Table of Contents

Supporting Methods

General Information

Unless otherwise noted, water was purified with ELGA Flex 3 purification system. DNA oligonucleotides without amine modification were purchased from Integrated DNA Technologies. DNA oligonucleotides with amine modification were synthesized on 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-pressure 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 quantitated by UV spectroscopy using a Nanodrop ND2000 spectrophotometer.

DNA sequences

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

Templates

TP(NNNNT)8P /5Phos/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 CCT

Primers

PR5 /5Phos/GG ATC CGA GCT CCA CGT G
PR6 /5Phos/TG CGA CGG CAG GCG AAT CT
iTruS_i7_D701 CAA GCA GAA GAC GGC ATA CGA GAT ATT ACT CGG TGA CTG GAG TTC AG
iTruS_i7_D702 CAA GCA GAA GAC GGC ATA CGA GAT TCC GGA GAG TGA CTG GAG TTC AG
iTruS_i7_D703 CAA GCA GAA GAC GGC ATA CGA GAT CGC TCA TTG TGA CTG GAG TTC AG
iTruS_i7_D704 CAA GCA GAA GAC GGC ATA CGA GAT GAG ATT CCG TGA CTG GAG TTC AG
iTruS_i7_D705 CAA GCA GAA GAC GGC ATA CGA GAT ATT CAG AAG TGA CTG GAG TTC AG
iTruS_i7_D706 CAA GCA GAA GAC GGC ATA CGA GAT GAA TTC GTG TGA CTG GAG TTC AG
iTruS_i7_D707 CAA GCA GAA GAC GGC ATA CGA GAT CTG AAG CTG TGA CTG GAG TTC AG
iTruS_i7_D708 CAA GCA GAA GAC GGC ATA CGA GAT TAA TGC GCG TGA CTG GAG TTC AG
iTruS_i7_D709 CAA GCA GAA GAC GGC ATA CGA GAT CGG CTA TGG TGA CTG GAG TTC AG
iTruS_i7_D710 CAA GCA GAA GAC GGC ATA CGA GAT TCC GCG AAG TGA CTG GAG TTC AG
iTruS_i7_D711 CAA GCA GAA GAC GGC ATA CGA GAT TCT CGC GCG TGA CTG GAG TTC AG
iTruS_i7_D712 CAA GCA GAA GAC GGC ATA CGA GAT AGC GAT AGG TGA CTG GAG TTC AG
PRIMER B AAT GAT ACG GCG ACC ACC GAG

Pentanucleotides

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

Synthesis of amino-modified pentanucleotides

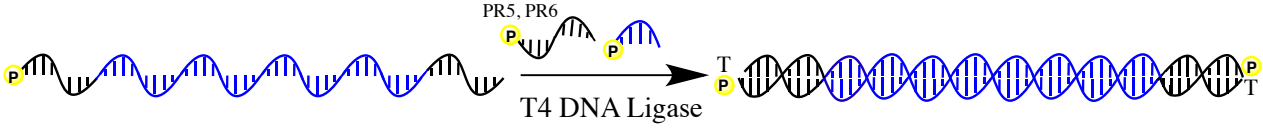
Pentanucleotides were synthesized on a Mermaid 12 DNA synthesizer using a DMT-ON protocol on a 1 µmol scale (1000 Å CPG column). Amine-modifier C6 dA (Glen Research 10-1089), dA+dC+dG+dT-CE Phosphoramindite (Glen Research 10-1000, 10-1010, 10-1020, 10-1030), Chemical Phosphorylation Reagent II (10-1901) 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 by filtration, followed by incubation at 60°C for 30 minutes to remove the protecting groups on 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, pH7] to [80% acetonitrile in 0.1 M TEAA, pH7] solvent gradient with a column temperature of 45°C. The purified oligonucleotide was then incubated at room temperature in 1mL of 40% aqueous acetic acid for 1 h 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 with carboxylic acids

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. Followed by addition 7.5 µL of amino modified pentanucleotides (10nmol 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 buffer (500 mM in H_2O) at room temperature for one hour and functionalized pentanucleotide was then purified with 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 pentanucleotide was dissolved in water and characterized with mass spectrometry.

T4-DNA Ligase-mediate adapter ligation protocols

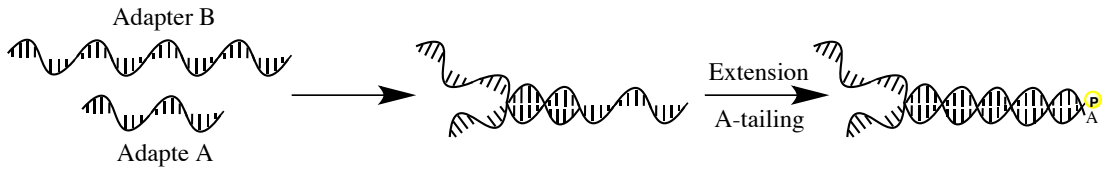
T4-DNA Ligase-mediate polymerization



In a PCR tube was added 1.5 µL DNA template (10 µM in H_2O), 2.25 µL PR5 (10 µM in H_2O), 2.25 µL PR6 (10 µM in H_2O), 5µL ligation reaction buffer 4X (40 mM $MgCl_2$, 24% w/v PEG6000, 40mM DTT, 264mM Tris pH 7.6), 2 µL ATP (0.25 mM in H_2O) and 4 µL H_2O. The mix was heated

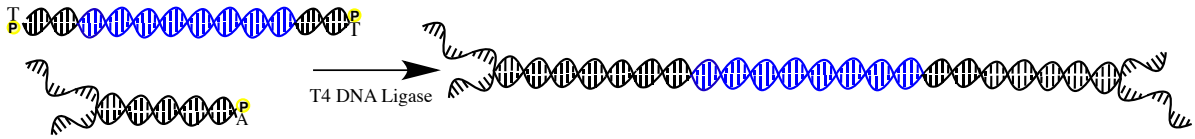
to 90 °C for 2 minutes and then cooled to 25 °C at the rate of 0.1 °C/s. In this PCR tube was then added 1 µL pentanuclotides (480 µM in H_2O), 1 µL BSA (2 mg/mL in H_2O) and 400 U T4 DNA ligase (New England Biolabs, M0202L). The polymerization was performed at 25 °C for 24 hours. The products were then purified with MinElute® PCR Purification Kit for adapter ligation.

Adapter duplex synthesis



In a PCR tube was added 15 µL of 100 µM adapterA and 15 µL of 100 µM adapterB, then the tube was heated to 95 °C for 5 minutes, and cooled to room temperature over one hour. Then in this PCR tube was added 4 µL NEBuffer2 10× (New England Biolabs, M0212L), 25 U Klenow Fragment (3'→5' exo$^-$, New England Biolabs, M0212L), 1 µL dNTP Mix (Thermo Scientific, 10 mM each). The extension was performed at 37 °C for 1 hour. The adapter duplex was purified with QIAquick Nucleotide Removal Kit, and then diluted in 30 µL water.
In a PCR tube was added 30 µl purified adapter duplex, 5 µl NEBuffer2 10× (New England Biolabs, M0212L), 25 U Klenow Fragment (3'→5' exo$^-$, New England Biolabs, M0212L), 5 µl dATP (Thermo Scientific, 10 mM), 5 µL H_2O. This PCR tube was incubated at 37 °C for 1 hour for A-tailing. Then product was purified with QIAquick Nucleotide Removal Kit, and then diluted in 30 µL water.

Adapter ligation



In a PCR tube was added 10pmol polymerization products, 200 pmol A-tailing adapter duplex, 10 µL NEBNext® Quick Ligation Reaction Buffer 5×, 2.5µL BSA (2mg/mL in H_2O), 1000U T4 DNA ligase (New England Biolabs, M0202L), the total volume of reaction was adjusted to 50 µL with H_2O. Then the ligation was performed at 16 °C for 16 hours. The ligated products were then gel purified.

PCR protocols

Each purified adapter ligation product was amplified with a different primer from iTrus_D701 to iTrus_D712.

In a PCR tube was added 50 attomole purified adapter ligation product in 10 µL H_2O, 1.25µL 10 µM Primer B, 1.25 µL 10 µM corresponding iTrus_D7XX primer, and 12.5 µL Q5® High-Fidelity 2× Master Mix (New England Biolabs). The tube was then transferred to a preheated themocylcler (98°C). The PCR cycle was 10 s of 98°C denature step, 30 s of primer annealing step (annealing temperature was 55°C for the first two cycles, and 71 °C for the rest of the cycles), and 30 s of 72°C extension step. The PCR products were then gel purified.

High-Throughput DNA sequencing Protocol

The concentrations of gel purified samples were determined with Kapa library quantification kit for Illumina libraries (KK4845) on Roche LightCycler 480. Paired-end Illumina sequencing was performed on an Illumina MiSeq system using the kit v2 with 300 cycles (150bp PE sequencing) at the Georgia Genomics Facility, University of Georgia, Athens, GA, USA

Supporting Tables

Table S1. Temperature influence on LOOPER with phenyl-monomodified ANNNN library

Entry	Temperature	Yield	Bias	Fidelity
1	10 °C	62%	0.37	94.91%
2	15 °C	61%	0.32	93.80%
3	20 °C	63%	0.30	94.49%
4	25 °C	63%	0.29	94.06%
5	30 °C	63%	0.41	93.94%

Table S2. ATP influence on LOOPER with phenyl-monomodified ANNNN library

Entry	ATP	Yield	Bias	Fidelity
1	1000 uM	63%	0.35	95.28%
2	150 uM	69%	0.31	95.19%
3	25 uM	62%	0.29	94.06%

Table S3. Enrichments and Fidelities of the 256 pentanucleotides in the heterofunctionalized library (16 different modifications for each sub-library are shown in Table 2).

Entry	**Pentanucleotide**	**Enrichment**	**Fidelity**
1	AAAAA	39.04%	94.67%
2	AAAAC	47.70%	79.20%
3	AAAAG	58.59%	93.94%
4	AAAAT	46.78%	89.78%
5	AAACA	76.27%	91.84%
6	AAACC	121.97%	96.43%
7	AAACG	81.17%	93.62%
8	AAACT	47.87%	95.79%
9	AAAGA	66.24%	92.00%
10	AAAGC	122.36%	97.14%
11	AAAGG	94.83%	98.43%
12	AAAGT	80.20%	89.84%
13	AAATA	40.28%	86.61%
14	AAATC	78.12%	93.84%
15	AAATG	95.43%	96.69%
16	AAATT	43.82%	90.72%
17	AACAA	96.27%	93.26%
18	AACAC	106.96%	86.39%
19	AACAG	106.93%	95.71%
20	AACAT	105.75%	92.65%
21	AACCA	96.43%	95.51%
22	AACCC	156.66%	97.00%
23	AACCG	123.99%	96.00%
24	AACCT	84.76%	92.44%
25	AACGA	116.04%	93.14%

26	AACGC	211.19%	95.29%
27	AACGG	151.85%	96.82%
28	AACGT	123.96%	95.00%
29	AACTA	72.60%	88.06%
30	AACTC	96.80%	93.22%
31	AACTG	172.32%	99.12%
32	AACTT	82.57%	92.68%
33	AAGAA	50.75%	93.52%
34	AAGAC	67.32%	84.40%
35	AAGAG	83.53%	94.74%
36	AAGAT	57.84%	90.72%
37	AAGCA	76.56%	94.12%
38	AAGCC	112.26%	95.74%
39	AAGCG	108.84%	95.33%
40	AAGCT	32.89%	90.24%
41	AAGGA	89.39%	92.24%
42	AAGGC	156.03%	96.82%
43	AAGGG	114.15%	94.68%
44	AAGGT	79.64%	94.05%
45	AAGTA	44.30%	89.87%
46	AAGTC	71.90%	78.95%
47	AAGTG	119.14%	95.45%
48	AAGTT	54.60%	89.04%
49	AATAA	62.85%	91.58%
50	AATAC	79.17%	75.00%
51	AATAG	88.27%	88.44%
52	AATAT	46.50%	87.13%
53	AATCA	87.01%	93.79%
54	AATCC	133.46%	95.71%
55	AATCG	92.39%	98.11%
56	AATCT	54.22%	95.95%
57	AATGA	99.18%	96.13%
58	AATGC	153.84%	96.30%
59	AATGG	150.11%	98.00%
60	AATGT	92.64%	88.33%
61	AATTA	61.26%	91.49%
62	AATTC	66.07%	95.65%
63	AATTG	117.72%	97.62%
64	AATTT	46.71%	87.80%
65	ACAAA	73.71%	96.63%
66	ACAAC	93.81%	83.85%
67	ACAAG	94.36%	95.42%
68	ACAAT	97.91%	93.82%
69	ACACA	123.44%	92.89%
70	ACACC	170.27%	94.65%
71	ACACG	154.28%	97.33%

72	ACACT	79.68%	95.37%
73	ACAGA	106.63%	95.76%
74	ACAGC	176.16%	98.07%
75	ACAGG	139.21%	95.07%
76	ACAGT	108.74%	95.12%
77	ACATA	94.14%	89.39%
78	ACATC	92.70%	87.07%
79	ACATG	154.97%	95.38%
80	ACATT	84.18%	88.80%
81	ACCAA	118.57%	96.51%
82	ACCAC	130.02%	89.00%
83	ACCAG	149.36%	95.56%
84	ACCAT	123.22%	97.74%
85	ACCCA	132.25%	95.43%
86	ACCCC	185.96%	98.54%
87	ACCCG	118.84%	97.41%
88	ACCCT	125.19%	97.12%
89	ACCGA	128.15%	95.18%
90	ACCGC	226.99%	98.47%
91	ACCGG	161.83%	99.27%
92	ACCGT	137.74%	96.35%
93	ACCTA	90.98%	92.56%
94	ACCTC	76.90%	89.02%
95	ACCTG	183.09%	97.91%
96	ACCTT	98.57%	91.23%
97	ACGAA	96.62%	96.15%
98	ACGAC	105.98%	95.24%
99	ACGAG	130.21%	97.66%
100	ACGAT	102.59%	92.06%
101	ACGCA	99.95%	95.93%
102	ACGCC	152.39%	97.87%
103	ACGCG	112.00%	90.32%
104	ACGCT	66.99%	93.75%
105	ACGGA	135.28%	97.20%
106	ACGGC	129.92%	98.32%
107	ACGGG	137.29%	97.87%
108	ACGGT	96.79%	98.67%
109	ACGTA	75.26%	93.62%
110	ACGTC	66.60%	90.16%
111	ACGTG	126.40%	98.32%
112	ACGTT	74.79%	93.15%
113	ACTAA	86.36%	91.88%
114	ACTAC	89.42%	85.83%
115	ACTAG	129.27%	93.38%
116	ACTAT	73.91%	88.68%
117	ACTCA	101.82%	96.03%

