Supplementary information for

Detection of internal N7-methylguanosine (m^7G) RNA modifications by mutational profiling sequencing

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CONTENT

Supplementary methods: Calculation of mutation frequencies and statistical analysis

We use the mpileup function from the samtools package to compile sequencing results for each base position present in the sequence file that was used for mapping. For each position, this function summarises the sequencing data into the observed bases with their corresponding quality score for each sample (1,2). We developed the getFreq tool to estimate the mutation frequencies for each position by taking the observed sequenced bases and possibility of sequencing/alignment error into account.

Formal description of the estimation of mutation frequencies

For each position, we first removed bases/indels that are observed at very low frequency (default=0.1%) or bases/indels that are only observed a low number of times (default=1). For the remaining bases/indels, we estimated the allele frequency based on a likelihood function that includes the possibility of sequencing error modelled based on the base quality score.

$$\hat{f} = argmax_f \, P(X|f),$$

where X is the sequencing data for the position and f in the frequency of the possible alleles including indels e.g. f=(f_A,f_G,f_{AGG}) if both A, G and indel AGG is observed at this loci. The likelihood assumes independence per read such that for N reads

$$P(X|f) = \prod_{i=1}^{N} P(X_i|f).$$

The probability of observed the sequencing data for a single read depends on the true allele that was sequenced and we assumed that the true allele, A, is one of the T observed ones such that

$$P(X_i|f) = \sum_{j=1}^{T} P\big(X_i\big|A = a_j\big)p(A = a_j|f)$$

Where we sum over all of the possible true alleles a_j. The probability of $p(A = a_j|f)$ is simply the frequency of the a_j allele. To obtain the probability of the sequencing data of the i^{th} read we convert the quality score in to a probability of error, e, based on the Phred scaling. We assumed that if we observe allele of type b that

$$P\big(X_i = b\big|A = a_j\big) = \begin{cases} 1 - e, & if\ b = a_j \\ e/3, & otherwise \end{cases}$$

Where the division of 3 is motivated by the fact that sequencing error of a base can results in three other bases with equal probability. Since we also allow for indels this should be seen as an approximation.

Formal description of the calculation of p-values for positions having mutations:

To test if a position is polymorphic (has a significant amount of mutations), we calculated the likelihood based on the estimates allele frequency $\hat{f}$ and under the null where all alleles are the same, f_0, using a likelihood ratio statistics

$$\boldsymbol{Y = 2log}\left(\frac{P\left(X\middle|\hat{f}\right)}{P(X|f_0)}\right).$$

If there are T types of possible alleles then $Y \sim \chi^2_{T-1}$, where T-1 is the number of degrees of freedom. When multiple samples are analysed, we can test for differences between samples or groups of samples by estimating the frequencies jointly and separately in the groups. The test statistics then become

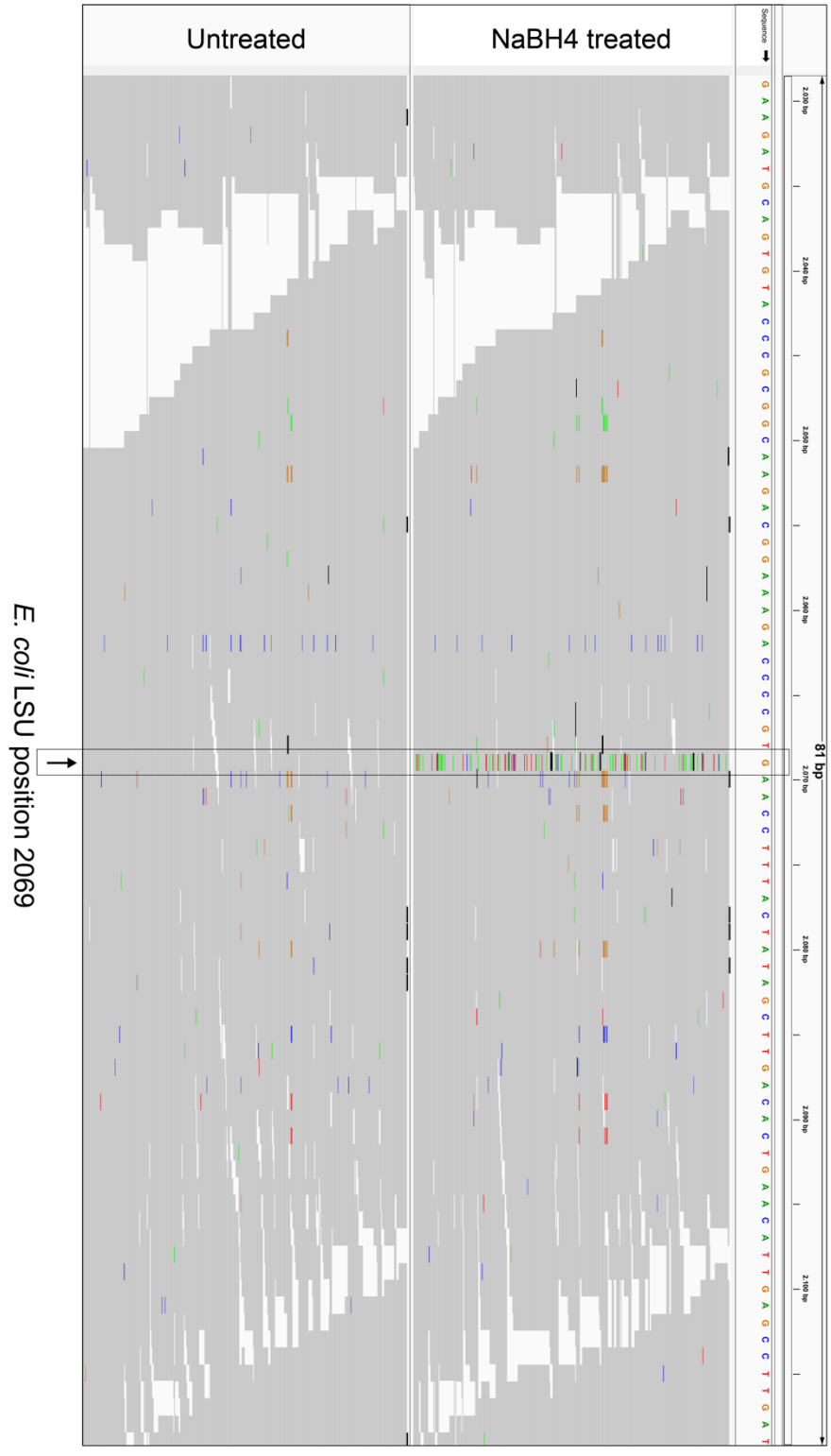
$$\boldsymbol{Y = 2log}\left(\frac{\prod_{k=1}^{k=K} P\left(X^k\middle|\widehat{f^k}\right)}{P\left(X\middle|\hat{f}\right)}\right)$$

