

SUPPLEMENTARY TABLES

Supplementary TABLE S1. Suggested RNA Type-specific alternative protocols for the preparation of sequencing libraries using standard RNA Type 1-specific methods of sRNA-Seq library preparation.

Suggested Ribomarker® Protocols	ST[1]	ST[2]	ST[1+2+3+4]	ST[2+3]	ST[3+4]	ST[4]	ST[1+2]	ST[3]
RNA Types in the sample	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4
Pretreatment # 1	–	Rnl1 + Rnl2 ligases (+ATP)	PNK (-ATP) pH 6.0	PNK (-ATP) pH 6.0	PNK, 3'-minus (+ATP) pH 7.6	RtcB ligase	PNK, 3'-minus (+ATP) pH 7.6	Terminator 5'-p-dependent exonuclease
RNA Types conversion	–	1 → 0	3 → 2 4 → 1	3 → 2 4 → 1	2 → 1 3 → 4	3 → 0	2 → 1	Degrade 1, 4
Pretreatment # 2	–	PNK, 3'-minus (+ATP) pH 7.6	PNK (+ATP) pH 7.6	Rnl1 + Rnl2 ligases (+ATP)	Rnl1 + Rnl2 ligases (+ATP)	PNK, 3'-minus (+ATP) pH 7.6	–	PNK, 3'-minus (+ATP) pH 7.6
RNA Types conversion	–	2 → 1	2 → 1	1 → 0	1 → 0	2 → 1	–	2 → 1 3 → 4
Pretreatment # 3	–	–	–	PNK (+ATP) pH 7.6	PNK (-ATP) pH 6.0	Rnl1 + Rnl2 ligases (+ATP)	–	Rnl1 + Rnl2 ligases (+ATP)
RNA Types conversion	–	–	–	2 → 1	4 → 1	1 → 0	–	1 → 0
Pretreatment # 4	–	–	–	–	–	PNK (-ATP) pH 6.0	–	PNK (-ATP) pH 6.0
RNA Types conversion	–	–	–	–	–	4 → 1	–	4 → 1
Sequencing library preparation Protocol	ST[1]	ST[1]	ST[1]	ST[1]	ST 1]	ST[1]	ST[1]	ST[1]
RNA Type(s) depleted from library	2, 3, 4	1, 3, 4	None	1, 4	1, 2	1, 2, 3	3, 4	1, 2, 4
RNA Type(s) enriched in library	1	2	1, 2, 3, 4 (All Types)	2, 3 (5'-OH Types)	3, 4 (3'-P Types)	4	1, 2 (3'-OH Types)	3

Supplementary TABLE S2. Specifications of human plasma samples used in this study. The plasma samples from 10 different individual donors were grouped into four (A, B, C and D) cohorts based on the plasma source vendor and known donor's pathology.

Plasma Sample	Vendor, Sample ID	Donor			
		Gender	Ethnicity	Age	Pathology Metadata
A1	Innovative Research, HMN756159	Male	Hispanic	41	None
A2	Innovative Research, HMN756160	Male	Hispanic	42	None
A3	Innovative Research, HMN756161	Male	Hispanic	29	None
B1	Innovative Research, 200944-PL-2	Female	Caucasian	58	Invasive carcinoma, Stage: IIA
B2	Innovative Research, 200969-PL-5	Female	Caucasian	66	Invasive carcinoma, Stage: II
B3	Innovative Research, 200979-PL-5	Female	Caucasian	53	Invasive carcinoma, Stage: IIA
C1	Innovative Research, IPLASNAE10ML-23 96153A	Female	Hispanic	40	None
D1	Cureline, CU170517-116-248BTB-3EVIIh BrCa P	Female	Caucasian	27	Infiltrating ductal carcinoma, Stage: IIIC
D2	Cureline, CU170517-116-652BTB-7CXh BrCa P	Female	Caucasian	35	Infiltrating ductal carcinoma, Stage: III
D3	Cureline, CU170517-116-312BTB-3LIh BrCa P	Female	Caucasian	38	Infiltrating ductal carcinoma, Stage: IIIA

Supplementary TABLE S3. Average percentage of RNA Types for stsRNA sequences of all lengths spiked into brain and plasma (A and B cohorts) RNA samples, in triplicate, for libraries prepared by protocol T[1+2]. Mock control (without input of naturally occurring RNA) libraries were prepared in duplicate.

RNA sample	Mock Control	Brain	Plasma (A1, A2, A3)	Plasma (B1, B2, B3)
stsRNA Type				
1	47.01 ± 0.48	53.64 ± 0.36	42.40 ± 0.30	43.24 ± 0.59
2	52.32 ± 0.56	45.97 ± 0.33	57.72 ± 0.42	56.60 ± 0.38
3	0.28 ± 0.03	0.14 ± 0.02	0.06 ± 0.01	0.07 ± 0.01
4	0.44 ± 0.04	0.25 ± 0.05	0.06 ± 0.01	0.09 ± 0.01

Supplementary TABLE S4. Average percentage of RNA Types for stsRNA sequences of all lengths spiked into brain and plasma (A and B cohorts) RNA samples, in triplicate, for libraries prepared by protocol T[2] under the indicated ligation reaction conditions. Mock control (without input of naturally occurring RNA) libraries were prepared in duplicate.

Reaction conditions	Rnl1_ 1h	2xRnl1_ 1h	2xRnl1_ 2h	Rnl1+Rnl2_ 1h	Rnl1+Rnl2_ 1h	Rnl1+Rnl2_ 1h	Rnl1+Rnl2_ 1h
RNA sample	Brain	Brain	Brain	Brain	Plasma (A1, A2, A3)	Plasma (B1, B2, B3)	Mock Control
stsRNA Type							
1	7.99 ± 0.11	8.89 ± 0.02	6.52 ± 0.21	8.31 ± 0.16	6.30 ± 0.51	5.71 ± 0.21	6.67 ± 0.23
2	90.59 ± 0.28	89.71 ± 0.02	83.80 ± 0.32	90.91 ± 0.19	93.50 ± 0.51	93.90 ± 0.24	91.53 ± 0.23
3	0.89 ± 0.09	1.07 ± 0.00	8.25 ± 0.03	0.56 ± 0.02	0.14 ± 0.01	0.29 ± 0.19	0.97 ± 0.03
4	0.33 ± 0.02	0.33 ± 0.00	1.43 ± 0.08	0.21 ± 0.00	0.07 ± 0.01	0.12 ± 0.04	0.82 ± 0.03

Supplementary TABLE S5. Average percentage of stsRNA Types of all lengths for mock control (in the absence of naturally occurring RNAs) libraries prepared by indicated RiboMarker[®] protocols in duplicate.

Protocol	T[1+2]	T[2]	T[1+2+3+4]	T[3+4]	T[3]	T[4]
stsRNA Type						
1	47.01 ± 0.48	6.67 ± 0.23	27.63 ± 0.68	8.56 ± 0.32	10.31 ± 0.24	9.00 ± 0.50
2	52.32 ± 0.56	91.53 ± 0.23	27.14 ± 0.45	6.90 ± 0.26	20.01 ± 0.85	7.08 ± 0.13
3	0.28 ± 0.03	0.97 ± 0.03	17.76 ± 0.25	32.63 ± 1.41	53.07 ± 1.61	27.94 ± 0.73
4	0.4 ± 0.04	0.82 ± 0.03	27.47 ± 0.89	51.91 ± 2.00	16.61 ± 2.22	55.98 ± 1.10

Supplementary TABLE S6. Average percentage of RNA Types for stsRNA sequences of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[1+2].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	56.39 ± 0.48	41.74 ± 0.30	31.31 ± 0.98	40.0 2± 0.22	50.96 ± 1.13	42.16 ± 0.48
2	43.54 ± 0.48	58.12 ± 0.29	68.58 ± 0.98	59.80 ± 0.21	48.83 ± 1.18	57.72 ± 0.48
3	0.02 ± 0.01	0.08 ± 0.01	0.04 ± 0.01	0.10 ± 0.01	0.09 ± 0.03	0.06 ± 0.00
4	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.12 ± 0.02	0.07 ± 0.00

Supplementary TABLE S7. Average percentage of RNA Types for stsRNAs of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[2].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	11.36 ± 1.38	6.97 ± 0.42	3.43 ± 0.23	4.49 ± 0.44	13.24 ± 0.76	6.30 ± 0.51
2	88.48 ± 1.38	92.78 ± 0.43	96.42 ± 0.22	95.21 ± 0.44	86.39 ± 1.00	93.50 ± 0.51
3	0.10 ± 0.01	0.19 ± 0.01	0.09 ± 0.01	0.18 ± 0.01	0.22 ± 0.06	0.14 ± 0.01
4	0.06 ± 0.02	0.06 ± 0.01	0.6 ± 0.01	0.12 ± 0.02	0.15 ± 0.01	0.07 ± 0.01

Supplementary TABLE S8. Reduction of the average percentage of Type 1 stsRNAs of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[2] in comparison to protocol T[1+2].

stsRNA length	20	30	40	50	60
RNA Type 1 in					
Protocol T[1+2] (from Table S6)	56.39	41.74	31.31	40.02	50.96
Protocol T[2] (from Table S7)	11.36	6.97	3.43	4.49	13.24
Reduction of RNA Type 1	$(56.39 - 11.36)$ $/ 56.39 \times 100\%$ = 79.85	$(41.74 - 6.97)$ $/ 41.74 \times 100\%$ = 83.30	$(31.31 - 3.43)$ $/ 31.31 \times 100\%$ = 89.05	$(40.02 - 4.49)$ $/ 40.02 \times 100\%$ = 87.90	$(50.96 - 13.24)$ $/ 50.96 \times 100\%$ = 74.02

Supplementary TABLE S9A. Reduction of the percentage of miRNA sequencing reads in a brain RNA sample, in triplicate, for libraries prepared by protocol T[2] in comparison to protocol T[1+2].

Replicate	1	2	3	Average
Protocol T[1+2]	20.07	21.70	20.18	20.65 ± 0.70
Protocol T[2]	2.84	1.61	1.53	1.99 ± 0.56
Reduced by fold	7.07	13.48	13.18	11.24 ± 2.80
Reduction by %	$(20.07 - 2.84) / 20.07 \times 100\% = 85.85$	$(21.70 - 1.61) / 21.70 \times 100\% = 92.59$	$(20.18 - 1.53) / 20.18 \times 100\% = 92.41$	90.28 ± 2.96

Supplementary TABLE S9B. Reduction of the percentage of piRNA sequencing reads in a brain RNA sample, in triplicate, for libraries prepared by protocol T[2] in comparison to protocol T[1+2].

Replicate	1	2	3	Average
Protocol T[1+2]	5.17	5.66	5.38	5.40 ± 0.17
Protocol T[2]	4.16	4.09	4.17	4.14 ± 0.03
Reduced by fold	1.24	1.38	1.29	1.30 ± 0.05
Reduction by %	$(5.17 - 4.16) /$ $5.17 \times 100\% =$ 19.54	$(5.66 - 4.09) /$ $5.66 \times 100\% =$ 27.73	$(5.38 - 4.17) /$ $5.38 \times 100\% =$ 22.50	23.26 ± 2.98

Supplementary TABLE S9C. Reduction of the percentage of miRNA reads in the A cohort plasma RNA samples, in triplicate, for libraries prepared by protocol T[2] in comparison to protocol T[1+2].

Sample	A1	A2	A3	Average
Protocol T[1+2]	8.53	7.34	4.54	6.80 ± 1.51
Protocol T[2]	0.41	0.19	0.20	0.27 ± 0.06
Reduced by fold	20.80	38.63	22.70	27.38 ± 7.50
Reduction by %	$(8.53 - 0.41) /$ $8.53 \times 100\% =$ 95.19	$(7.34 - 0.19) /$ $7.34 \times 100\% =$ 97.41	$(4.54 - 0.20) /$ $4.54 \times 100\% =$ 95.59	96.10 ± 0.88

Supplementary TABLE S9D. Reduction of the percentage of piRNA reads in the A cohort plasma RNA samples, in triplicate, for libraries prepared by protocol T[2] in comparison to protocol T[1+2].

