Towards high-throughput low-cost sustainable ecotoxicity testing

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Overview

Traditional whole animal-based toxicity testing is expensive, time-consuming, and unethical. Furthermore, these tests do not capture the full range of biological impacts[1]. There is growing interest in developing alternative methods (New Approach Methods, NAMs), that are less resource intensive (lower costs, quicker assays, fewer animals), while also providing more data on the mechanism of toxicity[2,3].

The objective of this work was to optimize and establish a high throughput in vitro transcriptomic-based toxicity testing platform for chemical and environmental risk assessment. The platform couples human Caco-2 and Hep G2 cell lines with QIAGEN's UPXome ultraplex technology, so that the combined test system can also derive transcriptomic points of departure (tPODs).

The advantages of this workflow are the microplate-based cell lysis, rRNA depletion and reverse transcription steps, and the incorporation of a unique sample index into the cDNA from each sample which result in a simpler workflow.

Methods

Human Caco-2 and Hep G2 cell lines were exposed for 24 h to solvents (dimethyl sulfoxide, DMSO and methanol, MeOH) and a positive control (hydrogen peroxide, H_2O_2) at a series of concentrations based on the OECD fish cell line test guideline-249[4].

The NGS libraries were prepared using the QIAGEN QIAseq UPXome RNA Library Kit (cat. number 334705) either manually or with the aid of the SPT Labtech mosquito® HV genomics liquid handler. The resulting libraries were assessed for quality and average library size on an Agilent Tapestation D1000 screen tape and quantified using qPCR. The sequencing was performed on Illumina NextSeq500 platform (280 samples sequenced in one lane).

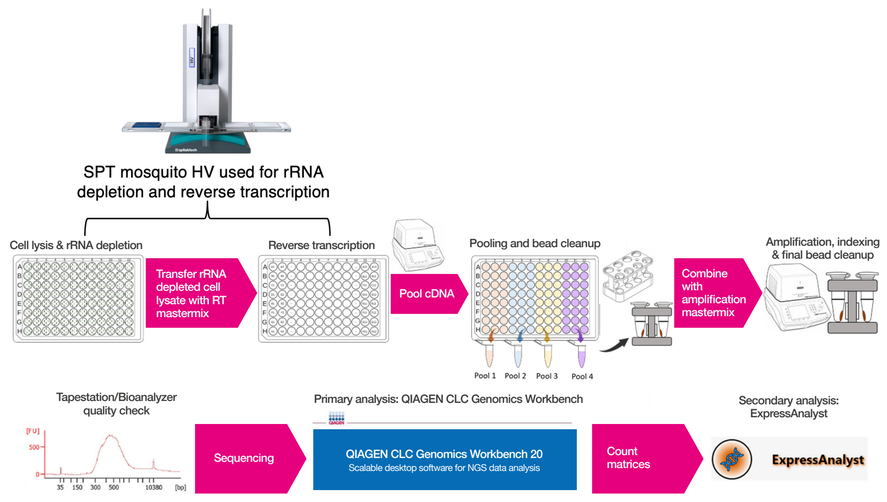

Figure 1 Schematic of the UPXome workflow (top) and downstream analysis (bottom). Caco-2 and Hep G2 cells were lysed and subjected to rRNA depletion, and reverse transcribed with oligo dT primers incorporating a unique sample index per sample, and subsequently pooled along with a bead clean-up. Pooled cDNA was indexed and amplified along with a final bead clean-up. Following a quality check, libraries were sequenced, and reads were submitted for primary (filtering and mapping) and secondary (differential expression and dose-response) analysis[5].

Results

- Overall mapping percentage of reads and percentage of reads mapping to protein coding genes using the semi-automated workflow ranged from 70-93% and 68-77% (Caco-2), and 26-82% and 72-79% (Hep G2), and using the manual workflow ranged from 69-81% and 74-77%, respectively. (Table 1)
- Based on the sequencing data, we were able to perform transcriptomic analysis, including identifying differentially expressed genes (DEGs), calculating gene benchmark doses (BMDs), and deriving tPODs.
- Many DEGs were involved in processes including stress response, cell signaling, motility and survival, and phase-II drug metabolism including heat shock proteins, selenoprotein P and angiopoietin-like 4.
- The strongest concentration-dependent transcriptomic response was observed upon exposure to DMSO with ~1,000 gene BMDs across tested cell lines while exposure to methanol resulted in the fewest gene BMDs. (Figure 2)
- While the tPODs were within 2-fold of each other, Hep G2 was generally more sensitive with lower tPODs than Caco-2 cells. (Table 2)
- Pathway enrichment analysis showed the most sensitive pathway in Caco-2 cells was Systemic lupus erythematosus upon H_2O_2 exposure (pathway-BMD = 0.42 mM), and in Hep G2 was Systemic lupus erythematosus and alcoholism upon exposure to DMSO (pathway BMD = 1.11%) and H_2O_2 (pathway-BMD = 0.78 mM).