Where X^k is the sequencing data in group k of K groups, f^k is the allele frequency in group k. Here we will have (K-1)*T degrees of freedom.

Outputs from the getFreq function:

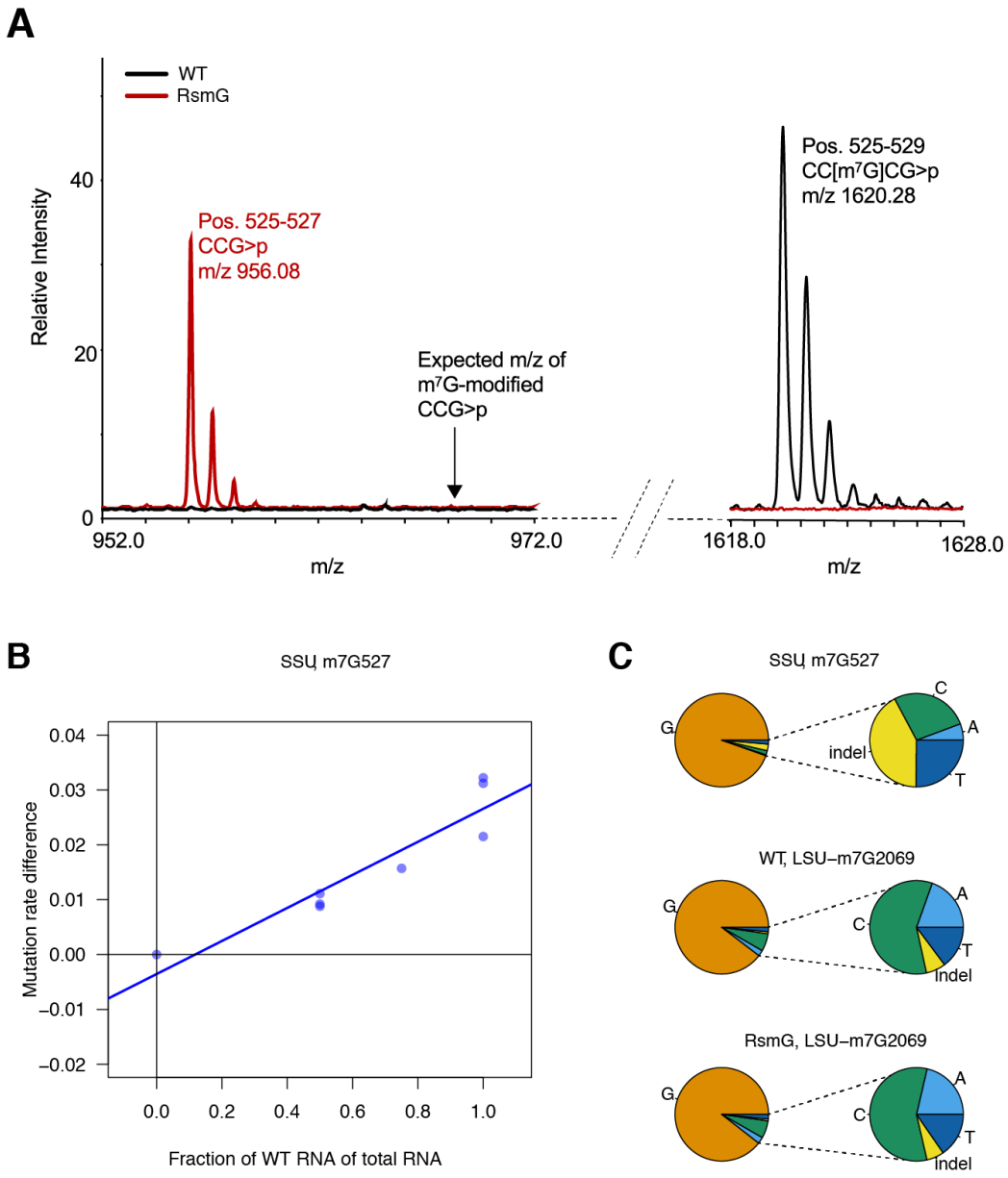
For each position, the getFreq function reports the estimated mutation frequencies for the control (AltFreqCC0) and treated (AltFreqCC1) samples as well as the mutation rate difference (relFreq). Moreover, the getFreq function reports p-values that have been log10 transformed and multiplied by -10. CCPval is the significance for there being a difference between the mutation rates observed in the treated and the control samples. The indPval is a p-values for each of the analysed samples testing whether the sample has a mutation frequency different from zero. For m7G positions, the individual p-values should be significant for treated samples, but not controls. CC0Pval and CC1Pval are the p-values for testing whether the observed mutation rates are different within the control and treated group, respectively. Preferably, the mutation rates obtained within the group should not be significantly different. Finally, the SNPPval in the p-value for a given position having a non-zero mutations frequency taking all samples into account. For a full description of the getFreq output see Supplementary table 2.

