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Supplemental information

ProtSeq: Toward high-throughput, single-molecule protein sequencing via amino acid conversion into DNA barcodes

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Supplemental Figures

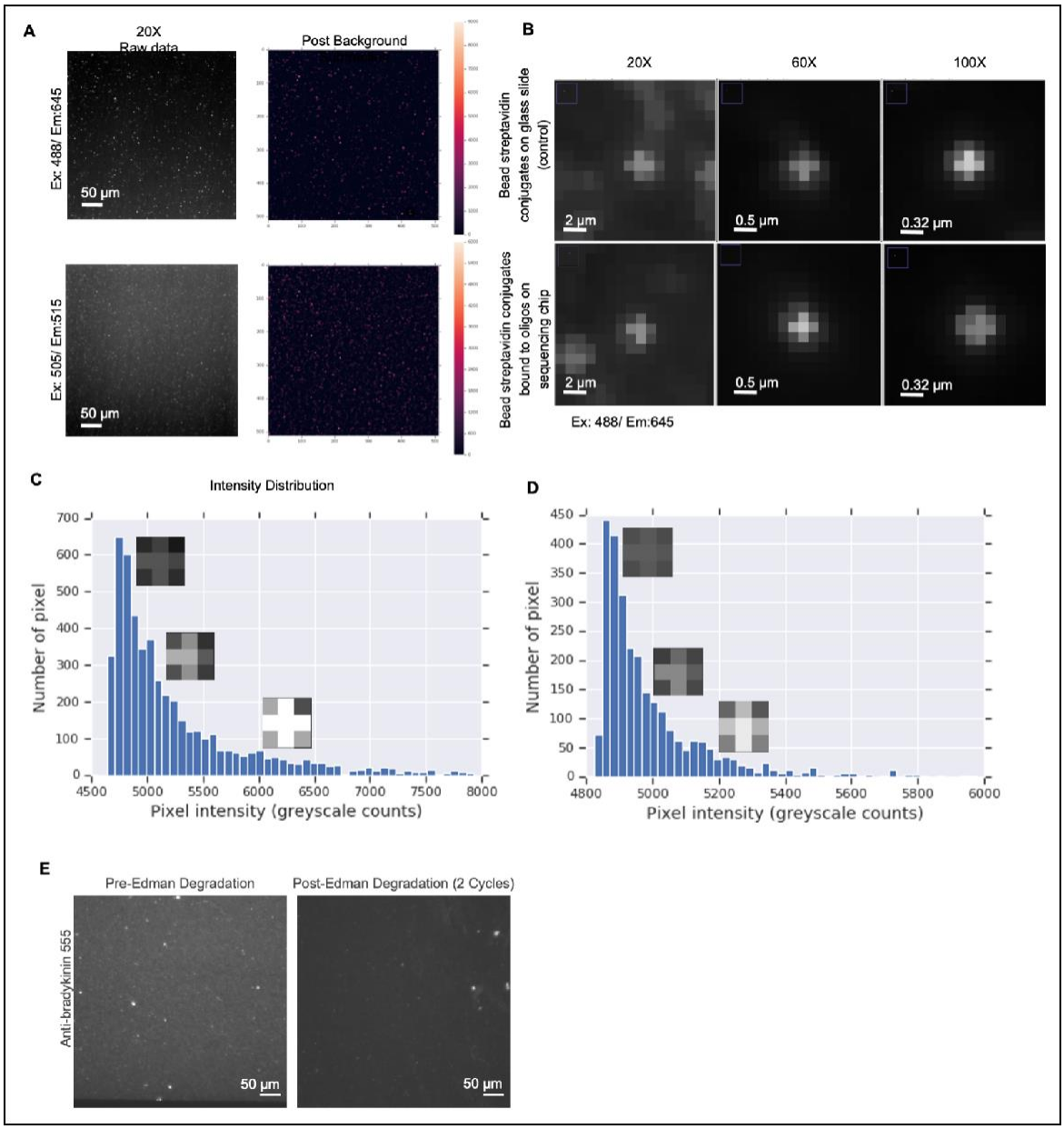

Figure S1 Single Molecule Imaging Quality Control and Edman Degradation, Related to Figure 1

(A) Fluorescent images of fluorescent bead-streptavidin conjugates on a sequencing chip and the intensity measurement after background subtraction using a local threshold. The threshold value is the median intensity for the local neighborhood (30 by 30 pixel) of pixels.

(B) Images of fluorescent bead-streptavidin conjugates on a glass slide (single molecule control) and bound to single oligos on a sequencing chip at 20x, 60x, and 100x magnification. The similarity of sizes of the observed spots between the fluorescent beads on the chip and sequencing chip suggests the observed spots on the sequencing chip are single molecules.

(C) Threshold intensity distributions for all of the fluorescent spots in S1A 488 excitation channel.

(D) Threshold intensity distributions for all of the fluorescent spots in S1A 505 excitation channel.

(E) Depicts fluorescent images of a flow cell with bradykinin attached to its surface prior to Edman degradation and after 2 cycles of Edman degradation. Flow cells were probed with fluorescent bradykinin antibody and imaged through the 555 channel. Diminishing but not absent signal indicates decreased antibody binding, which may suggest peptides are partially degraded while still remaining

attached to the flow cell surface.

