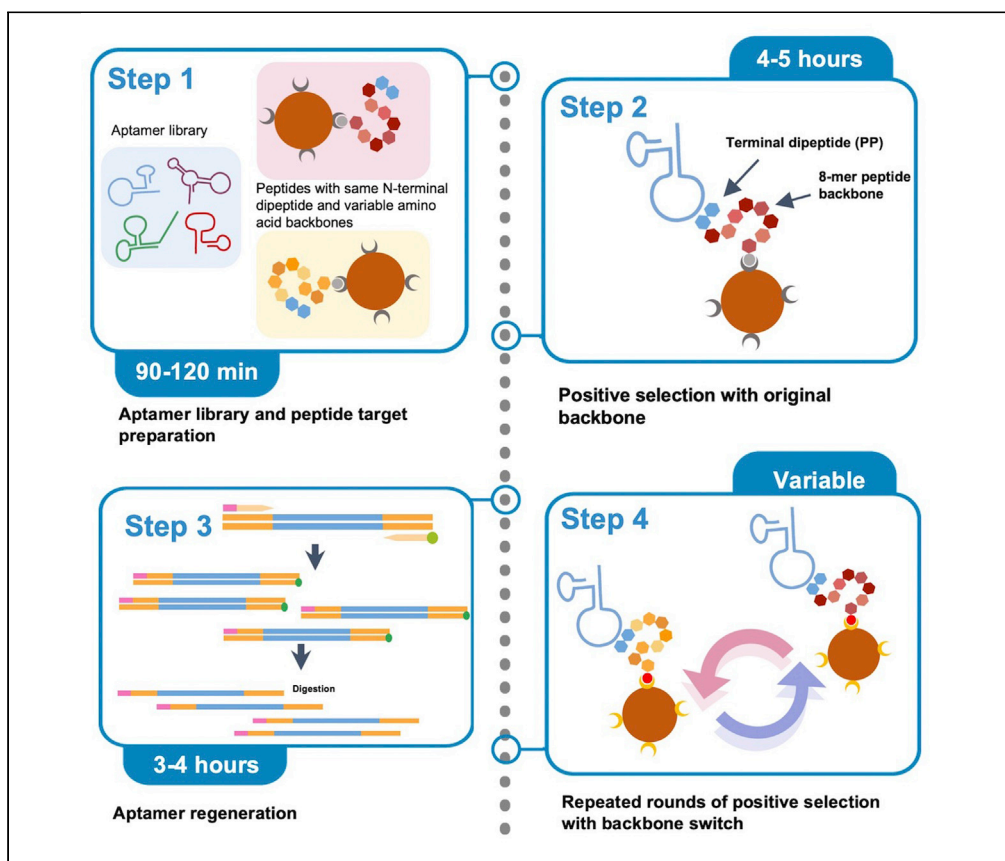**Protocol**

Target-switch SELEX: Screening with alternating targets to generate aptamers to conserved terminal dipeptides

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Highlights

SELEX method for
discovering aptamers
to N-terminal
dipeptide

Peptide targets with
shared 2-amino acid
(AA) N-terminus and
6-AA variable region

"Switch" runs
between 2 targets
over 5 selection
rounds (ex. target 1,
2, 1...)

Ran SELEX in parallel
with various targets
("switch", single
target, 2-target
mixture)

Systematic evolution of ligands by exponential enrichment (SELEX) encompasses a wide variety of high-throughput screening techniques for producing nucleic acid binders to molecular targets through directed evolution. We describe here the design and selection steps for discovery of DNA aptamers with specificity for the two consecutive N-terminal amino acids (AAs) of a small peptide (8–10 amino acids). This bead-based method may be adapted for applications requiring binders which recognize a specific portion of the desired target.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Target-switch SELEX: Screening with alternating targets to generate aptamers to conserved terminal dipeptides

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SUMMARY

Systematic evolution of ligands by exponential enrichment (SELEX) encompasses a wide variety of high-throughput screening techniques for producing nucleic acid binders to molecular targets through directed evolution. We describe here the design and selection steps for discovery of DNA aptamers with specificity for the two consecutive N-terminal amino acids (AAs) of a small peptide (8–10 amino acids). This bead-based method may be adapted for applications requiring binders which recognize a specific portion of the desired target.

For complete details on the use and execution of this protocol, please refer to Hong et al. (2022).

BEFORE YOU BEGIN

In traditional SELEX methods, 10^{15} random, unique DNA molecules are incubated with a target and screened for binding (Zhuo et al., 2017). The bound DNA is separated from the target and amplified to continue in the next round of selection. The 10^{15} sequence starting input allows for high diversity in the starting pool to encourage selection of successful aptamer candidates over multiple rounds of evolution, since pool diversity is reduced during each round. Typically, 5–15 rounds of selection are needed to reduce the pool diversity enough to identify the aptamer candidate through sequencing. One variation of SELEX is "toggle"-SELEX (White et al., 2001), which "toggles" between two different targets in alternating positive selections to isolate aptamers to either a conserved motif on both human and porcine thrombin or a unique motif on only human thrombin. Subsequently, "toggle"-SELEX is highly valuable for finding aptamers that can either bind to a broad range of similar targets or can distinguish between small structural differences in targets. Counter (Jenison et al., 1994), or subtractive, selections are also extremely common. Counter selection exposes the aptamer pool to molecules that closely resemble the target, with the intention of removing candidates that do not bind with high specificity to the desired target.

We incorporate aspects of both "toggle"-SELEX and counter-SELEX. This protocol is designed to discover binders to terminal dipeptides as part of a binder set for use in an approach for protein sequencing. The binder set may be flowed onto our chip-based, single molecule protein sequencing platform, where binding capture is used to identify the amino acid sequence of a protein. In Hong

