Supplemental Files for:

Physiological Magnesium Concentrations Increase Fidelity of Diverse Reverse Transcriptases from HIV-1, HIV-2, and Foamy Virus, but not MuLV or AMV

by

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Construction of plasmid pBS∇EcoRV$_{567}$ for *lacZα* complementation assays. Plasmid pBS∇EcoRV$_{567}$ was derived from pBSΔPVUII [1] by removing a portion of the plasmid between the lone remaining PVUII site (position 764 of the pBSM13+ parent plasmid) and the single NaeI site at position 564. These cleavages leave blunt ends and an insert was used to replace the removed segment. The insert was made using primers 5'-CTGGCGTAATGCGAAGAGG-3' and 5'-GATATCTTATTAGCGCCATTCGCCATTGAGGC-3' and performing PCR (using Q5 DNA polymerase and manufactures protocol) using the pBSΔPVUII plasmid as template. The underlined region of the second primer coded for the new EcoRV site and two consecutive stop codons to terminate *lacZ*α synthesis. Plasmid constructs were grown in GC5 bacteria and individual clones were sequenced to obtain plasmids with the insert in the correct orientation. The resulting pBS∇EcoRV$_{567}$ plasmid (see Fig. 1) retains the multiple cloning site (MCS) of pBSM13+ but the *lacZα* gene segment now mimics the gene in plasmid pNLZeoIN-R-E-.LZF/R used by others to analyze HIV RT fidelity in cell culture [2]. Nucleotides 3-162 of *lacZα* (forward) from pNLZeoIN-R-E-.LZF/R (numbering is as described in [2] where "1" designates the start of the first *lacZα* codon (6th actual codon)) after the multiple cloning site (MCS)) are the same in pBS∇EcoRV$_{567}$ while nucleotides 163-174 are not present in pBS∇EcoRV$_{567}$. Addition amino acid coding nucleotides upstream of nucleotides 3-162 (including the start of the *lacZα* gene and the MCS) are not identical to those in pNLZeoIN-R-E-.LZF/R. These differences had no noticeable effect on α-complementation.

Sources of mutations in the *α*-complementation assays. Two different systems were used to test fidelity (see Methods and Fig. 1). The first (termed "RNA templated" assay) was used for all RTs that were tested and started with an RNA template produced with T3 RNA polymerase from pBS∇EcoRV$_{567}$. This template was copied with RT to produce a DNA that, after isolation, was used as a template to make a second DNA strand. The second strand was used as a template

in a PCR reaction with Q5 DNA polymerase. The resulting purified product was processed as described above and used as a plasmid insert for the α-complementation assay. The second (termed "DNA templated" assay) system started with a DNA template produced by asymmetric PCR with Q5 DNA polymerase. This DNA was used as a template for RT to produce a second DNA that was then used for PCR as described above. The first system essentially mimics the entire reverse transcription process while the second mimics 2nd strand synthesis only. With respect to background mutations, the starting templates in each system would include mutations present in plasmids which are likely insignificant. The first system also includes mutations made by T3 RNA polymerase while the second has mutations made during asymmetric PCR with Q5 polymerase. Both systems would include background generated by the PCR steps as well as those related to the assay steps (e.g., restriction digest and ligation). The background controls (see Table S1) for each assay would correct for all mutations occurring after those present in the starting material. In the second system, the mutation rate in the starting DNA template is also accounted for as the PCR step uses that template to produce the control insert. However, errors made by T3 RNA polymerase are not accounted for in the first system. Importantly, recent results suggest that phage RNA polymerases contribute significantly to mutations in fidelity assays using RNA as a template [3]. Results in Table S1 with 0.5 mM Mg^{2+} and HIV RT wt showed colony mutation rates of 0.00435 and 0.00189 for the RNA and DNA template systems, respectively. However, it is not possible to directly assess what proportion of the higher error rate in the RNA system was due to errors in the original RNA template vs. those contributed by the extra RT-directed RNA to DNA synthesis step in the RNA system. Overall, we expect that the real background in the RNA templated system would be significantly higher than the DNA templated system and is higher than the 0.00087 frequency for the background control in Table S1. Therefore, the mutation frequencies for this RNA system in Table S1 (and Fig. 2), which subtracted the control background, would represent a maximum frequency. They are probably modestly high for the 6 mM Mg^{2+} conditions,

which had ~10-fold higher mutation frequencies than the background control, and more significantly high for the 0.5 mM Mg^{2+} conditions, which were only ~4-fold above background.

