Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Searching for avidity by chemical ligation of combinatorially self-assembled DNA-encoded ligand libraries

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SEQ ID #	Name/ Description	Sequence (5' to 3')	Spacer	Total Length	Other details/ remarks
1	TBA27-T0-c	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*AGACTGGTC CGCAAGTTC*aaatcca*ACCTACCTAc	T0	62	3'-propargyl dC
2	t-T0-G15D	tAGGTAGGT*ccctata*GAACTTGCGGACCAGTCT*GGTTGG TGTGGTTGG*	T0	49	5'-azide-T
3	t-T6-G15D	tAGGTAGGT*ctagtac*GAACTTGCGGACCAGTCTTTTTTT *GGTTGGTGTGGTTGG*	T6	55	5'-azide-T
4	t-T12-G15D	tAGGTAGGT*accttcg*GAACTTGCGGACCAGTCTTTTTTTT TTTTT*GGTTGGTGTGGTTGG*	T12	61	5'-azide-T
5	TBA27s-T0-c	*GTCCGTCCTACCGCAGCTTCCGTTGAC*AGACTGGTCC GCAAGTTC*aggtctc*ACCTACCTAc	T0	62	3'-propargyl dC
6	t-T12-G15Ds	tAGGTAGGT*tacacgt*GAACTTGCGGACCAGTCTTTTTTTT TTTTT*GTGTGTGTGTGTGTG*	T12	61	5'-azide-T
7 (1 + 2)	TBA27-ct-T0-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*AGACTGGTC CGCAAGTTC*aaatcca*ACCTACCTActAGGTAGGT*ccctata* GAACTTGCGGACCAGTCT*GGTTGGTGTGGTTGG*	T0	111	Clicked
8 (1 + 3)	TBA27-ct-T6-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*AGACTGGTC CGCAAGTTC*aaatcca*ACCTACCTActAGGTAGGT*ctagtac* GAACTTGCGGACCAGTCTTTTTTT*GGTTGGTGTGGTTG G*	T6	117	Clicked
9 (1 + 4)	TBA27-ct-T12-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*AGACTGGTC CGCAAGTTC*aaatcca*ACCTACCTActAGGTAGGT*accttcg* GAACTTGCGGACCAGTCTTTTTTTTTTTT*GGTTGGTG TGGTTGG*	T12	123	Clicked
10 (1 + 6)	TBA27-ct-T12-G15Ds	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*AGACTGGTC CGCAAGTTC*aaatcca*ACCTACCTActAGGTAGGT*tacacgt* GAACTTGCGGACCAGTCTTTTTTTTTTTT*GTGTGTGT GTGTGTG*	T12	123	Clicked
11 (5 + 2)	TBA27s-ct-T0-G15D	*GTCCGTCCTACCGCAGCTTCCGTTGAC*AGACTGGTCC GCAAGTTC*aggtctc*ACCTACCTActAGGTAGGT*ccctata*GA ACTTGCGGACCAGTCT*GGTTGGTGTGGTTGG*	T0	111	Clicked
12 (5 + 3)	TBA27s-ct-T6-G15D	*GTCCGTCCTACCGCAGCTTCCGTTGAC*AGACTGGTCC GCAAGTTC*aggtctc*ACCTACCTActAGGTAGGT*ctagtac*GA ACTTGCGGACCAGTCTTTTTTT*GGTTGGTGTGGTTGG*	T6	117	Clicked
13 (5 + 4)	TBA27s-ct-T12-G15D	*GTCCGTCCTACCGCAGCTTCCGTTGAC*AGACTGGTCC GCAAGTTCaggtctcACCTACCTActAGGTAGGT*accttcg*GA ACTTGCGGACCAGTCTTTTTTTTTTTT*GGTTGGTGTG GTTGG*	T12	123	Clicked
14 (5 + 6)	TBA27s-ct-T12-G15Ds	*GTCCGTCCTACCGCAGCTTCCGTTGAC*AGACTGGTCC GCAAGTTC*aggtctc*ACCTACCTActAGGTAGGT*tacacgt*GA ACTTGCGGACCAGTCTTTTTTTTTTTT*GTGTGTGTGT GTGTG*	T12	123	Clicked
15	G15D	*GGTTGGTGTGGTTGG*	NA	15	-
16	TBA27	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*	NA	27	-
17	G15D-T0-TBA27	*GGTTGGTGTGGTTGGGTCCGTGGTAGGGCAGGTTGG GGTGAC*	T0	43	directly synthesized

18	G15D-T6-TBA27	*GGTTGGTGTGGTTGG*TTTTTT*GTCCGTGGTAGGGCAG GTTGGGGTGAC*	T6	49	directly synthesized
19	G15D-T12-TBA27	*GGTTGGTGTGGTTGG*TTTTTTTTTTTT*GTCCGTGGTAG GGCAGGTTGGGGTGAC*	T12	55	directly synthesized
20	TBA27-T0-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGACTGGTTGGTG TGGTTGG*	T0	43	directly synthesized
21	TBA27-T6-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*TTTTTT*GGTT GGTGTGGTTGG*	T6	49	directly synthesized
22	TBA27-T12-G15D	*GTCCGTGGTAGGGCAGGTTGGGGTGAC*TTTTTTTTTTT T*GGTTGGTGTGGTTGG*	T12	55	directly synthesized
23	Reference	AGGGATATCACTCAGCATAATGTCGTAC	NA	28	Reference

Table S1: Synthesized DNA-Aptamer conjugates: *Aptamer sequences are in italics. Underlined regions become double helical after formation of the SABA complexes. Small letter italics indicate the identifier sequence (barcode) of each arm, small letters in a box indicate the position of the triazole linkage or the corresponding terminal nucleotide bearing the reactive groups. Small letter "s" indicates the original aptamer sequence was scrambled. Sequences ID #1-6 are the individual left and right "arms" covalently attached to an aptamer motif or a scrambled reference sequence. Sequences ID #7-14 are the SABA complexes formed after hybridisation and "Click"-reaction, presenting two individual potential binding sequences. Sequence ID #15 and 16 are the monomeric aptamer sequences, which are, together with #23, used as references in the blood clotting assay. Sequences ID #17-22 are directly synthesized, replacing the dsDNA scaffold with an oligo-thymidine linker and were also tested in the blood-clotting assay.*

1. Oligonucleotide Synthesis:

DNA sequences SEQ ID #1 and # 5 (Table S1) were synthesized by standard solid-phase phosphoramidite chemistry using 5'-Dimethoxytrityl-3'-propargyl-5-methyl-2'-deoxycytosine-N-succinyl-long chain alkylamino-CPG, from GlenResearch. The 5'-azide group for SEQ ID # 2, 3, 4 and 6 were introduced in a 2-stage process[2]. First 5'-iodo thymidine phosphoramidite was directly added during solid-phase synthesis. Then the resulting 5'-iodo oligonucleotides were reacted with sodium azide to complete the transformation. Cleavage of the oligonucleotide from this support requires 2 hr at room temperature with ammonium hydroxide and complete deprotection requires a further 5 hr at 55°C to remove the nucleobase protecting groups.

2. Synthesis and Analysis of Self-Assembled Bivalent Aptamers (SABAs):

2.i. Synthesis and Purification of SABAs:

CuAAC Reaction Protocol

Prior to chemical ligation, pairs of azide (e.g SEQ ID #2) and alkyne (e.g. SEQ ID #1) oligonucleotides (100.0 nmol of each) in 0.2 M NaCl (100.0 μL) were annealed by heating at 90°C for 5 min and cooling slowly to room temperature. A solution of Cu^{I} catalyst was prepared by adding the *tris*-hydroxypropyltriazole ligand[1] (35.0 μmol) to sodium ascorbate (50.0 μmol in 0.2 M NaCl, 100.0 μL) followed by the addition of $CuSO_4x5H_2O$ (5.0 μmol in 0.2 M NaCl, 50.0 μL) under argon. The Cu^{I} solution was added to the annealed oligonucleotide mixture and kept at room temperature for 2 hr under argon. Reagents were removed by NAP-25 gel-filtration (GE Healthcare) and the ligated product was purified by anion-exchange HPLC as described previously[2].

The self-assembled bivalent aptamer complexes SEQ ID #7-14 were analysed by 10% PAGE gel electrophoresis and purified by anion-exchange HPLC on a Gilson HPLC system using a Resource Q anion-exchange column (6 mL volume, GE Healthcare). The HPLC system was controlled by Gilson 7.12 software, and the following protocol was used: run time, 16 min; flow rate, 5 mL per min; binary system. Gradient (time in mins (% buffer B)): 0 (0), 3 (0), 4 (40), 9.5 (82), 10 (100), 12 (100), 13 (0), 15.5 (0), 16 (0). Elution buffers: (A) 0.01 M aqueous NaOH, 0.05 M aqueous NaCl, pH 12.0; (B) 0.01 M aqueous NaOH, 1 M aqueous NaCl, pH 12.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification oligonucleotides were desalted using a NAP-25 followed by a NAP-10 Sephadex column (GE Healthcare).

Yields of the purified products after HPLC were 45-57%.

Figure S1: Denaturing PAGE (10 % polyacrylamide/7 M urea gel) of ligated SABA complexes: *Lane 1: SEQ #5 (reference); lane 2: CuAAC reaction mixture (unpurified) to yield SEQ ID #11; lane 3: SEQ ID #1 (reference); lane 4: CuAAC reaction mixture (unpurified) to yield SEQ ID #7. Constant power of 20 W using 0.09 M Tris-borate-EDTA buffer (pH 8.0).*

SEQ ID #	Calc. Mass	Found. Mass
1	19212	19211
5	18930	18929
2	15271	15270
3	17136	17135
4	18937	18938
6	18936	18936
7	34482	34482
8	36348	36347
9	38149	38151
10	38148	38148
11	34201	34201
12	36066	36066
13	37867	37866
14	37866	37868

Table S2. Mass spectrometry analysis of the monovalent precursors SEQ ID #1-6 and the corresponding click-ligated products SEQ ID #7-14. *Mass spectra were recorded on a Bruker micrOTOF™ II focus ESI-TOF MS instrument in ES- mode and fit well with the calculated values.*

2.ii. Gel-electrophoretic Analysis of Self-Assembled Bivalent Aptamers SEQ ID #7-14

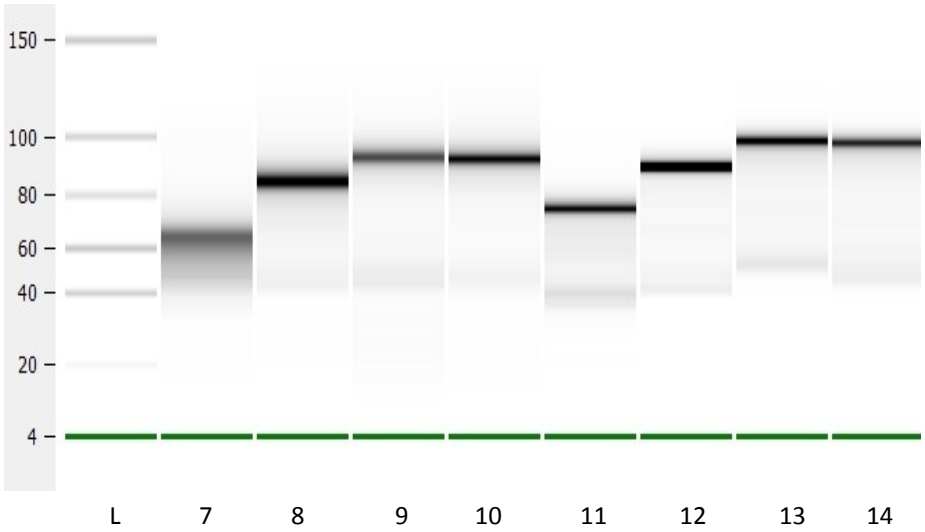


Figure S2: Gel-electrophoretic analysis of self-assembled bivalent aptamers: *The self-assembled bivalent aptamers SEQ ID #7-14 were dissolved in TE buffer (100 mM Tris pH 8.0, 1 mM EDTA). Their concentration was determined via UV absorption at 260 nm (NanoDrop, Thermo Scientific), then diluted to 10 ng/µl. 1 µl of each single-stranded DNA was loaded into an Agilent smallRNA chip and run on the Agilent BioAnalyzer 2100 capillary electrophoresis system (L= small RNA ladder). The self-assembled bivalent aptamers run faster than expected size. This is an indication for the stability of the self complementary assembly-region even under denaturing buffer conditions, which are sufficient to dissolve most of the secondary structures of RNA molecules(http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90094revB_QG_SmallRNA.pdf).*