Sample	A1	A2	A3	Average
Protocol T[1+2]	4.52	4.14	9.05	5.90 ± 2.10
Protocol T[2]	4.33	3.47	7.15	4.98 ± 1.44
Reduced by fold	1.04	1.19	1.27	1.17 ± 0.05
Reduction by %	$(4.52 - 4.33) /$ $4.52 \times 100\% =$ 4.20	$(4.14 - 3.47) /$ $4.14 \times 100\% =$ 16.18	$(9.05 - 7.15) /$ $9.05 \times 100\% =$ 21.00	13.79 ± 6.39

Supplementary TABLE S10. Average percentage of RNA Types for stsRNAs of all lengths spiked into either a brain RNA sample or plasma (A, B or C cohorts) RNA samples, in triplicate, for libraries prepared by different versions of protocol T[1+2+3+4] using various PNK pretreatment conditions.

Buffer	Tris-HCl pH 7.6 (+ATP)	Imidazole- HCl pH 6.5	MES-NaOH pH 6.0	MES-NaOH pH 6.0	MES-NaOH pH 6.0	MES-NaOH pH 6.0	MES-NaOH pH 6.0	MES-NaOH pH 6.0
PNK Units	10	10	10	10	10	10	10	20
PNK discharge	Column clean	Column clean	Column clean	Column clean	Column clean	Heat inactivation	Column clean	Column clean
RNA sample	Brain	Brain	Brain	Plasma (A1, A2, A3)	Plasma (B1, B2, B3)	Plasma (C1)	Plasma (C1)	Plasma (C1)
stsRNA Types								
1	29.88 ± 0.28	30.68 ± 0.23	30.69 ± 0.50	28.37 ± 0.24	28.40 ± 0.49	33.07 ± 0.53	27.10 ± 0.28	27.21 ± 0.04
2	32.27 ± 0.17	32.10 ± 0.23	31.93 ± 0.73	31.81 ± 0.34	31.54 ± 0.67	25.48 ± 0.12	31.61 ± 0.17	31.37 ± 0.31
3	15.98 ± 0.17	14.72 ± 0.23	14.76 ± 0.35	18.72 ± 0.19	18.74 ± 0.58	13.23 ± 0.51	19.29 ± 0.03	18.61 ± 0.14
4	21.88 ± 0.09	22.50 ± 0.06	22.62 ± 0.09	21.09 ± 0.29	21.31 ± 0.39	28.21 ± 0.08	22.00 ± 0.18	22.81 ± 0.21

Supplementary TABLE S11. Average percentage of RNA Types for stsRNAs of different lengths spiked into the D cohort (D1, D2, D3) plasma RNA samples, in triplicate, for libraries prepared by RiboMarker® protocol T[1+2+3+4].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	36.56 ± 3.66	25.57 ± 1.94	23.76 ± 1.18	19.79 ± 0.82	31.54 ± 3.07	26.56 ± 1.86
2	27.21 ± 1.78	30.01 ± 2.07	55.90 ± 4.90	29.21 ± 0.60	34.89 ± 1.45	35.15 ± 1.51
3	9.55 ± 2.03	20.24 ± 2.15	4.84 ± 2.09	27.06 ± 1.43	16.37 ± 2.17	15.80 ± 1.75
4	26.69 ± 3.23	24.18 ± 1.86	15.50 ± 2.85	23.94 ± 0.53	17.20 ± 0.79	22.49 ± 1.52

Supplementary TABLE S12. Average percentage of RNA Types for stsRNAs of different lengths spiked into the D cohort (D1, D2, D3) plasma RNA samples, in triplicate, for libraries prepared by Phospho-RNA-Seq.

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	58.18 ± 1.98	19.69 ± 0.87	32.59 ± 0.70	17.32 ± 0.62	45.43 ± 1.02	29.84 ± 0.44
2	11.27 ± 0.34	28.69 ± 1.49	17.35 ± 0.53	37.48 ± 1.76	28.35 ± 0.61	23.35 ± 0.56
3	10.34 ± 1.23	24.26 ± 0.39	17.29 ± 0.25	22.80 ± 1.20	14.76 ± 0.67	19.02 ± 0.32
4	20.21 ± 0.95	27.36 ± 0.86	32.77 ± 0.72	22.39 ± 0.34	11.46 ± 0.35	27.79 ± 0.49

Supplementary TABLE S13A. Average percentage of RNA Types for stsRNAs of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[3+4].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	9.12 ± 0.05	5.40 ± 0.29	3.88 ± 0.26	4.42 ± 0.24	28.48 ± 11.03	7.00 ± 0.79
2	2.37 ± 0.09	2.63 ± 0.22	38.53 ± 0.68	2.67 ± 0.35	2.67 ± 0.35	3.81 ± 0.16
3	32.72 ± 1.00	45.68 ± 0.32	38.53 ± 0.68	45.28 ± 0.90	25.58 ± 1.90	39.80 ± 0.17
4	55.79 ± 1.06	46.29 ± 0.29	50.61 ± 0.96	47.63 ± 1.39	40.87 ± 7.82	49.39 ± 0.94

Supplementary TABLE S13B. Average percentage of RNA Types for stsRNAs of different lengths spiked into the B cohort (B1, B2, B3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[3+4].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	9.55 ± 1.07	6.24 ± 1.03	4.23 ± 0.72	3.62 ± 0.48	12.52 ± 2.07	6.15 ± 0.70
2	2.37 ± 0.09	2.63 ± 0.22	6.98 ± 0.14	2.67 ± 0.35	2.67 ± 0.35	3.81 ± 0.16
3	31.17 ± 0.90	44.55 ± 1.69	39.77 ± 1.06	45.45 ± 1.46	30.11 ± 2.07	39.92 ± 0.81
4	56.26 ± 1.17	46.52 ± 0.63	48.75 ± 2.80	48.33 ± 2.27	52.45 ± 2.09	49.90 ± 0.51

Supplementary TABLE S14A. Average percentage of RNA Types for stsRNAs of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[3].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	3.49 ± 0.43	2.94 ± 0.47	1.78 ± 0.65	1.79 ± 0.49	3.80 ± 1.75	2.67 ± 0.24
2	5.81 ± 1.11	8.46 ± 1.56	19.01 ± 0.82	5.89 ± 0.89	8.31 ± 1.59	10.11 ± 1.06
3	81.00 ± 1.07	83.37 ± 2.32	67.92 ± 1.61	75.09 ± 2.58	74.21 ± 3.04	76.96 ± 1.68
4	9.70 ± 0.66	5.23 ± 0.35	11.28 ± 0.50	17.24 ± 1.53	13.68 ± 1.32	10.26 ± 0.61

Supplementary TABLE S14B. Average percentage of RNA Types for stsRNAs of different lengths spiked into the B cohort (B1, B2, B3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[3].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	4.43 ± 0.25	3.00 ± 0.51	2.79 ± 1.36	1.52 ± 0.42	5.11 ± 3.3	3.22 ± 0.73
2	7.10 ± 1.45	6.75 ± 1.01	15.46 ± 1.56	6.00 ± 1.37	9.87 ± 2.35	9.24 ± 0.63
3	74.86 ± 4.38	84.49 ± 0.54	67.54 ± 0.68	74.57 ± 1.75	69.35 ± 2.8	75.09 ± 1.42
4	13.61 ± 2.92	5.77 ± 0.08	14.20 ± 0.96	17.90 ± 0.04	15.67 ± 2.51	12.46 ± 1.26

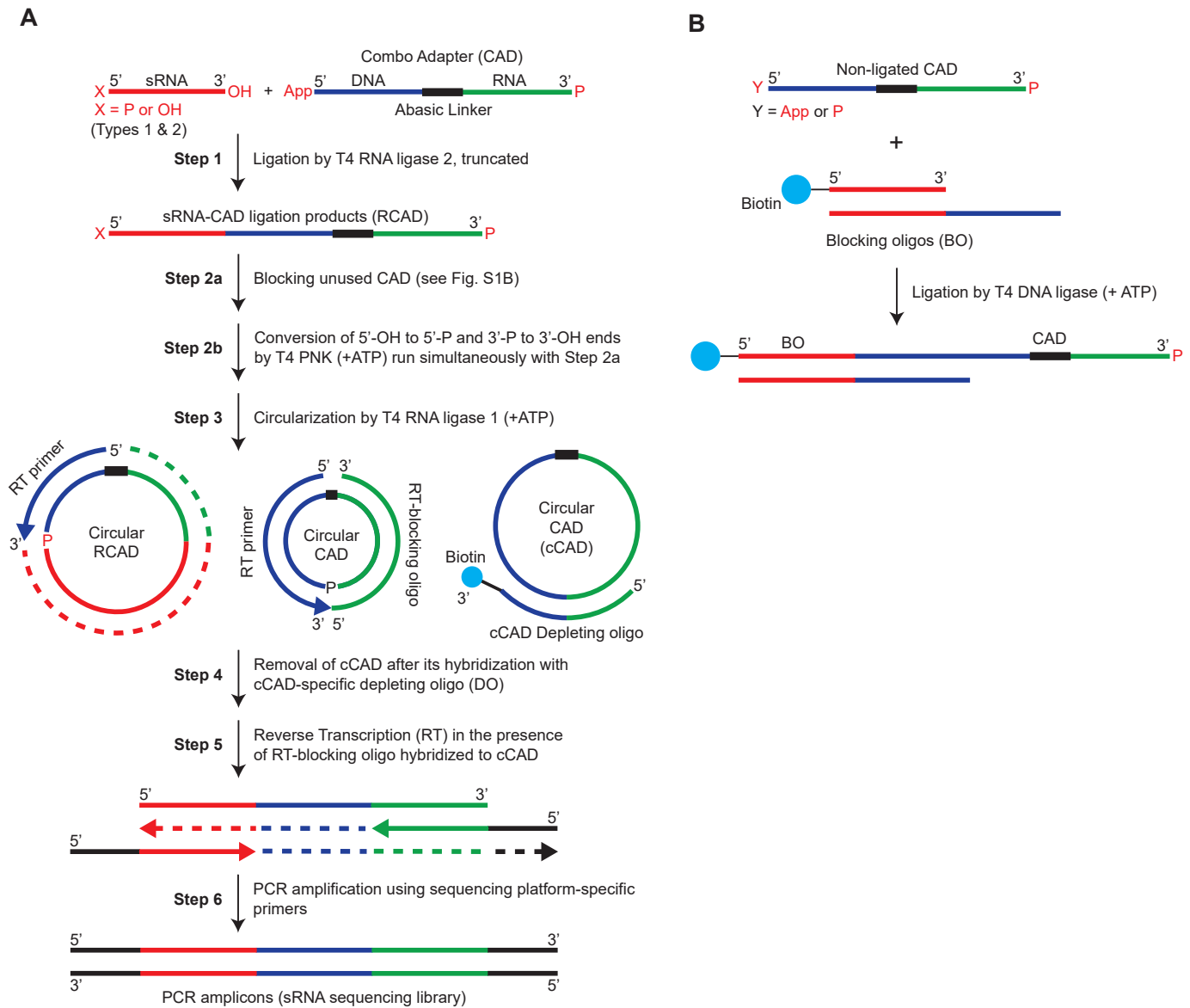
Supplementary TABLE S15A. Average percentage of RNA Types for stsRNAs of different lengths spiked into the A cohort (A1, A2, A3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[4].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	12.34 ± 0.46	7.05 ± 0.22	4.74 ± 0.15	4.62 ± 0.12	42.08 ± 8.61	10.13 ± 1.12
2	1.97 ± 0.22	2.26 ± 0.08	5.21 ± 0.33	2.25 ± 0.33	3.31 ± 0.91	3.00 ± 0.08
3	12.48 ± 1.42	30.70 ± 2.76	31.68 ± 2.19	39.01 ± 0.73	18.65 ± 3.23	27.89 ± 2.06
4	73.21 ± 1.36	59.99 ± 2.54	58.36 ± 2.40	54.12 ± 0.90	35.96 ± 4.48	58.98 ± 1.01