Table S1: Data from blue/white colony screening (α-complementation assay) of various reverse transcriptases (RT)

[Mg^{2+}] and RT	[a]Colony mutation freqeuncy ((white+faint blue)/total)	[b]Colony mutation frequency - BKG (relative to 0.5 mM HIV-1 wt)	[c]*p* value (*p*<0.05) vs. same enzyme at 0.5 mM Mg^{2+}	[c]*p* value (*p*<0.05) vs. HIV-1 wt at same [Mg^{2+}]
		[d]RNA→DNA→DNA assay		
Control (BKG)	16/18,371 (0.00087)			
0.25 mM HIV-1 wt	49/13,180 (0.00372)	0.00285 (0.82)	0.505255*	NA
0.5 mM HIV-1 wt	29/6,672 (0.00435)	0.00348 (1)	NA	NA
1 mM HIV-1 wt	36/6,608 (0.00545)	0.00458 (1.3)	0.365522*	NA
2 mM HIV-1 wt	65/9,816 (0.00662)	0.00575 (1.7)	0.058194*	NA
6 mM HIV-1 wt	79/9,305 (0.00849)	0.00762 (2.2)	0.001736	NA
12 mM HIV-1 wt	87/11,348 (0.00767)	0.00680 (2.0)	0.007477	NA
0.5 mM HIV-1 A/E wt	15/3,688 (0.00407)	0.00320 (0.92)	NA	0.834904*
6 mM HIV-1 A/E wt	40/4,965 (0.00806)	(0.00719) (2.1)	0.021726	0.787848*
12 mM HIV-1 A/E wt	32/4,005 (0.00799)	(0.00712) (2.0)	0.028338	0.842173*
0.5 mM PFV wt	27/11,052 (0.00244)	(0.00157) (0.45)	NA	0.029231
6 mM PFV wt	30/5,997 (0.00500)	(0.00413) (1.2)	0.005881	0.012866
12 mM PFV wt	36/6,431 (0.00560)	(0.00473) (1.4)	0.000827	0.112193*
0.5 mM HIV-2 wt	39/12,232 (0.00319)	0.00232 (0.67)	NA	0.205403*
6 mM HIV-2 wt	50/7,576 (0.00660)	0.00573 (1.6)	0.000517	0.163867*
12 mM HIV-2 wt	69/7,858 (0.00880)	0.00793 (2.3)	<0.00001	0.453543*
0.5 mM K103N HIV-1	22/5,819 (0.00378)	0.00291 (0.84)	NA	0.617957*
6 mM K103N HIV-1	39/5,321 (0.00733)	0.00646 (1.9)	0.011695	0.453918*
0.5 mM AZT^R HIV-1	11/3,000 (0.00367)	0.00280 (0.80)	NA	0.631232*
6 mM AZT^R HIV-1	28/2,953 (0.00948)	0.00861 (2.5)	0.005736	0.616860*
0.5 mM K65R HIV-1	26/8,960 (0.00290)	0.00203 (0.58)	NA	0.132730*
6 mM K65R HIV-1	40/7,663 (0.00522)	0.00435 (1.3)	0.018301	0.011638
0.5 mM M184V HIV-1	40/11,141 (0.00359)	0.00297 (0.85)	NA	0.433469*
6 mM M184V HIV-1	101/8,305 (0.01216)	0.01129 (3.2)	<0.00001	0.016707
0.5 mM Q151M HIV-1	9/2,588 (0.00349)	0.00262 (0.75)	NA	0.558781*
6 mM Q151 M HIV-1	19/2,462 (0.00772)	0.00685 (2.0)	0.043717	0.709775*
0.5 mM MuLV	19/4,940 (0.00385)	0.00298 (0.86)	NA	0.679027*
6 mM MuLV	12/2,836 (0.00423)	0.00336 (0.97)	0.7961*	0.022173
0.5 mM AMV	31/6,120 (0.00507)	0.00420 (1.2)	NA	0.554112*
6 mM AMV	34/5,497 (0.00619)	0.00532 (1.5)	0.421766*	0.122281*
		[d]DNA→DNA assay		
Control HIV-1 (BKG)	10/11,078 (0.00090)			
0.5 mM HIV-1 wt DNA	8/4,343 (0.00189)	0.00099 (1)	NA	NA
6 mM HIV-1 wt DNA	36/7,261 (0.00496)	0.00406 (4.1)	0.00844	NA
Control MuLV (BKG)	3/5,198 (0.00058)			
0.5 mM MuLV DNA	14/7,256 (0.00193)	0.00135 (1.4)	NA	0.916775*
6 mM MuLV DNA	8/5,928 (0.00135)	0.00077 (0.8)	0.417814*	0.001538

