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Article

Production and Application of Stable Isotope-Labeled Internal Standards for RNA Modification Analysis

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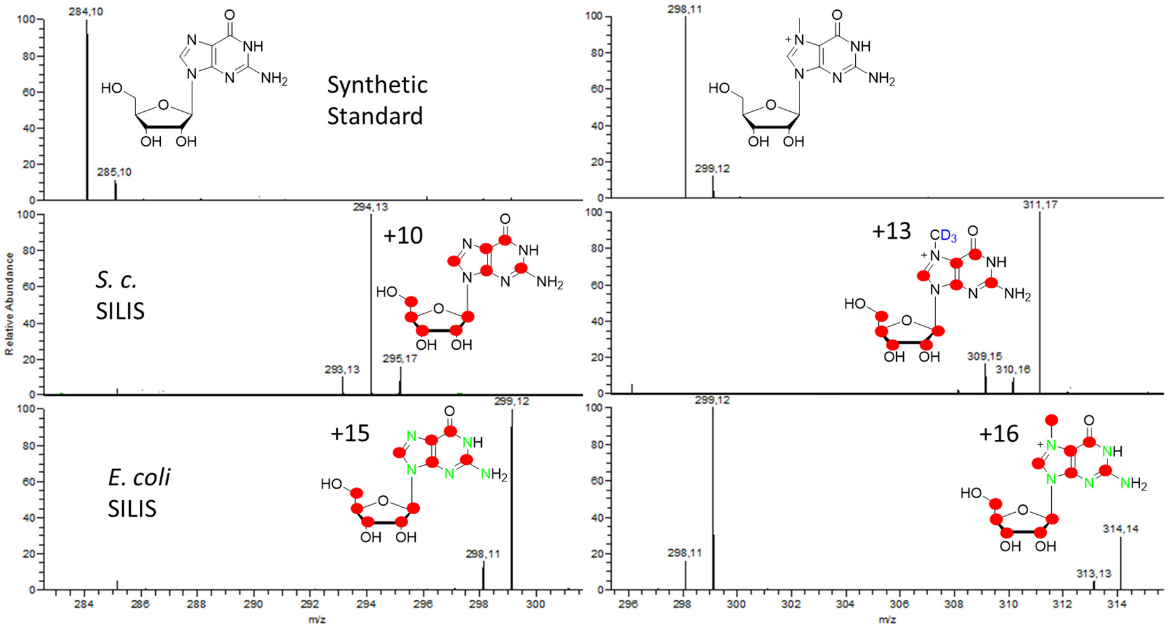
Content:

G and m^7G



C and Cm

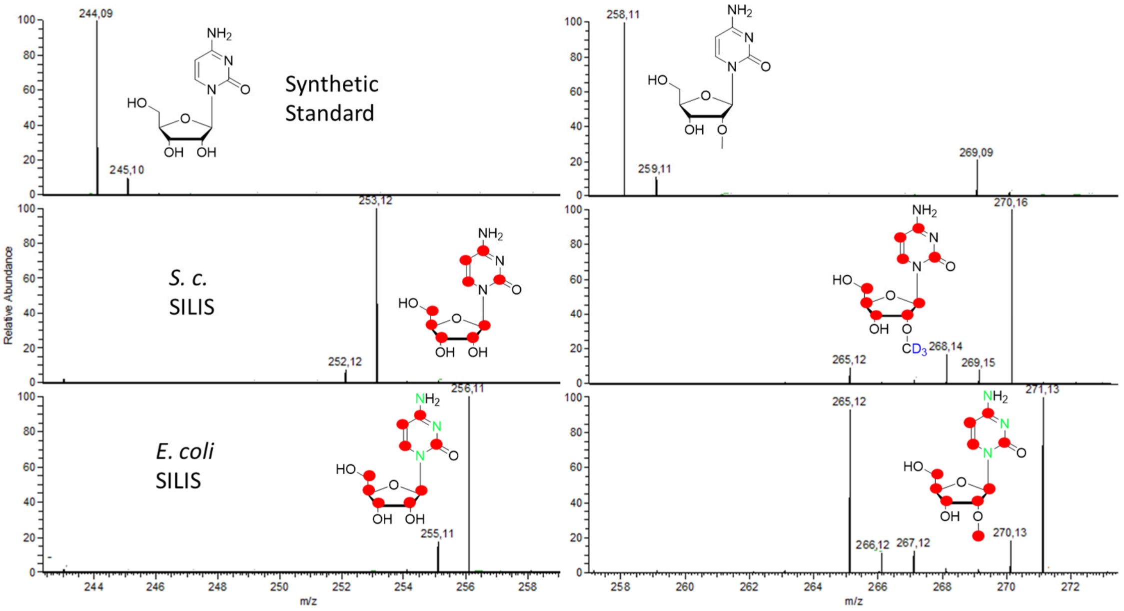


Figure S1. *Cont*

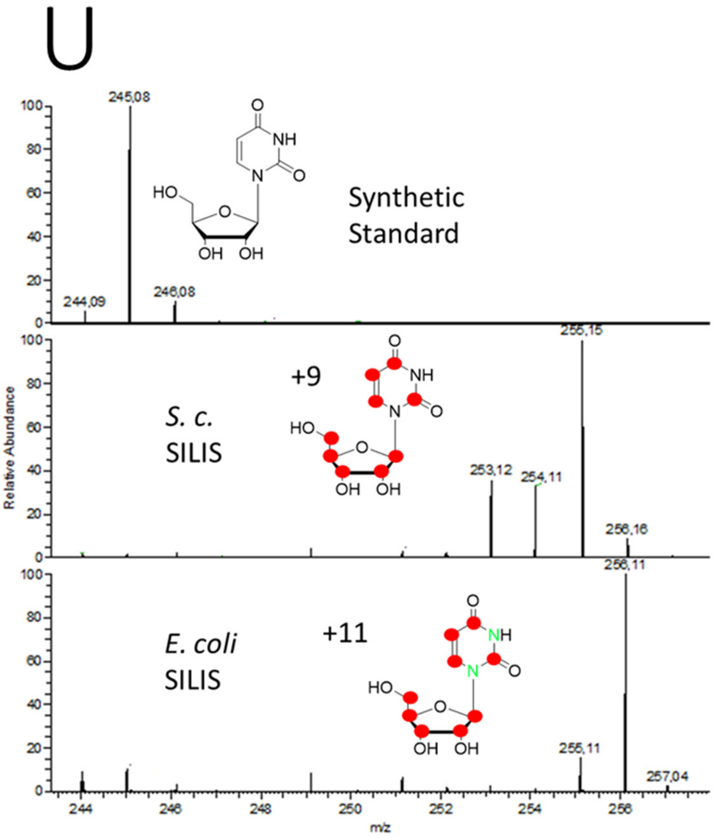


Figure S1: High-resolution mass spectra shows representative nucleosides from the produced labeled RNA digests.

