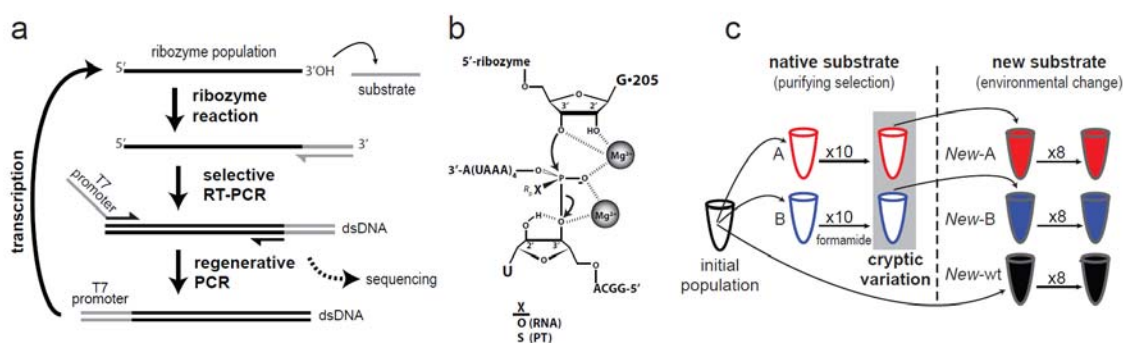
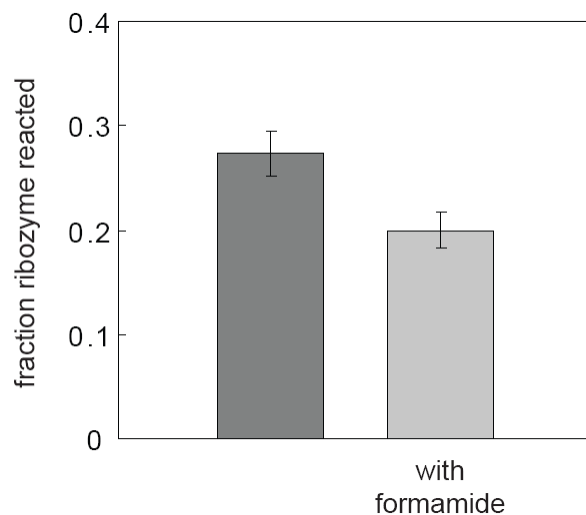
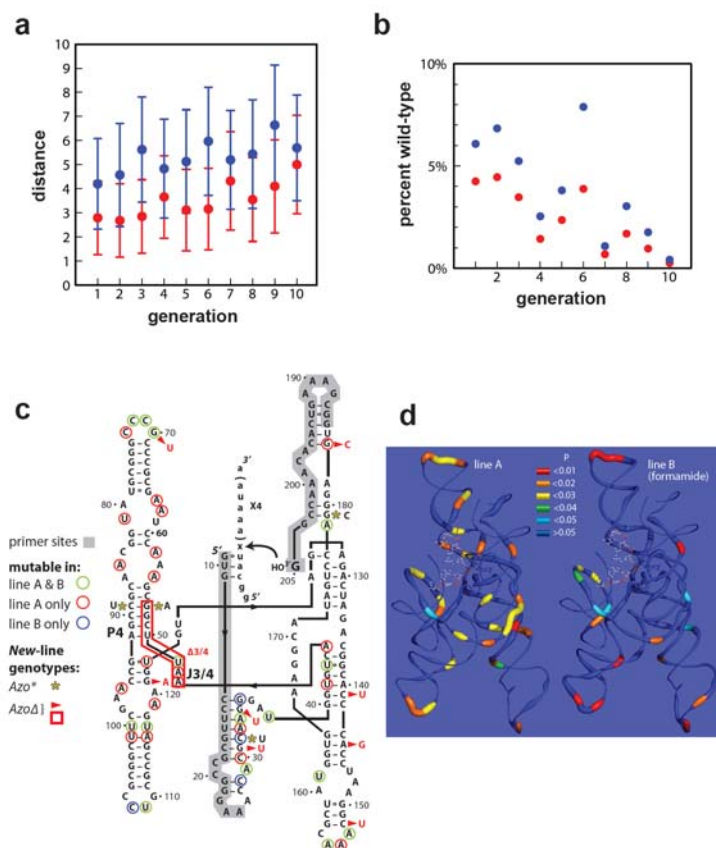