2.iii. PCR amplification and Sanger Sequencing of individual self-assembled bivalent aptamers:

PCR amplification Scheme for SEQ ID #8:

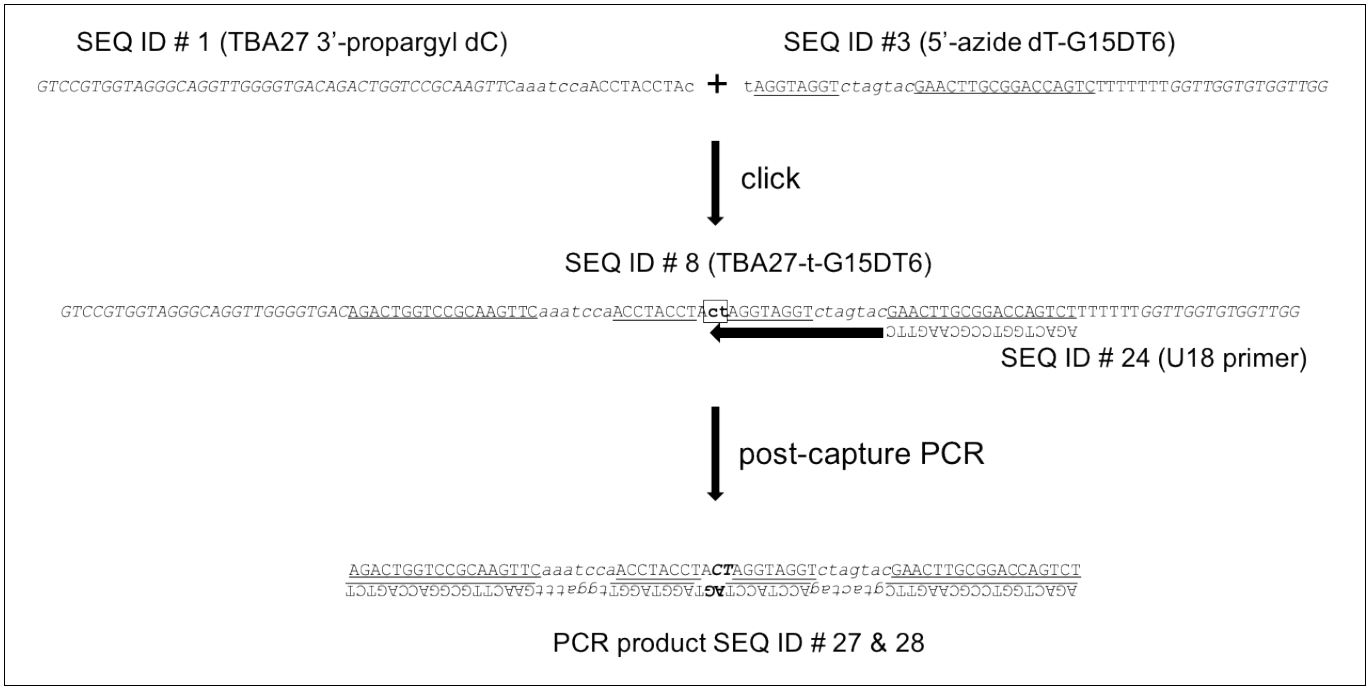


Figure S4: Example of a "Click"- reaction followed by PCR amplification: *SEQ ID #1 (left arm) is covalently conjugated with SEQ ID #3 (right arm) as described to yield SEQ ID #8, which folds into the corresponding self-assembled bivalent aptamer. Clicked termini in small litalics boxed. The corresponding amplification via PCR using only one universal primer sequence ID # 24 yields SEQ ID #27 (sense strand) and SEQ ID #28 (antisense strand). The self-complementary parts of the strands are underlined. Aptamer motif and spacer specific sequences (barcode) in small italics. Clicked site now indicated in bold capital italics.*

Analysis of PCR products SEQ IDs # 25-40

SEQ ID #	Name	Sequence (5' to 3')
24	U18_primer	AGACTGGTCCGCAAGTTC
25	PCR product(forward) from template SEQ ID #7	AGACTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCGGACCAGTCT
26	PCR product(reverse complement) from template SEQ ID #7	AGACTGGTCCGCAAGTTC*tatagggg*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCGGACCAGTCT
27	PCR product(forward) from SEQ ID #8	AGACTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCGGACCAGTCT
28	PCR product(reverse complement) from SEQ ID #8	AGACTGGTCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCGGACCAGTCT
29	PCR product(forward) from SEQ ID #9	AGACTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*accttcg*GAACTTGCGGACCAGTCT
30	PCR product(reverse complement) from SEQ ID #9	AGACTGGTCCGCAAGTTC*cgaaggt*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCGGACCAGTCT
31	PCR product(forward) from SEQ ID #10	AGACTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*tacacgt*GAACTTGCGGACCAGTCT
32	PCR product(reverse complement) from SEQ ID #10)	AGACTGGTCCGCAAGTTC*acgtgta*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCGGACCAGTCT
33	PCR product(forward) from SEQ ID #11	AGACTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCGGACCAGTCT
34	PCR product(reverse complement) from SEQ ID #11	AGACTGGTCCGCAAGTTC*tatagggg*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGACCAGTCT
35	PCR product(Fwd) from SEQ ID #12	AGACTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCGGACCAGTCT
36	PCR product(reverse complement) from SEQ ID #12	AGACTGGTCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGACCAGTCT
37	PCR product(forward) from SEQ ID #13	AGACTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*accttcg*GAACTTGCGGACCAGTCT
38	PCR product(reverse complement) from SEQ ID #13	AGACTGGTCCGCAAGTTCcgaaggtACCTACCT***AG***TAGGTAGGTgagacctGAACTTGCGGACCAGTCT
39	PCR product(forward) from SEQ ID #14	AGACTGGTCCGCAAGTTCaggtctcACCTACCTA***CT***AGGTAGGTtacacgtGAACTTGCGGACCAGTCT
40	PCR product(reverse complement) from SEQ ID #14	AGACTGGTCCGCAAGTTCacgtgtaACCTACCT***AG***TAGGTAGGTgagacctGAACTTGCGGACCAGTCT

Table S4: List of PCR products SEQ IDs #25-40 generated from templates SEQ IDs #7-14
All sequences listed in the 5' to 3' orientation. Self-complementary part of the strand underlined. Sequence barcodes in italics. "Clicked" termini in the sense as well as reverse complementary site in the antisense strand are annotated in bold capital italics.

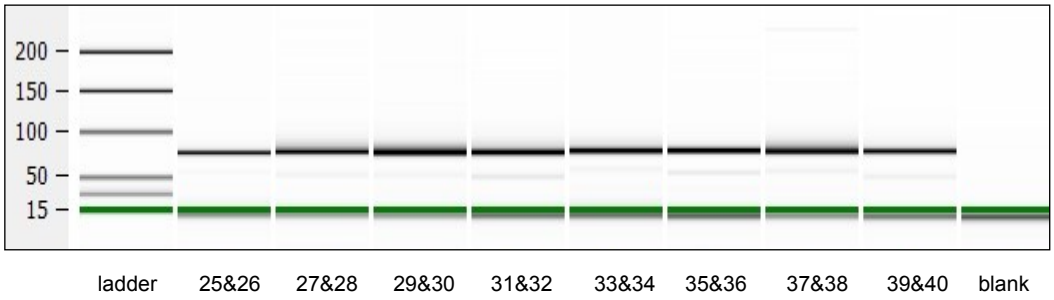
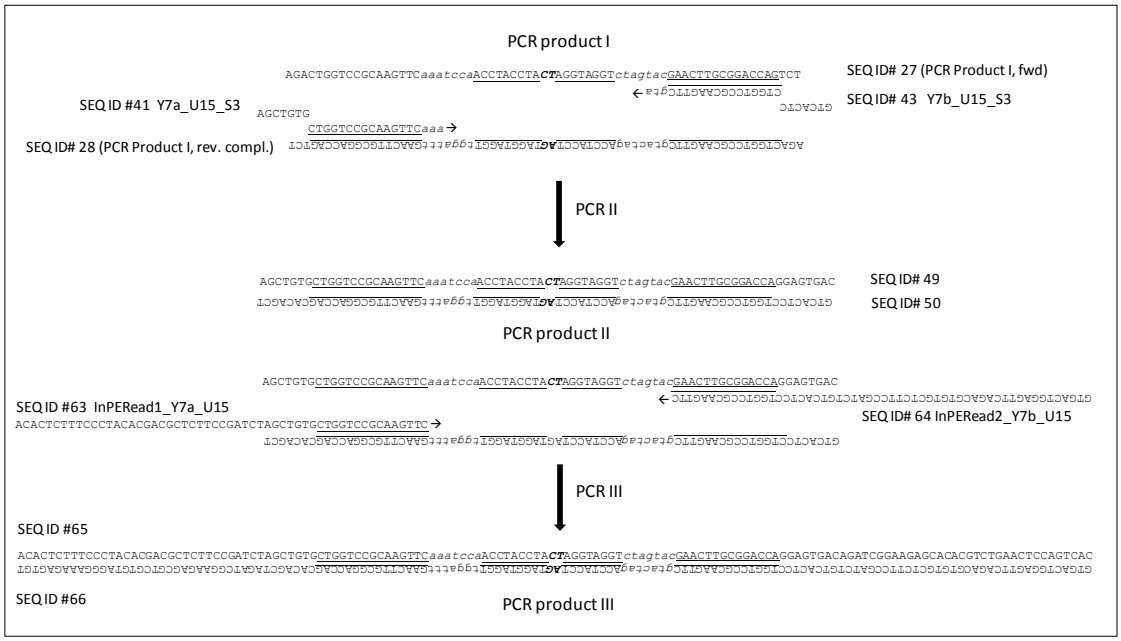
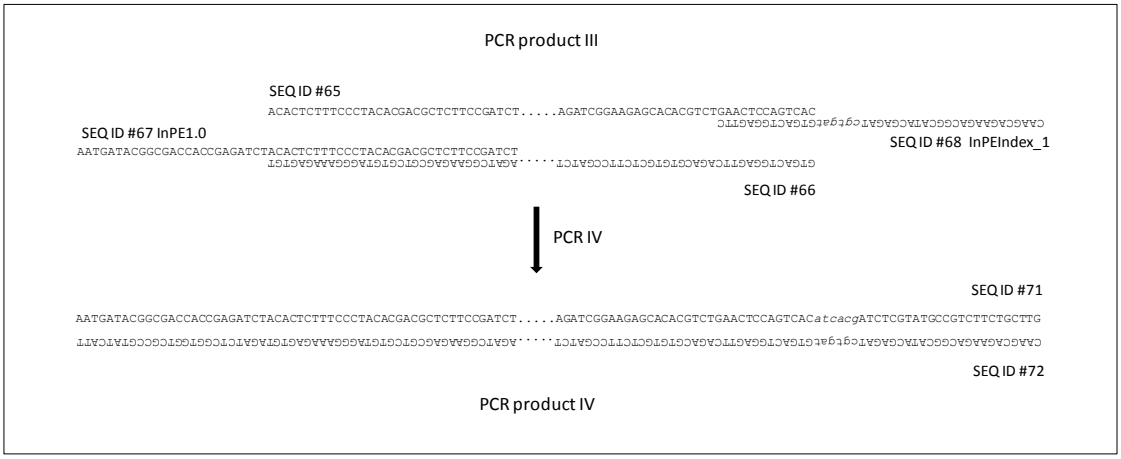


Figure S5: Gel-electrophoretic analysis of PCR products SEQ ID # 25-40 from SEQ ID #7-14.
Only one primer was used (sequence U18 (SEQ ID #24)).10 ng template, 1.2 µM primer, 0.5 mM triphosphates and 2.5 U thermostable DNA polymerase were mixed and PCR amplification was executed with 24 cycles at 60^0C annealing temperature. Gel-electrophoretic analysis was done on a Bioanalyzer 2100 DNA -1000 chip with 1 µl PCR product for each lane.