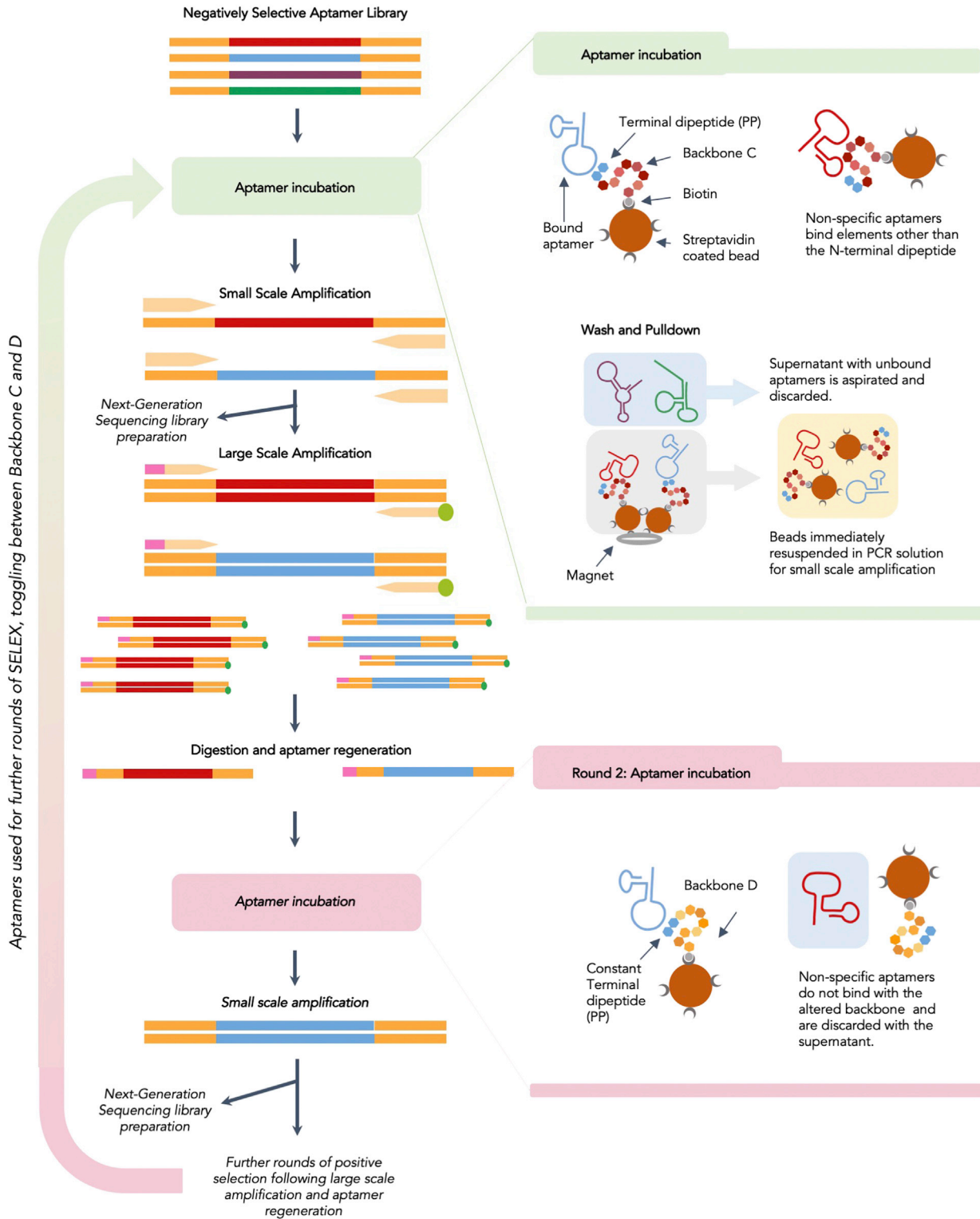Positive Selection of Aptamer Library

Figure 1. Target-Switch SELEX is designed to enrich aptamers with specific binding affinity to the terminal dipeptide

In odd-numbered rounds (green inset), incubations are done with a peptide with the dipeptide of interest at the N-terminus and an 8-mer peptide backbone designated backbone C. In even-numbered rounds (pink inset), the incubation is performed with a peptide containing the same N-terminus dipeptide, but an altered 8-mer backbone designated backbone D. The switch in peptide backbone between rounds reduces enrichment of aptamers with affinity for the backbone instead of the terminal dipeptide. After each small scale amplification, some of the sample is taken for NGS sequencing.

et al. (2022), we demonstrate target-switch SELEX on a proline dipeptide (PP) using two peptide targets named PP-C and PP-D, which have the same N-terminal dipeptide but different C-terminal backbones composed of 8 amino acids ("C" and "D" are the names of the backbones, not amino acids). Amino acid sequences are in the key resources table. Figure 1 illustrates the positive selection steps involved in target-switch SELEX for a single run following a single round of negative selection against streptavidin-coated beads. The aptamer pool is subjected to alternating rounds of positive selection against peptide PP-C and PP-D, with DNA sequencing of the aptamer pool after every round of selection.

For analysis purposes, we perform 12 parallel SELEX runs, where each run undergoes 5 rounds of selection (refer to section "selecting aptamer candidates" for analysis details). Three runs are conducted using the target switch technique, alternating between targets PP-C and PP-D from round to round. Three runs are conducted using target PP-C only, as a reference for enrichment without the target switch. Two runs are conducted on the specificity filter, which is created by conjugating beads to a 50-50 equimolar mixture of the C and D backbones only, minus the PP dipeptide. The purpose of the specificity filter is to eliminate binders to the backbones. Two runs are conducted on the enrichment reference, a target with known enrichment patterns against which to measure round-to-round enrichment as a positive control. Two runs are conducted on a beads-only negative control, where no peptide target is present.

This protocol is written here to be performed manually on the benchtop where use of multi-channel pipettes may be employed. In practice, several steps of this high-throughput method were performed using the Agilent Bravo liquid handling robot. These included conjugating peptide target to streptavidin beads (steps 1–7), positive selection (steps 17–29), small scale amplification (steps 30–35), large scale amplification (steps 36–41), aptamer regeneration (steps 42–48), and preparation for sequencing (steps 50–59). Where available, the use of lo-bind plastics (including tubes, plates, and pipette tips) is critical for equipment that comes into contact with solutions containing aptamers.

Aptamer library and primer design

⊙ **Timing: 20 min**

The purpose of this section is to design the aptamer library and primers. The aptamer library consists of a pool of 10^15 sequences, where each sequence contains a random oligonucleotide region flanked at the 5′ and 3′ ends by known sequences (Figure 2A). Primers are used to amplify the aptamer library in between rounds of selection and to prepare the sample for sequencing.
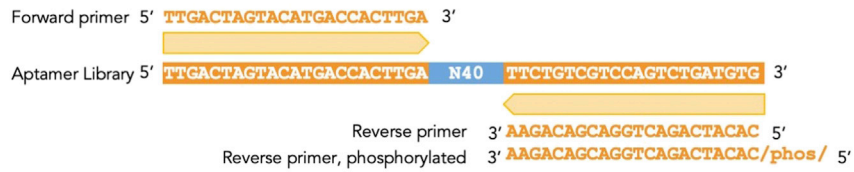
Note: All library and primer sequences are listed in the key resources table.

1. Design the forward and reverse primers for the aptamer library.
   a. Follow the general principles of appropriate primer design (ex. primer length, melt temperature, avoidance of secondary structures, etc.) (Rodríguez et al., 2015).
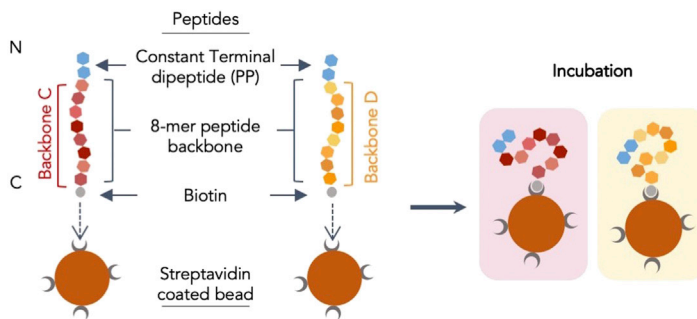
Note: The primers used for our aptamer library are 22–23 nt with 39%–50% GC content and melt temperatures of 53°C–56°C.

2. Order the primers: (1) forward primer, (2) protected forward primer with phosphodiester bond (3) reverse primer with unmodified bases, and (4) reverse primer with 5′ phosphorylation.
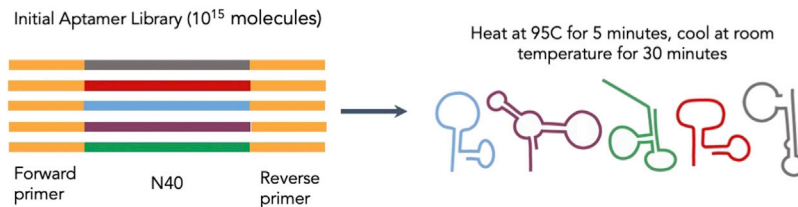   a. Order 250 nM scale and HPLC or PAGE-purification.

A Aptamer Library and Primer Design

Forward primer 5′ TTGACTAGTACATGACCACTTGA 3′

Aptamer Library 5′ TTGACTAGTACATGACCACTTGA N40 TTCTGTCGTCCAGTCTGATGTG 3′

Reverse primer 3′ AAGACAGCAGGTCAGACTACAC 5′
Reverse primer, phosphorylated 3′ AAGACAGCAGGTCAGACTACAC/phos/ 5′

B Conjugating peptide target to streptavidin beads

Peptides

N
Constant Terminal dipeptide (PP)
Backbone C
8-mer peptide backbone
Backbone D
C
Biotin
Streptavidin coated bead

Incubation

C Aptamer preparation

Initial Aptamer Library (10^15 molecules)

Heat at 95C for 5 minutes, cool at room temperature for 30 minutes

Forward primer   N40   Reverse primer

D Negative Selection of Aptamer Library

Aptamer incubation

Magnet Pulldown

Bound aptamer

Streptavidin coated bead

Supernatant aspirated and retained as negatively selected library

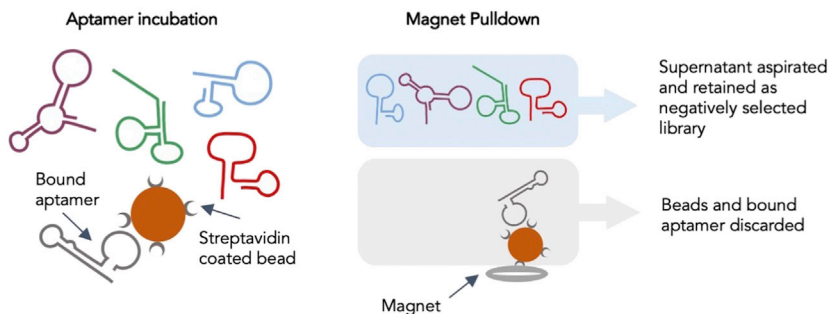Beads and bound aptamer discarded

Magnet

Figure 2. Preparation for target-switch SELEX
(A) Aptamer library and primer sequences. N40 indicates 40 random nucleotides. /phos/ indicates phosphorylation of the 5′ nucleotide (cytosine in this case).
(B) Peptides are conjugated to streptavidin-coated beads via biotin on the C′ terminus.
(C) Single-stranded aptamers are folded into their binding configurations through heating and slow cooling.
(D) The negative selection library is created by screening the refolded aptamers for binding against streptavidin-coated beads in the absence of peptides. Non-binding aptamers are collected and retained as the negative selection library.