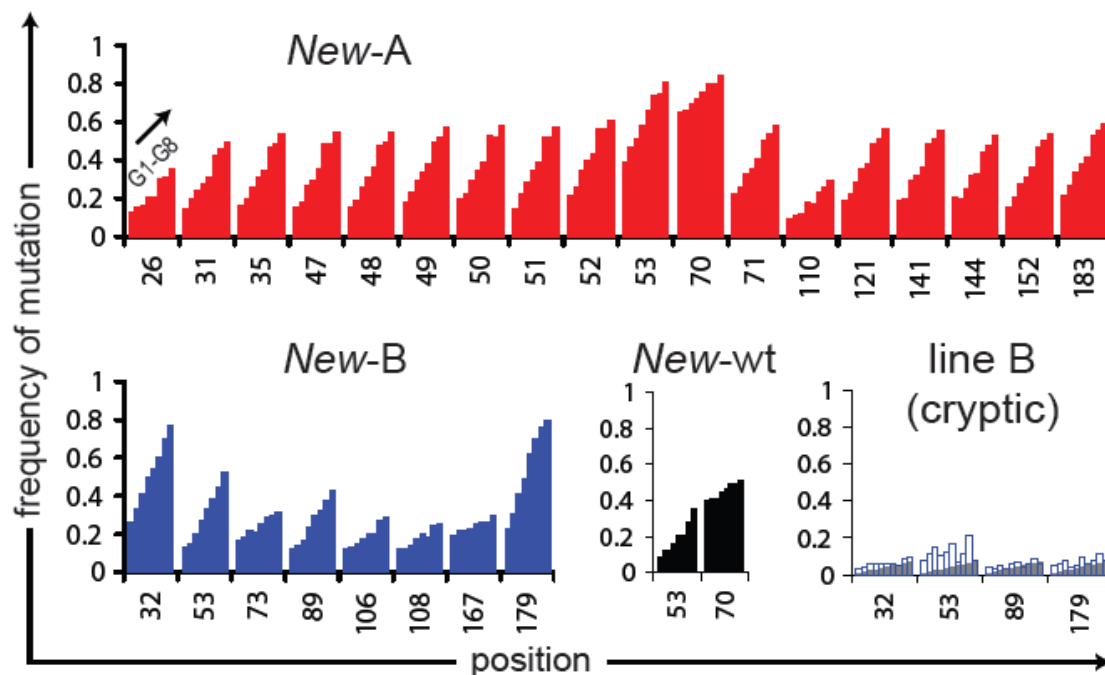
Supplementary Figure 1 | Selection procedure and experimental overview. **a**, Approximately 10^{13} ribozyme variants (single horizontal black line) are challenged to catalyze the cleavage of an exogenous nucleic acid substrate (single horizontal grey line). This reaction results in the ligation of a portion of the substrate to the 3' end of the ribozyme (grey line). Successful ribozymes are specifically amplified by reverse transcription (RT) PCR using a primer (grey arrow) specific to the ligated portion of the substrate. A second PCR regenerates the active form of the ribozyme using primers (black arrows) specific to its 5' and 3' ends. Transcription of this PCR product generates ribozymes for the next generation. **b**, A diagram of cleavage reaction chemistry. The chemical change (phosphorothioate; PT) for *New* lines is shown by an X bonded to the scissile phosphate. **c**, Experimental overview. The same initial population is used to start lines A (open red) and B (open blue). Cryptic variation is produced by selecting for activity on a native substrate (RNA oligonucleotide) for 10 generations (x10). The populations that have acquired cryptic variation are then used to start two lines, *New-A* (solid red) and *New-B* (solid blue). A third new line, *New-wt* (solid black) is started from the initial population that started A and B. The *New* lines experience an environmental change by requiring selection on a phosphorothioate substrate (b), carried out for 8 generations (x8). The color scheme used here is preserved in the main text.



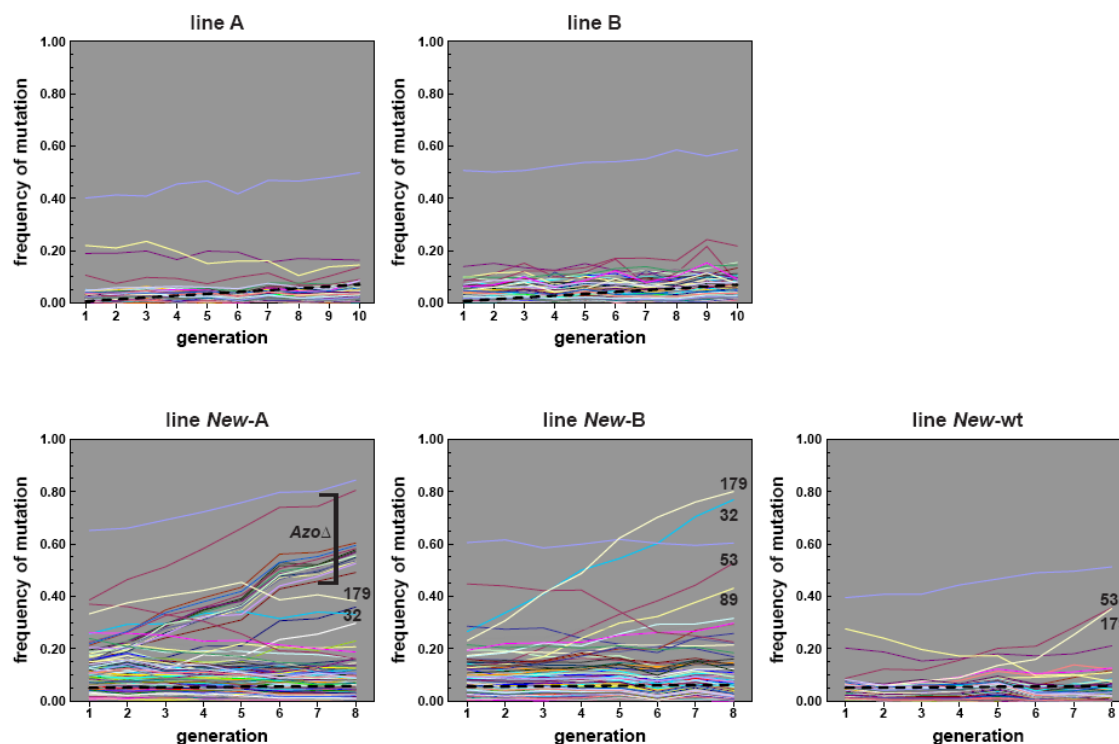
Supplementary Figure 2 | Affect of formamide on ribozyme activity. Plots of mean activity, calculated as the fraction of the ribozyme population reacted after 1 h under the native activity selection conditions. Error bars indicate standard error of three replicates. The decrease in activity is significant ($P = 0.03$, one-tailed t -test).



