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Comparative evaluation of HTG and TempO Seq targeted transcriptome profiling methods

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Transcriptomic profiling is a cornerstone of molecular biology and plays a central role in translational and molecular oncology, where reliable gene expression measurements are essential. Targeted RNA sequencing approaches offer several advantages over whole-transcriptome methods, including high sensitivity, reproducibility, and reduced turnaround time, particularly when working with formalin-fixed paraffin-embedded (FFPE) samples. HTG EdgeSeq HTP has previously addressed these needs; however, the recent discontinuation of this platform has created a technical gap for targeted transcriptome analysis in FFPE specimens. In this study, we perform a systematic technical comparison between HTG EdgeSeq HTP and an analogous targeted platform, TempO-Seq, to assess its suitability as an alternative for research applications in molecular oncology. We analyzed 21 FFPE endometrial cancer samples together with three RNA reference controls derived from cell lines and evaluated concordance across multiple levels of biomarkers, ranging from individual transcripts to complex multi-gene signatures comprising tens to thousands of genes. While single-gene measurements showed limited concordance between platforms, multi-gene biomarkers exhibited substantially higher agreement, indicating that aggregation across multiple probes mitigates platform-specific effects. Overall, our results demonstrate that TempO-Seq provides comparable performance to HTG EdgeSeq HTP at the level of multi-gene biomarkers, supporting its use as a robust alternative for targeted transcriptomic profiling of FFPE samples in molecular oncology research.

Keywords Endometrial cancer, FFPE samples, Biomarkers in molecular oncology, Targeted RNA-seq

Transcriptomics refers to the study of the total RNA present in a cell or tissue, providing a comprehensive approach to quantify RNA molecules and enabling high-throughput gene expression profiling. This methodology enables an improved understanding of genomic regulatory mechanisms, the assessment of genetic activity, and the dissection of the molecular mechanisms that control cellular processes[1]. There are three primary techniques used to assess gene expression: qRT-PCR-based techniques, which are considered the gold standard for low-throughput gene expression analysis, as well as microarray hybridization and RNA sequencing (RNA-seq), which are high-throughput techniques capable of analyzing thousands of genes or even the entire transcriptome. In particular, RNA-seq has been shown to be more accurate than microarray hybridization[2]. An advantage of this approach is that RNA is closer to the phenotype than DNA, making transcriptomics a more accurate representation than genomics in this regard. In addition, transcriptomic analysis avoids some of the limitations of direct protein analysis[2,3]. The data generated by RNAseq can be used to study differential gene expression between different tissues or pathological conditions, such as different types of tumors. This approach not only provides valuable insights into the molecular mechanisms underlying disease, but also aids in the identification of diagnostic, prognostic and predictive biomarkers[4,5]. The availability of archived formalin-fixed, paraffin-embedded (FFPE) samples provides an invaluable source of information for oncology research, as they represent the vast majority of biospecimens routinely processed in the pathology departments for diagnostic purposes[6]. However, formalin fixation is known to cause significant degradation of nucleic acids, making frozen

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samples the preferred option when available. Modern transcriptomic tools need to overcome the challenges posed by formalin treatment in order to fully exploit the wealth of FFPE samples archived in clinical pathology departments of pathology worldwide.

In this context, the development of a sensitive, affordable, reproducible and efficient RNA-seq approach for sequencing FFPE samples is of paramount importance. Until now, the HTG EdgeSeq System (HTG Molecular Diagnostics) has provided targeted RNA kits, including the Human Transcriptome Panel (HTP), which is a comprehensive approach in the context of translational research[7,8]. Even though there are other technologies assessing gene expression such as: NanoString nCounter® and Illumina AmpliSeq, there are some key differences that favor the HTG approach which does not require prior RNA extraction, furthermore, only a small scraped FFPE section is required to amplify the entire transcriptome, providing this method with higher sensitivity than the aforementioned approaches. Although these technologies share many features, they also exhibit important differences, as shown in Table S1. However, HTG EdgeSeq recently filed for bankruptcy, highlighting the need for alternative technologies.

In this setting, the TempO-Seq (TOS) technology (BioSpyder Technologies Inc.) provides a promising transcriptomic solution that may meet the above-mentioned translational needs and address some of the limitations of traditional RNA-seq techniques. Nevertheless, the performance of TOS needs to be rigorously tested, evaluated, and validated against the established gold standard in the field .

The aim of this study is to evaluate the comparability of HTP and TOS in a series of 21 endometrial carcinomas (EC) and to assess whether TOS represents a suitable alternative to HTP in the context of translational oncology research. In this regard, the study is conceived as a technical benchmarking analysis of two targeted transcriptomic platforms applied to FFPE samples, rather than as a biological discovery effort.

Material and methods

Patients' cohort

Twenty-one EC samples, previously characterized at the molecular level using a method based on 12 gene genotyping (Table 1)[9], and three synthetic Universal RNA controls (Agilent, Santa Clara, CA, USA) were studied. These samples were collected under the LBM-02–20 project, approved by the ethics committee of the Fundación Instituto Valenciano de Oncología and conducted in accordance with the Helsinki Declaration. Informed consent was obtained for all patients included in the study. These samples were reviewed by a pathologist to identify tumor-enriched areas for optimal tissue scraping.

HTG EdgeSeq

Samples were processed according to the manufacturer's protocol using EdgeSeq technology (HTG Molecular Diagnostics, Tucson, AZ, USA). This approach consists of targeted RNA-seq panels, specifically the Human Transcriptome Panel (HTP), which interrogates 19,616 mRNAs, covering virtually the entire transcriptome. Briefly, HTG Lysis Buffer was added to permeabilize the sample, making the RNA available for binding to the target-specific Nuclease Protection Probes (NPPs) for target capture. S1 Nuclease was then added to digest unhybridized mRNA and excess NPPs.

For library preparation, each processed sample was indexed by PCR and subsequently quantified using Kapa Library Quantification (Roche, Basel, Switzerland). All samples were then pooled equimolarly, denatured, and sequenced on a NextSeq 550 instrument (Illumina, San Diego, CA, USA).

Data were returned from the sequencer as demultiplexed FASTQ files, with four files per original well of the assay. HTG EdgeSeq host software was used to align the FASTQ files to the probe list for data matching. Data were provided as a raw data table, including quality control (QC) metrics, counts per million (CPM), and median-normalized values. A triplicate of Universal Human Reference RNA (Agilent, Santa Clara, CA, USA) was used as an internal control.