118	ACTCC	158.35%	96.71%
119	ACTCG	134.09%	97.41%
120	ACTCT	66.00%	96.67%
121	ACTGA	138.12%	94.08%
122	ACTGC	205.87%	97.62%
123	ACTGG	177.04%	99.28%
124	ACTGT	145.51%	94.93%
125	ACTTA	78.24%	93.22%
126	ACTTC	101.79%	95.88%
127	ACTTG	156.01%	98.19%
128	ACTTT	80.13%	90.11%
129	AGAAA	43.93%	89.47%
130	AGAAC	82.21%	93.10%
131	AGAAG	93.80%	89.09%
132	AGAAT	56.09%	87.64%
133	AGACA	92.60%	92.05%
134	AGACC	124.34%	96.03%
135	AGACG	101.55%	96.04%
136	AGACT	55.84%	84.85%
137	AGAGA	95.11%	95.04%
138	AGAGC	133.34%	96.09%
139	AGAGG	124.28%	95.74%
140	AGAGT	108.08%	94.23%
141	AGATA	66.91%	84.62%
142	AGATC	49.02%	84.00%
143	AGATG	125.02%	94.66%
144	AGATT	52.68%	80.88%
145	AGCAA	76.29%	95.45%
146	AGCAC	106.63%	97.67%
147	AGCAG	128.47%	94.78%
148	AGCAT	119.20%	93.28%
149	AGCCA	114.59%	94.70%
150	AGCCC	171.17%	96.91%
151	AGCCG	81.14%	92.54%
152	AGCCT	80.87%	93.59%
153	AGCGA	82.71%	95.45%
154	AGCGC	150.78%	88.28%
155	AGCGG	200.72%	94.87%
156	AGCGT	152.90%	94.83%
157	AGCTA	61.01%	85.19%
158	AGCTC	53.47%	93.62%
159	AGCTG	199.63%	98.09%
160	AGCTT	78.15%	89.87%
161	AGGAA	65.32%	94.05%
162	AGGAC	101.41%	96.84%
163	AGGAG	89.31%	92.75%

164	AGGAT	54.95%	71.70%
165	AGGCA	80.69%	93.83%
166	AGGCC	129.70%	96.75%
167	AGGCG	79.08%	95.00%
168	AGGCT	38.07%	87.10%
169	AGGGA	121.44%	100.00%
170	AGGGC	150.61%	80.00%
171	AGGGG	87.56%	91.67%
172	AGGGT	99.20%	100.00%
173	AGGTA	68.26%	89.39%
174	AGGTC	67.68%	100.00%
175	AGGTG	161.76%	97.35%
176	AGGTT	56.60%	76.60%
177	AGTAA	76.09%	90.91%
178	AGTAC	100.33%	80.00%
179	AGTAG	119.84%	89.72%
180	AGTAT	59.17%	90.67%
181	AGTCA	117.74%	91.60%
182	AGTCC	147.41%	98.51%
183	AGTCG	139.30%	95.40%
184	AGTCT	57.17%	91.67%
185	AGTGA	109.52%	92.11%
186	AGTGC	202.00%	99.39%
187	AGTGG	172.93%	100.00%
188	AGTGT	121.78%	97.00%
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190	AGTTC	68.26%	87.30%
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195	ATAAG	85.94%	94.62%
196	ATAAT	54.91%	81.51%
197	ATACA	83.63%	87.88%
198	ATACC	110.39%	93.41%
199	ATACG	118.36%	96.45%
200	ATACT	57.55%	91.46%
201	ATAGA	83.12%	94.24%
202	ATAGC	138.45%	96.93%
203	ATAGG	115.91%	95.50%
204	ATAGT	88.21%	93.69%
205	ATATA	52.38%	88.98%
206	ATATC	79.70%	95.28%
207	ATATG	112.94%	96.03%
208	ATATT	69.40%	88.99%
209	ATCAA	111.73%	95.98%

210	ATCAC	116.03%	92.99%
211	ATCAG	147.14%	93.21%
212	ATCAT	101.35%	94.53%
213	ATCCA	143.32%	92.82%
214	ATCCC	201.75%	95.85%
215	ATCCG	171.18%	97.81%
216	ATCCT	119.40%	94.83%
217	ATCGA	142.37%	93.90%
218	ATCGC	218.84%	99.02%
219	ATCGG	221.66%	100.00%
220	ATCGT	141.34%	97.48%
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223	ATCTG	181.84%	98.01%
224	ATCTT	100.94%	93.40%
225	ATGAA	81.13%	96.50%
226	ATGAC	116.15%	88.19%
227	ATGAG	99.27%	96.26%
228	ATGAT	93.73%	92.79%
229	ATGCA	114.01%	97.32%
230	ATGCC	147.72%	97.33%
231	ATGCG	138.09%	96.92%
232	ATGCT	94.03%	95.40%
233	ATGGA	92.19%	95.15%
234	ATGGC	204.97%	98.28%
235	ATGGG	184.61%	100.00%
236	ATGGT	116.40%	95.92%
237	ATGTA	75.30%	87.50%
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239	ATGTG	147.46%	100.00%
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241	ATTAA	73.04%	92.55%
242	ATTAC	92.35%	92.03%
243	ATTAG	118.82%	94.62%
244	ATTAT	37.17%	29.63%
245	ATTCA	106.59%	96.50%
246	ATTCC	165.74%	98.80%
247	ATTCG	165.23%	99.25%
248	ATTCT	61.45%	75.44%
249	ATTGA	106.87%	92.21%
250	ATTGC	155.82%	97.69%
251	ATTGG	173.68%	96.40%
252	ATTGT	96.52%	94.79%
253	ATTTA	67.39%	88.98%
254	ATTTC	86.65%	92.39%
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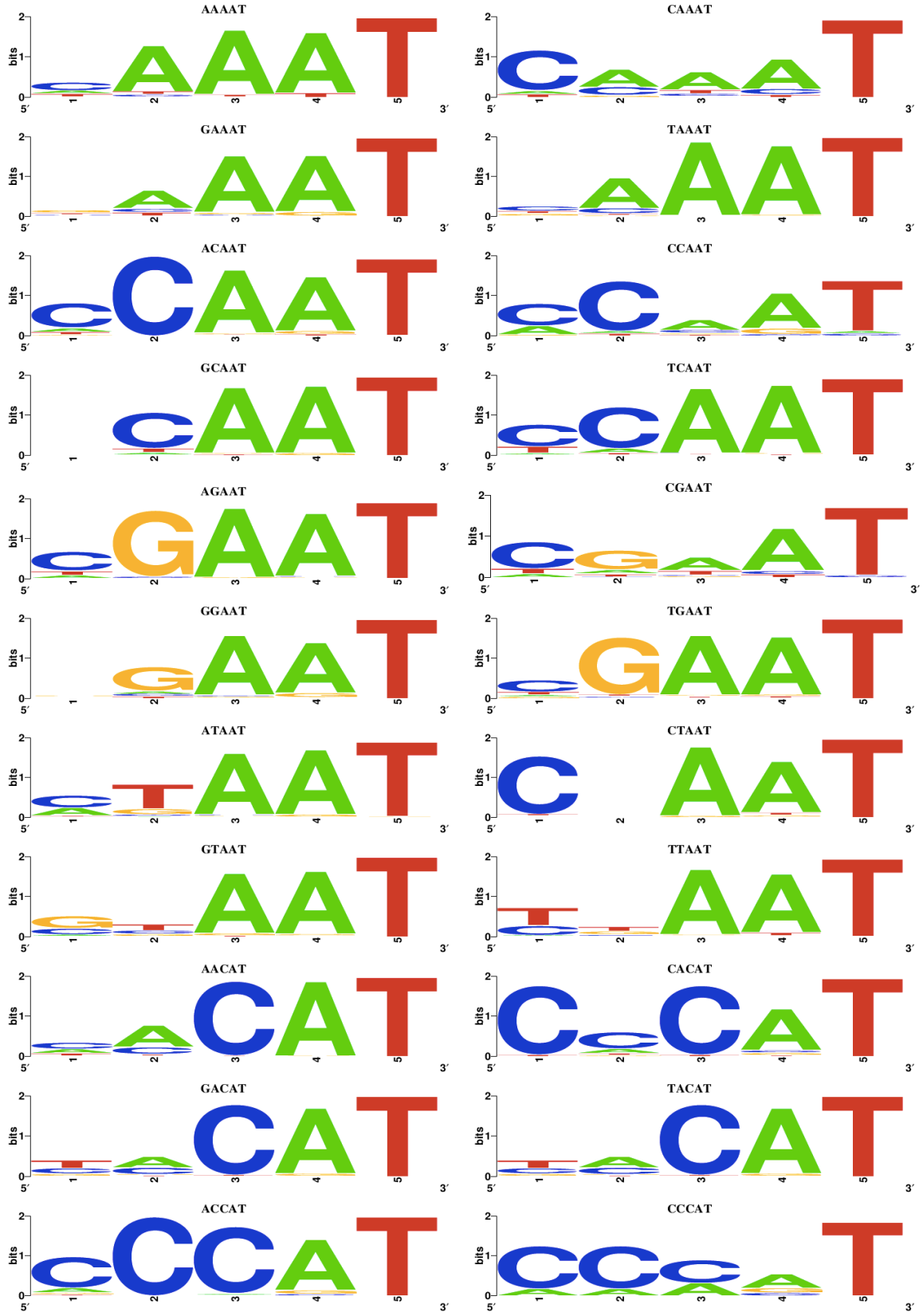
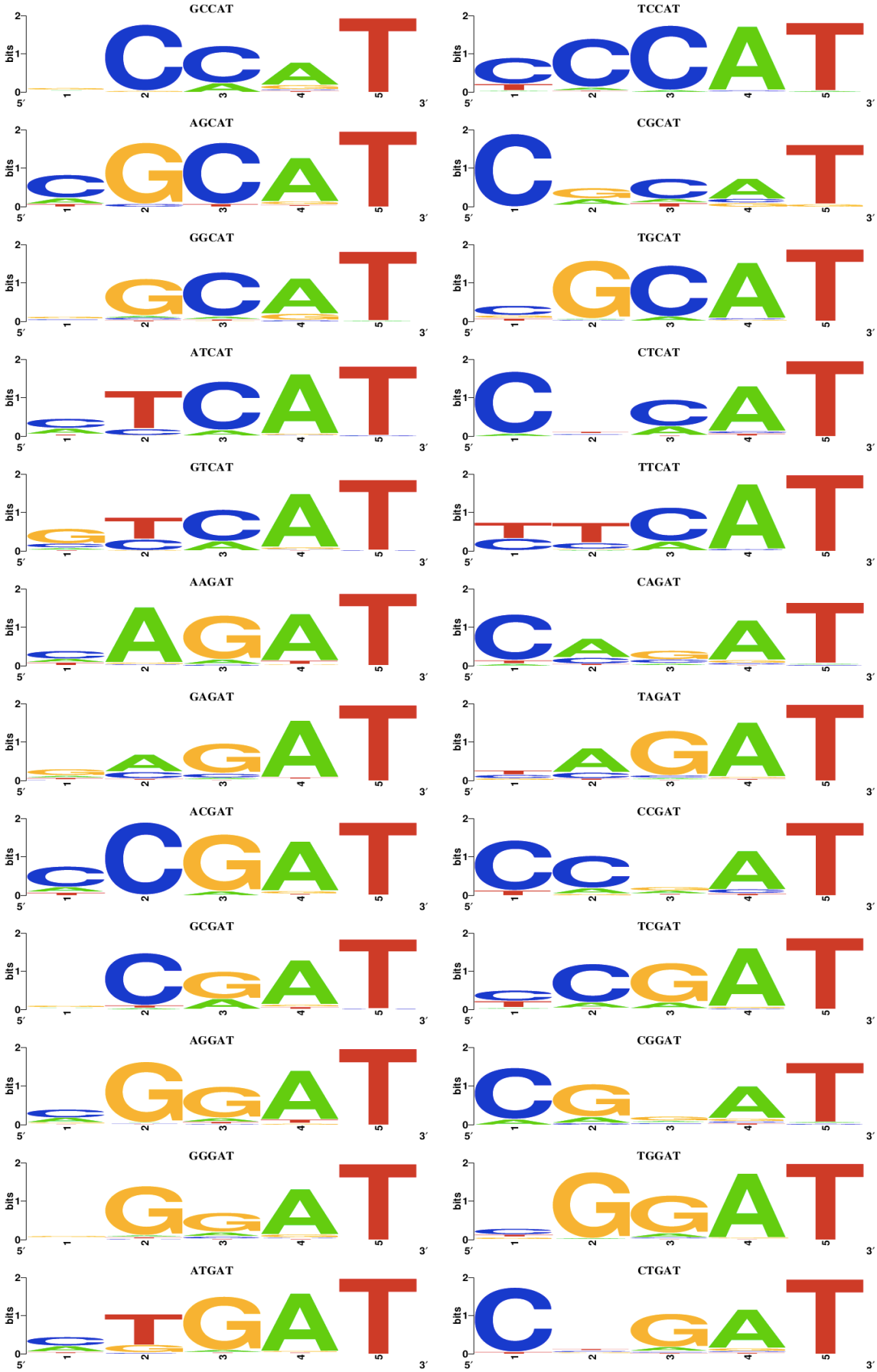
Supporting Figures