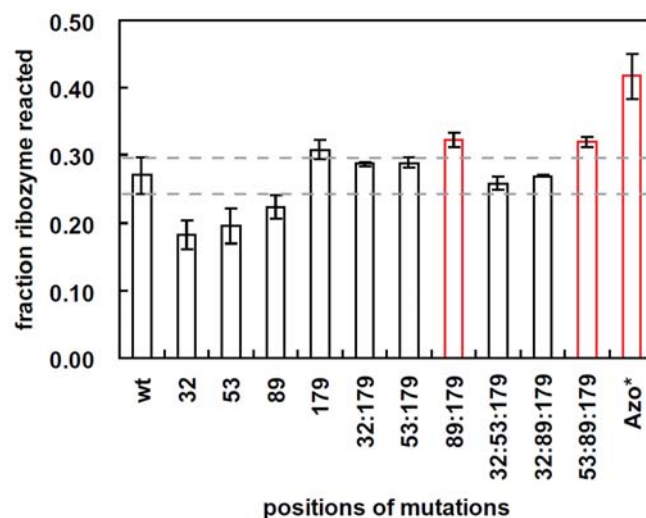
Supplementary Figure 3 | Distance measurements from lines A and B. **a**, Mean distance from wild-type for line A (red) and line B (blue) over ten generations of selection for the native activity of the ribozyme. Error bars represent one standard deviation. **b**, The fraction (percent) of the sampled sequences with the wild-type sequence (i.e. no mutations) in line A (red) and line B (blue) over ten generations of selection for the native activity. **c**, Secondary structure of the *Azoarcus* ribozyme according to¹, with nucleotide positions numbered according to². The substrate for selection is shown in lower case letters, with an “x” representing the location of the PT bond used in the *New* lines. Structural elements P4 and J3/4 are indicated. No positions showed a rate of accumulation significantly greater than the mutation rate, based on linear regression. Mutable positions showed a rate of mutational change significantly greater than zero. **d**, Representation of positions that accumulated mutations during selection for native activity. The crystal structure of the ribozyme (1ZZN)¹ is shown using the “PUTTY” function in Pymol (Schrödinger). “Mutable” positions are highlighted by scaling the thickness and color of the tube to the *P*-value found from linear regression (Methods). The active site of the ribozyme is indicated by the substrate (stick representation, colored by element).



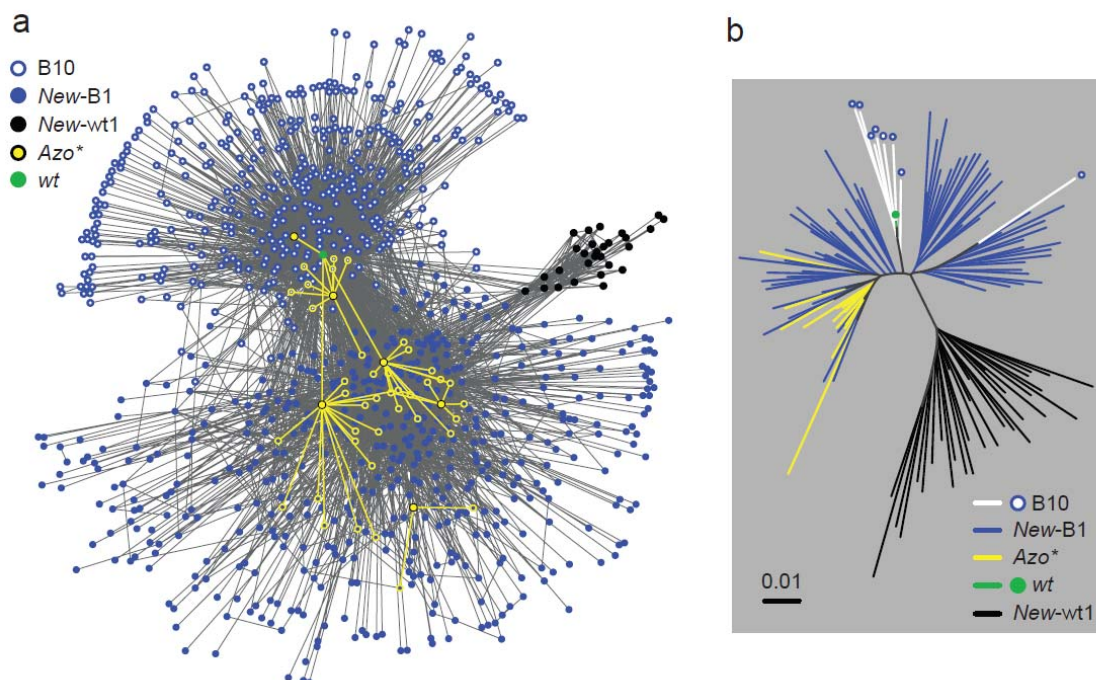
Supplementary Figure 4 | Mutation accumulation data at individual nucleotide positions determined significant by linear regression. For each nucleotide position numbered on the x-axis, the population frequency of mutants is represented as a bar on the vertical axis. Eight bars are shown above each position, and correspond to the eight generations (G1-G8). Also shown are the frequency of *Azo** mutations during selection for native activity in line B (cryptic); in the line B data only, the rate of change is not significantly different from the expected rate of mutation accumulation (grey bars).