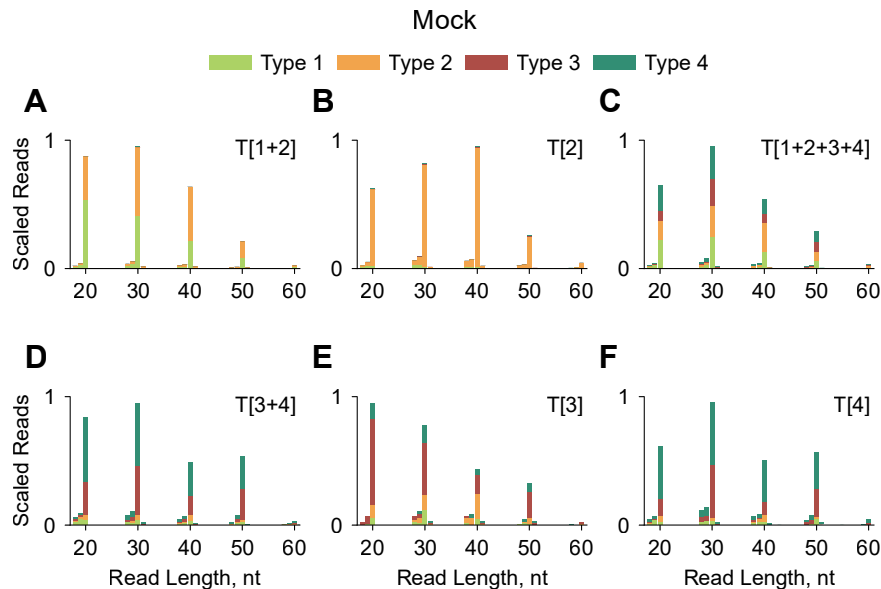
Supplementary TABLE S15B. Average percentage of RNA Types for stsRNAs of different lengths spiked into the B cohort (B1, B2, B3) plasma RNA samples, in triplicate, for libraries prepared by protocol T[4].

stsRNA length	20	30	40	50	60	All lengths
stsRNA Type						
1	12.52 ± 0.40	6.71 ± 0.25	5.39 ± 0.58	4.89 ± 0.66	12.63 ± 1.91	7.49 ± 0.63
2	2.28 ± 0.73	2.79 ± 0.44	6.02 ± 0.69	3.10 ± 0.16	4.48 ± 0.34	3.69 ± 0.42
3	12.57 ± 2.02	29.54 ± 4.51	29.27 ± 5.22	35.66 ± 5.78	22.20 ± 3.01	26.88 ± 4.60
4	72.64 ± 2.32	60.96 ± 4.62	59.32 ± 5.32	56.34 ± 5.19	60.69 ± 1.69	61.95 ± 4.40

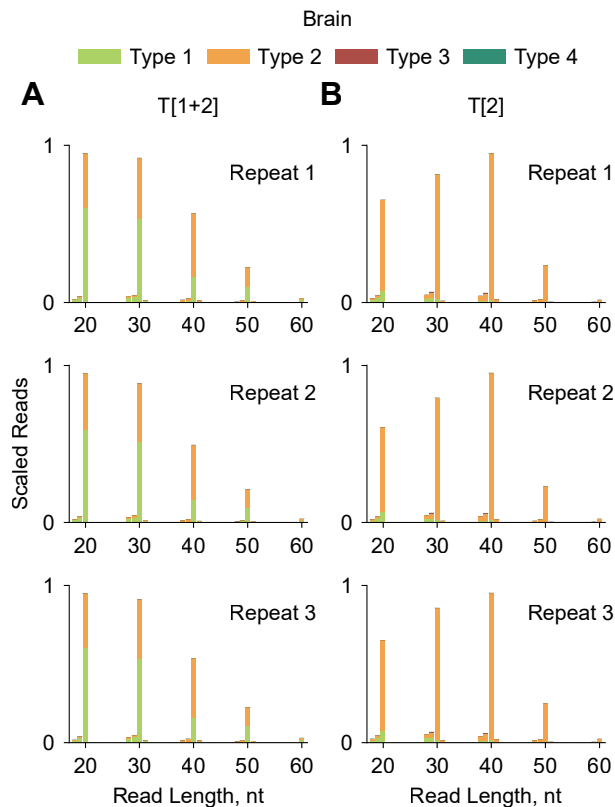
SUPPLEMENTARY FIGURES



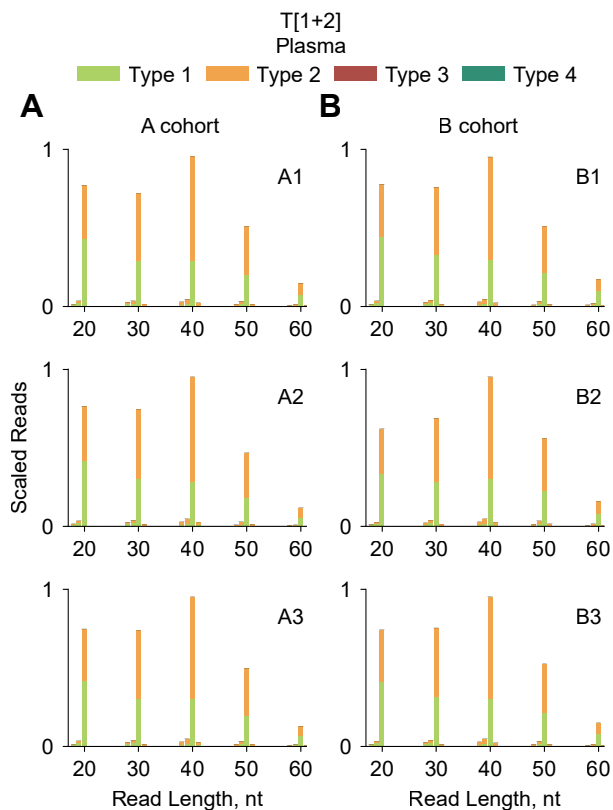
Supplementary FIGURE S1. Schematics of RiboMarker® protocol T[1+2] for sRNA sequencing library preparation. **(A)** General workflow including the ligation of a single combo adapter (CAD) to sRNA, circularization of the ligation products (RCAD), reverse transcription of the circularized RCAD, generating cDNA products, and PCR amplification of these cDNA. **(B)** Blocking of the unligated CAD.



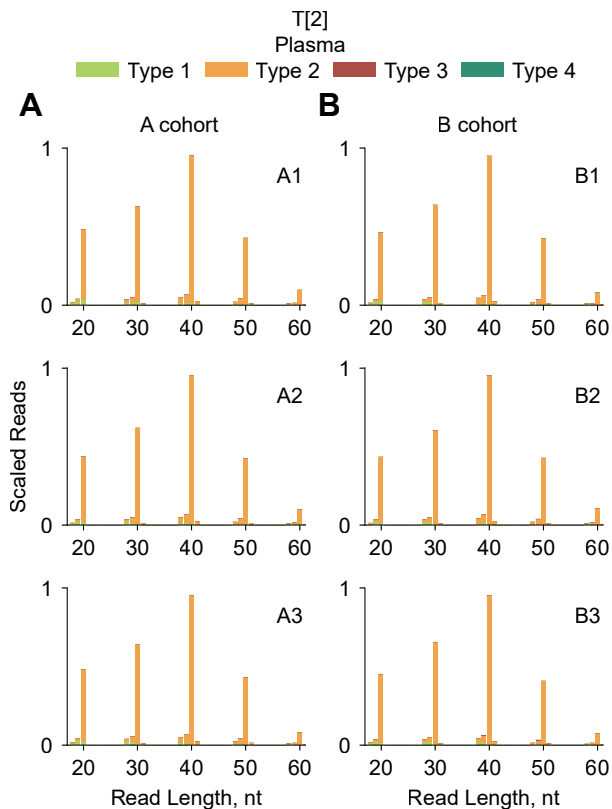
Supplementary FIGURE S2. Summary of sequencing length and RNA Type profiles for stsRNAs in the absence of naturally occurring sRNAs (mock controls) for libraries prepared by all RiboMarker® protocols. Note that these supplementary figures are identical to the following figures presented in different sections of the main article: Fig. S2A to Fig. 2A (left panel); Fig. S2B to Fig. 2A (right panel); Fig. S2C to Fig. 4A; Fig. S2D to Fig. 5A, Fig. S2E to Fig. 5B; and Fig. S2F to Fig. 5C, respectively.



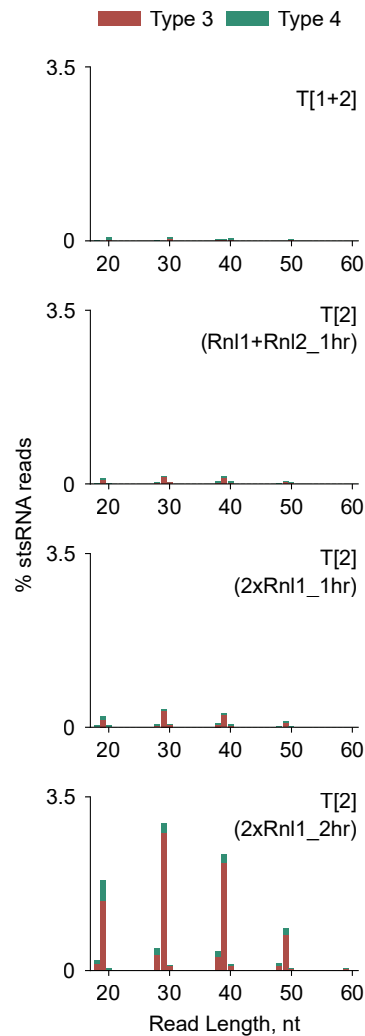
Supplementary FIGURE S3. Sequencing length and RNA Type profiles for stsRNAs spiked into brain RNA samples, in triplicate, for libraries prepared by **(A)** protocol T[1+2] or **(B)** protocol T[2].



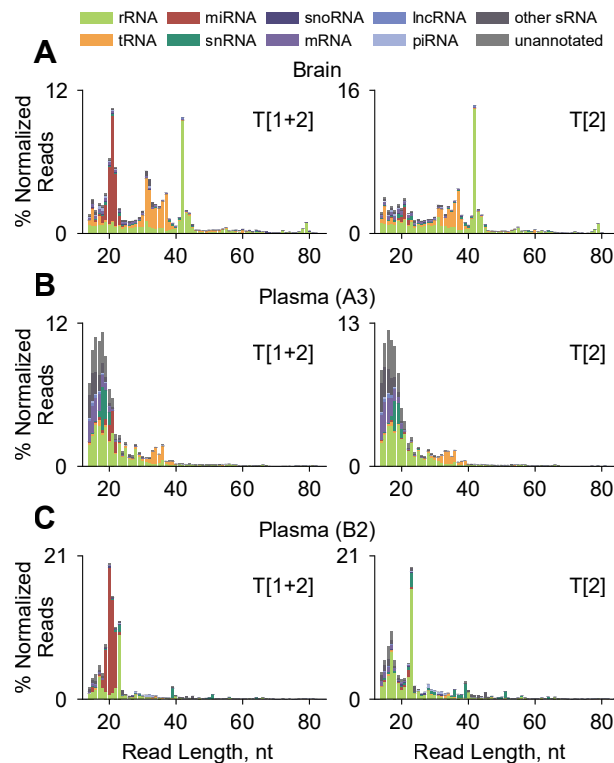
Supplementary FIGURE S4. Sequencing length and RNA Type profiles for stsRNAs spiked into plasma RNA samples of either **(A)** A cohort (A1, A2, A3) or **(B)** B cohort (B1, B2, B3) for libraries prepared by protocol T[1+2].