Sanger Sequencing of PCR products





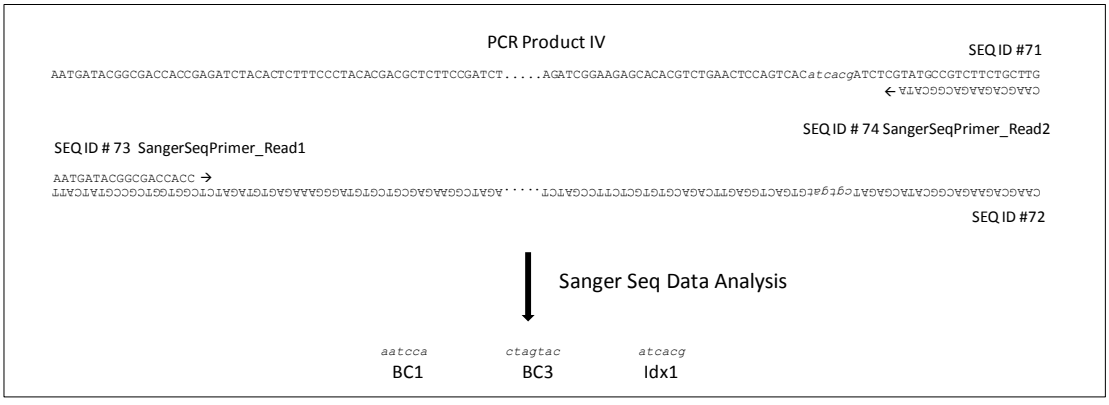


Figure S6: Sample Preparation prior to Sanger Sequencing. *As an example for sample preparation for Sanger sequencing the scheme for PCR product SEQ ID #27 and #28 is shown. PCR products SEQ ID #25, 26 and #29-40 are processed accordingly. Preparation of SEQ ID #71 and #72, which are sequenced using sequencing primers SEQ ID #73 and #74 is shown. Sequencing was done on a 96-capillary 3730xl DNA Analyzer (Life Technologies). The individual samples were processed using the corresponding BigDye® Direct Cycle Sequencing Kit and protocol.*

41	Y7a_U15_BC1f	AGCTGTGCTGGTCCGCAAGTTC*aaa*
42	Y7b_U15_BC2r	GTCACTCCTGGTCCGCAAGTTC*tat*
43	Y7b_U15_BC3r	GTCACTCCTGGTCCGCAAGTTC*gta*
44	Y7b_U15_BC4r	GTCACTCCTGGTCCGCAAGTTC*cga*
45	Y7a_U15_BC5f	AGCTGTGCTGGTCCGCAAGTTC*agg*
46	Y7b_U15_BC6r	GTCACTCCTGGTCCGCAAGTTC*acg*
47	PCR product II(forward)of PCR product I of SEQ ID#7 (SEQ ID #25 and #26) using primer SEQ ID #41	AGCTGTGCTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCG GACCAGGAGTGAC
48	PCR product II(reverse-complement) of PCR product I of SEQ ID #7 (SEQ ID #25 and #26. using primer SEQ ID #42	GTCACTCCTGGTCCGCAAGTTC*tatagggg*ACCTACCT***AG***TAGGTAGGT*tgcatttt*GAACTTGCG GACCAGCACAGCT
49	PCR product II(forward)of PCR product I of SEQ ID#8 (SEQ ID #27 and #28) using primer SEQ ID #41.	AGCTGTGCTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCG GACCAGGAGTGAC
50	PCR product II(reverse-complement) of PCR product I of SEQ ID #8 (SEQ ID #27 and #28) using primer SEQ ID #43.	GTCACTCCTGGTCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCG GACCAGCACAGCT
51	PCR product II(forward)of PCR product I of SEQ ID#9 (SEQ ID #29 and #30) using primer SEQ ID #41.	AGCTGTGCTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*accttcg*GAACTTGCG GACCAGGAGTGAC
52	PCR product II(reverse-complement)of PCR product I of SEQ ID #9 (SEQ ID #29 and #30)using primer SEQ ID #44.	GTCACTCCTGGTCCGCAAGTTC*cgaaggt*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCG GACCAGCACAGCT
53	PCR product II(forward) of PCR product I of SEQ ID#10 (SEQ ID #31 and #32) using primer SEQ ID #41.	AGCTGTGCTGGTCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*tacacgt*GAACTTGCG GACCAGGAGTGAC
54	PCR product II(reverse-complement) of PCR product I of SEQ ID#10 (SEQ ID #31 and #32)using primer SEQ ID #46.	GTCACTCCTGGTCCGCAAGTTC*acgtgta*ACCTACCT***AG***TAGGTAGGT*tggatttt*GAACTTGCG GACCAGCACAGCT
55	PCR product II(forward) of PCR product I of SEQ ID#11 (SEQ ID #33 and #34) using primer SEQ ID #45.	AGCTGTGCTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCG GACCAGGAGTGAC
56	PCR product II(reverse-complement) of PCR product I of SEQ ID#11 (SEQ ID #33 and #34)using primer SEQ ID #42.	GTCACTCCTGGTCCGCAAGTTC*tatagggg*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCG GACCAGCACAGCT
57	PCR product II(forward) of PCR product I of SEQ ID#12 (SEQ ID #35 and #36) using primer SEQ ID #45.	AGCTGTGCTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCG GACCAGGAGTGAC
58	PCR product II(reverse-complement) of PCR product I of SEQ ID#12 (SEQ ID #35 and #36)using primer SEQ ID #43.	GTCACTCCTGGTCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCG GACCAGCACAGCT
59	PCR product II(forward) of PCR product I of SEQ ID#13 (SEQ ID #37 and #38) using primer SEQ ID #45.	AGCTGTGCTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*accttcg*GAACTTGCG GACCAGGAGTGAC
60	PCR product II(reverse-complement) of PCR product I of SEQ ID#13 (SEQ ID #37 and #38)using primer SEQ ID #44.	GTCACTCCTGGTCCGCAAGTTC*cgaaggt*ACCTACCT***AG***TAGGTAGGTgagacctGAACTTGCG GACCAGCACAGCT
61	PCR product II(forward) of PCR product I of SEQ ID#14 (SEQ ID #39 and #40) using primer SEQ ID #45.	AGCTGTGCTGGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*tacacgt*GAACTTGCG GACCAGGAGTGAC

62	PCR product II(reverse-complement) of PCR product I of SEQ ID#14 (SEQ ID #39 and #40)using primer SEQ ID #46.	GTCACTCCTGGTCCGCAAGTTC*acgtgta*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCG GACCAGCACAGCT
63	InPEREad1_Y7a_U15	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTGTGCTGGTCCGCAAGTTC
64	InPEREAd2_Y7b_U15	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCACTCCTGGTCCGCAAGTTC
65	Pre-multiplexed PCR III product (forward)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTGTGCTGGTCCGCAAGTTCAAATCCAAC CTACCTA***CT***AGGTAGGTCTAGTACGAACTTGCGGACCAGGAGTGACAGATCGGAAGAGCACACG TCTGAACTCCAGTCAC
66	Pre-multiplexed PCR III product (anti-sense)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCACTCCTGGTCCGCAAGTTCGTACTAGA CCTACCT***AG***TAGGTAGGTTGGATTTGAACTTGCGGACCAGCACAGCTAGATCGGAAGAGCGTCG TGTAGGGAAAGAGTGT
67	InPE1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
68	InPEIndex_1 multiplex/index1	CAAGCAGAAGACGGCATACGAGAT*cgtgat*GTGACTGGAGTTC
69	InPEIndex_2 multiplex/index2	CAAGCAGAAGACGGCATACGAGAT*acatcg*GTGACTGGAGTTC
70	InPEIndex_3 multiplex/index3	CAAGCAGAAGACGGCATACGAGAT*gcctaa*GTGACTGGAGTTC
71	PCR product IV(forward)	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT.....A GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG
72	PCR product IV (reverse-complement)	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT .....AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT
73	Sanger Sequencing Primer InPE1-18	AATGATACGGCGACCACC
74	Sanger Sequencing Primer InPE2-18	CAAGCAGAAGACGGCATA

Table S5: Primers and corresponding PCR products used for Sanger Sequencing.
All sequences in 5' to 3' orientation. Small italics indicate the individual identifier sequence (barcode), underlined letters indicate reverse complementary sequences. Not all PCR products of PCR III and IV are listed, except #65, 66 (III) and #71, 72 (IV) used as examples for the general scheme above.

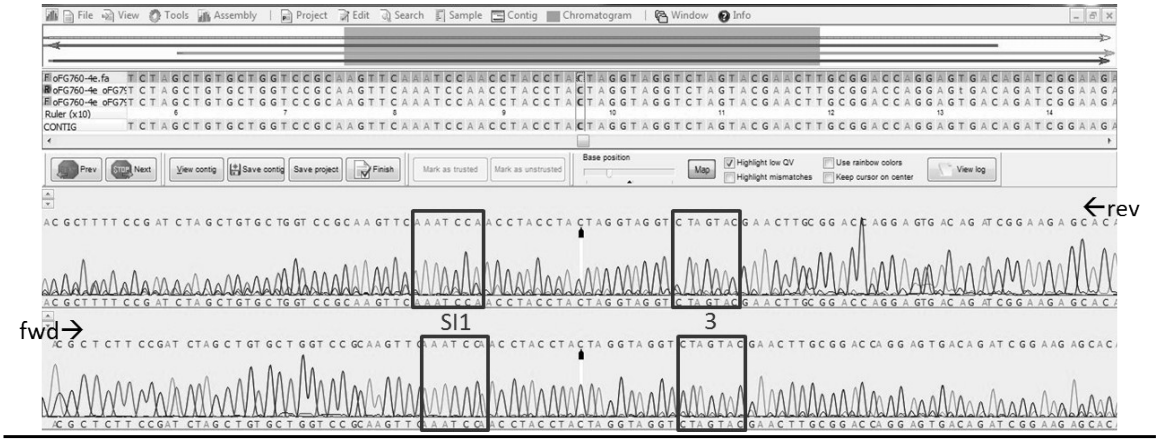


Figure S7: Results of Sanger-sequencing with Primer SEQ ID #73 and #74 of PCR products SEQ ID #71 and 72. *The corresponding identifier sequences (SI1 = aaatcca) and (SI3= ctagtac) can be read-out correctly on both strands. Indexing sequence (atcacg), which is introduced to allow next generation sequencing on an Illumina MiSeq Platform in a separate experiment after multiplexing, can be read-out correctly on forward strand (not shown).*

3. Biological Activity

3.i. Blood Clotting Experiments

Samples were obtained with consent from Sanquin Bloodbank. Pooled plasma from a pool of more than 32 healthy adult donors was used.

To evaluate the inhibitory potency of individual nucleic acid ligand or bivalent complex thereof, we measured the clotting time of each sample containing only thrombin, the ligand or ligand complex and fibrinogen substrate in physiological buffer. The mixture of sample becomes non fluidic when the fibrinogen is digested by thrombin. As a result, the different timepoints of this transition can be used as an indicator. Briefly, 1µl of 10 µM thrombin and 1 µl of 100 µM monovalent or bivalent aptamer were added to a disposable transparent plastic cuvette (Fisher Scientific) containing 200 µl buffer and incubated for 15 min. Then, 4 µl of 20 mg/ml fibrinogen was added, and samples in the cuvette were carefully examined by tilting the cuvette to record the time when the sample became non fluidic. Each experiment was performed in tandem. A reaction mixture containing only thrombin and fibrinogen was always tested together with other samples as an internal standard. All clotting times were normalized based on the internal standard and compared with it.