Supplementary Figure 5 | Rate of mutation accumulation. Each line represents a single position in the ribozyme sequence, and shows the change over time (generations) in the frequency of all mutations from wild-type at that position. Mutations corresponding to *Azo** and *AzoΔ* genotypes are indicated. The same positions have the same color in all plots. Mutations that produce an increased activity will be under directional selection, and accumulate in the population faster than expected from random mutation accumulation. The rate of mutation accumulation expected from the per generation mutation rate is represented by a dashed black line. The slope of each line (not the intercept) indicates the rate of mutation accumulation at that position, and was therefore used to identify positions with mutations that may increase ribozyme activity.



Supplementary Figure 6 | Activity of ribozymes with combinations of the Azo* mutations. Activity (fraction ribozyme reacted) of each ribozyme variant with the “native” (RNA) substrate is shown under the conditions used for selection in line A (no formamide). The introduced mutations are indicated by their position in the ribozyme sequence: 32 = C32U; 53 = G53A; 89 = C89U; 179 = G179C. Variants with a significantly higher activity than the *wild-type* ($p < 0.05$, two-tailed t-test) are shown in red. Error bars represent standard error from the mean based on at least three measurements. For reference, the error bars of the *wild-type* activity are extended as grey dashed lines. Eight measurements were made for the *wild-type*, and four for Azo*.



Supplementary Figure 7 | Clustering analysis of the proximity of individuals to the *Azo genotype.** **a**, A network graph composed of all sequences from the last generation of line B (B10), and the first generations of lines *New-B* (*New-B1*) and *New-wt* (*New-wt1*). Nodes represent several sequences clustered at 96% identity (Methods) and are colored based on the source population of their representative sequence (see color key). Edges connect clusters with ≤ 10 nucleotide differences between their most distant sequences. Nodes containing *Azo** individuals are shown as yellow circles with a black border. All connections to *Azo** nodes are shown as yellow edges, and the connecting node has a yellow border. The wild-type sequence is a green node. **b**, Unrooted maximum likelihood phylogenetic tree of sequences found to cluster within 96% sequence identity with *Azo** (sequences contained within the yellow nodes with black borders in **a**) and the representative sequences from the clusters of *New-wt1* (all $< 96\%$ sequence identity with *Azo**). Leaves represent individual sequences and are colored to match **a** (see key).