a- Approximately 200 colonies per 100 mm plate were visually scored. Total = white + faint blue + blue. Questionable faint blue colonies were replated with blue colonies to further access the color.

b- Colony mutation frequency was the (white+faint blue)/total values in column 2 and the BKG is from the "Control (BKG)" in column 2. For the RNA→DNA→DNA assay, BKG controls were made from PCR of plasmid sequences so they do not represent the "real" assay BKG. For DNA→DNA assays, BKG controls were from PCR of the starting DNA template and therefore represent the "true" BKG for the assay. See Methods and Results for an explanation of the BKG.

c-*p* values were calculated using Chi Square analysis and a significance level of $p < 0.05$. Values less than 0.05 were considered significant.

d- The RNA→DNA→DNA and DNA→DNA assays are described under Methods and Fig. 1 (main manuscript). The former includes two RT synthesis steps and the latter just 1. The former also starts with an RNA template made from T3 RNA polymerase. Therefore, more mutations would be expected in the RNA→DNA→DNA assay with the same reaction conditions.

*Value considered not statistically significant (>0.05) based on Chi-squared statistical analysis.

Table S2. Data from NGS experiment 2

Condition[a]	Control DNA and RNA	DNA 0.5 mM Mg^{2+}	DNA 6 mM Mg^{2+}	RNA 0.5 mM Mg^{2+}	RNA 6 mM Mg^{2+}
Total Tag Families[b]	303,378	301,642	185,208	149,329	388,824
Total nucleotides[c]	4.702×10^7	4.675×10^7	2.871×10^7	4.629×10^7	1.205×10^8
Substitution Type	**Number of recovered substitutions (substitution frequency per nucleotide x 10^6)[d]**				
A>C	729 (15.50)	1091 (23.33)	2001 (69.70)	964 (20.82)	3939 (32.68)
A>G	367 (7.805)	2073 (44.34)	1862 (64.86)	909 (19.64)	3761 (31.20)
A>T	456 (9.697)	721 (9.753)	1811 (15.88)	790 (9.851)	3954 (32.81)
*C>A	6669 (141.8)	6595 (141.1)	10448 (365.3)	4704 (101.6)	18361 (152.3)
C>G	613 (13.04)	2083 (44.55)	4178 (145.5)	1282 (27.69)	12996 (107.8)
C>T	1605 (34.13)	3252 (69.55)	14521 (505.8)	3029 (65.43)	26741 (221.9)
G>A	602 (12.80)	8091 (173.1)	13951 (486.0)	2547 (55.02)	20808 (172.6)
G>C	271 (5.763)	368 (7.871)	523 (18.22)	185 (3.996)	1034 (8.578)
*G>T	16002 (340.3)	11235 (240.3)	8044 (280.2)	5894 (127.3)	30484 (252.9)
T>A	745 (15.84)	1121 (23.98)	2170 (75.59)	1059 (22.88)	3569 (29.61)
T>C	251 (5.338)	600 (12.83)	7609 (265.1)	991 (21.41)	7300 (60.56)
T>G	897 (19.08)	1269 (27.14)	2041 (71.10)	843 (18.21)	3545 (29.41)
Insertion Type	**Number of recovered insertions (insertion frequency per nucleotide x 10^6)[d]**				
A	0	0	151 (5.359)	266 (5.746)	204 (1.693)
C	0	0	148 (5.155)	58 (1.253)	138 (1.145)
G	0	0	16 (0.5573)	0	24 (0.1992)
T	0	19 (0.4064)	1511 (52.63)	1357 (29.32)	1119 (9.286)
Deletion Type	**Number of recovered deletions (deletion frequency per nucleotide x 10^6)[d]**				
A	52 (1.106)	124 (2.652)	374 (13.03)	144 (3.112)	669 (5.552)
C	45 (0.9570)	183 (3.930)	914 (31.84)	501 (10.82)	2087 (17.32)
G	45 (0.9570)	48 (1.031)	199 (6.931)	131 (2.830)	640 (5.311)
T	61 (1.297)	113 (2.426)	787 (27.41)	246 (5.314)	1319 (10.95)
Mutation Type	**Percent of various types of mutations for each condition (mutation frequency x 10^4)[e]**				
*Substitutions		98.0 (2.975)	91.9 (15.78)	70.4 (1.286)	92.6 (5.883)
Insertions		0.1 (0.0030)	3.7 (0.6353)	19.9 (0.3637)	1.9 (0.1207)
Deletions		1.9 (0.0577)	4.4 (0.7555)	9.7 (0.1772)	5.5 (0.3494)
Overall mutation frequency		3.036×10^{-4}	1.717×10^{-3}	1.827×10^{-4}	6.353×10^{-4}

