Multiplex gene expression using the HyCEAD assay in CTCs isolated with the Parsortix™ system

David Englert, Mariya Kolesnikova, and Nishat Zaman, ANGLE Biosciences, Toronto, Ontario and Arianna Hustler, Gabrielle Wishart and Daniel J. O'Shannessy, ANGLE Europe Limited, 10 Nugent Road, Surrey Research Park, Guildford, Surrey GU2 7AF United Kingdom

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ABSTRACT

Background: Discrimination between benign and malignant pelvic masses by gene expression profiling of circulating tumor cells (CTCs) may provide information to assist in treatment decisions and improve outcomes. CTCs can be isolated and harvested from blood based on cell size and deformability using the Parsortix™ system. Because the number of CTCs in the blood of pelvic mass patients suspected of having ovarian malignancies is likely very low, gene expression profiling of these CTCs requires a highly sensitive detection system that is tolerant of the presence of normal nucleated blood cells.

Objective: To assess the suitability of the HyCEAD/Ziplex® assay for gene expression profiling of cells in Parsortix harvests. HyCEAD (**Hy**brid **C**apture **E**nrichment **A**mplification and **D**etection) is a fast and simple method for the simultaneous analysis of 100 or more mRNA species captured from cell lysates. The products of the multiplex amplification with HyCEAD can be quantified by hybridization on a flow-through chip on the Ziplex® instrument.

Methods: Multiple HyCEAD primer/probe sets were designed for 125 relevant genes, and purified total RNA or lysates from Parsortix harvests were processed with the HyCEAD assay. Primer/probe sets were screened using total RNA from cell lines and ovarian cancer tissues. Genes with significant expression in white blood cells were culled from the gene set. A set of non-human poly-adenylated mRNA molecules (separately quantified using digital droplet PCR) were used as spike-in standards to assess absolute sensitivity. CaOV3 cells spiked into blood from healthy normal volunteers (HNVs) were used as a model system for sensitivity analyses.

Results: Many genes were identified that yielded substantially greater signal intensities in harvests from HNV blood spiked with CaOV3 cells relative to the unspiked blood samples. The expression levels could be quantified over a signal intensity range of 2.5 log10 units. Small numbers of non-human standard molecules could be detected; stochastic detection failures were observed when five or fewer molecules of the standards were assayed (dropout level). CVs in repeatability experiments were typically less than 20% with numbers of standard molecules greater than the dropout level. CVs in repeatability measurements of human genes related to ovarian cancer in spiked Parsortix harvests averaged about 25%. CVs were greater between assays of Parsortix harvests of different HNVs, reflecting in part biological variability and variation of the number of cells actually spiked into the blood and the number of cells recovered in the individual harvests.

Conclusion: The HyCEAD assay provides high sensitivity sufficient to simultaneously profile expression levels of more than 100 genes in small numbers of CTCs directly from the lysates of Parsortix harvests. Expression profiling is focused on genes expressed at higher levels in CTCs than in normal nucleated blood cells.

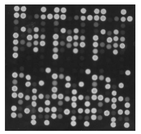
HyCEAD Process and Probe Design for CTCs

Lysis solution is added to cell suspensions (e.g., in PBS) and stored frozen if necessary. Multiple mRNA targets are captured from lysates on a solid support. Reverse transcription is performed after washing the support, and then cDNA recovered from the supernatant is amplified by PCR. Biotinylated PCR products are directly hybridized on a flow-through array and detected with chemiluminescence. Amplification of genomic DNA is minimized by capture of poly-A+ nucleic acids and by choosing primers that cross exon boundaries.

HyCEAD Process Hybrid Capture Extension and Detection

1) PolyA+ mRNA is captured directly from lysates.
2) Target sequences from many genes are amplified.
   - Two primers and one probe per target.
   - Primer-dimer formation and bias are minimized.
3) Products are sorted and quantified by chemiluminescent detection on a flow-through chip.