Figure S1. amine-modified ANNNN sequence logos

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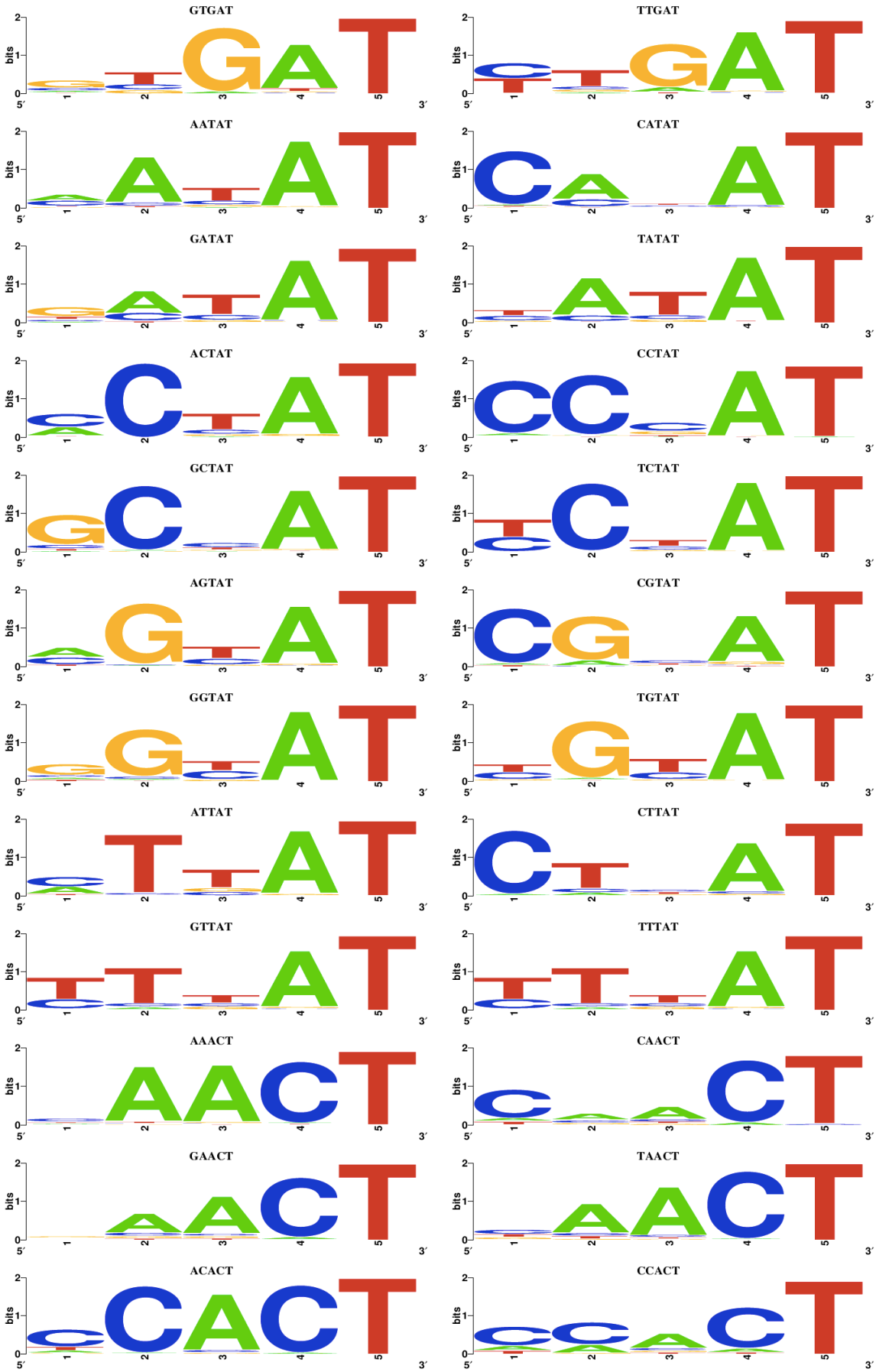
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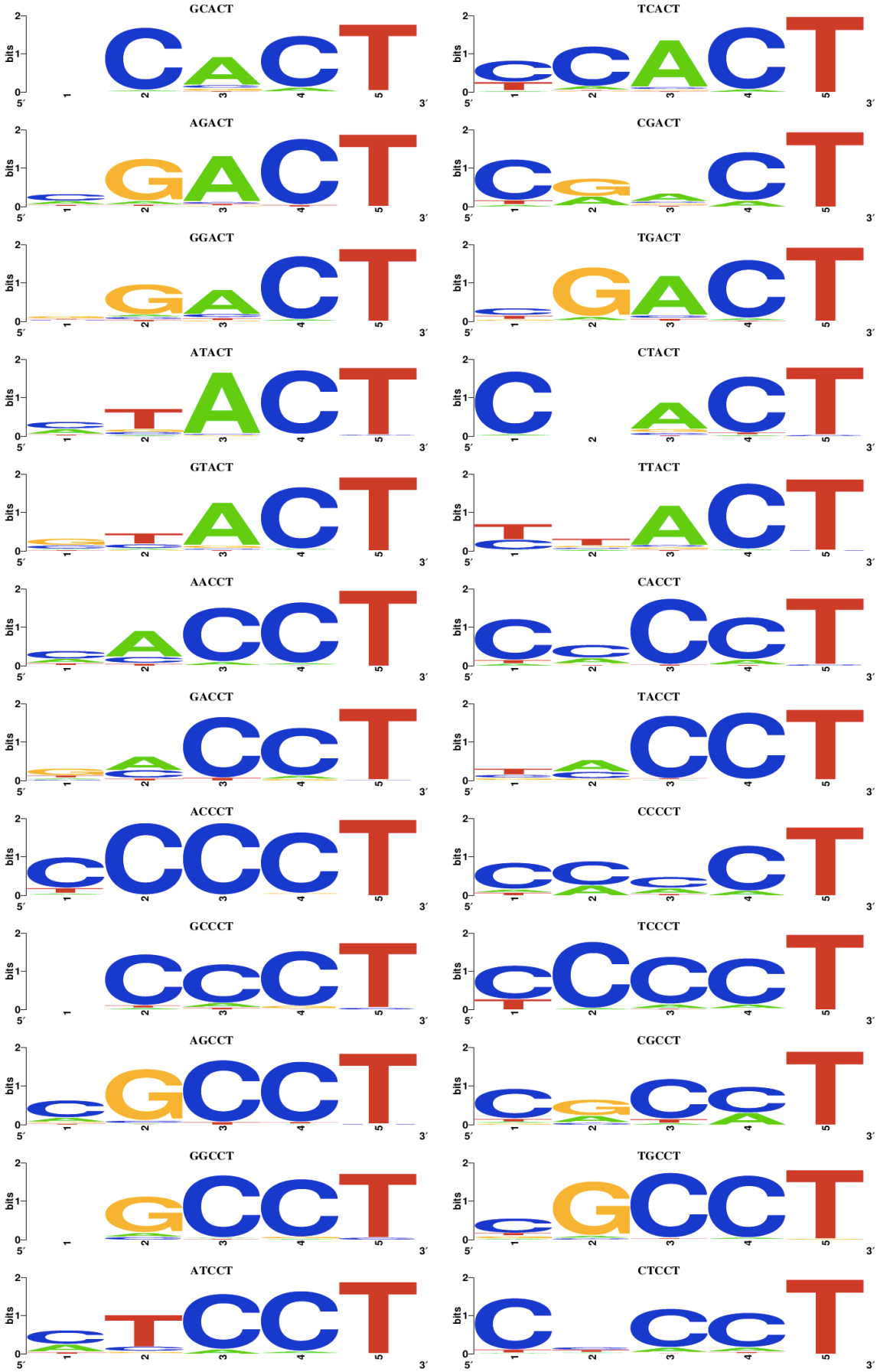
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ATACT
CTACT
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TTACT
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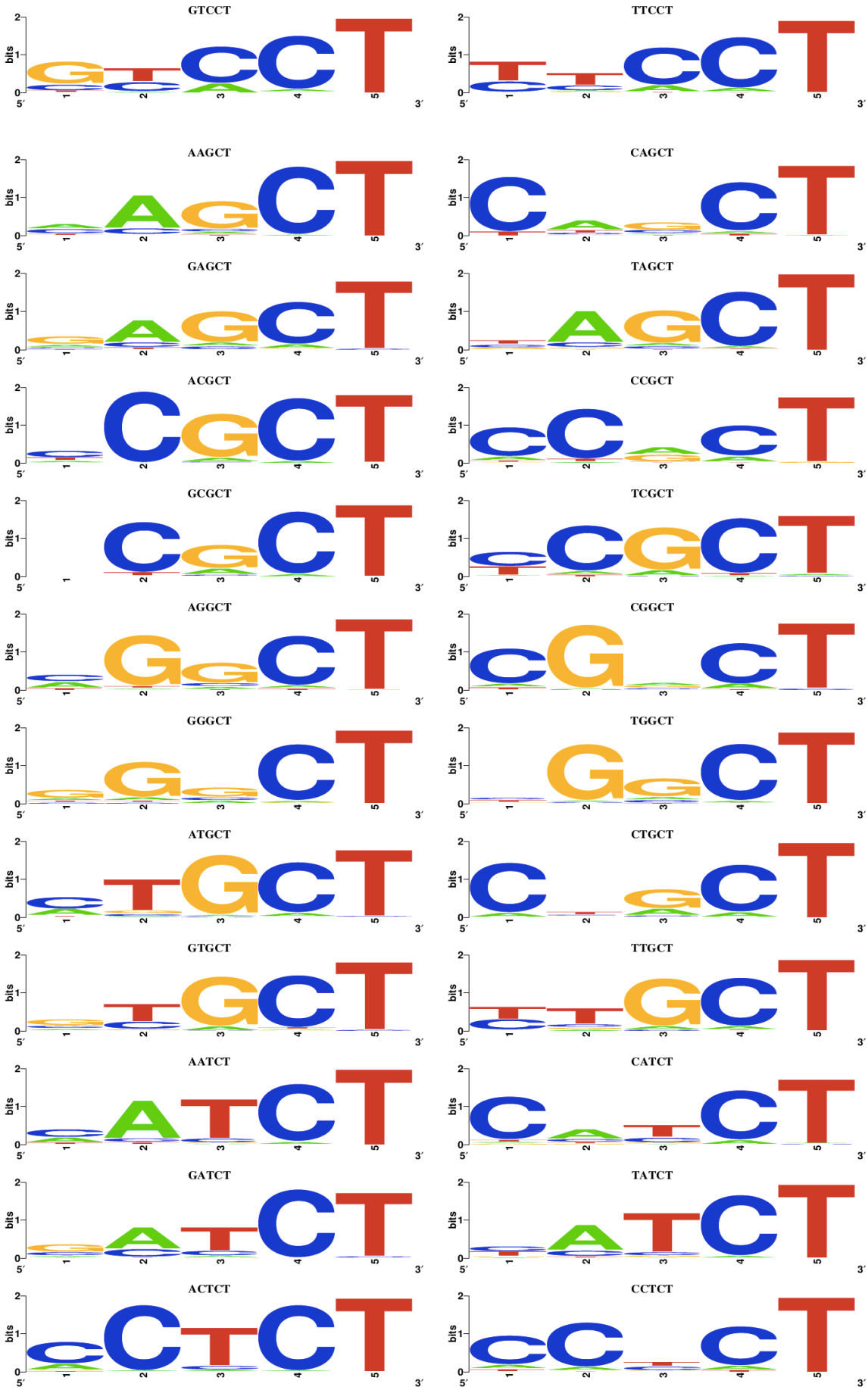
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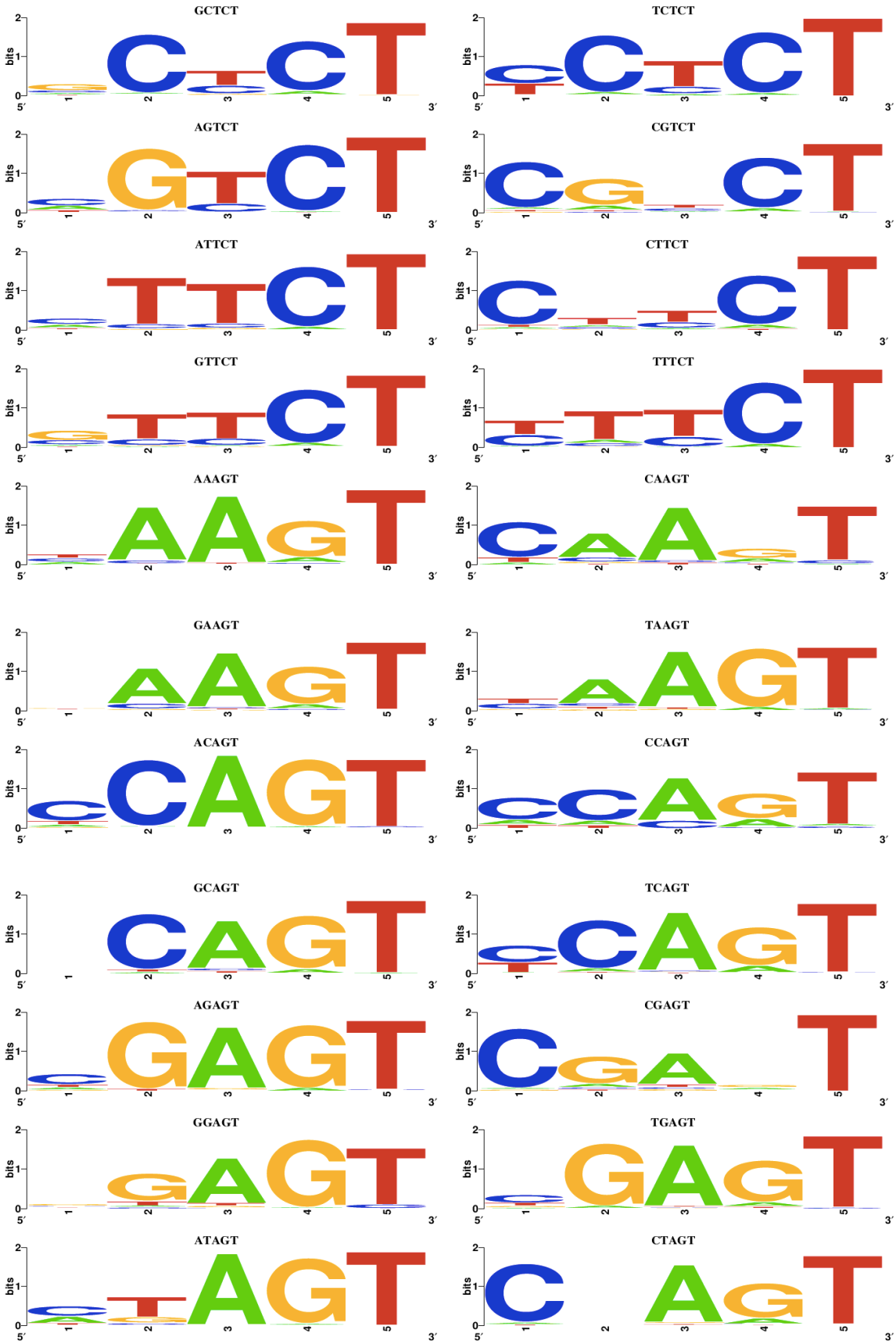
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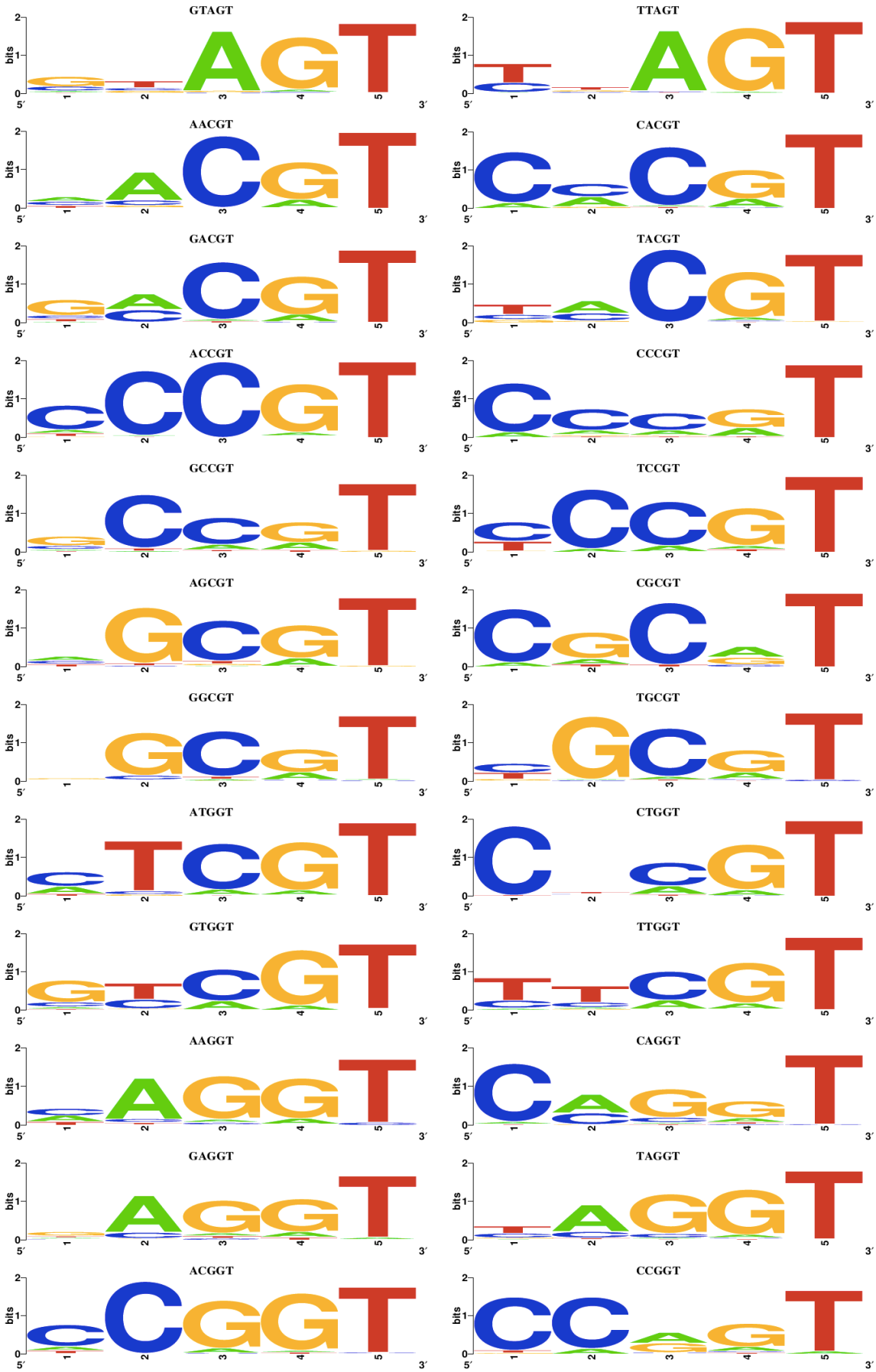