Primary data analysis was performed using HTG EdgeSeq Reveal software version 3.0 (HTG Molecular Diagnostics) to interrogate and visualize the generated data.

Clinical parameter	N (%)
Histology	
Endometrioid	15 (71.4)
Serous	6 (28.6)
Grade	
G1	10 (47.6)
G2	6 (28.6)
G3	5 (23.1)
TCGA group	
POLE	5 (23.8)
MSI	5 (23.8)
CNL	5 (23.8)
CNH	6 (28.6)

Table 1. Description of the main clinical and pathological characteristics of the twenty-one samples.

TempO-Seq technology

The other technique used to study gene expression at the whole transcriptome level was TOS (BioSpyder, Carlsbad, CA, USA) using the Whole Human Transcriptome v1 Kit. This assay interrogates 22,575 probes targeting all human coding genes. Library synthesis is based on the use of detector oligonucleotide (DO) pairs, each targeting a specific 25-nucleotide sequence. These DOs are hybridized to the tissue sample and lysis buffer is added to the reaction. Nuclease digestion is then performed to remove excess DOs. Finally, a unique sequence tag and sequencing adapters are ligated to each hybridized sample by PCR, enabling demultiplexing of the sequencing results.

Labeled amplicons were purified using NucleoSpin gel and PCR cleanup kits (Macherey–Nagel, Düren, Germany). Libraries were sequenced on a NextSeq 2000 instrument (Illumina, San Diego, CA, USA).

Alignment and extraction of initial read counts from FASTQ files were performed using the TempO-SeqR workflow, which provides summaries and raw data, including mapped and unmapped reads. Read counts were normalized to CPM.

TOS has 22,575 probes, but some of them map to different transcripts of the same gene; multiple transcripts of the same gene are collapsed before analysis.

It is important to note that HTP consists of 19,616 genes and that not all probes match between TOS and HTP. Therefore, subsequent analysis was performed on 18,506 mRNAs common to both kits.

Quality control

Three quality controls were implemented in HTG sequencing as outlined by the manufacturer. QC1 establishes the minimum number of reads required to ensure repeatability of results, with a cutoff of 1.5 million reads. QC2 sets the Relative Standard Deviation (RSD) cutoff (a value >0.15 is recommended), which is the ratio of the standard deviation to the mean of log2 (count + 1) for each probe. Finally, the number of unmapped reads provides an overall assessment of the quality of the run (QC3).

The TOS analysis implements two quality controls: the first is a coverage depth greater than 2 million reads per sample, and the second is the percentage of unmapped reads per sample. However, HTP QCs have also been applied to TOS to compare both approaches on an equal basis.

Batch effect control

Two different methods were implemented to control and correct batch effects between the two strategies tested. ComBat, which uses a Bayesian framework to adjust data for batch effects[10]; and RUV (Removal of Unwanted Variation), which fits a linear model using spike-in controls (RUVs), housekeeping genes (RUVg), or the residuals from the first-step linear model fit (RUVr)[11].

Tertiary analysis

The following tertiary analyses were performed to compare HTP and TOS:

Differential Expression (DE) Analysis: DE analysis was performed using linear models, with the limma package implementing the voom transformation of the raw data[12] , and using a model with a negative binomial distribution implemented in the DESeq2 package[13]. To compare DE from different perspectives, genes in both populations were compared using limma top 2000 DE genes analysis. With DESEq2, genes showing a false discovery rate <0.1 and an absolute log2 fold change greater than 0.6 are considered DE genes.

PAM50 is a robust prognostic gene signature developed to stratify breast cancer into five intrinsic subtypes: Luminal A, Luminal B, Basal-like, Her2-enriched and Normal-like, each with a different prognosis. *Absolute Intrinsic Molecular Subtyping (AIMS)* is a method for assigning these groups from RNA-seq data. The algorithm is implemented in the AIMS R package. Even though this complex biomarker lacks application in EC was chosen as a generic test to compare multi-gene reproducibility between both approaches[14]. Another valuable tool is the *ESTIMATE* (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) algorithm, which uses RNA-seq gene expression data to predict tumor burden and stromal and immune cell infiltration in tumor tissue samples. Three scores are calculated from this approach: stromal and immune, which measures the infiltration of stromal and immune cells in the sample; and the ESTIMATE score, which combines the above information. This algorithm can also infer tumor purity[15].

CIBERSORTx is a computational technique for the analysis of cellular composition in bulk RNA samples. This method uses deconvolution to infer the proportion of different cell lineages in RNA purified from mixed samples, such as those obtained from tumors[16].

Downsampling

To assess the impact of differences in flow-cell capacity between sequencers, downsampling per-sample library size by count-level rarefaction using the vegan package in R was performed. For each matched sample, rarefaction target was fixed to the total read count observed with the lower-capacity sequencer (NextSeq 550). The specified number of reads was then drawn, without replacement, from the corresponding count vector obtained on the higher-capacity sequencer (NextSeq 2000). This procedure yields integer downsampled counts whose totals match the target and preserves within-sample relative composition in expectation, enabling like-for-like comparisons of read counts between sequencers.

Statistics

Chi-squared and Fisher exact tests were used to compare categorical frequency variables. Non-parametric Wilcoxon and Kruskal–Wallis tests were used for continuous variables. Pearson correlation was used to assess the relationship between gene expression across samples. All statistical analyses were conducted using R v4.3.3.

Results

Quality control

Quality control metrics were applied to ensure the reliability of the results for both HTP and TOS approaches. QC1, which evaluates the number of mapped reads for each sample, showed satisfactory results for all 21 EC samples and three RNA controls for both techniques. QC2 evaluated the RSD of gene expression. While all samples processed with TOS showed an RSD greater than 0.15, two samples processed with HTP failed in this regard (Fig. 1). The main difference between the two approaches was the number of mapped reads. TOS showed an average mapping rate of 68.44%, with a median of 88.09% (range: 33.25–94.94), while HTP showed a higher average mapping rate of 92.15%, with a median of 92.42% (range: 89.9–93.1).

Batch effect removal

Both ComBat and RUVg methods were used to address the batch effect (Fig. S1). ComBat was found to be the best at removing the batch effect and achieving consistency in gene expression across matched samples (Fig. 2). Both methods, RUVg and ComBat, showed a mean Pearson correlation of 0.77 and a median of 0.81 (range: 0.55–0.93 and 0.64–0.89, respectively). However, ComBat showed a lower spread of consistency (0.085 standard deviation) compared to RUVg (0.1 SD). ComBat also had a higher minimum correlation (0.64) than RUVg (0.55) (Fig. 3), indicating slightly better performance in batch effect correction (Table 2). Principal Component Analysis (PCA), performed to visually test for batch effect, further confirmed the effective removal of batch effect for both tools (Fig. 2).