To evaluate the feasibility of the self-assembled bivalent aptamer as a potential anticoagulant reagent, Standard activated Partial Thromboplastin Time (aPTT)[3] and Prothrombin Time (PT)[4] values for each individual or ligand composition were determined by using human plasma samples. Procedures applied were those recommended by the supplier. For aPTT determination, 50 µl UCRP was preincubated at 37°C with a different amount of each ligand for 2 min; then 50 µl aPPT-L was added and incubated for another 200 sec. Next, 50 µl of pre-warmed $CaCl_2$ was added to initiate the intrinsic clotting cascade. Finally, the scattering signal was monitored until the signal was saturated. For PT determination, 50 µl of UCRP was pre-incubated at 37°C with a different amount of each ligand or ligand complex for 2 min; then 50 µl of thromboplastin-L was added to initiate the extrinsic clotting cascade. Finally, the scattering signal was monitored until the signal was saturated. For the calculation of aPTT and PT, the end time was determined to be the point where scattering signal reached half maximum between lowest and maximum points. This was repeated twice, and each set of experiments was done with one batch of plasma.

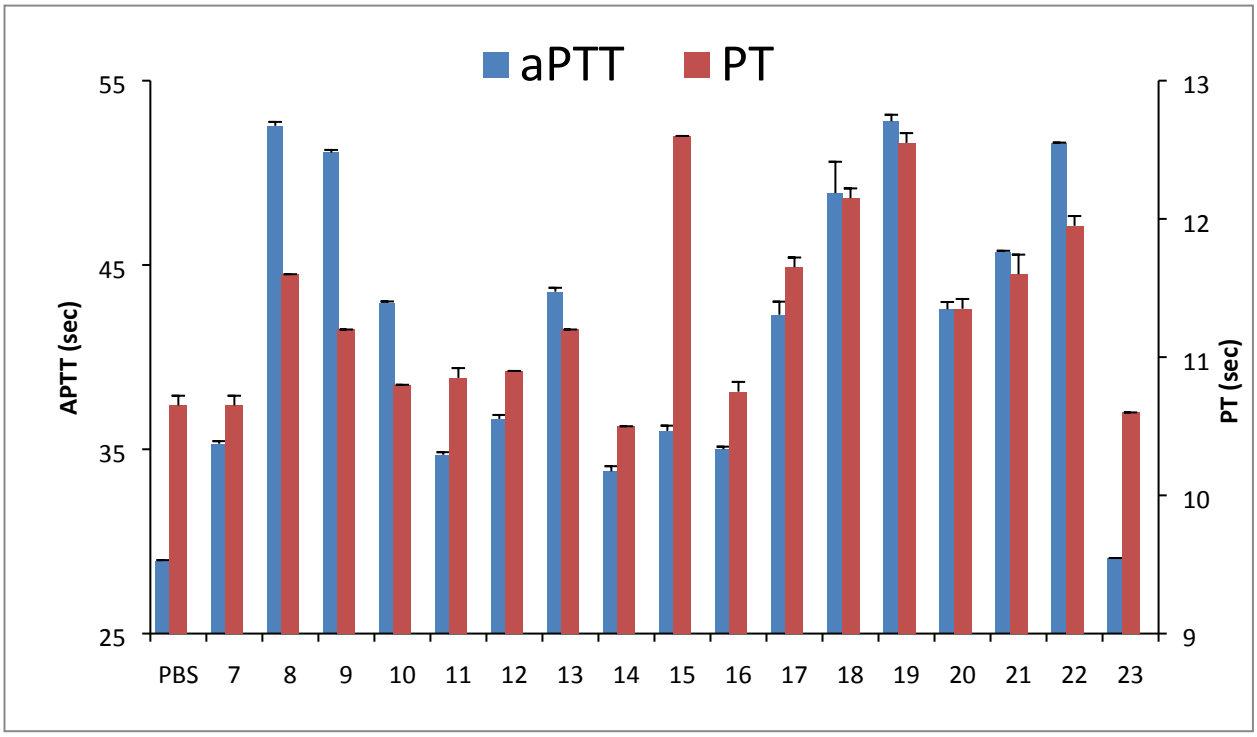


Figure S8: Standard Prothrombin Time (PT) and activated Partial Thromboplastin time (aPTT) values for each individual or aptamer ligand composition: *The values were determined by using human plasma samples. Whilst sequences do not show any significant difference in the PT values, except #15, the benefit of combining two binding motifs is clearly evident in the aPTT values. The aPTT value of a non-Thrombin binding oligonucleotide sequence SEQ ID # 23 is fully identical to the reference (buffer) below 30 sec. Combinations of SEQ ID #15 and #16 lead to values above 35 sec depending on the distance between the two aptamers. SEQ ID #17-19 and #20-22 are covalently conjugated aptamer motifs #15 and #16 without and with a T6 or T12 spacer. Similar aPTT values can be seen as confirmation of a stable dsDNA stem for the self-assembled bivalent ligand complexes (SABAs) of two binding motifs with varied distances (SEQ ID #7,8 and 9). Further more mutation of one of the binding motifs lead to decreased aPTT values as expected (SEQ ID #10-14).*

ID #	Sequence Description	PT	aPTT
7	TBA27-ct-T0-G15D	10.7	35.3
8	TBA27-ct-T6-G15D	11.6	52.6
9	TBA27-ct-T12-G15D	11.2	51.1
10	TBA27-ct-T12-G15Ds	10.8	43.0
11	TBA27s-ct-T0-G15D	10.9	34.7
12	TBA27s-ct-T6-G15D	10.9	36.7
13	TBA27s-ct-T12-G15D	11.2	43.6
14	TBA27s-ct-T12-G15Ds	10.5	33.8
15	G15D	12.6	36.0
16	TBA27	10.8	35.0
17	G15D-T0-TBA27	11.7	42.3
18	G15D-T6-TBA27	12.2	48.9
19	G15D-T12-TBA27	12.6	52.8
20	TBA27-T0-G15D	11.4	42.6
21	TBA27-T6-G15D	11.6	45.7
22	TBA27-T12-G15D	12.0	51.6
23	Reference	10.6	29.1

Table S6. *Sequences used for blood clotting experiments and the corresponding PT and aPTT values (average from at least two technical replicates, 2-4 data points in total).*

4. Aptamer selection, amplification and sequencing

4.i. Self-assembled bivalent aptamer (SABA) model library creation

A model library was prepared using SABA sequences ID #7-14. SEQ ID # 7-13 were mixed in 1:1 ratio and background DNA SEQ ID #14 (200 pmol) was added. The final mix comprised 99.125% background DNA and 0.125 % of each of the SEQ ID #7-13. 1 μM was the final concentration.

4.ii Protein Immobilisation

Magnetic Dynabeads® MyOne Carboxylic Acid (Invitrogen, cat. N0 65011) are resuspended by rolling the vial for 30 min on a rotor at 20 rpm, and 0.5 ml suspension was transferred to a new tube. The tube was placed close to a magnet for 1 min, the supernatant was removed and the beads were washed two times with 0.5 ml MEST buffer (50 mM 2-(N-morpholino)ethanesulfonic acid, 0.01 % Tween 20, pH 6.0). The beads were resuspended in 50 µl MEST buffer and activated by addition of 50 µl EDC (10 mg/ml N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma E6383) for 30 min at room temperature on a rotor at 10 rpm. The supernatant was removed, the beads were resuspended in 100 µl MEST buffer and split into two 50 µl aliquots. The first aliquot was incubated with 6 nmol corresponding to 200 µg α-thrombin (Tb, Haematologic Technologies, HCT-0020) in 250 µl MEST buffer (20 µM thrombin final), the second aliquot was blocked with 5 mM aminoethanol (EA) in 250 µl MEST buffer for 2.5 h on a rotor at 10 rpm at room temperature. The tubes were placed close to a magnet for 1 min, the supernatants were removed and the beads were each washed two times with 0.25 ml PBSMT buffer (137 mM sodium chloride, 2.7 mM potassium chloride and 10 mM sodium/potassium phosphate buffer solution pH7.4, Ambion AM9624, supplemented with 1 mM magnesium chloride and 0.01 % Tween 20).

4.iii. Selection by Bead Capture

Thrombin-coupled (Tb) beads and negative control (EA = deactivated with ethanolamine) beads were each re-suspended in 160 µl PBSMT, and to each aliquot 40 µl of 5 µM (corresponding to 200 pmol) SABA library was added (1 µM SABA library final). The tubes containing the mixtures were incubated for 1 h on a rotor at 10 rpm at room temperature. The tubes were placed close to a magnet for 1 min, the supernatants were removed and the beads were washed two times with 0.25 ml PBSMT buffer. The beads were boiled each in 100 µl of 1x thermostable DNA buffer (Roboklon) for 5 min at 95 °C in a thermocycler and cooled down to room temperature. The tubes were placed close to a magnet for 1 min, and 90 µl of the supernatants were transferred to new tubes for further analysis.

4.iv. Universal PCR Amplification

The bead-selected (Tb and EA) members of the SABA library were universally amplified by the following semi-quantitative PCR protocol:

20 µl of DNAse/RNAse free water (Life Technologies) was supplemented with 3 µl of 10x buffer for thermostable DNA polymerase (Roboklon), 6 µl of 10 µM primer U18 (SEQ ID # 24; Biolegio), 0.5 µl of 25 mM dNTP mix (25 mM of each deoxyribonucleotide dATP, dCTP, dGTP, dTTP; Invitrogen), 0.5 µl of thermostable DNA polymerase (5 U/µl; Roboklon) and 20 µl of template DNA in 1x thermostable DNA buffer (or 1 x thermostable DNA buffer alone for blank analysis). Template DNA for the standard curve was a serial dilution of the SABA library (0.5 µM to 0.5 pM) in 1x thermostable DNA buffer, template DNA for selection analysis consisted of bead eluate in 1x thermostable DNA buffer. The resulting 50 µl PCR premixes were incubated in a thermocycler instrument (BioRad iCycler) with the following program settings: 1x melt 03:00 min 95 ºC, 24x melt 00:30 min 95 ºC, 24x anneal 00:30 min 60 ºC, 24x elongate 00:30 min 72 ºC 1x elongate 03:00 min 72 ºC, 1x hold 4 ºC. 1 µl of the PCR mix was analyzed by capillary electrophoresis in a Bioanalyzer DNA-1000 Lab-on-a-Chip (Agilent). The concentration of the PCR products were determined by peak integration, performed by the Bioanalyzer 2100 Expert software, version B.02.08.SI648 (Agilent).