1) Capture polyA+ mRNA
2) Multiplex amplification/labeling
3) Flow-through chip hybridization
Automated analysis



Three sequence-specific oligonucleotides are designed for each target mRNA and screened against cell line and/or tissue RNAs to select probe sets with maximum signal intensity and high variance with tumor RNA and with minimum signal intensity with blood cell RNA; note that the mass of blood cell total RNA was 200 times that of cell line RNA. Good correlation between probe sets for the same target genes (e.g., probe sets A and B for Gene 1) indicates probe sets are probably targeting the desired targets. Signal intensities between different probe sets sometimes vary considerably (e.g., compare the **solid black** line of equality and the **dotted red** regression line for probe sets A and B for Gene 2).

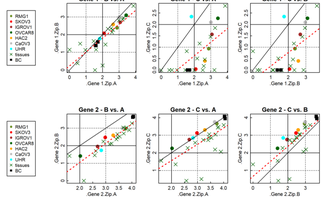
HyCEAD Probe Screening & Selectivity

Requirements for analysis of CTCs
- Verified selectivity for intended gene targets
- High sensitivity
- Low expression in nucleated blood cells compared to CTCs

HyCEAD probe design
- Primer pair and probe for each target sequence
- Target all RefSeq isoforms (if possible)
- Primers across exon boundaries (if possible)
- Multiple primer/probe sets per gene
- Primer/probe sets screened with relevant samples

Probe set evaluation for two target genes

	Expression in buffy coat RNA	High variance between cell lines & tumour tissues	Signal intensity	High correlation between probes	Potentially informative
Gene 1	low	high	A = B > C	A and B	yes
Gene 2	high	high	A > C > B	A, B, C	no

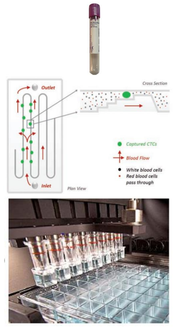


Parsortix/HyCEAD Workflow

Whole blood is processed through a Parsortix cassette to yield a cell suspension in a small volume of PBS enriched in Circulating Tumour Cells (CTCs).

The cells are lysed, and amplicons from multiple mRNA targets are amplified from the lysate. The mixture of amplicons are de-multiplexed on an array of target-specific probes on a flow-through microarray. Amplicons from eight HyCEAD amplifications (CTC samples) are automatically hybridized on separate flow-through arrays on the Ziplex® instrument to produce quantitative expression data for all targeted gene targets.

Draw blood
↓
Parsortix processing
↓
Recover harvest enriched for CTCs
↓
Lyse cells
↓
Multiplex HyCEAD processing
↓
De-multiplexing on array of probes
↓
CTC expression profiles



CTCs are held gently in narrow gaps within the cassette, whereas most blood cells and plasma pass through the gap and if required can be used for other analyses including ctDNA.

Target-specific oligonucleotide probes are printed on flow-through microarrays bonded to plastic tubes ("TipChips"). TipChips are immersed in solutions that are repeatedly passed through the arrays by application of pressure and vacuum. Images of chemiluminescent light emission are automatically analyzed to produce expression profiles.

Reproducibility of Expression Measurements

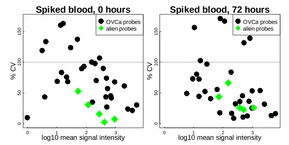
Blood from healthy normal volunteers was spiked with cells of an ovarian cancer cell line (CaOV3) and processed on Parsortix cartridges. The blood was processed immediately or stored for 72 hours before processing. Harvested cells were lysed, and the lysates were spiked with sub-femtogram amounts of synthetic mRNA control targets from non-human genes ("aliens"). The molar amounts of the spikes ("molecules") were estimated with digital-droplet PCR.

Coefficients of variation (%CVs) of the spiked-in control targets varied with the mean signal intensity, but were less than 20% with more than 100 molecules spiked into the lysate. %CVs were generally greater for genes expressed in harvested cells.

Parsortix/HyCEAD Reproducibility

Non-human spiked mRNA controls (◆)
- CVs in the high teens for >100 molecules and greater for fewer molecules.