Gene expression analysis: TOS vs. HTP

TOS and HTP were also compared in terms of their gene expression profiles. TOS examines the expression of 19,683 mRNAs (after collapsing all transcripts from the same gene), while HTP examines 19,616. Eighty-nine percent (18,506) of these overlap between the two approaches. To mimic the primary analyses, a DE analysis was performed using both limma and DESeq2 tools. Among the top 2000 genes (1005 up/995 down in TOS and 1072 up/928 down in HTP), 410 genes (20.5%; 270 up/140 down) were detected as differentially expressed in both methods using limma. Focusing on fold changes, the correlation between the two technologies was 0.857.

When DE analysis was performed with DESeq2, 808 mRNAs (536 up/272 down) were identified as differentially expressed with TOS, while only 26 mRNAs (12 up/14 down) were found with HTP. Among them, 6 common mRNAs (1 up/5 down for both TOS and HTP) were detected by both methods with a fold change correlation of 0.731 (Fig. 4). In this case, the top DE genes are considered as those with an adjusted p-value less than 0.1.

Tertiary analysis

To further investigate the performance of both methods, we applied several analyses commonly used in breast cancer research that are also relevant to endometrial cancer.

First, intrinsic subtype assignment was assesed. Because the PAM50 pipeline is frequently applied to data from such targeted platforms, comparing its output between HTP and TOS is of significant technical interest.

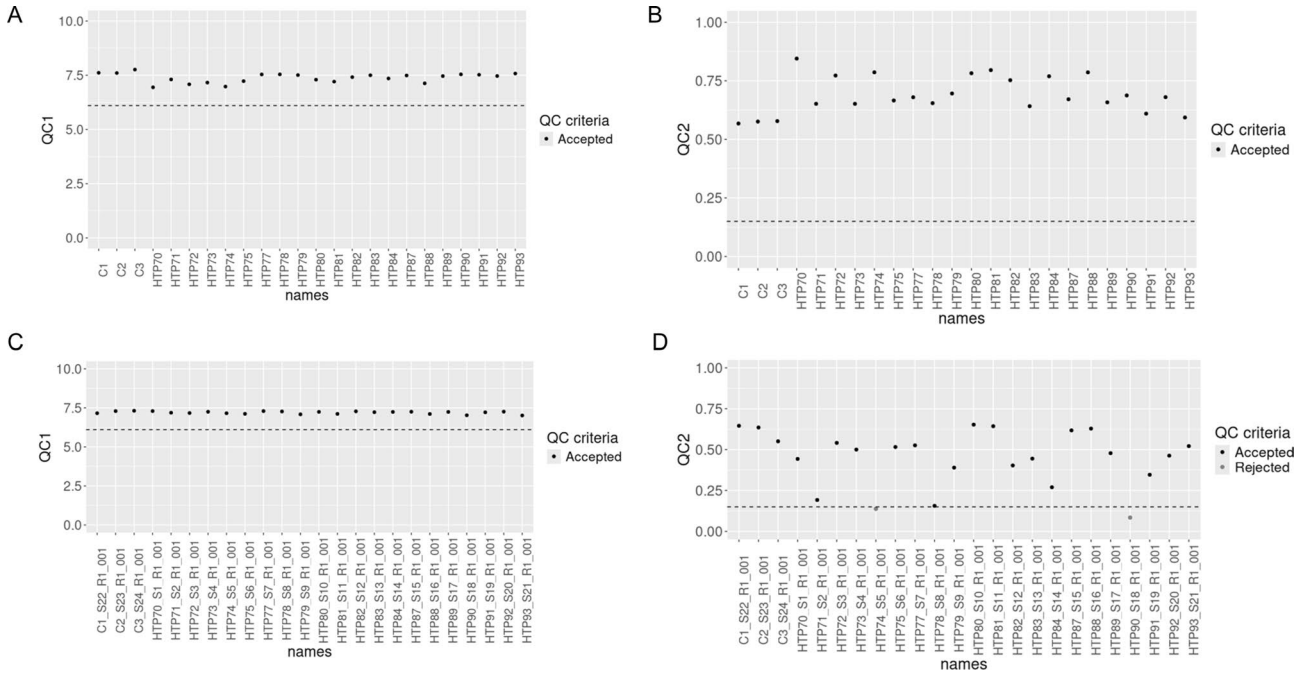

Fig. 1. Quality control of the two approaches used. (**A**) QC1 studying total number of reads using TOS. (**B**) QC2 studying relative standard deviation with TOS. (**C**) QC1 and (**D**) QC2 using HTP.