Supplementary figure 1: m7G-MaP-seq raw sequencing data



Supplementary figure 1: m7G-MaP-seq raw sequencing data
IGV browser view of 200 random m7G-MaP-seq reads from a NaBH4 treated and a control sample. The reads cover *E. coli* LSU position 2069, which is known to be m7G modified.

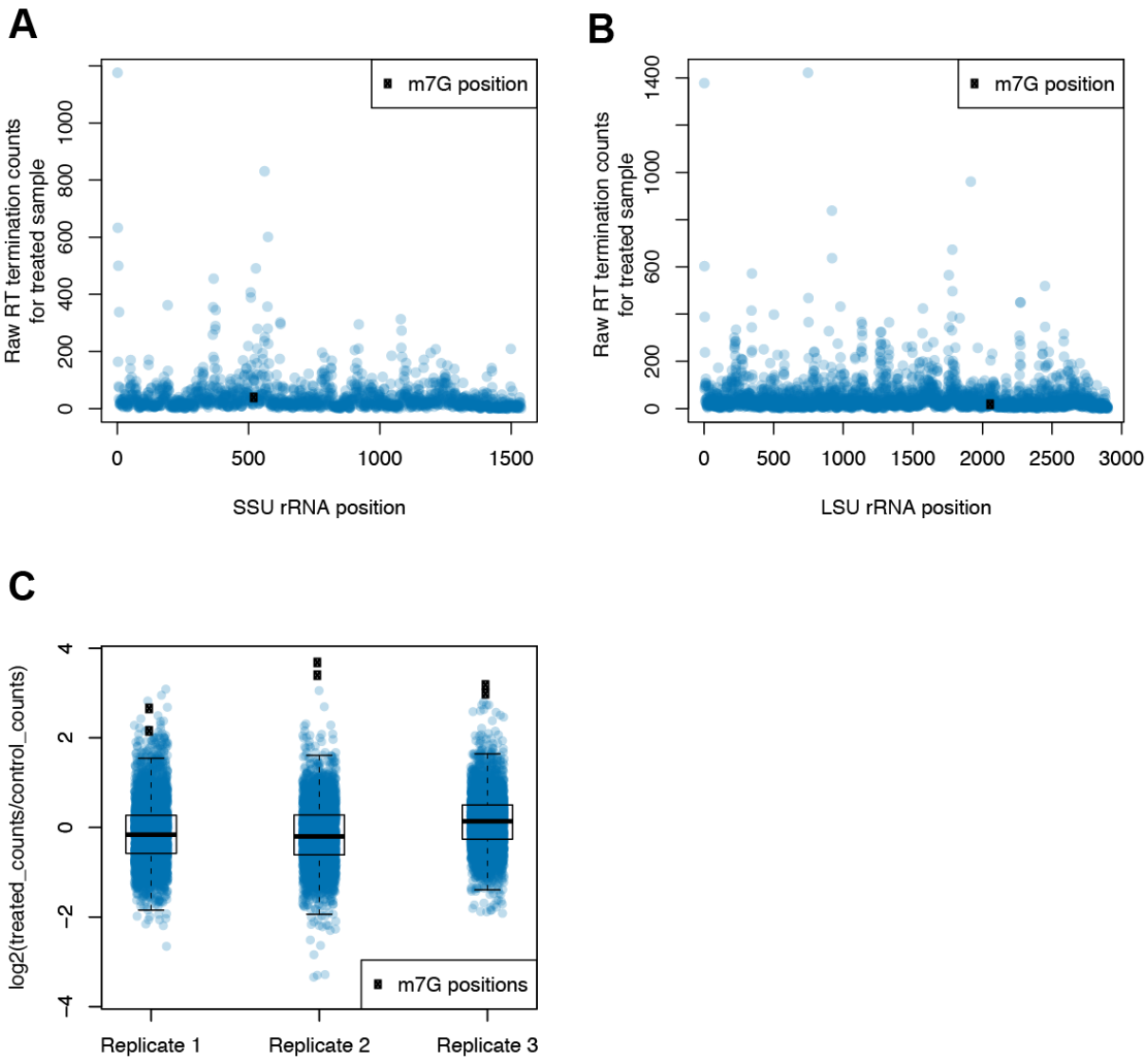
Supplementary figure 2: m7G-MaP-seq validation



