

Figure S1. Effects of kallisto pseudo-alignment efficiency on differences in TPM estimate Spearman's rank correlations between 2x40 vs. 1x125. Efficiency of 2x40 is presented as the total kallisto count for 2x40 for **(a)** transcripts and **(b)** genes, and contrasted with the efficiency of 1x125 in the form of count ratios for **(c)** transcripts and **(d)** genes. Symbol colors correspond to SRA accessions.

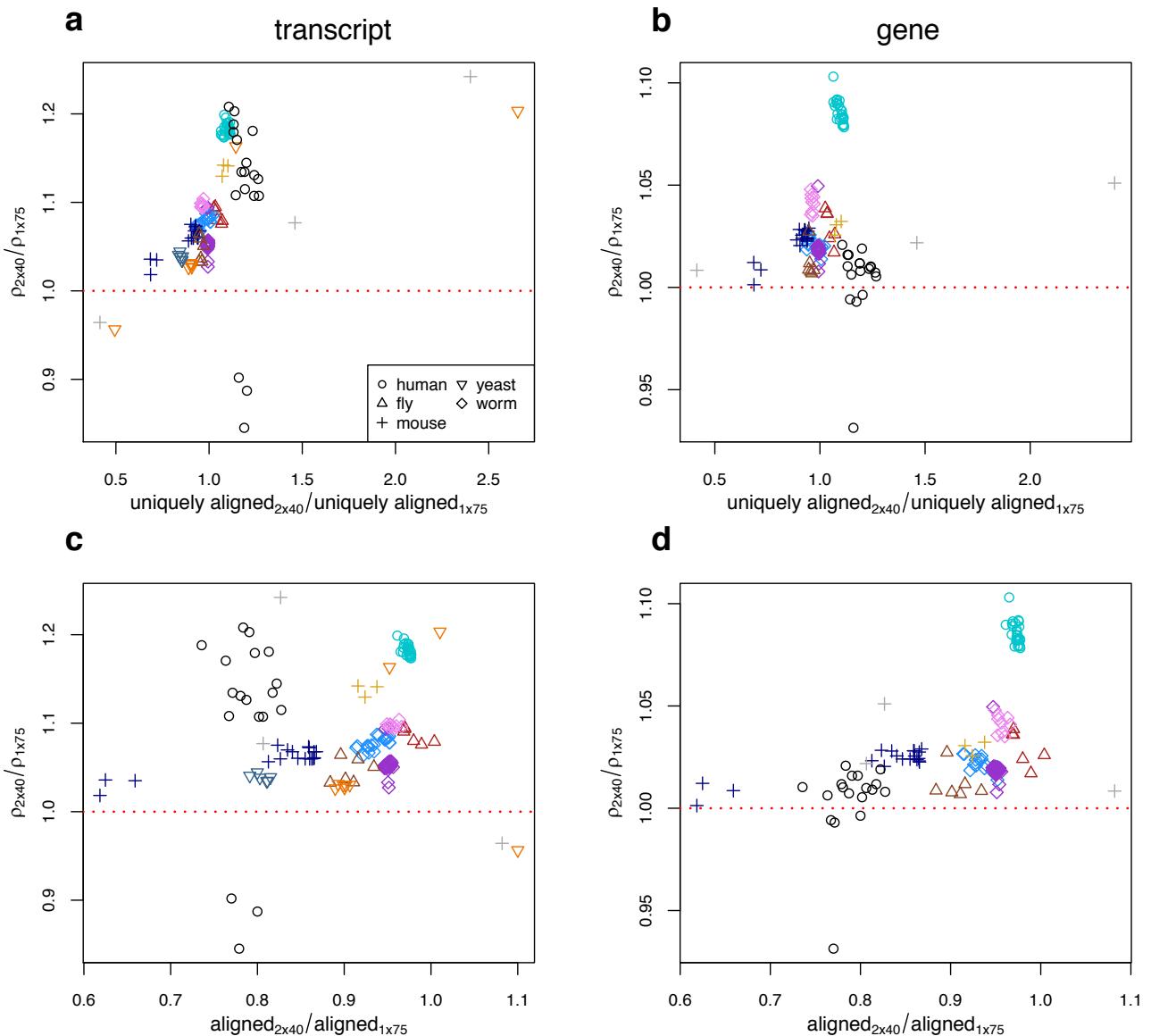


Figure S2. Effects of bowtie2 alignment rates on differences in RSEM TPM estimate Spearman's rank correlations between 2x40 vs. 1x75. Relative differences in efficiency are presented as the ratio of 2x40 to 1x75 TPM correlations with 2x125 TPM over the ratio of unique alignment rate for **(a)** transcripts and **(b)** genes, and over the ratio of overall alignment rates for **(c)** transcripts and **(d)** genes. Symbol colors correspond to SRA accessions.

