In Vitro Selection of Diversely-Functionalized Aptamers

Supporting information

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Supporting Information

Supporting Methods

General Information

Unless otherwise noted, water was purified with the ELGA Flex 3 purification system. DNA oligonucleotides without amine modifications were purchased from Integrated DNA Technologies. DNA oligonucleotides with amine modifications were synthesized using a Bioautomation Mermade 12 synthesizer. All materials and reagents used for oligonucleotide synthesis were purchased from Glen Research. All oligonucleotides were synthesized and deprotected according to the manufacturer's protocols. Oligonucleotides were purified by reverse-phase high-performance liquid chromatography (HPLC, Agilent 1260) using a C18 stationary phase (Eclipse-XDB C18, 5 µm, 9.4 x 200 mm) and an acetonitrile/100 mM triethylammonium acetate gradient. Oligonucleotide concentrations were determined by UV spectroscopy using a Nanodrop ND2000 spectrophotometer. Thrombin agarose slurry (EMD Millipore™ Thrombin, Immobilized, Human Plasma, Protein) was purchased from EMD Millipore™. Thrombin (HCT-0020 Human alpha-Thrombin) used for MST and SPR was purchased from Haematologic Technologies.

DNA sequences

The sequences below are written from 5'→3'
<Aam> = Amino-modifer C6 dA
<N>=A/T/C/G

Templates

LOOPERtemp: /5BioTinTEG/GA TTC GCC TGC CGT CGC ANN NNT NNN NTN NNN TNN NNT NNN NTN NNN TNN NNT NNN NTC ACG TGG AGC TCG GAT CC
bt-tempTBL1: /5BioTinTEG/GA TTC GCC TGC CGT CGC AAG CAT CGA ATA GGA TTG GAT AGG TTG GAA TTA GAT GCA GTC ACG TGG AGC TCG GAT CC
tempTBL1: GAT TCG CCT GCC GTC GCA AGC ATC GAA TAG GAT TGG ATA GGT TGG AAT TAG ATG CAG TCA CGT GGA GCT CGG ATC C

Primers

Poly_pr1: /5Phos/TG CGA CGG CAG GCG AAT C
Poly_pr2: GGA TCC GAG CTC CAC GTG
bt-prA: /5BioTinTEG/GA TTC GCC TGC CGT CGC A
PEG-prB: AAC AAC AAC AAC AA/iSp18/ GGA TCC GAG CTC CAC GTG
bt-prB: /5BioTinTEG/ GG ATC CGA GCT CCA CGT G
prD705: CAA GCA GAA GAC GGC ATA CGA GAT ATT CAG AA GTGACTGGAGTTCAG ACG TGT GCT CTT CCG ATCT GA TTC GCC TGC CGT CGC A
prC: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGG ATC CGA GCT CCA CGT G

Pentanucleotides

ANNNN: /5Phos/ANNNN
NH2-ANNNN: /5Phos/<Ama>NNNN

Synthesis of amino-modified pentanucleotides

Pentanucleotides were synthesized on a Mermaid 12 DNA synthesizer using a DMT-ON protocol on a 1 µmol scale (1000 Å CPG column). Amine-modified C6 dA (Glen Research 10-1089), dA+dC+dG+dT-CE Phosphoramindite (Glen Research 10-1000, 10-1010, 10-1020, 10-1030), Chemical Phosphorylation Reagent II (10-1901) were incorporated as specified by the manufacturer. Following synthesis, the oligonucleotide was cleaved from the resin by incubation at 25°C in 400 µL of a 1:1 mixture of ammonium hydroxide and methylamine for 25 minutes. The cleaved resin was filtered away, then incubated at 60°C for 30 minutes to remove the protecting groups on the phosphoramidites. The oligonucleotide was concentrated under reduced pressure using a speedvac. The residue was then taken up into 100 µL of H_2O, and purified using reverse-phase HPLC purification using a [10% acetonitrile in 0.1 M TEAA, pH 7] to [80% acetonitrile in 0.1 M TEAA, pH 7] solvent gradient with a column temperature of 45°C. The purified oligonucleotide was then incubated at room temperature in 1 mL of 40% aqueous acetic acid for 1 hour to cleave the DMT group, and then frozen and lyophilized. The oligonucleotide was incubated in 500 µL 30% ammonium hydroxide at room temperature for 15 minutes to cleave the CPRII linker. Following deprotection, the oligonucleotide was concentrated under reduced pressure using a speedvac. The dried product was dissolved into 100 µL H_2O and subjected to reverse-phase HPLC purification using a [10% acetonitrile in 0.1M TEAA, pH 7] to [80% acetonitrile in 0.1M TEAA, pH 7] solvent gradient with a column temperature of 45°C. The purified oligonucleotide was dissolved in water.

Functionalization of amino-modified pentanucleotides

A mixture of 25 µL carboxylic acid (100 mM in DMSO), 25 µL N-hydroxysuccinimide (NHS, 100 mM in 1:1 mixture of DMSO and H_2O), 5 µL 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 100 mM in DMSO) and 7.5 µL DMSO was incubated at room temperature for 30 minutes. This is followed by addition 7.5 µL of amino modified pentanucleotides (10 nmol in H_2O) and 30 µL Na_2CO_3 buffer (500 mM in H_2O, pH 9). The mixture was incubated at room temperature overnight with vortex. The reaction was then quenched by addition of 15 µL Tris-HCl buffer (500 mM, pH 8 in H_2O) at room temperature for one hour. Functionalized pentanucleotide was lyophilized and then purified with HPLC purification using a [10% acetonitrile in 0.1 M TEAA, pH 7] to [80% acetonitrile in 0.1 M TEAA, pH 7] solvent gradient with a column temperature of 45°C. The purified pentanucleotide was dissolved in water and characterized by mass spectrometry.

The acid-modified pentamer (A^{xR}NNCT) is synthesized with an anhydride instead of a carboxylic acid. A mixture of 25 µL succinic annhydride (100 mM in DMSO), 25 µL 1M $NaHCO_3$, 163 µL DMSO, 22.4 µL water and 14.6 µL of amino modified pentanucleotides (25 nmol in H_2O) was incubated at room temperature overnight with vortex. The reaction was then quenched by addition of 50 µL Tris-HCl buffer (500 mM, pH 8 in H_2O) at room temperature for one hour. Functionalized pentanucleotide was lyophilized and then purified with HPLC purification using a [10% acetonitrile in 0.1 M TEAA, pH 7] to [80% acetonitrile in 0.1 M TEAA, pH 7] solvent gradient with a column temperature of 45°C.

Each of the 16 pentanucleotides was dissolved to 480 µM in H_2O. Mixtures were assembled with equal volumes of each pentanucleotide.

SELEX protocol

In a PCR tube, a mixture of 1.5µL biotinylated DNA template (LOOPERtemp, 10 µM in H_2O), 2.25 µL Poly_Pr1 (10 µM in H_2O), 2.25 µL Poly_Pr2 (10 µM in H_2O), 5µL ligation reaction buffer 4X (40 mM $MgCl_2$, 24% w/v PEG6000, 40 mM DTT, 264 mM Tris pH 7.6), 2 µL ATP (0.25 mM in H_2O) and 4 µL H_2O was heated to 90 °C for 2 minutes and then cooled to 25°C at a rate of 0.1°C/s. This was followed by the addition of 1 µL 256-membered pentanuclotide mixtures (480 µM in H_2O), 1µL BSA (2 mg/mL in H_2O) and 400U T4 DNA ligase (New England Biolabs, M0202L). The mixture was allowed to polymerize for 24 hours at 25 °C. The products were then purified with a MinElute® PCR Purification Kit before strand separation.

For the first round of SELEX, LOOPER was done on a 120 pmol template scale. 8X reagents were mixed and then split into 8 aliquots. All subsequent rounds were done on a ~15pmol scale. Purified LOOPER product was strand separated: after 3 prewashes with 1X SA-Bt buffer (40 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% TWEEN-20), 5µL streptavidin beads (Dynabeads® MyOne™ Streptavidin C1) were added to the purified polymerization product. Following 30 minutes of incubation on a rotator, the complexes were washed 3 times and then eluted with 40 µL 150 mM NaOH. After adding 4µL 1.5 M HCl to neutralize the elution, Princeton separation was used to purify the elution.

Aptamer renaturation was performed on the purified strand separation product. After adding 11 µL 5X SELEX buffer to make final concentration 1X (20 mM Tris-HCl pH 7, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$), the aptamer was heated to 90°C for 4 minutes, snap-cooled on ice for 10 minutes and then maintained at room temperature for 30 minutes. 1 minute of centrifugation at 2000 RPM was used for prewash, wash and elution during the selection process. 50 µL human plasma thrombin immobilized agarose slurry (EMD Millipore™ Thrombin, Immobilized, Human Plasma, Protein) was thrice prewashed using 1X SELEX Buffer. Renatured product was then added to the thrombin agarose slurry. The complexes were incubated on a rotator at room temperature for 15 minutes. Another three washes were done after incubation. Elution buffer (40 mM Tris-HCl pH 8, 10 mM EDTA, 3.5 M urea and 0.02% TWEEN20) was added to the complexes followed by 4 minutes of heating at 90°C. Urea was removed from the elution product by Princeton Separation.

Purified selection winners were amplified by qPCR. 10 µL DNA was added to 40 µL qPCR Mix (Final concentration: 1X KOD DNA Polymerase Buffer, 1.5 mM $MgCl_2$, 0.5 µM bt-prA, 0.5 µM PEG-prB, 1X SYBR Green, 1 U KOD Hotstart DNA Polymerase, and 0.2 mM each dNTP). The mixture was amplified in a qPCR instrument (BioRad MiniOpticon Real-Time PCR System) with the following protocol: 95°C for 3 minutes, 95°C for 20 seconds, 54°C for 10 seconds, 70°C for 10 minutes, 95°C for 20 seconds (denaturation), 54°C for 10 seconds (annealing), 70°C for 1 second (extension), followed by a plate read at 70°C and a return to the denaturation step.

After qPCR, the biotinylated template was recovered by strand separation and gel purification. Biotinylated strand recovery from streptavidin beads was done after the PEG (complementary) strand was eluted with NaOH. Release immobilized biotinylated DNA with 2 minutes at 90°C in 10 mM EDTA pH 8.2 with 95% formamide. MinElute® PCR Purification Kit was used to remove any streptavidin from the released biotin strand. 10% denaturing PAGE was used to further purify the recovered biotin strand. Gel purified biotinylated template was added to the LOOPER in the next SELEX cycle.

Remelting curves

Diversity loss during SELEX be monitored by the default melting function of a qPCR instrument.[1] In the event of a successful cycle of SELEX, library diversity should decrease thus shifting the melt curve (**Figure S1**).

Sample preparations for High-Throughput DNA sequencing

The Round 6 winner was qPCR amplified with bt-prA and PEG-prB as mentioned in selection protocol. Barcode primers (prD705 and prC) were attached by another qPCR amplification. A mixture of 50 amol purified adapter ligation product in 10 µL H_2O, 1.25 µL 10 µM prC, 1.25 µL 10 µM corresponding prD705 primer, and 12.5 µL Q5® High-Fidelity 2× Master Mix (New England Biolabs) was transferred to a preheated themocycler (98°C). The first two PCR cycles were 10 seconds at 98°C (denaturation), 30 seconds at 55°C (annealing), and 30 seconds at 72°C (extension). For cycle 3 and onwards, annealing was changed to 71 °C for 30 seconds. The PCR products were then gel purified. The concentrations of gel purified samples were determined with Kapa library quantification kit for Illumina libraries (KK4845) on a Roche LightCycler 480. Paired-end Illumina sequencing was performed on an Illumina MiSeq system using the kit v2 with 300 cycles (150 bp PE sequencing) at the Georgia Genomics Facility at The University of Georgia in Athens, GA, USA.

Sequencing data analysis

Sequencing data was analyzed (**Table.S1**) by FASTAptamer.[2] Top 15 winners' random region sequences (**Figure S2**) were aligned by Weblogo.[3] Error rate (**Table S2**) and modifications analysis (**Figure S4**) was done using a custom Python2.7 script. The source of which is available at https://github.com/waylandy/LOOPalyzer.

MST protocol

There are two categories of Nanotemper MST instruments: labeled and label-free. For nucleic acids, Nanotemper recommends a labeled MST instrument (such as the Monolith NT.115). Labeled MST instruments require fluorescent tags such as Cy5 to be attached to the oligo while label-free type MST utilizes tryptophan fluorescence from the target protein. Different nucleic acid concentration will have some influence in tryptophan detection. Ideally, labeled MST should be used for characterization of nucleic acid binding affinity to avoid inhomogeneity caused by nucleic acid concentrations in label-free. Due to facility limitations, we used label-free. MST (Monolith NT.LabelFree) provided preliminary data for binding affinity and specificity (**Figure S3**). Bt-tempTBL1 was used and strand separation was done to generate an unbiotinylated version of modTBL1 (the winning LOOPER-modified sequence from selection). 20 µL of 100 nM renatured modTBL1 was used for serial dilutions. 10 µL 50 nM thrombin was added to each dilution series. MST was done with medium MST power and 40% excitation power.

SPR protocol

TempTBL1 and bt-prB were used for generating the double-stranded polymerization product. As previously mentioned, the biotin-strand recovery method was used to recover the biotinylated version of modTBL1.

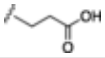
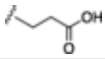
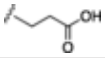
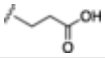
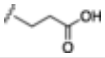
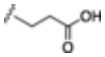
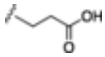
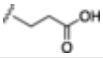
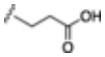
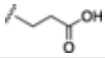
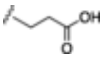
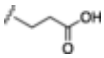
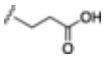
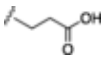
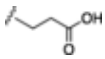
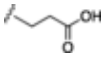
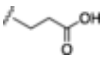
A Biacore T100 and Biotin CAPture Kit (GE Healthcare Life Sciences) were used in SPR interaction studies[4]. Sensor chip CAP was conditioned and regenerated according to manufacturer's specifications before initial use. Sensor chip CAP consists of a carboxymethylated dextran matrix modified with a pre-immobilized oligonucleotide. Complementary oligonucleotide conjugated with streptavidin (Biotin CAPture Reagent) was added later to build a streptavidin surface with an increase of ~3000 RU. A new sensor chip CAP would be used if the capture capacity (RU levels) decreased significantly. A reference channel was also built with streptavidin surface according to Biotin CAPture Kit specifications.

50 nM biotinylated modTBL1 in running buffer (identical to SELEX Buffer) was added at flow rate of 5 µL/min for 300 seconds with a net increase of 100~200 RU. Thrombin was serially diluted in running buffer to a concentration range of 5/10/20/30/50 nM and injected at 21°C at a flow rate of 5µL/min for 300 seconds and dissociated for 600 seconds. Single-cycle-kinetics mode[5] was used with thrombin injection from lowest concentration to highest concentration. Surface regeneration at the conclusion of each session as per Biotin CAPture Kit specifications. Using SPR of the same conditions as modTBL1, binding specificity was also evaluated for modTBL1 for BSA, unmodified winner sequence for thrombin, and complementary of the winner sequence for thrombin. 5 nM biotinylated modTBL2~5 were added at flow rate 5 µL/min for 300 seconds with a net increase of ~25 RU. Thrombin was serially diluted in running buffer to a concentration range of 0.5/2/5/10/20 nM and injected at 21°C at a flow rate of 5µL/min for 300 seconds and dissociated for 600 seconds.

Supporting Tables

Table S1. Top 15 reads: a) all primer regions removed and functional groups shown, b) sequences with Illumina primers removed.

a)

Name	Reads	(ANNNN)x8 Reading Region							
		pos1	pos2	pos3	pos4	pos5	pos6	pos7	pos8
TBL1	9525	ACTGC	ATCTA	ATTCC	AACCT	ATCCA	ATCCT	ATTCG	ATGCT
			*i*Bu			Bn			
TBL2	5596	AGTTT	ACTTA	AGTCC	AACCT	ACCCA	ACCTT	ACTTA	AGTCT
		*i*Pr	*i*Bu			Bn	*i*Pr		
TBL3	5445	ATGTC	ATCGC	ATTCC	AACCT	AATCC	ATCCT	ATGCG	ATAAT
									
TBL4	3021	ATGCT	AGTGC	AATCC	ACCCT	ACCCA	AACCT	ATTGC	ACCTT
						Bn			*i*Pr
TBL5	2796	AACCT	AAACT	ATCCC	ATCCT	ATCCA	ACCTA	AGTTT	AGTGT
						Bn	*i*Bu	*i*Pr	
TBL6	2657	ATTGA	ATCGA	ACCCA	ACCTG	ACCAA	ACCTT	ATTCG	ATTTT
		MOM	MOM	Bn		*n*Bu	*i*Pr		*i*Pr
TBL7	2526	AACAT	ACTTC	AGACC	ATCCT	ACCCA	AGCCT	ACTGA	AGTTT
						Bn		MOM	*i*Pr
TBL8	2500	ATGCA	ATCTC	ATACC	AACCT	ATCCA	ACCTT	ACTCT	ATTGC
		Bn				Bn	*i*Pr		
TBL9	2466	AATGT	ACGTA	AGACC	ATCCT	ACCCT	ACCAT	ATCTT	ACGTT
			*i*Bu					*i*Pr	*i*Pr
TBL10	2460	ATTTC	ATTCG	ATCCC	AACCT	ATCCA	ACCTT	ATCTC	ATGTT
						Bn	*i*Pr		*i*Pr
TBL11	2357	ACATG	ACTCA	ATTCC	AACCT	ACCCT	ACCTT	ATTTG	AGTCG
			Bn				*i*Pr		
TBL12	2351	ACTCT	ATCAA	ACTCC	AACCT	AACCA	ACCTA	ATTTG	ATCTT
			*n*Bu			Bn	*i*Bu		*i*Pr
TBL13	2292	AATGA	ATGTC	ACCCA	AACCT	AACCA	ACCTT	ATGCC	ATTCT
		MOM		Bn		Bn	*i*Pr		
TBL14	2111	AACTG	ACTGC	ATTCC	AACCC	AAGCC	AACCT	ATGTC	AGTTT
									*i*Pr
TBL15	2107	ATCTG	ATGTC	ATTCC	AACCA	ATCCA	AACCT	ATGCC	ATCAG
					Bn	Bn			

b)

Name	Reads	5' Primer Region	(ANNNN)x8 Reading Region	3' Primer Region
TBL1	9525	GGATCCGAGCTCCACGTG	ACTGC ATCTA ATTCC AACCT ATCCA ATCCT ATTCG ATGCT	TGCGACGGCAGGCGAATC
TBL2	5596	GGATCCGAGCTCCACGTG	AGTTT ACTTA AGTCC AACCT ACCCA ACCTT ACTTA AGTCT	TGCGACGGCAGGCGAATC
TBL3	5445	GGATCCGAGCTCCACGTG	ATGTC ATCGC ATTCC AACCT AATCC ATCCT ATGCG ATAAT	TGCGACGGCAGGCGAATC
TBL4	3021	GGATCCGAGCTCCACGTG	ATGCT AGTGC AATCC ACCCT ACCCA AACCT ATTGC ACCTT	TGCGACGGCAGGCGAATC
TBL5	2796	GGATCCGAGCTCCACGTG	AACCT AAACT ATCCC ATCCT ATCCA ACCTA AGTTT AGTGT	TGCGACGGCAGGCGAATC
TBL6	2657	GGATCCGAGCTCCACGTG	ATTGA ATCGA ACCCA ACCTG ACCAA ACCTT ATTCG ATTTT	TGCGACGGCAGGCGAATC
TBL7	2526	GGATCCGAGCTCCACGTG	AACAT ACTTC AGACC ATCCT ACCCA AGCCT ACTGA AGTTT	TGCGACGGCAGGCGAATC
TBL8	2500	GGATCCGAGCTCCACGTG	ATGCA ATCTC ATACC AACCT ATCCA ACCTT ACTCT ATTGC	TGCGACGGCAGGCGAATC
TBL9	2466	GGATCCGAGCTCCACGTG	AATGT ACGTA AGACC ATCCT ACCCT ACCAT ATCTT ACGTT	TGCGACGGCAGGCGAATC
TBL10	2460	GGATCCGAGCTCCACGTG	ATTTC ATTCG ATCCC AACCT ATCCA ACCTT ATCTC ATGTT	TGCGACGGCAGGCGAATC
TBL11	2357	GGATCCGAGCTCCACGTG	ACATG ACTCA ATTCC AACCT ACCCT ACCTT ATTTG AGTCG	TGCGACGGCAGGCGAATC
TBL12	2351	GGATCCGAGCTCCACGTG	ACTCT ATCAA ACTCC AACCT AACCA ACCTA ATTTG ATCTT	TGCGACGGCAGGCGAATC
TBL13	2292	GGATCCGAGCTCCACGTG	AATGA ATGTC ACCCA AACCT AACCA ACCTT ATGCC ATTCT	TGCGACGGCAGGCGAATC
TBL14	2111	GGATCCGAGCTCCACGTG	AACTG ACTGC ATTCC AACCC AAGCC AACCT ATGTC AGTTT	TGCGACGGCAGGCGAATC
TBL15	2107	GGATCCGAGCTCCACGTG	ATCTG ATGTC ATTCC AACCA ATCCA AACCT ATGCC ATCAG	TGCGACGGCAGGCGAATC

Table S2. Error rate analysis.