Supplementary figure 2: m7G-MaP-seq validation

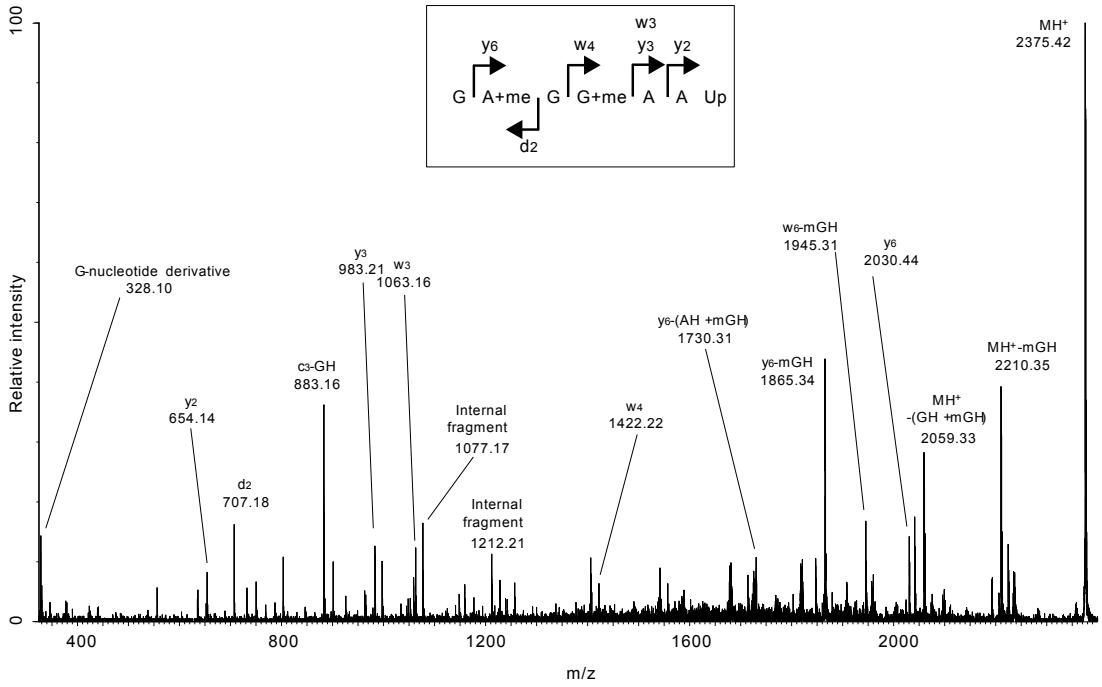
A) Overlaid MALDI mass spectra of *E. coli* 16S rRNA (position 500-549) digested with RNase T1; only m/z regions relevant to the m^{7}G527 modification are shown. Black trace: WT (strain BW25113); Red trace: In-frame RsmG deletion strain. m7G modification completely precludes RNase T1 cleavage resulting in the CC[m^{7}G]CG>p product in the wild type with a negligible signal for CCG>p, suggesting nearly complete methylation of G527. The RsmG deletion strain reveals the expected signal patter with G527 only being present in a CCG>p context. The intensity scale applies to all m/z traces. B) m7G-MaP-seq was applied to a mixtures of RNA isolated from the WT and the RsmG deletion strain and the mutational rate difference was calculated. C) Mutational signature of SSU rRNA m7G527 in the WT strain and LSU rRNA m7G2069 in the WT and RsmG strains.