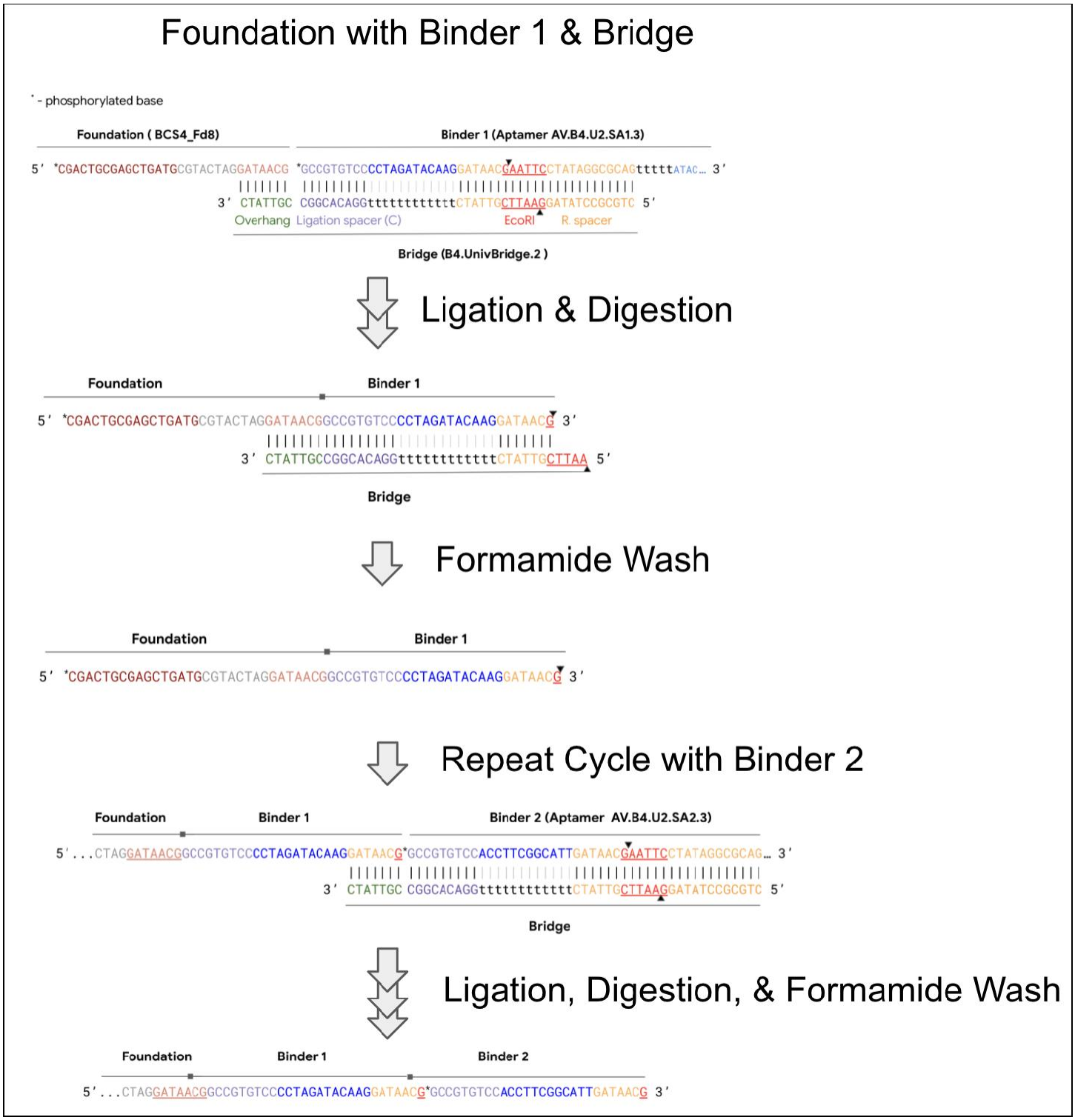

Figure S2 Building the DNA Barcode Chain, Related to Figure 1
This schematic provides the annealing, binding, ligation, and wash-off process for components (foundation, binders, and binder bridges) of the barcode chain on a nucleotide level. At the end of each cycle, a formamide wash is used to reset the barcode chain to a single-stranded oligo that serves as the foundation for the next binding cycle.

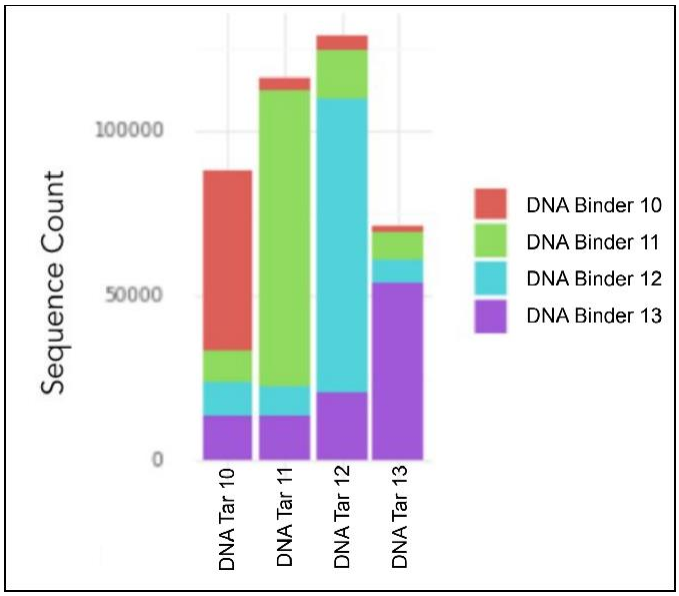

Figure S3 BCS Performance: Binder Consistency for DNA 6 Cycle Analysis, Related to Figure 5
DNA-DNA Binder consistency across cycles of a 6 cycle BCS experiment for 4 summed replicates. In each case the dominant binder is to the expected DNA barcode.