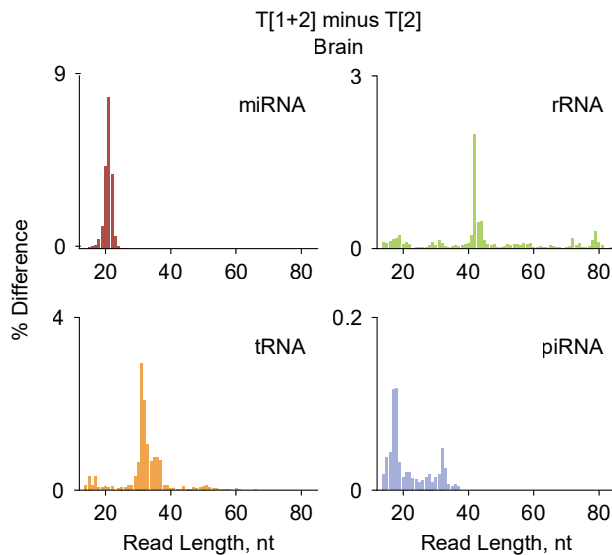
Supplementary FIGURE S5. Sequencing length and RNA Type profiles for stsrNAs spiked into plasma RNA samples of either **(A)** A cohort A (A1, A2, A3) or **(B)** B cohort (B1, B2, B3) for libraries prepared by protocol T[2].



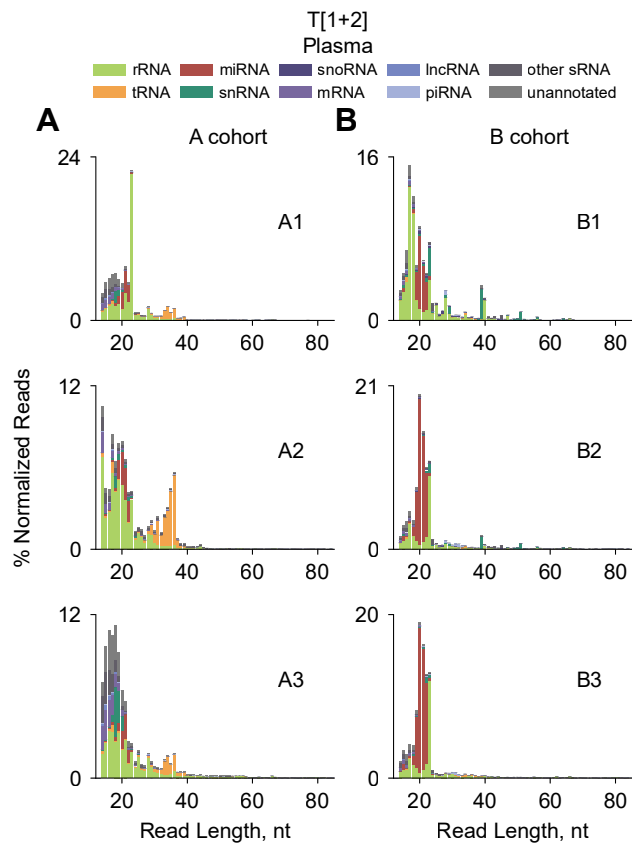
Supplementary FIGURE S6. Sequencing length and RNA Type profiles for stsRNAs spiked into brain RNA samples for libraries prepared by protocol T[1+2] and different versions of protocol T[2] performed under the indicated reaction conditions with T4 RNA ligase(s), including RNA ligase 1 (Rnl1) and RNA ligase 2 (Rnl2). The percentages for normalized stsRNA Type 3 and Type 4 reads are indicative of the efficiency of Rnl1 “reverse reaction” removing a single terminal nucleotide from the 3'-P ends of stsRNA Types 3 and 4 that makes them shorter by 1 nt and converts them to stsRNA Types 2 and 1, respectively, allowing their incorporation into sequencing libraries.



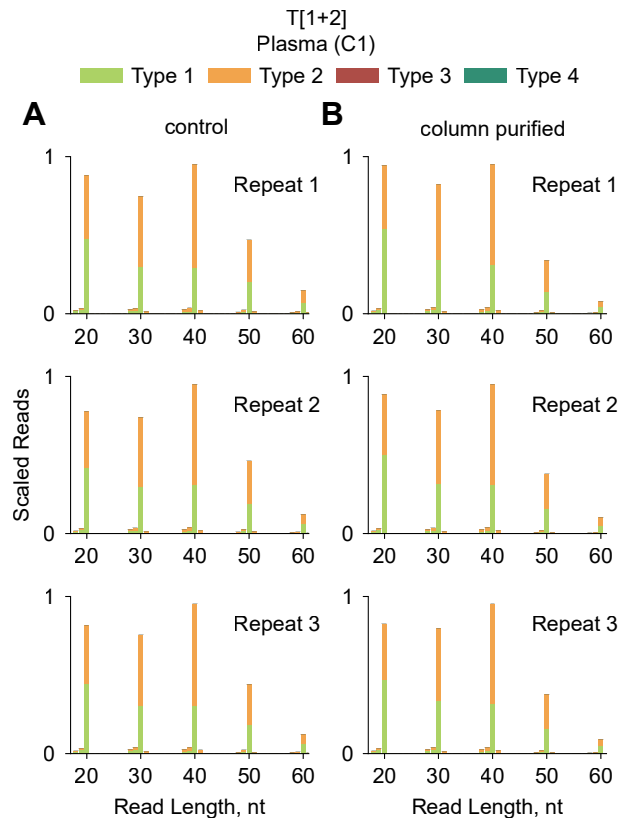
Supplementary FIGURE S7. Sequencing length profiles for indicated RNA classes of naturally occurring sRNAs found in either the **(A)** human brain sample, or **(B–C)** representative human plasma (from A and B cohorts: A3 and B2) RNA samples for libraries prepared by protocols T[1+2] (left panels) and T[2] (right panels).



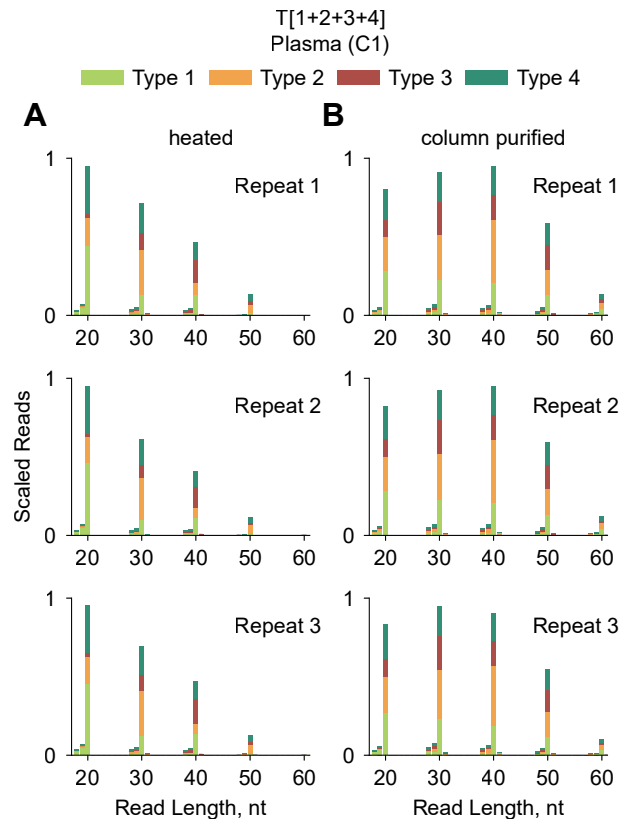
Supplementary FIGURE S8. Differential sequencing length profiles of naturally occurring sRNA Type 1 found in a brain RNA sample that were determined by subtracting percentages of normalized sequencing reads for libraries prepared by protocol T[2] from those prepared by protocol T[1+2]. The differential read length profiles were generated by calculating the proportion ($P_{T,L}$) of a given RNA class (T) (excluding unmapped reads) at a specified read length (L) for libraries prepared using different protocols. These were then subtracted from one another to yield the percent difference as shown below (where condition A is protocol T[1+2] and condition B is protocol T[2]).



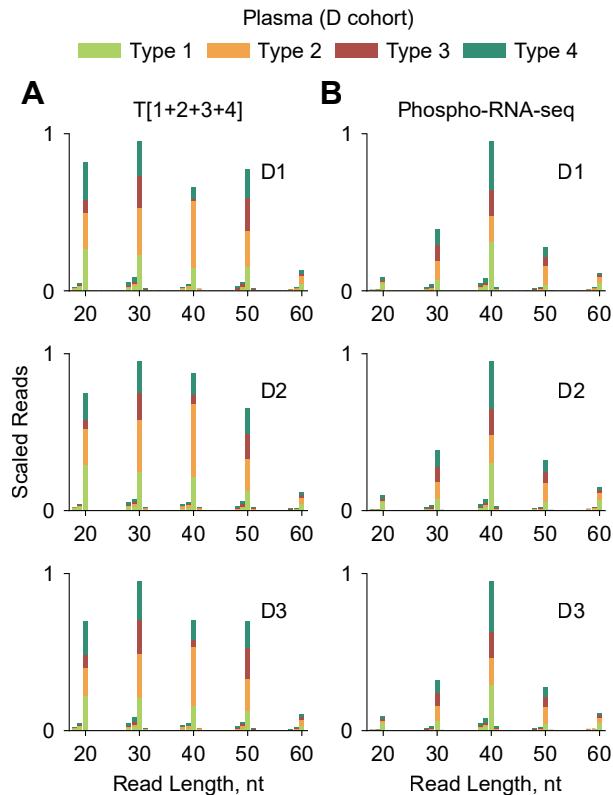
Supplementary FIGURE S9. Sequencing length profiles for indicated RNA classes of naturally occurring sRNAs found in plasma RNA samples of either **(A)** A cohort (A1, A2, A3) or **(B)** B cohort (B1, B2, B3) for libraries prepared by protocol T[1+2].



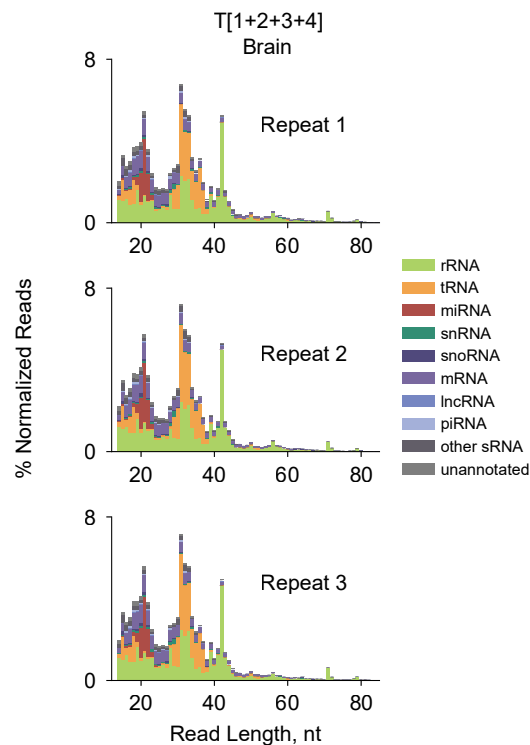
Supplementary FIGURE S10. Sequencing length and RNA Type profiles for stsRNAs spiked into C1 plasma RNA sample, in triplicate, for libraries prepared by protocol T[1+2] either **(A)** directly (control) or **(B)** after purification using the Zymo RNA clean and concentrator kit (column purified).