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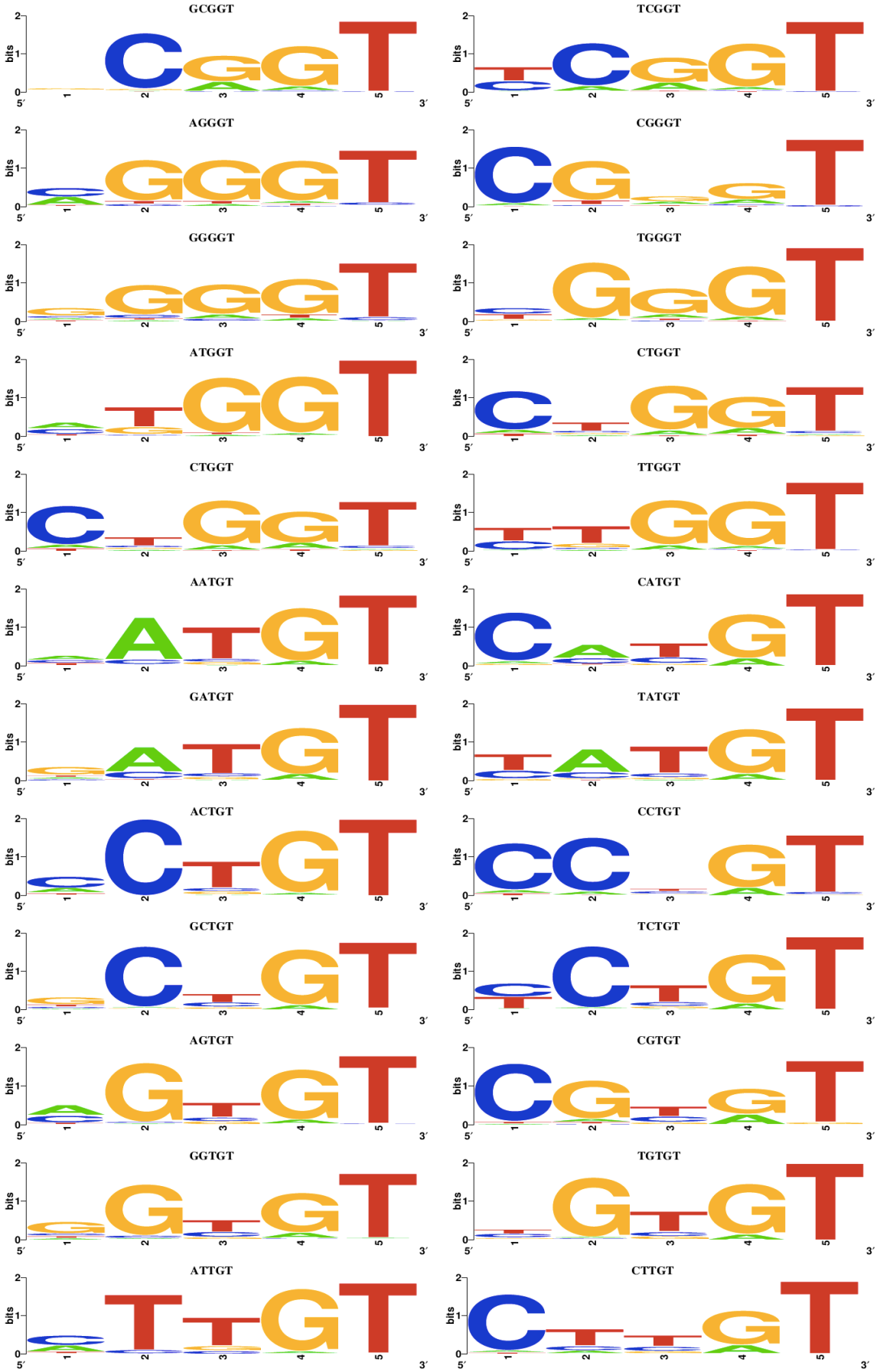
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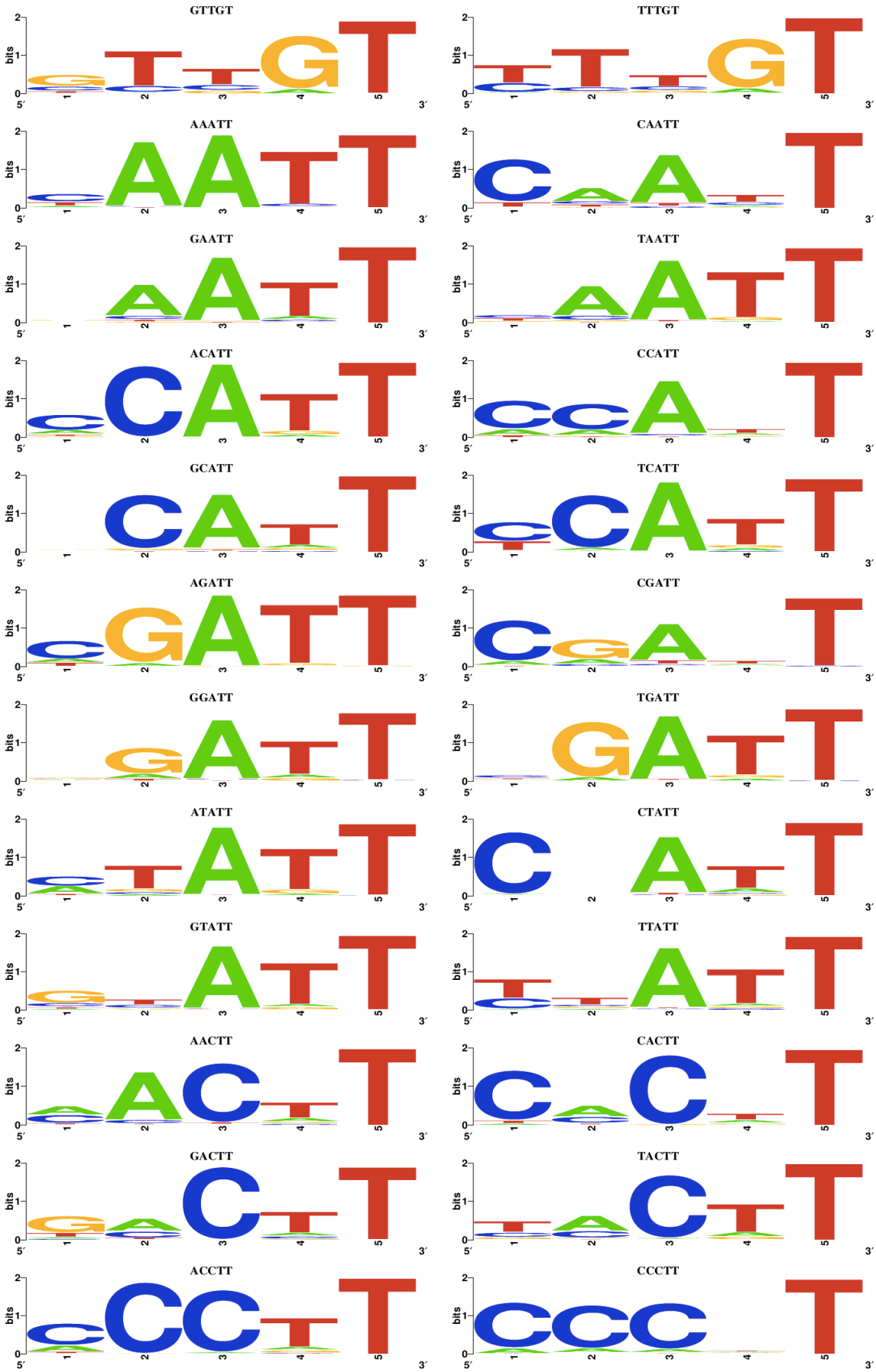
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TACTT
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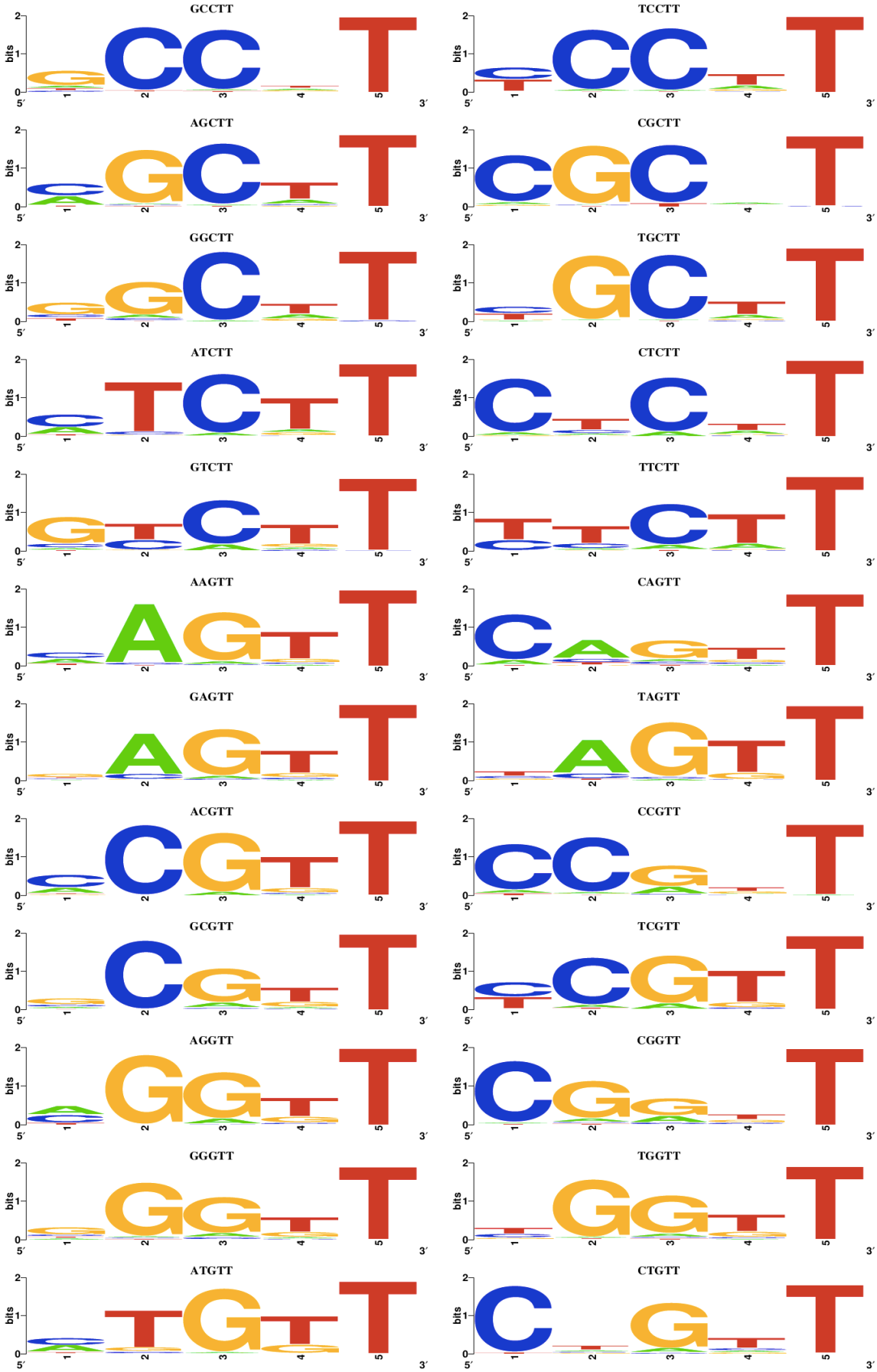
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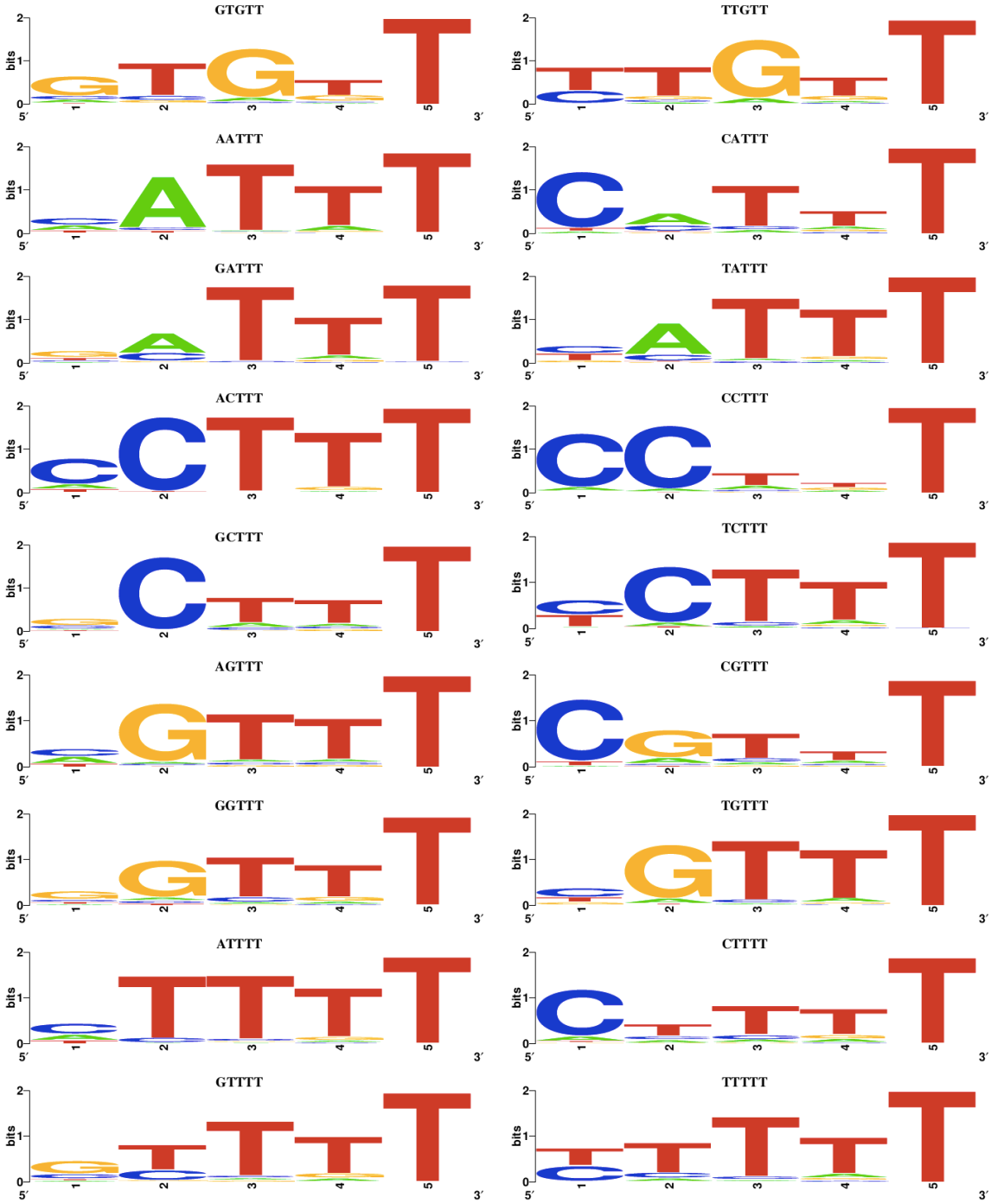
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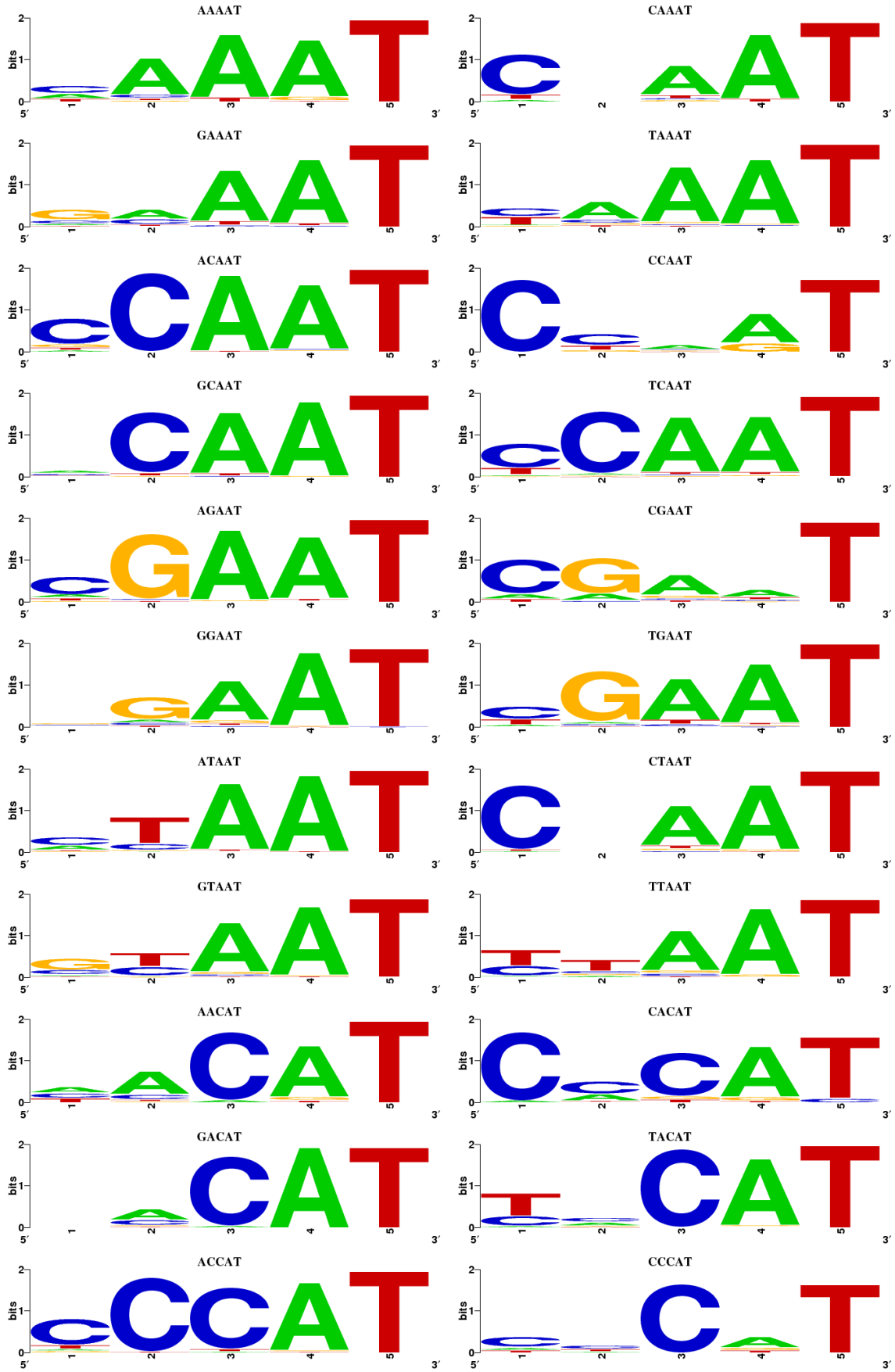
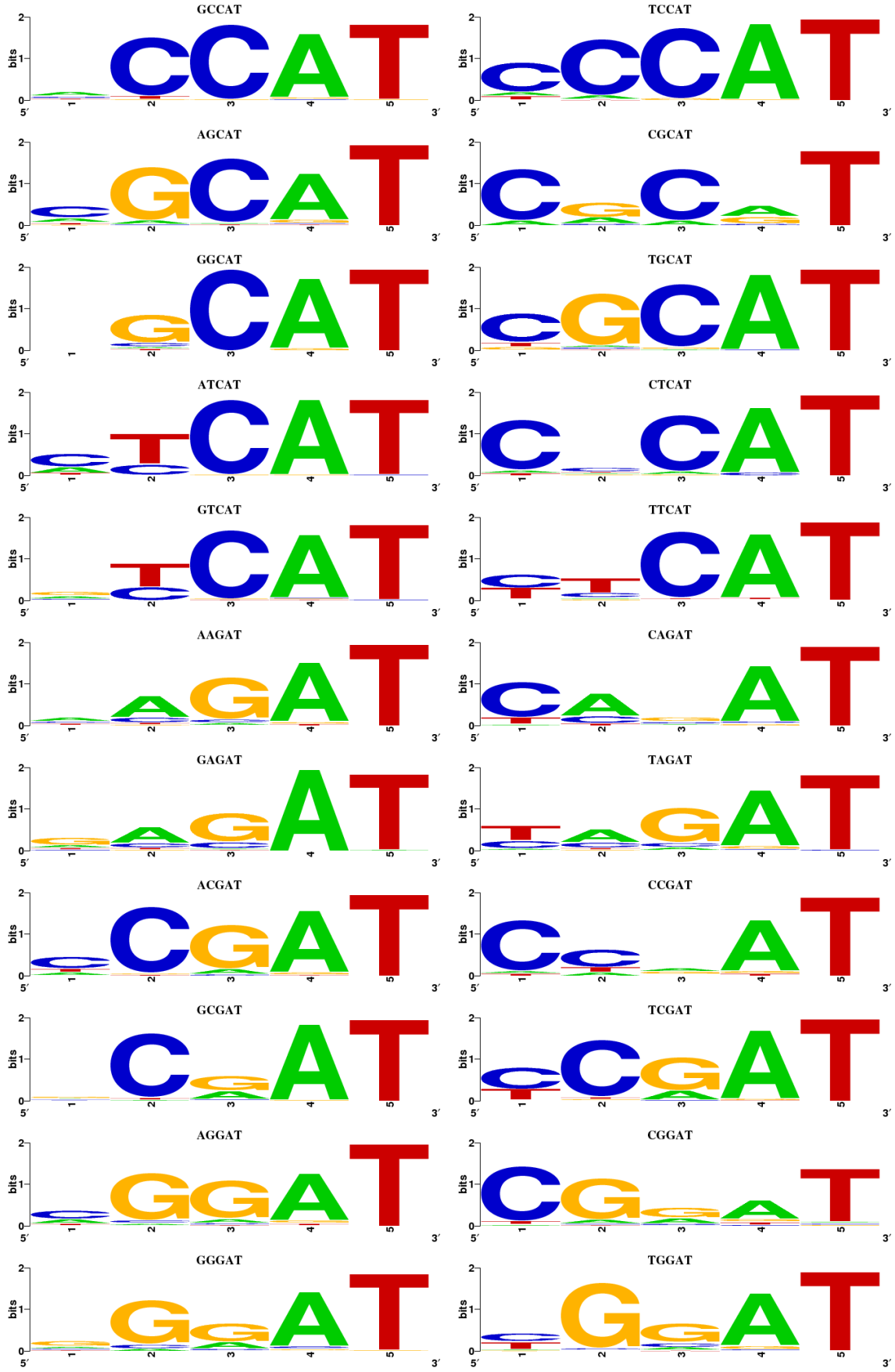
bits
5′
3′