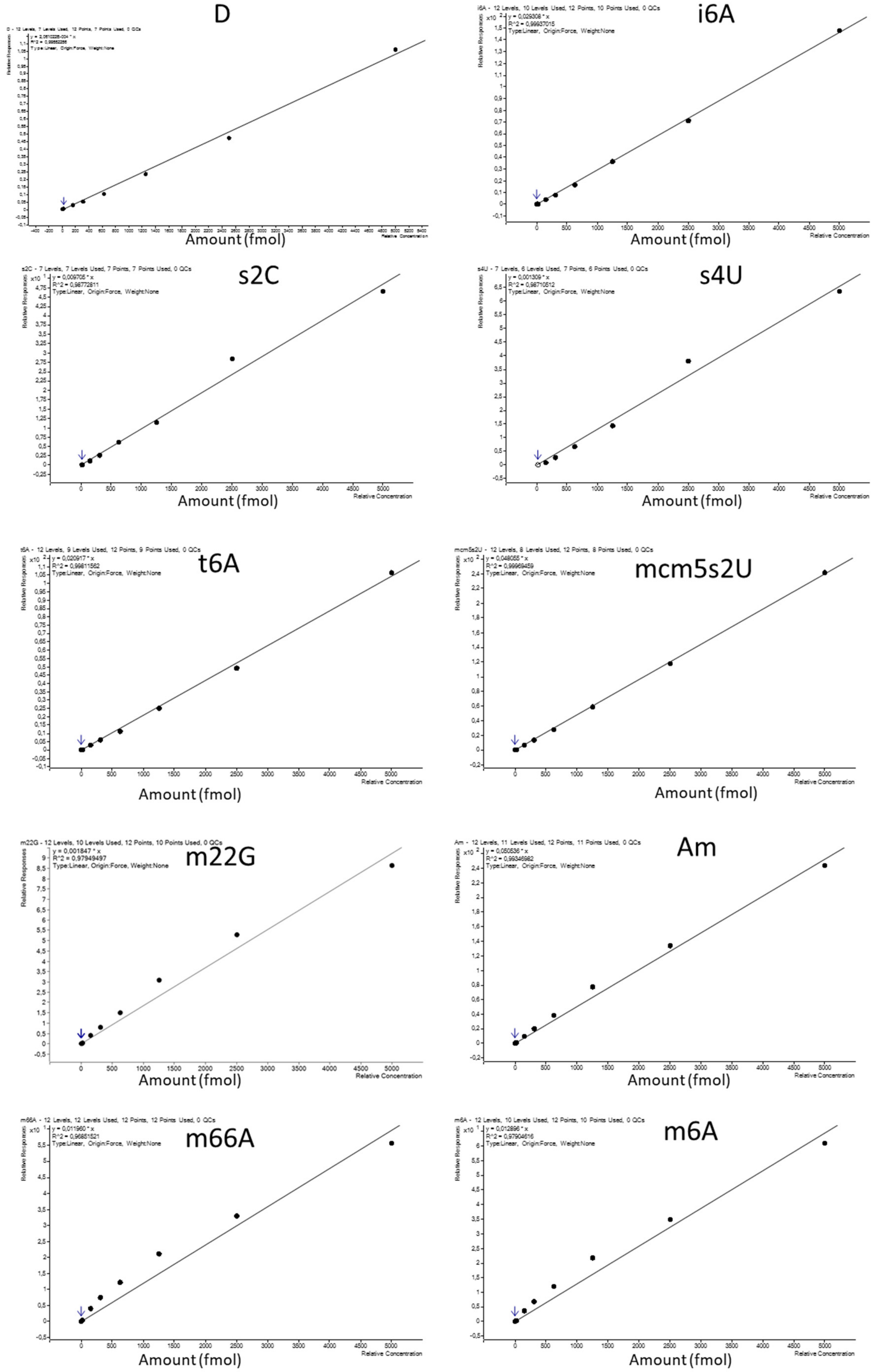


Figure S2. *Cont*

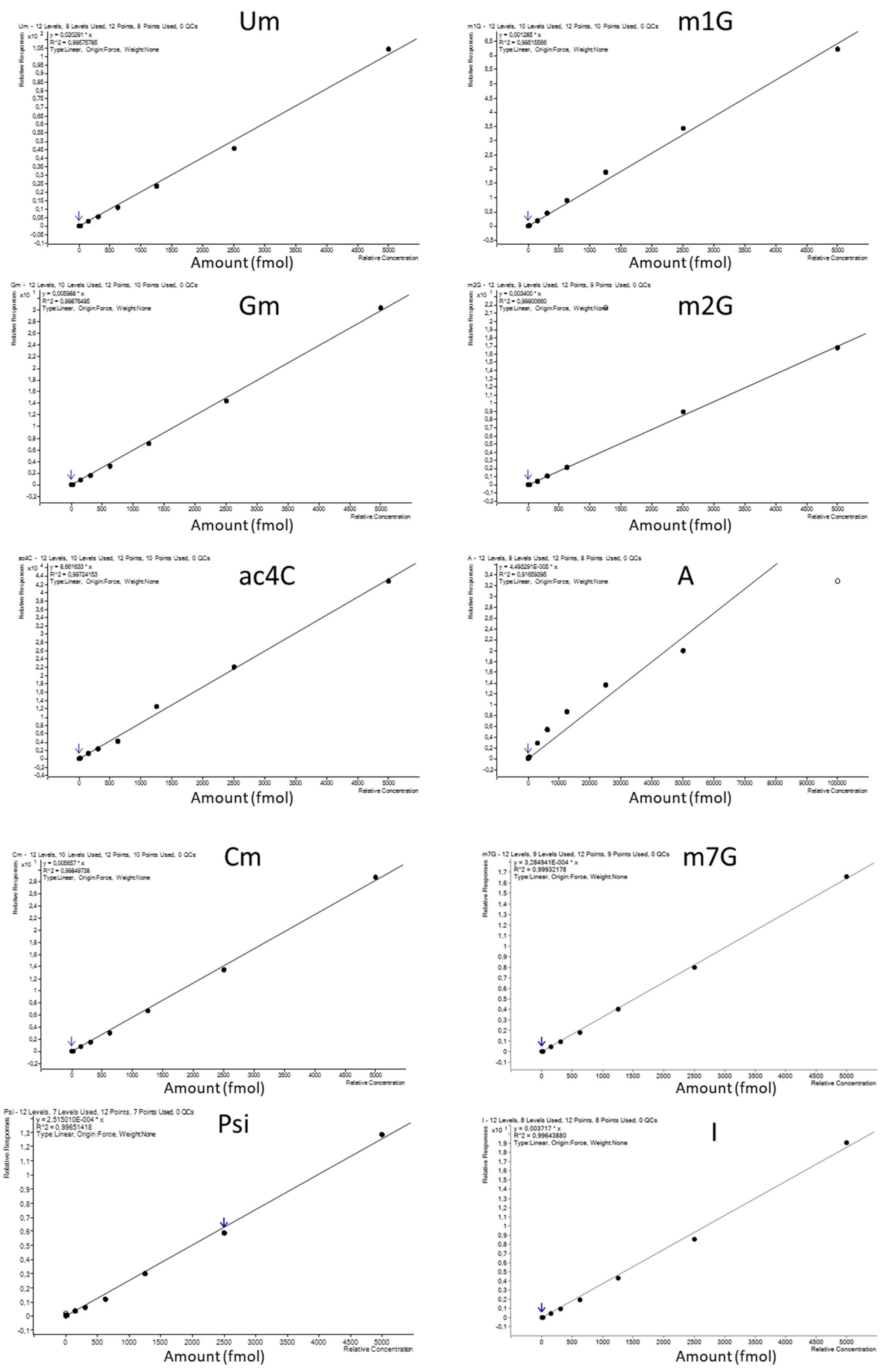


Figure S2. *Cont*

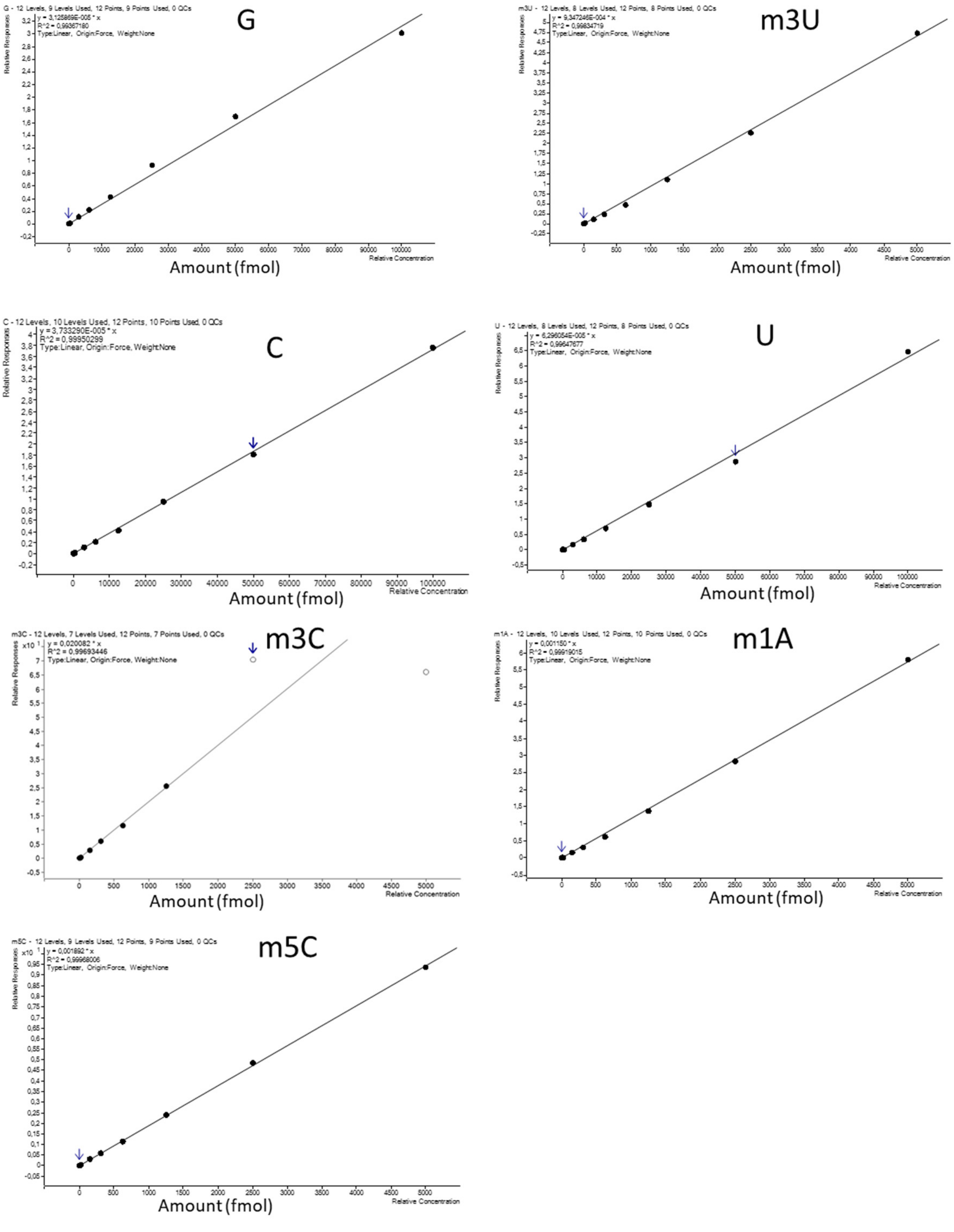


Figure S2: Calibration curves for all compounds in the calibration mix with relative response factor as the y axis and amount in fmol as the x axis. Most calibration curves shown are made with *S.c.* SILIS with the exception of s4U and s2C which were measured with *E. coli* SILIS.

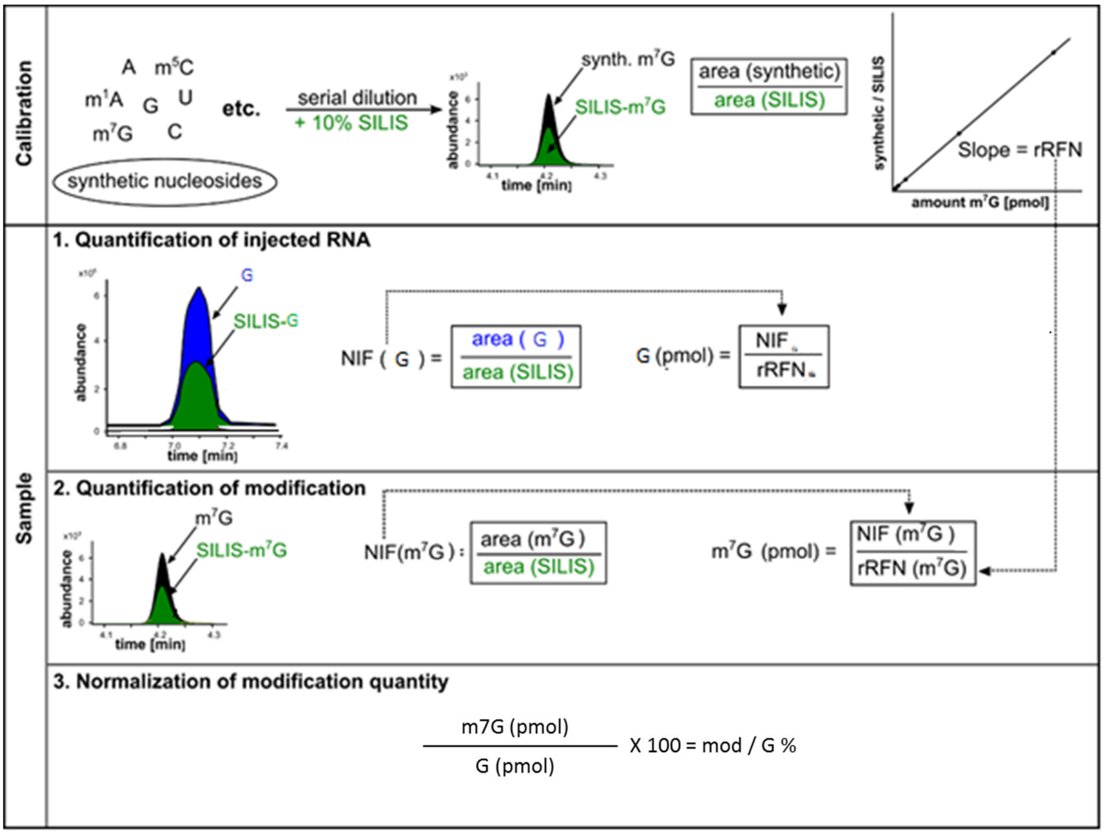


Figure S3: Calculations: The ratio of synthetic standard to SILIS area is plotted over the amount of injected synthetic standard. The slope of the calibration curve is the relative response factor nucleoside (rRFN) and is done for all calibrated nucleosides. The amount of sample RNA injected is quantified by calculation of the nucleoside-isotope-factor (NIF). The NIF is the area of the sample nucleoside peak over the corresponding SILIS peak area. The NIF is then divided by the rRFN to reveal the quantity in pmol.. The amount of modification is reported in percent G; calculated by the pmol of any modification over the pmol of G multiplied by 100.