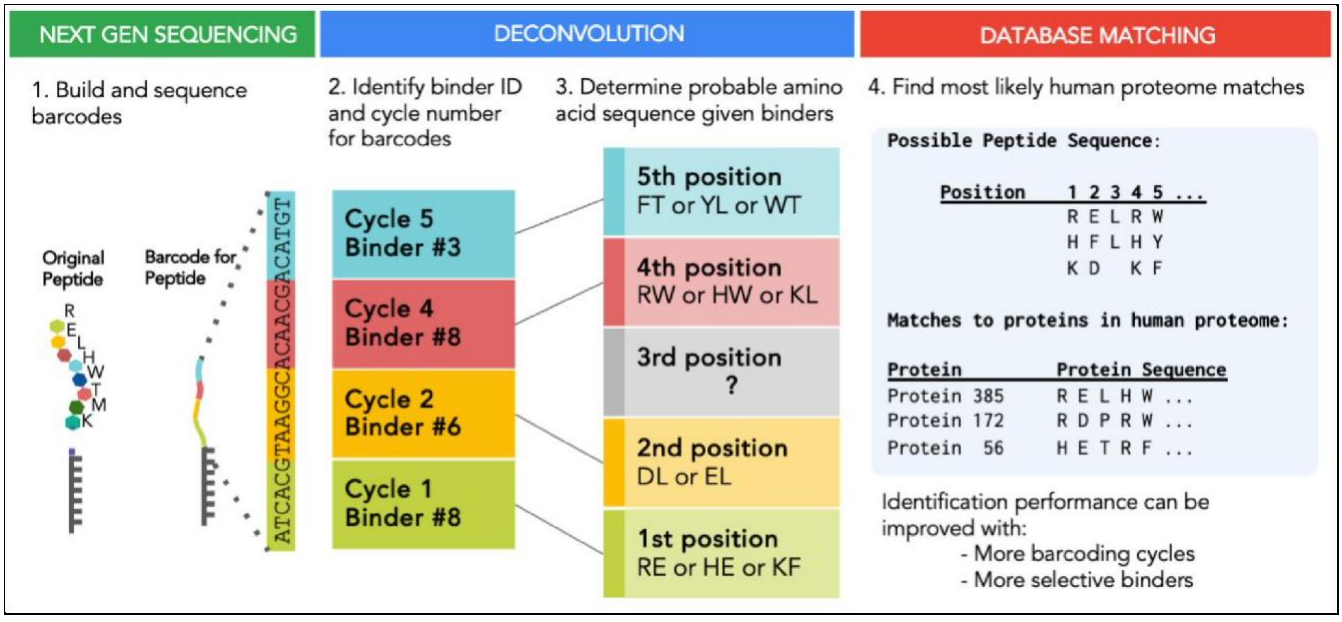

Figure S4 ProtSeq From a Computational Perspective, Related to Graphical Abstract
This schematic depicts the putative workflow for determining the identity of a protein from binder barcodes.
Next-generation sequencing: After all binding cycles are completed, the DNA barcodes assembled are sequenced with next generation sequencing.
Deconvolution: The binder barcodes and corresponding cycle numbers are extracted from the DNA sequence. In this example, cycle 3 had no binder leave a barcode.
Database matching: Given the binder barcode for each cycle number, the binder profiles (experimentally determined in advance) would be used to look up the probable amino acids at each position in the peptide fragment. In this example the profile for binder #8 would indicate that the amino acid is most likely R, H or K. With possible residues for each position, the fragment database (computed in advance) can be queried for the most probable protein matches given the probable amino acids at each position in the peptide fragment.

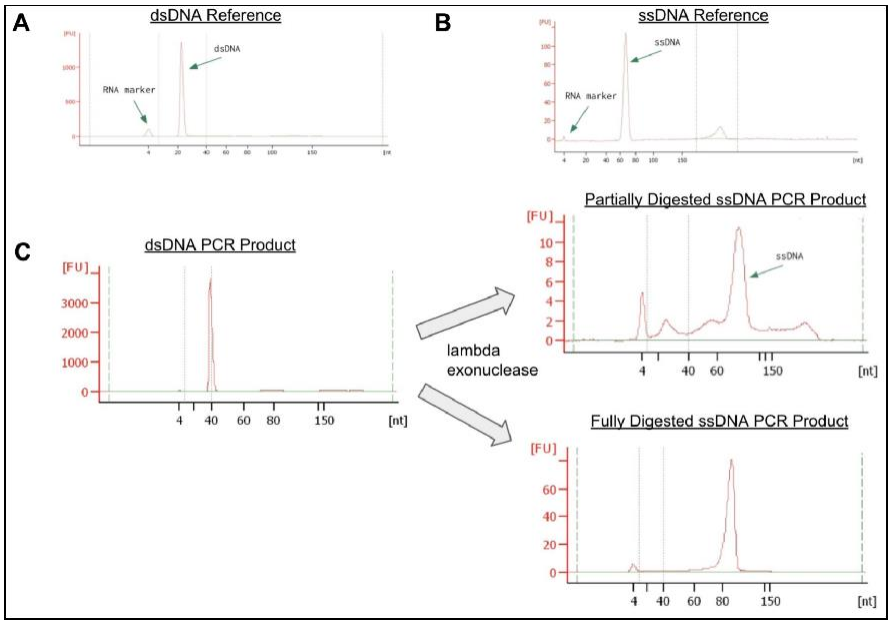

Figure S5 Digestion Quality Control Assay, Related to Figure 8
Bioanalyzer traces for (A) dsDNA, (B) ssDNA, (C) dsDNA PCR product, and ssDNA PCR product after partial and complete digestion with lambda exonuclease. Bioanalyzer traces are used to quality check aptamer input before each SELEX cycle.