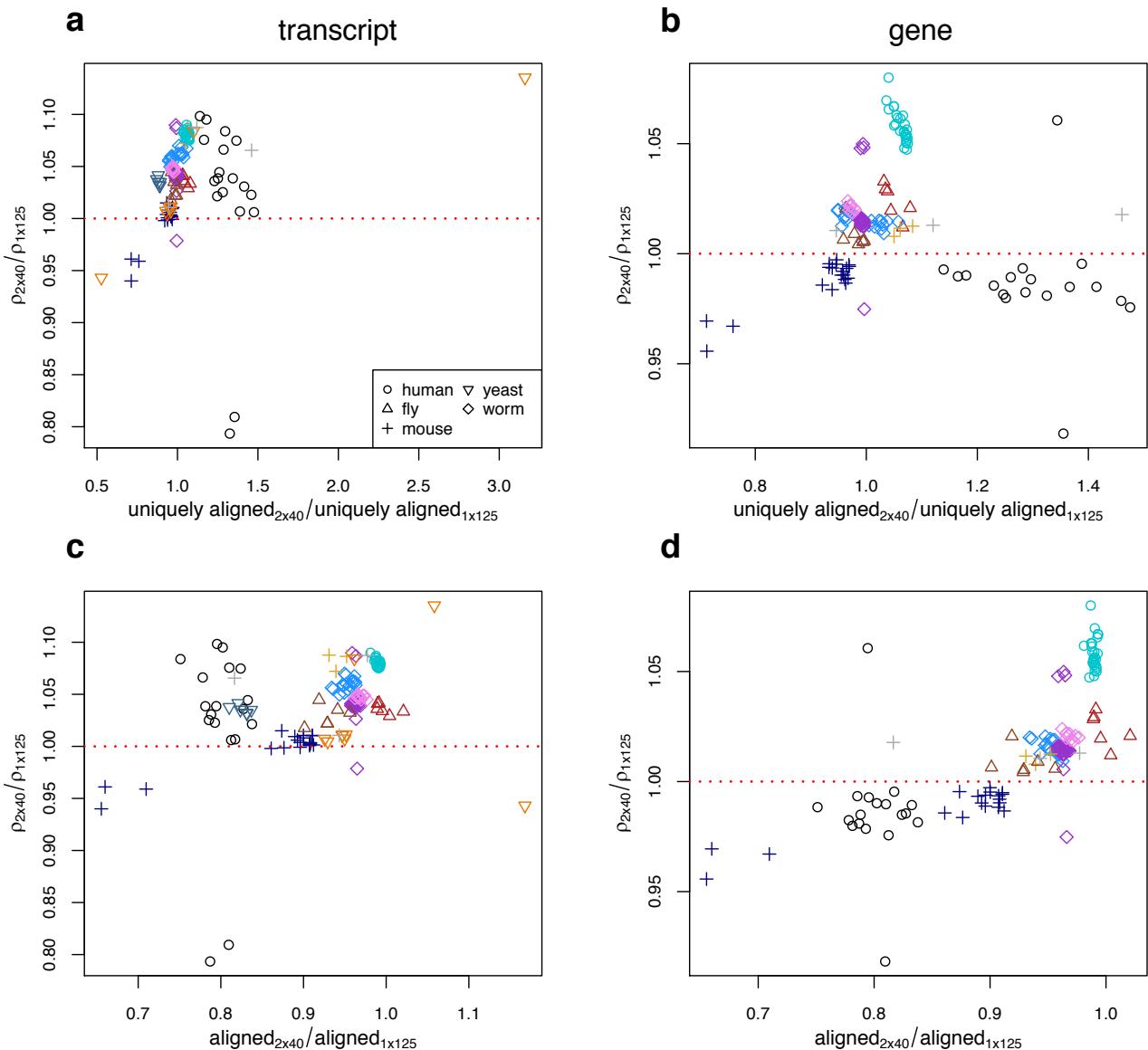


Figure S3. Effects of bowtie2 alignment rates on differences in RSEM TPM estimate Spearman's rank correlations between 2x40 vs. 1x125. Relative differences in efficiency are presented as the ratio of 2x40 to 1x125 TPM correlations with 2x125 TPM over the ratio of unique alignment rate for **(a)** transcripts and **(b)** genes, and over the ratio of overall alignment rates for **(c)** transcripts and **(d)** genes. Symbol colors correspond to SRA accessions.