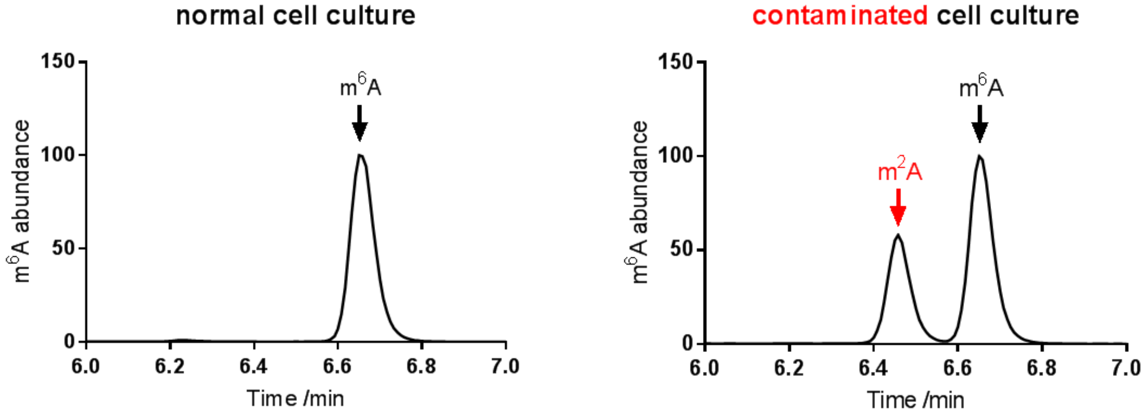


Figure S4: LC-MS/MS trace of the mass transition 282 →150 for methylated adenosines. On the left the chromatogram of a tRNA sample from a normal cell culture is shown. Only 6-methyladenosine (m^6A) can be detected. On the right, there are two peaks eluting. The first one is 2-methyladenosine (m^2A, red), which is a common bacterial tRNA modification and indicates the contamination of the cell culture with bacteria. Later testing revealed that the cell culture sample on the right was contaminated with mycobacteria.

Table S1: Overview of used synthetic standards, their vendors and alternative vendors. Sigma: Sigma, Aldrich, Munich, Germany; Carbosynth, Newbury, UK; TRC: Toronto Research Chemicals, Toronto, CA; Berry & Associates, Dexter, MI, USA.

Nucleoside	Abbreviation	Obtained from:	Also available from:
cytidine	C	Sigma	
2′-O-methylcytidine	Cm	Carbosynth	
3-methylcytidine	m3C	Carbosynth	Carbo and TRC
5-methylcytidine	m5C	Carbosynth	
2-thiocytidine	s2C	Berry & Associates	
N4-acetylcytidine	ac4C	Carbosynth	
pseudouridine	Y	Carbosynth	
uridine	U	Sigma	
dihydrouridine	D	Apollo scientific, UK	
2′-O-methyluridine	Um	Carbosynth	
3-methyluridine	m3U	Dedon Lab	
5-methyluridine	m5U	Carbosynth	
4-thiouridine	s4U	ordered from TRC	
5-methoxycarbonylmethyl-2-thiouridine	mcm5s2U	Helm Lab	
guanosine	G	Sigma	
1-methylguanosine	m1G		
2′-O-methylguanosine	Gm	Carbosynth	
N2-methylguanosine	m2G	Dedon Lab	
7-methylguanosine	m7G	Carbosynth	
N2,N2-dimethylguanosine	m2,2G	Carbosynth	
adenosine	A	Sigma	
inosine	I	Carbosynth	
1-methyladenosine	m1A		Carbosynth
2-methyladenosine	m2A	Dedon Lab	
2′-O-methyladenosine	Am	Carbosynth	Carbo and TRC
N6-methyladenosine	m6A	Carbosynth	Carbo, TRC and B&A
N6,N6-dimethyladenosine	m6,6A	Alfa Chemistry	TRC
N6-isopentenyladenosine	i6A	Dedon Lab	TRC
N6-threonylcarbamoyladenosine	t6A	TRC	TRC

90 Table S2a MRM method for sample quantification using the *E. coli* SILIS

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)
A eSILIS	283	146	5.1	1	200	40	5
Am eSILIS	298	146	6.0	1	110	17	5
C eSILIS	256	119	1.9	1	175	30	5
Cm eSILIS	271	119	3.7	1	180	9	5
D eSILIS	258	121	1.5	1	85	6	5
G eSILIS	299	162	4.0	1	95	30	5
Gm eSILIS	314	162	4.7	1	100	9	5
I eSILIS	283	146	3.8	1	100	9	5
i6a eSILIS	356	219	7.9	1.0	140	17	5
m1G eSILIS	314	177	4.7	1	105	13	5
m2G eSILIS	311	174	5	1	95	17	5
m3C eSILIS	271	134	2.5	2	88	14	5
m3U eSILIS	271	134	4.2	2	75	9	5
m5C eSILIS	271	134	3.4	1	185	13	5
m5U eSILIS	271	134	4.0	1	145	10	5
m66A eSILIS	313	176	7	1	130	21	5
m6A eSILIS	298	161	6.4	1	120	20	5
m7G eSILIS	314	177	3.6	2	105	14	5
Psi eSILIS	256	218	1.4	1	90	5	5
s2C eSILIS	272	135	3.2	2.0	85	13	5
s4U eSILIS	272	135	4.5	2	75	17	5
t6A eSILIS	434	297	5	2	130	9	5
U eSILIS	256	119	2.8	1	95	5	5
Um eSILIS	271	119	4.1	2.0	96	8	5
A	268	136	5.1	1	200	40	5
ac4C	286	154	4.8	1	85	9	5
Am	282	136	6.0	1.0	110	17	5
C	244	112	1.9	1	175	30	5
Cm	258	112	3.7	1	180	9	5
D	247	115	1.5	1	85	6	5
G	284	152	4.0	1	95	30	5
Gm	298	152	4.7	1	100	9	5
I	269	137	3.8	1	100	9	5
i6A	336	204	7.9	1.0	140	17	5
m1A	282	150	2.9	2	110	21	5
m1G	298	166	4.7	1	105	13	5
m22G	312	180	5.5	1	102	15	5
m2G	298	166	5	1	95	17	5

m3C	258	126	2.5	2	88	14	5
m3U	259	127	4.2	2	75	9	5
m5C	258	126	3.4	1	185	13	5
m5U	259	127	4.0	1	145	10	5
m66A	296	164	7	1	130	21	5
m6A	282	150	6.4	1	120	20	5
m7G	299	167	3.6	2	105	14	5
mcm5s2U	333	201	6	2	92	8	5
ms2A	314	182	5	2	125	21	5
Psi	245	209	1.4	1	90	5	5
Q	410	278	5.8	2	100	11	5
s2C	260	128	3.2	2.0	85	13	5
s4U	261	129	4.5	2	75	17	5
t6A	413	281	5	2	130	9	5
U	245	113	2.8	1	95	5	5
Um	259	113	4.1	2	96	8	5