Supplementary Table 1**Mutational Co-occurrence in the *New* lines.**

New-A8		D	D'	r²	χ^2
-42	-44	0.2438	0.9920	0.9745	1994.746
-39	-40	0.2442	0.9881	0.9612	1967.61
-40	-41	0.2385	0.9859	0.9217	1886.674
-41	-42	0.2372	0.9798	0.9192	1881.657
-41	-44	0.2346	0.9639	0.8984	1839.005
-39	-41	0.2338	0.9736	0.8848	1811.207
-43	-44	0.2284	0.9915	0.8688	1778.514
-42	-43	0.2279	0.9852	0.8664	1773.446
-40	-42	0.2298	0.9731	0.8598	1760.033
-40	-44	0.2277	0.9589	0.8431	1725.845
-39	-42	0.2252	0.9604	0.8245	1687.701
-39	-44	0.2231	0.9461	0.8081	1654.139
-41	-43	0.2200	0.9699	0.8039	1645.497
-44	-45	0.2214	0.9913	0.7934	1624.11
-42	-45	0.2192	0.9868	0.7786	1593.728
27U	-40	0.2197	0.9240	0.7757	1588.731
27U	-39	0.2183	0.9104	0.7649	1566.594
-40	-43	0.2131	0.9625	0.7508	1536.803
27U	-41	0.2140	0.9261	0.7390	1514.241
27U	-42	0.2135	0.9460	0.7384	1513.744
27U	-44	0.2118	0.9334	0.7260	1488.296
-39	-43	0.2085	0.9489	0.7183	1470.375
-41	-45	0.2104	0.9248	0.7142	1462.026
133U	144U	0.2089	0.8375	0.7000	1432.844
113A	144U	0.2085	0.8643	0.6963	1425.246
-43	-45	0.2052	0.9859	0.6937	1419.947
-40	113A	0.2044	0.8747	0.6712	1375.203
-40	144U	0.2042	0.8445	0.6711	1373.775
-39	113A	0.2044	0.8674	0.6704	1372.89
-42	175C	0.2025	0.8866	0.6652	1361.646
-39	144U	0.2029	0.8317	0.6613	1353.739
-40	-45	0.2028	0.8664	0.6611	1353.187
-44	175C	0.2018	0.8788	0.6599	1350.821
-40	62U	0.1997	0.8539	0.6503	1331.89
144U	175C	0.2006	0.8074	0.6455	1321.31
113A	133U	0.2004	0.8300	0.6434	1317.076
-41	113A	0.1992	0.8774	0.6404	1311.609
27U	113A	0.1998	0.8133	0.6386	1309.226
27U	-43	0.1969	0.9308	0.6383	1308.5
-41	144U	0.1989	0.8462	0.6390	1308.059
-39	62U	0.1979	0.8527	0.6384	1306.83
-39	-45	0.1989	0.8427	0.6352	1300.334
27U	144U	0.1986	0.8089	0.6317	1293.179
23U	-40	0.1956	0.9570	0.6247	1278.736
-41	175C	0.1963	0.8392	0.6224	1274.003
-40	133U	0.1962	0.8120	0.6192	1267.604

-42	144U	0.1948	0.8489	0.6157	1260.334
-42	113A	0.1949	0.8789	0.6152	1260.001
-41	62U	0.1938	0.8085	0.6148	1259.207
62U	113A	0.1941	0.8843	0.6117	1252.191
23U	-39	0.1934	0.9384	0.6101	1248.945
-40	175C	0.1943	0.8072	0.6072	1242.984
-39	133U	0.1943	0.7975	0.6068	1242.133
-44	144U	0.1926	0.8349	0.6015	1231.245
-44	113A	0.1927	0.8642	0.6008	1230.392
23U	27U	0.1919	0.8931	0.5989	1225.969
27U	62U	0.1918	0.8583	0.5973	1225.598
23U	-41	0.1908	0.9607	0.5971	1222.848
62U	144U	0.1911	0.8409	0.5935	1214.939
-43	24U	(0.1727)	0.9973	0.5619	1213.113
27U	133U	0.1920	0.7813	0.5905	1208.672
-42	62U	0.1887	0.7722	0.5858	1199.776
-39	175C	0.1910	0.7867	0.5858	1199.202
133U	175C	0.1911	0.7683	0.5857	1198.834
-41	133U	0.1904	0.8107	0.5854	1198.226
-43	175C	0.1862	0.8699	0.5715	1169.937
113A	175C	0.1887	0.7783	0.5702	1167.191
-42	24U	(0.1732)	0.9973	0.5545	1166.631
-44	62U	0.1862	0.7652	0.5696	1166.462
27U	-45	0.1885	0.7661	0.5689	1166.194
23U	-42	0.1857	0.9572	0.5676	1162.449
27U	175C	0.1881	0.7624	0.5666	1159.878
-44	24U	(0.1724)	0.9973	0.5492	1155.494
136G	144U	0.1873	0.8099	0.5632	1152.858
-42	133U	0.1858	0.8104	0.5600	1146.363
23U	-44	0.1833	0.9399	0.5527	1131.966
62U	24U	(0.1678)	1.0000	0.5252	1128.627
-41	136G	0.1845	0.8474	0.5502	1126.328
23U	136G	0.1832	0.8048	0.5467	1119.192
-44	133U	0.1836	0.7966	0.5465	1118.675
-41	24U	(0.1689)	0.9916	0.5257	1105.99
62U	133U	0.1817	0.8001	0.5364	1099.068
-42	136G	0.1803	0.8483	0.5280	1080.734
-40	136G	0.1811	0.8081	0.5277	1080.119
-43	144U	0.1779	0.8274	0.5221	1068.708
-43	113A	0.1780	0.8567	0.5218	1068.599
23U	144U	0.1788	0.8472	0.5203	1064.967
-44	136G	0.1791	0.8377	0.5200	1064.379
23U	113A	0.1787	0.8169	0.5191	1063.043
-40	24U	(0.1657)	0.9914	0.5042	1057.811
-39	136G	0.1785	0.7903	0.5126	1049.212
-39	24U	(0.1650)	0.9943	0.4994	1047.691
-45	175C	0.1784	0.7345	0.5098	1043.557
133U	136G	0.1778	0.7679	0.5073	1038.407
175C	171C	(0.1640)	1.0000	0.4945	1017.7
27U	136G	0.1759	0.7469	0.4962	1015.68