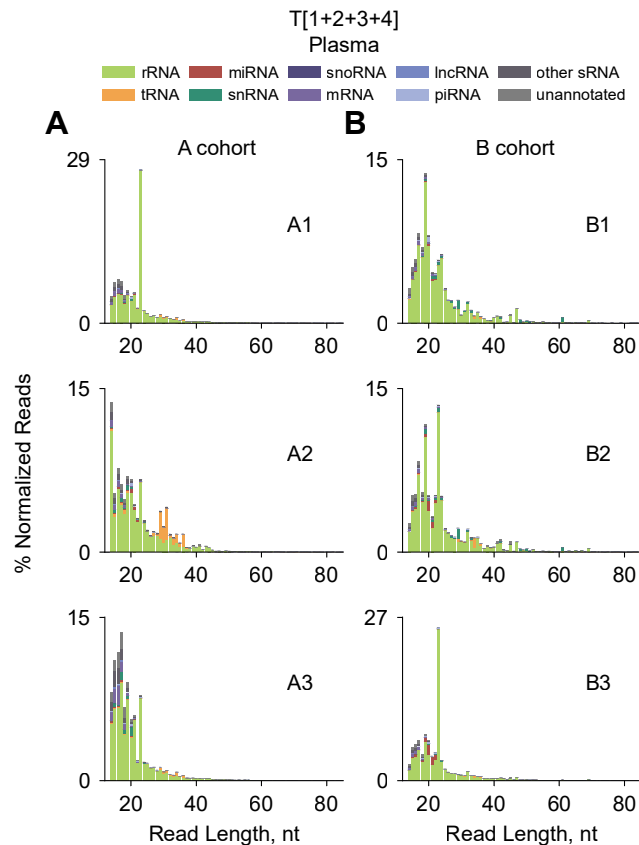
Supplementary FIGURE S11. Sequencing length and RNA Type profiles for stsRNAs spiked into C1 plasma RNA sample, in triplicate, for libraries prepared by protocol T[1+2+3+4] with either **(A)** heat-inactivation of PNK (heated) or **(B)** removal of PNK using the Zymo RNA clean and concentrator kit (column purified).



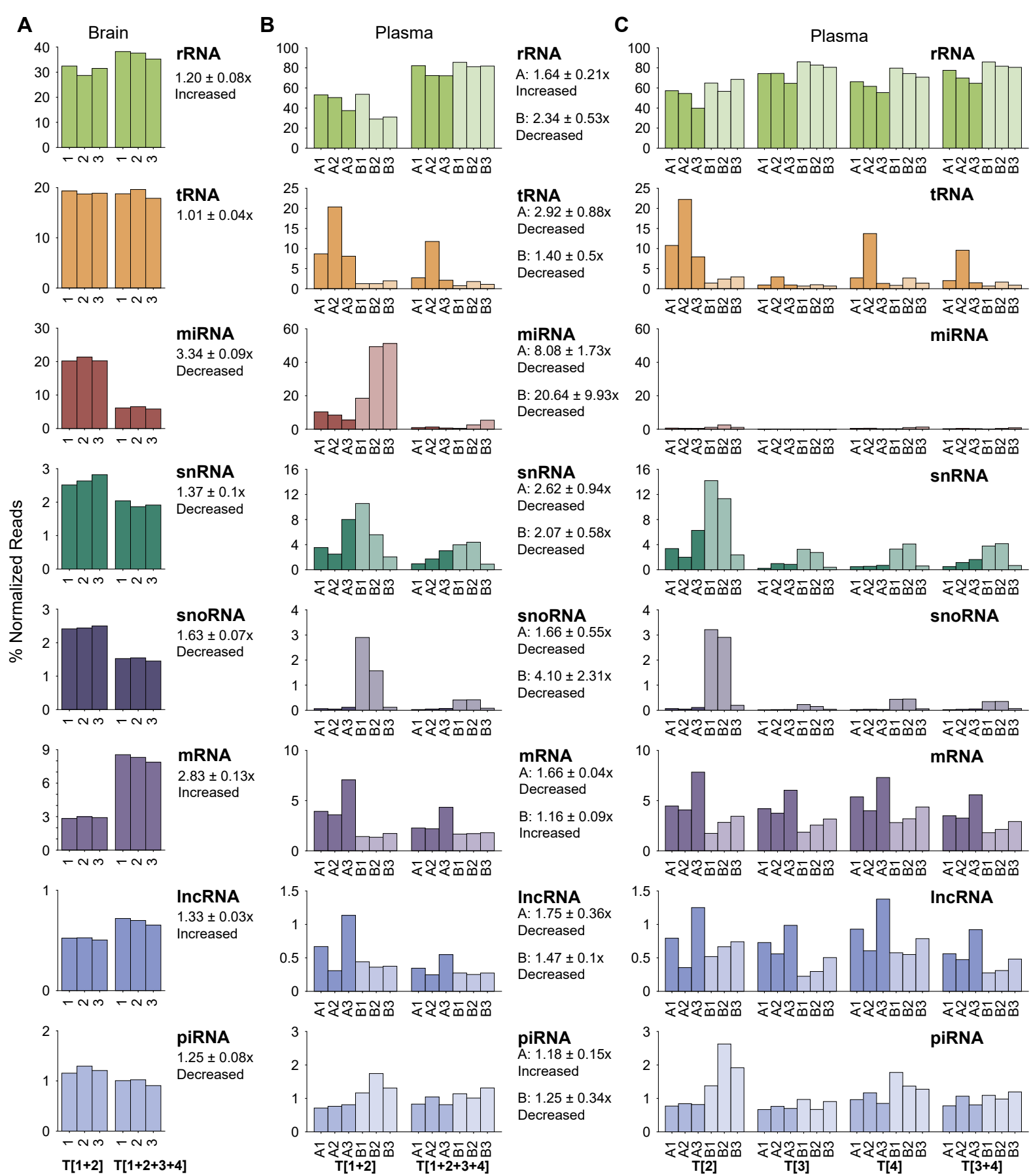
Supplementary FIGURE S12. Sequencing length and RNA Type profiles for stsRNAs spiked into D cohort (D1, D2, D3) plasma RNA samples for libraries prepared by either **(A)** RiboMarker[®] protocol T[1+2+3+4] or **(B)** Phospho-RNA-Seq.



Supplementary FIGURE S13. Sequencing length profiles for indicated RNA classes of naturally occurring sRNAs found in a brain RNA sample, in triplicate, prepared with protocol T[1+2+3+4].

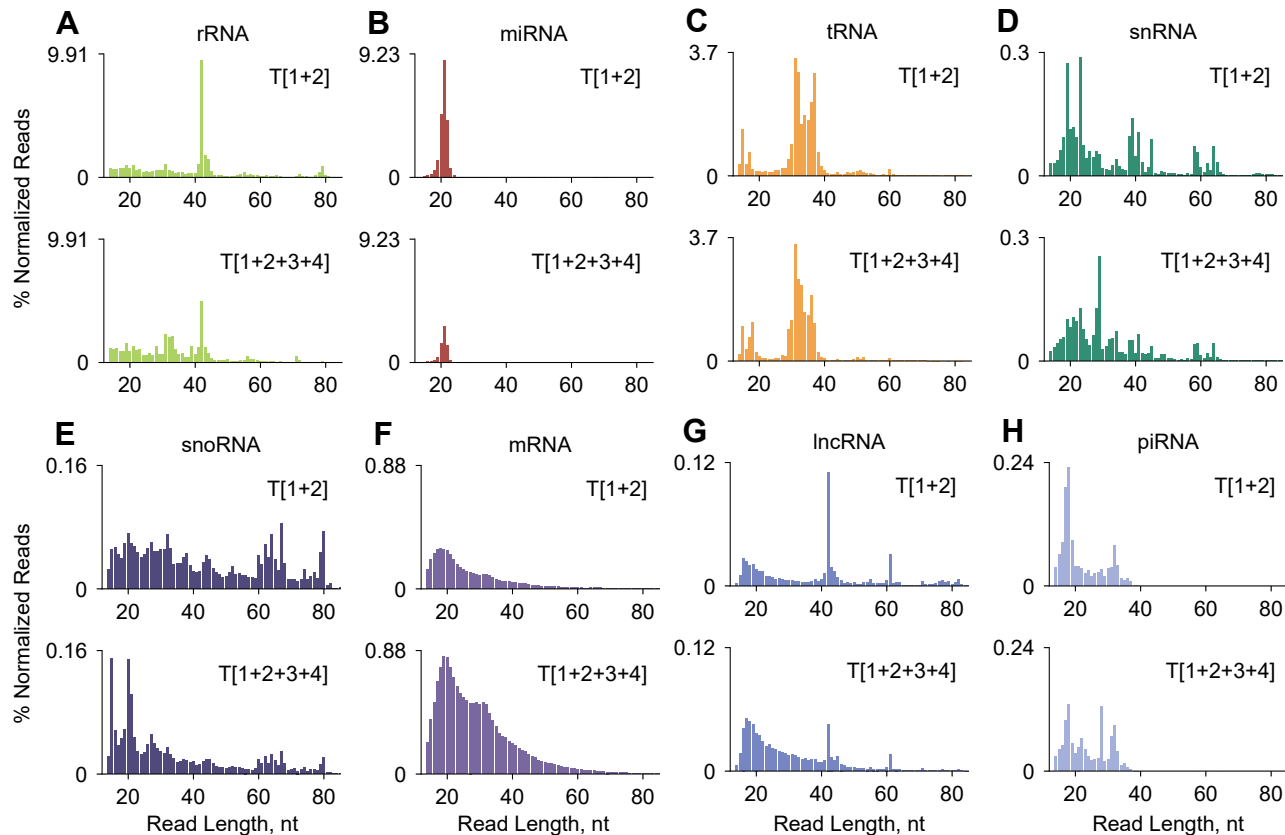


Supplementary FIGURE S14. Sequencing length profiles for indicated RNA classes of naturally occurring sRNAs found in plasma RNA samples either from **(A)** A cohort (A1, A2, A3) or **(B)** B cohort (B1, B2, B3), in triplicate, for libraries prepared by protocol T[1+2+3+4].

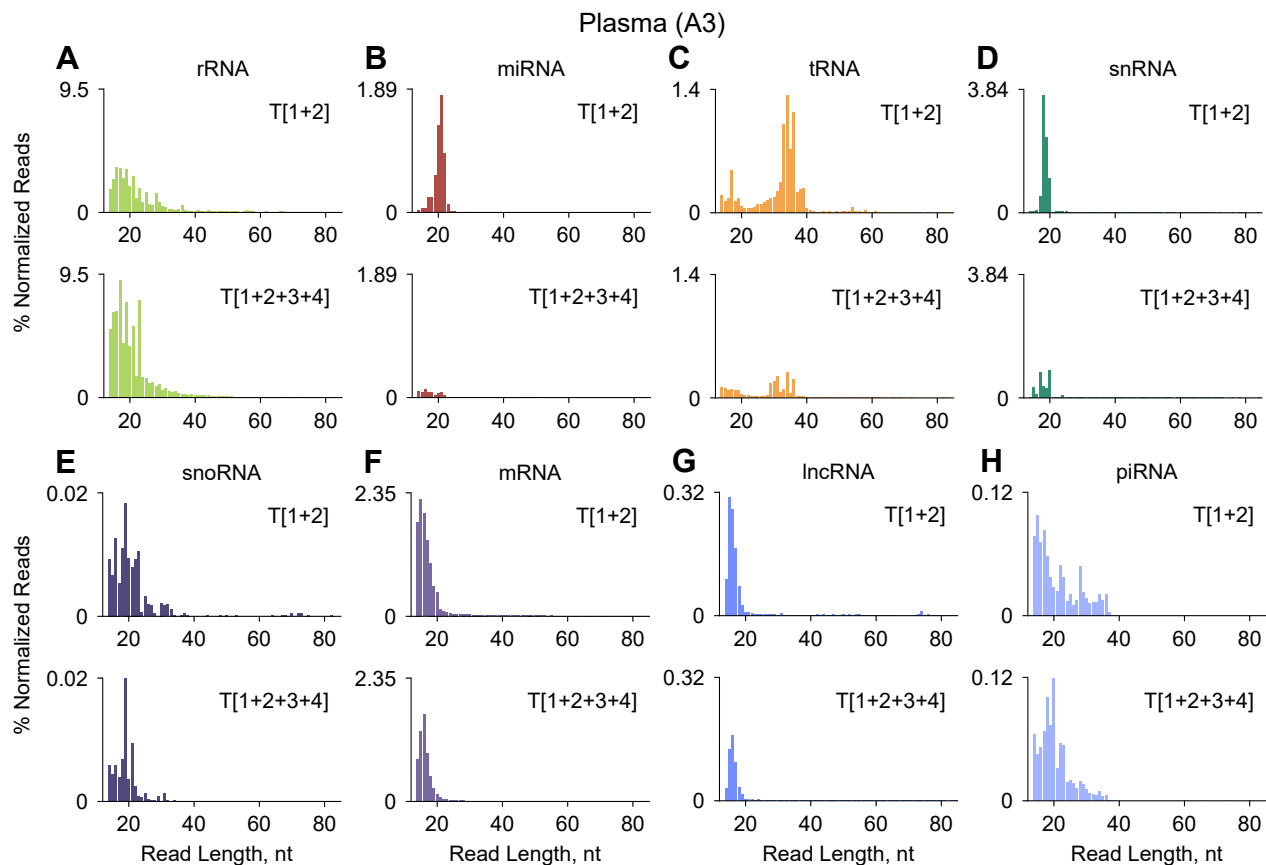


Supplementary FIGURE S15. Percentage of normalized sequencing reads of main RNA classes of naturally occurring sRNAs found in RNA samples from **(A)** brain (1, 2, 3); or **(B–C)** A cohort: (A1, A2, A3) plasma and B cohort (B1, B2, B3) plasma, in triplicate, for libraries prepared by indicated protocols. In panels **A** and **B**, we indicated the fold change for the percentage of sRNAs derived from individual RNA classes between protocols T[1+2] and T[1+2+3+4]. The average fold change for the triplicates was calculated relative to the protocol with the lowest percentage of reads (which varied among different RNA classes). If protocol T[1+2+3+4] yielded a higher relative percentage compared to protocol T[1+2], the fold change is labelled as “increased”. Conversely, if the percentage is lower, the fold change is labeled as “decreased”. In panel **C**, the libraries were prepared by protocols T[2], T[3], T[4] or T[3+4].

