Supplementary Figure Legends

Figure S1 Heatmaps of pairwise Pearson correlation and scatter plot of DI scores between samples (a-f) Shown are results for DI scores for SEQC-AA, SEQC-AB, PBMC-S01, GBM, DLPFC-Br1729 and Breast Tumor data respectively. SEQC-AB shows a strong between-condition correlation in degradation pattern, so does GBM R10 vs. R6+R4. (g-i) Scatter plot of DI scores between samples of same RIN or different RIN in GBM data.

Figure S2 Choice of empirical controls genes and factors in RUVg normalization method may dramatically affect differential expression analysis (a-c) Coefficient of variation (CV) vs. mean read count (in log scale) from RUVg method for GBM R10 vs. R4, PBMC-S01 and SEQC-AB respectively. The mean counts for each data were scaled to 0-1 range by a linear transformation (i.e., \( \frac{X_t - \min_j(X_j)}{\max_j(X_j)} \)). Compared are results when different number of empirical genes were used in estimation of the unwanted variation. For each data set, we excluded \( L \) most significant genes and used the remaining as empirical controls to estimate the unwanted variation factor for \( L=1000, 3000, 5000, 8000 \) and 10000 respectively. In all four data sets presented, the first factor from the factor analysis was used in the estimation of unwanted variation. The CV curve was smoothed using the built-in smoothing spline function in R. In the GBM R10 vs. R4 and PBMC-S01 comparison, the CV curves were extremely sensitive to the choice of \( L \), while not for the SEQC-AB data. (d-f) Empirical CDF of the p-value from DE analysis for each data set under each setting as specified in (a-c). Again ECDF curves show sensitivity to the choice of empirical control genes in GBM R10 vs. R4, PBMC-S01 and SEQC-AB data. (g, h) ECDF of the p-value for GBM-R10 vs. R4 and PBMC-S01 data when the second factor from factor analysis...
was used to estimate the unwanted variation for $L = 5000$. The curves were dramatically changed compared to results in (e, f) where the first factor was used.

**Figure S3 Comparing different global normalization methods in Differential expression analysis (a-e)** Comparison of ECDF plots of the p-values of DE analysis under different normalization methods including DegNorm, upper quartile (UQ), trimmed mean of m-values (TMM), relative log expression (RLE) and total read count (TC). The curves from the latter four global normalization methods are highly consistent and almost overlap perfectly for all the six comparisons under investigation.

**Figure S4 DE analysis of DLPFC data** (a) ECDF of p-values for DE analysis of DLPFC subject Br1729 mRNA-seq data T0+T15 vs T30+T60. (b) ECDF of p-values for DE analysis of DLPFC subject Br1729 mRNA-seq (T0+T15+T30+T60) vs. Ribo-Zero-seq data (T0+T15+T30+T60).

**Figure S5 Results for Simulations II-IV** (a-f) Box plot of true vs. estimated DI scores for each simulation. Condition A and B stand for control and treatment samples respectively. (g-l) Heatmap of Pearson correlation of true vs. estimated DI scores between samples.

**Figure S6 Results for Simulations II-IV** (a-d) Scatter plot of normalized read count vs. latent read count in log2 scale with diagonal line imposed for Simulation III under different normalization methods. The latent count was adjusted for sequencing depth using UQ method. $R^2$ is the regression coefficient of determination. (e-h) Same as (a-e) but for Simulation IV.
Fig S3

a) SEQC-AA

b) PBMC-S01

c) GBM-R6 vs R4

d) GBM-R10 vs R4

e) SEQC-AB

f) Br1729-Ribo-zero
Simulation II--true

Simulation III--true

Simulation IV--true

Simulation II--estimated

Simulation III--estimated

Simulation IV--estimated

Simulation II--true

Simulation III--true

Simulation IV--true

Simulation II--estimated

Simulation III--estimated

Simulation IV--estimated