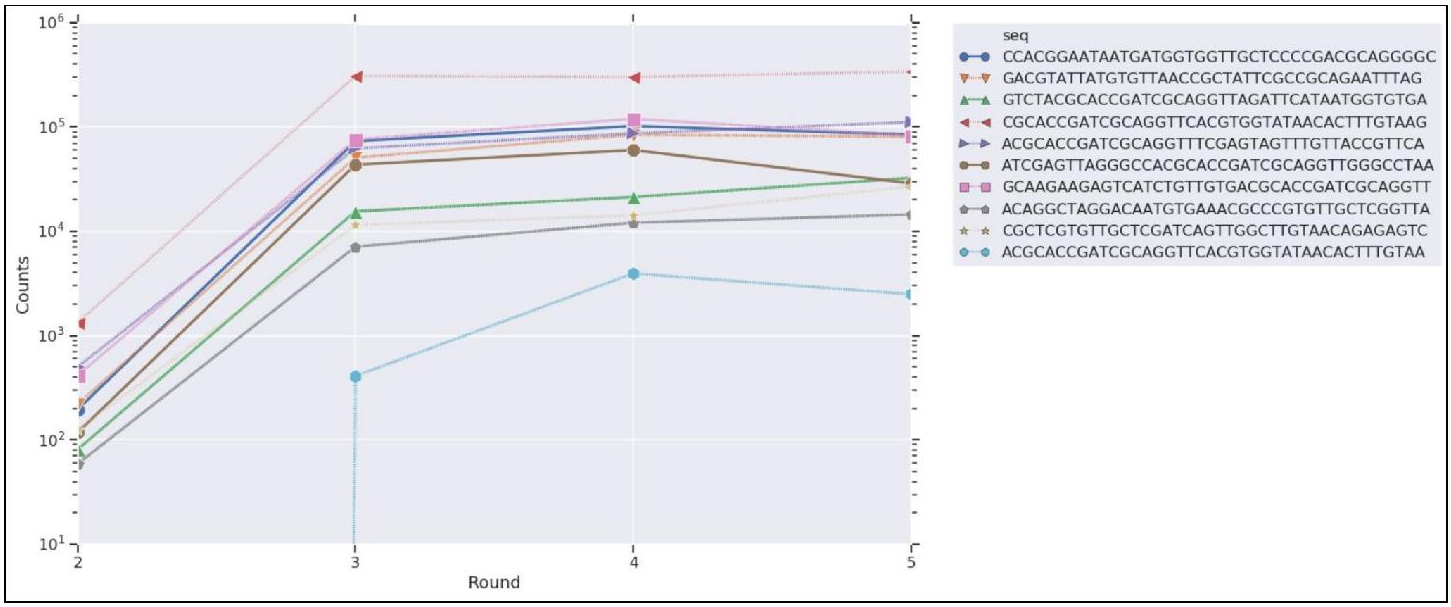

Figure S6 Enrichment Profile from Target-Switch SELEX, Related to Figure 8
Enrichment profile reports the sequencing counts of the top 10 most enriched sequences per round for target PPCD. X axis is the round of SELEX, Y axis is the number of counts seen during sequencing for the 10 sequences. The 10 sequences displayed were chosen because of their calculated enrichment values.

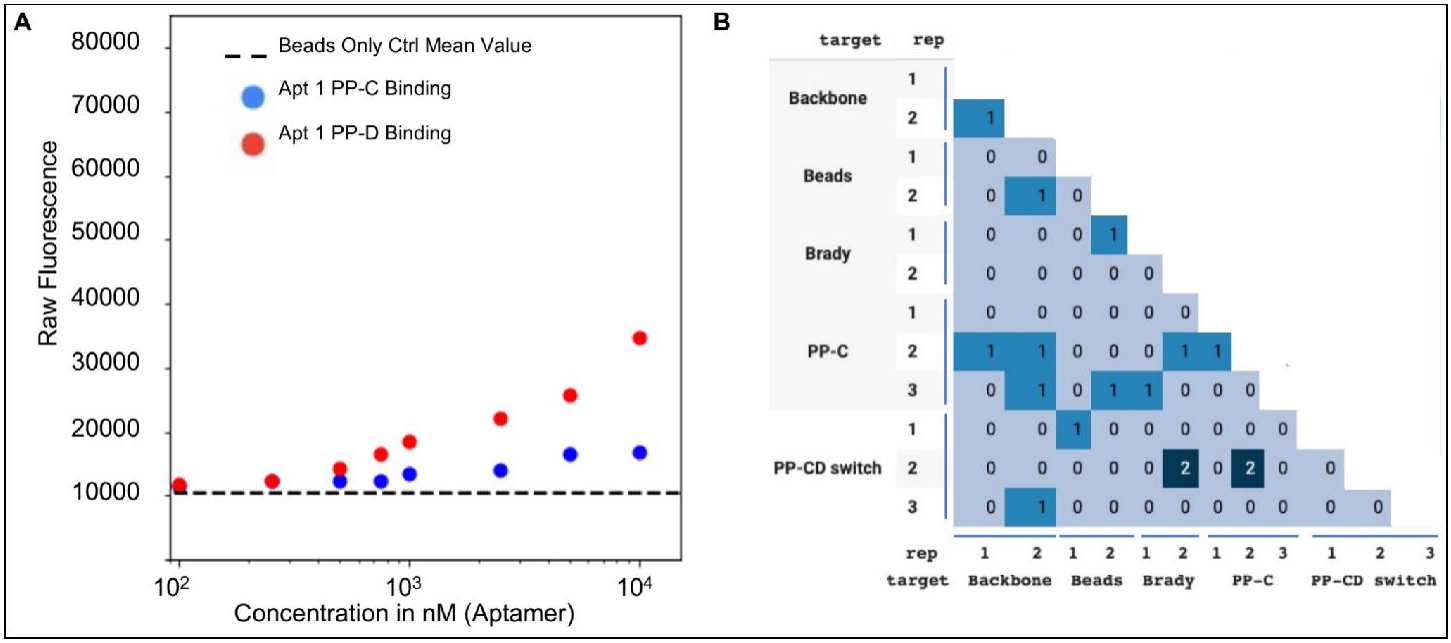

Figure S7 Contamination analysis from Target-Switch SELEX, Related to Figure 8

(A) Binding curves for Apt 1. Apt 1 shows increasing signal against PP-D, much greater than against PP-C. It looks to saturate against PP-C, while not saturating against PP-D, indicative of non-specific binding and influence of backbone switches.