	Reads	% Reads
Total Reads	887430	100.00%
5' Primer is mutated	20899	2.36%
3' Primer is mutated	11775	1.33%
Both primers are mutated	543	0.06%
Codon 1 error	642	0.07%
Codon 2 error	493	0.06%
Codon 3 error	341	0.04%
Codon 4 error	351	0.04%
Codon 5 error	369	0.04%
Codon 6 error	397	0.04%
Codon 7 error	381	0.04%
Codon 8 error	555	0.06%
Usable Reads	850684	95.86%

The FASTQ sequencing data was analyzed. A mutated primer was defined as a primer that does not exactly match the original primer sequence. Codon errors refer to LOOPER polymerization errors but also include errors accumulated through pre-sequencing PCR amplifications. These errors were defined as pentanucleotide segments that do not match the ANNNN format and were checked in the direction of polymerization. Usable reads were defined as sequences that contained the original primer sequence as well as a reading region that follows the (ANNNN)x8 format.

Table S3. Frequency of 16 pentamers on 8 positions after 6 rounds of SELEX. Pos refers to the position number of the codon.

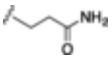
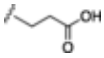
pentamer	modification	pos1	pos2	pos3	pos4	pos5	pos6	pos7	pos8	pos1-8	pos3-7
ANNAA	*n*Bu	2.40%	2.75%	1.19%	0.93%	2.02%	1.73%	1.19%	1.65%	1.73%	1.41%
ANNAC		4.83%	4.27%	2.12%	2.58%	2.37%	3.28%	3.62%	2.98%	3.26%	2.80%
ANNAG		2.55%	2.05%	0.74%	0.95%	1.30%	1.58%	2.48%	2.51%	1.77%	1.41%
ANNAT		4.14%	3.39%	2.55%	3.45%	3.93%	5.20%	3.37%	4.89%	3.86%	3.70%
ANNCA	Bn	5.68%	5.26%	11.25%	12.12%	30.47%	7.27%	3.87%	4.19%	10.01%	12.99%
ANNCC		7.07%	12.95%	45.01%	17.36%	21.17%	11.80%	7.42%	1.87%	15.58%	20.55%
ANNCG		4.95%	4.38%	2.07%	3.34%	2.28%	3.46%	6.78%	4.96%	4.03%	3.59%
ANNCT		9.08%	8.60%	9.17%	31.50%	8.95%	19.10%	10.15%	13.78%	13.79%	15.78%
ANNGA	MOM	3.42%	3.12%	1.01%	1.01%	1.13%	1.86%	2.72%	0.72%	1.87%	1.54%
ANNGC		9.15%	8.34%	2.74%	2.42%	2.47%	3.44%	7.63%	5.38%	5.20%	3.74%
ANNGG	CH_2OH	2.13%	2.23%	0.95%	1.26%	1.21%	1.61%	2.93%	2.78%	1.89%	1.59%
ANNGT		6.39%	6.42%	3.16%	3.18%	3.41%	4.76%	7.06%	11.47%	5.73%	4.31%
ANNTA	*i*Bu	4.77%	6.33%	2.36%	3.11%	2.78%	6.23%	4.84%	4.90%	4.42%	3.86%
ANNTC		12.08%	12.59%	5.62%	5.63%	5.69%	8.20%	12.52%	9.13%	8.93%	7.53%
ANNTG		9.07%	6.95%	2.98%	4.08%	3.42%	4.59%	9.89%	7.53%	6.06%	4.99%
ANNTT	*i*Pr	12.30%	10.35%	7.07%	7.08%	7.41%	15.89%	13.53%	21.28%	11.86%	10.20%

Table S4. SPR summaries from Biacore evaluation software. Sensorgrams are fit into a 1:1 binding model.

Ligand	Analyte	K_D/nM	Rmax/RU	Chi^2/ RU^2
TBL1	thrombin	1.570	205.5	8.24
tempTBL1	thrombin	306.7	176.2	12.0
TBL1	BSA	No binding	3.024	20.6
unmod-TBL1	thrombin	No binding	4.341	12.1
TBL2	thrombin	16.720	7.533	0.091
TBL3	thrombin	No binding	0.7824	0.302
TBL4	thrombin	14.446	9.465	0.119
TBL5	thrombin	29.03	14.26	0.105

Supporting Figures

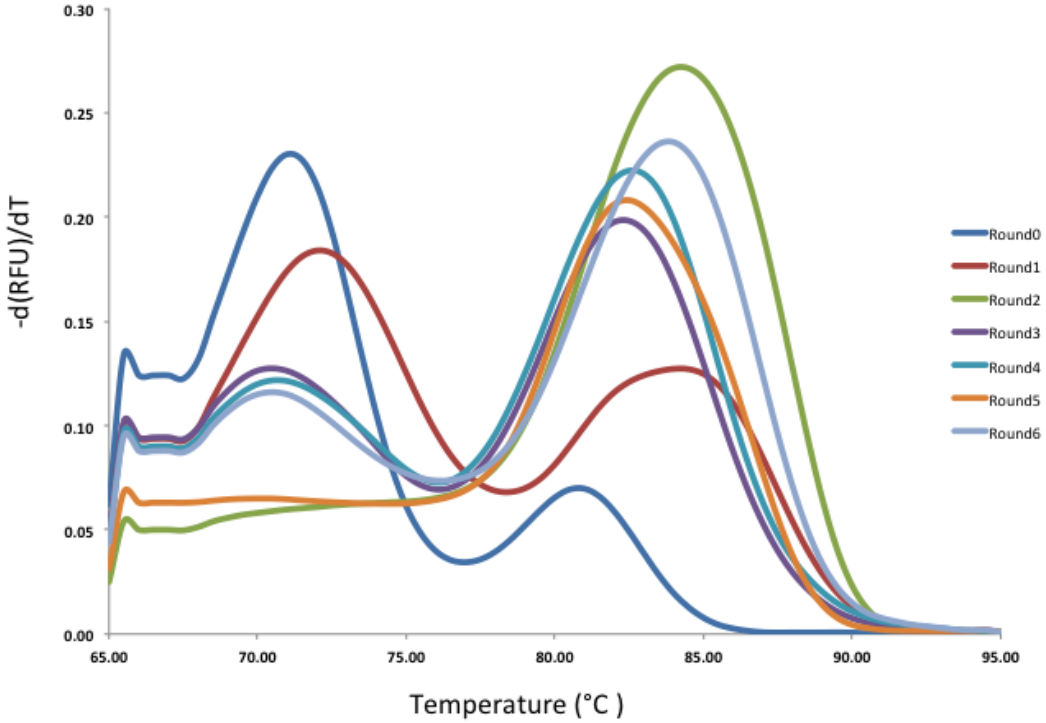


Figure S1. Remelting peaks from Round0 to Round6.

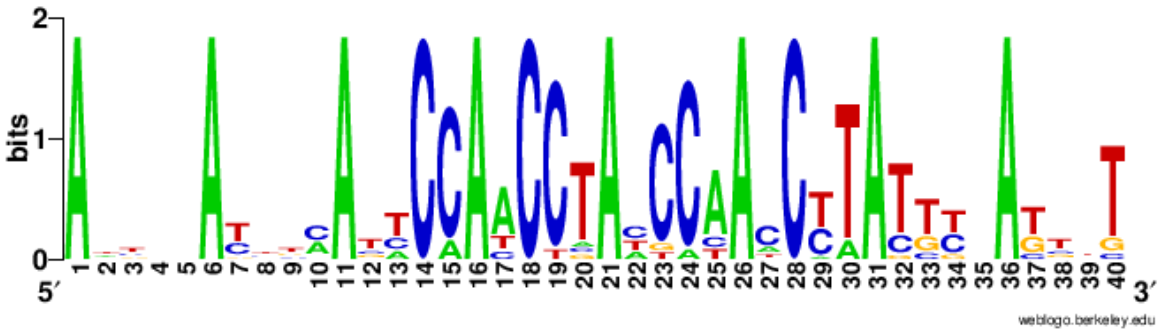


Figure S2. DNA logo of the reading frame for TBL1 to TBL15. Primer regions were cut out.

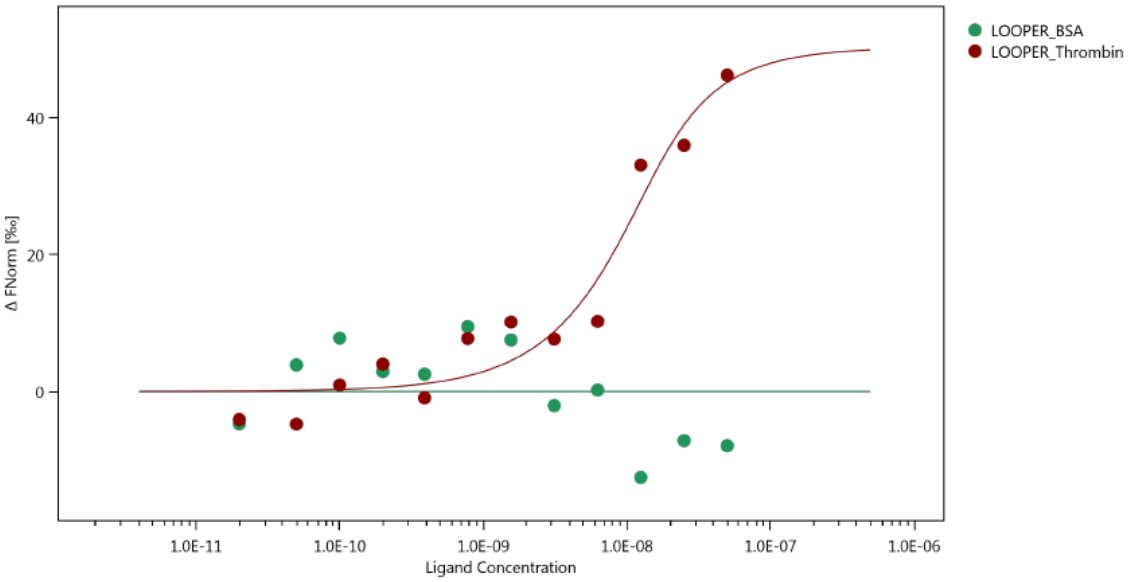


Figure S3. MST overlay of LOOPER-generated, modified TBL1 (modTBL1) for binding to thrombin (red data) and BSA (green data). ModTBL1 Kd for thrombin was measured as 4.49 nM in 1:1 binding model in Nanotemper MST evaluation software. Label-free MST instrument measures Trp thermophoresis. Thrombin and BSA have different Trp contents, which caused slightly different starting Fnorms in Y axis. Y axis starting point was set as zero in overlay version. No binding was observed in modTBL1 for BSA. SPR was used later to further confirm the results.

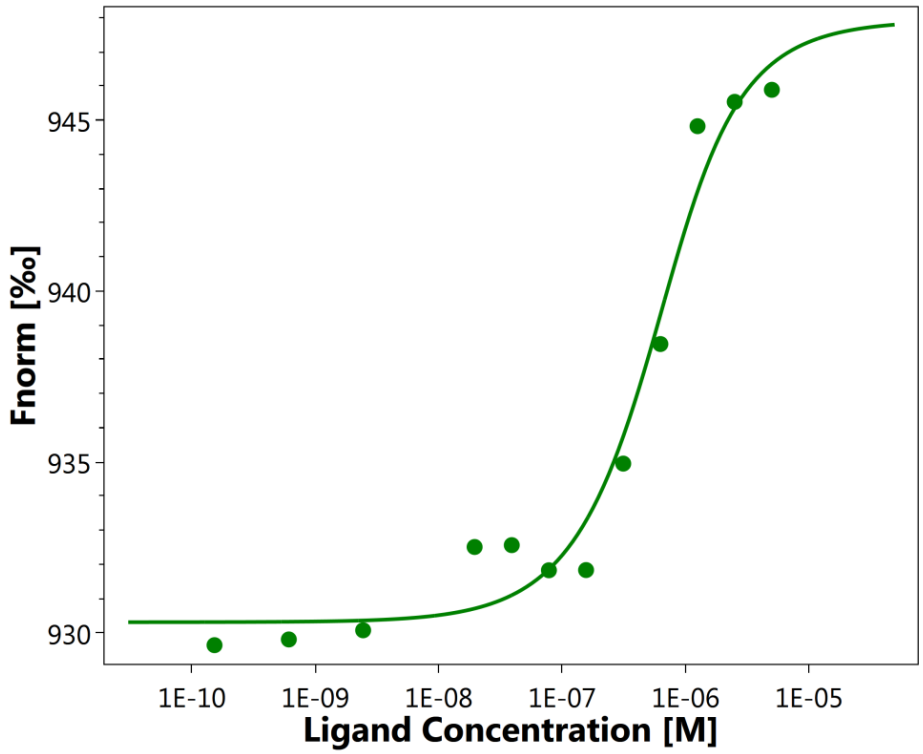


Figure S4. MST of the antisense of TBL1 (tempTBL1) binding for thrombin (green data). TempTBL1 Kd was measured as 354 nM in 1:1 binding model in Nanotemper MST evaluation software. SPR was used later to further confirm the result.

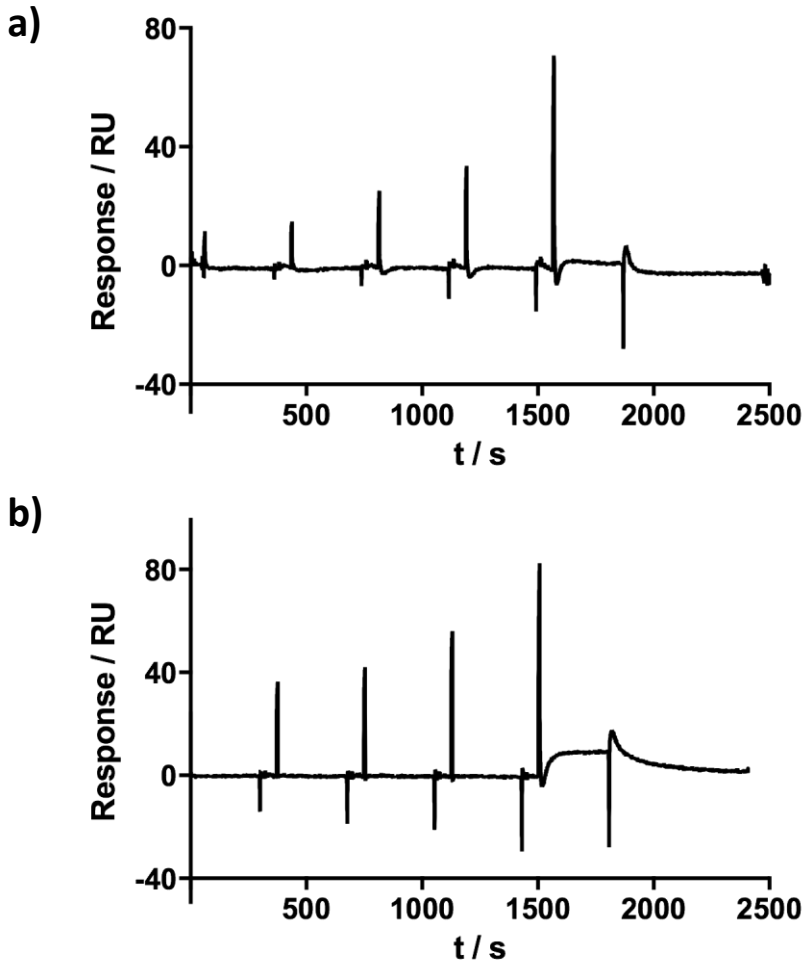
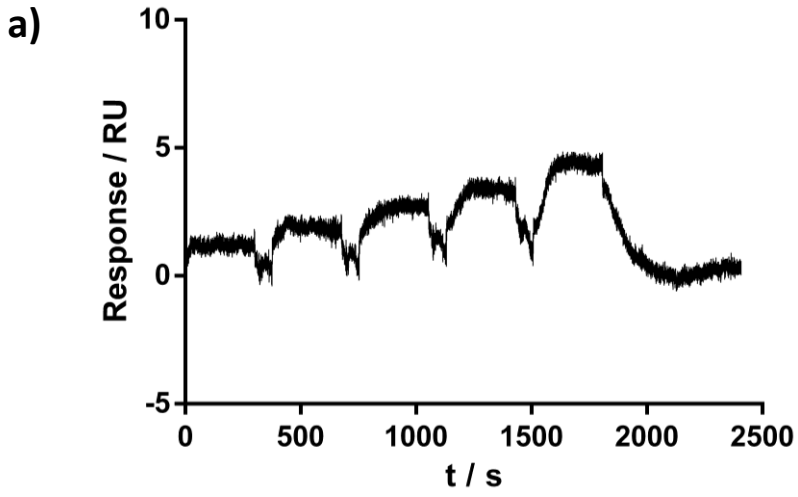


Figure S5. Single cycle kinetics SPR for a) unmodified LOOPER strand (TBL1) binding for thrombin, and b) LOOPER-generated, modified TBL1 (modTBL1) for BSA.