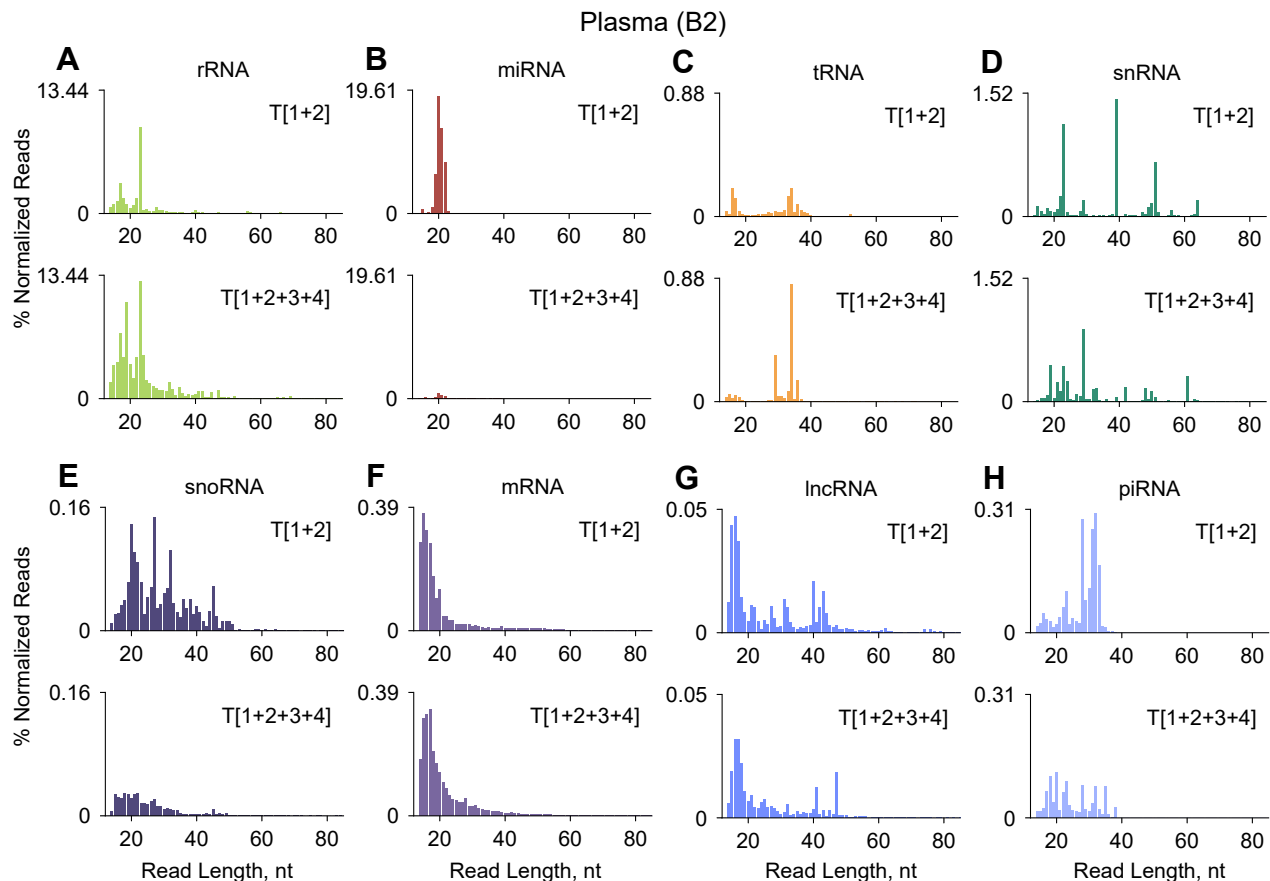
Brain



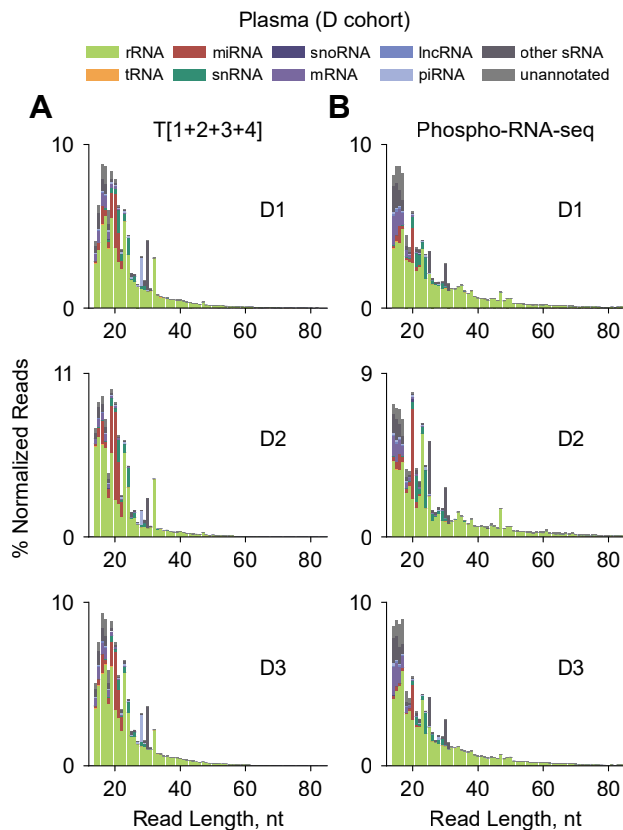
Supplementary FIGURE S16. Comparison of sequencing length profiles for naturally occurring sRNAs found in a brain RNA sample among libraries prepared by protocols T[1+2] and T[1+2+3+4] for the main RNA classes: **(A)** rRNA, **(B)** miRNA, **(C)** tRNA, **(D)** snRNA, **(E)** snoRNA, **(F)** mRNA, **(G)** lncRNA and **(H)** piRNA.



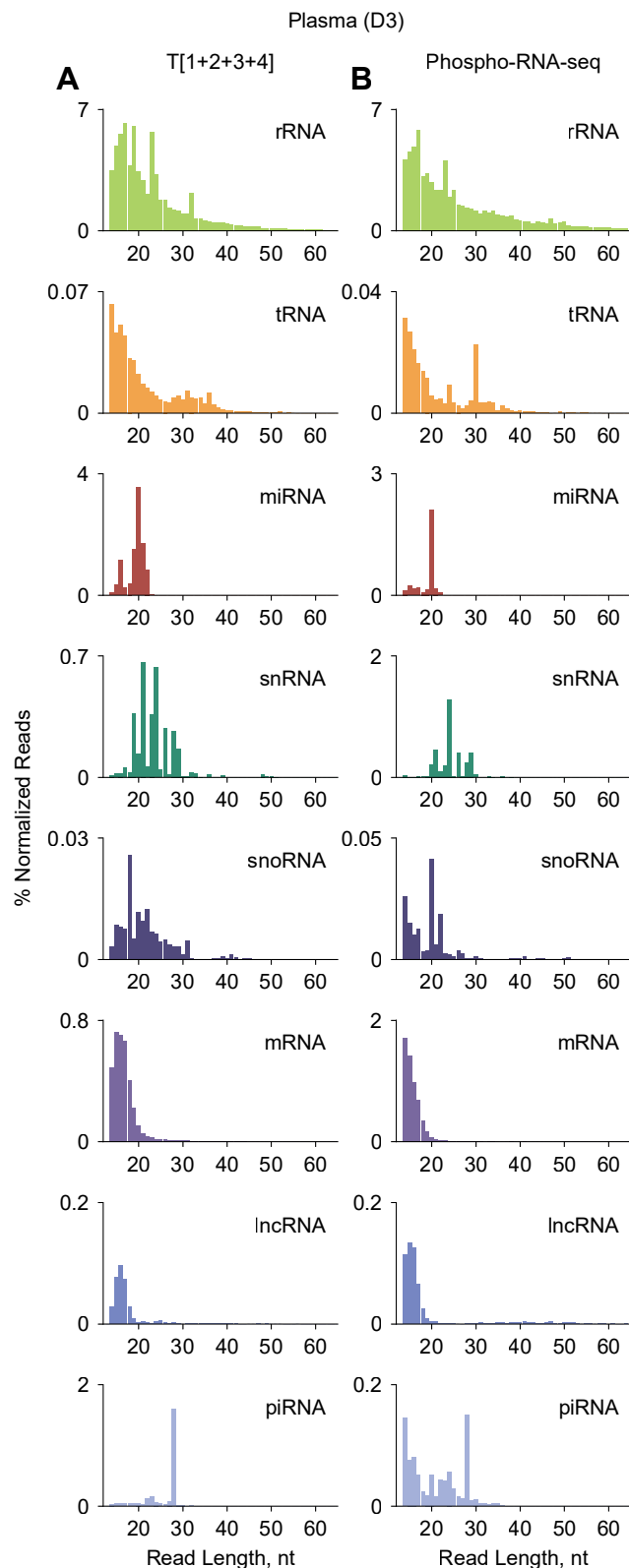
Supplementary FIGURE S17. Comparison of sequencing length profiles for naturally occurring sRNAs found in the representative A cohort plasma (A3) RNA sample between libraries prepared by protocols T[1+2] and T[1+2+3+4] for the following RNA classes: **(A)** rRNA, **(B)** miRNA, **(C)** tRNA, **(D)** snRNA, **(E)** snoRNA, **(F)** mRNA, **(G)** lncRNA and **(H)** piRNA.