(B) Our experimental design inclusion of replicates allows us to uniquely understand the effect of cross contamination during our selection process. When we compare top aptamers for targets and replicates (Figure 9) we can see that several sequences are observed across multiple samples. This is unlikely considering the size of the initial pool and thus suggests that there is some level of cross contamination between samples. We can observe this and account for it in our analysis when selecting aptamers that bind our target specifically versus targets that are enriched via the selection process via alternative means (generally sticky, selectively amplified during PCR).

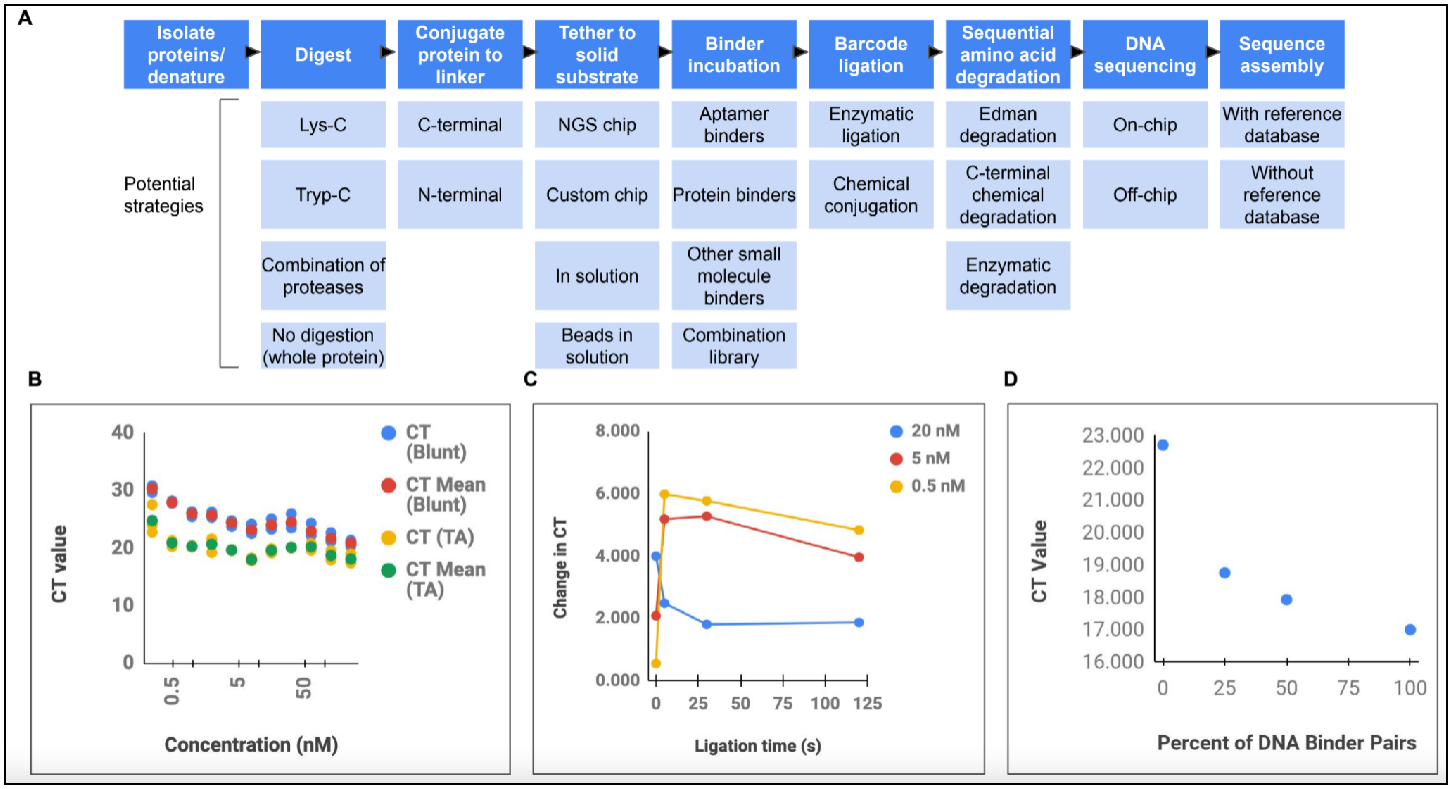

Fig S8 Alternative Approaches for ProtSeq, Related to Figure 1

A) Alternative approaches by step (B-D) illustrates that ligation kinetics can be optimized to favor spatially-associated oligos in solution (oligos with TA overhang vs blunt-ended oligos). Refer to Table S14 for sequences.

B) Effect of substrate concentration on qPCR threshold cycle number (CT) following ligation for blunt-ended dsDNA (red) and TA-overhang dsDNA (green). High CT values indicate lower DNA concentration. Ligation of blunt-ended oligonucleotides is weaker at lower DNA concentrations (slope of red) compared to oligonucleotides bound by a single TA overhang (slope of green).

C) Delta CT at various time points for 0.5 nM and 5 nM, where delta CT represents the difference in CT values between ligated associated and ligated unassociated oligonucleotides. Highest preferential ligation between associated DNA-DNA pairs over unassociated pairs (CT = 6.0) occurred using 0.5 nM DNA concentration at 5 second timemark.

D) CT decreases as the ratio of associated to unassociated pairs mixed together in solution decreases. Graph displays average CT, where each condition was performed in triplicate.