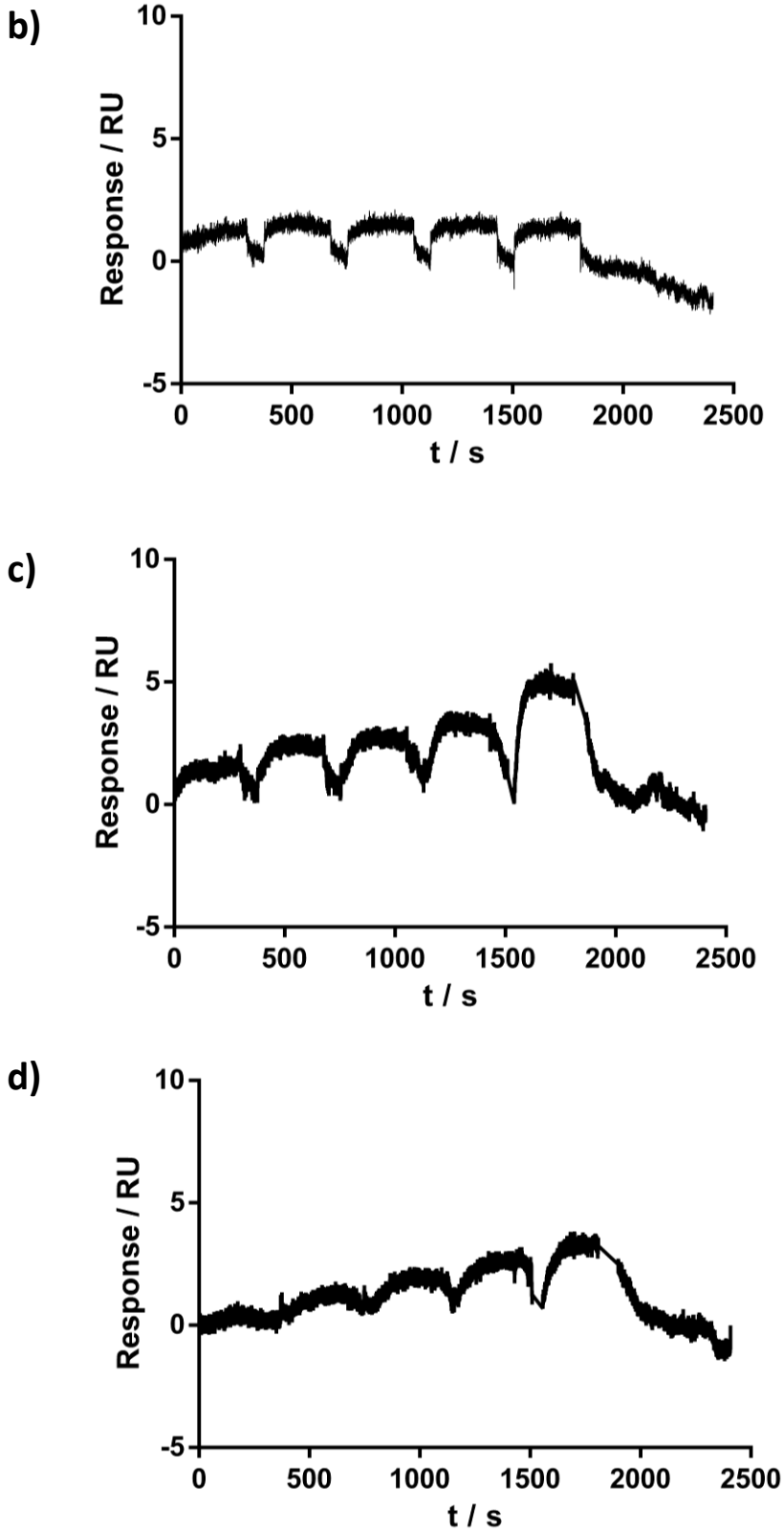


Figure S6. Single cycle kinetics SPR of select LOOPER-generated, modified aptamers for thrombin a) modTBL2 b) modTBL3 c) modTBL4 d) modTBL5. Binding affinities and chi-squared values are described in Table S4. Injection spikes were removed from sensorgrams using graphpad "exclude" function.

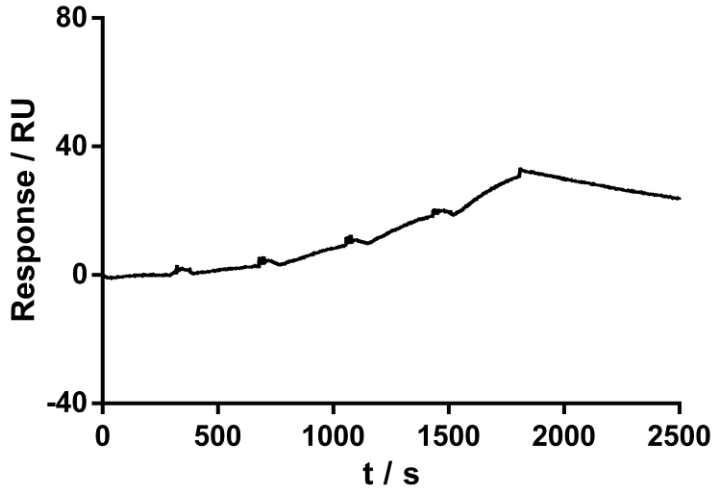


Figure S7. A duplicate experiment: single cycle kinetics SPR of the antisense of TBL1 (tempTBL1) binding for thrombin.

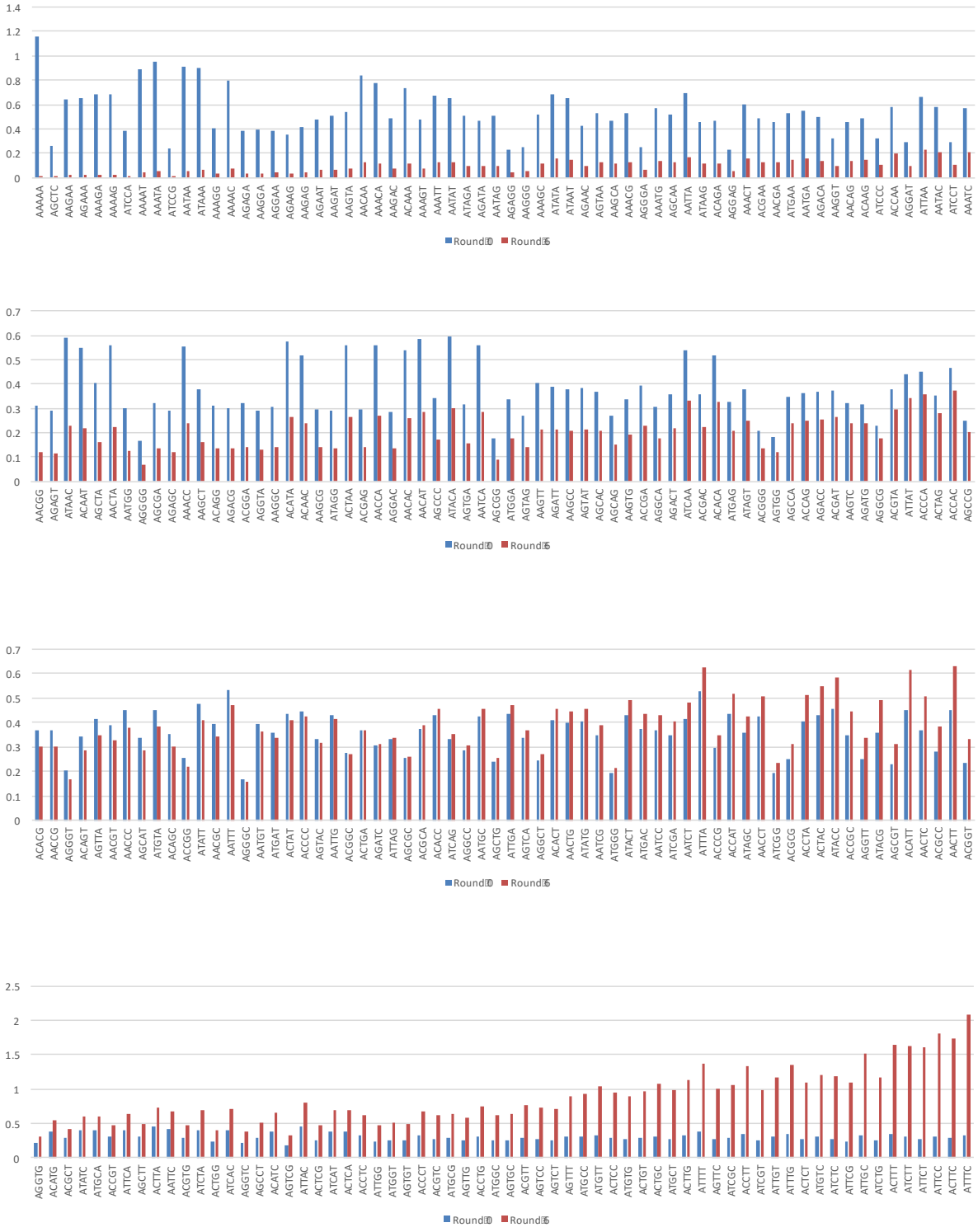
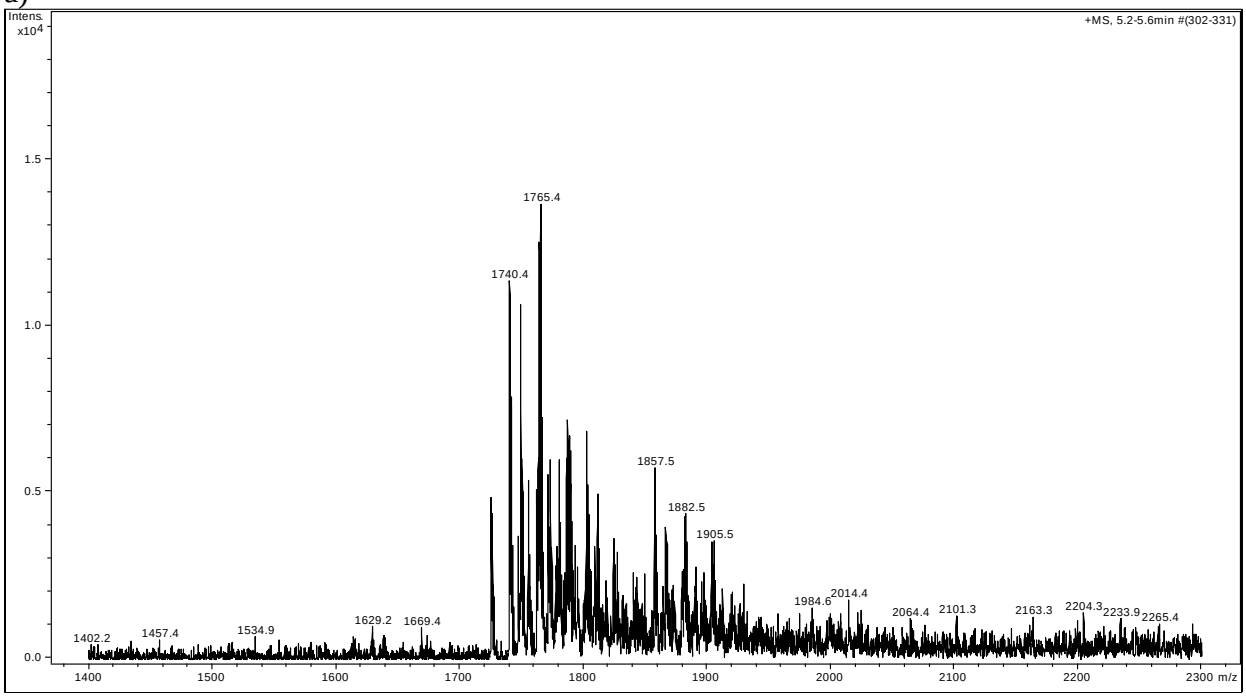


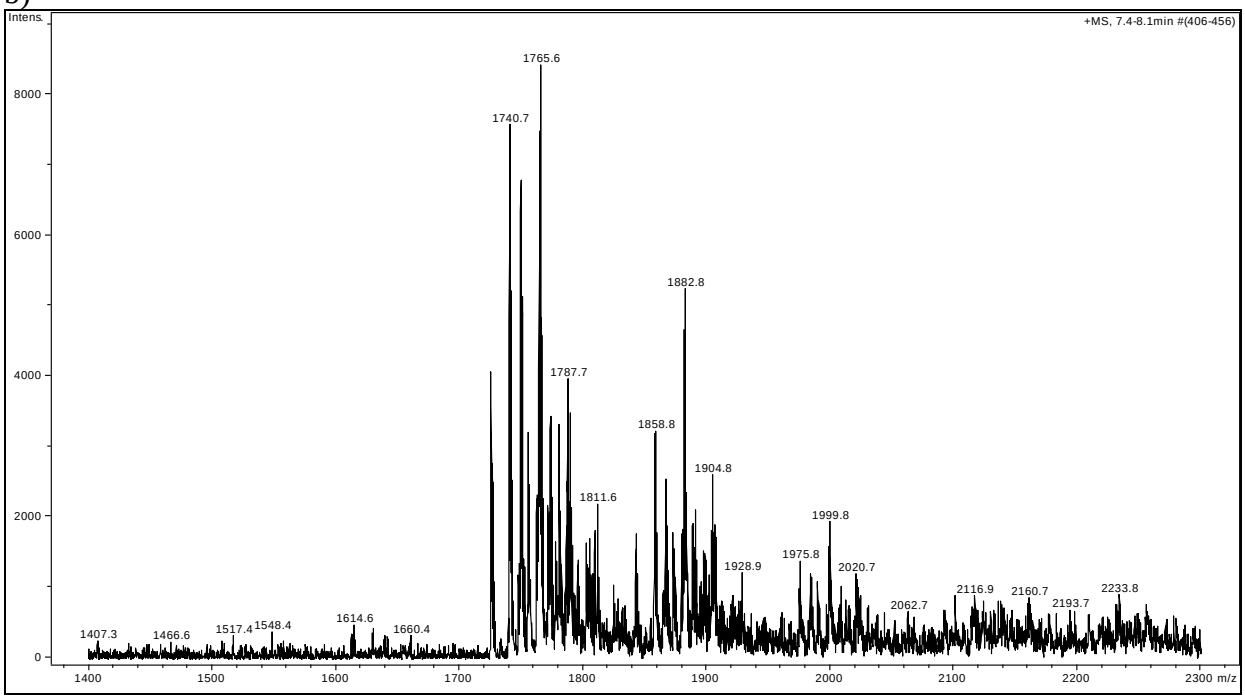
Figure S8. Comparison of building block frequencies between round 0 and round 6

Pentanucleotides were analyzed by Dr. Dennis Phillips using ESI in the UGA Proteomics and Mass Spectrometry Core Facility. Note that earlier samples were all done using ESI positive ion mode and causing a secondary set of ions about 117 m/z higher than primary ions from the adduct formation. Later we found that the second set of peaks were only formed in ESI positive ion mode and not in negative ion mode. The same batch of A^{xR}NNTA was analyzed in both modes (Figure S9). Mass spectra data of 16 different small molecule modified pentanucleotide libraries are shown in the following pages.

a)



b)



c)

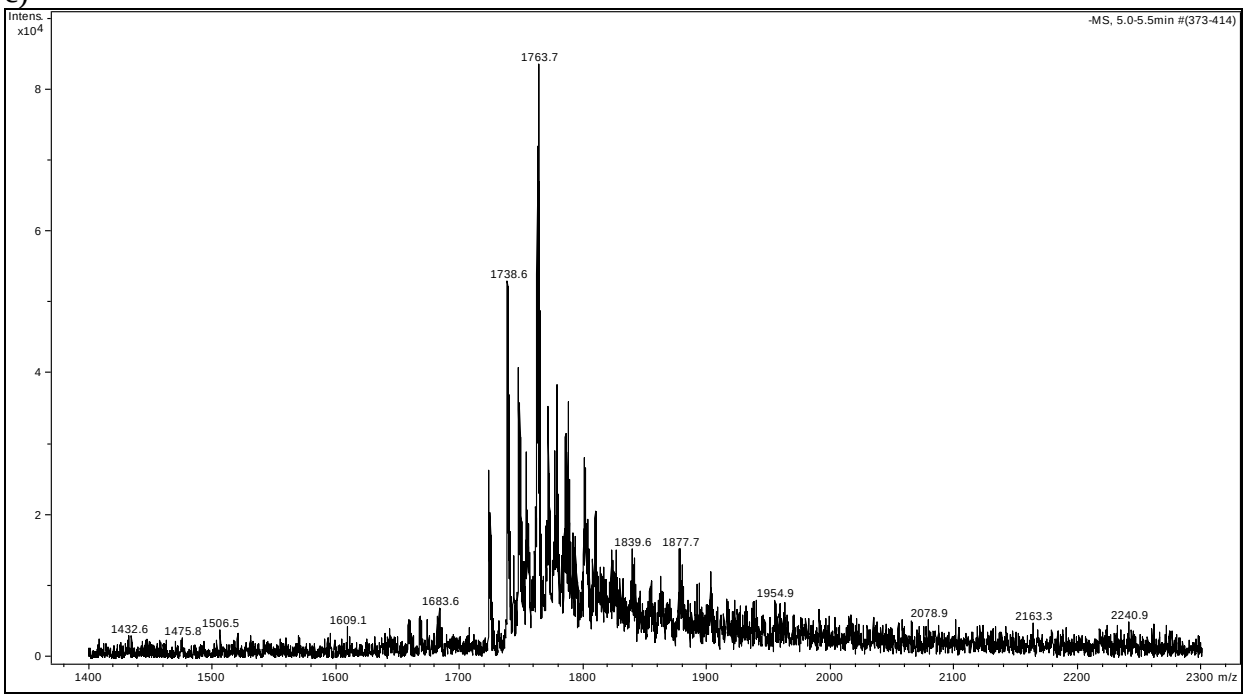
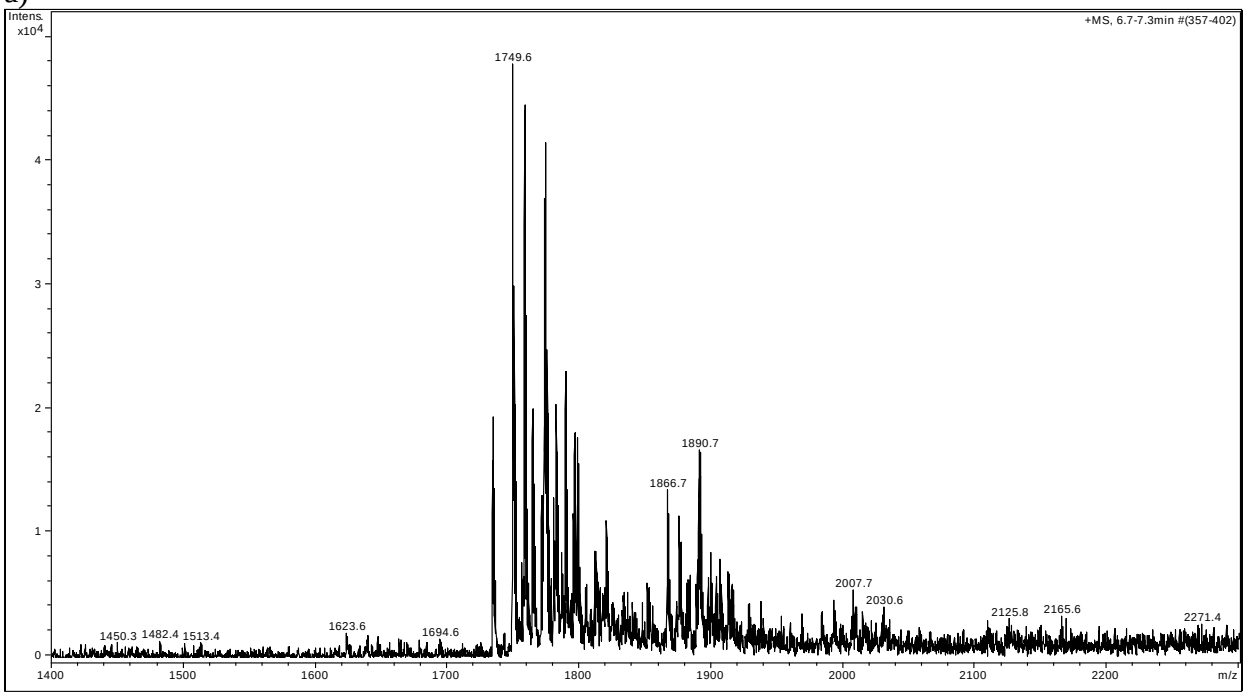