Human genes expressed in the harvested cells (●)
- CVs were generally greater than for the aliens, reflecting variability of cell spiking and of the recovery of cells in the Parsortix harvests.



	molecules	mean signal	% CV (N = 51)
Alien.1	322	300	16.7
Alien.2	106	436	18
Alien.3	154	1085	14.6
Alien.4	14	182	32.5
Alien.5	28	79	37.7

Analytical Sensitivity and Dynamic Range

HyCEAD Sensitivity and Dynamic Range

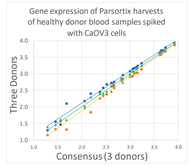
Sensitivity
- Assessed with non-human mRNA standards spiked into lysates (copy numbers estimated by ddPCR)
- Dropouts occurred at five molecules and fewer in lysates

Dynamic range
- Multiplex gene expression in CTC harvests of spiked blood
- 2.5 log signal intensity range for genes expressed in harvested cells

		No background		With background	
	molecules (ddPCR)	27 PCR cycles	31 PCR cycles	27 PCR cycles	31 PCR cycles
Alien.1	11	100%	100%	100%	100%
	21	100%	100%	100%	100%
	41	100%	100%	100%	100%
Alien.2	4	75%	75%	100%	100%
	7	100%	100%	100%	100%
	14	100%	100%	100%	100%
Alien.3	5	75%	100%	100%	100%
	10	100%	100%	100%	100%
	20	100%	100%	100%	100%
Alien.4	1	25%	50%	0%	75%
	1	75%	75%	50%	75%
	2	75%	50%	100%	75%
Alien.5	1	50%	100%	25%	100%
	2	75%	75%	25%	75%
	4	75%	100%	100%	75%

Sub-femtogram amounts of five synthetic mRNA control targets from non-human genes ("aliens") were processed with HyCEAD either with or without background total RNA (50 pg of CaOV3 RNA and 50 ng of WBC RNA). cDNA was amplified for either 27 or 31 PCR cycles.

The molar amounts of the spikes ("molecules") were estimated with digital-droplet PCR of cDNA reverse transcribed from the alien mRNA (which may underestimate the mRNA copy number due to the efficiency of reverse transcription). The fractions of four replicates in which the alien targets were detected are indicated by the percentage values.



Four EDTA tubes of blood were drawn from each of three healthy normal volunteers to create three pooled blood samples. Four aliquots of each pooled blood sample were spiked with CaOV3 ovarian cancer cells and processed on Parsortix cartridges. Lysates of each Parsortix harvest were analyzed with HyCEAD multiplex gene expression.

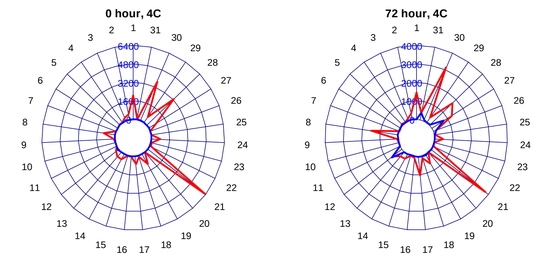
Mean expression values from the four replicate bloods from each donor are plotted vs. the consensus of the three donors. A consistent range of 2.5 $\log_{10}$ signal intensity units was observed with the three spiked donor pools.

Blood Storage

Blood from healthy normal volunteers was either unspiked or spiked with cells of an ovarian cancer cell line (CaOV3) and processed on a Parsortix cartridge. The blood was processed immediately or stored for 72 hours before processing. Lysates of each Parsortix harvest were analyzed with HyCEAD multiplex gene expression.

The radar plots show the expression levels of 31 genes expressed in harvested cells (mean values of three blood donors). The **blue** traces indicate unspiked blood and **red** traces indicate spiked blood.

Although the expression levels changed somewhat after 72 hours, the pattern of increased expression in spiked samples was largely maintained.



Conclusions

- HyCEAD combined with hybridization on flow-through microarrays enables multiplex gene expression directly from a single cellular lysate without prior RNA extraction.
- Probe sets may be screened with representative sample sets to select probes that provide relatively strong signal intensities and that are likely to accurately target the intended targets (based on correlations between probe sets).
- Cell suspensions enriched for CTCs with the Parsortix system may be readily processed for multiplex gene expression profiling with HyCEAD.
- Reproducible expression profiles can be obtained from lysates to capture the variance in expression levels of specific gene transcripts in Parsortix harvests.
- Small numbers of mRNA molecules can be detected in Parsortix lysates with HyCEAD.
- Expression can be quantified over at least 2.5 $\log_{10}$ intensity units.
- Expression profiles of tumour cells in blood samples persist when blood is stored for up to 72 hours.