Supplementary figure 3: Stop-rate analysis



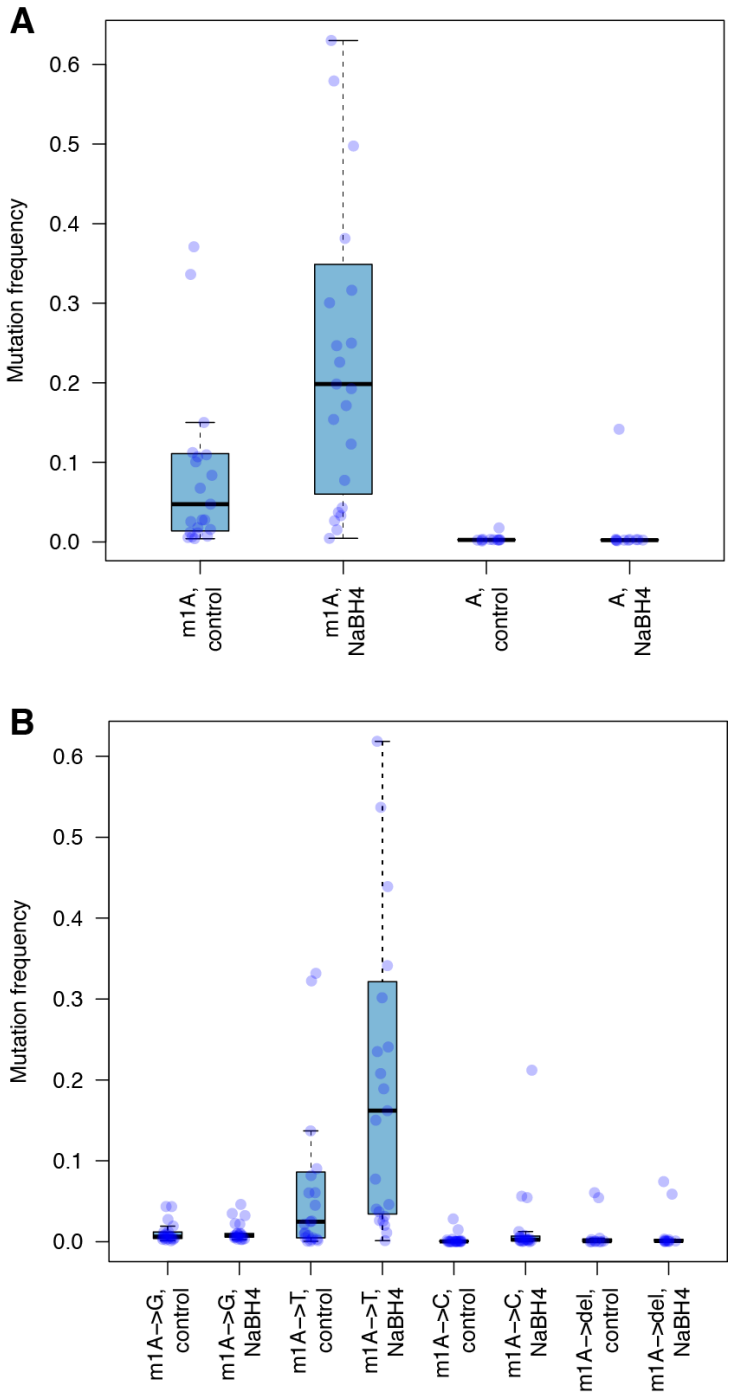
Supplementary figure 3: Stop rate analysis
Plot of the raw reveres transcription termination counts (collapse on barcodes to remove potential PCR duplicates) for $NaBH_4$ treated sample across A) *E. coli* SSU rRNA and B) LSU rRNA as determined by the RNAprobR R package. C) Log2 ratio of counts from $NaBH_4$ treated samples divided by counts from control samples for the same three replicates shown in Figure 1D). The analysis in D was performed as previously described using barcode counts (3).

Supplementary figure 4: Tandem mass spectrometry of Arabidopsis SSU position 1578-1584



Supplementary figure 4: MALDI Tandem mass spectrum of expected di-methylated RNase A product of *A. thaliana* SSU rRNA (position 1578-1584). Sequence (backbone cleavage) ions and other major signals are assigned. The insert places the observed sequence ions onto the expected RNase A product. MH^+: Parent ion selected for tandem MS. AH: adenine. GH: guanine. mGH: methylated guanine. a, b, c, d and w, x, y, z: 5' and 3' backbone fragment ions, respectively. Digit in subscript indicates number of nucleotides in fragment. According to nomenclature in (4).

