Evaluation of Sequencing Approaches for High-Throughput Transcriptomics

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Science and Decision Context

Whole-genome in vitro transcriptomics has shown the capability to identify mechanisms of action and estimates of potency for chemical-mediated effects in a toxicological framework, but with limited throughput and high cost. The generation of high-throughput global gene expression profiles using RNA-sequencing technologies for the evaluation of chemically-mediated effects could greatly advance the current toxicogenomics knowledgebase. We present the evaluation of three transcriptomics platforms for potential application to high-throughput screening: 1. TempO-Seq utilizing custom designed paired probes per gene; 2. Targeted sequencing utilizing Illumina's TruSeq RNA Access Library Prep Kit containing tiled exon-specific probe sets; 3. Low coverage whole transcriptome sequencing using Illumina's TruSeq Stranded mRNA Kit. In summary, the three transcriptomics platforms showed the ability to measure whole-genome transcript levels with good technical reproducibility and show promise for the integration of transcriptomics into high-throughput screening.

Approach

Three high-throughput sequencing (HTS-Seq) approaches were evaluated to assess technical and functional performance in order to characterize the limitations and possible applications of HTS-Seq technologies.

(MAQC= Microarray Quality Control; SEQC = Sequencing Quality Control)

Low Coverage Sequencing

- Omega Bio-Tek Mag-Bind Total RNA kit to **isolate total RNA**
- Library prep with **Illumina Stranded mRNA Sample Prep :** isolate mRNA using poly-T oligo attached to magnetic beads, fragment purified mRNA, copy first strand cDNA with random primers, purify, enrich with PCR
- Pooled libraries **sequenced on Illumina HiSeq 2500**

~ 11 million aligned reads per sample

Targeted Sequencing

- Omega Bio-Tek Mag-Bind Total RNA kit to **isolate total RNA**
- Library prep with **Illumina TruSeq RNA Access Library Prep Kit:** fragmentation, cDNA generation by random priming, ligate polyA sequencing adaptors, coding regions captured using optimized probe set
- **Pooled libraries sequenced on Illumina HiSeq 2500**

~20 million aligned reads per sample

TempO-Seq

- **Cell lysate** input (ie. capture-free method)
- **Detector oligos annealed** (50 base probes, two per gene, designed to target a gene-specific region; have adaptor sequences allowing sample-specific barcodes to be used)
- Illumina adaptors on ligated detector oligos ultimately enable standard dual index sequencing

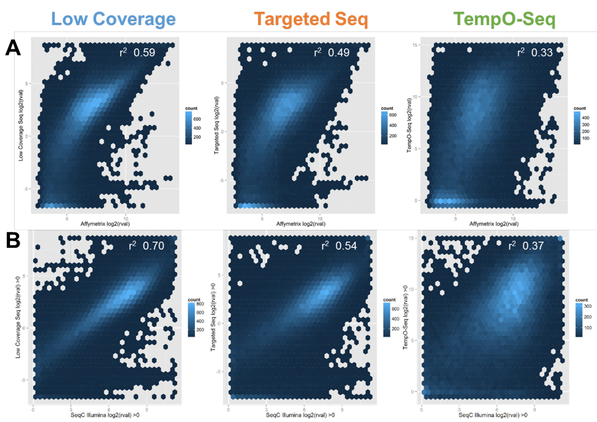
~34 million mapped reads per sample

Use all three sequencing technologies to quantify gene expression for MAQC control samples A and B as well as samples from MCF7 cells treated with a single concentration of five chemicals for 6 hrs.

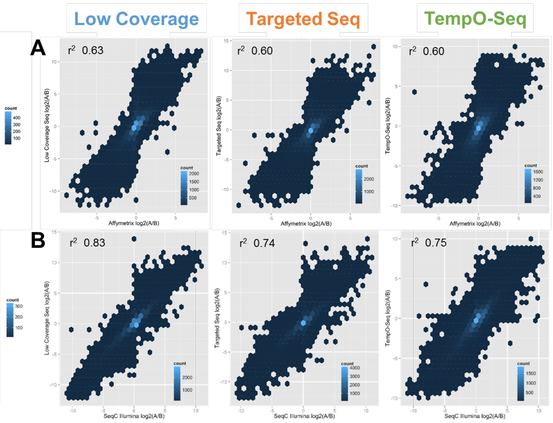
Objectives for the evaluation of three HTS-Seq platforms:

- ► Assess technical and inter-replicate reproducibility
- ► Identify chemical-mediated differential gene expression signatures
- ► Evaluate output from Connectivity Mapping to assess functional utility for toxicogenomics screening

Evaluating Technical Performance



▲ ***Figure 1. Correlation of normalized expression values (rval) to MAQC Affymetrix (A) and SEQC Illumina (B) datasets****: The mapped reads were normalized and log2 transformed to obtain "rval". For low coverage and targeted sequencing FPKM was used. For TempO-Seq each gene was normalized as total reads relative to the average of the sum of total reads for that gene across all replicates. The lower r2 values for Targeted Seq and TempO-Seq may be due to differing probe efficiencies across genes and may not be appropriate for measuring absolue transcript abundance.*