Figure S2. phenyl-modified ANNNN sequence logos

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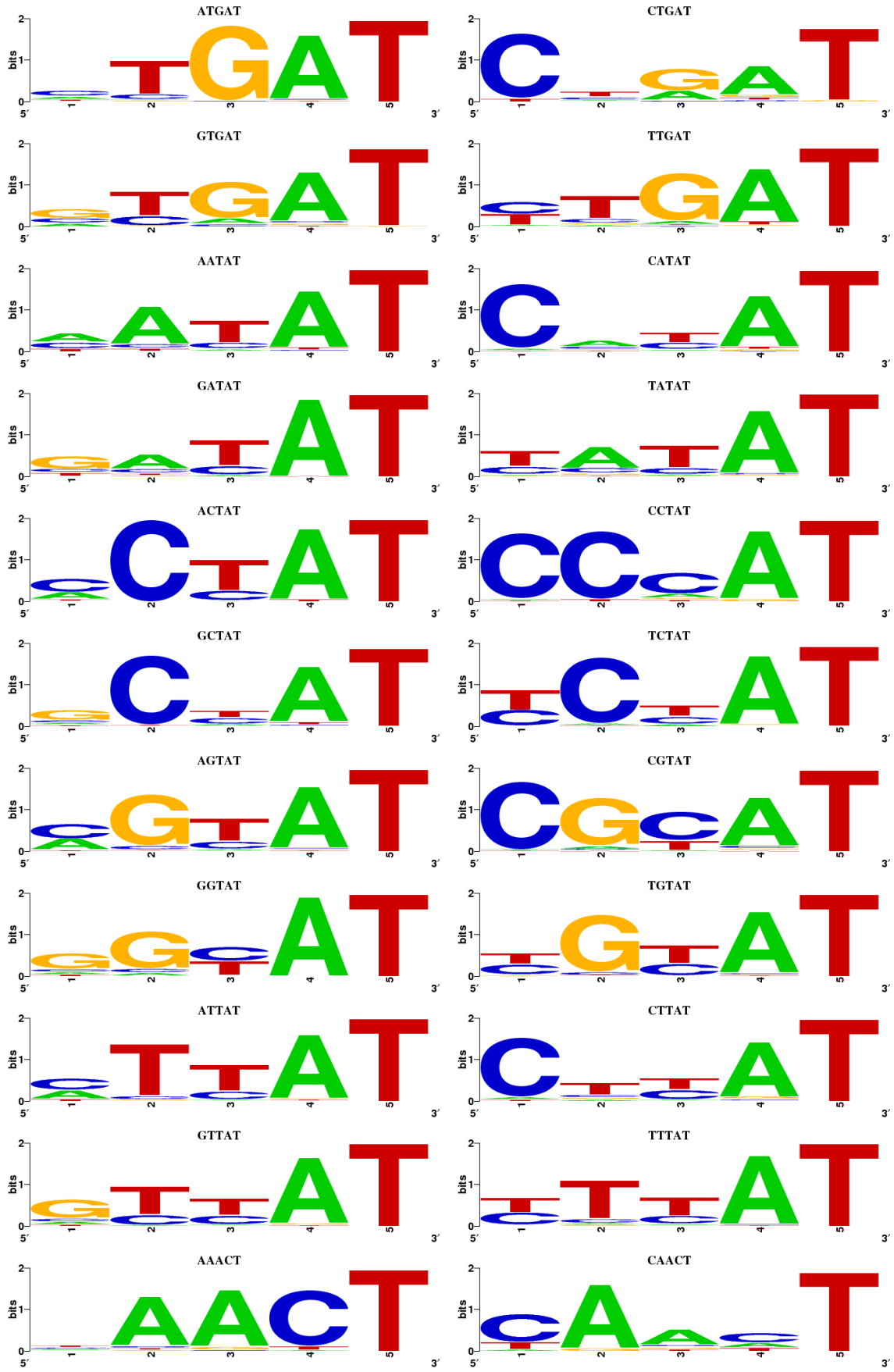
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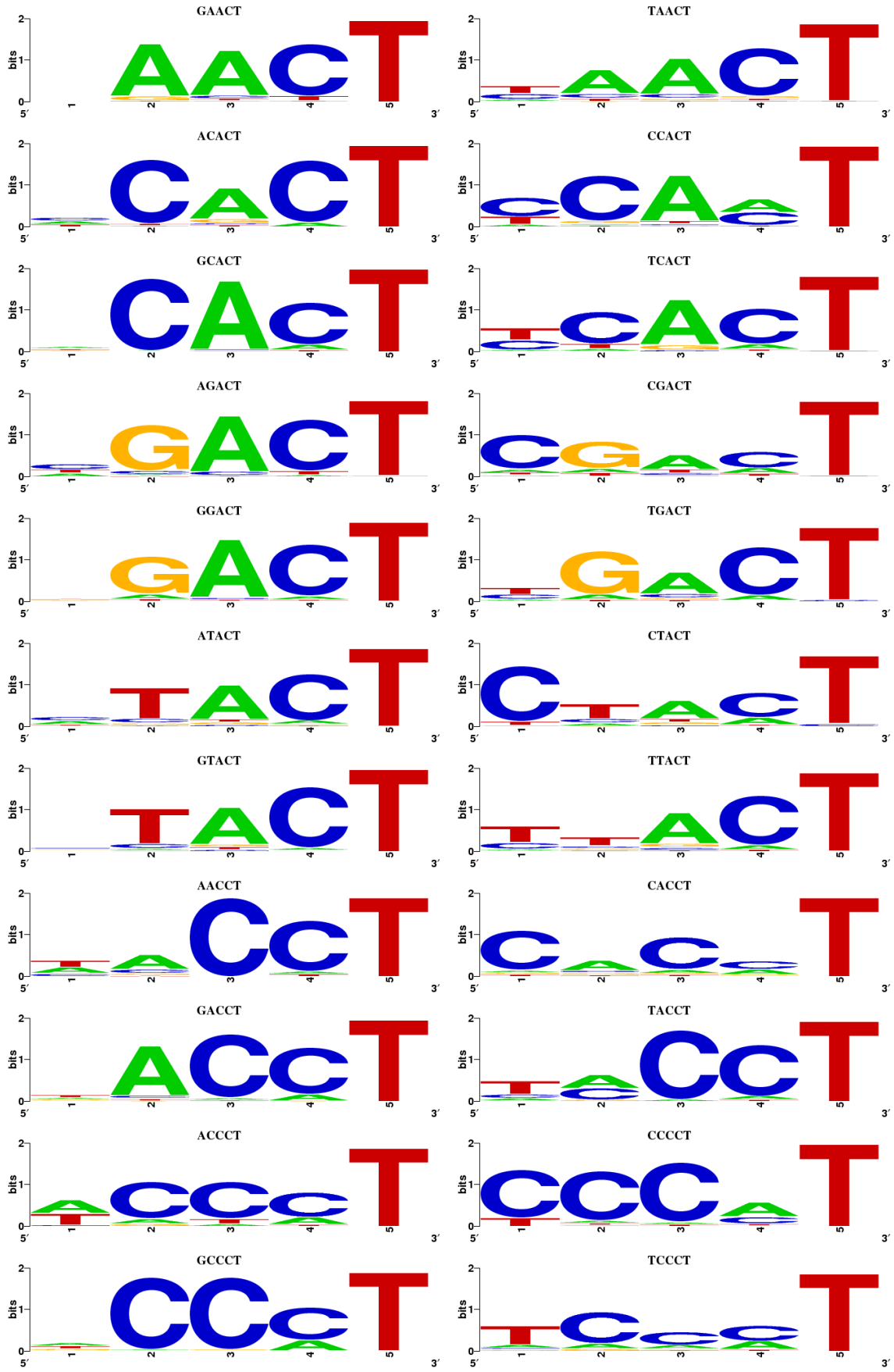
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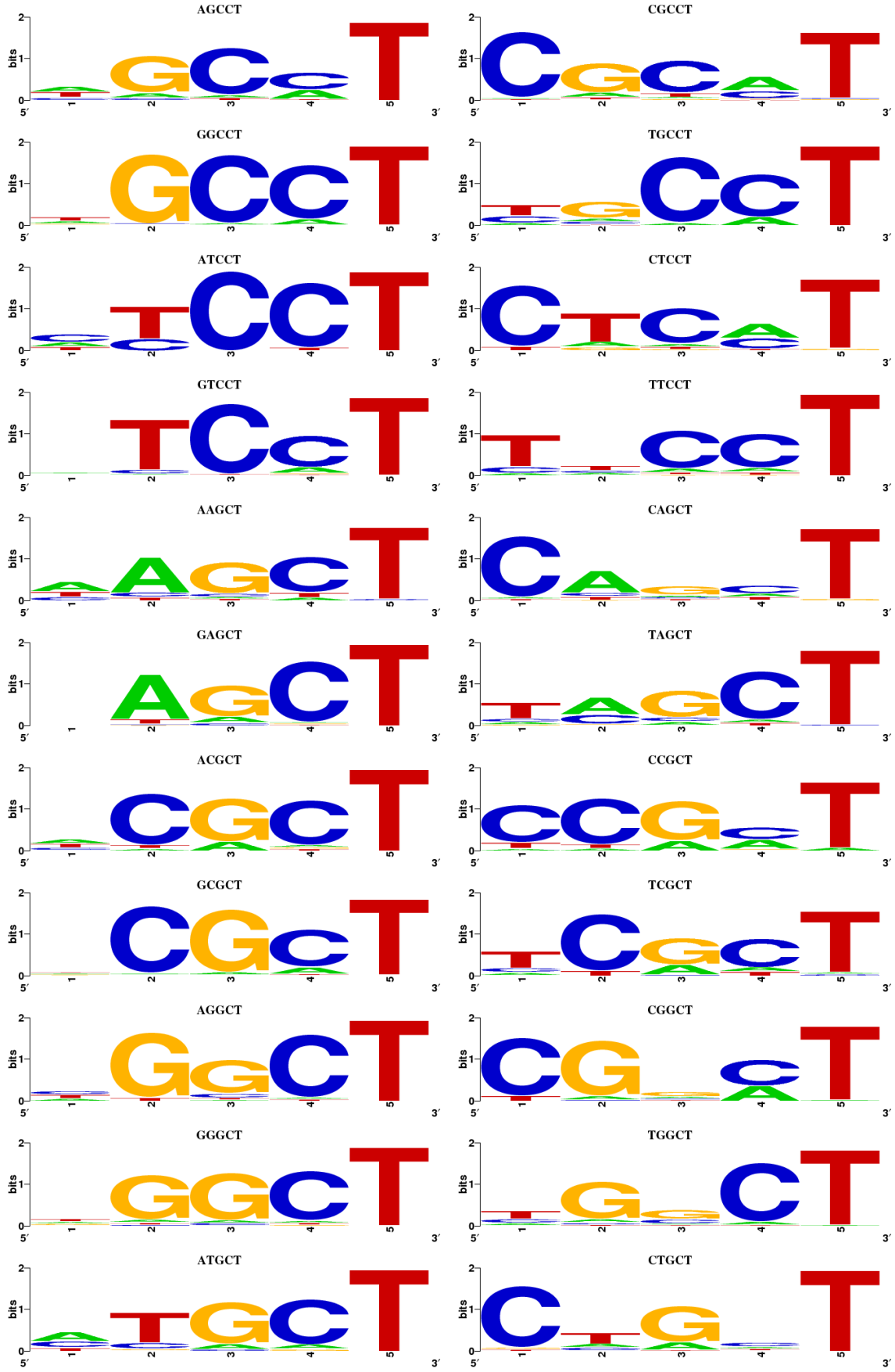