Metadata mosquito automated vs. manual

Percentage of mapped reads	SPT Labtech mosquito HV semi-automated 0.5x volume		Manual 0.5x volume*
	Caco-2	Hep G2	Caco-2
Overall mapping	70 to 93	26 to 82	69 to 81
Mapped to genes	84 to 98	72 to 98	91 to 96
Mapped to introns	11 to 26	16 to 31	15 to 22
Mapped to exons	60 to 84	44 to 82	70 to 80
Mapped to intergenic region	2 to 13	3 to 27	4 to 9
Mapped to rRNA	0.04 to 0.89	0.03 to 0.59	0
Mapped to protein coding	68 to 77	72 to 79	74 to 77

Table 1 Range of percentage mapped reads from human Caco-2 and Hep G2 cells for the UPXome libraries prepared at 0.5x reaction volumes using the SPT Labtech mosquito HV liquid handler.*an initial trial of Caco-2 cells was conducted by manual library preparation at 0.5x volume for comparison.

Dose-response analysis

Cell lines	Caco-2 (%v/v)			Hep G2 (%v/v)		
Exposure	DMSO (%v/v)	H_2O_2 (mM)	Methanol* (%v/v)	DMSO (%v/v)	H_2O_2 (mM)	Methanol* (%v/v)
# of DEGs	1807	977	414	1802	917	653
# of DEGs fitted to curves	987	576	96	1171	488	167
# of DEGs with BMDs	964	575	69	1159	482	114
$tPOD_{20th\ gene}$	0.26	0.17	1.8	0.11	0.220	0.9
$tPOD_{mode}$	1.1	1	1.6	0.74	0.85	1.40
$tPOD_{10th\ percentile}$	0.53	0.52	0.6	0.51	0.39	0.29

Table 2 Overall number of differentially expressed genes (DEGs), number of DEGs fitted to curves and those for which a benchmark dose (BMD) could be derived, and transcriptomic points of departure (tPOD) values. Genes were considered differentially expressed for log2FC > 1 and FDR adj p-value < 0.05.*due to low number of DEGs for these exposures, the FDR adjusted filter was turned off to be able to perform dose-response analysis.