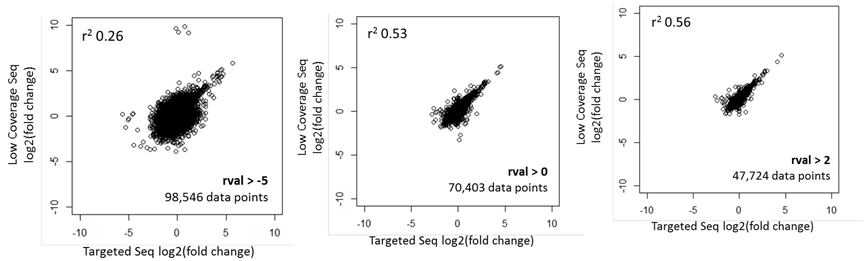


▲ ***Figure 2. Evaluation of MAQC control sample A:B ratio correlation between sequencing technologies and MAQC Affymetrix (A) and SEQC Illumina (B)****: As a surrogate for fold change, the ratio of control sample A vs. B was evaluated. Pearson's correlations (r^2) show better concordance with SEQC than microarray. The dynamic range achieved among platforms was more similar to the SEQC dataset compared with Affymetrix. All three platforms show similar performance for measuring fold-change gene expression changes.*

Table 1: Inter-replicate correlations for raw normalized values (rval)

Technology	MAQC Control A	MAQC Control B	10 µM Chlorpromazine	10 µM Ciclopirox	10 µM Genistein	100 nM Sirolimus	1 µM Tanespimycin	DMSO
Affymetrix	0.99	0.99	-	-	-	-	-	-
SeqC Illumina	0.99	0.99	-	-	-	-	-	-
Low Coverage	0.95	0.96	0.96	0.96	0.95	0.96	0.96	0.96
Targeted	0.96	0.96	0.95	0.95	0.95	0.95	0.95	0.95
TempO-Seq	0.97	0.97	0.95	0.95	0.95	0.96	0.94	0.95

Note: Chemical treatments were chosen from the Connectivity Map, encompassing unique modes of action. The inter-replicate correlations reflect Pearson's correlation (r^2) across 5 replicates. These correlations were calculated for the normalized expression values (rval). All treatments were conducted independently for each technology for 6 hrs in MCF7 cells.



◄ ***Figure 3. Chemical-treated gene expression correlation among the sequencing technologies****: Chemical-elicited gene expression (log2 fold change) from Targeted Seq and Low Coverage Seq were compared, revealing low correlation (Figure 3A). To address the effect of low rval, as determined based on the Bland-Altman plot in Figure 3B, a filter was applied requiring rval to be greater than log2(-5), log2(0), and log2(2). This filtering resulted in increased correlation, reaching r^2 0.56.*

Evaluating Functional Performance

Table 2: Number of matching mechanisms in top 10 CMAP results

Chemical	Low Coverage	Targeted Seq	TempO-Seq
Genistein	0	0	3
Ciclopirox	0	0	3
Sirolimus	0	0	2
Tanespimycin	1	1	4
Chlorpromazine	1	1	1

Note: Differentially expressed genes for CMAP were identified using filtering criteria: |fold change| > 2 and t-test p < 0.01

▲ ***Connectivity Mapping (CMAP) Analysis****: Genes identified as differentially expressed were used as input for CMAP. The resulting output was ranked based on p-value and the top ten profiles were evaluated for mechanism of action (MOA) that match the reference chemicals. Overall, TempO-Seq resulted in the most matching MOAs among the top CMAP outputs.*

Impacts

Gene expression profiles were successfully generated using three high-throughput sequencing technologies

- ► The technical reproducibility across replicates within a technology for all sequencing platforms was very high with Pearson's correlations of r^2 >0.95.
- ► The normalized expression values for MAQC control samples A and B were highly correlated to results from MAQC Affymetrix and SEQC Illumina datasets. Furthermore, the A:B gene expression demonstrated good dynamic range comparable to SEQC for all technologies, outperforming Affymetrix microarrays.
- ► Due to differing probe efficiencies across genes, Targeted Seq and TempO-Seq showed lower performance for measuring absolute transcript abundance; however, all three platforms showed high technical performance for measuring fold change gene expression changes.
- ► The TempO-Seq platform showed better functional performance for correctly identifying chemical MOA than the other two platforms; however, this may be due to differences in read depth or slight differences in cell and treatment protocols among vendors.
- ► Future work will seek to define a minimum mapped read requirement, refine how significant differential expression is identified, establish an automated in-house CMAP algorithm, and incorporate concentration-response modeling for chemical-mediated gene expression.