Figure S9. ESI mass spectrometry analysis for a single batch of sample ANNTA in a) positive ion mode, b) positive ion mode duplicate and c) negative ion mode.

a)



b)

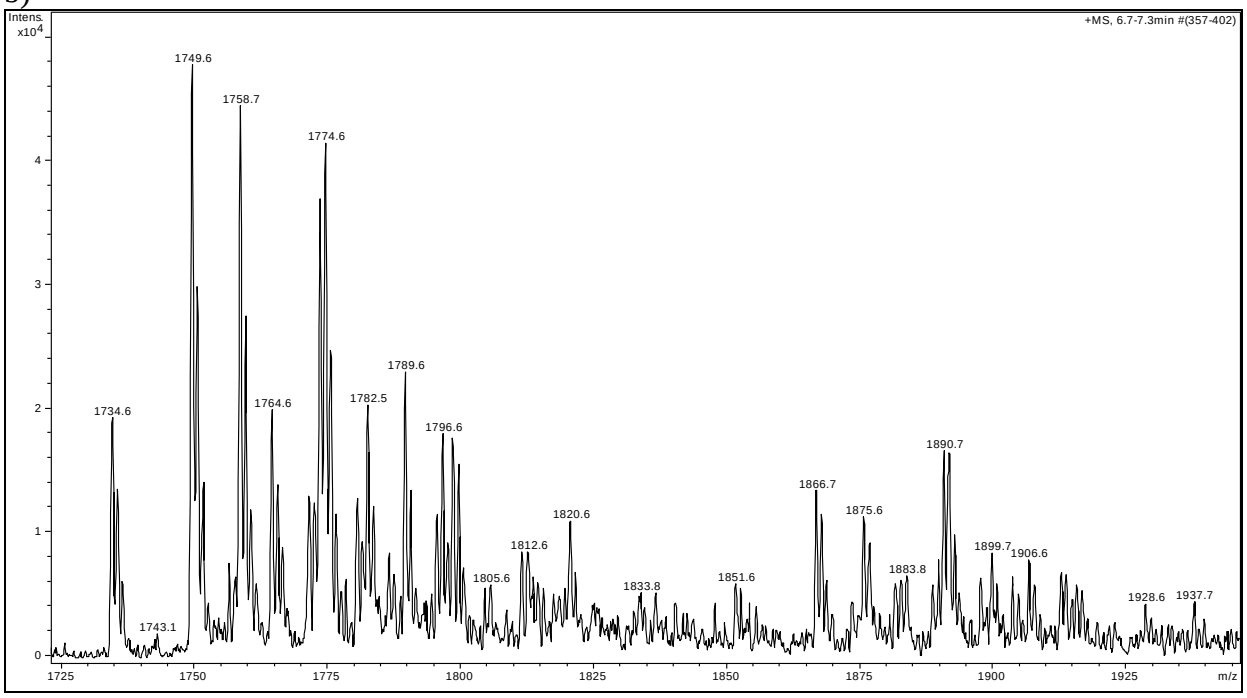
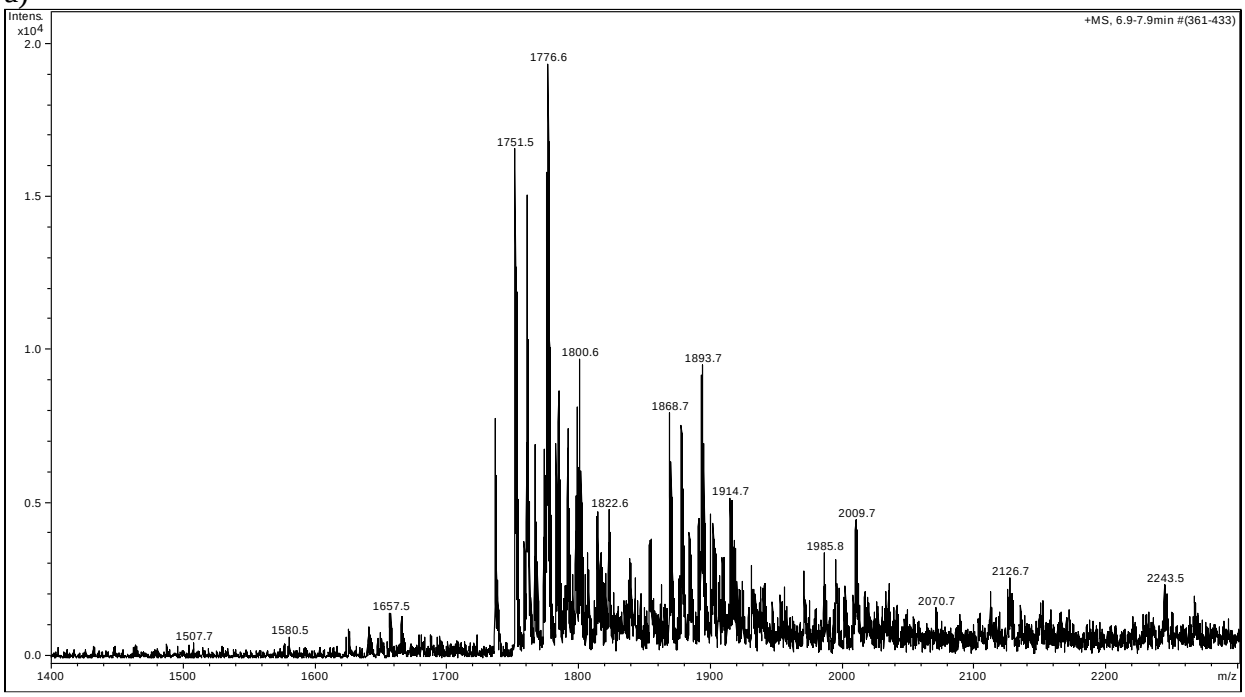


Figure S10. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNAA. Calculated range: 1733.5-1813.5 a) original graph and b) magnified version.

a)



b)

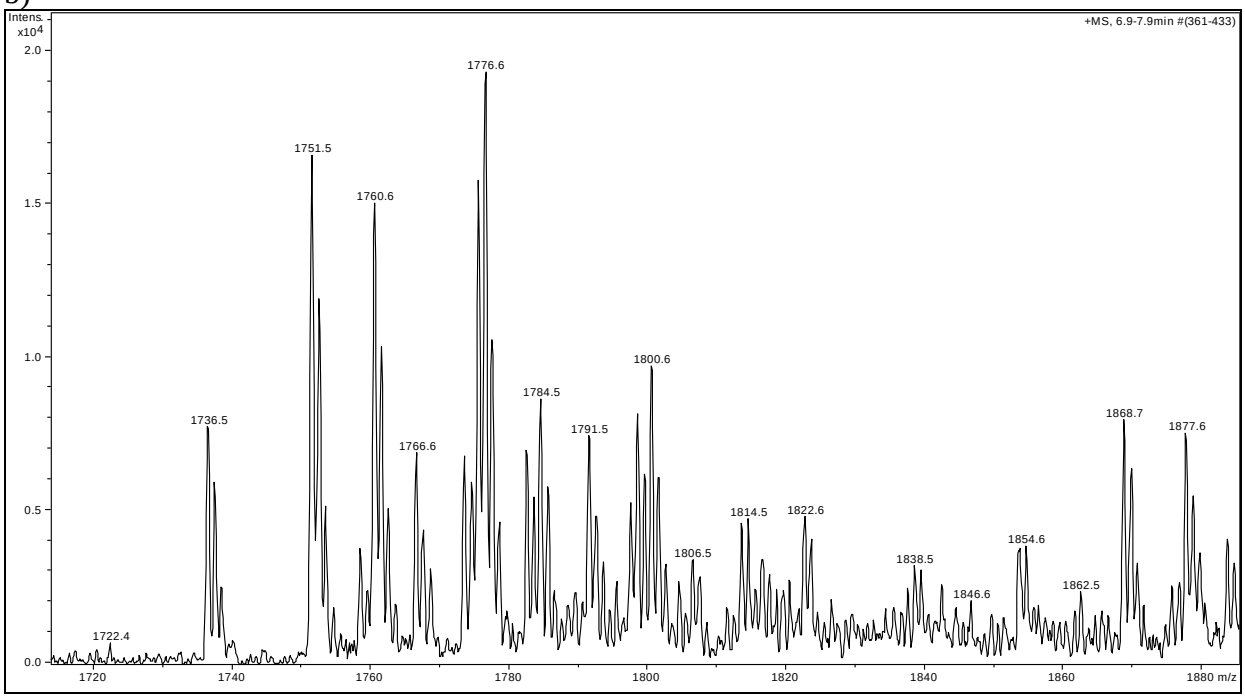
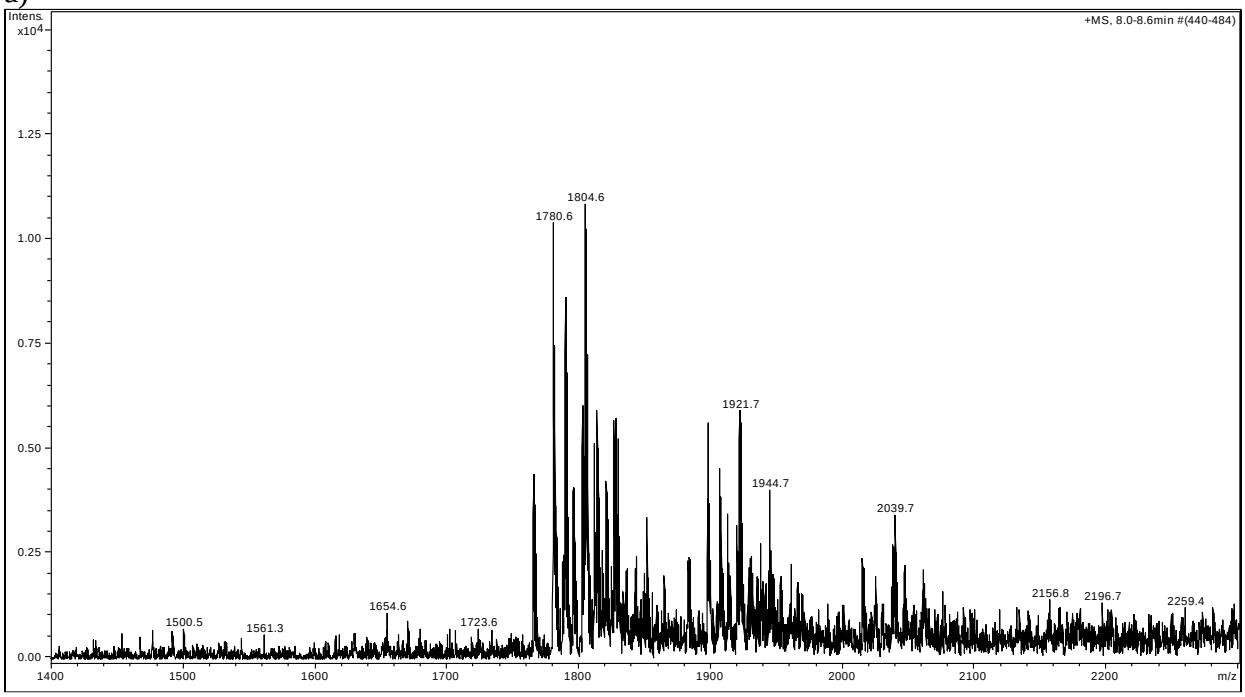


Figure S11. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNAC. Calculated range: 1735.4-1815.4 a) original graph and b) magnified version.

a)



b)

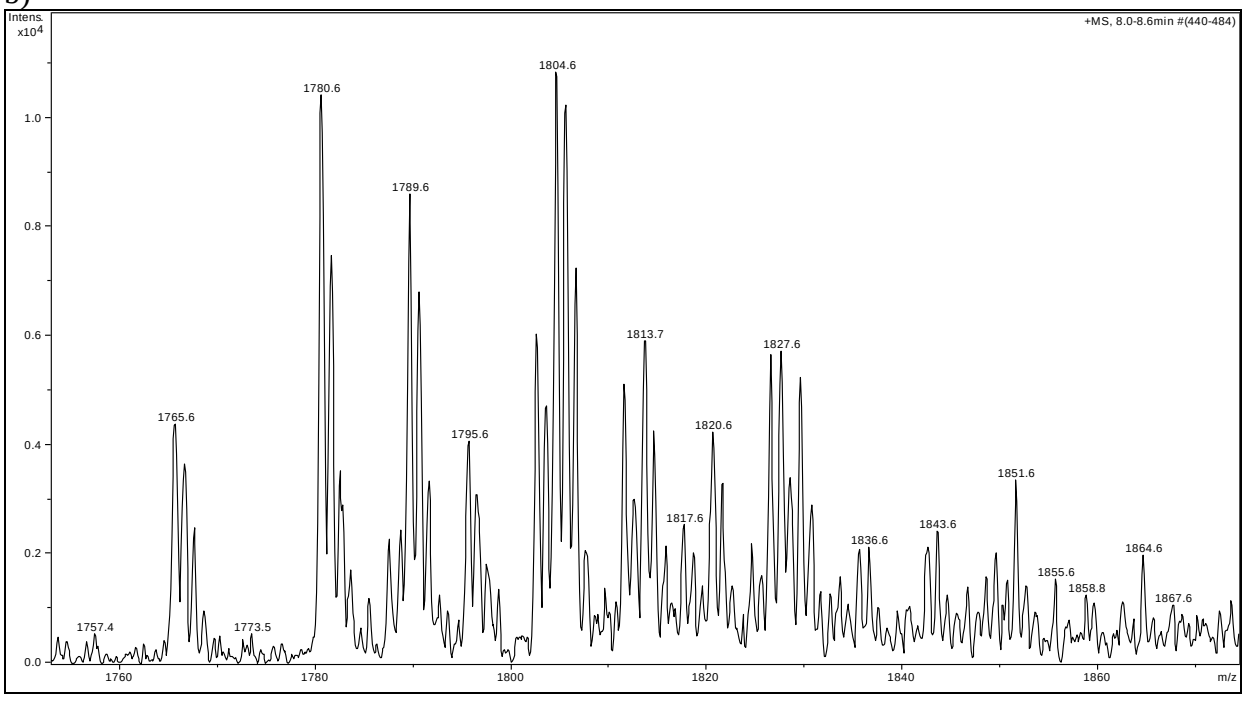
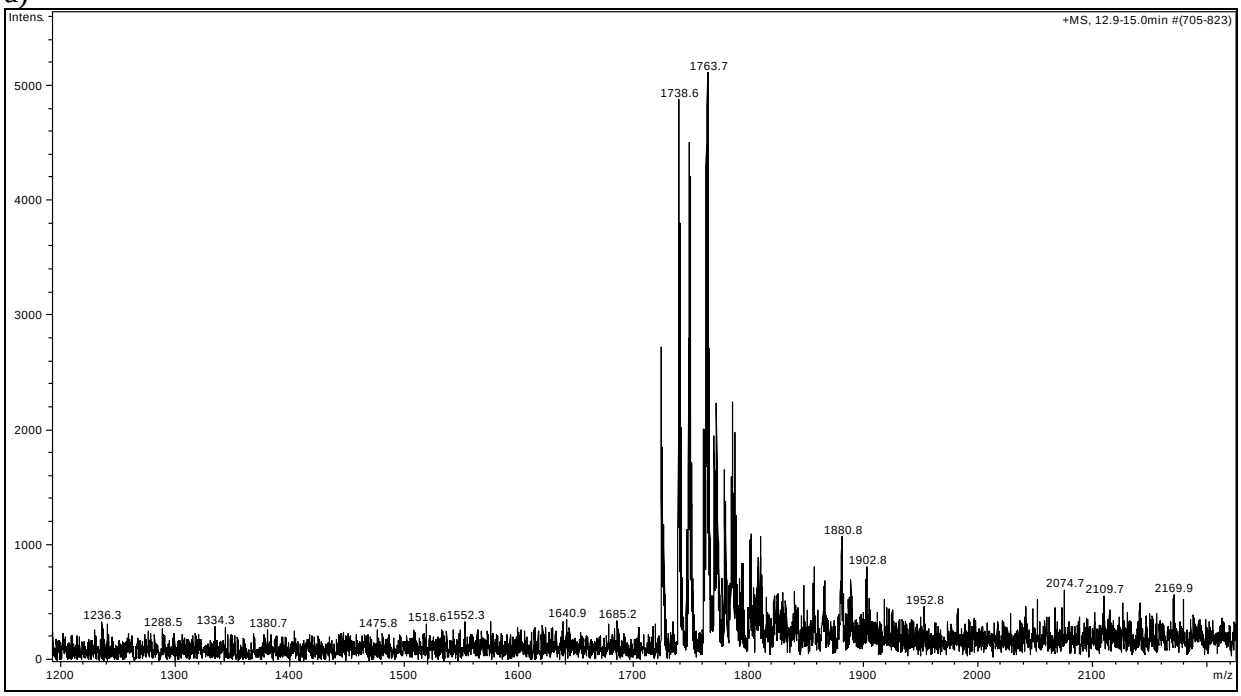