Note: PCR amplification using the 5′ phosphorylated primer is required for successful enzymatic digestion during aptamer regeneration.

3. Order the aptamer library.
   a. Order a library with random region length 30–60 (our experience is with 40 nt length).

   Note: We selected a library with a 40 nt length random region due to commercial availability. Aptamer libraries may be 15–60 nt long, with longer random regions associated with higher binding affinity (Zhu et al., 2021). While our aptamer library was purchased from Trilink Biotech, aptamer libraries are available from many vendors such as IDT.

   b. Order a hand mixed library to ensure equal nucleotide distribution.

   Note: Phosphoramidite-based synthesis skews the pool toward a G and T preference (Komarova and Kuznetsov, 2019). The quality of the nucleic acid library depends on many parameters, starting with chemical synthesis. During phosphoramidite-based synthesis, nucleotides are unequally incorporated into an oligonucleotide chain, with a preference for G and T. For equal nucleotide distribution, the molar ratio of A:C:G:T phosphoramidites should be optimized—for example, a 1.5:1.5:1.0:1.2, 1.30:1.25:1.45:1.00, or 1.50:1.25:1.15:1.00 A:C:G:T molar ratio can be applied (Hall et al., 2009). The same libraries obtained from different manufacturers can differ significantly in sequence heterogeneity and, consequently, in structure complexity, which has been proved by the analysis of high-throughput sequencing results (Takahashi et al., 2016).

4. Resuspend all oligonucleotides including the aptamer library, forward primer, and reverse primers at 100 μM concentration in TE buffer.
5. Oligonucleotides may be stored at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SELEX peptide PP-C N′- PPNHFENEIK-bt - C′	GenScript	Custom
SELEX peptide PP-D N′- PPTKYVGTGK-bt - C′	GenScript	Custom
Specificity filter peptide C N′- NHFENEIK-bt - C′	GenScript	Custom
Specificity filter peptide D N′- TKYVGTGK-bt - C′	GenScript	Custom
SELEX peptide Bradykinin N′- RPPGFSPFRK bt - C′	PubChem	439201
Bovine Serum Albumin (BSA)	Thermo Scientific	A2153-50G
Phosphate Buffered Saline (PBS)	Thermo Scientific	AM9624
Tween-20	Sigma-Aldrich	P9416
Dynabeads™ M-280 Streptavidin	Thermo Fisher Scientific	60210
Lambda exonuclease	NEB	M0262L
Mag-Bind Total Pure NGS beads	Omega Bio-tek	M1378-02
Herculase II Phusion polymerase and herculase buffer	Agilent	600679
70% ethanol	Fisher Scientific	BP8201500
200 proof ethanol	Fisher Scientific	BP2818500
MgCl2 (1 M)	Thermo Fisher Scientific	AM9530G
dNTP mix (25 mM each)	Thermo Fisher Scientific	R1122
5′ end FAM-labeled aptamers	IDT	Custom
Fluorescein biotin	Biotium	#80019
DMSO, Anhydrous	Thermo Scientific	D12345
Critical commercial assays		
Qubit ssDNA kit	Thermo Fisher Scientific	Q10212
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agilent Small RNA kit	Agilent	5067-1548
TapeStation DNA ScreenTape	Agilent	5067-5582
Deposited data		
Raw sequencing data for Target-Switch SELEX	Harvard Dataverse	https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/W903IJ
Oligonucleotides		
N40 Aptamer Library TTGACTAG TACATGACCACTTGA-N40-TTCT GTCGTCCAGTCTGATGTG	TriLink BioTechnologies	Custom
Aptamer Library Forward Primer TTG ACTAGTACATGACCACTTGA	IDT	Custom
Aptamer Library Forward Primer, protected T*T*G*A*C*T*AGTACATGACCACTTGA (* indicates a phophodiester bond)	IDT	Custom
Aptamer Library Reverse Primer CACAT CAGACTGGACGACAGAA	IDT	Custom
Aptamer Library Reverse Primer, phosphorylated / 5phos/CACATCAGACTGGACGACAGAA	IDT	Custom
NGS Forward Primer for Library Preparation CAA GCAGAAGACGGCATACGAGATNNNNNNNN TTGACTAGTACATGACCACTTGA	IDT	Custom
NGS Reverse Primer for Library Preparation AAT GATACGGCGACCACCGAGATCTACACNNNN NNCACATCAGACTGGACGACAGAA	IDT	Custom
NGS Forward Primer for Sequencing AATGATACG GCGACCACCGAGATCTACAC-XXXXXX-GCATG CAGCCGGTTGACTAGTACATGACCACTTGA	IDT	Custom
NGS Reverse Primer for Sequencing CAAGCAGA AGACGGCATACGAGAT-XXXXXXX-GTGCGTGC GTGCTTCTGTCGTCCAGTCTGATGTG	IDT	Custom
Other		
4200 TapeStation System	Agilent	G2991AA
PippinHT	Sage Science	none
2100 Bioanalyzer	Agilent	G2939BA
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33238
Bravo liquid handler	Agilent	G5574AA
1.5 mL microfuge tubes, DNA LoBind	Eppendorf	cat#022431021
96-well plates, DNA Lo-Bind	Eppendorf	30129512
Magnetic Stand-96	Thermo Fisher Scientific	AM10027
Nunc plates	VWR	73520-120
Mastercycler® nexus gradient, 115 V/50 – 60 Hz (US)	Eppendorf	6331000025
Mastercycler® nexus eco, 115 V/50 – 60 Hz (US)	Eppendorf	6332000029
Mastercycler® nexus flat eco, 110 V/50 – 60 Hz (JP/South America/TW/US)	Eppendorf	1010015267
Adhesive PCR Plate Seals	Thermo Fisher Scientific	AB0558
Plate reader	BioTek Synergy HTX	S1LFA

MATERIALS AND EQUIPMENT

Refold Buffer

Reagent	Final concentration	Amount
PBS (10×)	1×	5 mL
Tween-20*	0.025%	12.5 μL
$MgCl_2$ (1 M Stock)	1 mM	50 μL
ddH_2O	N/A	937.5 μL and 44 mL
Total	N/A	50 mL

*Assemble Tween-20 and all small-volume reagents in a 1.5 mL tube. Add ddH2O to a total volume of 1 mL, and mix slowly by pipette to avoid agitating the Tween-20. Add remaining ddH2O to achieve 50 mL final volume.
Refold buffer may be stored stably at 4°C for 1 month.

SELEX Buffer

Reagent	Final concentration	Amount
Refold Buffer	1×	50 mL
BSA	0.1 mg/mL	5 mg
Total	N/A	50 mL

SELEX buffer may be stored stably at 4°C for 1 month.

STEP-BY-STEP METHOD DETAILS
Conjugating peptide target to streptavidin beads

⏱ Timing: 30 min

In this section, biotinylated peptides are conjugated to streptavidin-coated magnetic beads (Figure 2B). These beads will be used to pull down aptamers in solution.

Note: Peptide biotinylation may be performed using a commercial kit such as Thermo Scientific™

EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Catalog number: 21435).

Note: Prepare separate conjugation reactions for each of the desired targets (PP-C, PP-D, specificity filter, and the enrichment reference target). We prepared many reactions simultaneously in a lo-bind deep well plate.

Note: Peptide-bead conjugates can be prepared ahead of time and aliquoted in 96-well plates for freezing (1 freeze/thaw cycle maximum). Large batch preparation will reduce variation, as beads from the same conjugation batch can be used throughout a single experiment.