Supplemental Tables

Table S1. BCS Buffer Solutions, Related to Figure 1

Buffer	Formulation (5' - 3')
Hybridization Buffer	0.025% TWEEN20 in 1x PBS
Blocking Buffer	0.025% TWEEN20 in 1x PBS + 10 mg/mL BSA
Chip Blocking Buffer	10 uM of P5 Complementary oligo (5'-TCTCGGTGGTCGCCGTATCATT-3')/P7 Complementary oligo (5'-ATCTCGTATGCCGTCTTCTGCTTG-3') sequences + 10 µM POC Tail blocking sequence (5'-TAGGGAAGAGAAGGACATATGATTATCCACGTGCATCTAAG-3') in 60 µL of Blocking Buffer
Aptamer Incubation Buffer	0.025% TWEEN20 in 1x PBS + 0.1mg/mL BSA

Table S2. Foundation Sequences And DNA Components Used In BCS Experiments, Related To Figure 2

Foundation Name	Sequence
Fd7	/5Phos/CGACTGCGAGCTGATGGCCTTGATGATAACG
Fd8	/5Phos/CGACTGCGAGCTGATGCGTACTAGGATAACG
Fd11*	/5Phos/CGACTGCGAGCTGATGTGTACGCAGATAACG
Fd12	/5Phos/CGACTGCGAGCTGATGCGTTTGCAGATAACG
Fd13	/5Phos/CGACTGCGAGCTGATGTCTTTCCGGATAACG
Fd14	/5Phos/CGACTGCGAGCTGATGTTGCTCACGATAACG
Fd15	/5Phos/CGACTGCGAGCTGATGGAGTTACGGATAACG
Fd16	/5Phos/CGACTGCGAGCTGATGTGATATAGGATAACG
Fd17	/5Phos/CGACTGCGAGCTGATGACCTTAGAGATAACG
Fd18	/5Phos/CGACTGCGAGCTGATGAGTTGCTTGATAACG
Fd19	/5Phos/CGACTGCGAGCTGATGAGGTACCAGATAACG
Fd20	/5Phos/CGACTGCGAGCTGATGCACTTACGGATAACG
Fd21	/5Phos/CGACTGCGAGCTGATGTTGGGCAAGATAACG
Fd22*	/5Phos/CGACTGCGAGCTGATGTTGGGCAAGATAACG
Fd23	/5Phos/CGACTGCGAGCTGATGTTCCACGTGATAACG
Fd24	/5Phos/CGACTGCGAGCTGATGAGGAGCAAGATAACG
Fd25	/5Phos/CGACTGCGAGCTGATGTTCCCTTCGATAACG
Fd26	/5Phos/CGACTGCGAGCTGATGTCTGAGGTGATAACG
Fd27	/5Phos/CGACTGCGAGCTGATGTCATGTGGGATAACG
Fd28	/5Phos/CGACTGCGAGCTGATGCACCAAACGATAACG
Fd29	/5Phos/CGACTGCGAGCTGATGATTGTCCCGATAACG
Fd31	/5Phos/CGACTGCGAGCTGATGTGGCATCTGATAACG
Fd32*	/5Phos/CGACTGCGAGCTGATGCTTCTAGCGATAACG
Fd43*	/5Phos/CGACTGCGAGCTGATGCAGCACATGATAACG
Forward Cololinker (FC)	CATCAGCTCGCAGTCGATCTCGTATGCCGTCTTCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCAGCCACCGCCAACCATCC
Reverse Cololinker (RC)	ATTATCCACGTGCATCTAAGATCTCGTATGCCGTCTTCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGATGGTTGGCGGTGGCTGG
Bridge	CTGCGCCTATAGGAATTCGTTATC/i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd//i5NitInd/GGACACGGCCGTTATC

*Four sequences which demonstrated greatest consistency in target deposition and rate of binder barcode capture
Fd=Foundation
i5NitInd=5-Nitroindole

Table S3. DNA Cololinkers Used In Fluorescence Colocalization Experiment, Related To Figure 3

Name	Sequence (5' - 3')
Forward Cololinker (FC)	/Atto 488/ CATCAGCTCGCAGTCGATCTCGTATGCCGTCTTCTGTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCAGCCACCGCCAACCATCC
Reverse Cololinker (RC)	/Atto 647/ ATTATCCACGTGCATCTAAGATCTCGTATGCCGTCTTCTGTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGATGGTTGGCGGTGGCTGG

Table S4. DNA Binders and Targets Used in 5ingle and Multiple Cycle DNA Binder-DNA Target Experiments, Related to Figures 4 and 5

Name	**5equence**
DNA Binder 4.1	ATACATGGAATCCTAT
DNA Binder 4.2	ATACATGGAATCCTAT
DNA Binder 6	TCAGGTTAGTACTTCAT
DNA Binder 9	CTTGACTAGTACATGACCACTTGA
DNA Binder 10	/5Phos/TCTTGGTAGATAACGAATTCGTATAGGCGCAGttttttATACATGGAATCCTAT
DNA Binder 11	/5Phos/GATACTCAGATAACGAATTCGTATAGGCGCAGttttttTCAGGTTAGTACTTCAT
DNA Binder 12	/5Phos/TGAACAGCGATAACGAATTCGTATAGGCGCAGttttttCTTGACTAGTACATGAC CACTTGA
DNA Binder 13	/5Phos/GTTACGAAGATAACGAATTCGTATAGGCGCAGttttttTGCTGGTATGGCTTAAA TCC
Binder to Thrombin	/5Phos/GCCGTGTCCGCGTGTTGGATCGATAACGAATTCCTATAGGCGCAGAGTCC GTGGTAGGGCAGGTTGGGGTGACT
DNA Target 6	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttTCAAGTGGTCATGTACTAGTCAAG
DNA Target 9	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttATGAAGTACTAACCTGA
DNA Target 10	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttATAGGATTCC
DNA Target 11	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttATGAAGTACTAACCTGA
DNA Target 12	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttTCAAGTGGTCATGTACTAGTCAAG
DNA Target 13	/5Phos/cttagatgcacgtggataatttttttttttttttttttttttttttttGGATTTAAGCCATACCAGCA

Table S5. DNA target-DNA binder counts, Related to Figure 4

Target	5Phos	5Phos	5Phos	5Phos	Empty	DT 6	DT 6	DT 6	DT 6	DT 9	DT 9	DT 9	DT 9
Target Foundation	Fd11	Fd12	Fd7	Fd8	Empty	Fd17	Fd18	Fd19	Fd20	Fd13	Fd14	Fd15	Fd16
DNA Binder 4.1 (Neg. Ctr)	0	0	0	0	0	0	0	0	1	0	1	0	1
DNA Binder 4.2 (Neg. Ctr)	0	0	2	0	0	1	0	1	0	1	1	0	0
DNA Binder 6	117	84	935	76	0	19140	11384	29377	18751	83	100	54	21
DNA Binder 9	205	152	1768	191	0	277	135	375	252	28256	62962	11395	6234