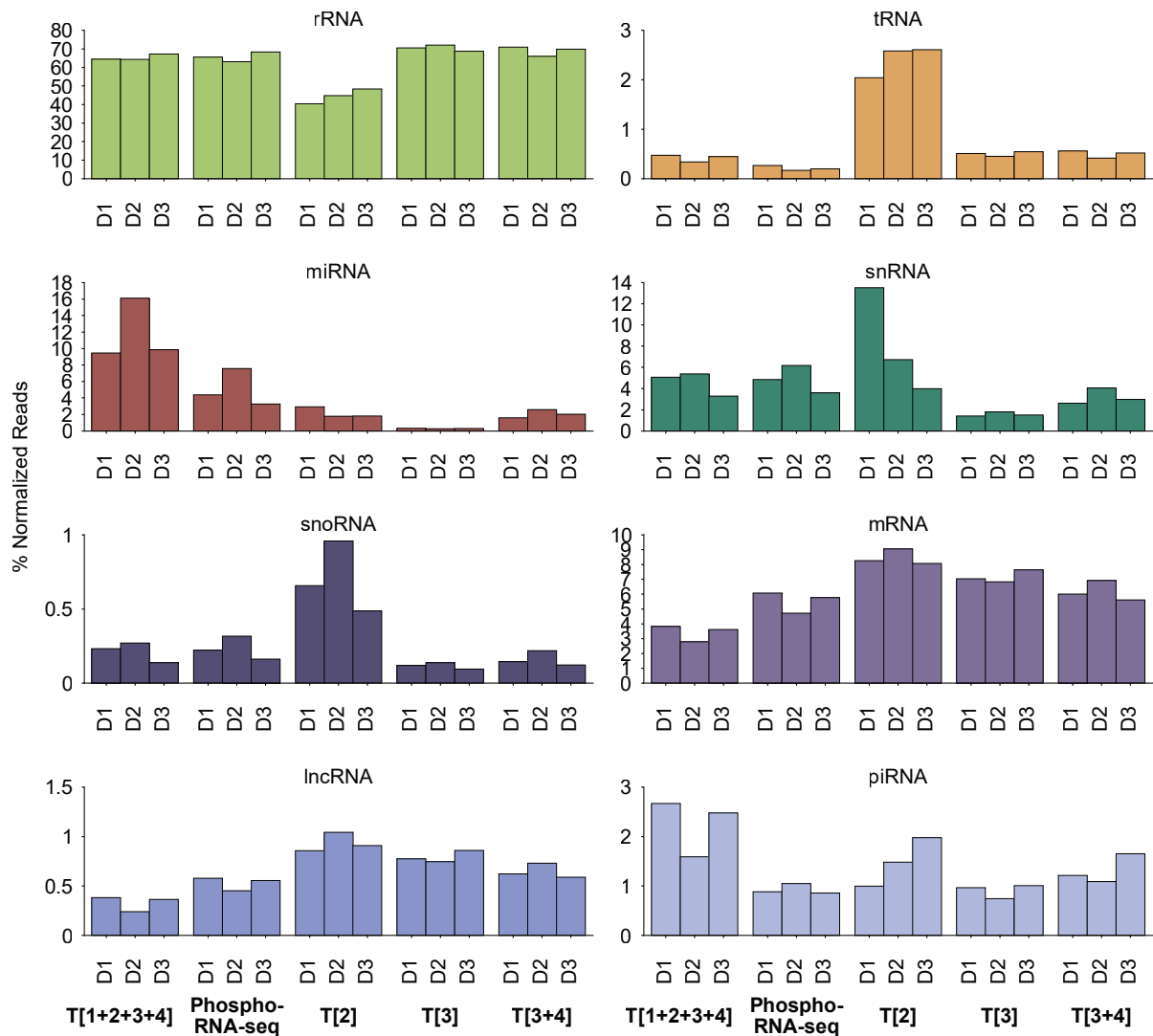
Supplementary FIGURE S18. Comparison of sequencing length profiles for naturally occurring sRNAs found in the representative B cohort plasma (B2) RNA sample between libraries prepared by protocols T[1+2] and T[1+2+3+4] for the following RNA classes: **(A)** rRNA, **(B)** miRNA, **(C)** tRNA, **(D)** snRNA, **(E)** snoRNA, **(F)** mRNA, **(G)** lncRNA and **(H)** piRNA.



Supplementary FIGURE S19. Sequencing length and RNA Type profiles for naturally occurring sRNAs of the indicated RNA classes found in D cohort plasma (D1, D2, D3) RNA samples for libraries prepared by **(A)** RiboMarker® protocol T[1+2+3+4] or **(B)** Phospho-RNA-Seq.

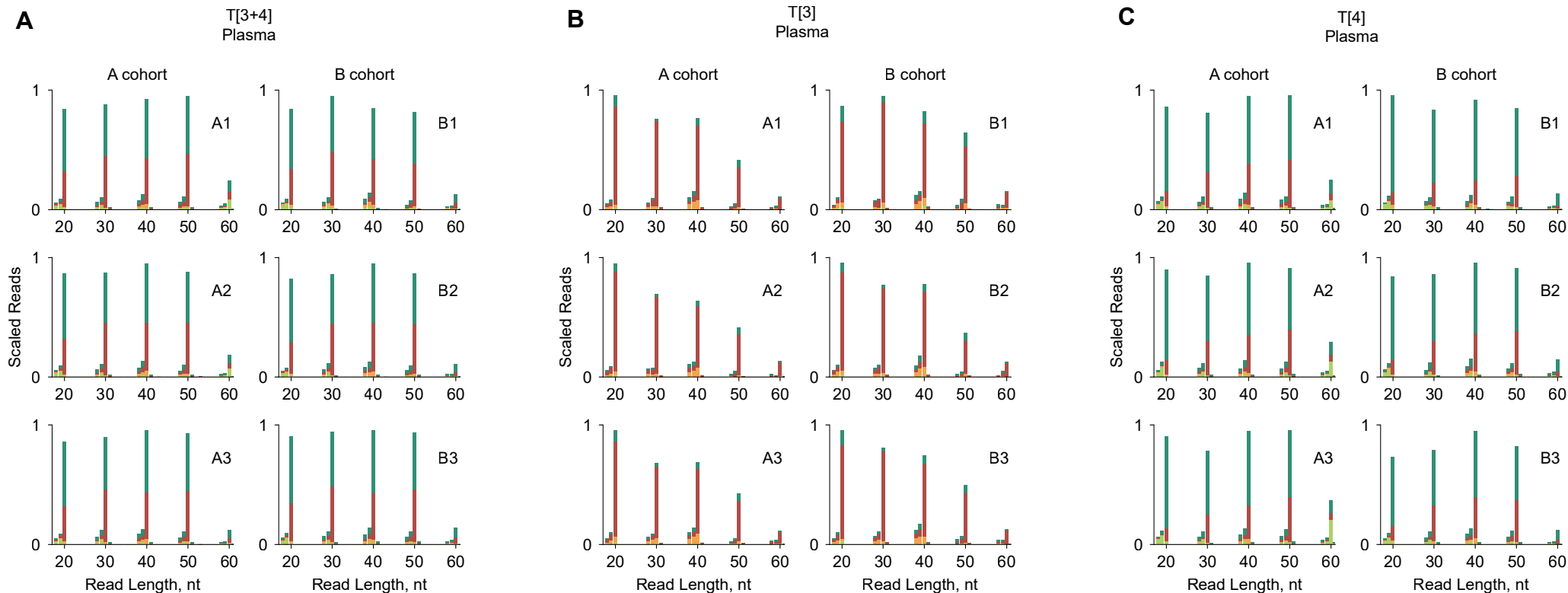


Supplementary FIGURE S20. Comparison of sequencing length profiles of naturally occurring sRNAs of the indicated RNA classes found in the representative D cohort plasma (D3) RNA sample between libraries prepared by **(A)** RiboMarker® protocol T[1+2+3+4] or **(B)** Phospho-RNA-Seq.



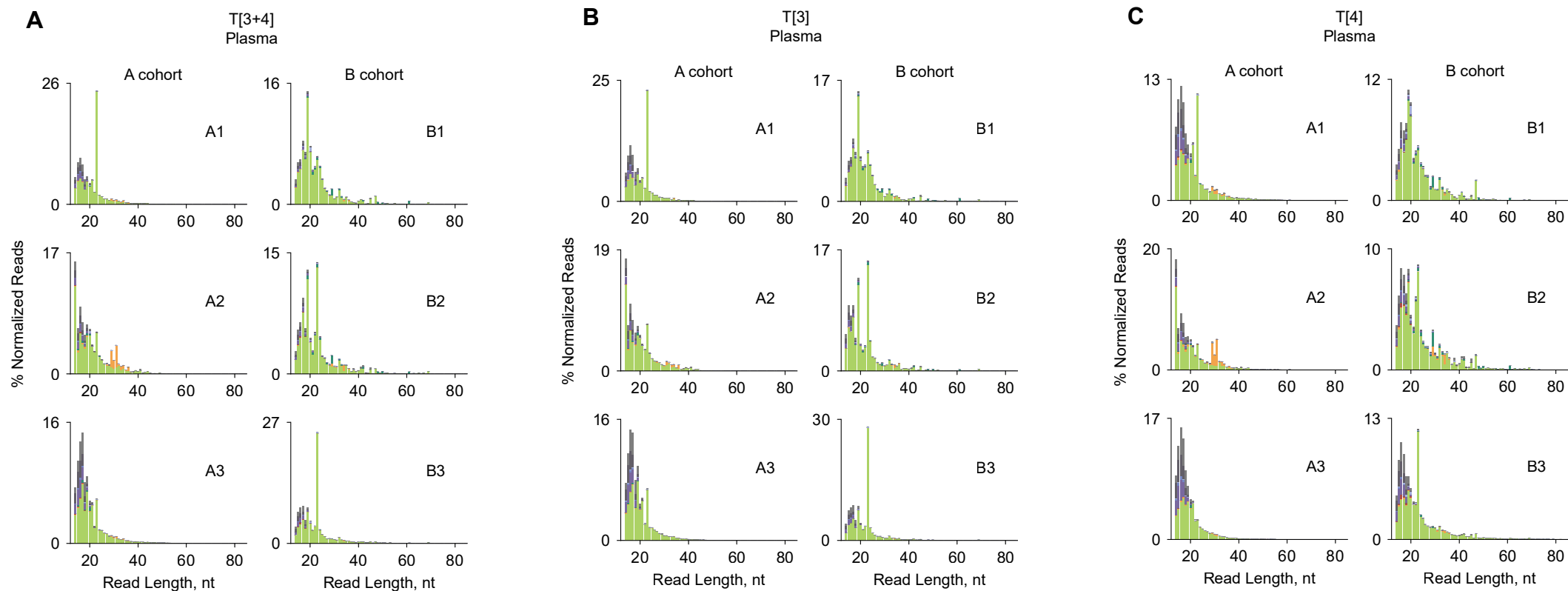
Supplementary FIGURE S21. Percentages of normalized sequencing reads for the main sRNA classes found in the D cohort (D1, D2, D3) plasma RNA samples among libraries prepared by the indicated RiboMarker® protocols and Phospho-RNA-Seq.