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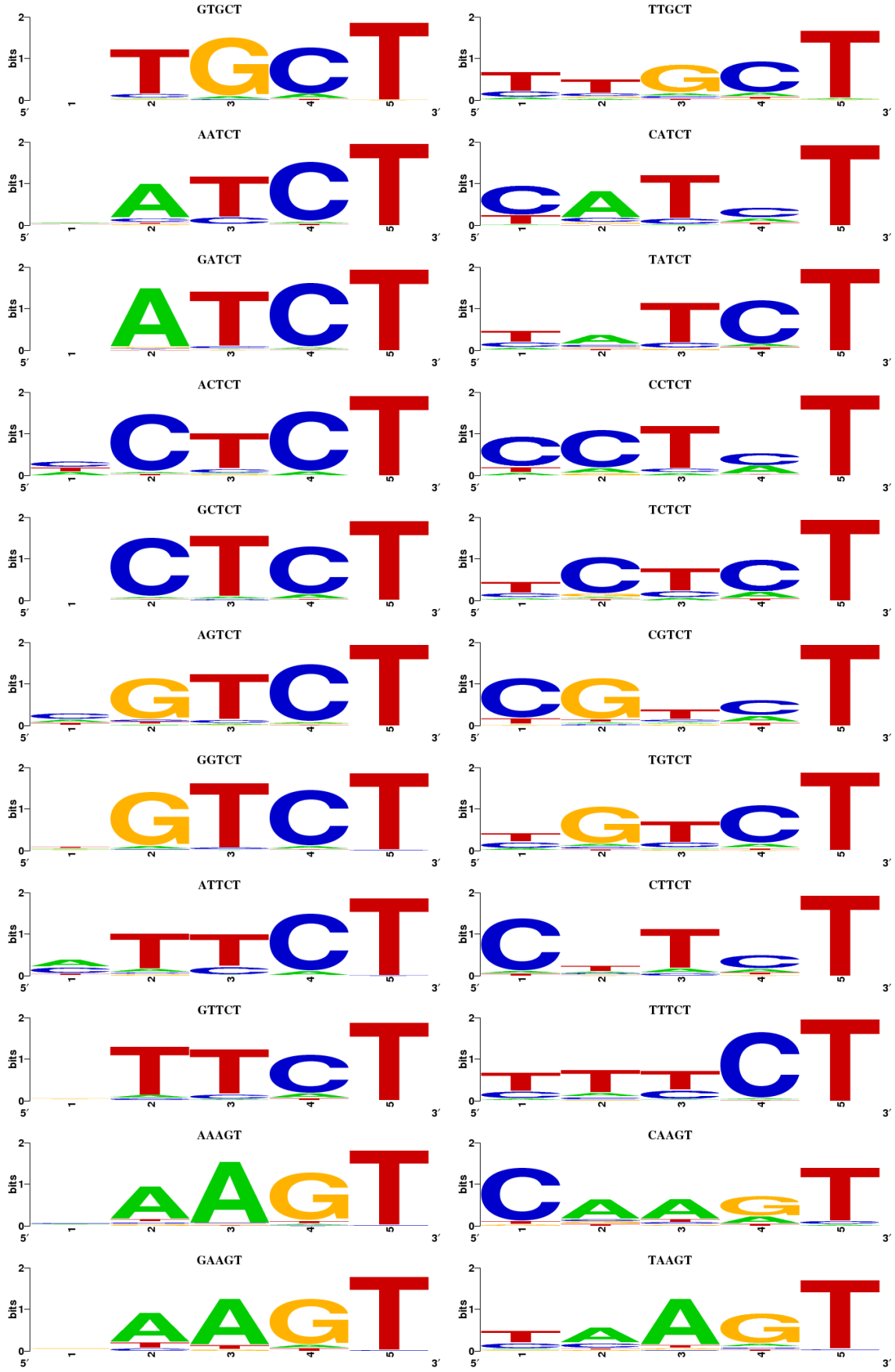
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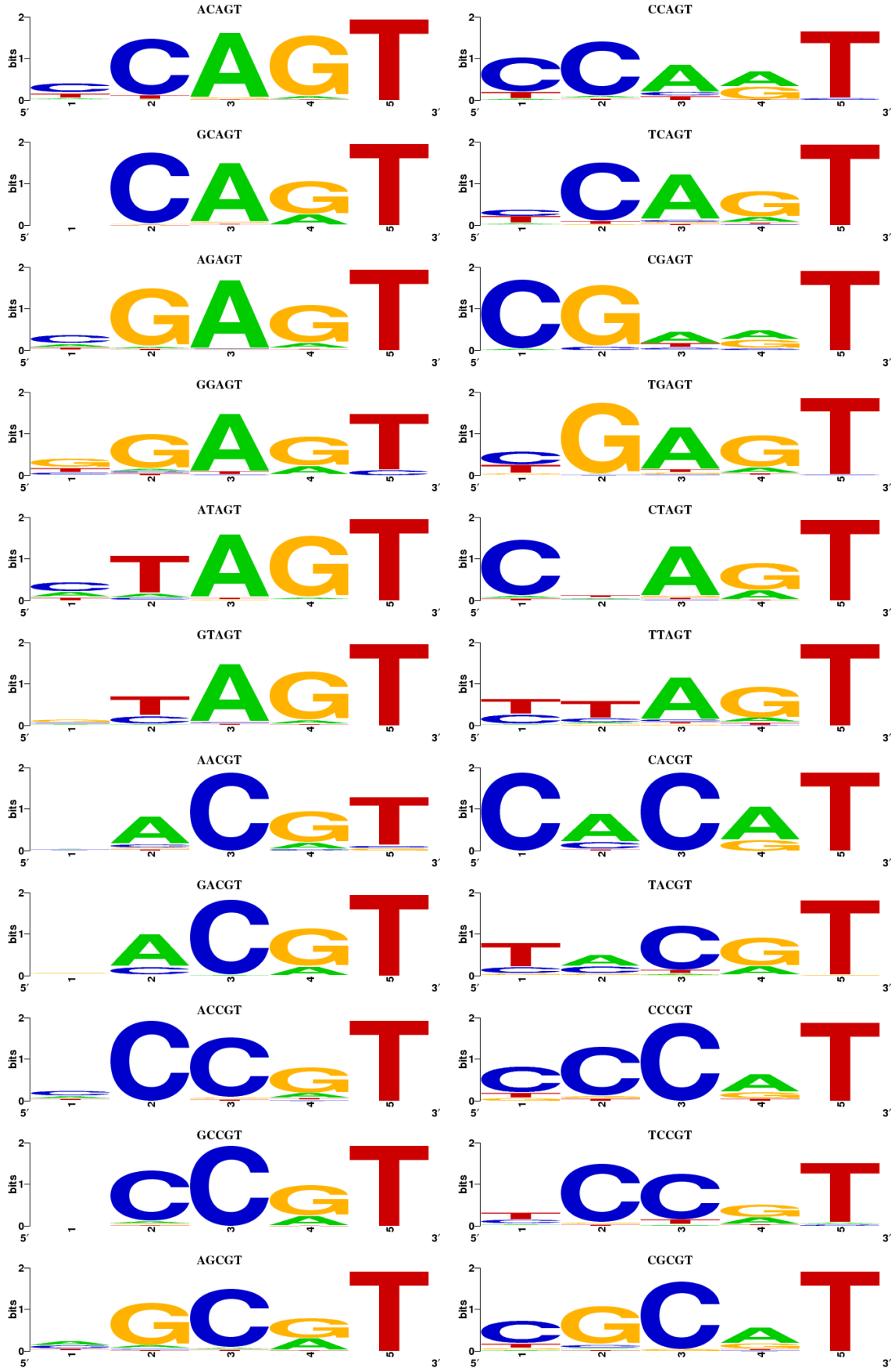
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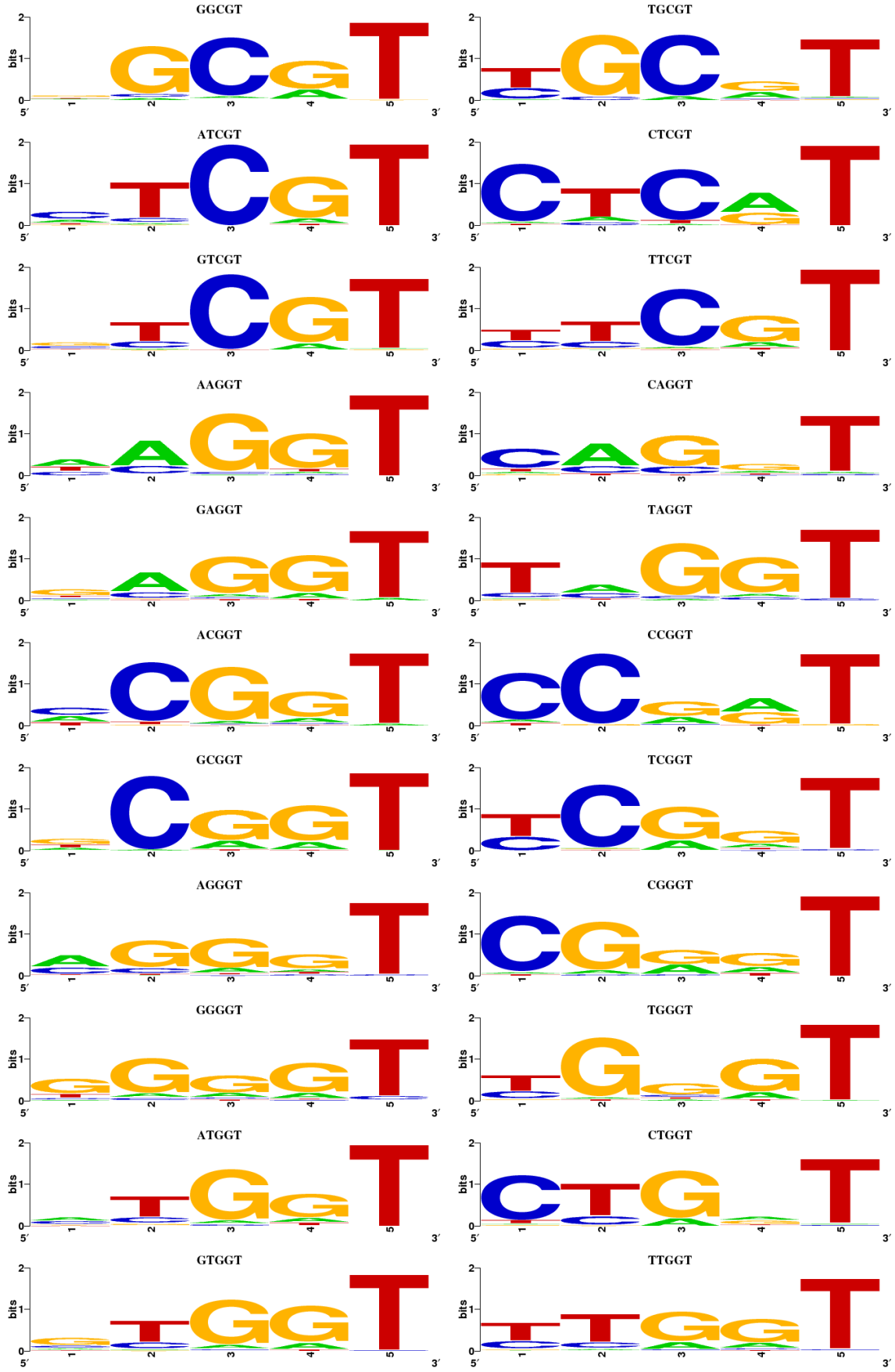
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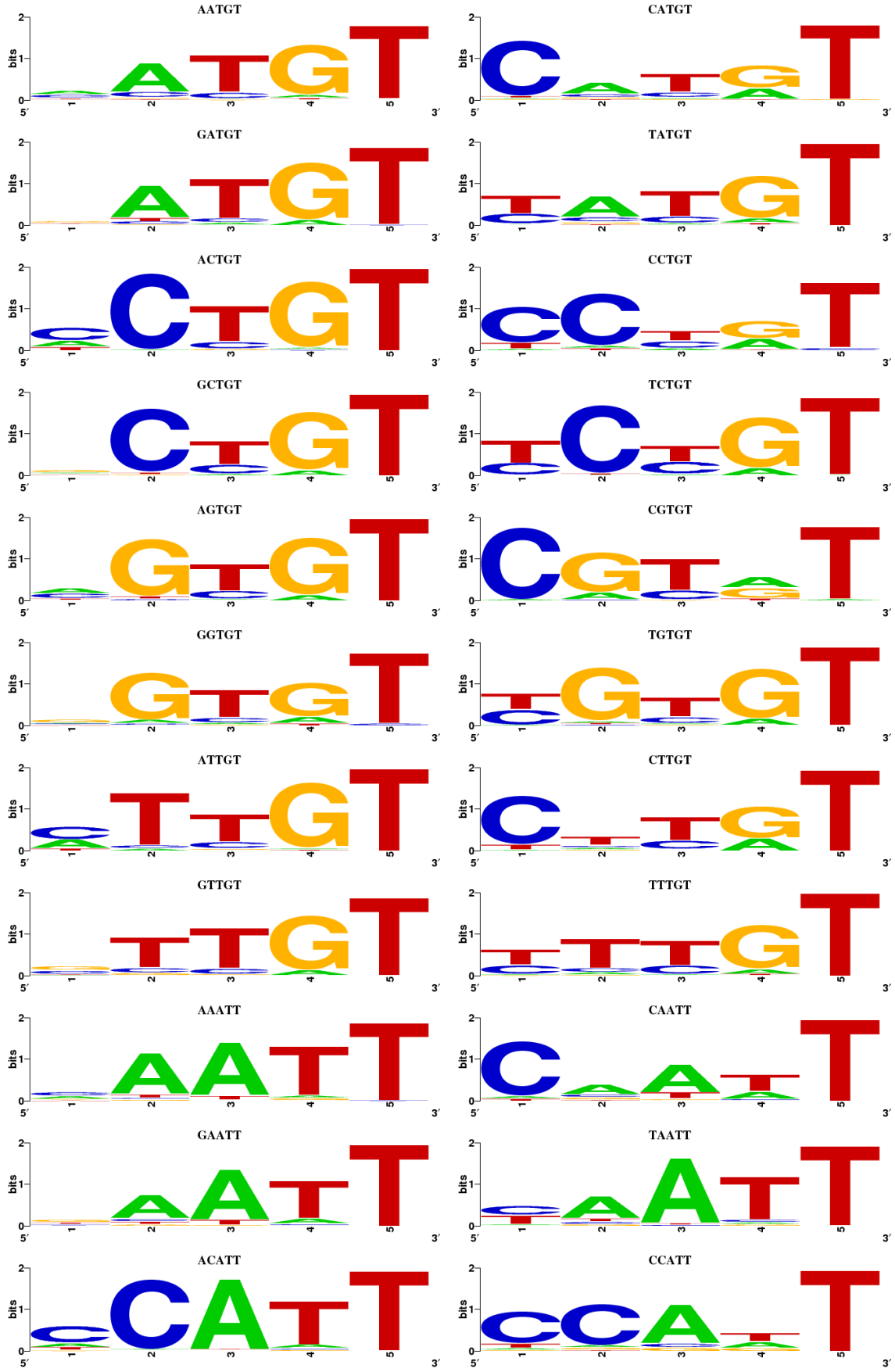