DT=DNA Target

Table S6. 5equencing Counts for DNA Target-DNA Binder 6 Cycle Experiment, Related to Figure 5

Binder	Cycle	DNA Target 1	DNA Target 11	DNA Target 12	DNA Target 13
DNA Binder 10	1	6539	2096	1007	885
DNA Binder 10	2	8835	116	345	107
DNA Binder 10	3	11316	155	298	179
DNA Binder 10	4	11440	317	595	242
DNA Binder 10	5	10406	235	1218	177
DNA Binder 10	6	5829	325	1430	257
DNA Binder 11	1	4885	16295	5540	5520
DNA Binder 11	2	263	12517	314	113
DNA Binder 11	3	424	16513	495	189
DNA Binder 11	4	614	13933	1219	400
DNA Binder 11	5	1424	15798	3137	839
DNA Binder 11	6	2211	14751	4209	1333
DNA Binder 12	1	2982	2824	17822	1786
DNA Binder 12	2	449	217	19332	178
DNA Binder 12	3	1554	1745	5444	1227
DNA Binder 12	4	1662	1265	18408	1094
DNA Binder 12	5	2034	2167	15733	1626
DNA Binder 12	6	1399	946	12292	675
DNA Binder 13	1	6081	6385	6884	13344
DNA Binder 13	2	157	67	275	6867
DNA Binder 13	3	51	11	126	702
DNA Binder 13	4	1616	1547	2899	12098
DNA Binder 13	5	3173	3500	5655	14169
DNA Binder 13	6	2835	2008	4672	6179

Table S7. Target Sequences and Foundations Used for Spot-Tag validation experiment, Related toFigure 6

Target Type	Target Name	Sequence	Foundations Used
Spot-Tag* (peptide target)	Spot-Tag.O1	(N-terminus)-PDRVRAVSHWSSGGG-Cys (C-terminus)-3'ATCCCTTCTCTTCCTGTAT ACTAATAGGTGCACGTAGATTC/5Phos/	Fd31, Fd19, Fd20, Fd27, Fd28, Fd29
Bradykinin* (peptide target control for non-specific binding)	Brady.O1	(N-terminus)-RPPGFSPFR-Cys (C-terminus)-3'ATCCCTTCTCTTCCTGTAT ACTAATAGGTGCACGTAGATTC/5Phos/	Fd12, Fd13, Fd14
DNA** (null control)	CLR.Null.Block	CTTAGATGCACGTGGATAAT	Fd24, Fd25, Fd26
DNA** (null control)	5'Phos.O1	/5Phos/CTTAGATGCACGTGGATA	Fd7, Fd8, Fd11
DNA** (positive control)	DNA Target 6.O1	/5Phos/CTTAGATGCACGTGGATAATCATA TGTCCTTCTCTTCCCTAATGAAGTACTAA CCTGA	Fd21, Fd22, Fd23
DNA** (positive control)	DNA Target 4.O1	/5Phos/CTTAGATGCACGTGGATAATCATA TGTCCTTCTCTTCCCTAATAGGATTCC	Fd15, Fd16, Fd17

*The C-terminal of the peptide targets is directly conjugated to the 3' end of one DNA tail via a cysteine
**Binding sequences and DNA tails of DNA targets are continuous oligos rather than conjugated through another chemical conjugation method.

Table S8. 5equencing Counts for Spot-Tag Binder-Target Experiment, Related to Figure 7

Target	Target Foundation	DNA Binder 4.2	DNA Binder 6	DNA Binder 9 (Neg. Ctr)	Binder Spot-Tag
5Phos	Fd11	180	970	1	288
5Phos	Fd7	679	1269	4	611
5Phos	Fd8	91	523	0	175
Brady	Fd12	24	116	0	42
Brady	Fd13	516	1611	4	1663
Brady	Fd14	222	1113	1	603
CLR	Fd24	224	1061	2	405
CLR	Fd25	233	930	3	558
CLR	Fd26	81	361	0	92
Empty	Empty	0	0	0	0
DNA Target 4	Fd15	7201	397	0	207
DNA Target 4	Fd16	8399	447	2	107
DNA Target 4	Fd17	11355	441	1	188
DNA Target 6	Fd21	83	28641	3	129
DNA Target 6	Fd22	82	28871	1	148
DNA Target 6	Fd23	50	21007	0	96
Spot-Tag	Fd19	132	485	2	7013
Spot-Tag	Fd20	138	406	0	9825
Spot-Tag	Fd27	114	453	38	3461
Spot-Tag	Fd28	124	455	1	4976
Spot-Tag	Fd29	161	458	1	10803
Spot-Tag	Fd31	130	428	1	5256

Table S9. Thrombin Binder Sequencing Counts

Target	5Phos	5Phos	5Phos	CLR	CLR	CLR	DT4	DT4	DT4	Thrombin	Thrombin	Thrombin
Target Foundation	Fd11	Fd7	Fd8	Fd12	Fd13	Fd14	Fd15	Fd16	Fd17	Fd22	Fd27	Fd31
DNA Binder 4.2	1412	2082	856	369	1797	1947	47936	52275	48420	2239	1724	1747
DNA Binder 9 (Neg. Ctr)	25	70	25	10	70	44	8	14	24	148	1470	72
Binder to Thrombin	73	99	30	13	132	132	36	70	40	2203	1500	1487

DT=DNA Target

Table S10. Dipeptide switch design of *A-D* groups and backbones (+, ,- : hydrophobicity), Related to Figure 8

i						Backbone (A-D)
A group	A+	W0	D-	C+	R-	ADRWADRK
B group	L+	P-	S0	Q-	M+	MSQPLQPK
C group	I+	F+	E-	N-	H-	NHFENEIK
D group	V+	Y-	G0	T0	K-	TKYVGTGK