■ Type 1 ■ Type 2 ■ Type 3 ■ Type 4

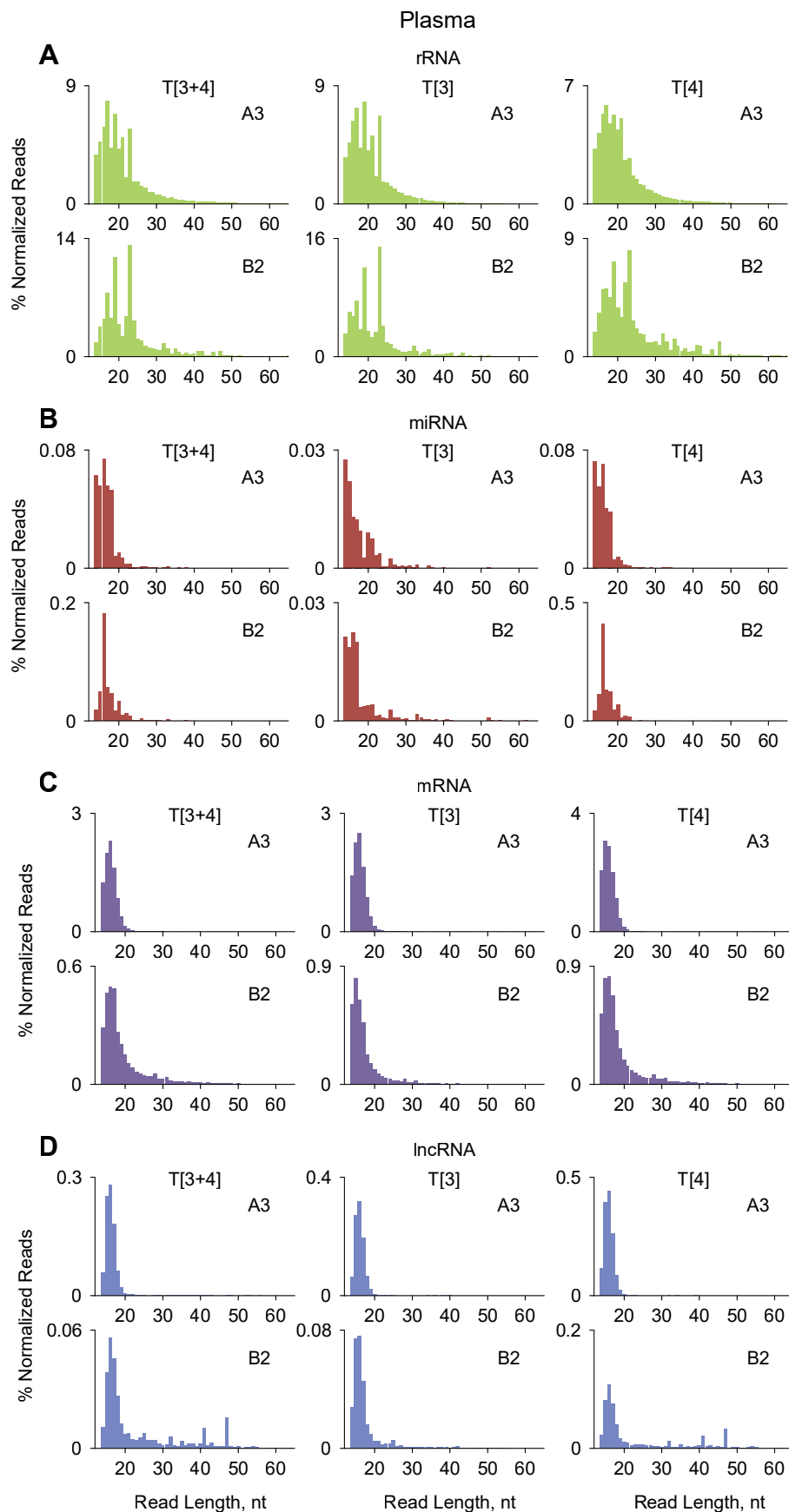


Supplementary FIGURE S22. Sequencing length and RNA Type profiles for stsRNAs spiked in either A cohort (A1, A2, A3; left panels) or B cohort (B1, B2, B3; right panels) plasma RNA samples for the libraries prepared by protocols (A) T[3+4], (B) T[3], or (C) T[4].

■ rRNA
 ■ miRNA
 ■ snoRNA
 ■ lncRNA
 ■ other sRNA
 ■ tRNA
 ■ snRNA
 ■ mRNA
 ■ piRNA
 ■ unannotated

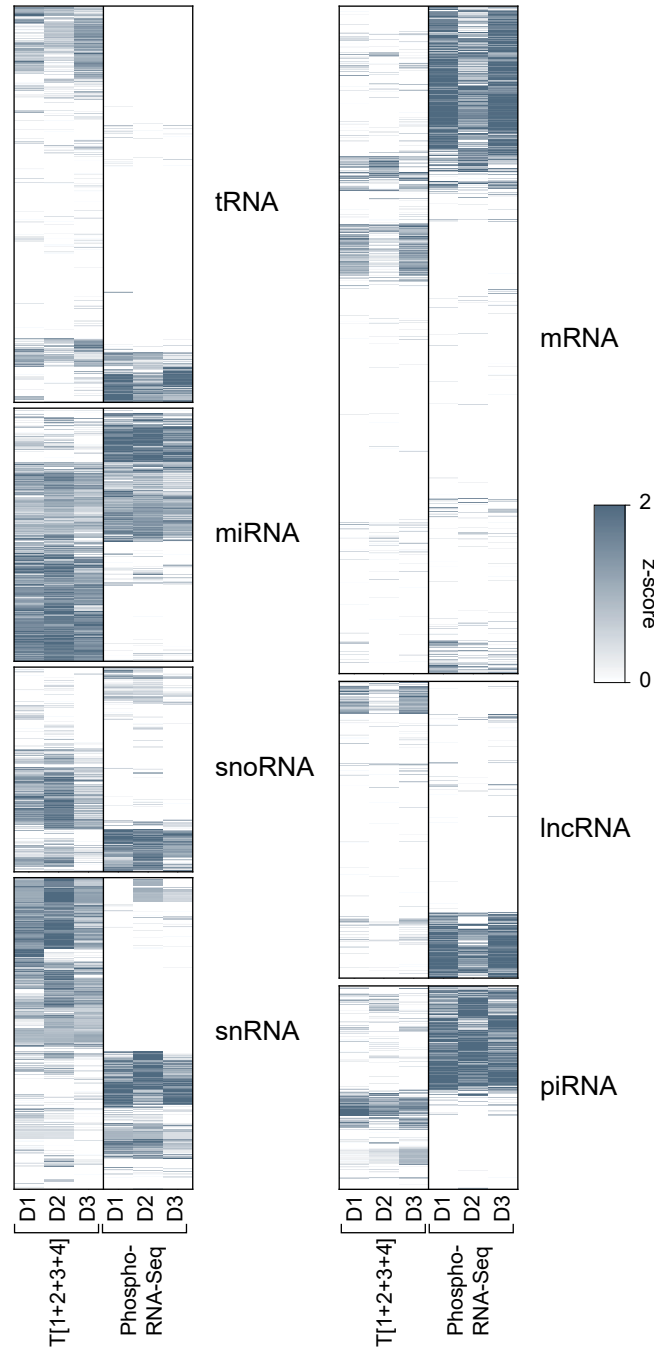


Supplementary FIGURE S23. Sequencing length and RNA Type profiles for naturally occurring sRNAs found in either A cohort (A1, A2, A3; left panels) or B cohort (B1, B2, B3; right panels) plasma RNA samples for libraries prepared by protocols **(A)** T[3+4], **(B)** T[3], or **(C)** T[4].

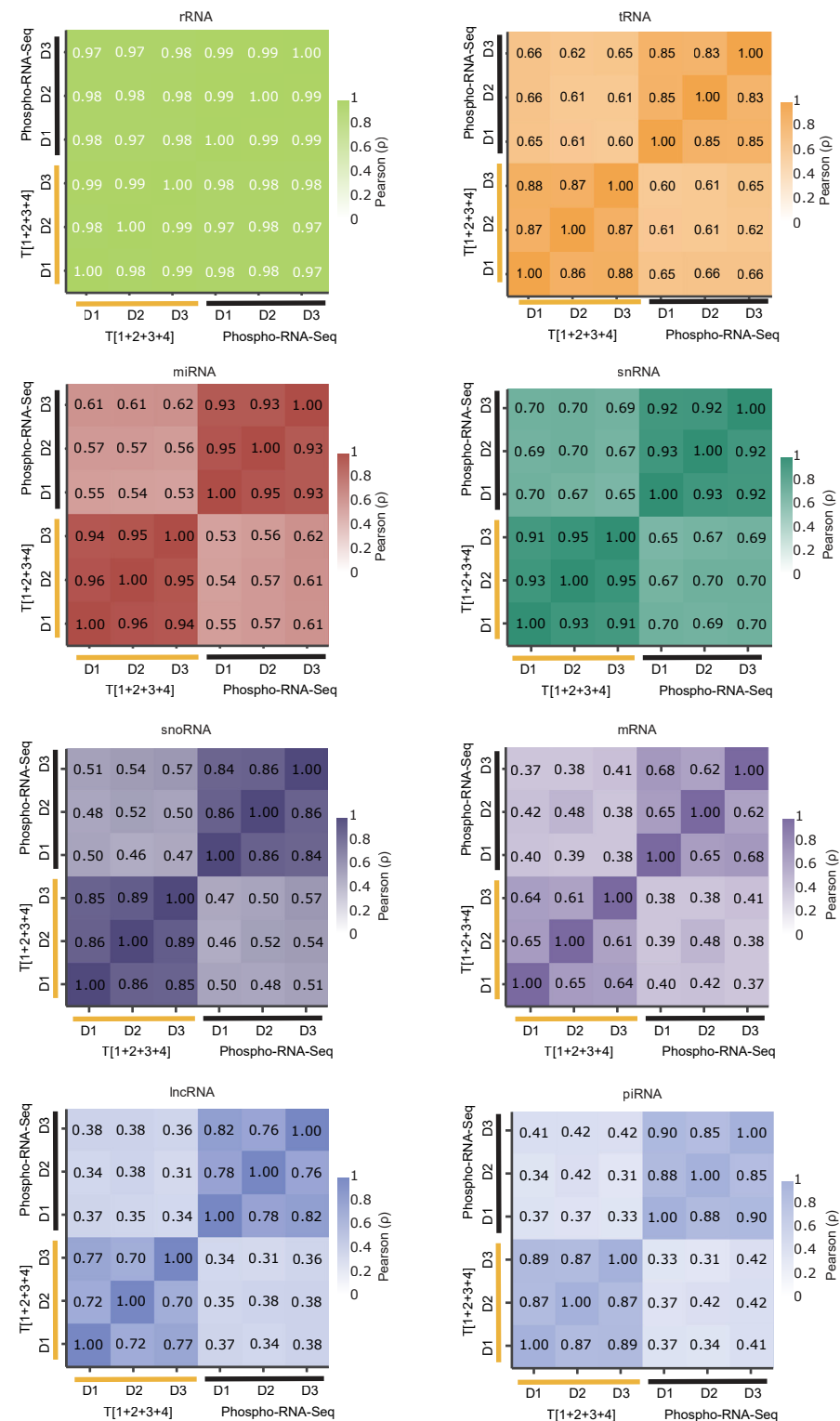


Supplementary FIGURE S24. Comparison of sequencing length profiles for naturally occurring sRNAs found in the representative A cohort plasma (A3) and B cohort plasma (B2) RNA samples between libraries prepared by protocols T[3+4], T[3], and T[4] for the following RNA classes: **(A)** rRNA, **(B)** miRNA, **(C)** mRNA and **(D)** lncRNA.

A



B



Supplementary FIGURE S25.

(A) Hierarchically clustered heatmap showing the relative abundance (column-wise z-score) of significantly variable RNA transcripts and/or fragments among RiboMarker® protocol T[1+2+3+4] and Phospho-RNA-Seq (DESeq2 LRT; padj < 0.05) from D cohort plasma (D1, D2, D3) RNA samples. Darker patterns indicate a higher relative abundance of corresponding RNA transcripts.

(B) Pearson correlation heatmaps for the main sRNA classes found in the D cohort (D1, D2, D3) plasma RNA samples among libraries prepared by RiboMarker® protocol T[1+2+3+4] or Phospho-RNA-Seq. Each heatmap shows the pairwise Pearson correlation coefficient (p) between plasma RNA samples, ranging from 0 to 1, with the color scale corresponding to the Pearson correlation. The darker shades of color indicate higher correlations, more similar expression profiles, whereas lighter shades of color represent weaker correlations, more differences in expression profiles.

Sample B1

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AAATGGATTTTGGAGCAGGGAGATGGAAT.....ATCCACTCCACGCATCGACCTGGTATTGCAGTACTCCAGGAACGGTGCACC
AAATGGATTTTGGAGCAGGGAG.....GTCCACTCCACGCATCGACCTGGTATTGCAGTACTCCAGGAACGGTGCACC
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CTCCACTCCACGCATCGACCTGGTATTGCAGTACTCCAGGAACGGTGCACC

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Sample B2

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CGGAGCTTGCTCCGTCCACTCCACGCATCGACCTGGTATTGCAGTACTCCAGGAACGGTGCACC

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Area

01

02

Supplementary FIGURE S26. Top 20 most abundant sRNA sequencing reads (5'-3') aligned to the RNU2-1 snRNA that were detected in the selected B cohort (B1, B2) plasma RNA samples for libraries prepared by protocol [T2]. The underlined 01 and 02 areas of the RNU2-1 transcript sequences correspond to the highlighted areas in Fig. 9C.