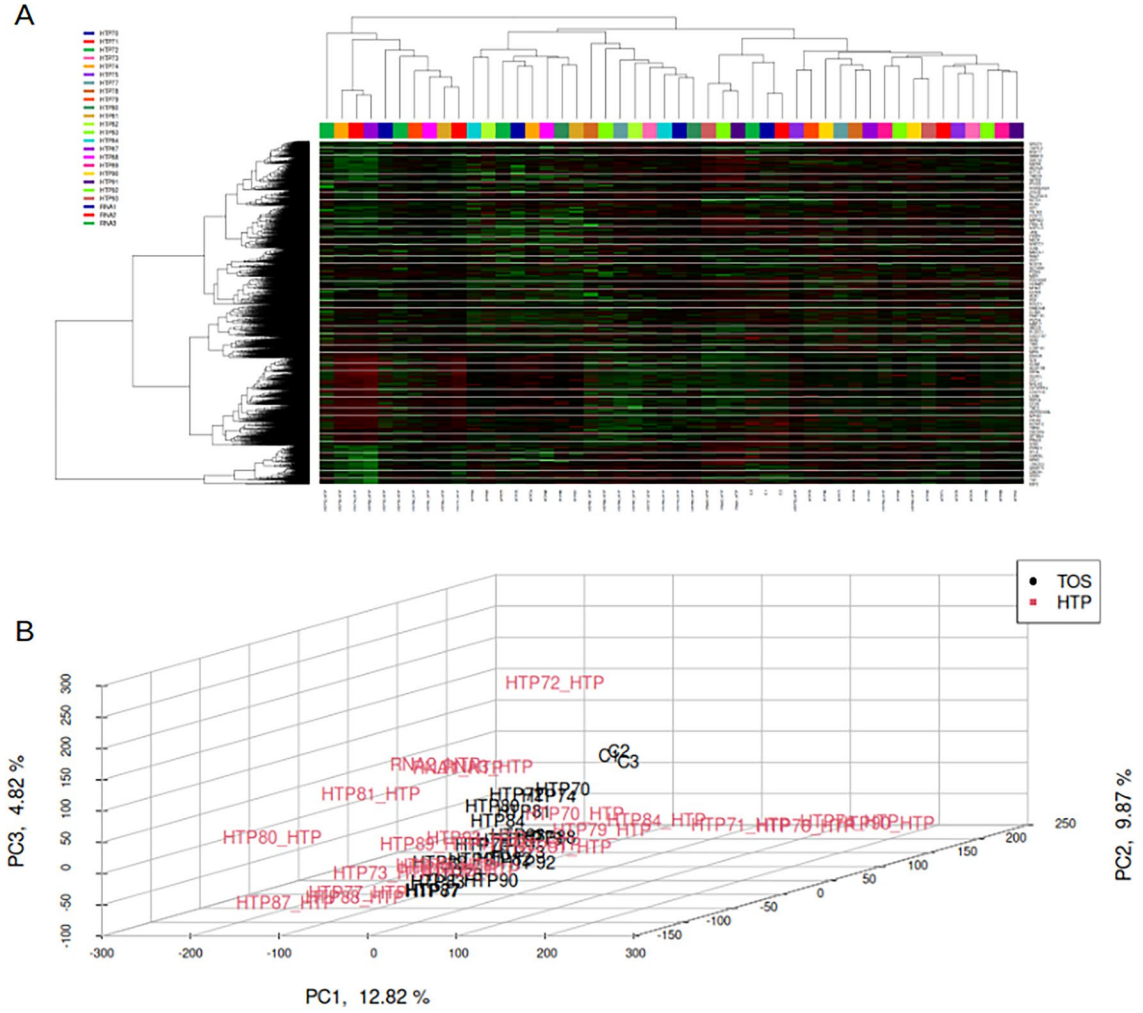

Fig. 2. (**A**) Principal components plot in 3 dimensions and (**B**) heatmap using bayesian framework (ComBat).

The TOS analysis classified 8 samples as Normal-like, 1 as Luminal A, 9 as HER2-enriched and 4 as Basal, while HTP classified 10 as Normal-like, 6 as HER2-enriched and 6 as Basal. The concordance between the two systems was 83.3%, with two samples migrating from the HER2-enriched subtype analyzed by TOS to normal by HTP, 1 sample migrating from Luminal A to Normal-like, and 1 sample migrating from Normal-like to Basal (Fig. 5).

Additionally, ESTIMATE was used to evaluate the tumor microenvironment. .No statistically significant differences were observed in ESTIMATE-derived scores between platforms ($p = 0.24–0.39$) (Fig. 6). However, given the limited sample size (n = 21), this analysis may be underpowered to detect small-to-moderate differences. Therefore, the absence of statistically significant differences should not be interpreted as formal equivalence between platforms, but rather as an indication that no systematic bias was detected in this dataset.

It is interesting to note that the ImmuneScore obtained from both platforms matches the expected values for the four EC molecular prognostic subtypes (POLE, MSI, CNL and CNH) as developed in the TCGA Endometrial Cancer Study (Figure S2)[17].

Finally, CIBERSORTx was used to assess the cellular composition of infiltrating immune cell subsets (22 cell lineages) in the 21 samples, using the LM22_source_GEPs.txt as a reference matrix for gene expression. This signature matrix was derived from microarray data of 22 immune cell types [18]. No significant differences were observed between TOS and HTP for the proportion of the 22 immune cell types, with a median p-value of 0.607 (0.051–0.991) as shown in Figure S3.

Downsampling analysis

To ensure that findings were not driven by differences in sequencer flow-cell capacity, biomarker analyses were repeated on a downsampled TOS dataset. We then compared TOS (NextSeq 2000)—both original and downsampled—against HTP (NextSeq 550).

Differential-expression (DE) analysis with limma identified 357 mRNAs (87.1%) shared between the two contrasts (TOS vs HTP and downsampled TOS vs HTP) (Figure S4). This result was corroborated with DESeq2, which showed good concordance, with 5 of 6 samples (83.3%) commonly deregulated (Figure S5).

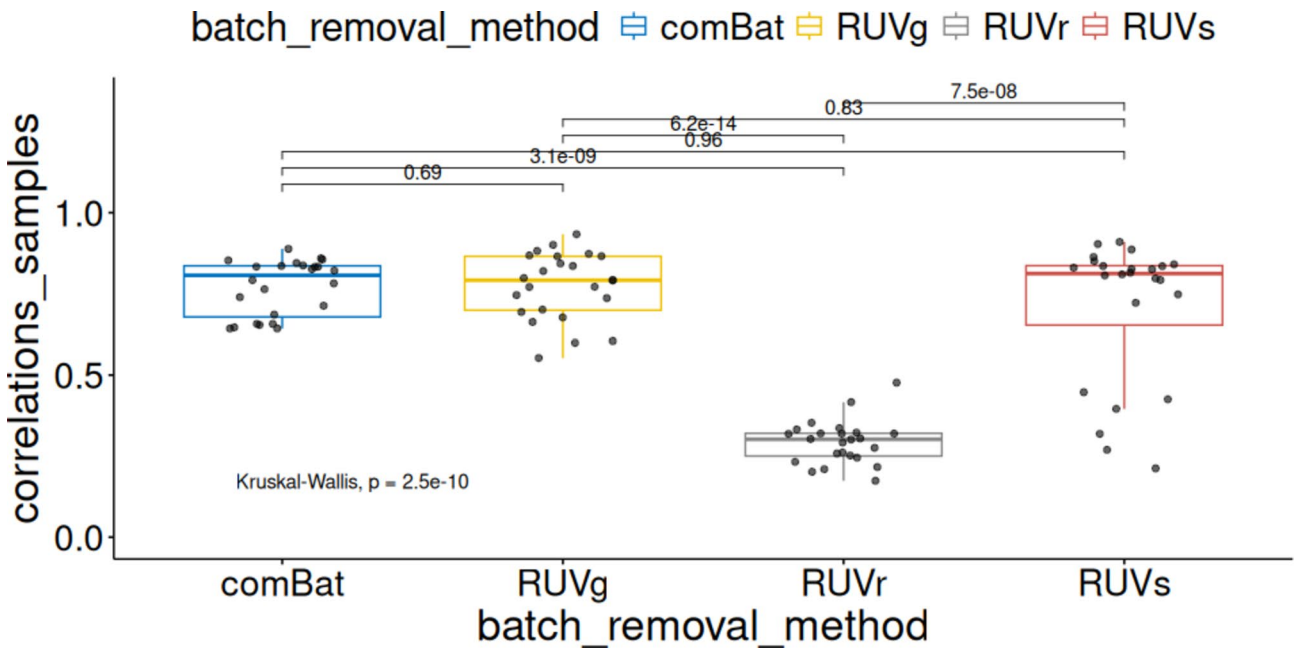

Fig. 3. Boxplot showing correlation between matched samples analyzed with both kits when different batch removal methods are applied.