113A	136G	0.1757	0.7331	0.4950	1013.277
136G	175C	0.1753	0.7541	0.4931	1009.355
144U	24U	(0.1606)	1.0000	0.4740	1000.063
23U	62U	0.1720	0.8958	0.4883	1000.01
27U	24U	(0.1621)	1.0000	0.4797	998.827
23U	-43	0.1706	0.9382	0.4868	996.893
-43	62U	0.1704	0.7303	0.4846	993.51
175C	24U	(0.1595)	1.0000	0.4680	988.316
23U	133U	0.1722	0.8150	0.4824	987.431
133U	24U	(0.1579)	1.0000	0.4601	978.617
-42	171C	(0.1596)	0.8909	0.4727	967.661
62U	175C	0.1700	0.7516	0.4695	961.077
-43	133U	0.1685	0.7842	0.4681	958.739
-45	144U	0.1708	0.7069	0.4676	957.143
-44	171C	(0.1588)	0.8904	0.4676	957.103
-45	113A	0.1704	0.6828	0.4644	950.676
-45	24U	(0.1566)	0.9970	0.4496	943.815
113A	24U	(0.1564)	1.0000	0.4492	943.772
23U	175C	0.1672	0.7881	0.4546	930.59
-43	136G	0.1650	0.8284	0.4493	919.744
-43	45A	(0.1413)	0.9966	0.4450	914.909
-45	62U	0.1652	0.7512	0.4432	907.232
-41	171C	(0.1542)	0.8776	0.4392	899.123
144U	171C	(0.1543)	0.9267	0.4370	894.548
62U	136G	0.1629	0.7732	0.4313	884.937
-40	171C	(0.1519)	0.8860	0.4245	868.972
-45	133U	0.1628	0.6729	0.4245	868.903
136G	24U	(0.1480)	1.0000	0.4055	857.645
-39	171C	(0.1506)	0.8852	0.4171	853.817
23U	-45	0.1597	0.7316	0.4146	849.182
-44	45A	(0.1346)	0.9964	0.3968	813.906
-45	136G	0.1572	0.6570	0.3961	810.725
-42	45A	(0.1327)	0.9784	0.3865	792.619
62U	45A	(0.1297)	1.0000	0.3741	787.766
27U	171C	(0.1437)	0.8777	0.3784	774.676
-45	171C	(0.1431)	0.8879	0.3754	768.402
113A	171C	(0.1428)	0.8877	0.3737	765.027
-41	45A	(0.1300)	0.9780	0.3691	757.394
-43	171C	(0.1383)	0.7351	0.3606	738.064
23U	24U	(0.1387)	0.9627	0.3537	724.349
-40	45A	(0.1265)	0.9812	0.3488	717.832
133U	171C	(0.1371)	0.8238	0.3447	705.656
-39	45A	(0.1252)	0.9810	0.3416	703.987
144U	45A	(0.1224)	1.0000	0.3280	682.978
175C	45A	(0.1228)	1.0000	0.3291	682.587
133U	45A	(0.1203)	1.0000	0.3186	668.819
136G	171C	(0.1328)	0.8607	0.3237	662.534
-45	45A	(0.1221)	1.0000	0.3229	662.29
62U	171C	(0.1312)	0.7267	0.3202	655.431
27U	45A	(0.1214)	0.9764	0.3192	653.716

113A	45A	(0.1192)	1.0000	0.3100	642.97
45A	81U	0.0941	0.6344	0.3035	621.265
23U	171C	(0.1256)	0.8924	0.2938	601.331
24U	171C	0.1181	0.5472	0.2890	591.498
136G	45A	(0.1129)	1.0000	0.2806	585.369
23U	63A	0.1201	0.7342	0.2843	582.021
-40	63A	0.1203	0.8850	0.2819	577.342
63A	136G	0.1196	0.7902	0.2781	569.189
-39	63A	0.1194	0.8717	0.2777	568.406
63A	144U	0.1193	0.8503	0.2764	565.772
-41	63A	0.1169	0.8854	0.2676	547.957
62U	63A	0.1164	0.9120	0.2670	546.899
102A	136G	0.1137	0.8203	0.2653	543.105
63A	113A	0.1161	0.7984	0.2615	535.245
-42	63A	0.1148	0.8902	0.2590	530.378
-44	63A	0.1145	0.8833	0.2575	527.338
63A	133U	0.1136	0.8087	0.2505	512.777
27U	63A	0.1136	0.7949	0.2503	512.626
175C	81U	(0.0964)	1.0000	0.2397	502.587
144U	81U	(0.0960)	1.0000	0.2386	502.532
133U	81U	(0.0952)	1.0000	0.2353	497.335
23U	45A	(0.1036)	0.9680	0.2359	482.955
-45	63A	0.1099	0.7576	0.2345	480.073
-39	102A	0.1060	0.8445	0.2310	473.229
113A	81U	(0.0932)	1.0000	0.2245	471.754
27U	102A	0.1059	0.8097	0.2300	470.953
-40	102A	0.1055	0.8471	0.2288	468.768
102A	113A	0.1049	0.7873	0.2251	460.792
23U	102A	0.1039	0.6935	0.2246	459.829
-43	63A	0.1057	0.8748	0.2232	457.162
102A	144U	0.1041	0.8099	0.2221	454.559
136G	81U	(0.0910)	1.0000	0.2132	443.894
-41	102A	0.1024	0.8466	0.2166	443.651
63A	175C	0.1053	0.7471	0.2155	441.08
-43	81U	(0.0892)	0.7822	0.2079	426.86
-42	102A	0.1000	0.8470	0.2076	425.224
45A	171C	0.0912	0.5496	0.2062	422.056
-44	102A	0.0987	0.8314	0.2020	413.767
24U	45A	0.0908	0.5533	0.2016	412.777
102A	175C	0.0989	0.7655	0.2003	410.044
62U	102A	0.0974	0.8330	0.1972	403.966
63A	24U	(0.0934)	1.0000	0.1941	402.572
102A	133U	0.0979	0.7607	0.1963	401.819
-42	81U	(0.0844)	0.7720	0.1827	373.98
-44	81U	(0.0839)	0.7710	0.1804	369.335
63U	24U	(0.0856)	1.0000	0.1716	366.427
-41	81U	(0.0831)	0.7765	0.1767	361.851
-43	102A	0.0912	0.8236	0.1752	358.827
102A	24U	(0.0858)	1.0000	0.1727	357.635
-40	81U	(0.0816)	0.7804	0.1695	347.029