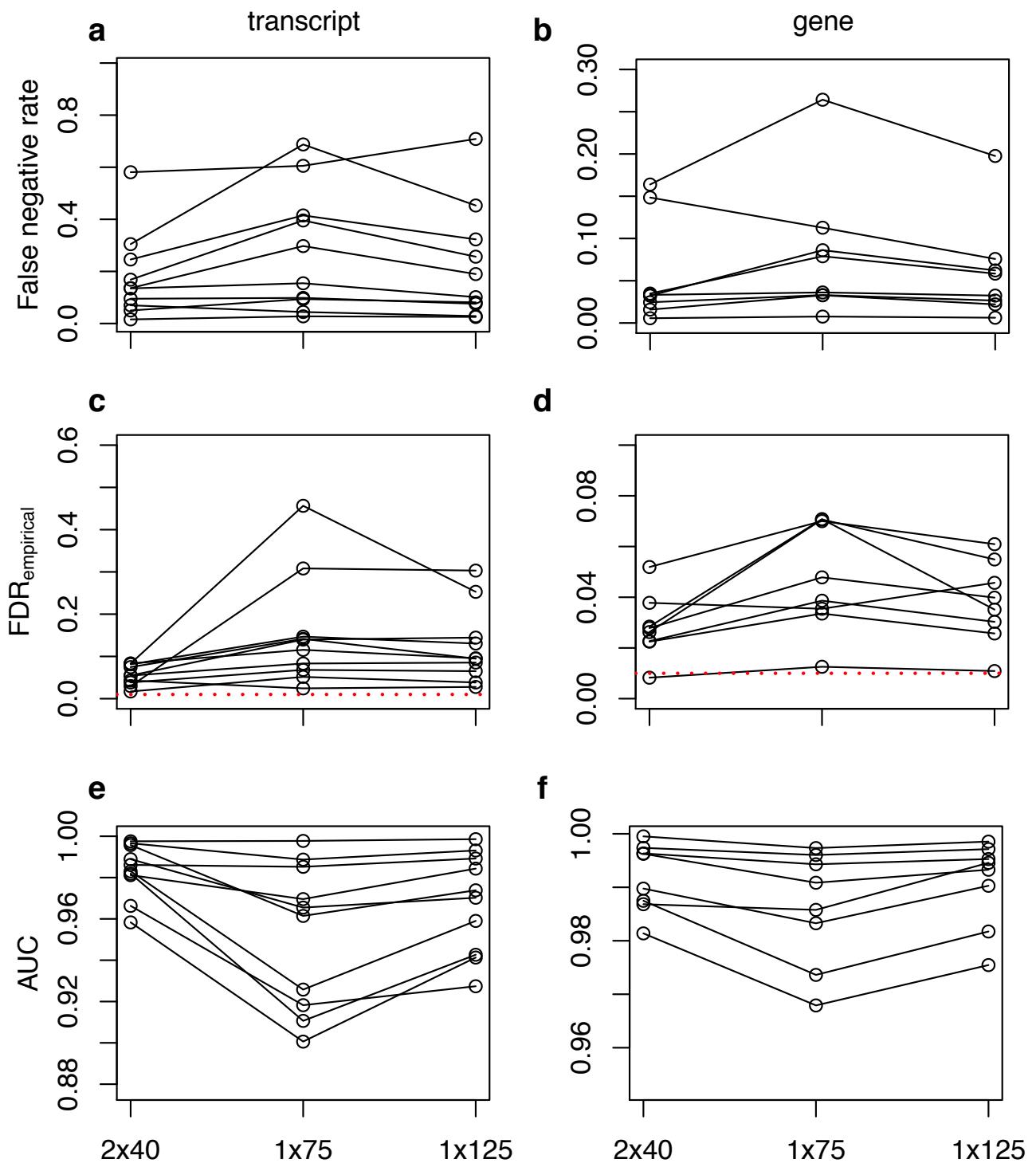


Figure S4. For limma-voom Wald tests, variation across SRA accessions, in **(a,b)** false negative rate, **(c,d)** empirical false discovery rate (red line indicates FDR threshold of 0.01 for calling tests significant), and **(e,f)** AUC between 2x40, 1x75 and 1x125 sequencing strategies. Differences are plotted as means for Wald tests within accessions, for **(a,c,e)** transcripts and **(b,d,f)** genes.