Method	Mean	Sd	Min	Pctl. 25	Pctl 50	PCTL 75	Max
No batch removal	0.59	0.110	0.34	0.52	0.63	0.66	0.74
ComBat	0.77	0.085	0.64	0.68	0.81	0.84	0.89
RUVg	0.77	0.100	0.55	0.70	0.79	0.87	0.93
RUVr	0.29	0.068	0.17	0.25	0.30	0.32	0.48
RUVs	0.71	0.220	0.21	0.65	0.81	0.84	0.91

Table 2. Main characteristics of the methods used to remove batch effect.

Tertiary analyses yielded consistent results as well. First, AIMS subtype assignments were concordant in 79.2% (19/24) of samples; subtype transitions are shown in Figure S6. Second, ESTIMATE scores did not differ significantly between platforms: stromal score $p = 0.2405$ (TOS vs HTP) and $p = 0.2897$ (downsampled TOS vs HTP); immune score $p = 0.3900$ and $p = 0.4908$; ESTIMATE score $p = 0.2768$ and $p = 0.3748$; and tumor purity $p = 0.3029$ and $p = 0.3902$, respectively (Wilcoxon tests; Figure S7).

Finally, comparisons of the 22 immune cell fractions (CIBERSORTx) are presented in Figure S8; notably, only M2 macrophages reached nominal significance ($p = 0.0475$, unadjusted).

Discussion

The analysis of the whole transcriptome in FFPE tumor samples is an unmet need. Traditionally, HTG EdgeSeq addressed this need with exonuclease protection targeted probes with the so-called HTP (Human Transcriptome Panel), which interrogates 19,616 mRNAs, covering virtually the entire human transcriptome. With the bankruptcy of the company, the unavailability of this approach has given rise to new solutions from other manufacturers. Among these, BioSpyder's TOS (TempO-Seq) kit is an interesting substitute that fills the scientific gap left by HTP. Both technologies perform only targeted sequencing, which offers two main advantages: eliminating the need for RNA isolation and optimizing sequencing capacity thereby reducing costs. The aim of this study was to evaluate whether the results obtained with TOS are comparable to those of HTP, and to demonstrate that there is a reliable alternative for researchers who previously used HTG technology. To our knowledge, there are few articles comparing TOS with other techniques such as qRT-PCR[19], but the study comparing two RNA-seq approaches using targeted probes is novel.

Both approaches had slightly different performances. Both provided a reasonable number of reads per sample to draw valid biological conclusions. Interestingly, TOS showed better performance in terms of RSD, being more efficient in this aspect. All samples analyzed with TOS met this criterion, while two HTP samples showed RSD below the threshold set by EdgeSeq to compare both technologies. These results may be related to the different flow cell types used (NextSeq 550 high-capacity for HTP vs. NextSeq 2000 P3 for TOS kit). However, it is important to consider the different capacity of these two kits: 400 million reads for the former and 1200 million reads for the latter.

