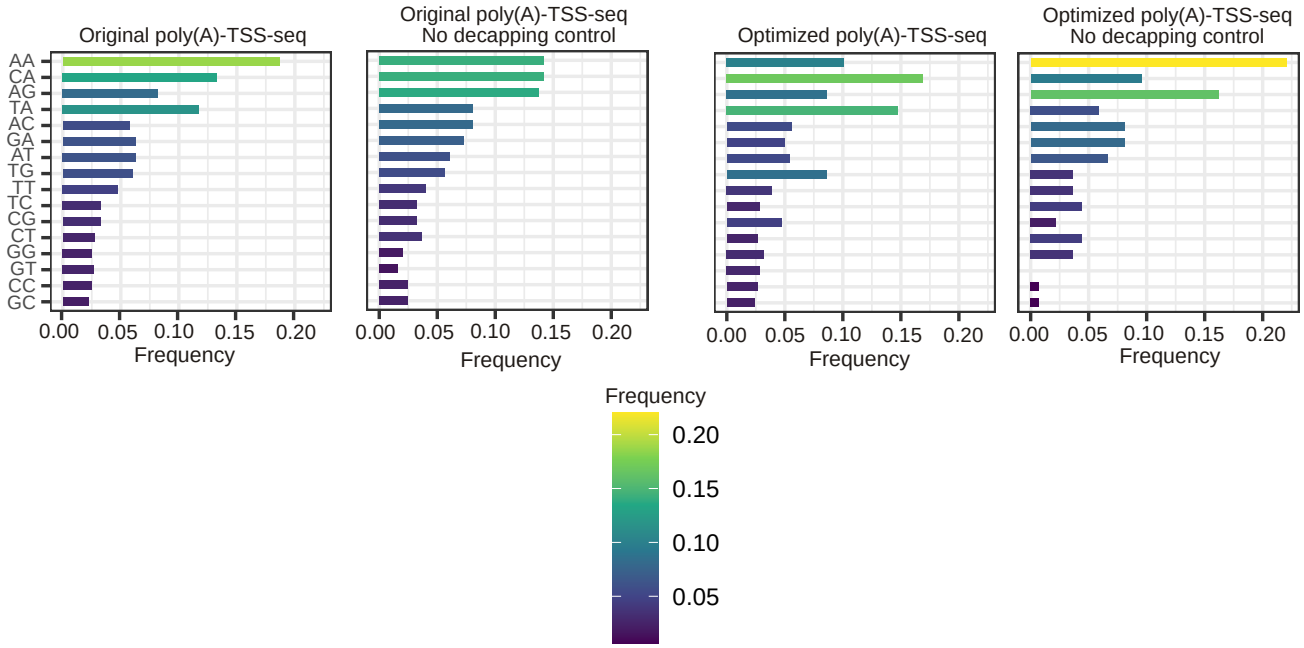
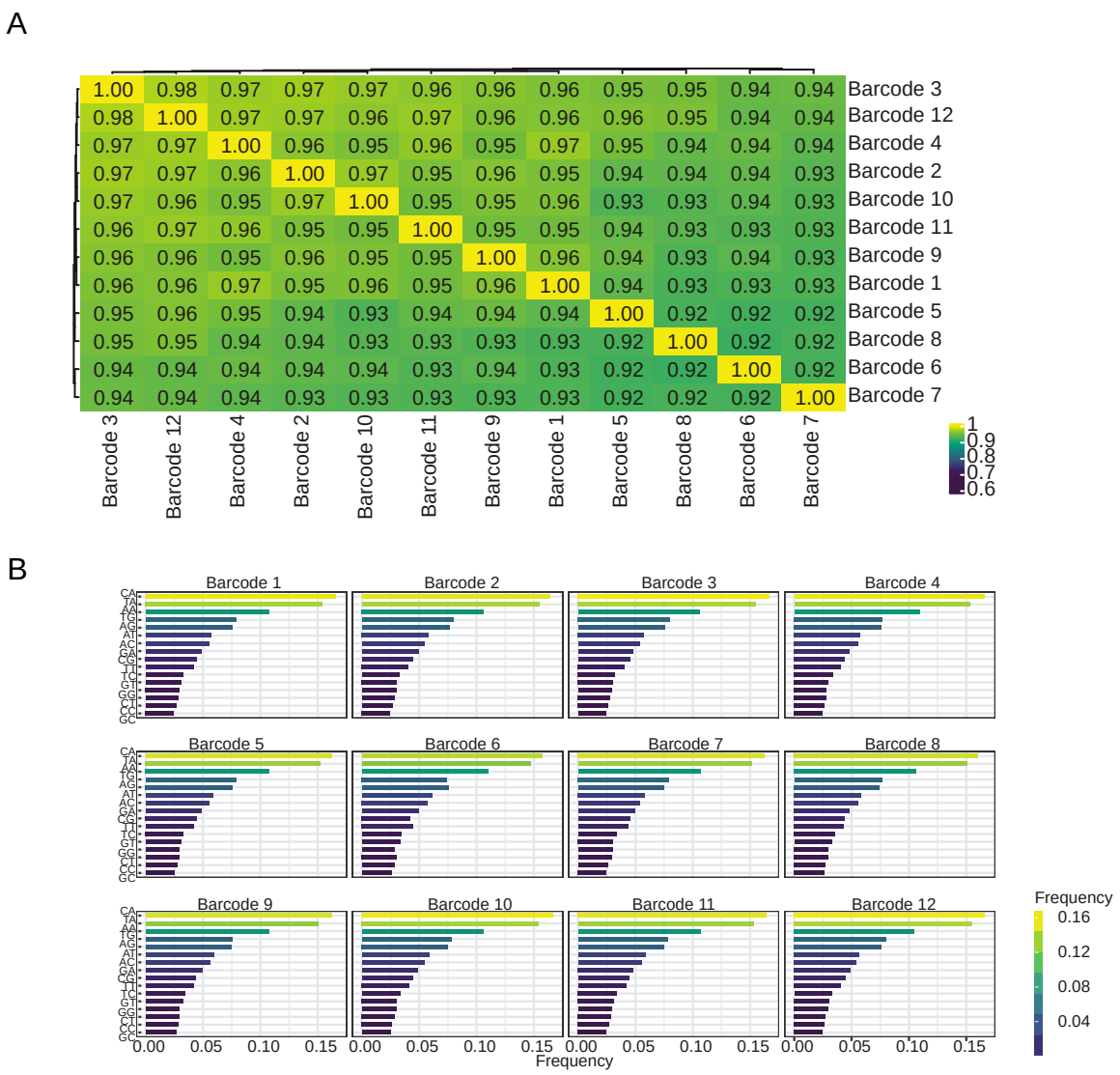
Figure S1