a- Refer to Material and Methods for details. DNA assays included 1 round of RT DNA-template-directed DNA synthesis while RNA assays include an RT RNA-template-directed round and a second RT DNA-template-directed DNA synthesis round.

b- Tag families are defined in Materials and Methods. To qualify as an "tag family", the same 14-nucleotide barcode had to be recovered in 5 or more reads in NGS. Specific mutations were recorded when they appeared at a specific position in all the reads that constituted a particular index.

c- Total number of RT-directed nucleotides catalysis events evaluated in the assay. For DNA templated assay, this was the number of tag families x 155 nucleotide positions (see Fig. 1, main manuscript). For RNA templated assays, the tag family number was multiplied by 310 because this assay had an additional round of RT-directed synthesis (see Materials and Methods).

d- Insertion frequency per nucleotide was calculated by dividing the raw number of mutations of the specified type by the total number of nucleotides that were assessed for that condition. Unlike the raw number of mutations, this value allows a direct relative comparison of the data in the Table with higher numbers representing a greater frequency for that mutation. Note that the DNA and RNA templated assays cannot be directly compare with this number the as the templates was synthesized by Q5 DNA polymerase or T3 RNA polymerase in the DNA and RNA assays, respectively. The Q5 synthesis product was used as the Control for both assays. Given the very low mutation rate of Q5, it is likely the RNA assays contain a significant proportion of mutations derived from T3 RNA polymerase that are not accounted for by the Control (see Results for further discussion).

e- Values were determined after subtracting background Control values from each mutation type using the frequency per nucleotide data above. These values were then summed for each of the 3 categories (i.e. substitutions (S), insertions (I), and deletions (D)). The percent values listed were calculated by dividing individual values by the sum of the 3 categories and multiplying by 100 (e.g. for Substitutions: (S/(S+I+D)) x 100). Mutation frequencies in parentheses were the individual S, I, and D values and the Overall Mutation Frequency was S+I+D for each category.

*Due to high numbers in the Controls, C>A and G>T substitutions were excluded from data calculations (see Results).