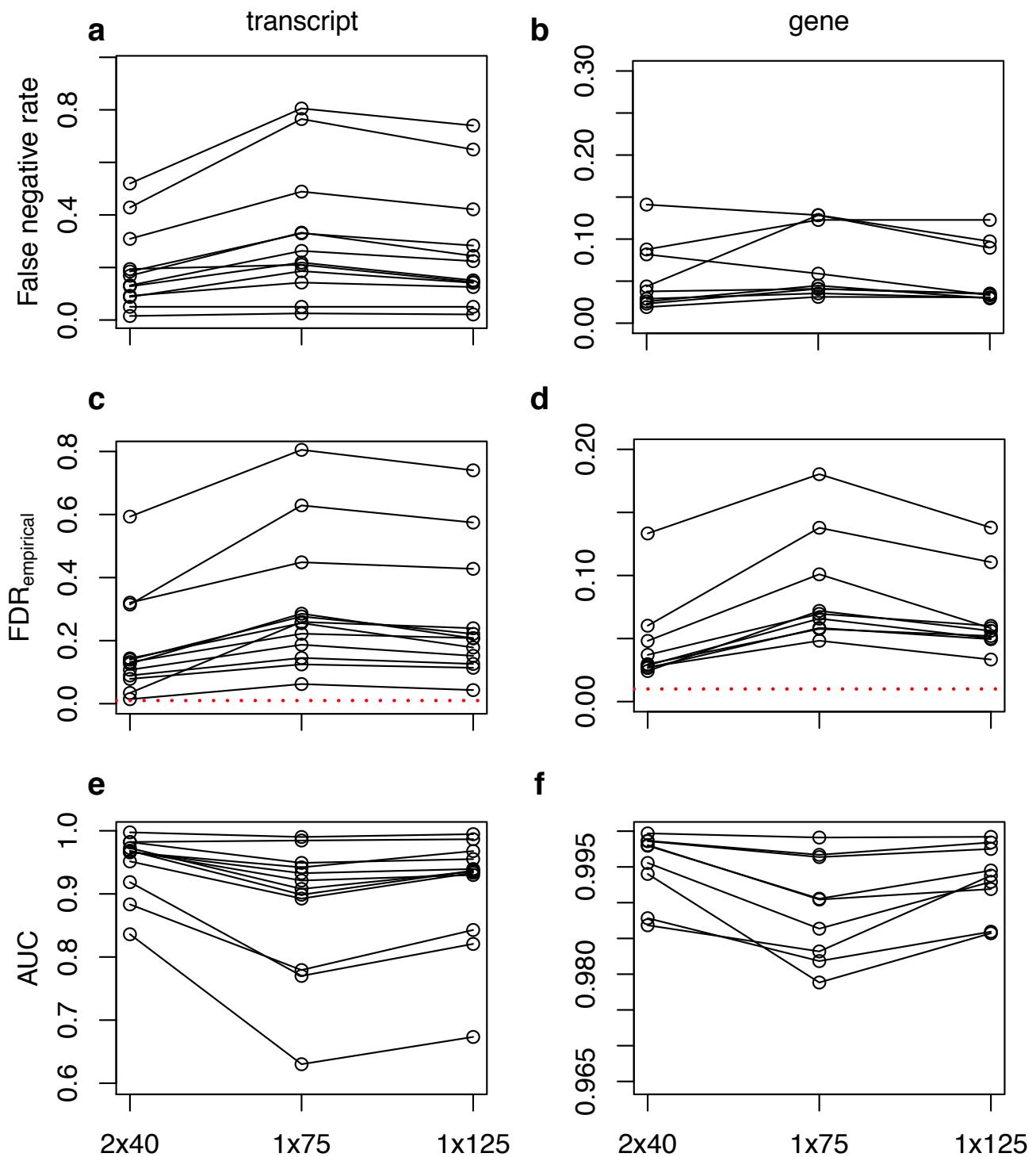


Figure S5. For DESeq2 Wald tests, variation across SRA accessions, in (a,b) false negative rate, (c,d) empirical false discovery rate (red line indicates FDR threshold of 0.01 for calling tests significant), and (e,f) AUC between 2x40, 1x75 and 1x125 sequencing strategies. Differences are plotted as means for Wald tests within accessions, for (a,c,e) transcripts and (b,d,f) genes.

Table S1. SRA accessions used in this study.

Accession	Organism	Instrument	Paired-end read length	Conditions	Biological replicates per condition		Kallisto	RSEM
SRP133853	<i>H. sapiens</i>	HiSeq 2500	125	6	3		x	x
SRP115815	<i>H. sapiens</i>	HiSeq 2500	125-126	9	3		x	
SRP105271	<i>M. musculus</i>	HiSeq 2000	125	5	3-4		x	
SRP143508	<i>M. musculus</i>	HiSeq X Ten	150	2	3		x	
SRP096374	<i>M. musculus</i>	HiSeq 4000	150	2	3		x	x
ERP017328	<i>D. melanogaster</i>	HiSeq 2500	126	2	3		x	
SRP128516	<i>D. melanogaster</i>	HiSeq 4000	151	2	3		x	x
SRP089981	<i>C. elegans</i>	NextSeq 500	151	4	5		x	
SRP092256	<i>C. elegans</i>	HiSeq 2500	126	7	5		x	
SRP129557	<i>C. elegans</i>	HiSeq 3000	126	4	3		x	x
SRP133093	<i>S. cerevisiae</i>	HiSeq 2000	151	4	3		x	x
SRP142501	<i>S. cerevisiae</i>	HiSeq X Ten	150	2	3		x	

Table S2. Across all assayed SRA accessions, percentages of pairwise differential expression tests between conditions where a performance metric is greater for 2x40 compared to 1x75 or 1x125. Gene-level analyses are not included for yeast accessions (SRP133093 and SRP142501) as this species has no alternative splicing, such that there is only one annotated transcript per gene. Bold values indicate those where 2x40 outperforms the evaluated single-end strategy. NA indicates there were < 50 differentially expressed features such that performance metrics were not calculated.