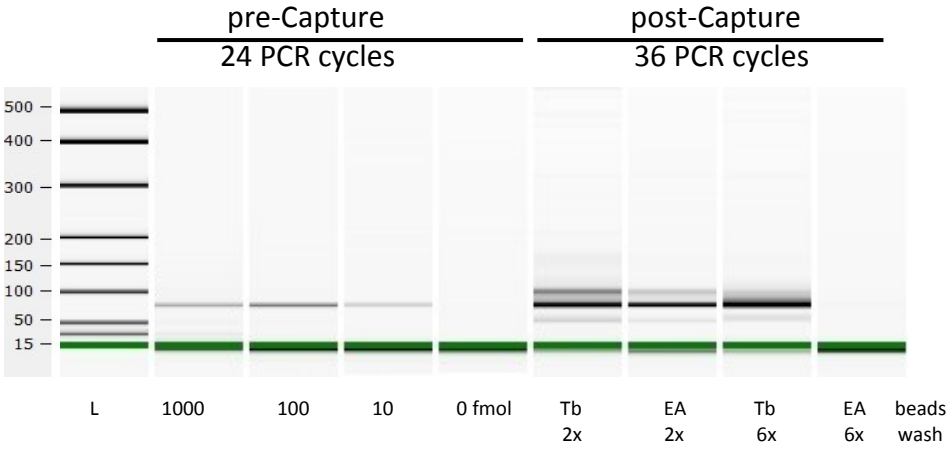


Figure S9: Semi-quantitative 1st PCR with Single Primer U18: *According to the Agilent BioAnalzyer Data (described above) the PCR detection limit for SABA hairpins with single universal U18 primer is 10 fmol. Apparently selection of a SABA library on DynaBeads coupled to 20 µg thrombin results in captured material in the low fmol range (2x wash) and much lower range (6x wash). Negative selection on beads blocked with ethanolamine results in very little (2x wash) and nearly non detectable material (6x wash).*

4.v. Quantitative PCR (qPCR) Analysis

The compositions of the universal PCR products of the bead-selected SABAs (Tb and EA) were determined by amplification of individual SABA barcodes by the following quantitative PCR (qPCR) protocol: 6 µl of DNAse/RNAse free water (Life Technologies) were supplemented with 10 µl of iQ™ SYBR®Green Supermix (Bio-Rad, cat. # 170-8882), 1 µl of 10 µM forward primer (SEQ ID # 75, 79 and 81; Biolegio), 1 µl of 10 µM reverse primer (SEQ ID #76-78, 80, 82; Biolegio) and 2 µl of 60 nM (corresponding to 120 fmol) template DNA. The resulting 20 µl PCR premixes were incubated in a thermocycler instrument (Bio-Rad iCycler) with the following program settings: 1x melt 03:00 min 95 ºC, 40x melt 00:30 min 95 ºC, 40x anneal 00:30 min 60 ºC, 40x elongate 00:30 min 72 ºC. The cycle threshold (Ct) values were calculated by PCR baseline subtraction curve fits of the SYBR®Green amplification charts, performed by the iQ™5 Optical System Software, Version 2.1 (Bio-Rad).

Enrichment factors (Ef) were calculated in Microsoft Excel as follows: Ef=POWER(1,7;ΔCt(EA-Tb))=POWER(1,7;AVERAGE(Ct(EA))-AVERAGE(Ct(Tb))).

SEQ ID #	Name	Sequence (5´ to 3´)
24	U18_primer	AGACTGGTCCGCAAGTTC
75	U11_S7-BC1f barcode 1 specific	TCCGCAAGTTC*aaatcca*
76	U11_S7-BC2r barcode 2 specific	TCCGCAAGTTC*tataggg*
77	U11_S7-BC3r barcode 3 specific	TCCGCAAGTTC*gtactag*
78	U11_S7-BC4r barcode 4 specific	TCCGCAAGTTC*cgaaggt*
79	U11_S7-BC5f barcode 5 specific	TCCGCAAGTTC*aggtctc*
80	U11_S7-BC6r barcode 6 specific	TCCGCAAGTTC*acgtgta*
81	Y7a_U15_U3f background specific	AGCTGTGCTGGTCCGCAAGTTC*ctg*
82	Y7b_U15_U3r background specific	GTCACTCCTGGTCCGCAAGTTC*gtc*
83	PCR product II(forward)from SEQ ID #25 and 26 and primer #75	TCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCGGA
84	PCR product II(reverse complement) from SEQ ID #25 and 26 and primer #76	TCCGCAAGTTC*tataggg*ACCTACCT***AG***TAGGTAGGTtgcatttGAACTTGCGGA
85	PCR product II(forward) from SEQ ID #27 and 28 and primer #75	TCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCGGA
86	PCR product(reverse complement)from SEQ ID #27 and 28 and primer #77	TCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*tggattt*GAACTTGCGGA
87	PCR product (forward)from SEQ ID #29 and 30 and primer #75	TCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*tggattt*GAACTTGCGGA
88	PCR product (reverse complement)from SEQ ID #29 and 30 and primer #78	TCCGCAAGTTC*cgaaggt*ACCTACCT***AG***TAGGTAGGT*tggattt*GAACTTGCGGA
89	PCR product (forward)from SEQ ID #31 and 32 and primer #75	TCCGCAAGTTC*aaatcca*ACCTACCTA***CT***AGGTAGGT*tacacgt*GAACTTGCGGA
90	PCR product (reverse complement)from SEQ ID #31 and 32 and primer #80	TCCGCAAGTTC*acgtgta*ACCTACCT***AG***TAGGTAGGT*tggattt*GAACTTGCGGA
91	PCR product (forward)from SEQ ID #33 and 34 and primer #79	TCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ccctata*GAACTTGCGGA
92	PCR product (reverse complement)from SEQ ID #33 and 34 and primer #76	TCCGCAAGTTC*tataggg*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGA
93	PCR product (forward)from SEQ ID #35 and 36 and primer #79	TCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*ctagtac*GAACTTGCGGA
94	PCR product (reverse complement)from SEQ ID #35 and 36 and primer #77	TCCGCAAGTTC*gtactag*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGA
95	PCR product (forward)from SEQ ID #37 and 38 and primer #79	TCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*accttcg*GAACTTGCGGA
96	PCR product (reverse complement)from SEQ ID #37 and 38 and primer #78	TCCGCAAGTTC*cgaaggt*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGA
97	PCR product (forward) from SEQ ID #39 and 40 and primer #81	AGCTGTGTCCGCAAGTTC*aggtctc*ACCTACCTA***CT***AGGTAGGT*tacacgt*GAACTTGCGGAC
98	PCR product (reverse complement)from SEQ ID #39 and 40 and primer #82	GTCACTGTCCGCAAGTTC*acgtgta*ACCTACCT***AG***TAGGTAGGT*gagacct*GAACTTGCGGACCAGTCT

Table S7: List of primer sequences used for qPCR (SEQ ID #24, 75-82) and corresponding PCR products (forward and reverse complement). *All sequences in 5' to 3' orientation. Small italics indicate the individual identifier sequence (barcode), underlined letters indicate reverse complementary sequences.*

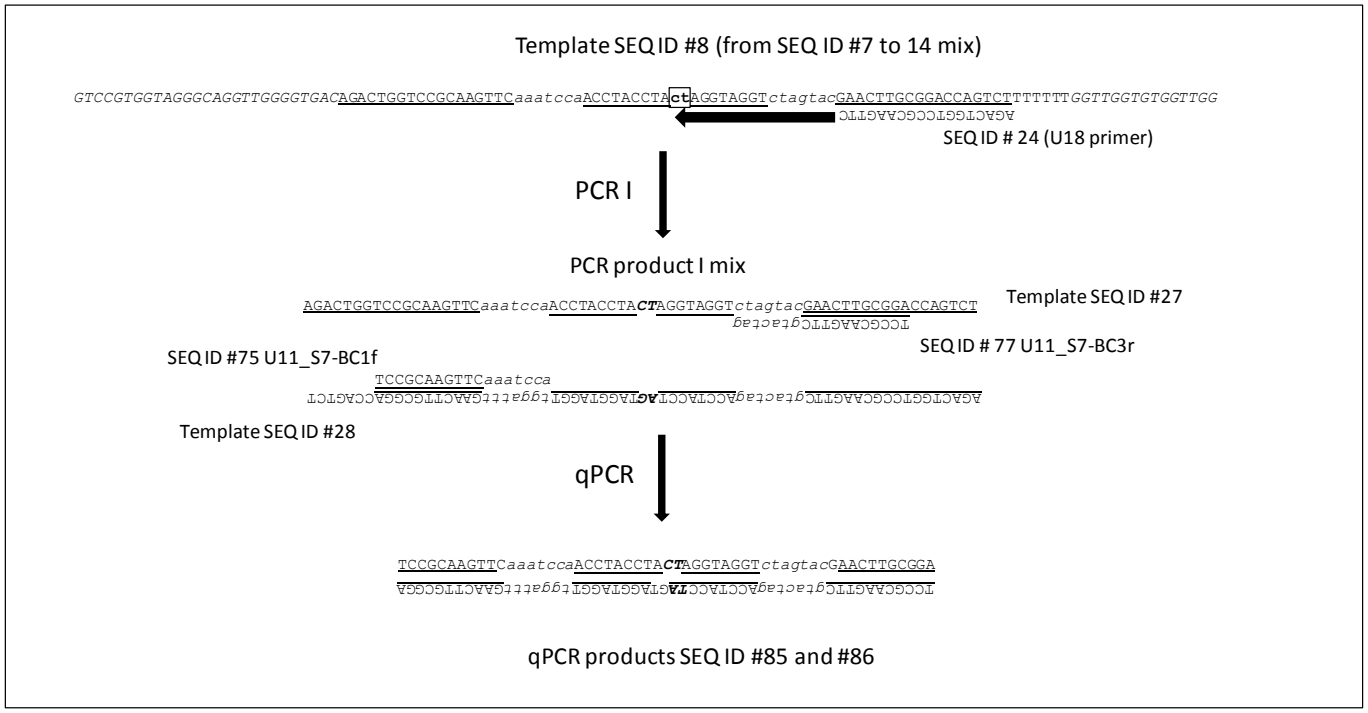


Figure S10: *Sample Preparation for quantitative PCR was done according to the above scheme and protocol. As an example the scheme is shown in detail starting with template SEQ ID #8 from the mix SEQ ID#7-14.*

Results:

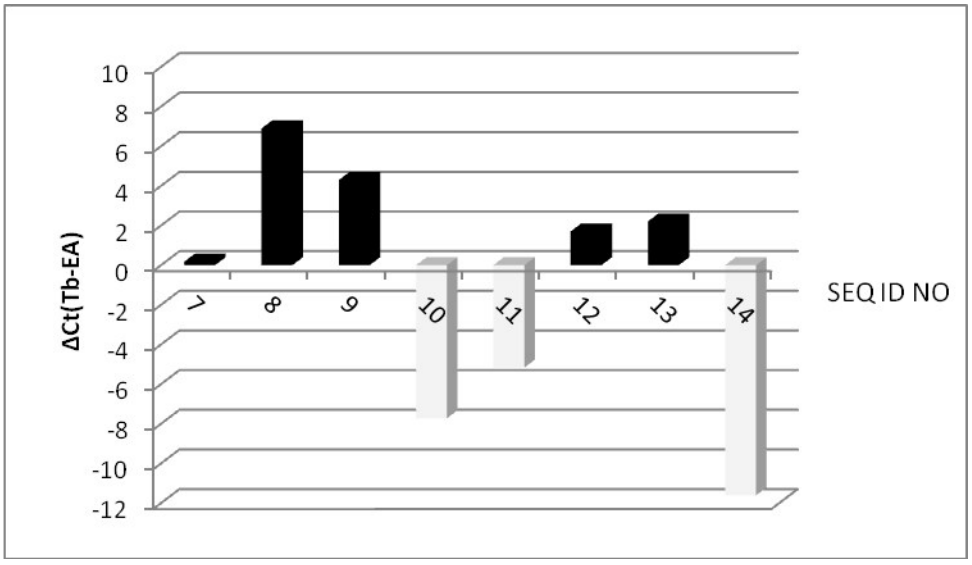


Figure S11: qPCR Data: *ΔCt (Tb-EA) values of the different SABAs show enrichment for SEQ ID # 7-9 as well as for #12 and #13. From this data it can be concluded that SEQ ID #8 with a T6 spacer and #9 with a T12 spacer is most efficient combination of binding motifs. If only one binding motif is scrambled the numbers of the corresponding SABA after selection are less. Modification (scrambled sequence) of TBA27 (#12,13) is less effective than modification of G15D (#10 and #11). The relative abundance of SEQ ID #14, where both sequences have been modified, is dramatically reduced.*

4.vi. Illumina Multiplexing PCR

The universally amplified members of the SABA library (Tb and EA) were further amplified by the following PCR protocol: 36 µl of DNAse/RNAse free water (Life Technlogies) was supplemented with 5 µl of 10x thermostable DNA Buffer (Roboklon), 3 µl of each 10 µM primer (Biolegio), 0.5 µl of 25 mM dNTP mix (25 mM of each deoxyribonucleotide dATP, dCTP, dGTP, dTTP; Invitrogen), 0.5 µl of thermostable DNA polymerase (5 U/µl; Roboklon) and 2 µl of template DNA (or 2 µl PCR buffer alone for blank analysis). The resulting 50 µl PCR premixes were incubated in a thermocycler instrument (BioRad iCycler) with the following program settings: 1x melt 03:00 min 95 ºC, 18x melt 00:30 min 95 ºC, 18x anneal 00:30 min 65 ºC, 18x elongate 00:30 min 72 ºC 1x elongate 03:00 min 72 ºC, 1x hold 4 ºC. 1 µl of the PCR mix was analyzed by capillary electrophoresis in a Bioanalyzer DNA-1000 Lab-on-a-Chip (Agilent). The concentration of the PCR products were determined by peak integration, performed by the Bioanalyzer 2100 Expert software, version B.02.08.SI648 (Agilent).