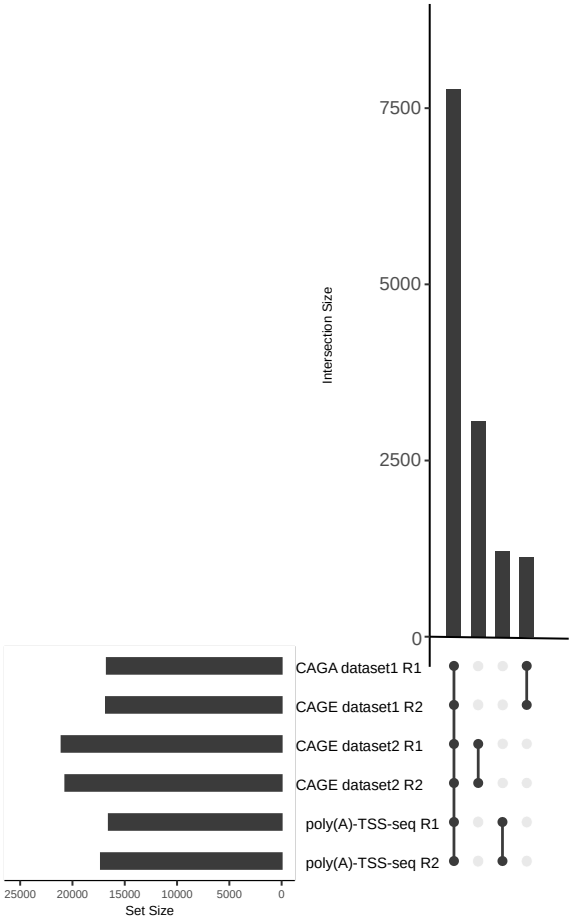


Supplemental Figure S1. Dinucleotide frequency analysis of the original and optimized TSS-seq protocol with yeast RNA. The first nucleotide represents the -1 nucleotide, and the second nucleotide represents the +1 tag/TSS identified by TSS-seq. The analysis was performed using TSRexploreR.