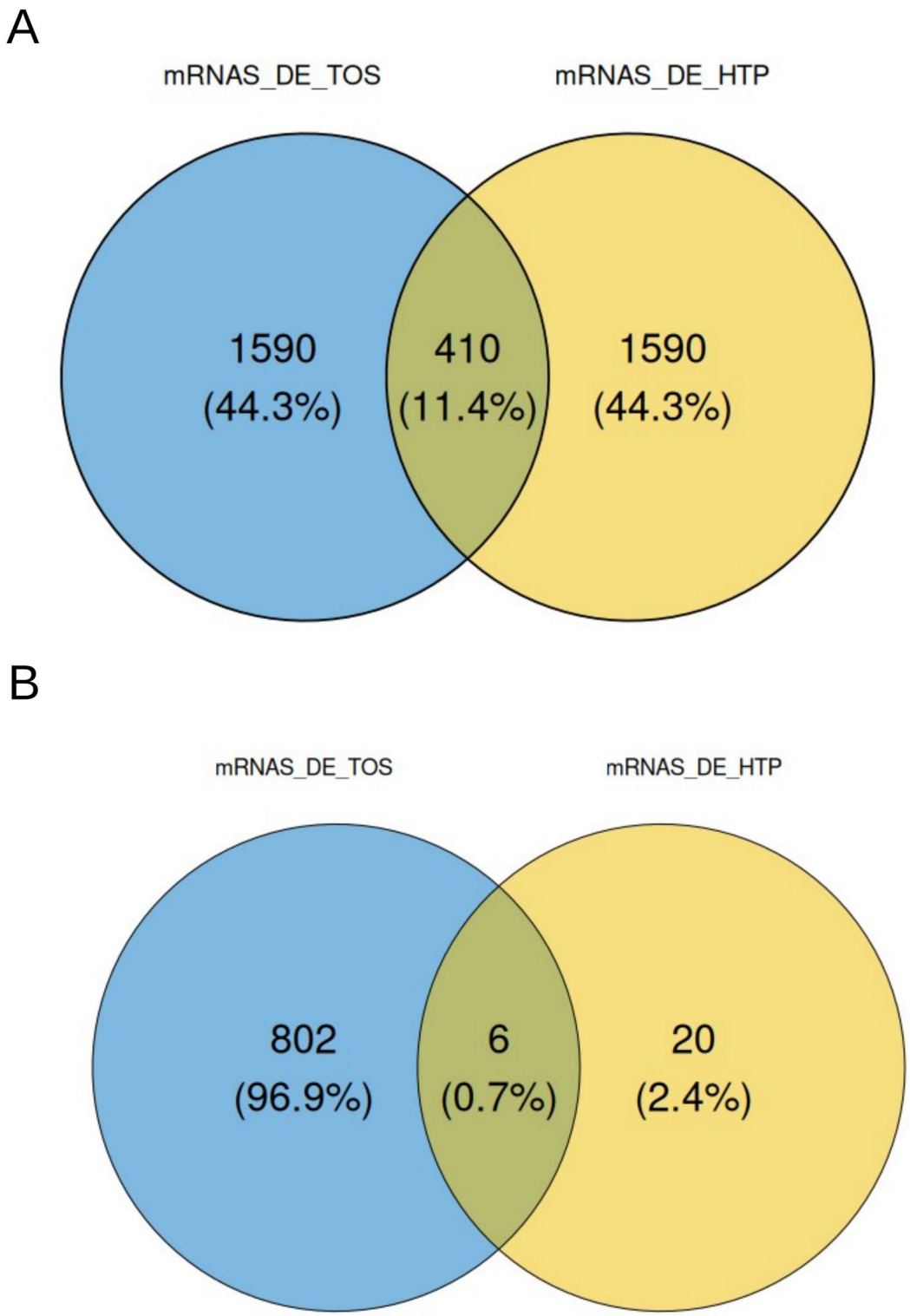

Fig. 4. results of the DE analyses (**A**) common mRNAs between both approaches using limma. (**B**) Using DESeq2 method.

On the other hand, the remarkable difference in the number of mapped reads, which was significantly higher in HTP, should be highlighted. This could be partly due to the fact that the TOS protocol is entirely manual, while the HTP protocol automates the hybridization part, making HTP sequencing more productive for the same flow cell capacity. To mitigate this effect, BioSpyder incorporates a proprietary attenuation technology in its TOS kit to prevent overrepresentation of the most highly expressed genes, saving a significant amount of sequencing capacity. To account for the potential source of confounding regarding different capacity of

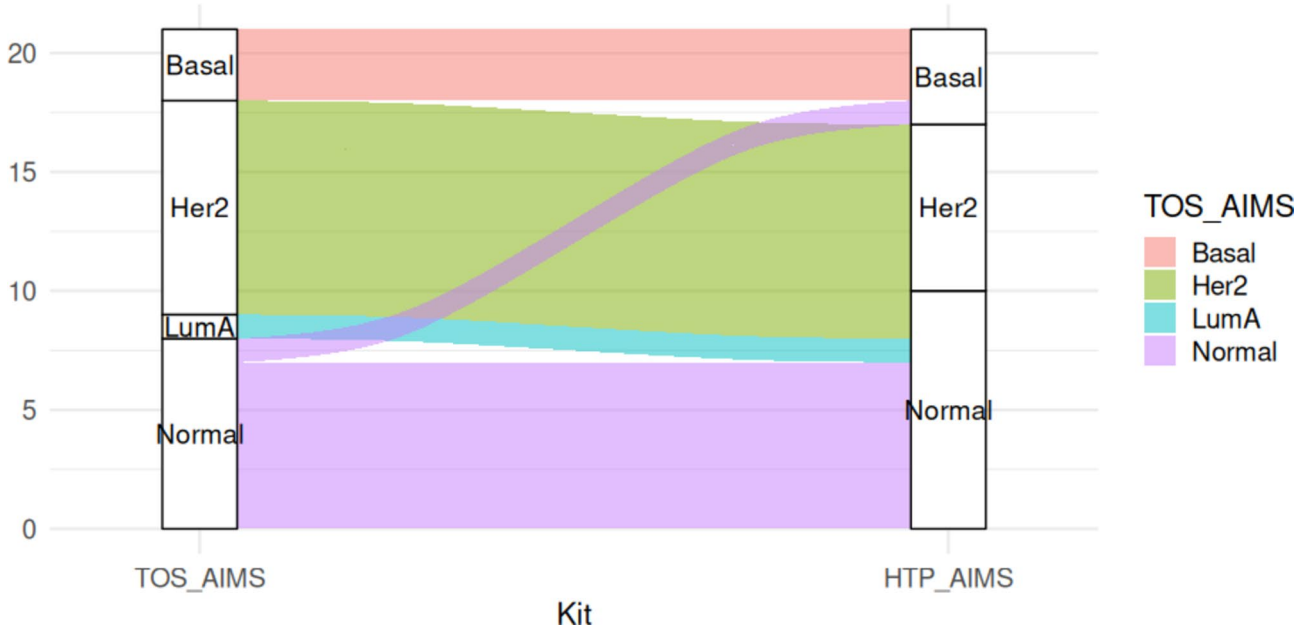

Fig. 5. PAM50 intrinsic subtype migration analyzing samples with both TOS and HTP.

sequencers used, we conducted all comparisons in parallel using the downsampled TOS dataset against HTP, which yielded concordant results.

In RNA-seq experiments, the batch effect is related to the experimental conditions. In particular, the different probes used to interrogate gene expression introduce additional bias into the results. Therefore, it is essential to properly normalize the data and to test and compare different batch effect removal algorithms[20,21]. Comparison was made in terms of Pearson correlation between paired samples analyzed with both genes and their standard. ComBat and RUVg (using housekeeping genes) show a median correlation of 0.77 (range: 0.55–0.93 and 0.64–0.89, respectively), but the standard deviation is 0.085 for the first method and 0.1 for the second. Furthermore, the minimum correlation is 0.64 with ComBat and 0.55 with RUVg. Therefore, for this scenario, the removal of the batch effect from the Bayesian framework was chosen with ComBat. RUVr was included in the comparison even though there are no biological replicates in the experiment. Considering this issue, its performance could be diminished in this scenario[22].