SEQ ID	Name	Sequence (5' to 3')
68	InPEIndex_1 multiplex/index1	CAAGCAGAAGACGGCATACGAGAT*cgtgat*GTGACTGGAGTTC
69	InPEIndex_2 multiplex/index2	CAAGCAGAAGACGGCATACGAGAT*acatcg*GTGACTGGAGTTC
70	InPEIndex_3 multiplex/index3	CAAGCAGAAGACGGCATACGAGAT*gcctaa*GTGACTGGAGTTC
99	IllumSeqPrimer_Read1	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
100	IllumSeqPrimer_Read2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
101	IllumSeqPrimer_IndexRead	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC

Table S8: Primers used for NGS sample preparation after selection and multiplexing. *Other sequences mentioned in the scheme (Figure S12) are listed previously in Table S5. All sequences in 5' to 3' orientation. Small italics indicate the individual identifier sequence (index).*

Illumina Sequencing:

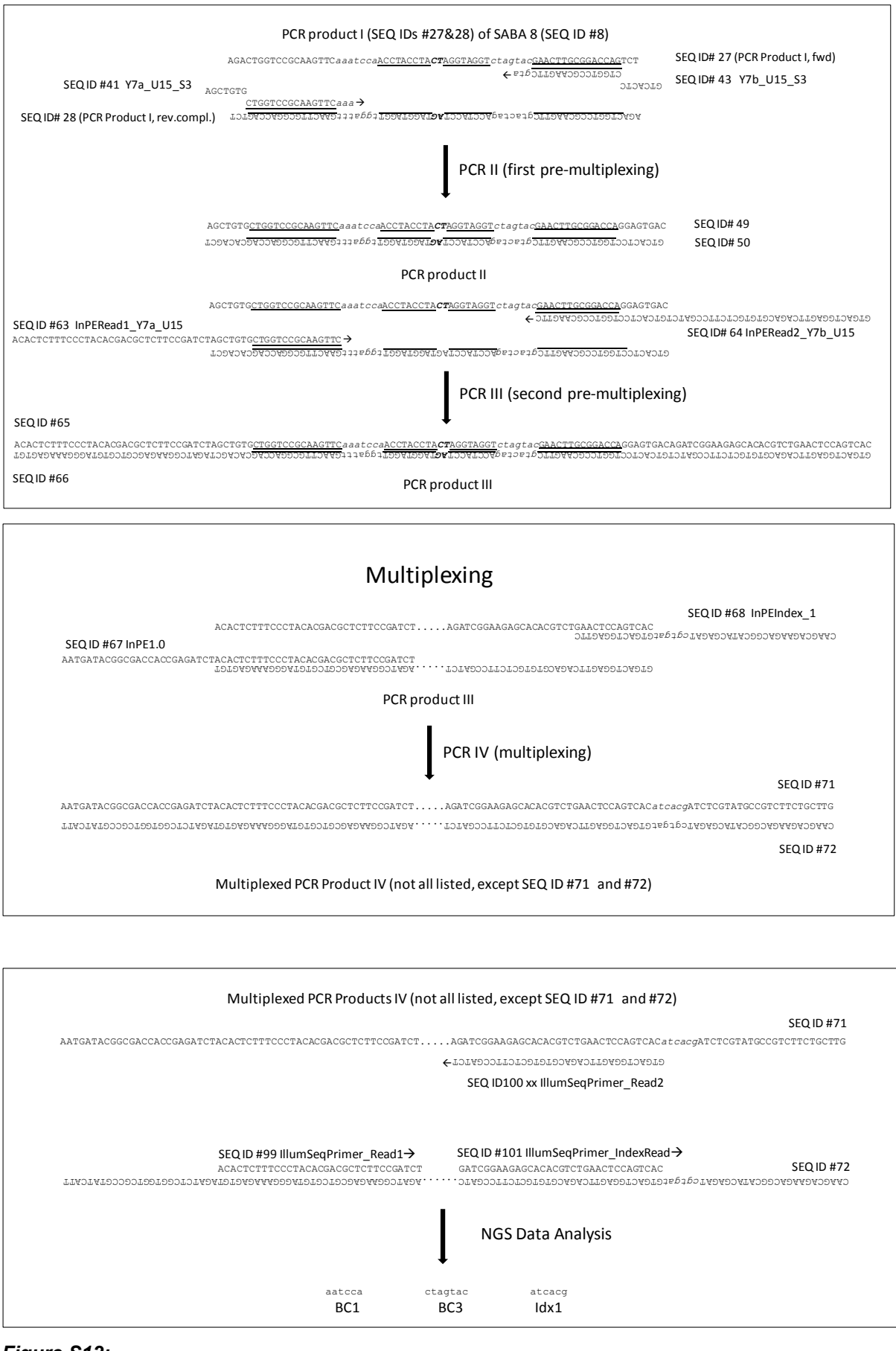


Figure S12:

As an example for sample preparation for Illumina NGS sequencing the scheme for PCR amplification is of SABA oligonucleotide #8 is shown. After selection a first PCR amplification with universal primer U18 (SEQ ID #24) is performed to yield PCR product I (SEQ IDs #27 and #28, see Figure S10.) For a second PCR amplification eight aliquots of PCR product I were amplified with eight primer pairs (forward and reverse,e.g. SEQ IDs #41 and #43) to yield PCR product II (SEQ IDs #49 and #50). This is followed by a third PCR amplification with universal primer pairs (SEQ IDs #63 and 64) to create PCR product III (SEQ IDs #65 and #66). For multiplexing standard Illumina index primers #67 and #68) are introduced to yield PCR product IV (SEQ IDs #71 and #72) according to standard Illumina NGS sample preparation protocol.

Results:

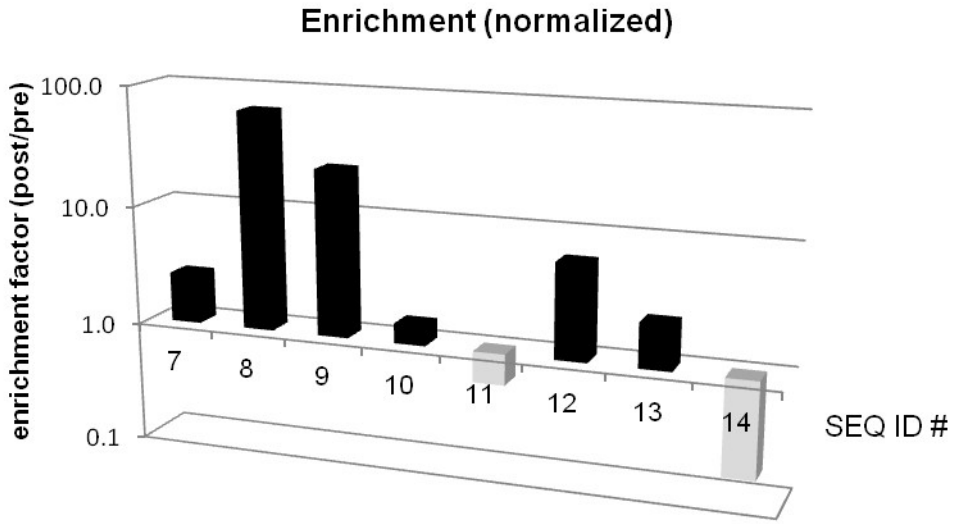


Fig S13: Results of NGS sequencing of the model library before and after selection
Burrows-Wheeler Alignment and Mapping; NGS platform is an Illumina MiSeq instrument. Data is shown in logarithmic scale, normalized). According to the counts SEQ ID # 8 with a T6 spacer between the binding motifs is most abundant, whilst a SEQ ID #9 with a T12 spacer is slightly diminished. If no spacer is used the enrichment in the final pool is negligible (SEQ ID #7) probably due to steric hinderance. Especially a modification in the G15D motif reduces the abundance in the final pool after selection (SEQ ID # 9 versus #10). Modification in the other binding motif TBA27 also reduces the counts (SEQ ID #11,12 and #13), but with a less significant impact. Modifications in both binding motifs lead to a significant reduced number of reads (SEQ ID #14).

5. Disassociation rates by surface plasmon resonance

Disassociation rates were determined using a G.E. Healthcare Biacore X100. Biotinylated human alpha-thrombin (2 µg/µl, Thermo Fisher Scientific cat. no. RP43103) was diluted with running buffer (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.01% Tween) to ~1µg/ml and immobilized on the active lane of the Sensor Chip SA (G.E. Helathcare cat. no. BR100032) using the in-built wizard application. Two separate chips were immobilized with different levels of thrombin (615.5 and 2452.4 RU; 'low' and 'high' respectively) and the reference lane was left blank. SEQ ID 15, 16, 20, 21 and 22 were freezed dried and resuspended in running buffer before serial two-fold dilution (max-min concentration range = 1000 to 7.8 nM for 'low' immobilization chips and 125 to 7.8 nM for 'high' immobilization chips). Samples were injected over both surfaces at a flow rate of 30 µl/min for 180 s before disassociation was monitored for 600 s. For all sequences, expect 15, the surface was regenerated using a 100 mM sodium acetate (pH 4.4) solution containing 4 M $MgCl_2$ at a flow rate of 30 µl/min for 30 s (high immobilization surface) or 15 s (low immobilization surface) before post-regeneration running buffer equilibration time of 180 s.

For data analysis, the active lane signal was reference subtracted and blank injection subtracted. The decay component for each aptamer was then normalized to the 180 s RU (start of decay) and averaged over the various concentration injections (error in the decay was less than 0.04 of the normalized response). Decays were fitted to the equation $Responce = A\left((1-\propto)e^{-tk_{d,1}} + \propto e^{-tk_{d,2}}\right)$ where the fractional contribution $\alpha < 1$, A is the pre-exponential constant, t is time and k_d is the disassociation rate. The goodness of fit was evaluated by considering the weighted residual deviation from the model. Least squared minimization was performed using trust-region-reflective algorithm and custom written code in MatLab 2016a. The decay curves from the high and low immobilization chips were analysed separately.