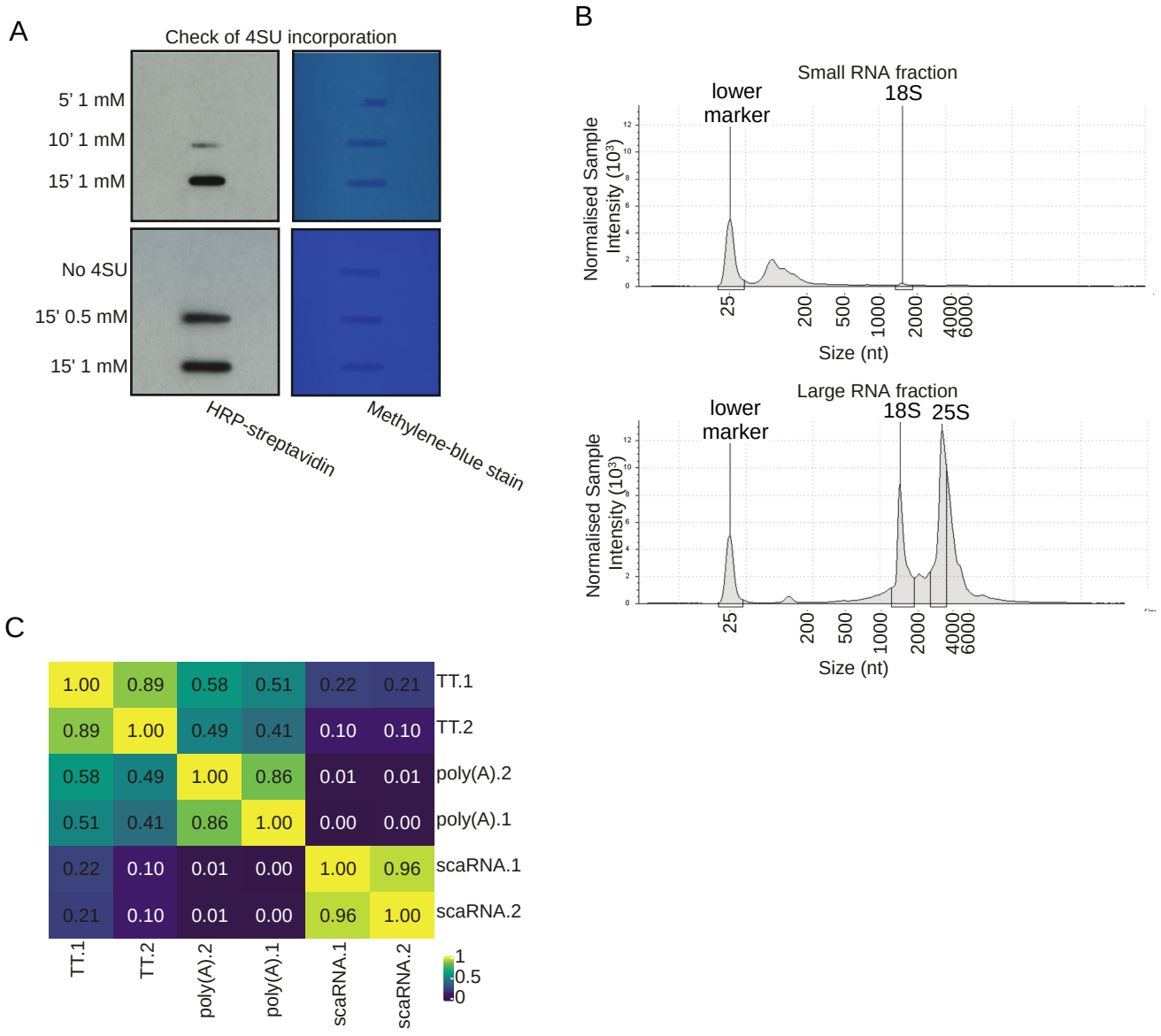


Supplemental Figure S2. Testing of sample multiplexing in optimized TSS-seq protocol, using yeast RNA.

(**A**) Hierarchically clustered heatmap of Pearson correlation coefficients (r values) between the determined TSSs of the libraries produced using the different 5' adaptor sequences. (**B**) Dinucleotide frequency analysis. The first nucleotide represents the -1 nucleotide, and the second nucleotide represents the +1 tag/TSS identified by poly(A)-TSS-seq.



Supplemental Figure S3. Poly(A)-TSS-seq protocol in mESCs and compared to CAGE. Upset plot comparing tag locations as identified by poly(A)-TSS-seq and two independent CAGE datasets in mESCs. Two replicates (R1 and R2) are included for each approach.



Supplemental Figure S4. TT-TSS-seq protocol development and comparisons.
(**A**) Assessment of 4SU incorporation in mESCs using slot blot, using different labelling times and concentrations. 4SU-containing RNA was labelled using a biotin linker, and HRP-conjugated streptavidin. Methylene blue staining was used to assess RNA loading levels. (**B**) RNA electropherogram, as measured using TapeStation, showing the sizes of RNA in the flow-through and eluate after size selection. A 3:1 ratio of RNA to ethanol was used. The 25 nt peak represents the ladder. (**C**) Hierarchically clustered heatmap of Pearson correlation coefficients (*r* values) between the determined TSRs of the different samples. Tag counts were normalised using the DESeq2 median-of-ratios approach using a threshold of three counts. Tags within 25 bp, and to a maximum distance of 250 bp, were merged to identify TSRs.