23U	81U	(0.0805)	0.9374	0.1668	341.518
-39	81U	(0.0808)	0.7788	0.1662	340.207
63A	63U	(0.0796)	0.9702	0.1516	310.411
27U	81U	(0.0761)	0.7647	0.1470	301.397
-45	102A	0.0841	0.6325	0.1447	296.263
63A	171C	(0.0794)	0.8486	0.1401	286.797
-45	81U	(0.0739)	0.7516	0.1385	283.422
81U	171C	0.0677	0.5084	0.1330	272.334
63A	45A	(0.0702)	1.0000	0.1303	268.642
62U	81U	(0.0701)	0.6398	0.1267	259.918
102A	171C	(0.0730)	0.8518	0.1250	255.876
-41	63U	0.0785	0.6120	0.1228	251.631
63U	45A	(0.0659)	1.0000	0.1194	250.48
-40	63U	0.0778	0.5895	0.1201	245.914
27U	63U	0.0772	0.5567	0.1179	241.373
-39	63U	0.0770	0.5782	0.1174	240.338
-42	63U	0.0764	0.6102	0.1168	239.293
102A	45A	(0.0640)	1.0000	0.1147	236.985
-44	63U	0.0747	0.5930	0.1114	228.199
63U	175C	0.0740	0.5399	0.1081	221.206
63U	133U	0.0721	0.5285	0.1029	211.192
63U	113A	0.0696	0.4928	0.0958	196.583
24U	81U	0.0579	0.4396	0.0960	196.472
-43	63U	0.0669	0.5693	0.0910	186.988
63U	144U	0.0671	0.4926	0.0892	183.222
102A	81U	(0.0518)	1.0000	0.0876	180.097
63A	81U	(0.0532)	0.9315	0.0869	177.88
63U	102A	0.0577	0.3021	0.0842	172.395
62U	63U	0.0620	0.5000	0.0771	157.832
-45	63U	0.0589	0.4181	0.0686	140.504
23U	63U	0.0534	0.3360	0.0572	117.015
63U	171C	(0.0464)	0.5110	0.0488	99.837
18C	27U	0.0216	0.9170	0.0409	83.72
63U	136G	0.0447	0.3042	0.0396	81.351
63A	102A	0.0384	0.2033	0.0366	74.901
18C	-42	0.0203	0.9540	0.0365	74.761
18C	-44	0.0199	0.9314	0.0351	71.967
18C	-45	0.0200	0.8366	0.0351	71.892
18C	175C	0.0188	0.8107	0.0311	63.73
18C	102A	0.0166	0.4715	0.0308	63.075
18C	-43	0.0184	0.9264	0.0307	62.928
18C	-40	0.0185	0.8255	0.0301	61.643
18C	144U	0.0182	0.7886	0.0292	59.716
18C	-41	0.0178	0.8204	0.0282	57.743
18C	-39	0.0177	0.7837	0.0276	56.433
18C	23U	0.0172	0.6375	0.0263	53.888
18C	133U	0.0173	0.7466	0.0262	53.625
18C	62U	0.0166	0.7910	0.0247	50.489
18C	113A	0.0166	0.6942	0.0243	49.695
18C	24U	(0.0153)	0.9691	0.0232	47.553

18C	171C	(0.0144)	0.9366	0.0209	42.869
18C	136G	0.0147	0.5885	0.0189	38.738
63U	81U	(0.0236)	0.4268	0.0175	35.797
18C	45A	(0.0112)	0.9583	0.0150	30.632
18C	81U	(0.0089)	0.9480	0.0110	22.607
18C	63A	0.0090	0.2663	0.0087	17.818
18C	63U	0.0070	0.2039	0.0053	10.882