Table S11. SELEX Aptamer, Peptide Target, and NGS 5equences, Related to Figures 8 and 9

Name	Sequence
Aptamer Screening Library (Forward Primer FP - N40 - Reverse Primer RP)	TTGACTAGTACATGACCACTTGA-N40-TTCTGTCGTCCAGTCTGATGTG
SELEX Peptide PP-C	PPNHFENEIK bt
SELEX Peptide PP-D	PPTKYVGTGK bt
SELEX Peptide Bradykinin	RPPGFSPFRK bt
NGS Forward Sequencing Primer*	AATGATACGGCGACCACCGAGATCTACAC-**XXXXXX-**GCATGCAGCCGGTTGACTAGTACATGACCACTTGA
NGS Reverse Sequencing Primer*	CAAGCAGAAGACGGCATACGAGAT-**XXXXXXXX**-GTGCGTGCGTGCTTCTGTCGTCCAGTCTGATGTG
NGS N40 Library preparation Forward Primer	5'-CAAGCAGAAGACGGCATACGAGATNNNNNNNN-(Forward primer)-3
NGS N40 Library preparation Reverse Reverse	5'-AATGATACGGCGACCACCGAGATCTACACNNNNNN-(Reverse primer)-3'
Aptamer 1	FP - GACGGTACAGCTTAGTGAATTGCCCCCCGACGCAGGGGTT - RP
Aptamer 2	FP - TTTGCCGCTGTCTGACGCAAGACCACATCAACTTTATTTC - RP
Aptamer 3	FP - CGCTCGTGTTGCTCGATCAAGGGTCTGTGCGTCTAGCTGG - RP
Aptamer 4	FP - ACACCCAGACACCGCTGTCCGACGCAGGACTGACTGGGGC - RP
Aptamer 5	FP - AACGACCGGTTAGACTGTGACCGCTTATCGCCGCAGATAT - RP
Aptamer 6	FP - CGCATCCGGCGCAGGATTCAAGCGGGATTGTAAGGTAAGA - RP
Aptamer 7	FP - GACATTGCCCTTCGCCGCAGAAGTGATGAAAGGGTTTGTG - RP
Aptamer 8	FP - CGCTCGTGTTGCTCGATCAAGTGGACTAGAATTTGCTTCT - RP
Aptamer 9	FP - CCACGGAATAATGATGGTGGTTGCTCCCCGACGCAGGGGC - RP
Aptamer 10	FP - ACGCACCGATCGCAGGTTCACGTGGTATAACACTTTGTAA - RP

*Unique barcodes for each individual barcode listed in Table S12.

bt = biotinylated

NGS = Next Generation Sequencing

Table S12. Individual NGS Barcodes (XXXXXX) from Table S11, Related to Figures 8 and 9

Forward Primer	Barcode	Reverse Primer	Barcode
Fw1 NGS	ATCACG	Rv1 NGS	TCGCCTTA
Fw2 NGS	TAAGGC	Rv2 NGS	CTAGTACG
Fw3 NGS	ACATGT	Rv3 NGS	TTCTGCCT
Fw4 NGS	GATCAG	OMB63 Rv4 NGS	GCTCAGGA
Fw5 NGS	CGATCT	Rv5 NGS	AGGAGTCC
Fw6 NGS	TTAGGC	Rv6 NGS	CATGCCTA
Fw7 NGS	GCGAAC	Rv7 NGS	GTAGAGAG
Fw8 NGS	GTGCCT	Rv8 NGS	CCTCTCTG
Fw9 NGS	AACTCT	Rv9 NGS	AGCGTAGC
Fw10 NGS	TTGAGA	Rv10 NGS	CAGCCTCG
		Rv11 NGS	TGCCTCTT
		Rv12 NGS	TCCTCTAC

Table S13. Target-Switch SELEX Stringencies by Round and Target Type, Related to Figure 8

Round	"Non-Switch" Stringency	"Switch" Stringency	"Switch" Backbone
1	1:1	1:1	C
2	1:2	1:1	D
3	1:5	1:2	C
4	1:10	1:2	D
5	1:25	1:5	C

Targets following the "non-switch" stringency gradient include the negative control, enrichment reference, specificity filter, and PP-C. The only target following the "switch" stringency gradient is the PP-CD switch target.

Table S14. DNA and Peptide 5equences for Alternative Ligation Methods, Related to Figure 1 andFigure S8

Name	Sequence (5' to 3', N-terminus to C-terminus)
50A	AACATGACTTTACAACCCAAGAGCTTTGTAGGGAAGAGAAGGACATATGATACG AATTCtcggtcgcagatcctacgaaGCCAATggT
50A partial complement	/5Phos/CCATTGGCTTCGTAGGATCTGCGACCGAGAATTCGTATCATATGTCCTT CTCTTCCCTA
50B	CAAAGCTCTTGGGTTGTAAAGTCATGTTTCAAGTGGTCATGTACTAGTCAAacg GCCAATcA
50B partial complement	/5Phos/GATTGGCCGTTTGACTAGTACATGACCACTTGA
0A	TAGGGAAGAGAAGGACATATGATACAAGCTTtcggtcgcagatcctacgaaCAGATCggT
0A partial complement	/5Phos/CCGATCTGTTCGTAGGATCTGCGACCGAAAGCTTGTATCATATGTCCTT CTCTTCCCTA
0B	TCAAGTGGTCATGTACTAGTCAAacgCAGATCcA
0B partial complement	/5Phos/GGATCTGCGTTTGACTAGTACATGACCACTTGA
Trilink forward	TAGGGAAGAGAAGGACATATGAT
Trilink reverse	TCAAGTGGTCATGTACTAGTCAA
NC1	KQNTSQNTSC
NC2	KQNTYQNTSC