1. For each conjugation reaction, prepare 50 picomoles (suggested volume 50 μL) of biotinylated peptide suspended in ddH$_2$O.

   Note: To create conjugated beads for the specificity filter, create a 50-50 equimolar mixture of targets C and D (no PP dipeptide).

2. Vortex M280 streptavidin beads until homogenous.
3. Add 25 μL of M280 beads to 50 picomoles of biotinylated peptide.
4. Mix thoroughly.
5. Incubate the bead-peptide mixture on a chilled plate for 2 min.
6. Wash the conjugated beads using SELEX buffer.
   a. To the bead-peptide mixture, add 360 μL of SELEX buffer. Mix well by pipetting up and down.
   b. Use a magnetic rack (Magnetic Stand-96) to separate the beads to the bottom of the well.
   c. Let sit for 4 min.
   d. Aspirate and discard the supernatant.
   e. Repeat bead washing (steps 6a–6d) 2 additional times.
7. After the final wash, resuspend the M280 beads in 25 μL of SELEX buffer.

   ⏸ Pause point: Use the conjugated beads in the "Positive Selection" step or store at −20°C.

   Note: Using the Bravo liquid handler, we tested 4 bead types (Dynabeads™ M-280 Streptavidin, Dynabeads™ M-270 Streptavidin, Dynabeads™ MyOne™ Streptavidin C1, and Dynabeads™ MyOne™ Streptavidin T1) under BSA blocked and non-BSA blocked conditions

with several peptide concentrations ranging from 25 picomolar to saturation. We performed a single round of positive selection using beads conjugated under each condition. The most uniform results by k-mer analysis on sequencing were created using beads conjugated under the conditions described in this section.

Negative selection against streptavidin beads

⏱ Timing: 1 h 30 min

The negative selection step is intended to eliminate aptamers from the library with high binding affinity toward the streptavidin bead substrate (Figures 2C and 2D). The library generated through negative selection will be divided for use in 12 parallel SELEX runs (3 sets of target with switch, 3 sets of target without switch, 2 sets of specificity filter, 2 sets of enrichment reference, and 2 sets of bead-only control).

8. Suspend the aptamer library in 150 µL at 100 µM using Refold Buffer (equal to 10^15 aptamer molecules).
9. Perform aptamer refolding.
   a. To refold the aptamer library, divide the library into 50 µL per thermocycler tube.
   b. Heat tubes at 95°C for 5 min in the thermocycler.
   c. Cool tubes at 22°C (room temperature) for 30 min.
10. Prepare the M280 beads by washing the beads 3 times in SELEX buffer.
    a. To 55 µL of M280 beads, add 500 µL of SELEX buffer.
    b. Use a magnetic rack to separate the beads to the bottom of the well.
    c. Aspirate and discard the supernatant.
    d. Repeat bead washing steps (5a–5c) three times.
    e. After the final wash, resuspend the M280 beads in 55 µL of SELEX buffer.
11. Transfer the washed M280 beads (55 µL) to a 1.5 mL lo-bind tube.
12. To the resuspended M280 beads, add the refolded aptamer library (150 µL).
13. Incubate the solution on a rotator at 22°C (room temperature) for 30 min.
14. Place the tube in a magnetic rack and wait 1 min for beads to fully aggregate next to the magnet.
15. Collect the supernatant. The supernatant is ready for use as the negative selection library.
16. Measure the library concentration using the Qubit ssDNA kit.

⏸ **Pause point:** Use the negative selection library in the "Positive Selection" step or store at −20°C.

Positive selection

⏱ Timing: 2 h

In this positive selection step, aptamers are incubated with peptide targets conjugated to streptavidin beads. The goal is to collect the aptamers which bind to the peptide targets and wash away non binding DNA molecules. The aptamers which are collected will be directly amplified off the beads in the next step. Below are described the steps for a single run. All 12 runs may be completed simultaneously in one row of a 96-well plate.

17. Perform aptamer refolding on the negative selection library (see step 9).
18. Divide the negative selection library into 12 aliquots to perform 12 runs.
19. In a NUNC deepwell plate, add the appropriate volume of conjugated beads according to the desired peptide to aptamer ratio. Volume of beads may be calculated according to the formula below.

$$\# \text{ DNA input in ng} \times \frac{1000 \, pg}{1 \, ng} \times \frac{1}{\text{aptamer length in nt}} \times \frac{1 \, pmol}{327 \, pg} \times \text{stringency} \times \frac{25 \, \mu L \text{ beads}}{50 \, pmol \text{ peptide}}$$

$$= \# \, \mu L \text{ conjugated beads}$$

Note: Stringency refers to the ratio of peptide molecules to DNA molecules $\left(\frac{pmol \, peptide}{pmol \, DNA} \right)$.

Below is an example calculation using our aptamer library (length 85 nt), assuming a starting aptamer input of 2,250 ng and desired stringency of 1:1.

$$2,250 \, ng \text{ DNA} \times \frac{1,000 \, pg}{1 \, ng} \times \frac{1}{85 \, nt} \times \frac{1 \, pmol}{327 \, pg} \times \frac{1 \, pmol \text{ peptide}}{1 \, pmol \text{ DNA}} \times \frac{25 \, \mu L \text{ beads}}{50 \, pmol \text{ peptide}}$$

$$= 40.5 \, \mu L \text{ conjugated beads}$$

20. Add the conjugated beads to the aliquoted negative selection library.
21. Add SELEX buffer to reach a total volume of 400 μL.
22. Incubate the mixture on a rotator at 22°C (room temperature) for 1 h.
23. Use a magnetic rack to separate the beads to the bottom of the well.
24. Let sit for 2 min.
25. Discard the supernatant.
26. Complete two bead wash cycles using SELEX buffer.
    a. For each wash:
        i. Add 150 μL of SELEX buffer.
        ii. Pipette up and down to mix.
        iii. Place beads on a magnetic rack for 4 min.
        iv. Discard the supernatant.

Note: While completing these wash steps, simultaneously begin assembling the PCR reagents for Small Scale Amplification. The PCR reagents will need to be added immediately after the last step in this section.

27. Resuspend the beads in 150 μL of PBS.
28. Place beads on a magnetic rack for 4 min.
29. Discard the supernatant.

⚠ CRITICAL: Proceed immediately to the next section. Do not allow the beads to dry out between Positive Selection and Small Scale Amplification.

Small scale amplification

⏱ Timing: 1 h 20 min

The purpose of this step is to amplify the aptamers captured in the Positive Selection step. PCR is performed directly off of the peptide-bound aptamers (on beads). All beads from one run are put into a single PCR reaction.

30. To the beads, add PCR reaction reagents.
    a. PCR Reaction for Small Scale Amplification.

Reagent	Per 50 µL reaction
Beads from Positive Selection	All
Aptamer Library Forward primer	3 µM
Aptamer Library Reverse primer	3 µM
Herculase buffer	1×
dNTP	0.2 mM
Herculase polymerase	0.5 µ/uL

31. Transfer the solution to a lo-bind thermocycler tube and mix well.
32. Cycle according to the table.
    a. PCR Cycling Conditions for Small Scale and Large Scale Amplification.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	13 cycles
Annealing	55°C	30 s	
Extension	72°C	30 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

33. Perform PCR purification.
    a. Vortex Mag-Bind TotalPure NGS beads before use.
    b. In a lo-bind deep well plate, prepare 30 µL beads for each PCR reaction.
    c. To the beads, add 50 µL of 100% ethanol and mix by pipetting.

    Note: The addition of ethanol here is a departure from the manufacturer's instructions. In pilot tests, we found that the addition of ethanol at this step increases product yield.

    Note: Bead and ethanol volumes are determined based on sample volume and desired DNA size. The concentration of DNA input is not considered. For a volume of N uL digested product (before addition of ethanol in step 33c), 0.6*N uL of beads and 1.0*N uL of 100% ethanol should be used. Example: 50 µL PCR reaction + 30 µL beads + 50 µL 100% ethanol.

    d. Add PCR product (50 µL) to the bead-ethanol mixture.
    e. Complete 2 ethanol wash cycles according to the manufacturer's instructions.
    f. Elute PCR product in 32 µL of NF dH$_2$O.