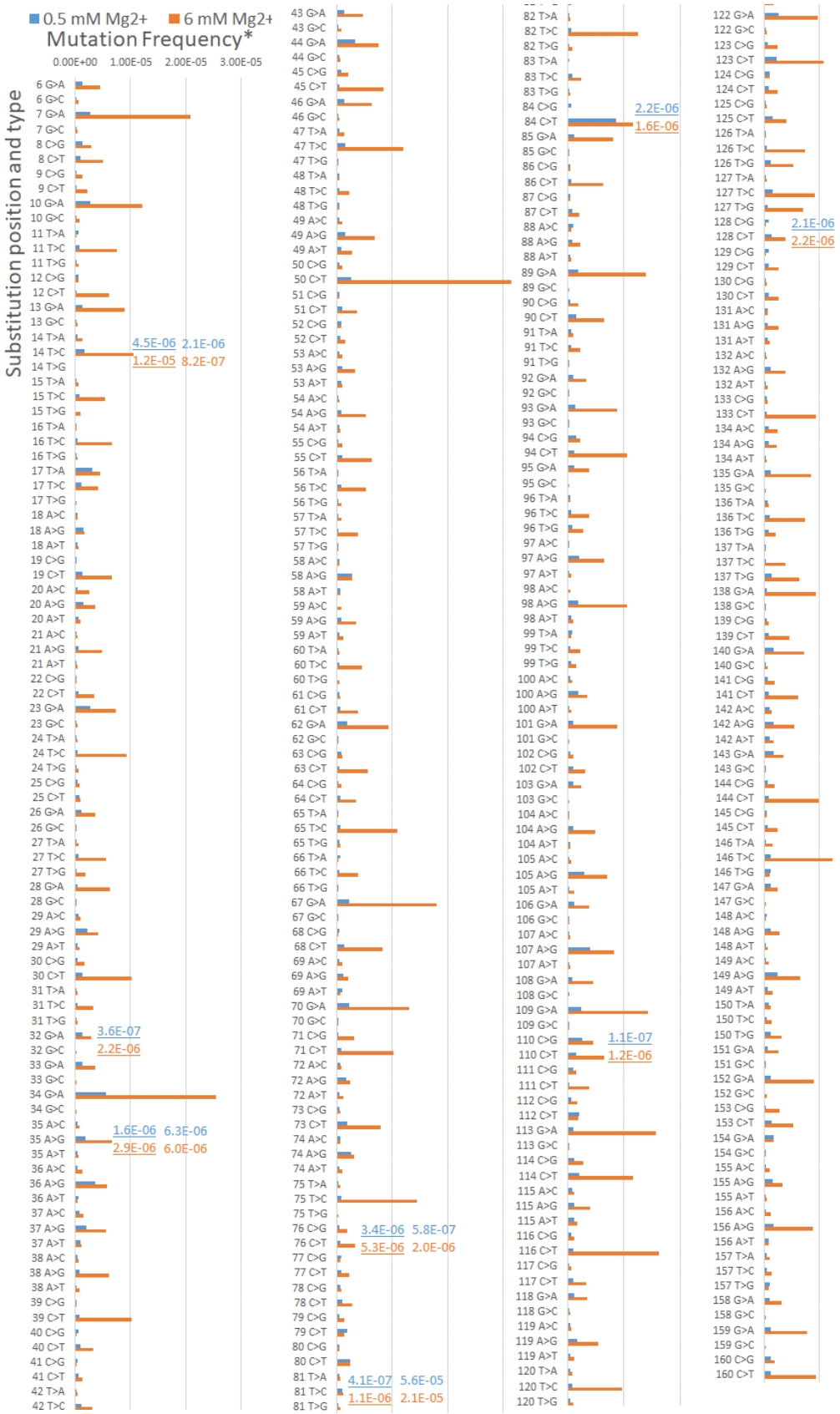


Figure S1a. Mutation frequency with 0.5 or 6 mM Mg^{2+} in the RNA templated assay (Exp. 1). The mutation frequency per nucleotide at specific positions (see Table 1 and Fig. 1) for specific types of recovered substitution mutations at the two different Mg^{2+} concentrations (blue, 0.5 mM and orange 6 mM Mg^{2+}) is plotted. Mutation frequencies for deletions (underlined) and insertions (not underlined) are given only for those positions where the rate at 0.5 or 6 mM was at least 1 x 10^{-6} for one or both of the conditions. Indel values were arbitrarily placed above the first nucleotide of a run of nucleotides although it is not known at which position the indel occurred. *Mutation Frequencies are for the specific mutation type and position and were calculated after subtraction of background rates at the same template position. See Table 1 for the total number of nucleotides scored for each condition.