Figure S12. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNAG. Calculated range: 1764.4-1844.4 a) original graph and b) magnified version.

a)



b)

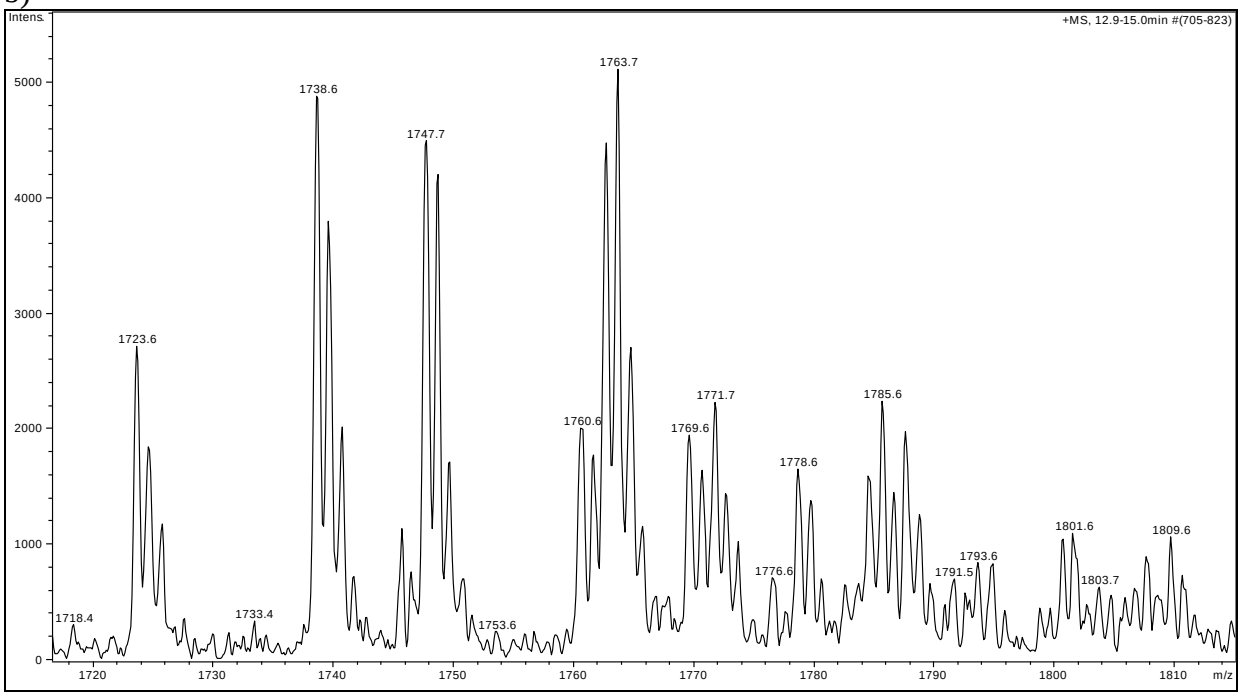
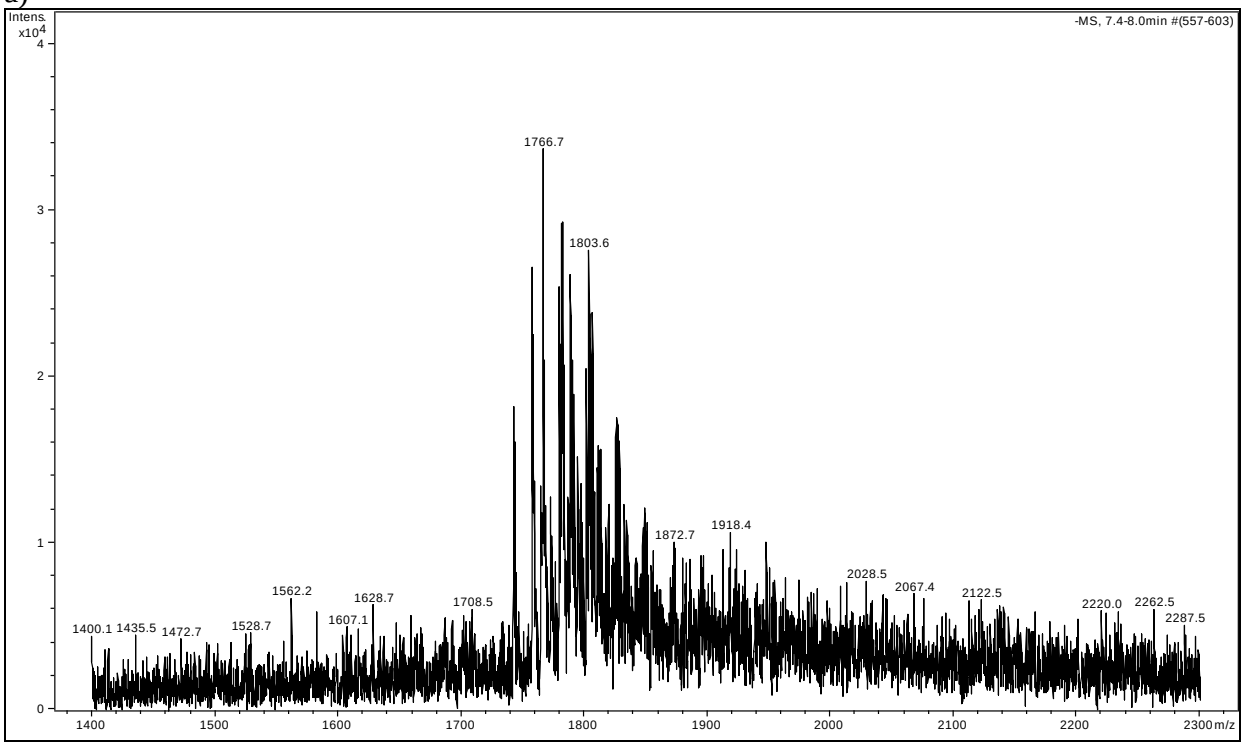


Figure S13. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNAT. Calculated range: 1722.4-1802.4 a) original graph and b) magnified version.

a)



b)

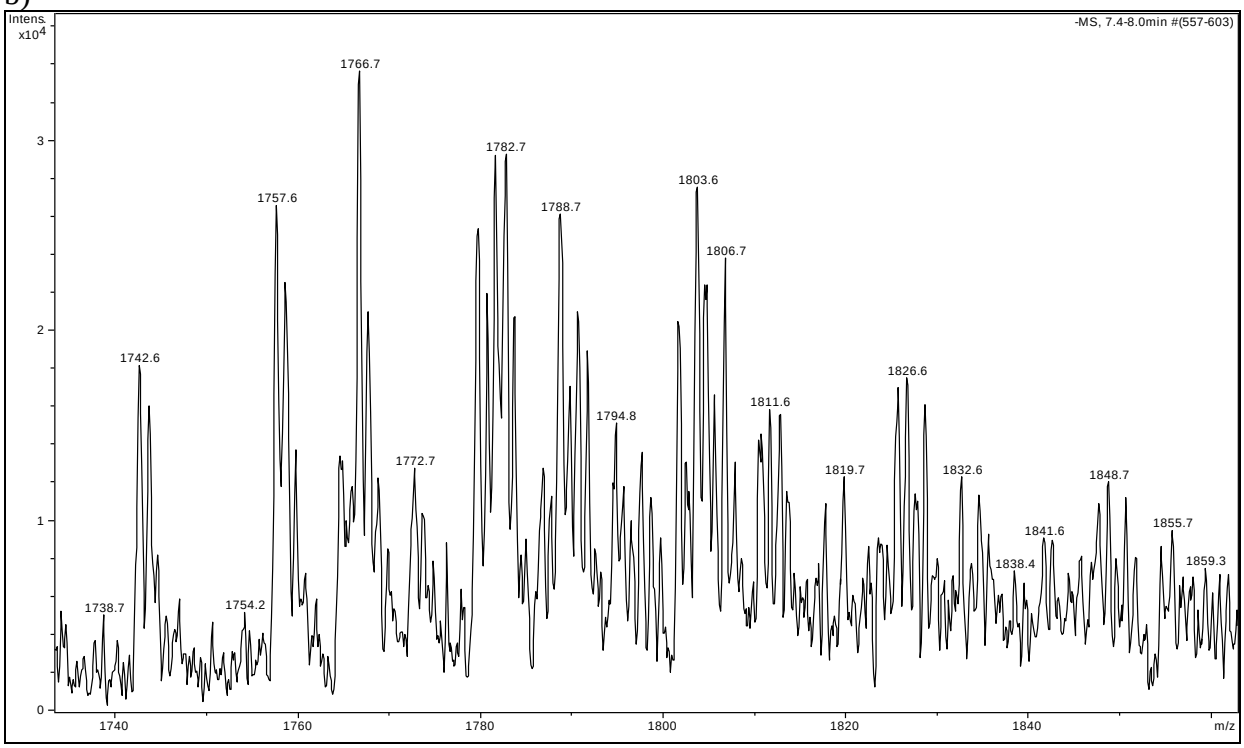
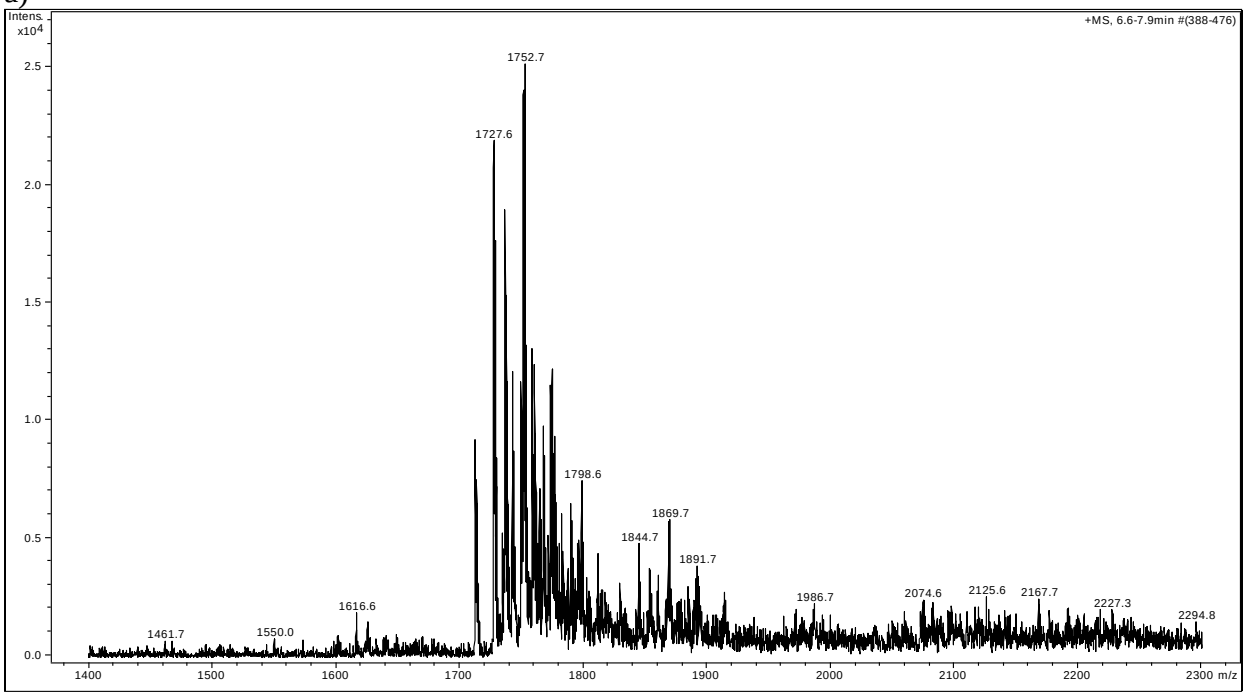


Figure S14. ESI negative ion mode mass spectrometry analysis for modified pentanucleotide ANNCA. Calculated range: 1743.4-1823.4 a) original graph and b) magnified version.

a)



b)

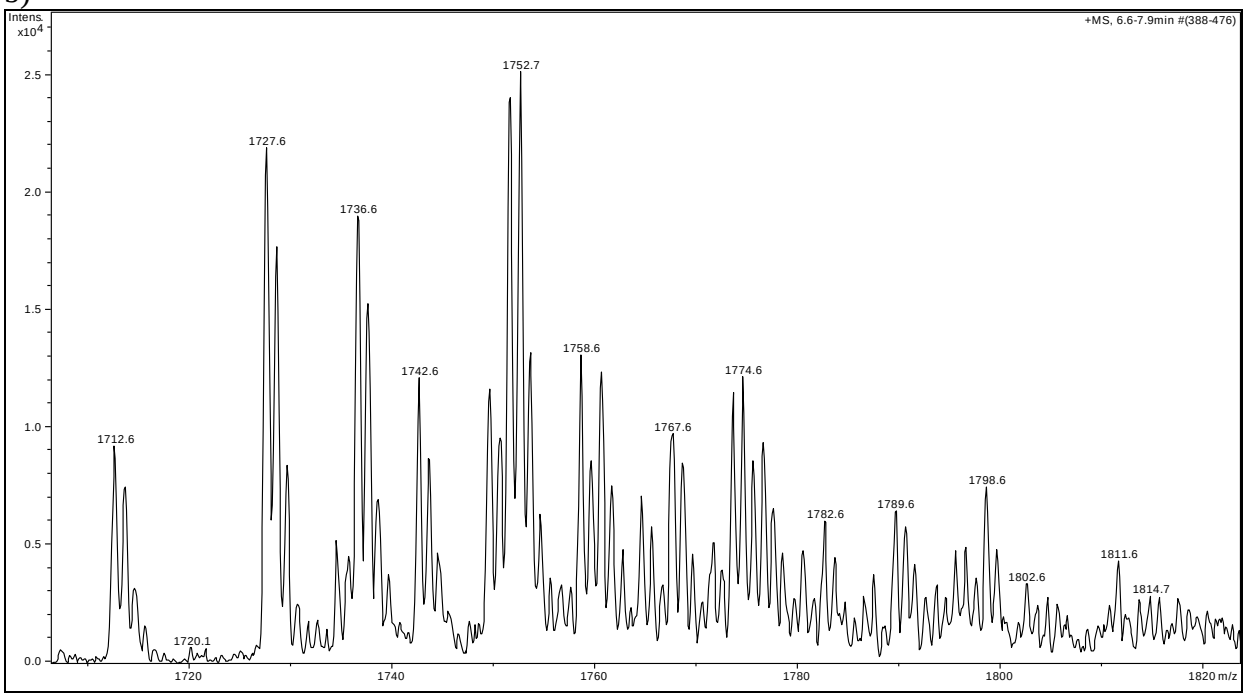
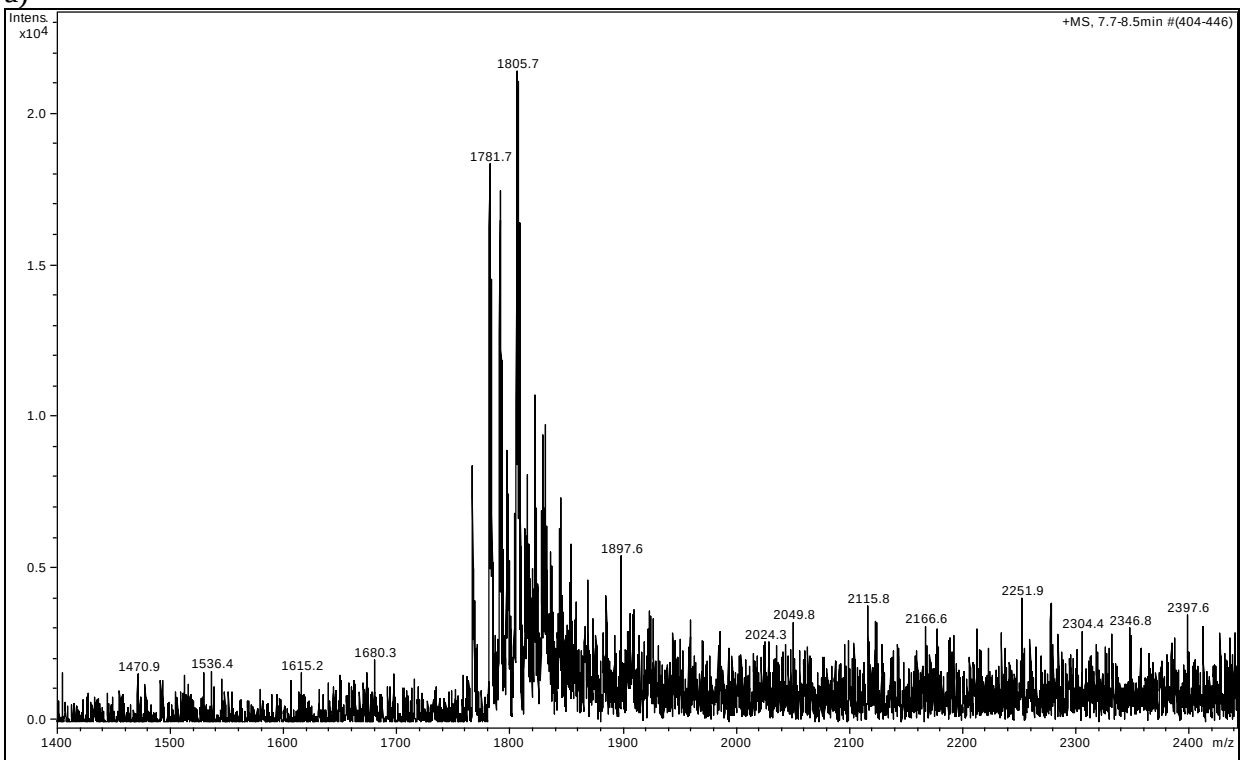


Figure S15. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNCC. Calculated range: 1711.4-1791.5 a) original graph and b) magnified version.

a)



b)

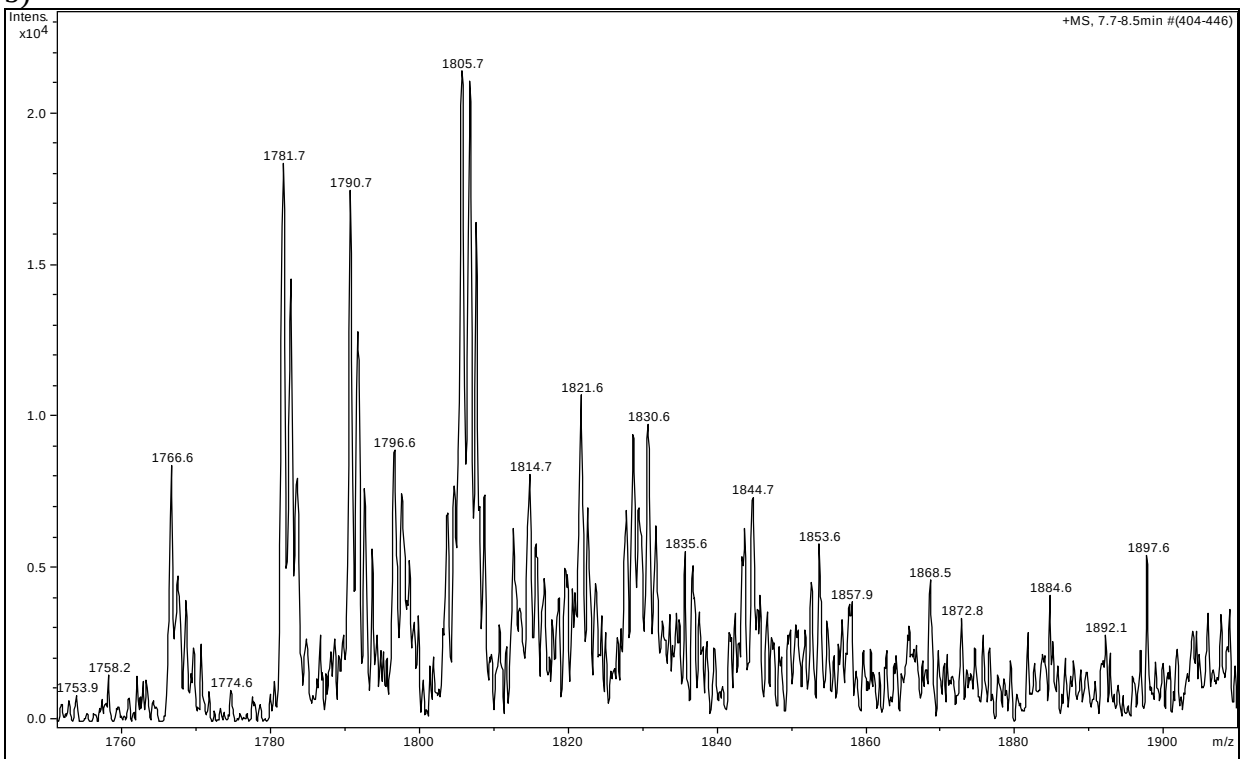


Figure S16. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNCG. Calculated range: 1765.4-1845.4 a) original graph and b) magnified version.