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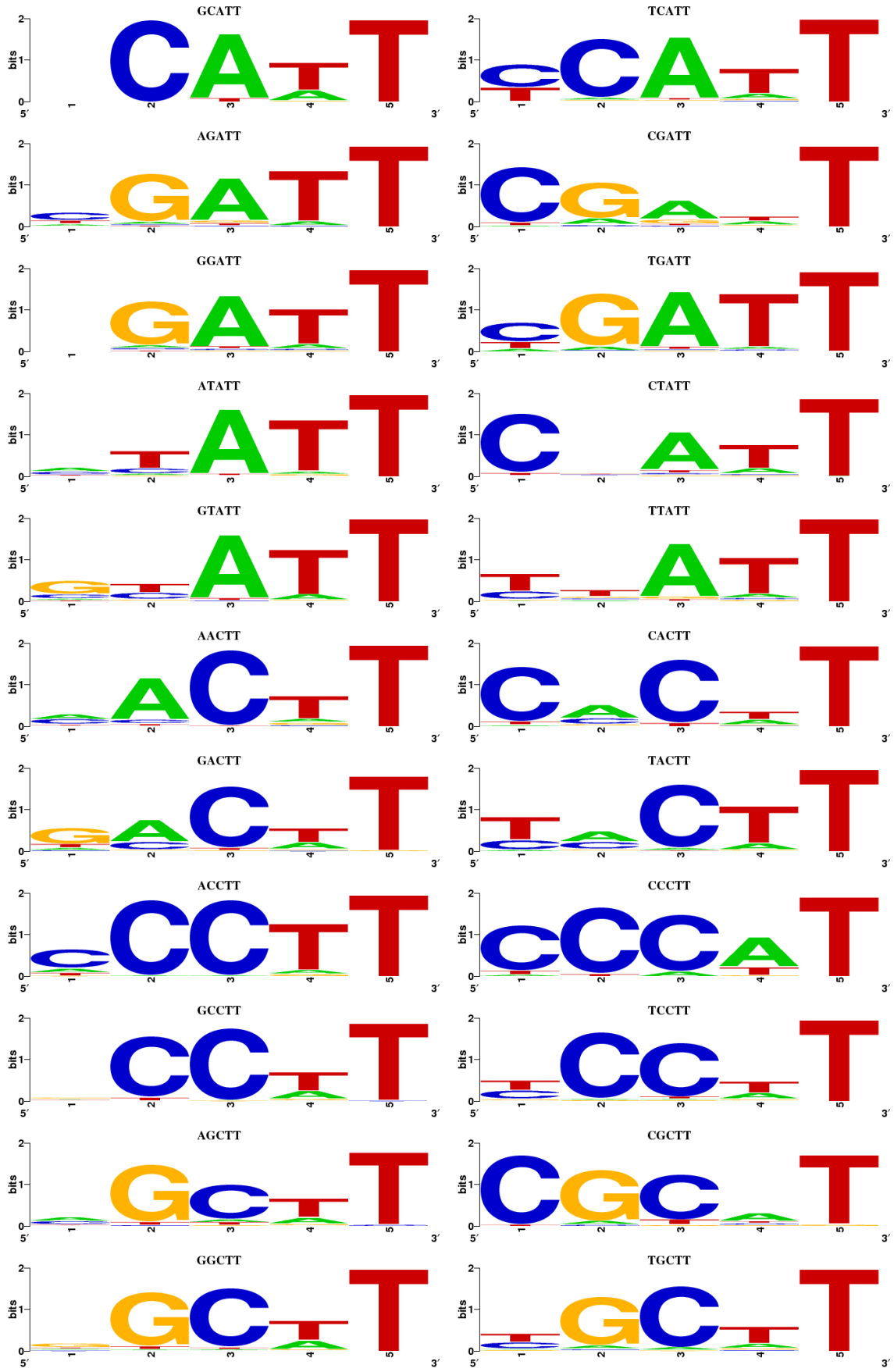
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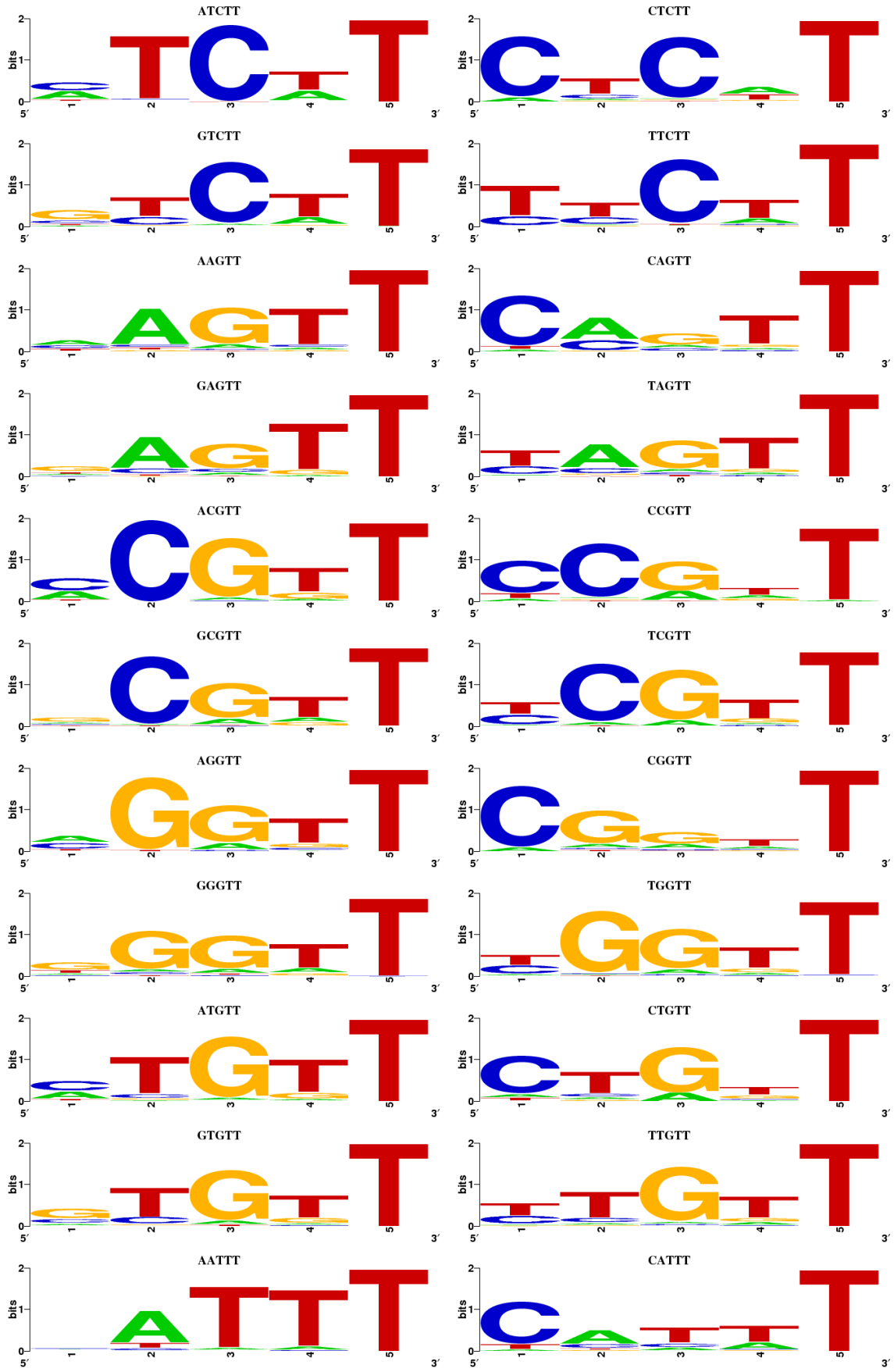
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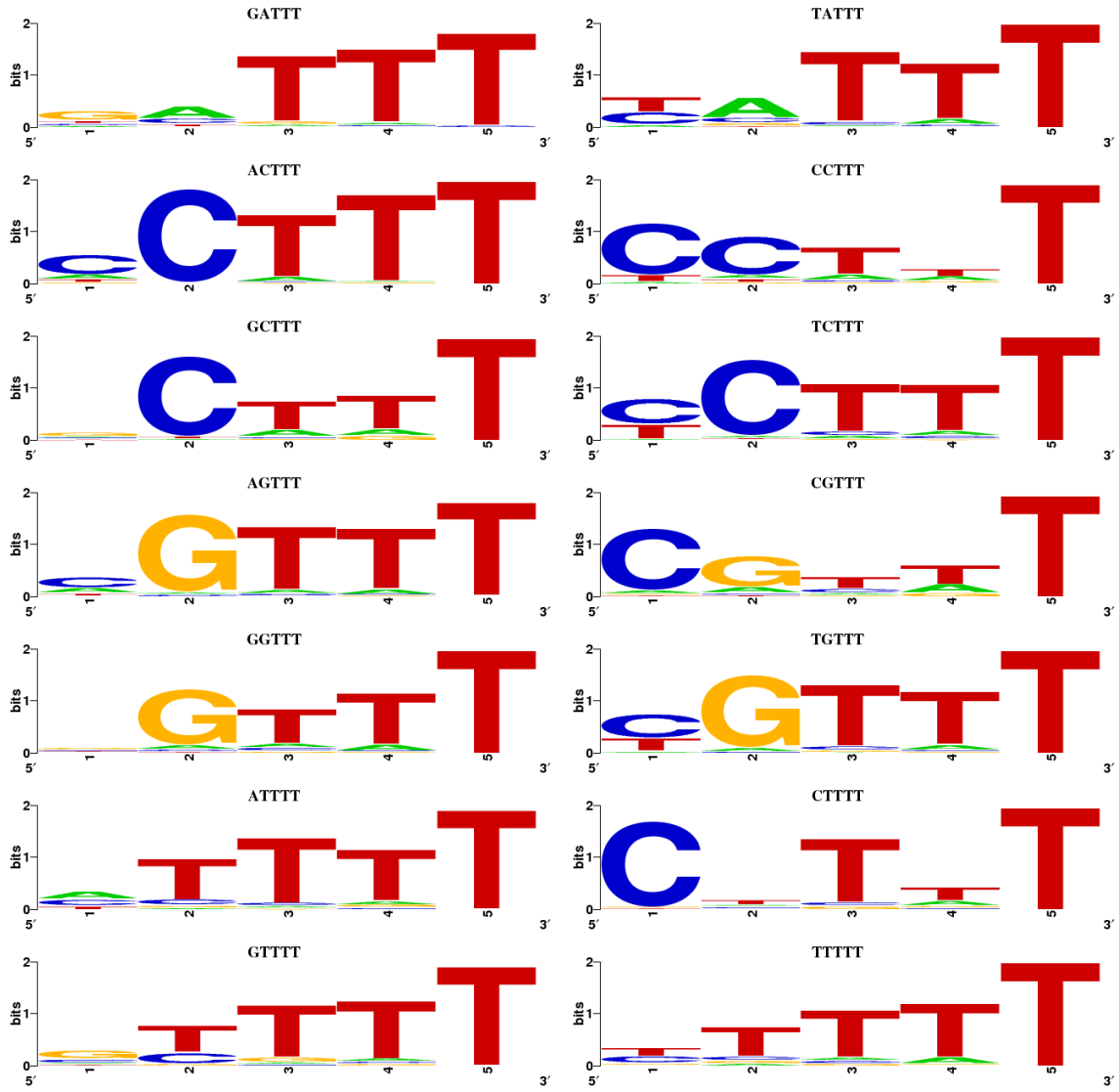
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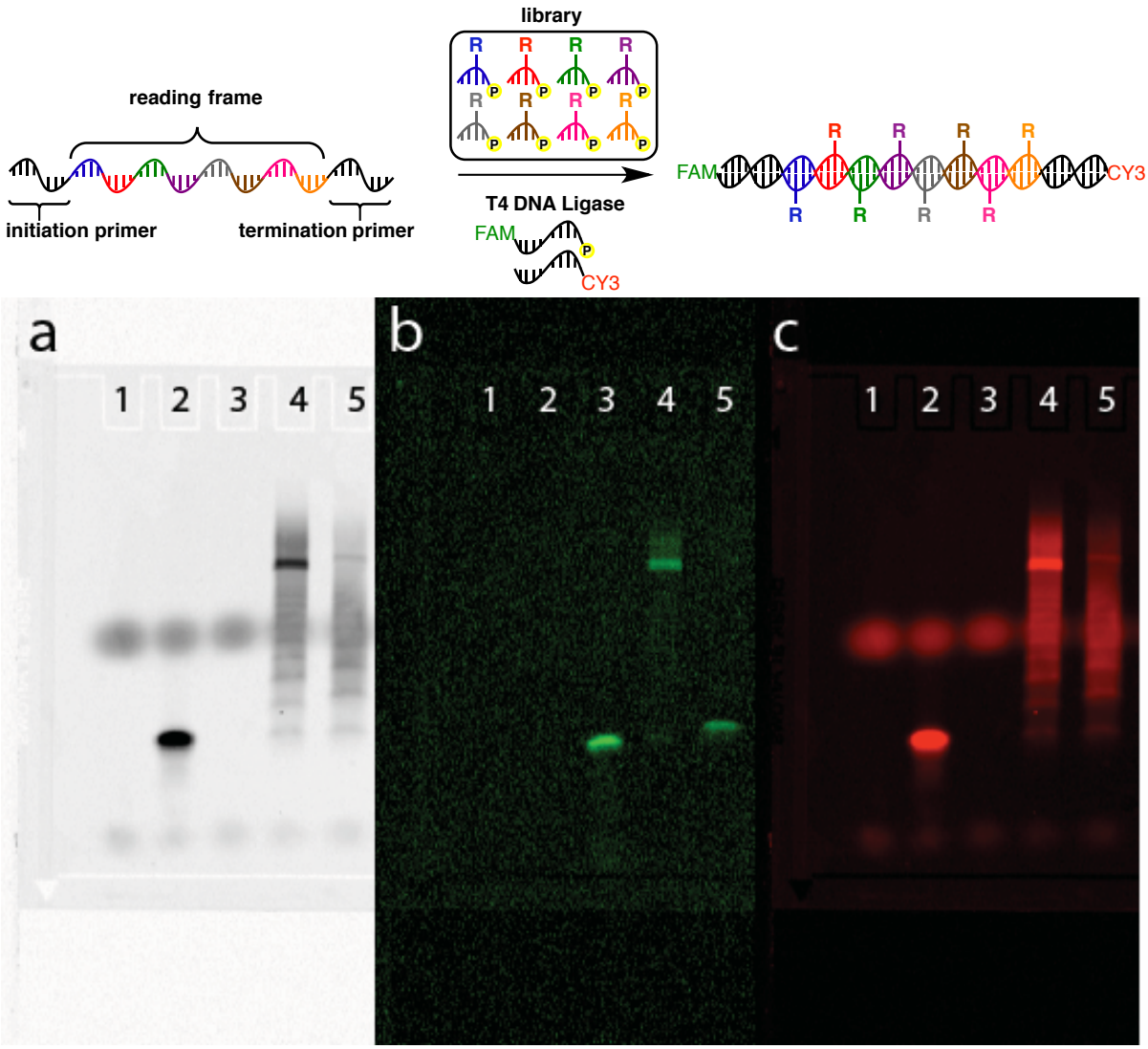