Table S2b MRM method for sample quantification using the yeast SILIS

Compound Name	**Precursor Ion**	**Product Ion**	**Ret Time (min)**	**Delta Ret Time (min)**	**Fragmentor (V)**	**Collision Energy (eV)**	**Cell Accelerator Voltage (V)**
A ySILIS	278	141	5.1	1	200	40	5
ac4C ySILIS	297	160	4.8	1	85	9	5
Am ySILIS	295	141	6.0	1	110	17	5
C ySILIS	253	116	1.9	1	175	30	5
Cm ySILIS	270	116	3.7	1	180	9	5
D ySILIS	256	119	1.5	1	85	6	5
G ySILIS	294	157	4.0	1	95	30	5
Gm ySILIS	311	157	4.7	1	100	9	5
I ySILIS	279	142	3.8	1	100	9	5
i6a ySILIS	351	214	7.9	1	140	17	5
m1A ySILIS	295	158	2.9	2	110	21	5
m1G ySILIS	311	174	4.7	1	105	13	5
m22G ySILIS	328	191	5.5	1	102	15	5
m2G ySILIS	311	174	5	1	95	17	5
m3C ySILIS	270	133	2.5	2	88	14	5
m3U ySILIS	271	134	4.2	2	75	9	5
m5C ySILIS	270	133	3.4	1	185	13	5
m5U ySILIS	271	134	4.0	1	145	10	5
m66A ySILIS	312	175	7	1	130	21	5

m6A ySILIS	295	158	6.4	1	120	20	5
m7G ySILIS	311	174	3.6	2	105	14	5
mcm5s2U ySILIS	347	210	6	2	92	8	5
Psi ySILIS	254	218	1.4	1	90	5	5
Q ySILIS	427	290	5.8	2	100	11	5
t6A ySILIS	428	291	5	2	130	9	5
U ySILIS	254	117	2.8	1	95	5	5
Um ySILIS	271	117	4.1	2	96	8	5
A	268	136	5.1	1	200	40	5
ac4C	286	154	4.8	1	85	9	5
Am	282	136	6.0	1	110	17	5
C	244	112	1.9	1	175	30	5
Cm	258	112	3.7	1	180	9	5
D	247	115	1.5	1	85	6	5
G	284	152	4.0	1	95	30	5
Gm	298	152	4.7	1	100	9	5
I	269	137	3.8	1	100	9	5
i6A	336	204	7.9	1	140	17	5
m1A	282	150	2.9	2	110	21	5
m1G	298	166	4.7	1	105	13	5
m22G	312	180	5.5	1	102	15	5
m2G	298	166	5	1	95	17	5
m3C	258	126	2.5	2	88	14	5
m3U	259	127	4.2	2	75	9	5
m5C	258	126	3.4	1	185	13	5
m5U	259	127	4.0	1	145	10	5
m66A	296	164	7	1	130	21	5
m6A	282	150	6.4	1	120	20	5
m7G	299	167	3.6	2	105	14	5
mcm5s2U	333	201	6	2	92	8	5
ms2A	314	182	5	2	125	21	5
Psi	245	209	1.4	1	90	5	5
Q	410	278	5.8	2	100	11	5
s2C	260	128	3.2	2.0	85	13	5
s4U	261	129	4.5	2	75	17	5
t6A	413	281	5	2	130	9	5
U	245	113	2.8	1	95	5	5
Um	259	113	4.1	2.0	96	8	5

Table S3a Nucleosides found in the heavy labeled *E. coli* RNA and their mass transitions

Compound	**Precursor Ion (m/z)**	**Product Ion (m/z)**
A eSILIS	283	146
Am eSILIS	298	146
C eSILIS	256	119
Cm eSILIS	271	119
D eSILIS	258	121
G eSILIS	299	162
Gm eSILIS	314	162
I eSILIS	283	146
i6a eSILIS	356	219
m1G eSILIS	314	177
m2G eSILIS	311	174
m5C eSILIS	271	134
m5U eSILIS	271	134
m66A eSILIS	313	176
m6A eSILIS	298	161
m7G eSILIS	314	177
Psi eSILIS	256	218
s2C eSILIS	272	135
s4U eSILIS	272	135
t6A eSILIS	434	297
U eSILIS	256	119
Um eSILIS	271	119

Table S3b Nucleosides found in the heavy labeled yeast RNA and their mass transitions

Compound	Precursor Ion (m/z)	Product Ion (m/z)
A ySILIS	278	141
ac4C ySILIS	297	160
Am ySILIS	295	141
C ySILIS	253	116
Cm ySILIS	270	116
D ySILIS	256	119
G ySILIS	294	157
Gm ySILIS	311	157
I ySILIS	279	142
i6a ySILIS	351	214
m1A ySILIS	295	158
m1G ySILIS	311	174
m22G ySILIS	328	191
m2G ySILIS	311	174
m3C ySILIS	270	133
m3U ySILIS	271	134
m5C ySILIS	270	133
m5U ySILIS	271	134
m66A ySILIS	312	175
m6A ySILIS	295	158
m7G ySILIS	311	174
mcm5s2U ySILIS	347	210
mcm5U ySILIS	331	194
ncm5s2U ySILIS	329	192
Psi ySILIS	254	218
Q ySILIS	427	290
t6A ySILIS	428	291
U ySILIS	254	117
Um ySILIS	271	117