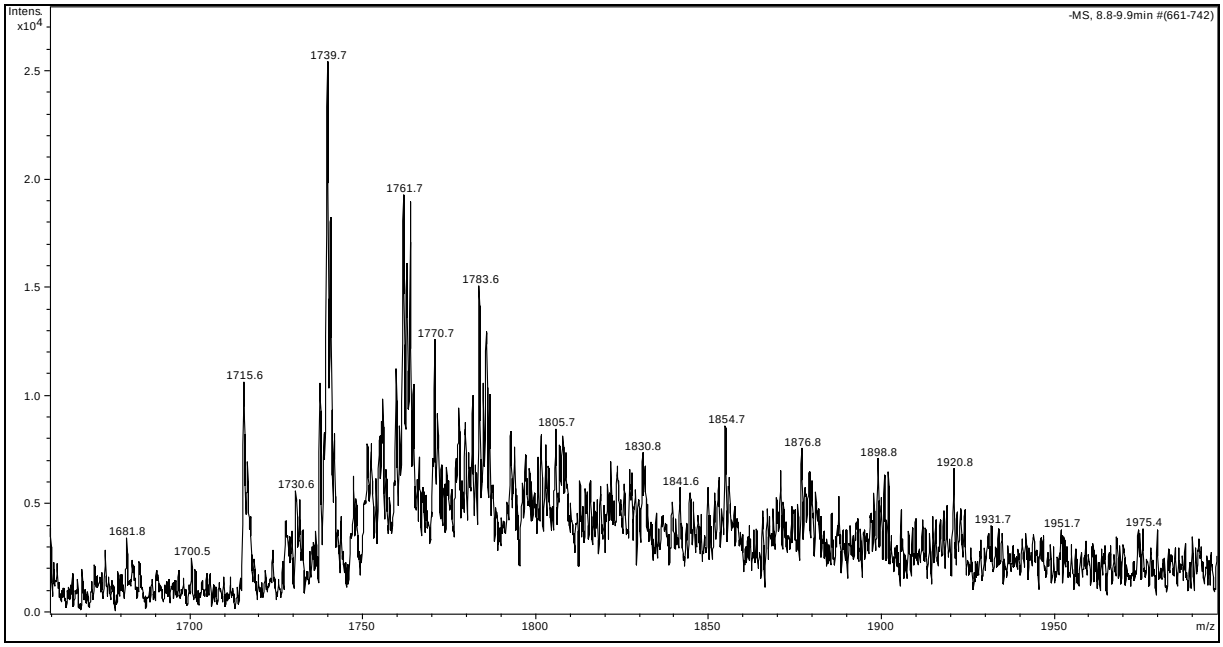
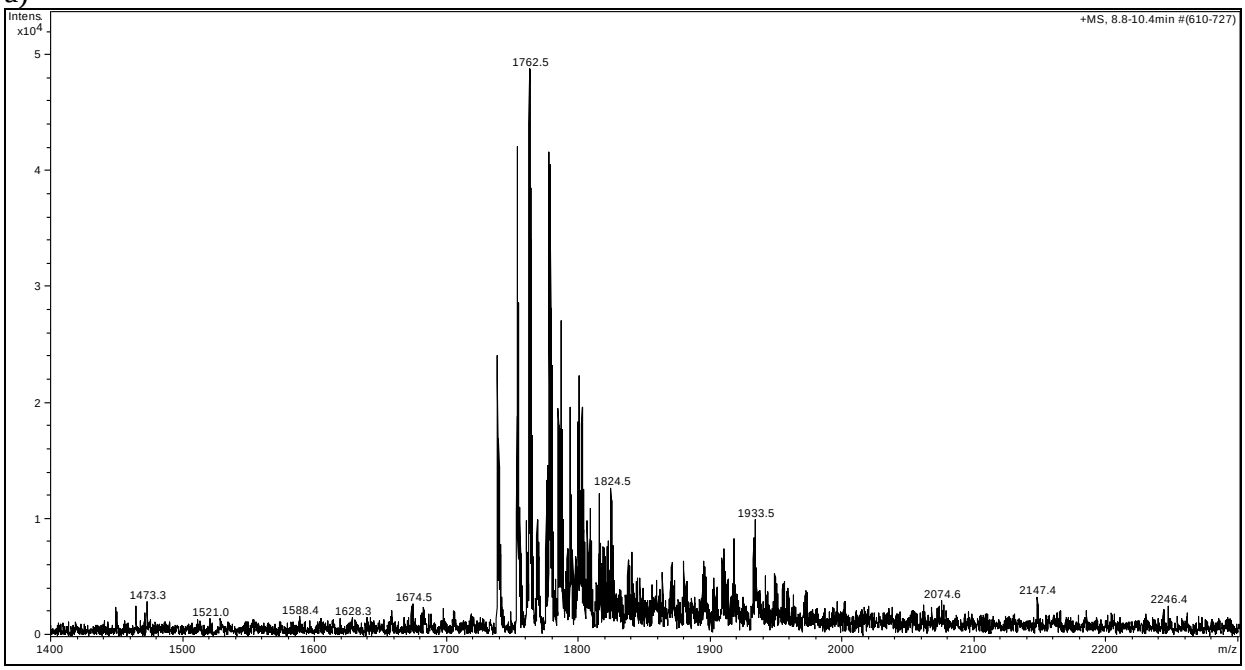


Figure S17. ESI negative ion mode mass spectrometry analysis for modified pentanucleotide ANNCT. Calculated range: 1716.4-1796.4.

a)



b)

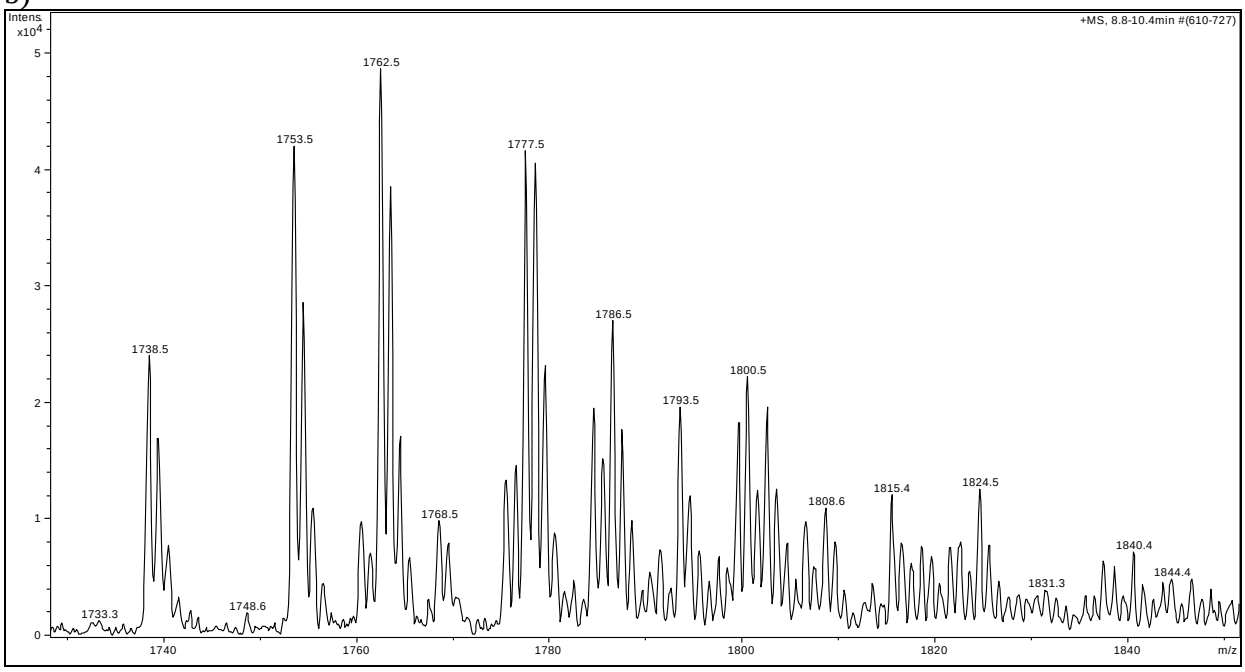
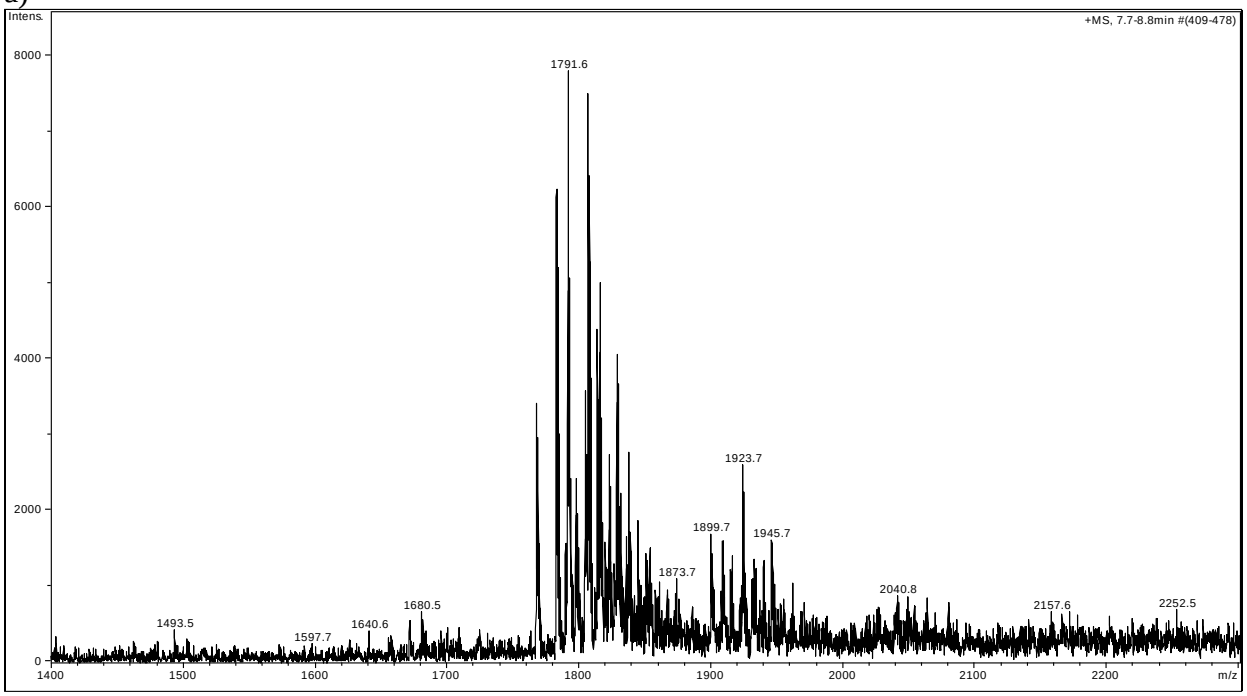


Figure S18. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNGA. Calculated range: 1737.4-1817.4 a) original graph and b) magnified version.

a)



b)

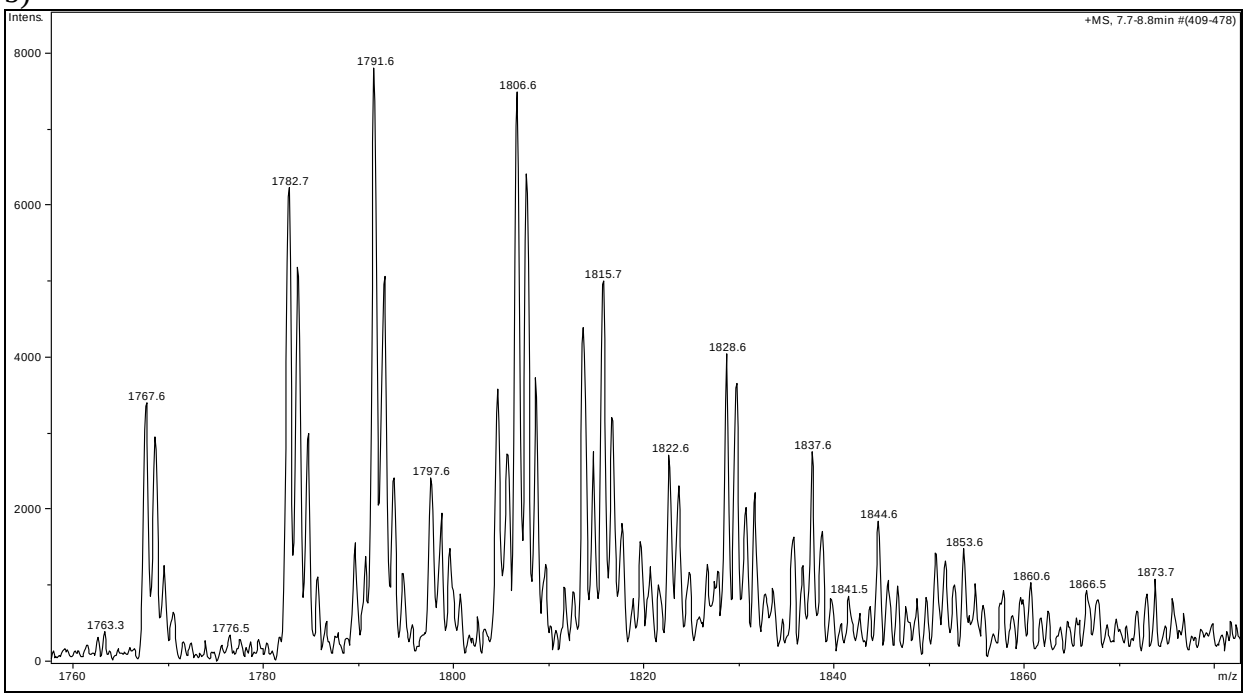
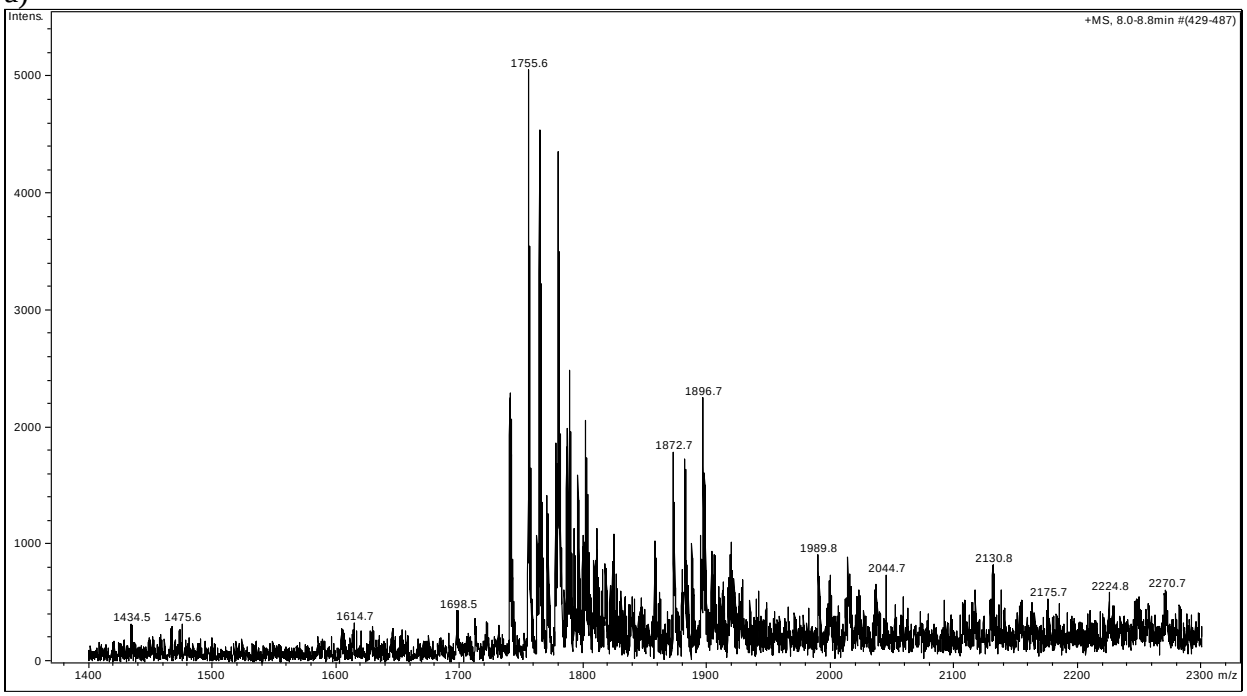


Figure S19. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNGC. Calculated range: 1766.5-1846.5 a) original graph and b) magnified version.

a)



b)