DE studies are a mainstay of RNAseq and microarray studies. There is a common strategy to study pathological conditions using case–control transcriptomic studies. Therefore, two common methods were used and compared: DESeq2 package (based on negative binomial distribution) and limma package (linear method with voom transformation). DESeq2 identified 808 DE mRNAs in TOS with an adjusted p-value < 0.1, while only 26 DE genes were identified in HTP. Of these, 6 were common to both approaches. On the other hand, using limma, 410 (20.5%) of the top 2000 DE mRNAs were common to both kits. This second strategy showed better concordance as no p-value adjustment was applied. However, the shared dysregulated genes identified using limma exhibited a strong correlation between platforms ($R = 0.75$, $p < 0.001$), whereas those analyzed with DESeq2 showed a more moderate relationship ($R = 0.37$, $p = 0.5$), likely due to the lower number of dysregulated mRNAs detected by this method (Figure S9). . The limited concordance observed between limma- and DESeq2-based differential expression results at the single-gene level is not unexpected in the context of this study. The analysis was not designed to detect strong biological contrasts, but rather to assess technical comparability between platforms using a relatively small cohort and targeted transcriptomic panels. In such settings, differences in probe design, transcript collapsing strategies, normalization procedures, and statistical modeling assumptions can result in variability in individual gene-level calls. Importantly, this variability does not indicate poor performance of either method, but rather reflects the inherent sensitivity of single-gene differential expression analyses to technical and methodological factors in low-contrast datasets. Consistent with this interpretation, concordance between platforms and methods improved markedly when expression was evaluated using multi-gene biomarkers and pathway-level scores, which are inherently more robust to probe-level noise and platform-specific effects. Furthermore, the probes and the sequencing they map to are different in both kits. All map to mRNA of coding genes, but TOS has probes for more than 1 transcript of 2892 genes. In these cases, the expression of all transcripts is averaged, which is a source of bias, especially when inference is done gene-by-gene. Another reason for the relatively low concordance is the previously reported fact that concordance in DE genes is related to the difference between the groups being compared[23]. In this case, differences are not warranted because there are user-defined groups being compared with no biological significance. At this level, validation with a well-established low-throughput technique such as qRT-PCR would be helpful but is beyond the scope of this two-way comparison. . It should be noted that both platforms rely on proprietary probe sets whose exact exon positions and targeted transcript regions are not publicly disclosed. This restriction prevents the design of qPCR primers that specifically match the same target regions, limiting the interpretive value of a conventional

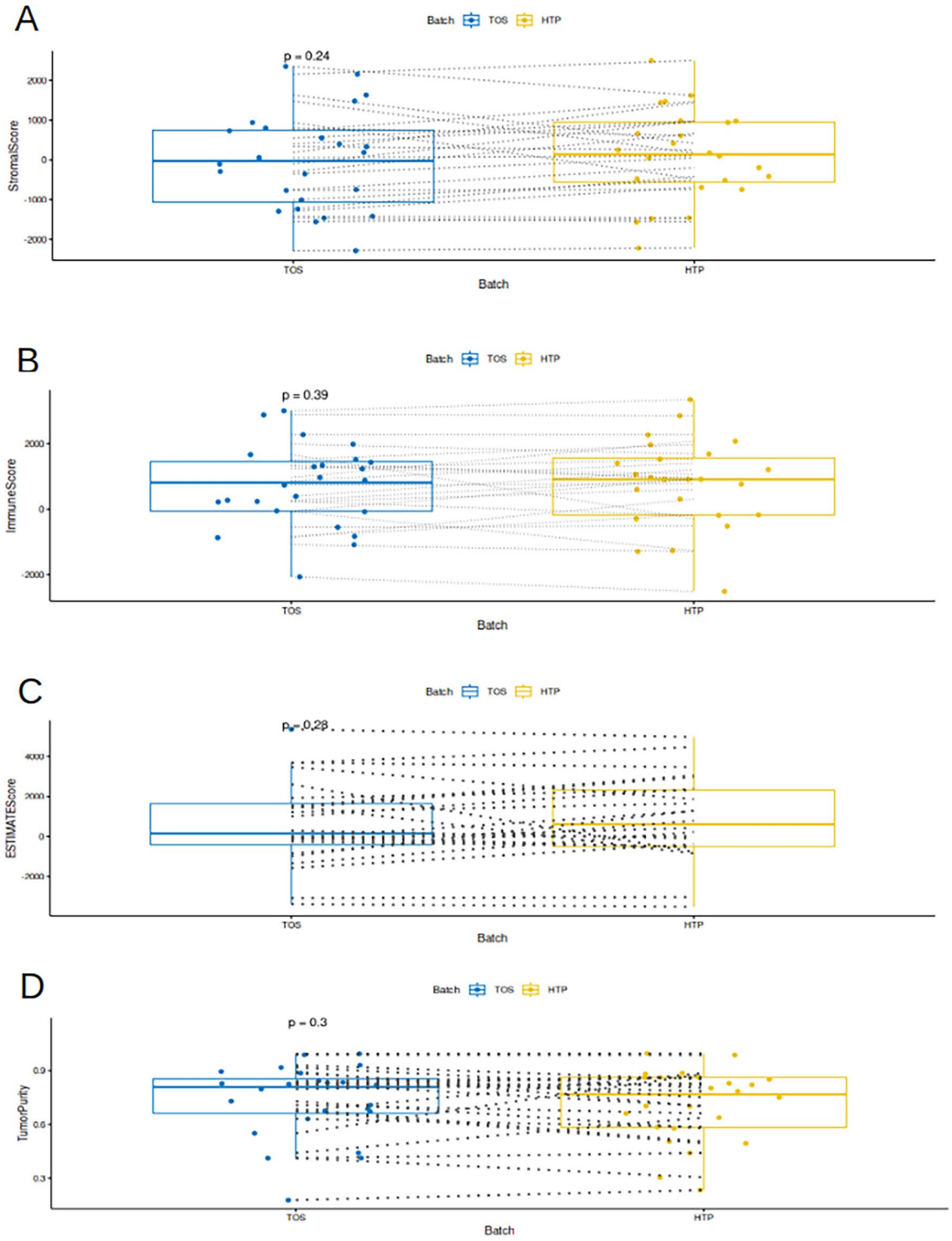

Fig. 6. comparison of results of ESTIMATE algorithm, (**A**) stromal score (**B**) immune score (**C**) ESTIMATE score (**D**) tumor purity.

qPCR validation. Consequently, alternative validation approaches that are independent of platform-specific probe design—such as cross-platform correlation analysis, complex biomarker preservation, or comparisons of global expression patterns—may provide a more objective benchmark for assessing concordance.