Metric	transcript		gene	
	2x40 > 1x75	2x40 > 1x125	2x40 > 1x75	2x40 > 1x125
sleuth				
empirical false discovery rate				
ERP017328	0	0	0	0
SRP089981	0	80	0	0
SRP092256	0	0	0	0
SRP096374	0	0	0	0
SRP105271	0	90	0	0
SRP115815	0	0	0	11.1
SRP129557	0	0	0	0
SRP133093	0	0	0	0
SRP133853	46.7	46.7	46.7	46.7
SRP142501	0	0	0	0
false negative rate				
ERP017328	0	0	0	0
SRP089981	0	20	0	0
SRP092256	0	0	0	0
SRP096374	0	0	0	0
SRP105271	0	100	0	100
SRP115815	0	8.3	0	27.8
SRP129557	0	0	0	0
SRP133093	0	0	—	—
SRP133853	0	0	13.3	13.3
SRP142501	0	0	—	—
false positive rate				
ERP017328	0	0	0	0
SRP089981	80	80	60	80
SRP092256	0	0	0	0
SRP096374	0	0	0	0
SRP105271	0	90	20	80
SRP115815	0	0	5.6	16.7
SRP129557	0	0	0	0
SRP133093	0	0	—	—
SRP133853	60	60	53.3	53.3
SRP142501	0	0	—	—

sensitivity

ERP017328	100	100	100	100
SRP089981	100	80	100	100
SRP092256	100	100	100	100
SRP096374	100	100	100	100
SRP105271	100	0	100	0
SRP115815	100	91.7	100	72.2
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	100	93.3	86.7	86.7
SRP142501	100	100	—	—

specificity

ERP017328	100	100	100	100
SRP089981	20	20	40	20
SRP092256	100	100	100	100
SRP096374	0	100	100	100
SRP105271	100	10	80	20
SRP115815	100	100	94.4	83.3
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	40	40	46.7	46.7
SRP142501	100	100	—	—

precision

ERP017328	100	100	100	100
SRP089981	40	20.0	100	40
SRP092256	100	100	100	100
SRP096374	100	100	100	100
SRP105271	100	10	100	20
SRP115815	100	100	100	88.9
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	53.3	53.3	53.3	53.3
SRP142501	100	100	—	—

auc

ERP017328	100	100	100	100
SRP089981	100	80	100	100
SRP092256	100	100	100	100
SRP096374	100	100	100	100
SRP105271	100	0	100	30
SRP115815	100	100	100	100
SRP129557	100	100	100	100
SRP133093	100	100	—	—

SRP133853	100	100	100	100
SRP142501	100	100	—	—
limma-voom				
empirical false discovery rate				
ERP017328	0	0	0	0
SRP089981	0	0	0	0
SRP092256	0	0	0	5
SRP096374	100	100	100	0
SRP105271	0	0	0	40
SRP115815	0	0	5.6	25
SRP129557	0	0	0	0
SRP133093	0	0	0	0
SRP133853	25	25	42.9	50
SRP142501	0	0	0	0
false negative rate				
ERP017328 (1)	0	0	0	0
SRP089981 (4)	0	0	0	25
SRP092256 (19)	21	89.5	0	30
SRP096374 (1)	0	0	0	0
SRP105271 (9)	22.2	77.8	70	100
SRP115815 (36)	0	0	0	0
SRP129557 (3)	0	33.3	16.7	33.3
SRP133093 (6)	33.3	50	—	—
SRP133853 ()???	50	25	35.7	42.9
SRP142501 (1)	100	100	—	—
false positive rate				
ERP017328	0	0	0	0
SRP089981	0	0	0	0
SRP092256	0	0	0	5.0
SRP096374	100	100	100	0
SRP105271	0	0	0	20
SRP115815	11.1	0	8.3	30.6
SRP129557	0	0	0	0
SRP133093	0	0	—	—
SRP133853	50	75	57.1	50
SRP142501	0	0	—	—
sensitivity				
ERP017328	100	100	100	100
SRP089981	100	100	100	75
SRP092256	78.9	10.5	100	60
SRP096374	100	100	100	100
SRP105271	77.8	11.1	30	0