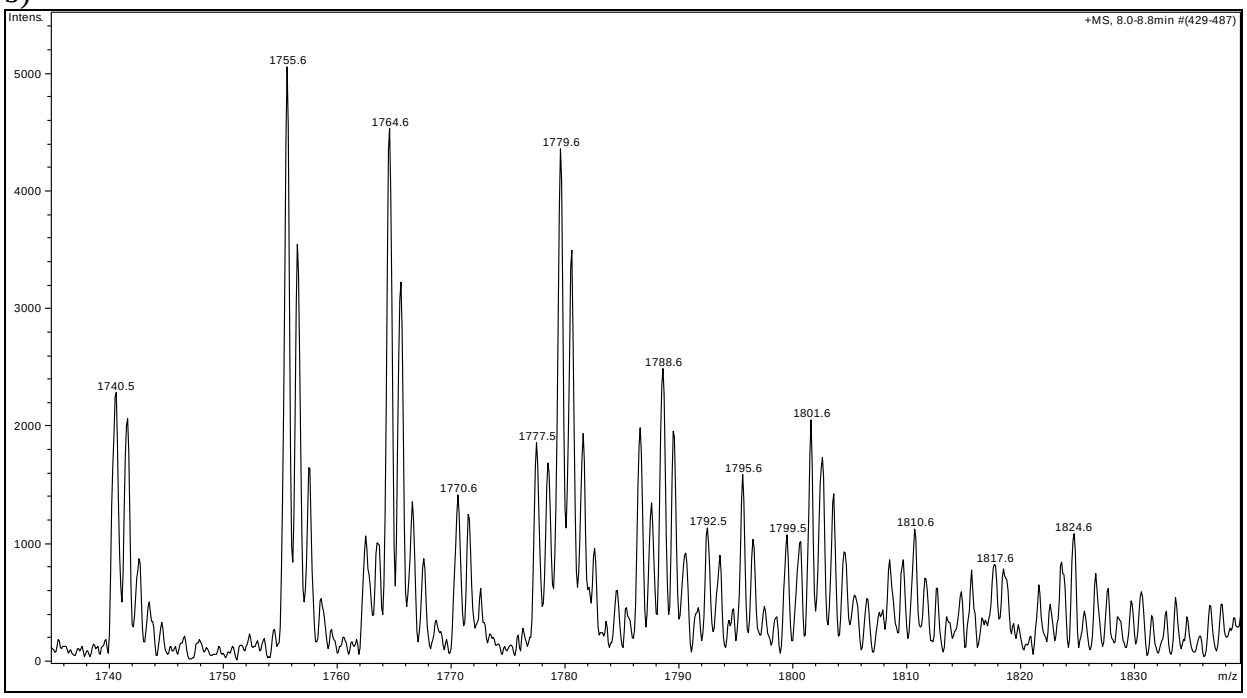
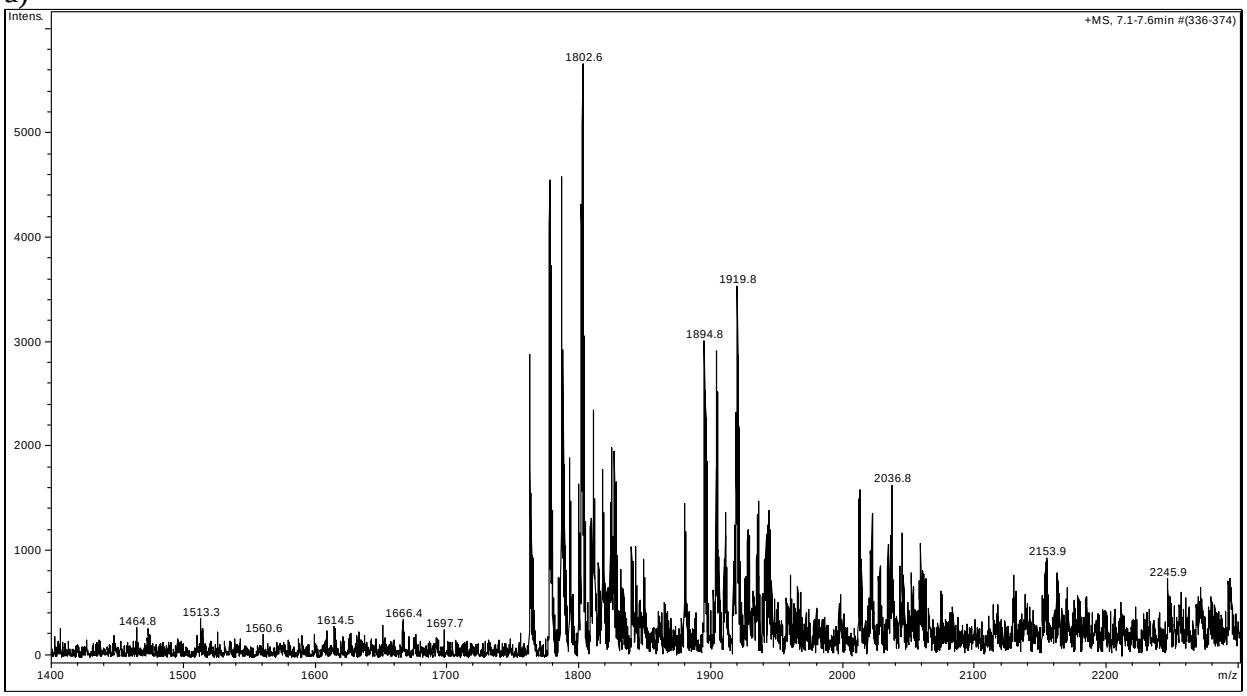


Figure S20. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNGG. Calculated range: 1739.4-1819.4 a) original graph and b) magnified version.

a)



b)

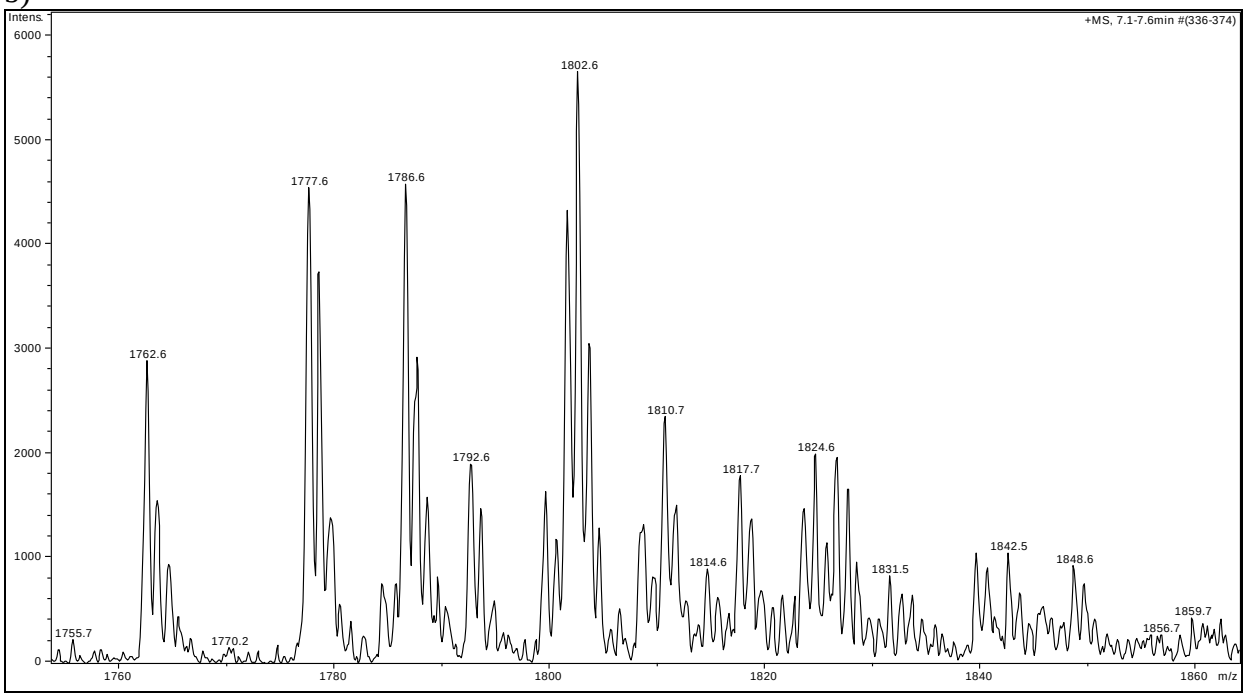
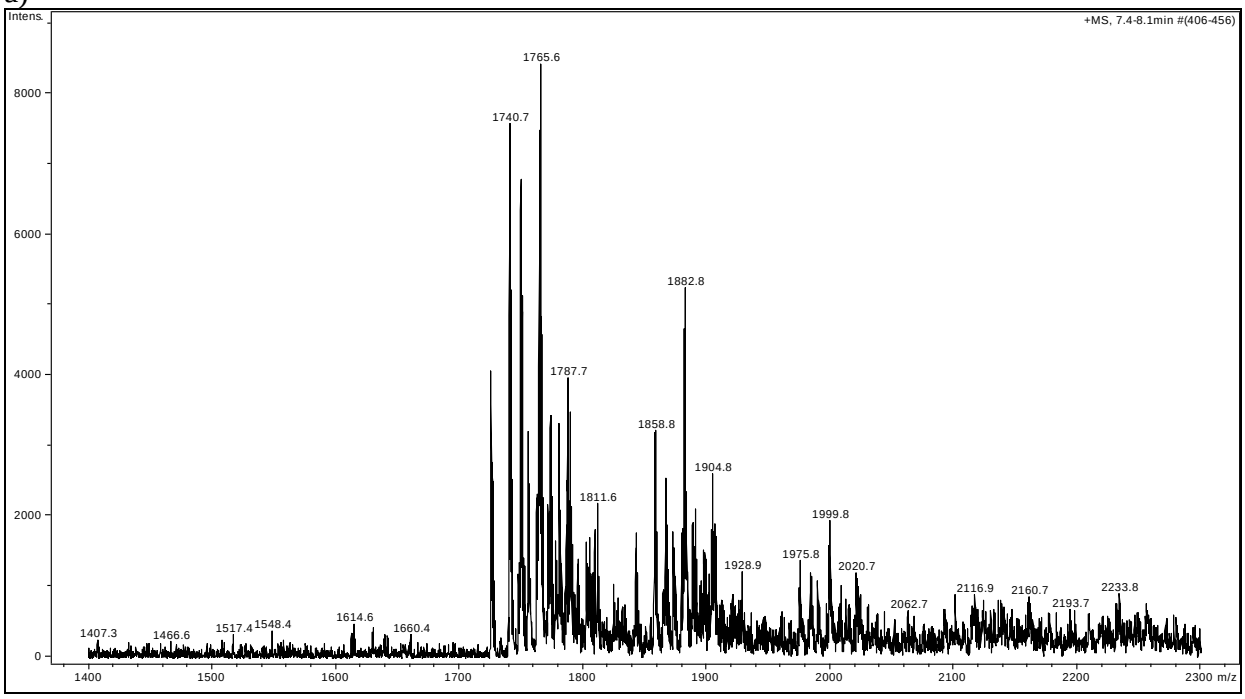


Figure S21. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNGT. Calculated range: 1762.4-1841.4 a) original graph and b) magnified version.

a)



b)

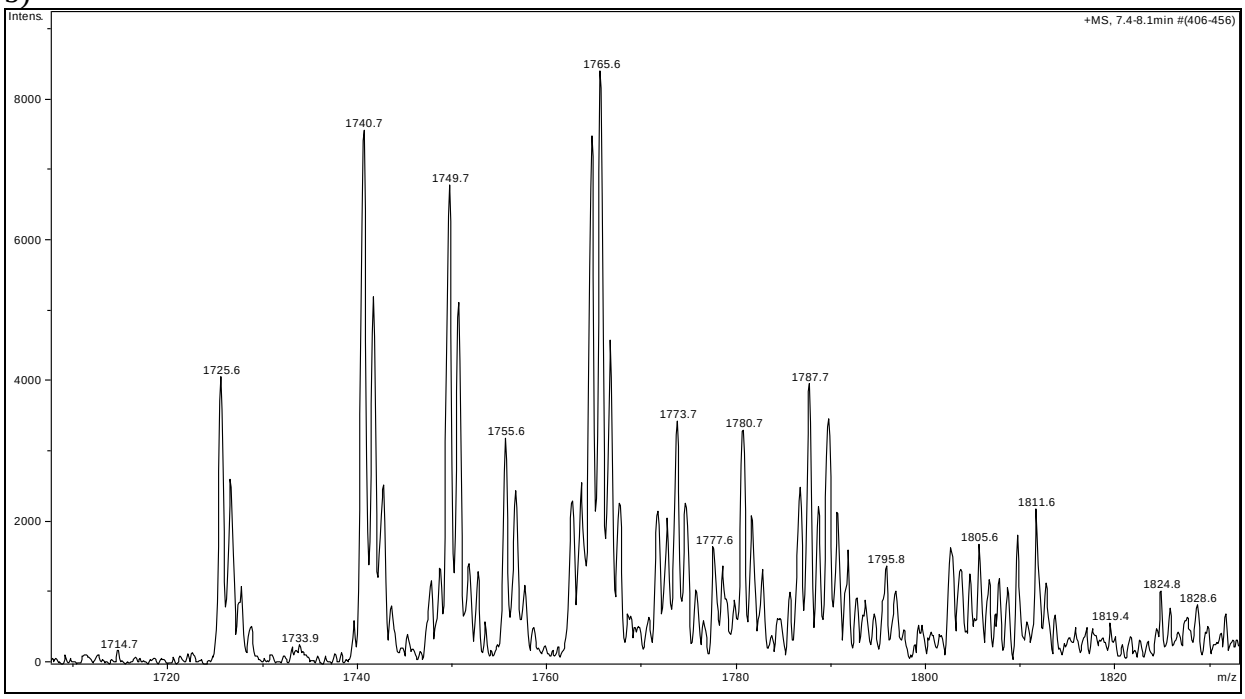
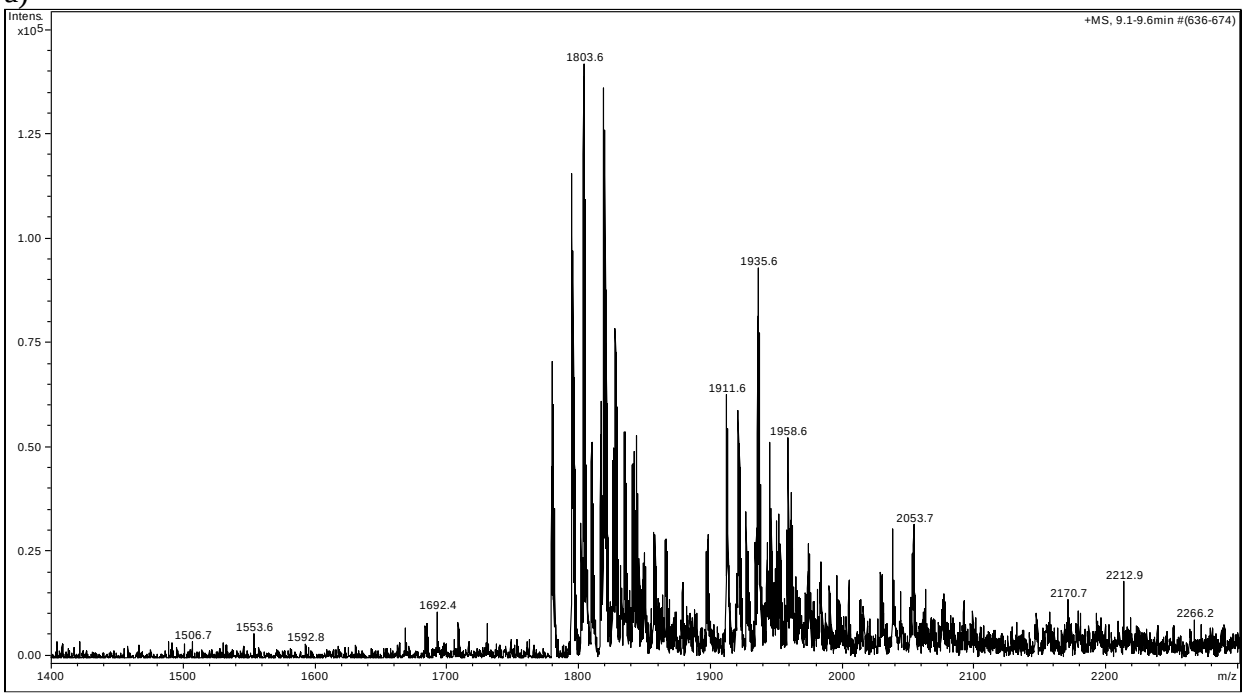


Figure S22. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNTA. Calculated range: 1724.4-1804.5 a) original graph and b) magnified version.

a)



b)

