differentially expressed genes and variability in spike-in performance among replicates—provide researchers with defined objectives to create and implement tools to improve data normalization and remove unwanted variation[3,4,6]. Surprisingly, spike-ins do not behave predictably and do not perform well for normalizing data between samples or sites[3,4,6]. As the researchers note, once these biases and errors are identified and compensated for, RNA-seq will become a more powerful and sensitive tool moving beyond the basic researcher's bench.

Further addressing differential gene expression, Li *et al.*[3] characterize reproducibility and false-positive rates on replicates of the same sample across multiple sites. Importantly, the authors identify library preparation as a major source of these false positives and put forward several metrics that should be monitored, including GC content distribution, gene-body coverage uniformity, average error rate and insert size. Each group[2–6] assessed the utility of several tools to mitigate the false-positive rate and increase detection of real differential expression. Moreover, they evaluate approaches that improve the sensitivity and validity of differentially expressed genes (DEGs) identified by RNA-seq[2–6].

From both a regulatory and practical perspective, establishing diagnostic utility requires that tests repeatedly produce a similar result when conducted by the same laboratory on the same platform, and are reproducible when conducted by other laboratories on alternate diagnostic platforms[7]. Platform-specific biases must be characterized and understood as a fundamental aspect of establishing the concept of reproducibility.

One of the clear points that emerged from these efforts was that there can be biases between sites, and that it is critical to carefully examine the sources of bias at each site. Many of these bias types, once identified, can be compensated for through analysis and normalization. For example, although library preparation is considered a potential source of significant variation, Li *et al.*[5] demonstrated that even between different qualities of RNA (degraded vs intact) and library preparation methods (poly-A selection vs ribodepletion) there could be good correlation after adjusting for factors such as read depth.

The study design feature of using commercially available Universal Human and Human Brain Reference RNAs and External RNA Control Consortium (ERCC) spike-ins ensures every center or institute has the ability to compare their site-specific biases to the data generated in these papers. This publicly available resource will be immensely valuable to groups wanting to transition RNA-seq technology to clinical settings, providing benchmark methods and data necessary to develop clinical laboratory tests.

These studies highlight many areas that will need to be investigated and improved before RNA-seq can be adopted in the clinic and in regulatory settings. First, future protocols must better account for the biases introduced by sample collection, RNA isolation, library preparation and differences between sequencing platforms. Second, new analytical tools need to be developed to address known biases and remove them. Third, there is a clear need for improved assays to validate DEGs and new questions about which assays measure the ground truth most accurately.For instance, Wang *et al.*[4] clearly show a higher rate of qPCR validation for RNA-seq–defined DEGs than those identified by DNA microarray, particularly for genes expressed at low levels; furthermore, Li *et al.*[5] show the correlation between RNA-seq and qPCR is improved when RNA-seq expression estimates are derived from the same interval tested by qPCR. These studies pinpoint low-level gene expression as a limitation in the accuracy and reproducibility of DEG measurements.

> "We anticipate new problems and terminology, such as "transcript of unknown significance" mirroring the established "variant of unknown significance."

Clearly, RNA-seq works well for discovering a huge number of transcripts, isoforms and base changes all at once. The early hope is that the studies described in this issue will help set the stage for wider adoption in regulatory and clinical settings by rigorously defining the strengths and weaknesses of the assay plus critical controls and quality metrics that will improve accuracy and reproducibility. These first milestones must be passed before the long-term potential of RNA-seq to both measure established clinical biomarkers and discover rare transcripts in the context of clinical disease treatment can be fulfilled. Nevertheless, looking forward we anticipate new problems and terminology, such as "transcript of unknown significance" mirroring the established "variant of unknown significance," as a testament to the transformative and disruptive potential of RNA-seq.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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