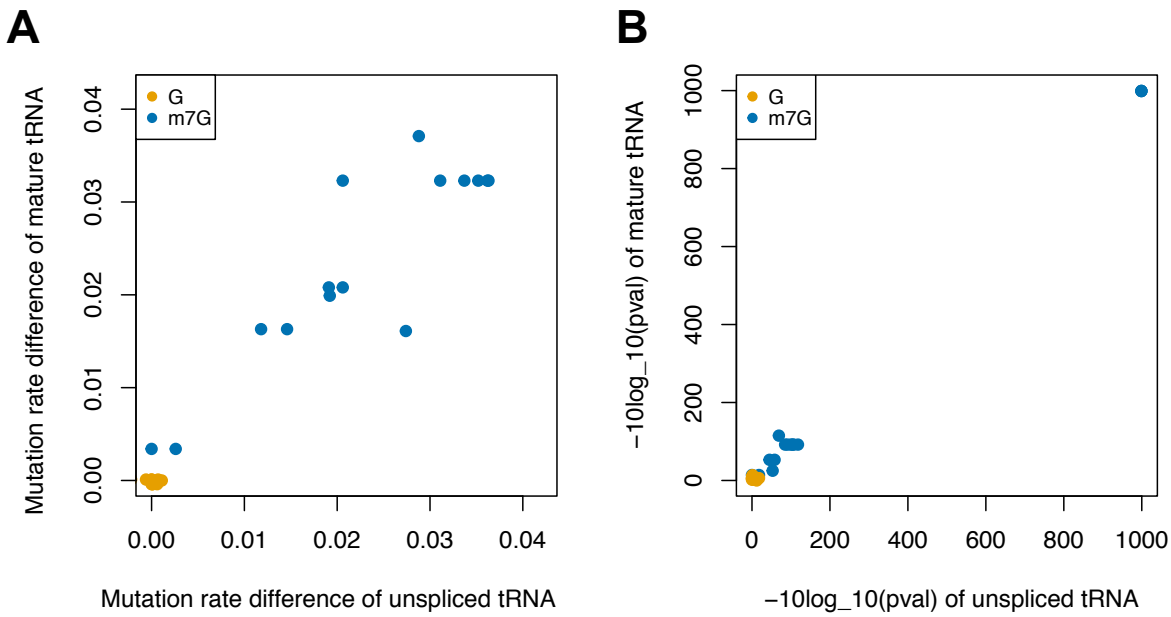
Supplementary figure 5: m1A sensitivity to $NaBH_4$ treatment



Supplementary figure 5: m1A sensitivity to $NaBH_4$ treatment

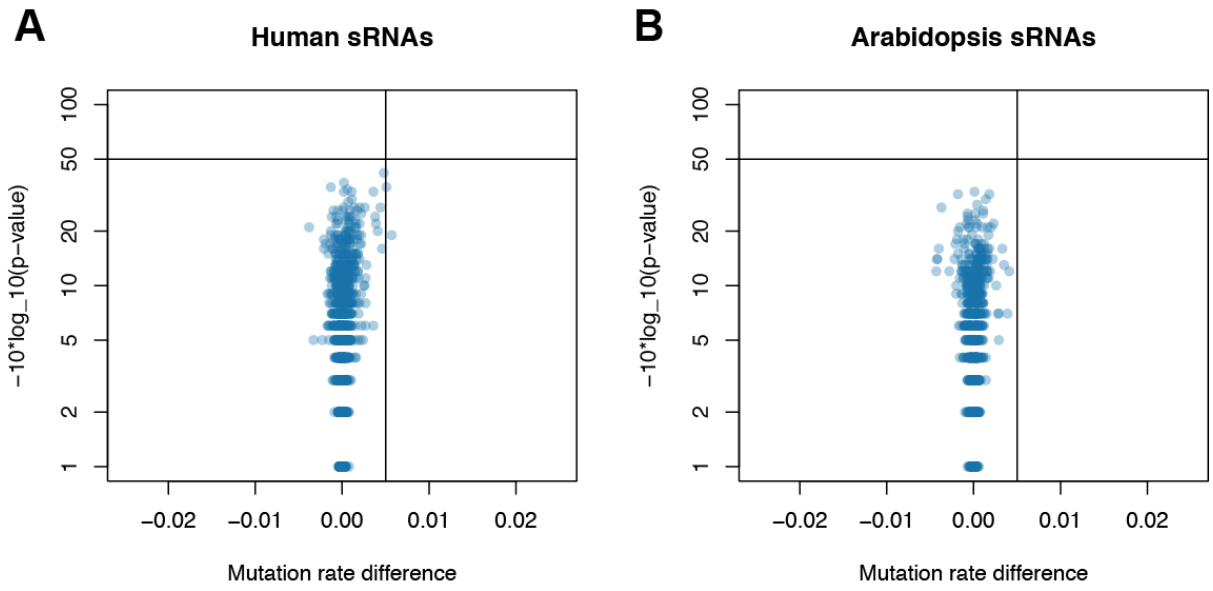
A) Mutational frequencies for yeast tRNA A positions and annotated m1A positions (5) in the control and $NaBH_4$ treated samples. B) Specific mutation frequencies observed for yeast tRNA annotated m1A positions (Modomics database). The figures show all A positions having sequencing depths of more than 1500 and no significant difference in mutation rates (p-value < 10^{-5}) within the control or NaBH4 treated replicates.