34. Measure DNA concentration using plate reader.
35. Prepare a portion of the small scale PCR sample for sequencing (see section preparation for sequencing). Using the remaining sample, proceed to Large Scale Amplification.

    ▐▐ Pause point: Store stably at −20°C.

Large scale amplification

⏱ Timing: 2 h

The purpose of this step is to amplify the DNA as much as possible without introducing excess PCR bias. The amplified products will be used in the next round of positive selection.

Note: Below are described the steps for a single selection. Large scale amplification for all twelve selections may be completed simultaneously in three 96-well plates.

Note: It is highly recommended to use the protected forward primer, which includes six nuclease resistant phosphodiester bonds at the 5′ end, to retain the sense strand of the aptamer during the single strand generation in the aptamer regeneration step.

36. For the purified PCR product from a single small scale amplification reaction, assemble 24 PCR reactions containing 50 μL each.
    a. PCR Reaction for Large Scale Amplification.

Reagent	Per 50 μL reaction
Purified PCR product	0.17 ng
N40 protected forward primer	3 μM
N40 Reverse primer, phosphorylated	3 μM
Herculase buffer	1×
dNTP	0.2 mM
Herculase polymerase	0.5 μ/uL
H2O	up to 50

Note: Save remaining purified PCR product from Small Scale Amplification that is not used in this PCR in −20°C. In the case of future mistakes, you may return to this point.

37. Transfer the solution to a lo-bind thermocycler tube and cycle according to protocol in step 32a.
38. After thermocycling, pool the 24 PCR reactions into 8 tubes of 150 μL each.
39. Perform PCR purification.
    a. Vortex Mag-Bind TotalPure NGS beads before use.
    b. In a lo-bind deep well plate, prepare 90 μL beads for each PCR reaction.
    c. To the beads, add 150 μL of 100% ethanol and mix by pipetting.

Note: For sample volume N, 0.6*N uL of Mag-Bind beads and 1.0*N uL of 100% ethanol should be used (volume N refers to reaction volume before addition of ethanol in step 39c).

    d. Add DNA to the bead-ethanol mixture.
    e. Complete 2 ethanol wash cycles according to manufacturer instructions.
    f. Elute PCR product in 32 μL of NF dH$_2$O.

40. Pool 8 tubes of cleaned PCR products (8 tubes × 32 μL/tube =256 μL) into a single tube and mix well.
41. Measure the DNA concentration of the purified product using the plate reader.

⫸ **Pause point:** Store purified DNA product stably at −20°C.

⚠ CRITICAL: During PCR, use a reverse primer with 5′ phosphorylation. In the following aptamer regeneration step, lambda exonuclease will only recognize and degrade 5′ phosphorylated DNA strands.

Aptamer regeneration (single-strand generation)

⏱ Timing: 1.5 h

This step converts the double-stranded PCR product from large scale amplification into ssDNA for use as aptamers in the next round of selection using digestion by lambda exonuclease (Figure 3). To avoid overdigestion or incomplete digestion, the reaction is first tested on an aliquot of PCR product to determine the appropriate incubation time. The remainder of the PCR product is then digested following the optimal incubation time.

42. Digest 44 μL of purified PCR product from large scale amplification using lambda exonuclease according to manufacturer instructions.
43. On the Agilent bioanalyzer, visualize the digested product. Dilute product if needed to be within manufacturer's specifications.
    a. Follow manufacturer instructions for the small RNA protocol.

    Note: Successful conversion of dsDNA to ssDNA is indicated by a shift in peak from 20-40 nt to 60–80 nt. Incomplete digestion will result in multiple varied peaks (Figure 4B).

    Note: The small RNA protocol was not designed by the manufacturer for the purpose of distinguishing proportions of dsDNA and ssDNA in a sample. We ran several dsDNA, ssDNA, and mixed DNA controls using various kits made for the Agilent bioanalyzer and found that the small RNA protocol resulted in an acceptable separation in run size between the two.

44. If the DNA is partially digested, extend the digestion reaction according to steps 44a–c below. Otherwise, proceed to step 45.
    a. To extend the digestion reaction, add 1 μL lambda exonuclease and incubate for 2–3 additional minutes.
    b. Visualize the product using the bioanalyzer small RNA kit.
    c. Repeat steps 44a–b until the bioanalyzer tracing resembles ssDNA.

    Note: Achieving the ideal digestion time may require optimization based on sample concentration. See "troubleshooting" for additional bioanalyzer traces.

45. Digest the remainder of the purified PCR product.
    a. If the aliquot required extended digestion times, the total incubation time (all extended times added together) should be used. Do not add additional lambda exonuclease.
        i. For example, if the aliquot required a 7 min incubation, followed by 1 μL lambda and 3 min additional incubation, followed by 1 μL lambda and 2 min additional incubation, then the optimized regimen is 1 μL lambda over a single 12 min incubation.
    b. Visualize products using the bioanalyzer small RNA kit.
46. Combine the digested aliquot with the remainder of the digested PCR product.

47. Perform ssDNA purification of all digested products.
    a. Vortex Mag-Bind TotalPure NGS beads before use.
    b. In a lo-bind deep well plate, prepare 30 μL beads for each digestion reaction.
    c. To the beads, add 50 μL of 100% ethanol and mix by pipetting.

    Note: For a volume of N uL digested product, 0.6*N uL of beads and N*1 μL of 100% ethanol should be used (volume N refers to reaction volume before addition of ethanol in step 47c).

    d. Add products of digestion (50 μL) to the bead-ethanol mixture.
    e. Complete 2 wash cycles according to the manufacturer's instructions.
    f. Elute PCR product in 32 μL of NF dH$_2$O.
48. Measure ssDNA concentration using the qubit ssDNA kit. Dilute samples as necessary to remain within the assay reference range.
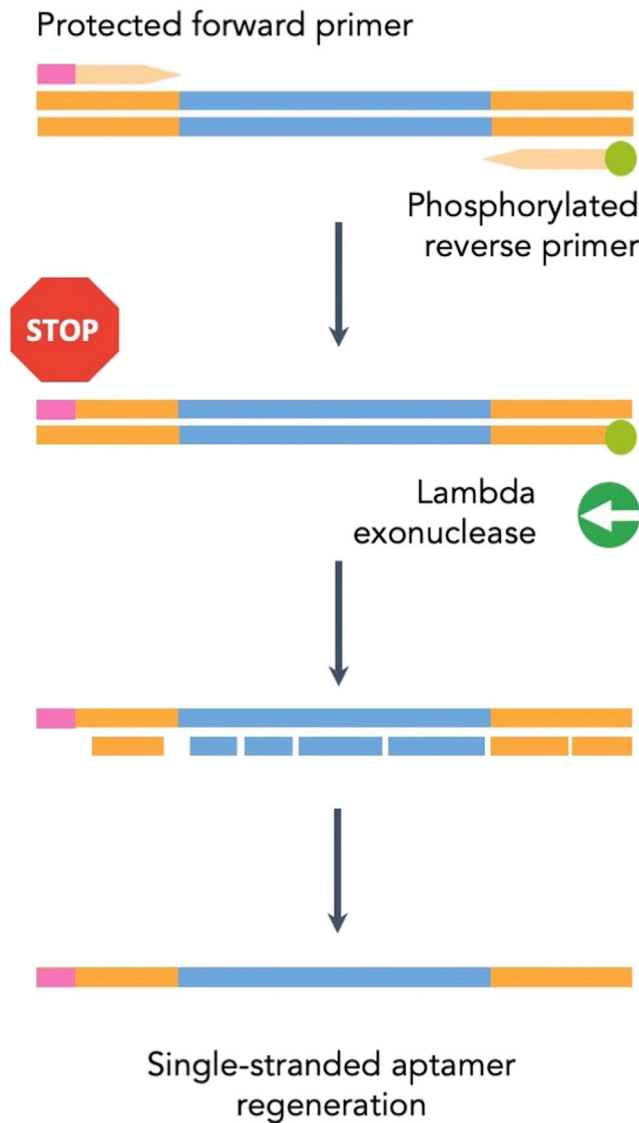
Figure 3. Aptamer regeneration Digestion with lambda exonuclease removes the 5′ phosphorylated strand of a double-stranded construct
Six phosphodiester bonds at the 5′ end of the sense primer allows preferential preservation of the sense strand.