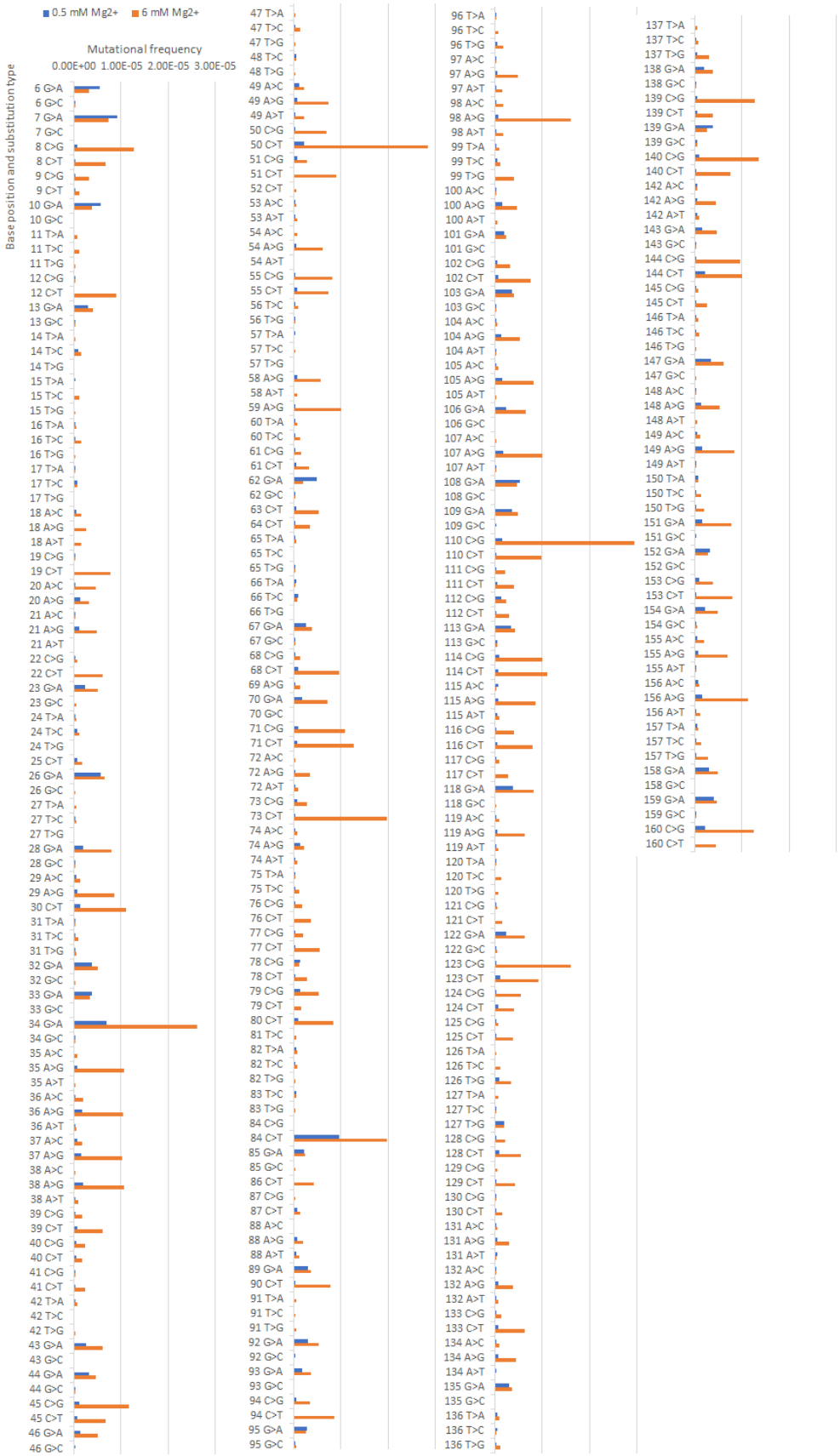


Figure S1b. Substitution mutation frequencies using the DNA templated system in the NGS assay (Exp. 1). The mutation frequency per nucleotide at specific positions (see Table 1 in main text) for specific types of recovered substitution mutations at the two different Mg^{2+} concentrations (blue, 0.5 mM and orange 6 mM Mg^{2+}) is plotted. Mutation frequencies are for the specific mutation type and position and were calculated after subtraction of background rates at the same template position. See Table 1 for the total number of nucleotides scored for each condition.