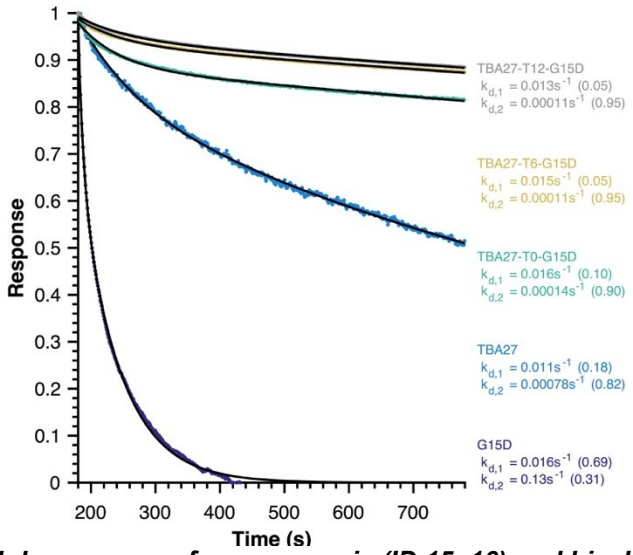
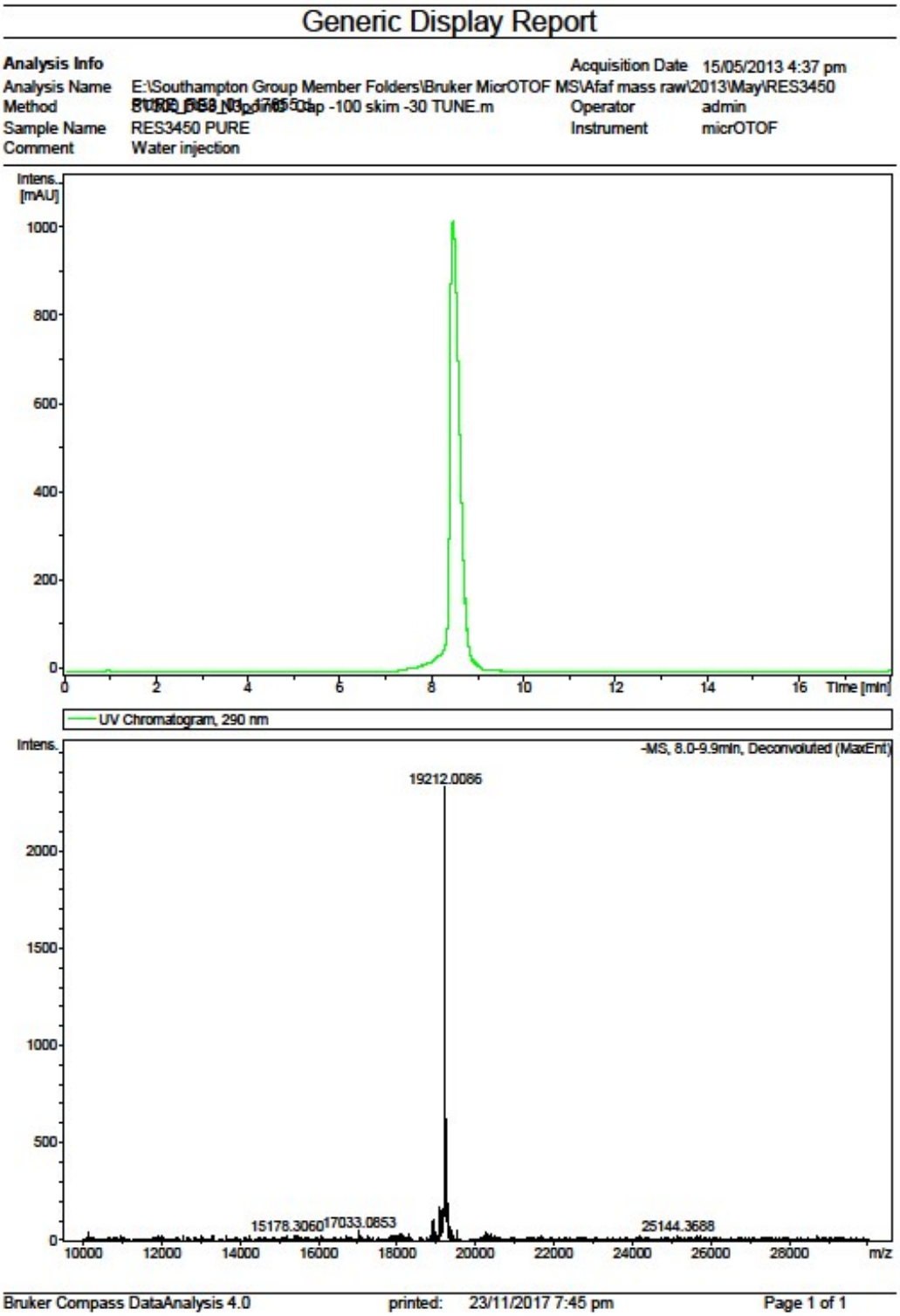
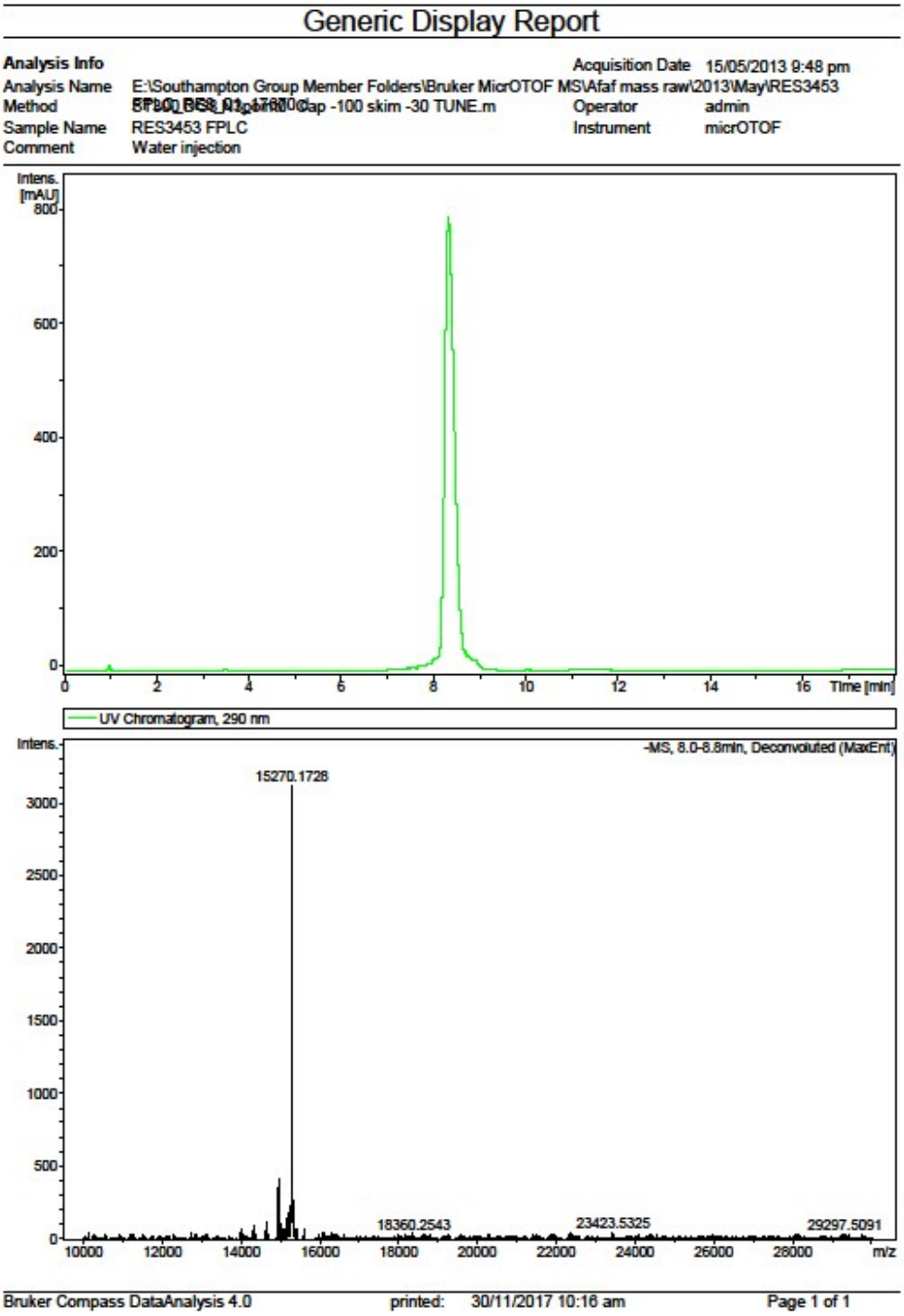


Fig S14: Average normalised decay curves for monomeric (ID 15, 16) and bivalent aptamers (ID 20-22) from surface plasmon resonance using a high immobilisation surface.
The aptamers and raw data are colour-coded, with the modelled fit to a bi-exponential decay in black. The fractional contribution of each disassociation component is in brackets. Immobilized biotinylated human alpha-thrombin = 2452.4 RU

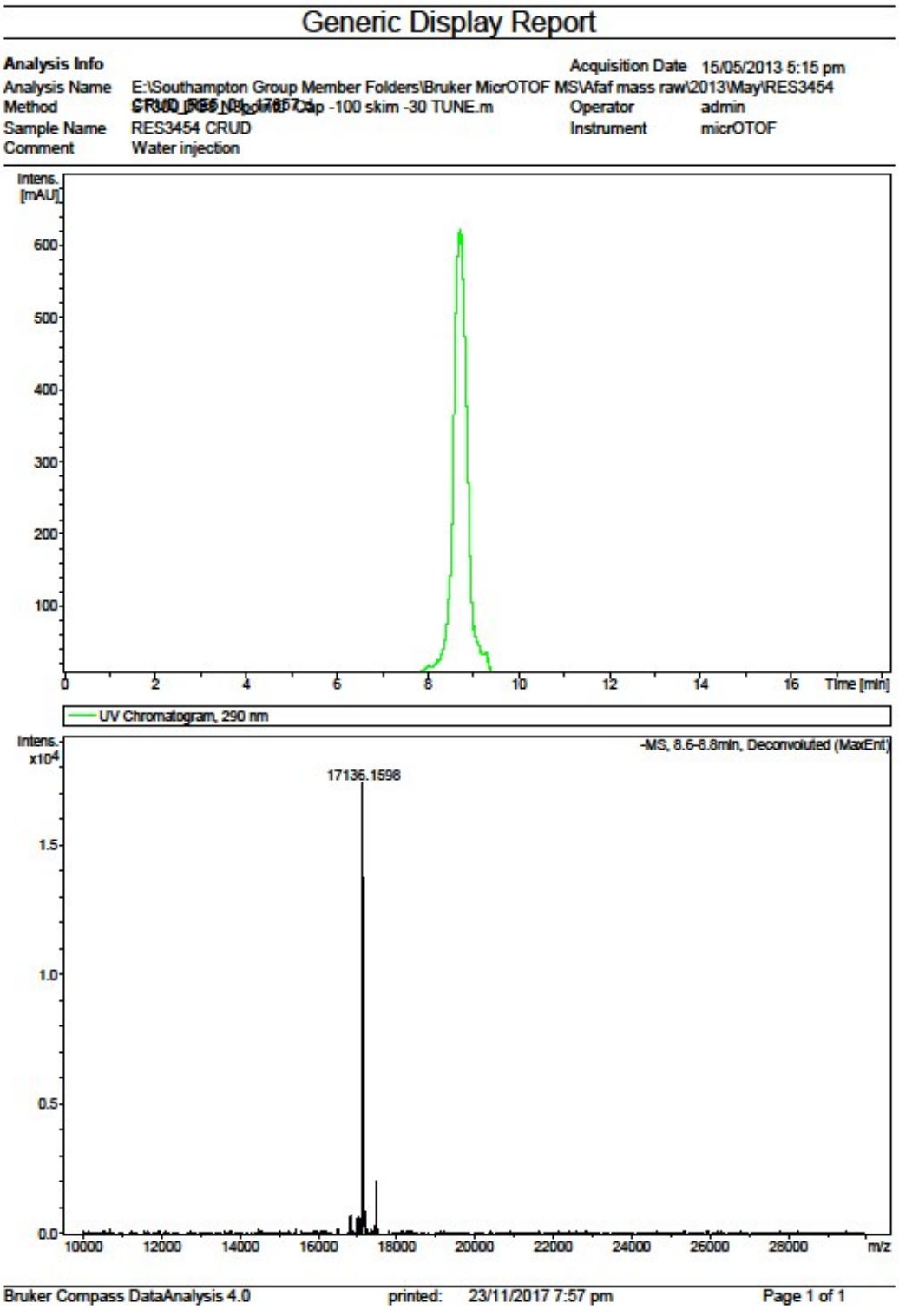
Mass spectrometry and UV analysis of modified oligonucleotides used in this study:



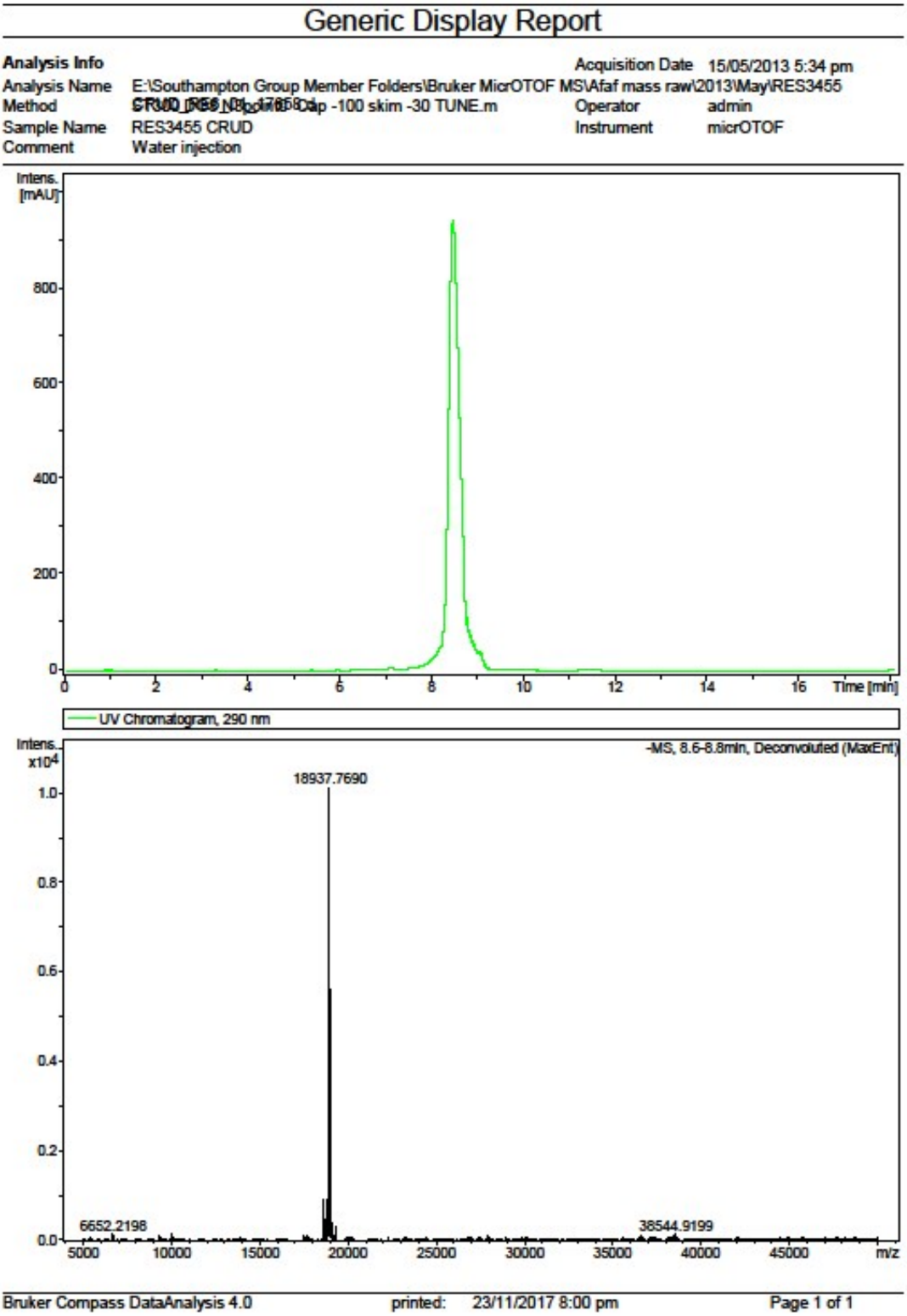
Mass spectrum and UV analysis of oligonucleotide 1 in table S1. Calculated; 19212, found; 19212.



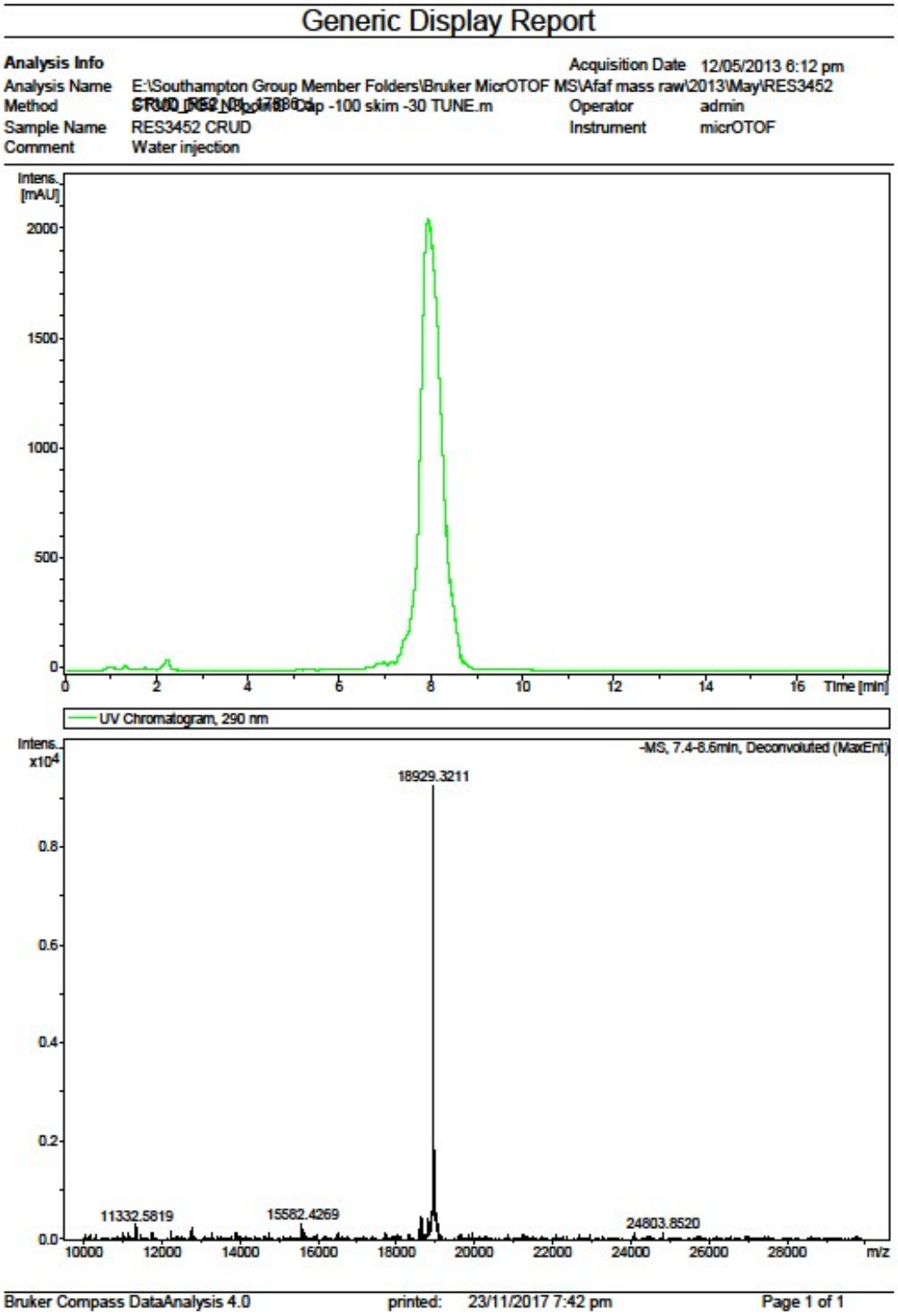
Mass spectrum and UV analysis of oligonucleotide 2 in table S1. Calculated; 15271, found; 15270.



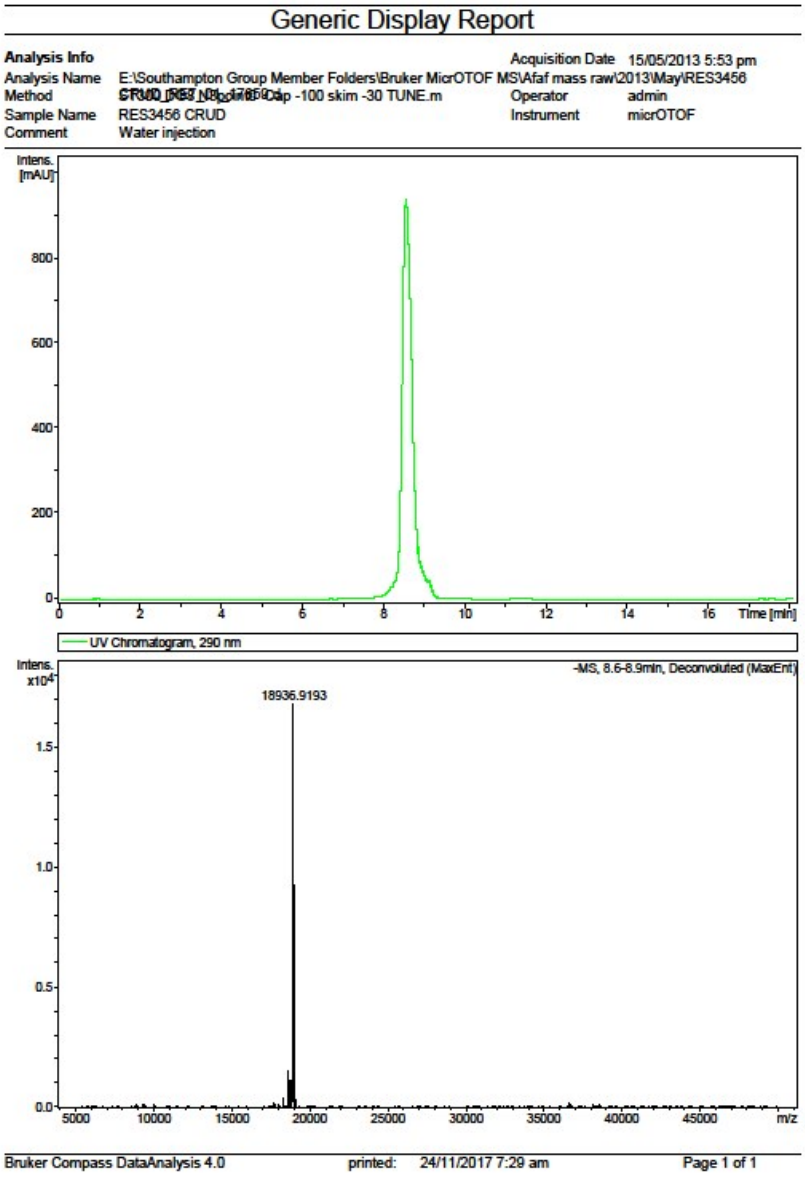
Mass spectrum and UV analysis of oligonucleotide 3 in table S1. Calculated; 17136, found; 17136.



Mass spectrum and UV analysis of oligonucleotide 4 in table S1. Calculated; 18937, found; 18937.



Mass spectrum and UV analysis of oligonucleotide 5 in table S1. Calculated; 18930, found;18929.



Mass spectrum and UV analysis of oligonucleotide 6 in table S1. Calculated; 18936, found; 18936.

References

[1] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853-2855.

[2] A. H. El-Sagheer, A. P. Sanzone, R. Gao, A. Tavassoli, T. Brown, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 11338–11343.

[3] R. D. Langdell, R. H. Wagner, K. M. Brinkhous, *J. Lab. Clin. Med.* **1953**, *41*, 637-647.

[4] A. J. Quick, M. Stanley-Brown, F. W. A. Bancroft, *Am. J. Med. Sci.* **1935**, *190*.