Repeated rounds of positive selection

Positive selection, small scale amplification, large scale amplification, and aptamer regeneration (steps 17–48) should be repeated for 5 rounds with each of the 12 target conditions with varying stringencies. Stringency is defined as the ratio of peptide molecules to aptamer molecules.

49. Repeat Positive selection, Small scale amplification, Large scale amplification, and Aptamer regeneration for a total of 5 selection rounds. In the 5th selection round, stop after small scale amplification and proceed to NGS preparation.
    a. Adjust the stringency in each round according to Figure 5 by changing the volume of beads added in step 19.
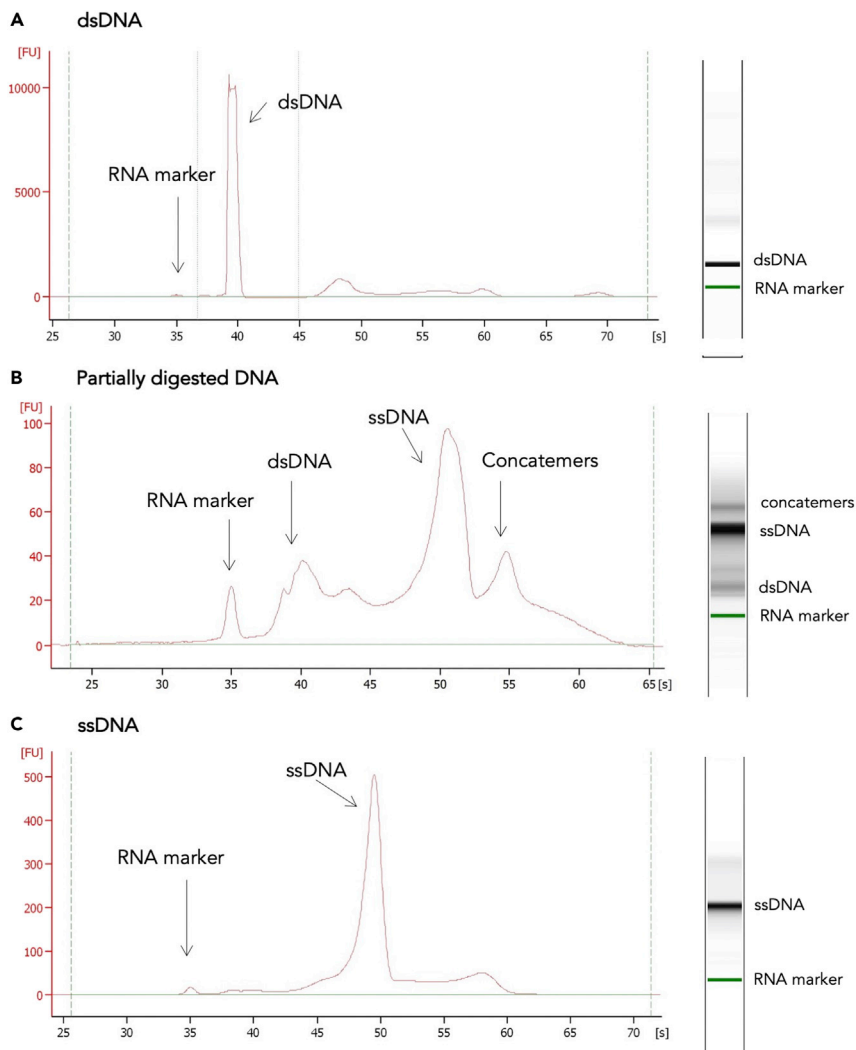
Figure 4. Interpretation of bioanalyzer tracings using the small RNA protocol
(A) Sample containing dsDNA only.
(B) Sample containing ssDNA, dsDNA, and dsDNA partially digested by lambda exonuclease.
(C) Sample containing ssDNA only.

Preparation for sequencing

⏱ Timing: 2 h

This step explains how to prepare libraries for next-generation sequencing (Figure 6).

Note: Libraries may also be prepared using standard Nextera XT indices, if an initial PCR is performed with Illumina sequencing primer FP and Illumina sequencing primer RP. If libraries are prepared using the Illumina forward and reverse primers, the forward and reverse primers will be sequenced and can be trimmed off during analysis. Examples of this type of preparation can be found in the methods section of Gordon et al. (2019) and Wu et al. (2022). Alternatively, Illumina library preparation guides can be adapted to suit your needs.

Round	Target-Switch (3 runs)	Non-Switch (3 runs)	Specificity filter (2 runs)	Enrichment reference (2 runs)	Beads Only (2 runs)
1	Stringency 1:1 Target PP-C	1:1 PP-C	1:1 C, D	1:1 Bradykinin	1:1 No target
2	Stringency 1:1 Target PP-D	1:2 PP-C	1:2 C, D	1:2 Bradykinin	1:2 No target
3	Stringency 1:2 Target PP-C	1:5 PP-C	1:5 C, D	1:5 Bradykinin	1:5 No target
4	Stringency 1:2 Target PP-D	1:10 PP-C	1:10 C, D	1:10 Bradykinin	1:10 No target
5	Stringency 1:5 Target PP-C	1:25 PP-C	1:25 C, D	1:25 Bradykinin	1:25 No target

Figure 5. Stringency gradients and targets for each run across five rounds of selection

50. Normalize the sample to a concentration of 10 ng/uL.
51. For each sequencing sample, set up a PCR reaction using the reagents and cycling conditions below.
    a. PCR Reaction for NGS Preparation.

Reagent	Per 50 μL reaction
DNA sample (10 ng/uL)	10 μL
NGS Forward Primer for Library Preparation (6.25 μM)	2 μL
NGS Reverse Primer for Library Preparation (6.25 μM)	2 μL
Herculase buffer (5×)	10 μL
Herculase enzyme	1 μL
dNTPs (24 mM)	2 μL
NF H2O	23 μL

Note: Two sets of NGS primers are listed (NGS primers for library preparation and NGS primers for sequencing). This PCR reaction uses the library preparation primers to attach the Illumina flow cell primers to the aptamers.

    b. PCR Cycling Conditions for NGS Preparation.

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	5 min	1
Denaturation	98°C	30 s	10 cycles
Annealing	65°C	30 s	
Extension	72°C	30 min	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

52. Perform dsDNA purification of PCR product.
    a. Vortex Mag-Bind TotalPure NGS beads before use.
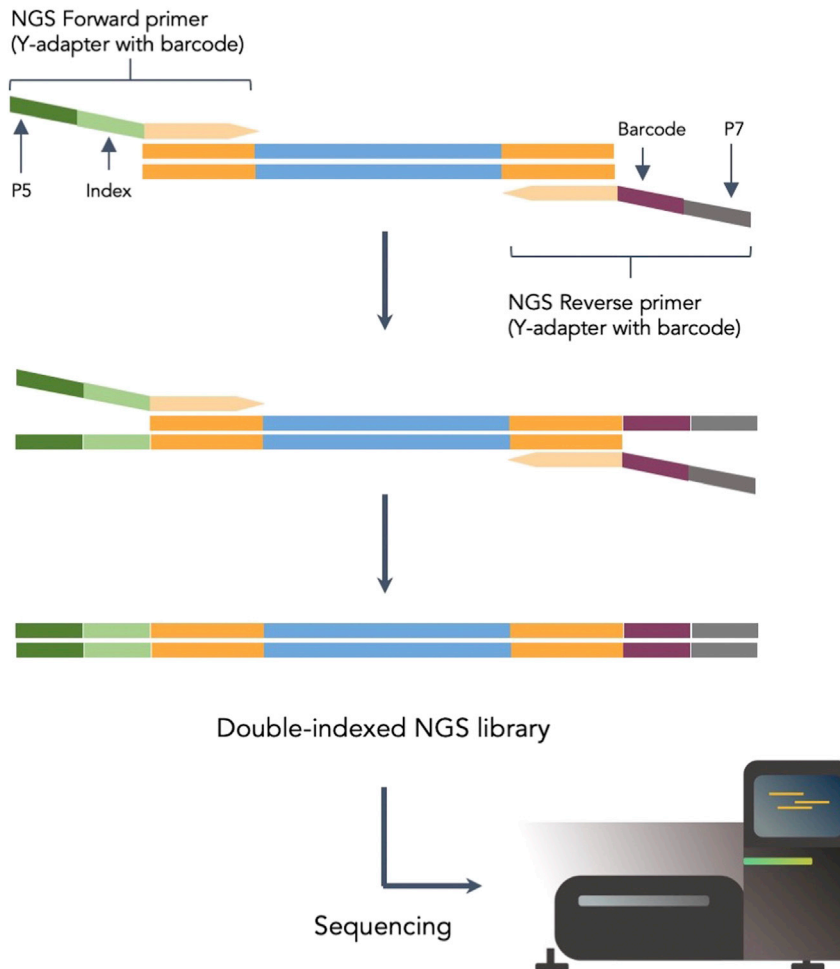
Next Generation Sequencing Library Prep

Figure 6. NGS library preparation
Aptamers were prepped for NGS every round using custom primers.

b. In a lo-bind deep well plate, prepare 30 μL beads for each digestion reaction.
c. To the beads, add 50 μL of 100% ethanol and mix by pipetting.