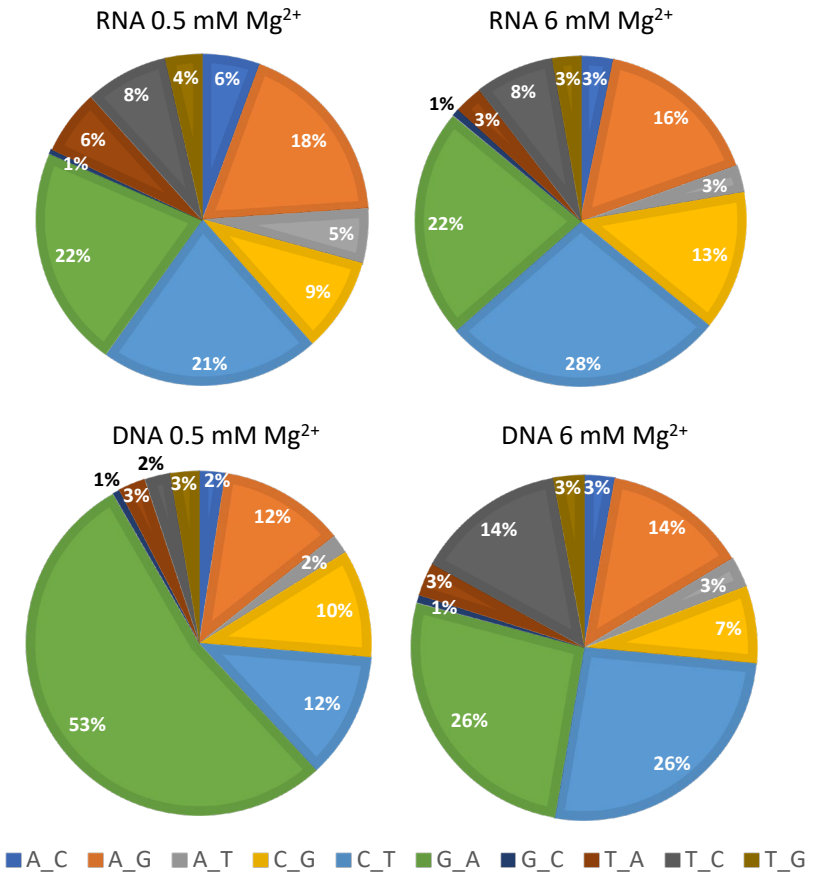
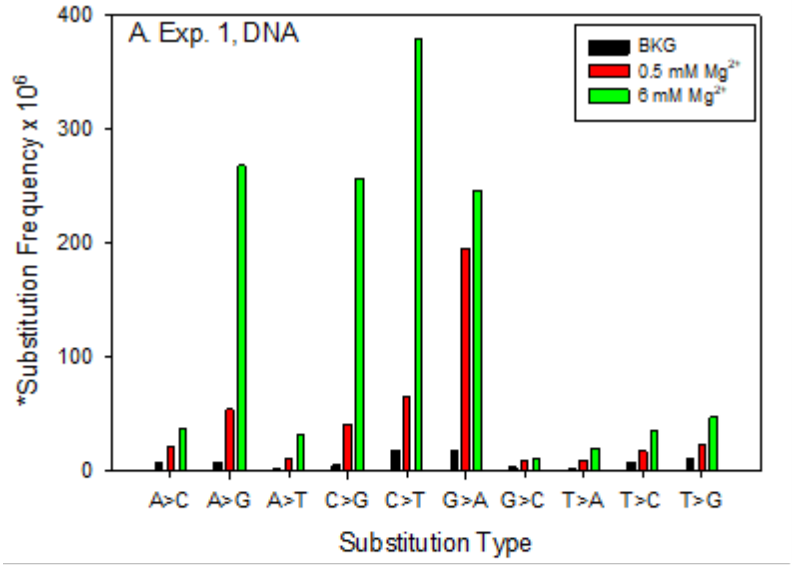
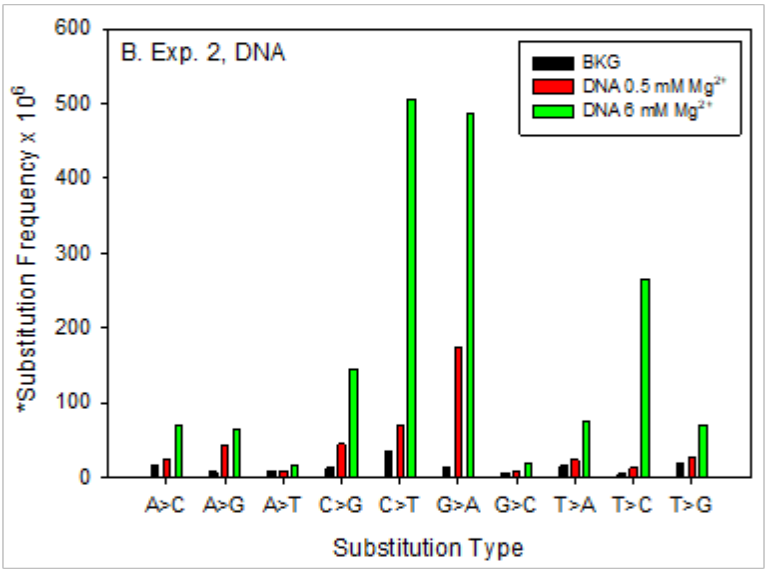