Supplementary figure 6: m^7G modifications in precursor and mature tRNA



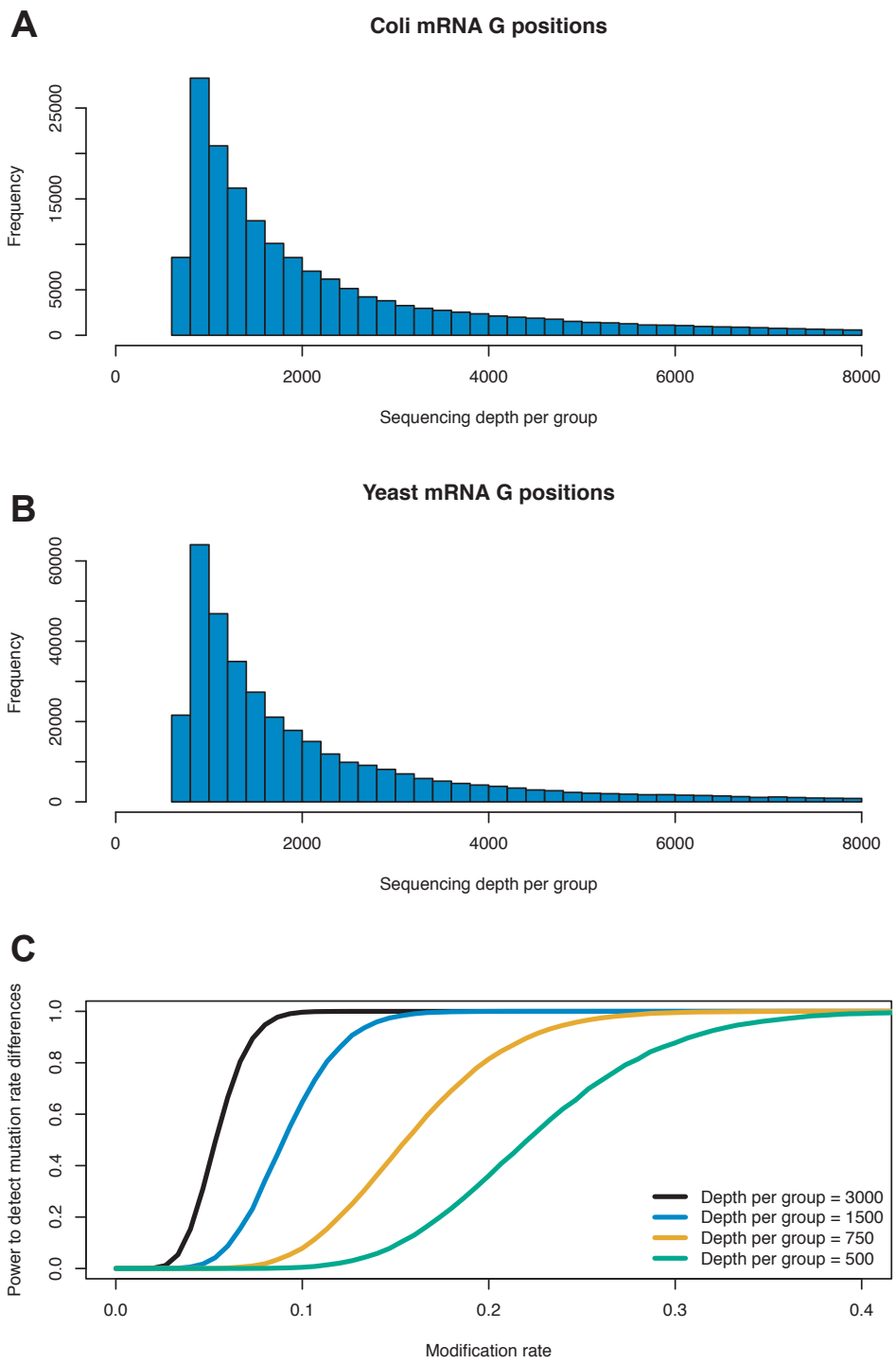
Supplementary figure 6: m^7G modifications in precursor and mature tRNA. A) Mutation rate difference between the control and the NaBH4 treated samples observed for mature tRNAs known to have either m^7G or G in position 46 in the variable loop and the corresponding unspliced precursor tRNA. For some mature tRNAs, several different genes exist. B) P-values for mutation rate difference between the control and the $NaBH_4$ treated samples observed for mature tRNAs known to have either m^7G or in position 46 in the variable loop and the corresponding unspliced precursor tRNA. For some mature tRNAs, several different genes exist.

Supplementary figure 7: Analysis of sRNA and miRNA m7G modification



Supplementary figure 7: Analysis of sRNA m^7G modifications. A) Small RNA reads were mapped to human snoRNAs and sRNAs. 4184 Gs in 245 different sRNAs passed the cut-offs described in the methods section. B) Small RNA reads mapped to arabidopsis snoRNAs and sRNAs. 3315 Gs in 217 different sRNAs passed the cut-offs described in the methods section.

Supplementary figure 8: Power analysis of mRNA m^{7}G detection



Supplementary figure 8: Power analysis of mRNA m^{7}G detection. A) Sequencing depth of Gs analysed in E. coli mRNA experiment. B) Sequencing depth of Gs analysed in yeast mRNA experiment. C) Simulated power to detect an m^{7}G modification with a p-value of 10^{-5} for 4 different sequencing depths. The simulations assume that a 100% modified position has a 15% mutation rate (as observed for ribosomal m7G RNA modifications in this experiment), sequencing depth is equal in the two groups, errors gives rise to the same observed base and a base error rate of 0.1 %.