SRP115815	100	100	100	100
SRP129557	100	66.7	83.3	66.7
SRP133093	66.7	50	—	—
SRP133853	50	75	64.3	57.1
SRP142501	0	0	—	—
specificity				
ERP017328	100	100	100	100
SRP089981	100	100	100	100
SRP092256	100	100	100	95
SRP096374	0	0	0	100
SRP105271	100	100	100	80
SRP115815	88.9	100	91.7	69.4
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	50	25	42.9	50
SRP142501	100	100	—	—
precision				
ERP017328	100	100	100	100
SRP089981	100	100	100	100
SRP092256	100	100	100	95
SRP096374	0	0	0	100
SRP105271	100	100	100	60
SRP115815	100	100	94.4	75
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	75	75	57.1	50
SRP142501	100	100	—	—
auc				
ERP017328	100	100	100	0
SRP089981	100	100	100	100
SRP092256	100	100	100	75
SRP096374	0	0	100	100
SRP105271	100	33.3	60	10
SRP115815	100	100	100	94.4
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	100	100	100	71.4
SRP142501	100	0	—	—
DESeq2				
false discovery rate				
ERP017328	0	0	0	0
SRP089981	0	0	0	20

SRP092256	4.8	9.5	0	4.8
SRP096374	0	0	0	0
SRP105271	0	0	0	20
SRP115815	0	0	2.8	8.3
SRP129557	0	0	0	0
SRP133093	0	0	0	0
SRP133853	0	0	13.3	20
SRP142501	0	0	0	0
SRP128516	0	0	0	0
SRP143508	0	0	0	0

false negative rate

ERP017328	0	0	0	0
SRP089981	0	0	0	0
SRP092256	0	0	9.5	42.9
SRP096374	0	0	0	100
SRP105271	20	100	90	100
SRP115815	0	0	0	0
SRP129557	0	0	0	16.7
SRP133093	0	33.3	—	—
SRP133853	0	20	66.7	93.3
SRP142501	0	0	—	—
SRP128516	0	0	0	0
SRP143508	0	0	NA	NA

false positive rate

ERP017328	0	0	0	0
SRP089981	0	0	0	20
SRP092256	9.5	14.3	0	4.8
SRP096374	0	0	0	0
SRP105271	0	0	0	20
SRP115815	0	0	8.3	11.1
SRP129557	0	16.7	0	0
SRP133093	0	0	—	—
SRP133853	46.7	40	20	20
SRP142501	0	0	—	—
SRP128516	0	0	0	0
SRP143508	0	0	NA	NA

sensitivity

ERP017328	100	100	100	100
SRP089981	100	100	100	100
SRP092256	100	100	90.5	52.4
SRP096374	100	100	100	0
SRP105271	80	0	10	0

SRP115815	100	100	100	100
SRP129557	100	100	100	66.7
SRP133093	100	66.7	—	—
SRP133853	100	80	33.3	6.7
SRP142501	0	0	—	—
SRP128516	100	100	100	100
SRP143508	100	100	NA	NA
specificity				
ERP017328	100	100	100	100
SRP089981	80	100	100	80
SRP092256	90.5	85.7	100	95.2
SRP096374	100	100	100	100
SRP105271	100	100	100	80
SRP115815	100	100	91.7	88.9
SRP129557	100	83.3	100	100
SRP133093	100	100	—	—
SRP133853	53.3	60	80	80
SRP142501	100	100	—	—
SRP128516	100	100	100	0
SRP143508	100	100	NA	NA
precision				
ERP017328	100	100	100	100
SRP089981	100	100	100	80
SRP092256	95.2	90.5	100	95.2
SRP096374	100	100	100	100
SRP105271	100	100	100	80
SRP115815	100	100	97.2	91.7
SRP129557	100	100	100	100
SRP133093	100	100	—	—
SRP133853	100	100	86.7	80
SRP142501	100	100	—	—
SRP128516	100	100	100	100
SRP143508	100	100	NA	NA
auc				
ERP017328	100	100	100	100
SRP089981	100	100	100	100
SRP092256	100	100	100	100
SRP096374	100	100	100	100
SRP105271	100	40	90	0
SRP115815	100	100	100	100
SRP129557	100	100	100	100
SRP133093	100	100	—	—

SRP133853	100	100	93.3	73.3
SRP142501	0	0	—	—
SRP128516	100	100	100	100
SRP143508	100	100	NA	NA