Figure S3. Monitoring LOOPER progression from differentially labelled primers. a) fluorescence overlay; b) FAM signal; c) CY5 signal. Lane 1: template; lane 2: CY5 primer; lane 3: FAM primer; lane 4: LOOPER with unmodified NNNAN library; lane 5: LOOPER with amine-modified NNNAN library.

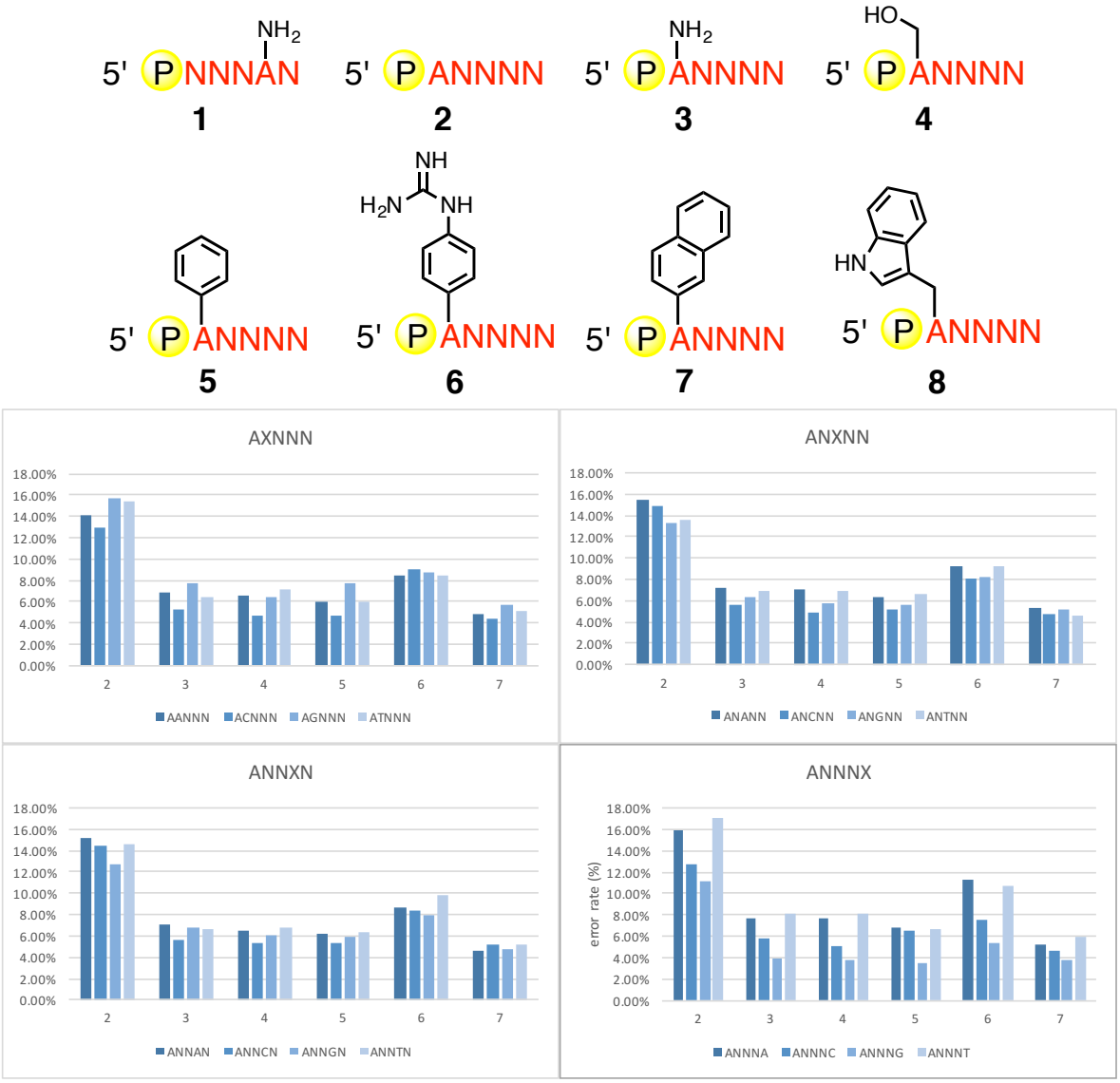

Figure S4. Error Rate of unmodified and modified pentanucleotide incorporation parsed by nucleotide identity at various positions.

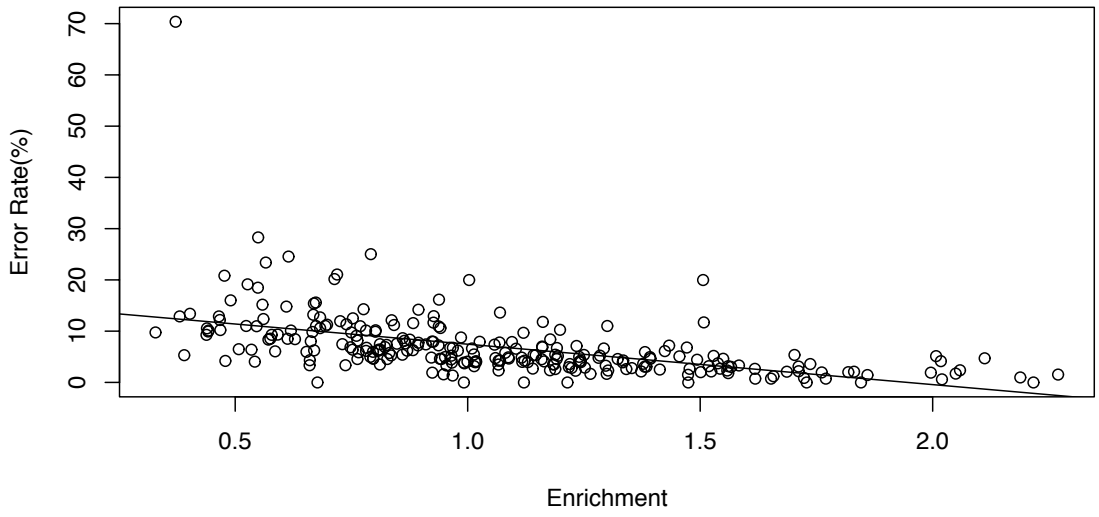

Figure S5. Error Rate vs Enrichment of LOOPER with a heterofunctionalized library

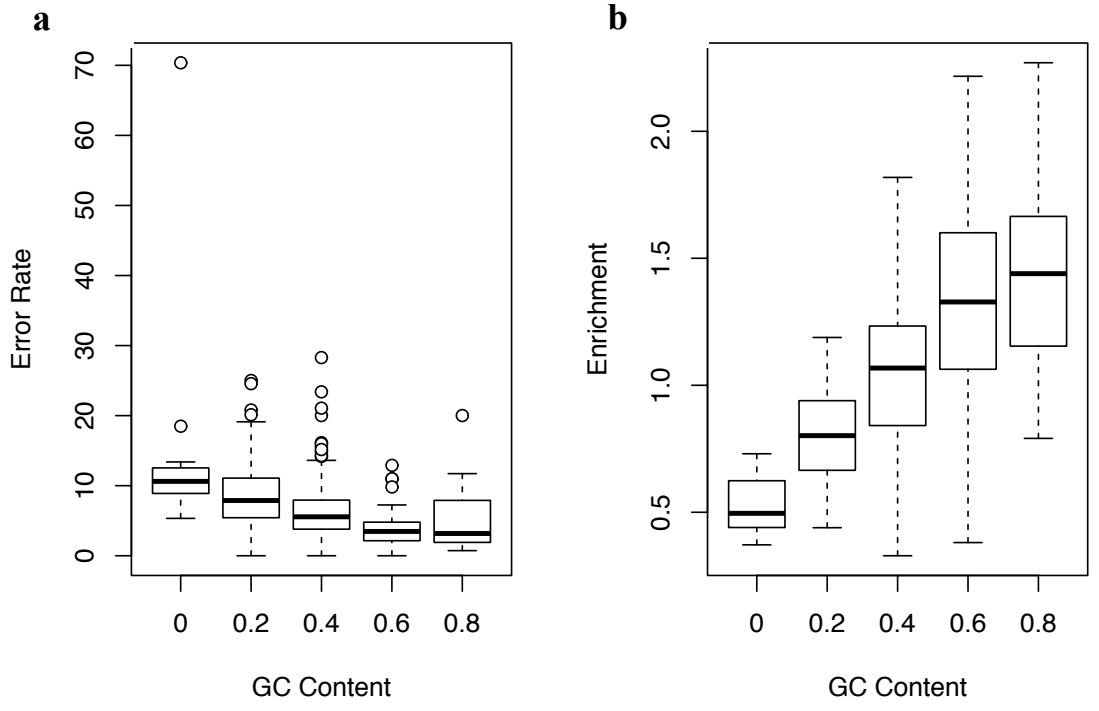

Figure S6. a) Error Rate vs GC Content boxplot of LOOPER with a heterofunctionalized library. b)Enrichment vs GC Content boxplot of LOOPER with a heterofunctionalized library.