Figure S2. Proportion of various substitution mutations in the NGS assay for Exp. 2. The proportions of substitution mutations recovered using the indicated conditions in the NGS assay are shown. G>T and C>A mutations are excluded from the analysis (see Results). See Table S2 for more information on the mutation rates for the various mutation types.





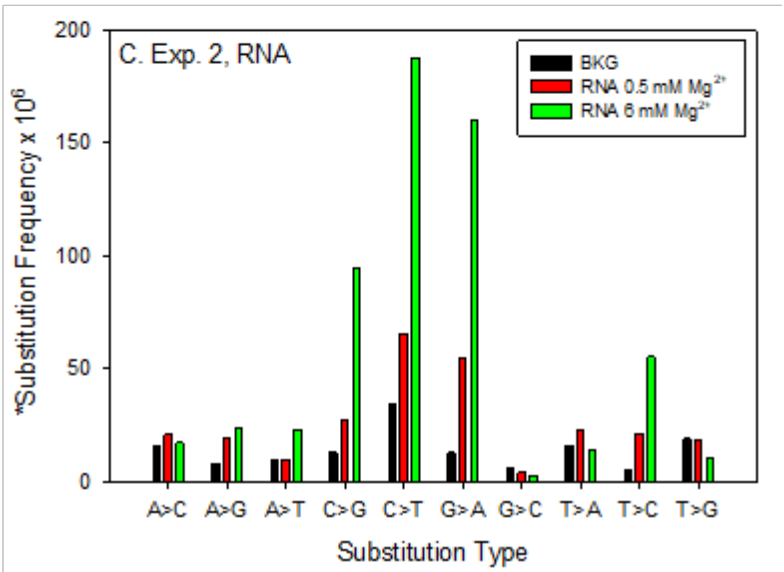


Figure S3a, b, and c. Mutation frequencies of different types of substitution mutations in the DNA templated assay for Exp. 1 (A), or DNA and RNA templated assays for Exp. 2 (B and C, respectively) at 0.5 or 6 mM Mg^{2+} using NGS analysis. A plot of background, 0.5 mM and 6 mM Mg^{2+} results are shown for the various NGS assays. The mutation frequency numbers were taken directly from Tables 1 or S1 and exclude C>A and G>T mutations (see Results). *This parameter is defined in Table 1 and S1 and represents the substitution frequency per nucleotide for the particular type of substitution indicated.

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