Note: For a volume of N uL digested product, 0.6*N uL of beads and N*1 μL of 100% ethanol should be used (volume N refers to reaction volume before addition of ethanol in step 47c).

d. Add PCR product (50 μL) to the bead-ethanol mixture.
e. Complete 2 ethanol wash cycles according to the manufacturer's instructions.
f. Elute PCR product in 32 μL of NF dH$_2$O.

53. Quantify the PCR product size using the Tapestation.
    a. Samples should have DNA size of 170–190 bases.
54. Measure the DNA concentration using the qubit dsDNA assay.
55. Pool PCR products together in equimolar concentrations.
56. Measure the DNA concentration of the pooled products using the qubit dsDNA assay.
57. Purify PCR product by selecting DNA size using the Pippin Prep system by Sage Science, according to the manufacturer instructions.

    a. Select for the expected size of the aptamer library plus the NGS adapters.

       i. For our protocol, 81 bases are added to the aptamer library.

       ii. P5 adaptor [29] + custom barcode [6] + filler sequence [12] =37 bases.

       iii. P7 adaptor [24] + custom barcode [8] + filler sequence [12] = 44 bases.

58. Measure the DNA concentration of purified product using the qubit dsDNA assay.

59. Submit 10 μL of >10 nM purified product per SELEX round for next generation sequencing.

    a. Depending on your library preparation, the sequencing format will vary. Specify the lengths of index 1, index 2, read 1 and read 2. Example:R1/R2/i7/i5 = 40/38/8/6.

    b. We performed 400 million reads total. However, depending on the downstream analysis pipeline, you may choose to sequence more shallowly- aptamers can be identified with ~10–20 million reads or less if there is sequence convergence.

    c. If using custom sequencing primers, these will need to provided and spiked into the kit.

Pre-processing of sequencing data

The purpose of this section is to prepare sequencing results for analysis and select final aptamer candidates to be used in a downstream assay (affinity testing, etc).

There are a number of different softwares available for aptamer analysis, or custom code can be written. Published software includes: AptCompare (Shieh et al., 2020), FASTAptamer (Alam et al., 2015), AptaCluster (AptaTools; Hoinka et al., 2014), MPBind (Jiang et al., 2014), APTANI (Caroli et al., 2016) and RNAmotifAnalysis (Ditzler et al., 2013). For a comparison of these softwares, see SI Table 1 of Shieh et al. (2020).

For our work, we wrote custom python scripts to perform frequency counts for each sequence and calculate round over round enrichment. Please contact the authors for details regarding custom scripts.

After importing FASTA sequences, these software will perform some or all of the following: Trim off forward and reverse primers, align the random regions, frequency counting of individual sequences (either in the final round or in each round, depending on the sequencing input), sequence enrichment from round to round, sequence family clustering, motif identification, and secondary structure analysis.

Selecting aptamer candidates

In this step, custom code or published software will be used to choose final aptamer candidates.

Previous studies have shown that high affinity aptamer sequences can be identified in early rounds of SELEX (Schütze et al., 2011). However, round to round enrichment of sequences is also highly correlated with an aptamer that binds to the target of interest (Cho et al., 2010).

In our selection, we filtered our candidates by: (1) high copy number in rounds 3,4,5, (2) high round to round enrichment, (3) appearance in the target switch selections, (4) not appearing in the specificity filter selections (indicating they do not have a preference for the C and D backbones), and (5) not appearing in the enrichment reference or beads only selection (indicating it is not contamination sequence).

Our filters resulted in 26 candidate aptamers. We chose 10 candidates to characterize for binding further.

Characterizing aptamer candidate binding

The purpose of this section is to characterize the binding affinity of the candidate aptamers for the desired target.

Methods to measure binding affinity (Kd) include microscale thermophoresis (MST), biolayer interferometry (BLI), surface plasmon resonance (SPR), and fluorescence assays, where each method has advantages and disadvantages (Plach and Schubert, 2020). We recommend testing aptamer binding in a manner consistent with the ultimate application of the aptamer. In Hong et al. (2022), we performed target-switch SELEX on fluorescently labeled aptamers using biotinylated peptide targets immobilized on streptavidin beads. In their final application for ProtSeq, aptamers would require an oligonucleotide barcode sequence added to the 5′ end. The barcoded aptamers would be screened against peptide-oligonucleotide conjugates immobilized on a chip.

When choosing an assay to characterize aptamers: 1) immobilizing the target on a solid surface versus keeping the target in solution, which may impact the availability of the target to the aptamer (steric hindrance) 2) immobilizing the aptamer to a solid surface versus keeping the aptamer in solution, which may impact how the aptamer secondary and tertiary structure is available to bind to the target and 3) fluorescently labeling the target or binder if either is in solution. Assay availability and throughput may also be a consideration.

To determine Kd, a dilution series of either the target or aptamer should be performed. The steps described below will yield Kd measurements for a single 5′ FAM-labeled aptamer sequence. The steps may be repeated for every aptamer candidate that requires Kd testing.

> *Note:* If performing fluorescence assay, make sure the target and aptamer are incubated to reach equilibrium. For SPR and BLI, Kd can be measured based on and off rates along with steady state equilibriums. Ensure that the reagent that is not being varied is in extreme excess so that saturation is reached.

60. Conjugate 200 pmol peptide target to 100 μL Dynabeads M-280 Streptavidin following the manufacturer's protocol and resuspend to the original concentration in SELEX buffer.
61. Resuspend 5 mg of fluorescein biotin in DMSO.
62. Conjugate 650 pmol fluorescein biotin to 100 μL Dynabeads M-280 Streptavidin following manufacturer's protocol (as a positive control) and resuspend to original concentration in SELEX buffer.
63. Dilute peptide-conjugated beads to 0.03 mg/mL, or 1:320 of original concentration for the binding assay.
64. Aliquot 100 μL of diluted peptide-conjugated beads or fluorescein-conjugated beads into 10 individual wells of a 96 well plate.
65. Place the plate on a magnetic rack for 2 min.
66. Remove the supernatant.
67. To each of 10 wells containing magnetic beads, add 100 μL of 5′ end FAM-labeled aptamers at one of these concentrations: 0 nM (blank control which is SELEX buffer only), 100 nM, 250 nM, 500 nM, 750 nM, 1 mM, 2.5 mM, 5 mM, 10 mM, and 20 mM. Repeat steps 64–67 for replicate measurements of each peptide target. Repeat steps 60–67 for each different peptide target.
68. Seal the 96-well plate. We used a plate-sealer machine but this may also be done manually.
69. Perform aptamer refolding on the sealed plate by following the aptamer refolding steps (step 9).
70. Rotate the plate in the dark at 22°C (room temperature) for 1 h.
71. Spin down the plate in a centrifuge so no product remains on the seal.
72. Remove the seal.
73. Wash the beads three times.
    a. Add 100 μL SELEX buffer to each bead well and pipette up and down to mix.
    b. Place the plate on a magnetic rack and let sit for 2 min.
    c. Remove the supernatant.
74. Resuspend the beads in 100 μL of SELEX buffer.
75. Transfer the beads to a compatible 96-well plate and read out single endpoint fluorescence using a plate reader.

76. Perform computational analysis (fit to Kd Curve) following the outline published by Jarmoskaite et al. (2020).
    a. The Hyperbola formula is from https://github.com/jimrybarski/biofits/blob/master/biofits/function.py.