Table S4 Absolute lower limits of quantification (fmol) of modified nucleosides (LLOQ) and the LLOQ per injected mRNA (normalized to G)

	LLOQ	
	amount (fmol)	LLOQ (fmol)/ average G (fmol) x100
Psi	20	0.022853
D	3	0.003428
Cm	3	0.003428
m3C	0.6	0.000686
m5C	3	0.003428
Um	10	0.011427
m3U	20	0.022853
m5U	2	0.002285
s2C	2	0.002285
s4U	3	0.003428
I	3	0.003428
m1A	0.6	0.000686
Am	0.2	0.000686
m6A	0.2	0.000229
ac4C	0.6	0.000686
m66A	0.1	0.000114
m1G	0.6	0.000686
Gm	3	0.003428
m2G	3	0.003428
m7G	3	0.003428
m22G	0.6	0.000686
mcm5s2U	3	0.003428
i6A	0.6	0.000686
t6A	3	0.003428

Table S5a: Data table from Figure 4

	E. c. SILIS rep1	*E. c.* SILIS rep2	*E. c.* SILIS rep3	*S. c.* SILIS rep1	*S. c.* SILIS rep2	*S. c.* SILIS rep3	
Present in both SILISs (Figure 4a)							
D	10.311	10.871	10.768	10.620	10.019	10.394	
m1G	2.557	2.746	2.818	2.910	2.972	3.127	
m7G	2.287	2.301	2.254	2.389	2.258	2.303	
t6A	1.396	1.304	1.385	1.345	1.257	1.326	
Gm	0.857	0.859	0.821	0.870	0.802	0.803	
I	0.779	0.746	0.755	0.770	0.746	0.767	
Cm	0.285	0.237	0.181	0.537	0.475	0.476	
m6A	0.113	0.119	0.116	0.135	0.125	0.124	
i6A	0.020	0.024	0.025	0.015	0.015	0.016	
Am	0.017	0.019	0.021	0.022	0.023	0.026	
m66A	0.000	0.000	0.001	0.001	0.001	0.001	
Only present in the *S. c.* SILIS (Figure 4b)							Specialty
m2G	0.812	0.807	0.767	0.539	0.543	0.536	Reference to m1G
m1A	2.693	3.447	3.470	2.977	3.879	4.583	External cal
m5C	5.313	5.972	5.980	2.681	2.763	2.899	Reference to m5U
m22G	1.571	1.946	1.865	1.820	2.047	2.319	External cal
m5U	2.328	2.149	2.144	1.494	1.471	1.288	External cal
Um	0.754	0.822	0.812	1.732	1.738	1.893	Reference to Cm
m3C	1.843	1.670	1.426	0.893	0.861	0.846	Reference to m5U
ac4C	0.626	0.709	0.622	0.326	0.386	0.292	External cal
mcm5s2U	0.215	0.213	0.169	0.223	0.201	0.191	External cal

Table S5b: Data table from Figure 5

	E. c. LB_1	*E. c.* LB_2	*E. c.* LB_3	*E. c.* M9_1	*E. c.* M9_2	*E. c.* M9_3	*P. a.* M9 1	*P. a.* M9 2	*P. a.* M9 3
Psi	6.672	7.347	7.031	6.076	6.316	6.233	5.588	5.478	5.346
D	8.108	8.383	8.008	8.837	9.223	8.159	5.669	8.304	7.946
m2G	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
m1A	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
m5C	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
m1G	0.650	0.576	0.574	0.610	0.652	0.648	0.705	0.676	0.683
m22G	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
m7G	2.452	2.374	2.258	2.433	2.368	2.375	3.243	3.137	3.135
m5U	5.114	5.158	4.902	4.942	4.934	4.798	4.855	4.951	4.724
Um	0.376	0.395	0.307	0.243	0.192	0.222	0.134	0.137	0.109
t6A	0.373	0.389	0.313	0.305	0.318	0.307	0.206	0.209	0.216
Gm	0.804	0.826	0.850	0.675	0.692	0.659	0.176	0.176	0.179
m3C	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
I	0.204	0.215	0.202	0.170	0.178	0.156	0.154	0.145	0.153
Cm	0.057	0.059	0.051	0.049	0.042	0.048	0.080	0.075	0.073
ac4C	0.004	0.008	0.004	0.009	0.008	0.008	below LLOQ	below LLOQ	below LLOQ
mcm5s2U	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
m6A	0.065	0.132	0.124	0.130	0.128	0.123	0.101	0.102	0.103
i6A	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.005
Am	0.017	0.015	0.018	0.016	0.016	0.016	0.030	0.030	0.031
m66A	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ	below LLOQ
s4U	0.054	0.626	0.855	1.180	0.315	0.937	1.040	0.811	0.878
s2C	0.167	0.205	0.186	0.194	0.191	0.167	0.208	0.186	0.307