Supplementary table 1: Oligonucleotides used in this study

Oligo name	Oligo sequence (5'- 3')
Ara_LSU_1581	GATGACTCGCGCTTACTAGGAATTCCTCGTTGAAGACCAACAATTGCAATGA
RT_random_primer	AGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNN
Ligation_adapter	PHO-NNNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3NHC3
PCR_forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCT
PCR_reverse_index1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index13	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index14	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index15	CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index16	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index17	CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index18	CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index19	CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index20	CAAGCAGAAGACGGCATACGAGATGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index21	CAAGCAGAAGACGGCATACGAGATCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index22	CAAGCAGAAGACGGCATACGAGATCGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index23	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR_reverse_index24	CAAGCAGAAGACGGCATACGAGATGCTACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Supplementary table 2: Explanation of output from getFreq function

Variable	Description	Example
chr	Seqence/chr/scaffold name.	Yeast_LSU_rRNA_U53879
pos	Position.	1870
ref	Reference base.	C
totalCounts	Total number of reads that cover position (including deletions)*.	15675
relFreq	Difference in mutation frequency (AltFreqCC1-AltFreqCC0).	0,0083
CCPval	P-value for frequency difference between the treated and control samples.	145
indPval	P-values of individual samples having mutations.	1;1;14;999;147;999
CC0Pval	P-value for differences in mutation frequencies within control samples.	2
CC1Pval	P-value for differences in mutation frequencies within treated samples.	12
SNPPval	P-value for position being variable.	999
countsCC0	Counts of alleles within control samples*.	.=10481,A=1,T=8
countsCC1	Counts of alleles within treated sample*.	.=5137,A=2,T=46
typeCountCC0	Number of different types of mutation in control samples*.	2
typeCountCC1	Number of different types of alleles in treated samples*.	2
totalCountsCC0	Total reads covering controls inc. deletions*.	10490
totalCountsCC1	Total reads covering treated samples inc. deletions*.	5185
AltFreq	Frequency of alt alleles (1-Frequency of ref allele).	0,0034
AltFreqCC0	Frequencies of alleles (1-Frequency of ref allele) within control samples.	0,0089
AltFreqCC1	Frequencies of alleles (1-Frequency of ref allele) within treated samples.	0,0006
SNPfreq	Estimated frequencies (for all samples).	.=0.9966,T=0.0033,A=0
CC0freq	Estimated frequencies for control samples.	.=0.9994,T=6e-04,A=0
CC1freq	Estimated frequencies for treated samples.	.=0.9911,T=0.0087,A=2e-04
counts	Counts of alleles for all samples*.	.=15618,A=3,T=54
indCounts	Counts of alleles for each sample*.	.=4915,A=1,T=4;.=4943,T=3;.=623,T=1;.=3092,T=27;.=1010,A=2,T=7;.=1035,T=12
indFreq	Estimated allele frequencies for each sample.	.=0.9995,T=5e-04,A=0;.=0.9994,T=6e-04,A=0;.=0.9984,T=0.0016,A=0;.=0.9915,T=0.0085,A=0;.=0.9916,T=0.0068,A=0.0015;.=0.9886,T=0.0114,A=0

* Only alleles that are observed with more than "minCount" in at least one sample are counted. The getFreq function takes the “minCount” as an input and will only perform analysis of the position if the total number of observed mutations at the position (totalCounts) > minCounts.

Supplementary table 3: m7G modification in yeast tRNA

GtRNAdb Gene symbol	Modomics**	Enroth *et. al*	Marchand *et. al*
Yeast_tRNA-Ala-AGC-1	-	-	+
Yeast_tRNA-Cys-GCA-1	+	+*	+
Yeast_tRNA-Ile-TAT-1	+	+	
Yeast_tRNA-Ile-TAT-2	+	+	
Yeast_tRNA-iMet-CAT-1	+	+	+
Yeast_tRNA-Lys-CTT-1	+	+	+
Yeast_tRNA-Lys-TTT-1	+	+*	+
Yeast_tRNA-Met-CAT-1	+	+*	+
Yeast_tRNA-Phe-GAA-1	+	+	
Yeast_tRNA-Phe-GAA-2	+	+	+
Yeast_tRNA-Pro-AGG-1		+	
Yeast_tRNA-Pro-TGG	+	+	+
Yeast_tRNA-Thr-TGT-2		+	
Yeast_tRNA-Trp-CCA-1	+	+	+
Yeast_tRNA-Val-CAC-1	+	+	+
Yeast_tRNA-Val-AAC-1	+	+	+

*The three tRNAs, met-CAU, Lys-UUU and Cys-GCA all have increased relative frequency and -10*log transformed p-value, but does not reach our cut-off, most likely due to low coverage. Conversely, these tRNA obtain the highest normalized cleavage values in Marchand et. al, which may indicate that abasic sites created in these tRNA are more prone to strand breakage.
** MODOMICS: a database of RNA modification pathways (5).

Supplementary references

1. Li, H. (2011) Improving SNP discovery by base alignment quality. *Bioinformatics*, **27**, 1157-1158.
2. Li, H. (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, **27**, 2987-2993.
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4. McLuckey, S.A., Van Berkel, G.J. and Glish, G.L. (1992) Tandem mass spectrometry of small, multiply charged oligonucleotides. *J Am Soc Mass Spectrom*, **3**, 60-70.
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