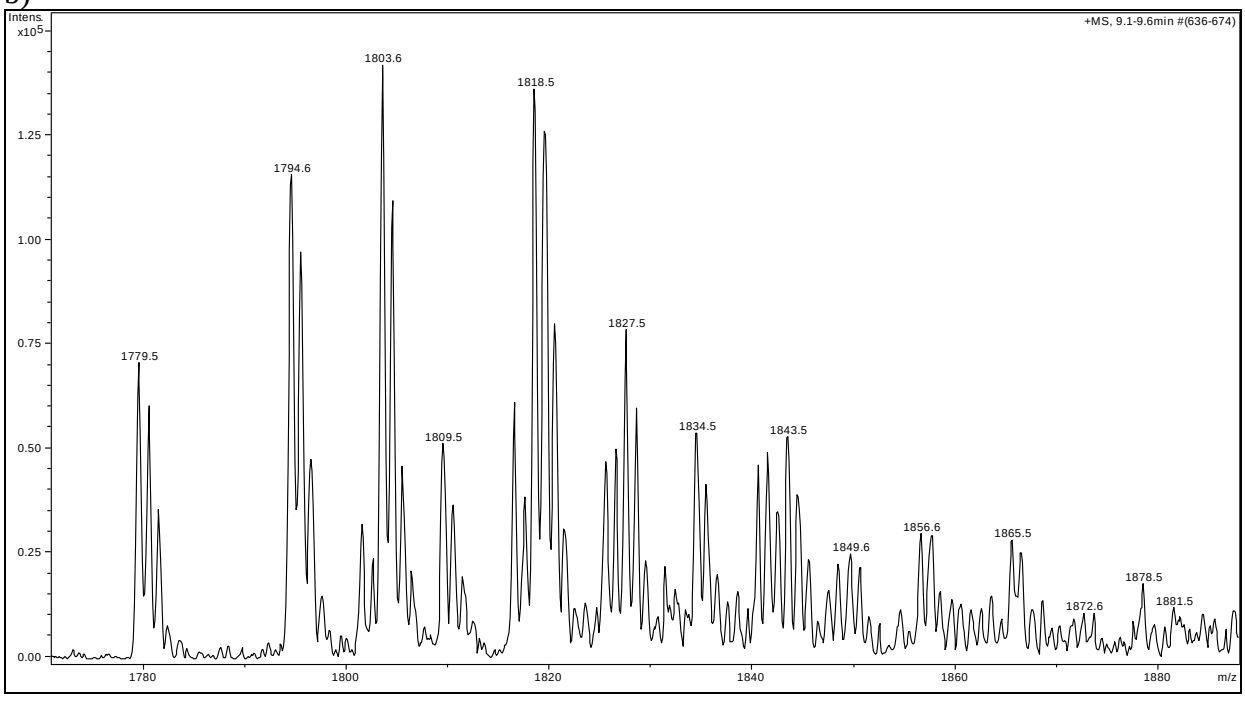
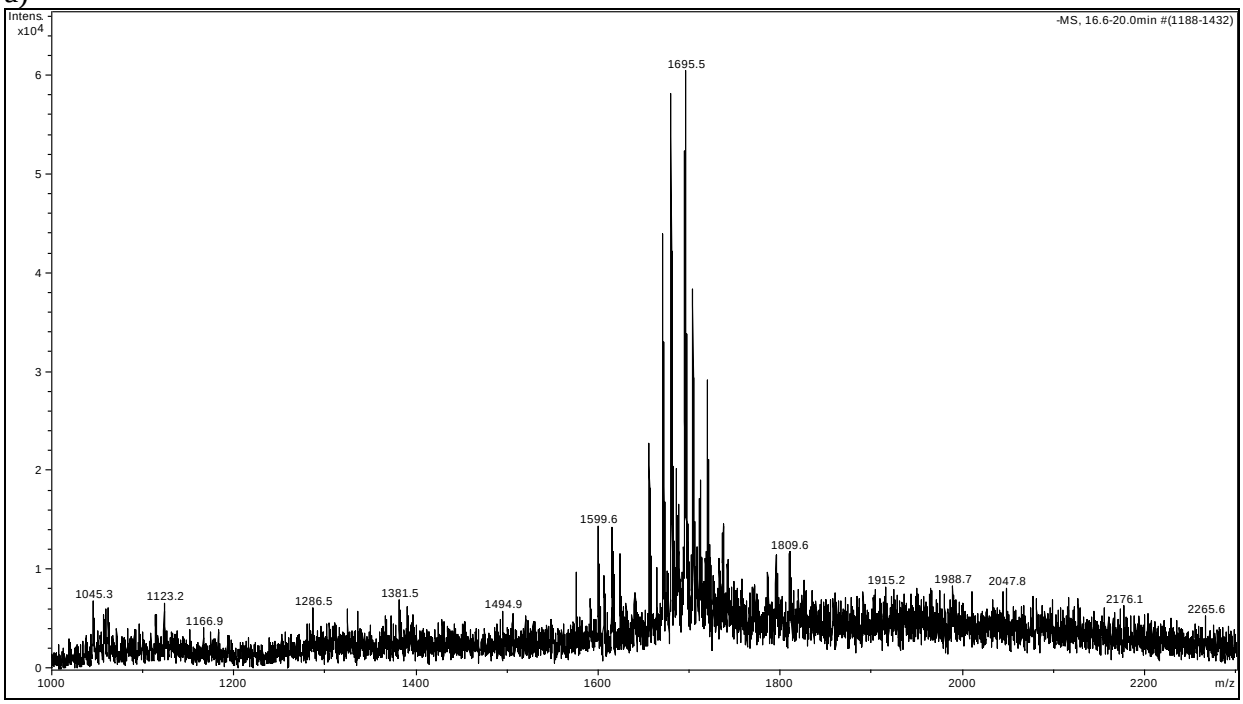


Figure S23. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNTC. Calculated range: 1778.4-1858.4 a) original graph and b) magnified version.

a)



b)

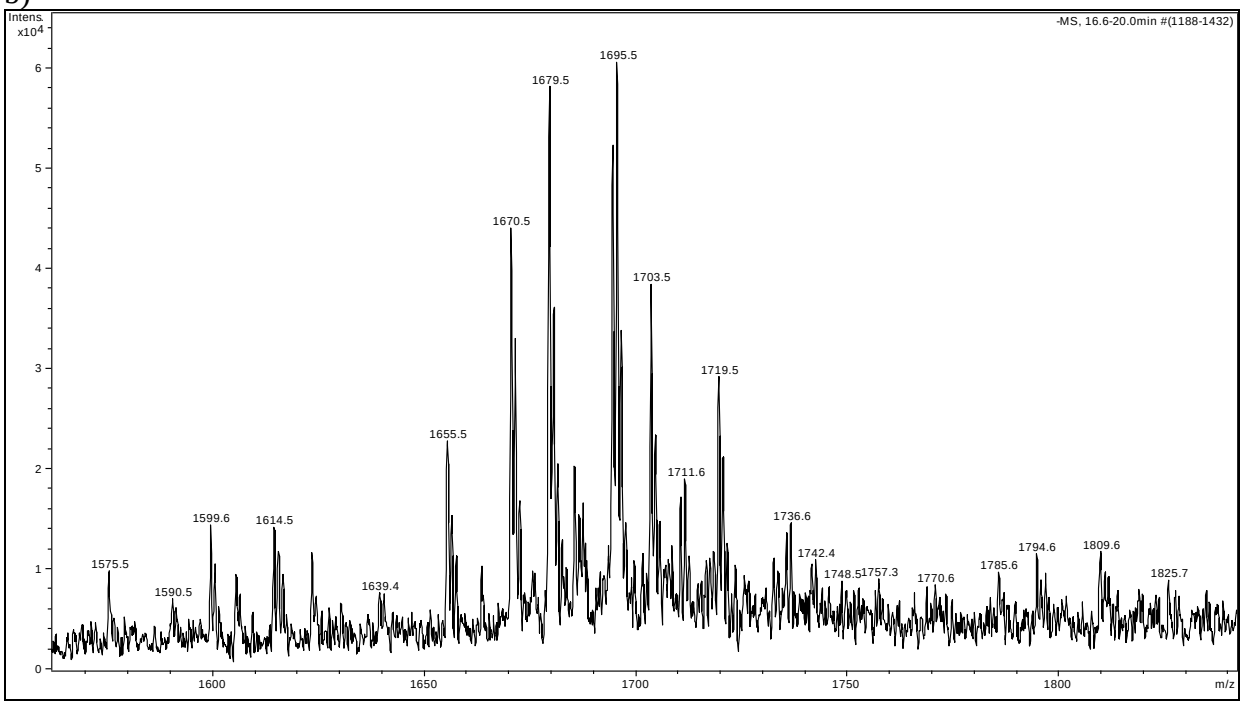
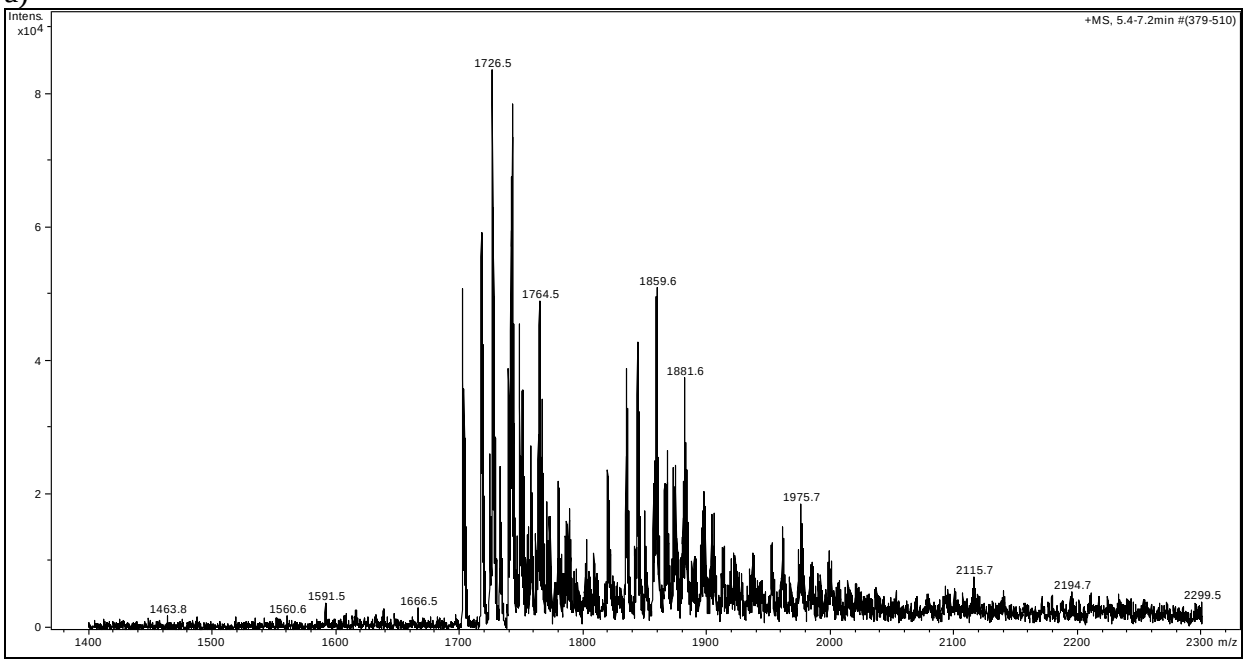


Figure S24. ESI negative ion mode mass spectrometry analysis for modified pentanucleotide ANNTG. Calculated range: 1656.4-1736.4 a) original graph and b) magnified version.

a)



b)

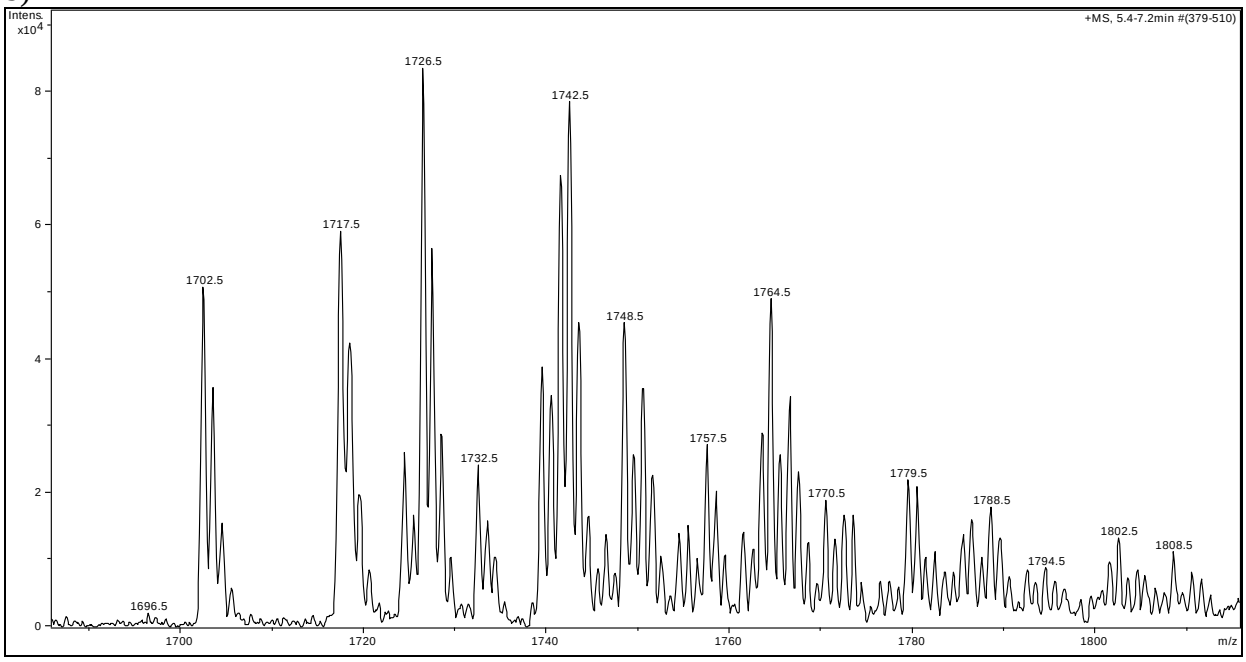


Figure S25. ESI positive ion mode mass spectrometry analysis for modified pentanucleotide ANNTT. Calculated range: 1701.4-1781.4 a) original graph and b) magnified version.

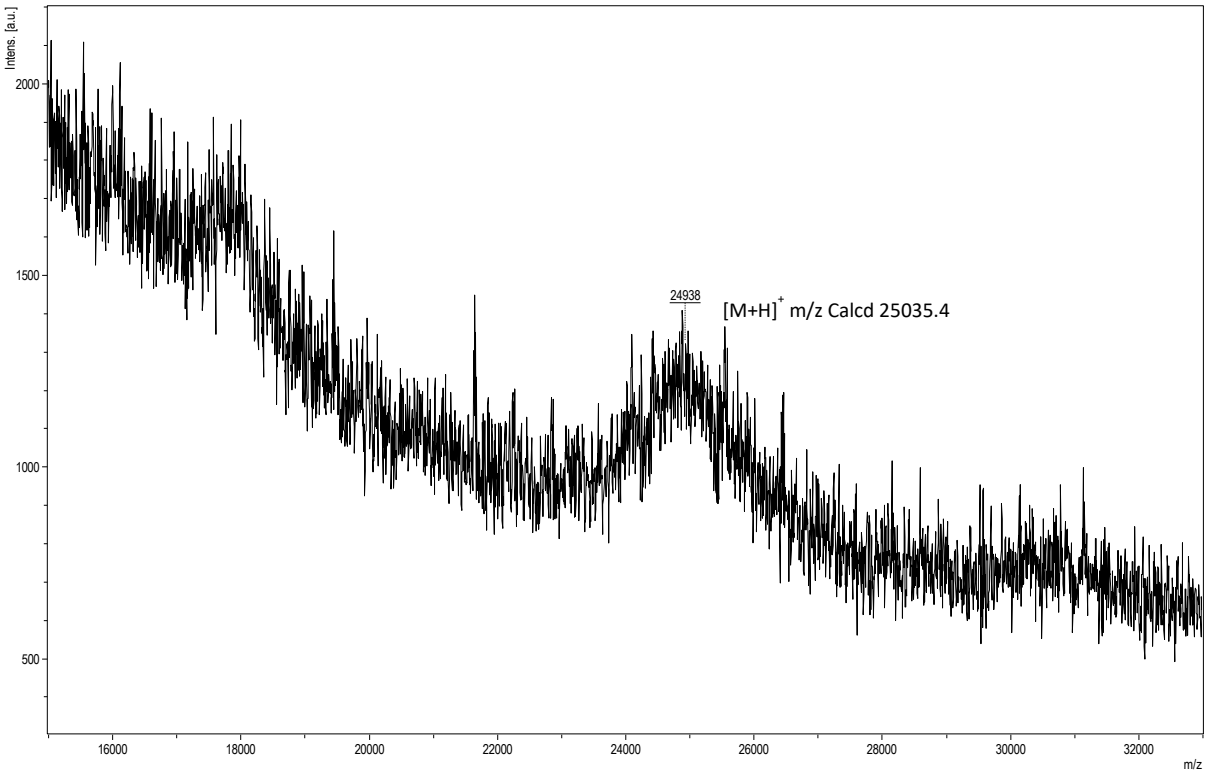
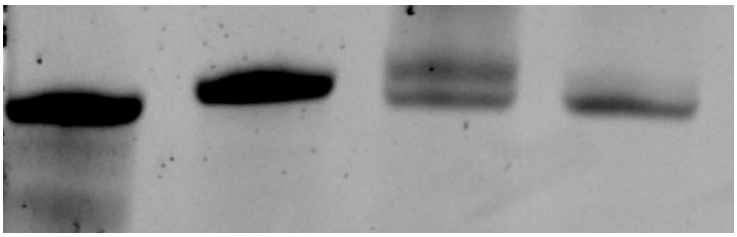
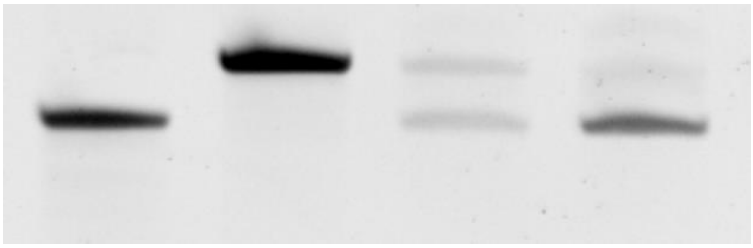


Figure S26. MALDI mode mass spectrometry analysis for modified TBL1(modTBL1).



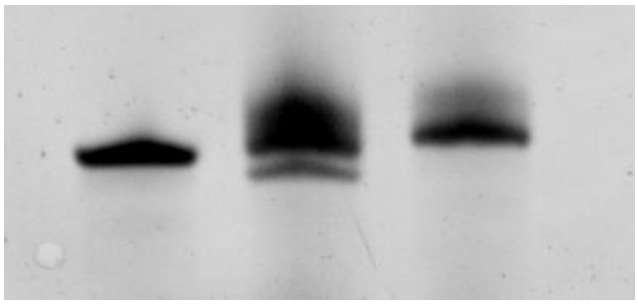
1 2 3 4

Figure S27. Denaturing PAGE of strand separation in pretest stage using unmodified pentanucleotide ANNNN. In this case, biotinylated template is the top strand. No observable biotinylated template leaching was shown after strand separation. Lane 1: Unmodified 76mer strand; Lane 2: Biotinylated 76mer template; Lane 3: LOOPER using unmodified ANNNN; Lane 4: Strand separation product of the polymerized strand.



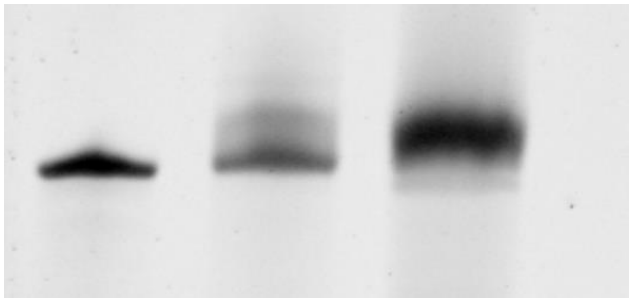
1 2 3 4

Figure S28. Denaturing PAGE of PCR using biotinylated and PEGylated primers in pretest stage. In this case, biotinylated template is the bottom strand. Lane 1: Biotinylated 76mer template; Lane 2: PEGylated 76mer; Lane 3: PCR using biotinylated and PEGylated primers; Lane 4: Biotinylated strand recovery after PCR. Gel cut will be done on Lane 4 in SELEX stage.



1 2 3

Figure S29. Denaturing PAGE of LOOPER on Round One in SELEX stage. In this case, biotinylated template is the bottom strand. Lane 1: Biotinylated 76mer template; Lane 2: LOOPER on Round One using heterofunctionalized pentanucleotides; Lane 3: LOOPER on Round One using homofunctionalized pentanucleotides NH2-ANNNN.



1 2 3

Figure S30. Denaturing PAGE of LOOPER on Round Six in SELEX stage. In this case, biotinylated template is the bottom strand. Lane 1: Biotinylated 76mer template; Lane 2: LOOPER on Round Six using heterofunctionalized pentanucleotides; Lane 3: LOOPER on Round Six using homofunctionalized pentanucleotides NH2-ANNNN.

References

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