EXPECTED OUTCOMES

After negative selection, the expected DNA concentrations using the Qubit ssDNA kit ranges from 8-20 ng/uL (step 16). After small-scale amplification, the expected concentration of dsDNA is 4–20 ng/uL using the Qubit dsDNA kit on the plate reader (step 34). After large-scale amplification, the expected concentration of DNA using the Qubit dsDNA kit ranges from 30-90 ng/uL (step 41). After aptamer regeneration (single-stranded generation), the expected DNA concentration using the Qubit ssDNA kit is at or above 30 ng/uL (step 48). Expected aptamer affinities can range from millimolar to nanomolar range depending on the size of the small molecule target (McKeague and DeRosa, 2012) (step 76).

LIMITATIONS

One limitation to the target-switch protocol includes the inability to monitor binding function as selection cycles are being performed.(Wu et al., 2022) As opposed to other methods such as labeled binding pairs or particle display (Wang et al., 2017), sequencing enrichment is the only readout for this method. To combat this limitation, we included an enrichment reference selection for a target known to be aptagenic, and monitored the DNA quantity after every round, to ensure enrichment was occurring.

Another limitation to this protocol is that target switching between only two backbones may not be sufficient to achieve truly backbone-agnostic binding under all conditions. Further testing will need to be performed to determine enrichment behavior when using a larger variety of backbones.

Lastly, several of the parameters described in this protocol (such as PCR conditions) are library dependent and have been optimized for our library. These parameters may require optimization for custom libraries. In our experience, SELEX is highly sensitive to components of the binding apparatus, including plastics (pipette tips, tubes, plates) and streptavidin beads. As such, performing target-switch SELEX on more complicated targets, such as peptide-oligonucleotide conjugates, may prove more challenging.

TROUBLESHOOTING

Problem 1
Difficulty with aptamer amplification (side product formation, low yield, etc.) (steps 30–41).

Potential solution
PCR of random libraries can be challenging and vary with library design. We observed formation of multiple products of different lengths when attempting to amplify our library. Sequencing of these products suggested that the primers were binding to and amplifying from random regions instead of their intended primer sites. Our solution was to increase primer concentrations to encourage the intended amplification reactions. One approach to improve on low yield during Small scale amplification is to pause the thermocycler reaction midway through on an extension step and agitate the beads to prevent them from settling at the bottom of the tube. PCR conditions may be optimized by testing different primer concentrations and annealing temperatures.

Problem 2
Incomplete conversion of dsDNA to ssDNA for aptamer regeneration or high concentration of side products formed after digestion with lambda exonuclease (steps 42–48).

Potential solution
We encountered wide variation in lambda exonuclease activity, reaction timing, and the formation of unknown peaks on bioanalyzer tracings depending on sample volume and concentration. During

initial establishment of an acceptable digestion protocol, we recommend visualizing a small amount of digested product on the bioanalyzer following digestion but before DNA purification. If digestion is incomplete, consider spiking in additional lambda exonuclease and adding time to the reaction as needed (Figure 7).

Problem 3
Lack of aptamer enrichment from round to round (step 49).

Potential solution
The switch protocol specifies the stringencies used in our selections. If enrichment is not observed with the desired target, consider reducing the stringency, performing the target switch every two rounds instead of every other round, or increasing the number of SELEX cycles.

Problem 4
Contamination of a dominating sequence in PCR (from automation, from pipetting, from buildup of environmental DNA) (steps 30–41).

Potential solution
At one point, we observed the same sequences reappearing in multiple PCR reactions. In an attempt to resolve this issue, we cleaned thermocyclers, pipettes, and PCR equipment, designated a specific PCR workstation, and set up PCR reactions in a separate room. These did not alleviate the issue. The contamination issue was ultimately resolved by designing and using a different aptamer library with different primer sequences to prevent undesired amplification.

Problem 5
Low yield or side product formation during NGS preparation PCR (steps 50–59).

Potential solution
PCR products which display significant bands of unexpected nt length may indicate concatemer formation. In step 57, we use a size-based DNA purification step to isolate products of the correct nt length. For low yield products, sequencing depth for detecting enrichment should be deep enough to detect multiple copies of the same sequence. For 1 library over 5 rounds, we recommend a minimum MiSeq run of 12–25 million single reads. We performed 400 million reads on the NextSeq instrument.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Annalisa Pawlosky (apawlosky@google.com).

Materials availability
There are no materials available at this time. Example buffers and sequences of oligos, peptides, and oligo-peptides used in this protocol are listed in the key resources table, though these should be interchanged with materials compatible with the user's subject(s) of study.

Data and code availability
Datasets generated from these experiments are available and located at Mendeley Data: https://data.mendeley.com/datasets/f9hdn5xc3v.
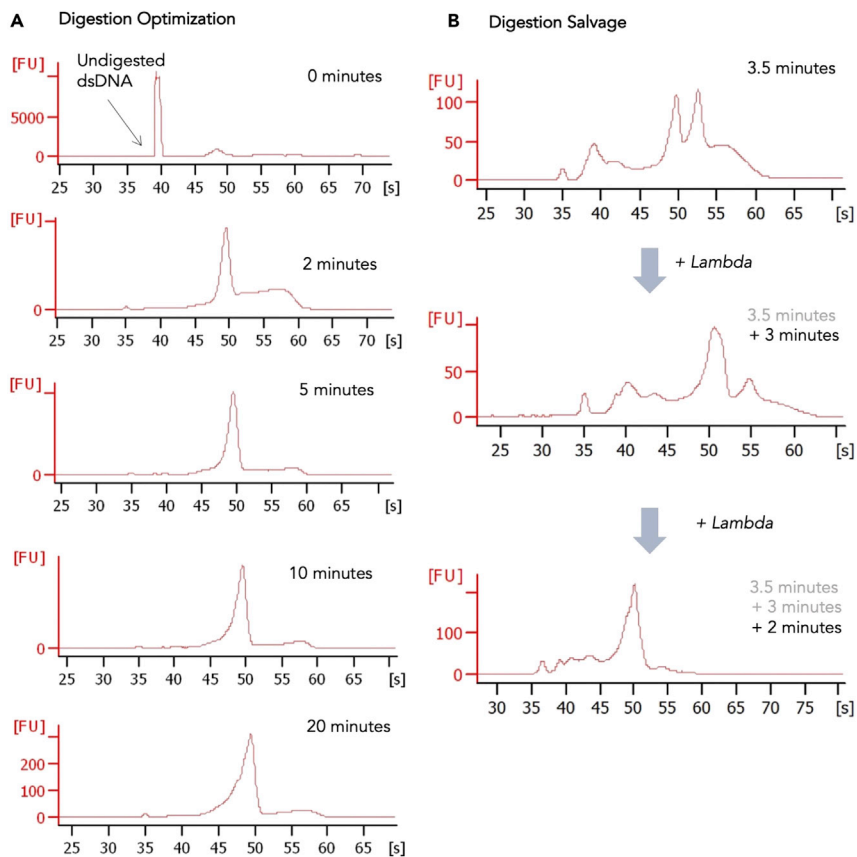
Figure 7. Optimization of lambda digestion
(A) Incubation of the same dsDNA sample at different time periods leads to different degrees of digestion, which can be assessed with a unit test. Over-digested DNA at 20 min has a distinctive triangular peak with a shark-fin shape.
(B) Chronological bioanalyzer runs where additional lambda exonuclease and incubation time was added until digestion completion.

AUTHOR CONTRIBUTIONS

Conceptualization, D.W., A.P.; methodology, Z.W.C., J.M.H., D.W., M.D., M.B., A.P.; validation, D.W., S.S., Z.W.C., J.M.H., A.P.; formal analysis, D.W.; investigation, Z.W.C., A.T., D.W.; writing—original draft, J.M.H., S.S., D.W., A.P.; writing—reviews, J.M.H., D.W., A.P.; visualization, S.S., J.M.H., D.W., A.P.; supervision, A.P.; project administration, A.P.

DECLARATION OF INTERESTS

M.D., M.B., and A.P. are employees and shareholders of Alphabet. Google has filed patent applications related to this work, including PCT/US2020/050574, PCT/US2020/053716, PCT/ US2020/ 040130, PCT/US2020/053715, US20210079398A1, US20210079557A1, US20210102248A1, and WO2021051011A1.

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