An intermediate step between gene-based DE analysis and total correlation between paired samples is the use of complex biomarkers involving a larger number of mRNAs. We use these complex biomarkers merely as technical benchmark. Even though the first of them is specific to breast cancer. First, the AIMS algorithm was used to infer PAM50 intrinsic molecular subtypes using 100 decision rules[14]. Concordance between the 5 intrinsic subtypes is 83.3%, with 4 samples migrating between subtypes. Since this algorithm is based on 100

	Rho Spearman TOS HTP (*p*-value)	Concordance rate TOS HTP (*p*-value)	Kappa index TOS HTP (*p*-value)	Rho Spearman TOS downsampled HTP (*p*-value)	Concordance rate downsampled TOS HTP (*p*-value)	Kappa index TOS HTP (*p*-value)
AIMS	0.256 (0.2274)	0.833	0.733	0.255 (0.2282)	0.792	0.667
ESTIMATE	–	–	–	–	–	–
Stromal Score	0.797 (3.08 e−6)	–	–	0.793 (3.87 e−6)	–	–
Immune Score	0.796 (3.2 e−6)	–	–	0.811 (1.5 e−6)	–	–
Estimate Score	0.615 (0.0013)	–	–	0.638 (0.0008)	–	–
Tumor Purity	0.615 (0.0013)	–	–	0.638 (0.0008)	–	–
CIBERSORTx	–	–	–	–	–	–
B cells naive	− 0.051 (0.813)	–	–	− 0.294 (0.164)	–	–
B cells memory	− 0.226 (0.288)	–	–	− 0.290 (0.170)	–	–
Plasma cells	0.495 (0.014)	–	–	0.521 (0.009)	–	–
Tcells CD8	0.373 (0.072)	–	–	0.416 (0.043)	–	–
T cells CD4 naive	− 0.406 (0.049)	–	–	− 0.443 (0.030)	–	–
T cells CD4 resting memory	0.109 (0.612)	–	–	0.229 (0.283)	–	–
T cells CD4 memory activated	− 0.188 (0.379)	–	–	− 0.159 (0.459)	–	–
T cells follicular helper	0.302 (0.152)	–	–	0.199 (0.351)	–	–
T cells regulatory	0.214 (0.315)	–	–	0.083 (0.701)	–	–
T cells gamma delta	0.112 (0.602)	–	–	0.028 (0.895)	–	–
NK cells resting	0.448 (0.028)	–	–	0.430 (0.036)	–	–
NK cells activated	0.229 (0.282)	–	–	0.233 (0.273)	–	–
Monocytes	0.338 (0.107)	–	–	0.413 (0.045)	–	–
Macrophages M0	0.491 (0.015)	–	–	0.498 (0.013)	–	–
Macrophages M1	0.295 (0.161)	–	–	0.077 (0.721)	–	–
Macrophages M2	0.085 (0.694)	–	–	0.150 (0.483)	–	–
Dendritic cells resting	0.240 (0.259)	–	–	0.186 (0.384)	–	–
Dendritic cells activated	0.525 (0.008)	–	–	0.314 (0.135)	–	–
Mast cells resting	0.473 (0.020)	–	–	0.667 (3.7e−04)	–	–
Mast cells activated	0.155 (0.470)	–	–	0.071 (0.741)	–	–
Eosinophils	0.260 (0.219)	–	–	0.319 (0.129)	–	–
Neutrophils	0.484 (0.016)	–	–	0.375 (0.071)	–	–

Table 3. Metrics of the complex biomarkers comparison of TOS HTP data and TOS downsampled HTP.

decision rules, it appears that correlations with a greater number of mRNAs involved are better preserved when samples are analyzed with both approaches as the number of genes studied increases.

Additionally, the ESTIMATE algorithm[15], which infers immune, stromal and ESTIMATE scores and tumor purity based on the expression of 282 genes, showed no significant differences in score assignment between the TOS and HTP. This complex biomarker was evaluated by nonparametric statistical inference using the paired sample Wilcoxon signed rank test, with p-values of 0.24, 0.39 and 0.28 for the stromal, immune, and ESTIMATE scores, respectively. In addition, tumor purity predictions were similar using both methods ($p = 0.3$). However An important limitation of this study is the relatively small sample size, which limits statistical power and precludes formal equivalence testing between platforms. While ESTIMATE scores did not show statistically significant differences in this cohort, larger studies will be required to robustly assess equivalence and to determine the sensitivity of these metrics to platform-specific effects.

Cibersortx is a deconvolution method for immune cell type assignment from RNA-seq data[24,25]. This analysis showed no differences between the median frequencies of cell types in paired samples analyzed with TOS and HTP. Notably, this method relies on the expression of 548 mRNAs, underscoring its sensitivity to technical and platform-specific variation.

Overall, several key considerations emerge from this comparison. First, effective batch-effect removal is critical when comparing platforms. Second, the reliability of transcriptomic biomarkers strongly depends on the number of genes involved: while global correlations provide the most stable comparisons, single-gene analyses are the most sensitive to technical variability. Complex biomarkers represent an intermediate solution with acceptable and reproducible performance, as summarized in Table 3. In this context, ESTIMATE-based scores showed a high level of consistency between platforms, supporting their robustness as multi-gene biomarkers, whereas CIBERSORTx-derived cell-type estimates exhibited more variable agreement across platforms, reflecting the increased sensitivity of deconvolution-based approaches to technical and platform-specific factor.

Therefore, best strategy for combining the analysis of two different approaches is to analyze the data generated for the discovery and validation steps separately, rather than merging datasets directly.

In conclusion, TOS is a good alternative for analyzing samples in the scenarios previously covered by HTG's HTP approach, i.e. a targeted RNA-seq panel covering the entire transcriptome. Besides substantially lower read-depth requirements of TOS inherently reduce sequencing costs. However, precautions should be taken: DE analysis may be biased by the probe composition of both kits, and the number of DE genes depends on intrinsic differences between the biological groups being compared. The best strategy is to focus on transcriptomic biomarkers that involve multiple genes. Such multi-genes signatures proved to be more stable and thus more transferable between both approaches that single-gene readouts. In summary, complex multi-gene signatures can be reliably compared across platforms, whereas single-gene differential expression analyses are not recommended for direct cross-platform comparison due to probe composition and design differences. Importantly, this study was designed as a technical benchmarking analysis rather than a biological discovery effort. Consequently, the observed differences and concordances between platforms should be interpreted in terms of methodological performance, probe design, and data processing strategies, rather than as indicators of biological variability or disease-associated expression changes. Overall, this study should be considered an initial validation demonstrating the potential utility of TOS as an alternative to HTP. It provides a foundation for broader, large-scale, or orthogonal validation efforts aimed at establishing full equivalence between both approaches. TOS performs well in this scenario, providing high dynamic range in a cost-effective manner. Another evident limitation from this study is that it only validates relative agreement between both techniques, since HTG was widely used in transcriptomic analyses and thus has intrinsic value.

Data availability

The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, under the accession number GSE300263.

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Author contributions
R.L.R., A.F.S. and J.A.L.G. contributed to the study design. I.R. and J.A. contributed to the data acquisition. R.L.R. and A.F.S. contributed to data analysis and statistics. R.L.R., A.F.S. and J.A.L.G. contributed to manuscript preparation, editing and reviewing.

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The authors declare no competing interests.

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