Gene-level accumulation curves

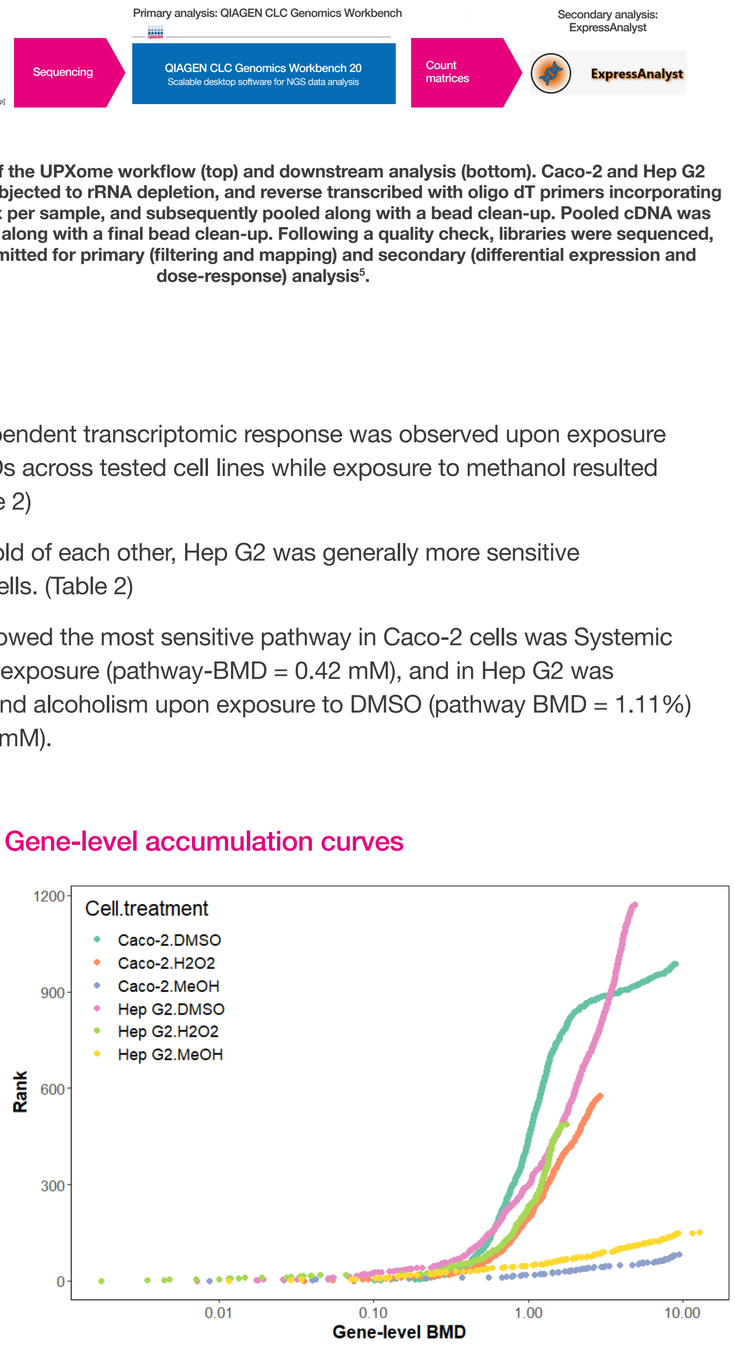

Figure 2 Gene accumulation plots on the differentially expressed genes for which benchmark doses could be calculated for Caco-2 and Hep G2 cells exposed to dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2) and methanol (MeOH).

Sustainability impact

		traditional RNAseq	UPXome technology	
		manually	manually	mosquito HV
rRNA depletion and reverse transcription	Reaction volume	1X	1X	0.5X ▼
	Reaction plate(s)	2x 96w PCR plates	2x 96w PCR plates	half 384w PCR plate
	Reagent reservoir(s)	variable, at least 2 reservoirs	2 reservoirs	partial 384w LVSD
	Tips	1168	288 ▼▼	248 ▼▼
	Pre-amplification pooling	no	yes, 24 samples per library pool ▲	yes, 48 samples per library pool ▲▲
Amplification and bead clean-up	Reaction volume	1X	1X	0.5X ▼
	Reaction plates	4x 96w PCR plates	0 ▼▼	0 ▼▼
	Tubes	0	16	8 ▼
	Tips	960	80 ▼▼	40 ▼▼
Quality check	Assay reagent	24 µl	24 µl	12 µl ▼
	Tubes	192	8 ▼▼	4 ▼▼
	Tips	200	9 ▼▼	5 ▼▼
Time (h)	Up to reverse transcription	24	9 ▼	7 ▼▼
	Amplification to the end	16	5 ▼	4 ▼▼

Table 3 Comparison of single-use plastic, reagents and time required for library preparation of 192 samples using traditional RNA-seq kit, UPXome technology manually at 1X reaction volumes or with SPT mosquito HV genomics at half reaction volumes.

Conclusions

The proposed workflow enables cost-effective, large-scale gene expression studies and subsequently generation of tPODs, aiding in chemical screening and prioritization in a more ethical and sustainable way:

- The semi-automated and miniaturized approach allowed for library preparation in one 384-well, as opposed to two 96-well PCR plates and resulted in cost savings in terms of reagents used and time needed for library preparation, and further downstream steps such as quality checks and quantification.
- We have shown that reduction of the reaction volumes does not affect the quality of the NGS libraries. The percentage of reads mapping to the genome and specifically to genes, exons, protein coding regions were comparable across the manual and semi-automated workflow.
- Combining the mosquito-enabled miniaturization with the streamlined UPXome protocol, where the cDNA is pooled before amplification, led to >85% reduction in single-use plastic (reservoirs, plates, tips) use and waste compared to the traditional RNA library preparation workflow.

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Acknowledgements

We would like to thank the staff at Department of Natural Resource Sciences, Faculty of Agricultural and Environmental Sciences, McGill University, Canada for their collaboration and providing data presented in this poster, and Samuel Rulli from QIAGEN for scientific support.

We would like to thank Jenny Eng for administrative support, Sophie Emberley-Korkmaz for assistance with exposures and library preparation, and Drs. Jessica Ewald, Peng Liu, Guangyan Zhou, and Jeff Xia for assistance with data analysis. We would like to acknowledge funding from the NSERC Discovery Grants Program and Genome Quebec GIPP.

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