New-B8		D	D'	r^2	χ^2
45A	81U	0.1328	0.6485	0.2901	431.337
159U	171C	(0.0434)	0.2648	0.0528	78.493
81U	98U	0.0161	0.4528	0.0186	27.714
24U	45A	0.0275	0.2275	0.0168	24.917
98U	159U	0.0117	0.2656	0.0117	17.428
24U	81U	0.0198	0.1996	0.0089	13.219
24U	100U	(0.0104)	0.1521	0.0073	10.855
65A	171C	0.0107	0.4701	0.0073	10.806
98U	100U	(0.0055)	1.0000	0.0064	9.521
98U	171C	0.0072	0.5180	0.0052	7.68
65A	159U	(0.0094)	0.3331	0.0048	7.208
45A	98U	0.0078	0.2603	0.0042	6.316
100U	100A	(0.0037)	1.0000	0.0042	6.248
81U	171C	0.0132	0.1383	0.0040	6.019
45A	65A	0.0094	0.1923	0.0040	5.889
24U	171C	0.0104	0.0601	0.0034	5.087
24U	98U	0.0058	0.3982	0.0032	4.785
100U	171C	0.0056	0.2744	0.0022	3.248
65U	98U	(0.0033)	0.4925	0.0019	2.855
100U	159U	(0.0050)	0.1995	0.0015	2.281
24U	65A	0.0050	0.2084	0.0015	2.239
65A	81U	(0.0055)	0.1316	0.0014	2.054
24U	65U	0.0049	0.1889	0.0013	2.006
24U	100A	0.0030	0.3094	0.0013	1.896
65A	100A	0.0019	0.0526	0.0011	1.581
100A	171C	0.0026	0.2809	0.0010	1.482
45A	100A	0.0026	0.1306	0.0007	1.043
65A	100U	(0.0022)	0.2496	0.0007	1.014
65U	159U	(0.0036)	0.1171	0.0007	0.969
65U	171C	0.0032	0.1282	0.0006	0.876
81U	159U	0.0040	0.0247	0.0003	0.495
65U	100U	(0.0016)	0.1661	0.0003	0.489
100A	159U	(0.0015)	0.1273	0.0003	0.404
45A	159U	(0.0031)	0.0215	0.0002	0.28
45A	65U	0.0020	0.0371	0.0002	0.239
24U	159U	0.0023	0.0349	0.0001	0.221
81U	100U	0.0017	0.0317	0.0001	0.205
65A	98U	(0.0008)	0.1226	0.0001	0.162
45A	100U	(0.0012)	0.0265	0.0001	0.107
65U	81U	0.0012	0.0193	0.0001	0.094
45A	171C	0.0012	0.0104	0.0000	0.05

65U	100A	0.0003	0.0073	0.0000	0.028
98U	100A	0.0002	0.0047	0.0000	0.021
81U	100A	0.0004	0.0148	0.0000	0.019
<i>New-wt8</i>		D	D'	r ²	χ ²
45A	62A	(0.0324)	0.3369	0.0229	28.4130
45A	81U	0.0119	0.1767	0.0077	9.6020
24U	81U	0.0069	0.0876	0.0068	8.4790
45A	171C	0.0115	0.0561	0.0029	3.5770
24U	62A	0.0061	0.1032	0.0022	2.6960
24U	45A	0.0060	0.0985	0.0021	2.6550
62A	171C	(0.0044)	0.0432	0.0004	0.5100
24U	171C	0.0020	0.0330	0.0002	0.2730
81U	171C	(0.0011)	0.0369	0.0001	0.0850
62A	81U	(0.0002)	0.0059	0.0000	0.0020

Text accompanying Supplementary Table 1. The table shows calculations of co-occurrence of pairs of mutations in several forms (see **Methods** for calculations). The first two columns are the position and base of the mutations under consideration, where “-” indicates a deletion. The numbers indicate the position relative to the first unique position of the alignment, not the ribozyme, and thus are shifted by eight nucleotides upstream relative to the numbering used in the main text. The value for *D* can take on a negative (red) or positive value, indicating whether the mutations occur together less or more frequently than expected by their individual frequencies.

The above data confirms the absence of significant association of the *Azo** mutations. For example, while the individual mutations G53A and G179C were quite frequent by generation 8 (each >30% of the sampled individuals), they co-occurred at only 5.6% of the theoretical maximum (*D'*). This suggests that the significant individual mutations from line *New-wt* are acting separately, not in concert. Further, the other individual mutations of *Azo** (C32U and C89U) did not show a significant increase in frequency in this line, as determined by linear regression, suggesting that they do not display a strong selective advantage individually.

References

1. Stahley, M.R. & Strobel, S.A. Structural evidence for a two-metal-ion mechanism of group I intron splicing. *Science* **309**, 1587-1590 (2005).
2. Tanner, M. & Cech, T. Activity and thermostability of the small self-splicing group I intron in the pre-tRNA(Ile) of the purple bacterium *Azoarcus*. *RNA* **2**, 74-83 (1996).