	S. c. 1	*S. c.* 2	*S. c.* 3	*D._d. 1*	*D._d. 2*	*D._d. 3*	*C._e. 1*	*C._e. 2*	*C._e. 3*
Psi	13.661	14.332	12.982	10.260	10.609	10.271	5.061	5.304	6.254
D	14.506	14.224	14.493	10.171	10.959	10.799	2.033	2.301	1.942
m2G	0.305	0.289	0.289	0.224	0.262	0.227	0.051	0.052	0.071
m1A	2.544	2.505	2.543	5.032	4.138	4.582	0.658	0.679	0.669
m5C	1.846	1.786	1.820	1.850	1.938	1.836	0.430	0.441	0.434
m1G	3.517	3.314	3.472	3.079	3.117	3.054	0.598	0.600	0.680
m22G	2.664	2.420	2.589	3.703	3.007	3.544	0.656	0.659	0.644
m7G	1.299	1.272	1.273	3.331	3.454	3.117	0.631	0.638	0.645
m5U	2.572	2.277	2.518	1.372	1.168	1.154	0.682	0.445	0.351
Um	0.255	0.181	0.235	0.966	0.965	0.610	0.866	0.812	0.822
t6A	1.019	0.896	0.931	1.009	0.818	0.784	0.231	0.237	0.260
Gm	0.848	0.781	0.829	1.353	1.410	1.246	0.951	0.932	0.920
m3C	0.306	0.285	0.301	0.727	0.746	0.701	0.068	0.072	0.067
I	0.906	0.856	0.873	0.781	0.773	0.786	0.036	0.038	0.035
Cm	0.695	0.668	0.678	0.669	0.704	0.737	0.477	0.481	0.481
ac4C	0.110	0.105	0.093	0.063	0.067	0.084	0.066	0.084	0.103
mcm5s2U	0.109	0.116	0.110	0.411	0.475	0.425	0.008	0.008	0.007
m6A	0.179	0.146	0.167	0.148	0.117	0.132	0.071	0.071	0.070
i6A	0.104	0.105	0.117	0.047	0.130	0.072	0.001	0.001	0.001
Am	0.113	0.103	0.116	0.355	0.376	0.388	0.960	0.970	0.959
m66A	0.001	0.001	0.001	0.177	0.193	0.120	0.064	0.059	0.056

	HEK 1	HEK 2	HEK 3	*M. m.* brain 1	*M. m.* brain 2	*M. m.* brain 3	*M. m.* liver 1	*M. m.* liver 2	*M. m.* liver 3
Psi	13.284	11.877	13.201	14.710	12.612	13.308	10.433	12.381	16.348
D	10.620	10.019	10.394	10.497	11.656	11.035	10.868	9.753	10.800
m2G	0.539	0.543	0.536	0.594	0.556	0.568	0.624	0.530	0.666
m1A	2.977	3.879	4.583	3.118	3.128	3.041	3.339	2.937	3.247
m5C	2.681	2.763	2.899	2.529	2.495	2.507	2.651	2.333	2.741
m1G	2.910	2.972	3.127	3.255	3.255	3.187	3.383	2.933	3.508
m22G	1.820	2.047	2.319	2.257	2.242	2.197	2.218	2.028	2.229
m7G	2.389	2.258	2.303	1.829	1.895	1.856	1.738	1.543	1.797
m5U	1.494	1.471	1.288	0.674	0.612	0.627	0.648	0.507	0.668
Um	1.732	1.738	1.893	1.437	1.613	1.475	1.208	1.413	1.085
t6A	1.345	1.257	1.326	1.416	1.274	1.332	1.293	1.122	1.304
Gm	0.870	0.802	0.803	0.789	0.854	0.877	0.404	0.489	0.385
m3C	0.893	0.861	0.846	1.051	0.926	0.888	0.812	0.750	0.888
I	0.770	0.746	0.767	0.602	0.612	0.584	0.580	0.497	0.568
Cm	0.537	0.475	0.476	0.582	0.656	0.654	0.602	0.641	0.586
ac4C	0.326	0.386	0.292	0.134	0.080	0.102	0.078	0.065	0.110
mcm5s2U	0.223	0.201	0.191	0.081	0.018	0.092	0.102	0.074	0.086
m6A	0.135	0.125	0.124	0.251	0.255	0.251	0.260	0.247	0.279
i6A	0.015	0.015	0.016	0.048	0.051	0.042	0.064	0.050	0.068
Am	0.022	0.023	0.026	0.037	0.103	0.128	0.106	0.324	0.038
m66A	0.001	0.001	0.001	0.002	0.014	0.010	0.005	0.020	0.002

Table S5c: Data table from Figure 6b

	***M. m.* brain 1**	***M. m.* brain 2**	***M. m.* brain 3**	***M. m.* liver 1**	***M. m.* liver 2**	***M. m.* liver 3**
m6A	0.343497	0.374741	0.36192	0.191708	0.229845	0.177888
m7G	0.077023	0.11138	0.098379	0.128558	0.083871	0.071753
Gm	0.043097	0.054056	0.047701	0.049877	0.055619	0.040185
Cm	0.032846	0.030672	0.033159	0.037945	0.087054	0.037659
m1A	0.043834	0.02216	0.014301	0.011702	0.025167	0.018145
Am	0.021215	0.023379	0.014492	0.028854	0.039273	0.027936
Um	0.025887	0.02572	0.025261	0.027112	0.050256	0.027751
I	0.02753	0.013562	0.020386	0.01576	0.069667	0.016231
m2G	0.04162	0.01978	0.01472	0.004941	0.019116	0.013516
m5U	0.013019	0.013915	0.024981	0.093623	0.195699	0.023944
m1G	0.012174	0.005405	0.004853	below LLOQ	0	0.00577
m66A	0.000792	0.000803	0.000555	0.000437	0.001041	0.000544
m22G	0.00444	0.00227	0.002168	below LLOQ	below LLOQ	below LLOQ
m5C	0.009534	0.006	0.005234	0.007464	below LLOQ	0.007353
t6A	0.005305	0.00367	0.005382	0.004546	0.006739	0.00271
m3C	0.004104	0.001624	below LLOQ	0.007423	0.024755	0.012224