Amplicon-based targeted sequencing of single circulating tumor cells

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Background

RareCyte has developed technology for visual identification and single cell retrieval of rare cells in blood, including circulating tumor cells (CTCs). There is increasing interest in mutational analysis of circulating tumor cells (CTCs) as a liquid biopsy application. Because of the miniscule amount of DNA present in a single cell (~6 pg), whole genome amplification (WGA) is typically performed prior to next generation sequencing (NGS) library preparation. Existing WGA methods have inherent amplification biases leading to non-uniform genome coverage that can cause dropout of desired targets, as well as elevated error rates that can lead to false positive mutations. Paragon Genomics has developed the CleanPlex® amplicon-based target enrichment technology for ultra-high multiplexing of amplicons with high coverage uniformity. Here we apply CleanPlex without pre-amplification for single CTC targeted DNA sequencing.

Methods

Cultured A549 cells as "model CTCs" were spiked into whole blood from healthy donors. Blood was processed by AccuCyte® Sample Preparation System onto slides. Multi-parameter immunofluorescence and automated imaging with the CyteFinder® Instrument were used to identify CTCs. CTCs were retrieved by CytePicker® into PCR tubes and either amplified by WGA (PicoPLEX®) or lysed in a PCR-compatible lysis buffer. In clinical samples, patient-matched plasma DNA from RareCyte Blood Collection Tubes or Streck Cell-Free DNA BCT® was also isolated. WGA products, cell lysates and plasma DNA were used as template for the CleanPlex OncoZoom® Cancer Hotspot Panel; additional PCR cycles were added during target amplification to compensate for low DNA input in the non-WGA single cell samples. Libraries were sequenced on the Illumina MiSeq.

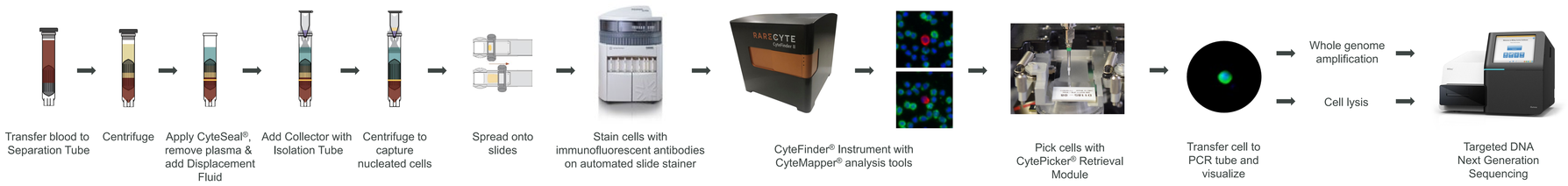
Results

Increased uniformity of coverage with decreased target dropout was observed with CleanPlex NGS library prep direct from cell lysate (non-WGA). Median read depth increased 48-fold when compared to the WGA method. On average, 23 out of 29 (79%) variants present in bulk A549 genomic DNA were observed with the non-WGA method in single mCTCs, while 15 of 29 (52%) were observed after WGA. Additionally, the false positive error frequency of non-WGA samples was 8-fold lower than in the WGA samples. CTCs and cell-free DNA from two metastatic breast cancer patients were also sequenced using OncoZoom. In one patient, PIK3CA E542K, a well-documented oncogenic mutation, was observed in 3 of 5 CTCs. In the other, ERBB2 L755S, a known HER2-reactivating mutation associated with chemoresistance, was found in 3 of 5 CTCs. Both patients' variants were also seen at similar allelic frequencies in RareCyte and Streck cell-free DNA.

Conclusions

Single-cell CleanPlex amplicon-based sequencing without prior WGA resulted in libraries with more complete and consistent coverage and lower error frequencies, enabling efficient and accurate assessment of somatic mutations in CTCs. This approach shows promise for cell-based liquid biopsy diagnostic applications.

Sample preparation and analysis workflow



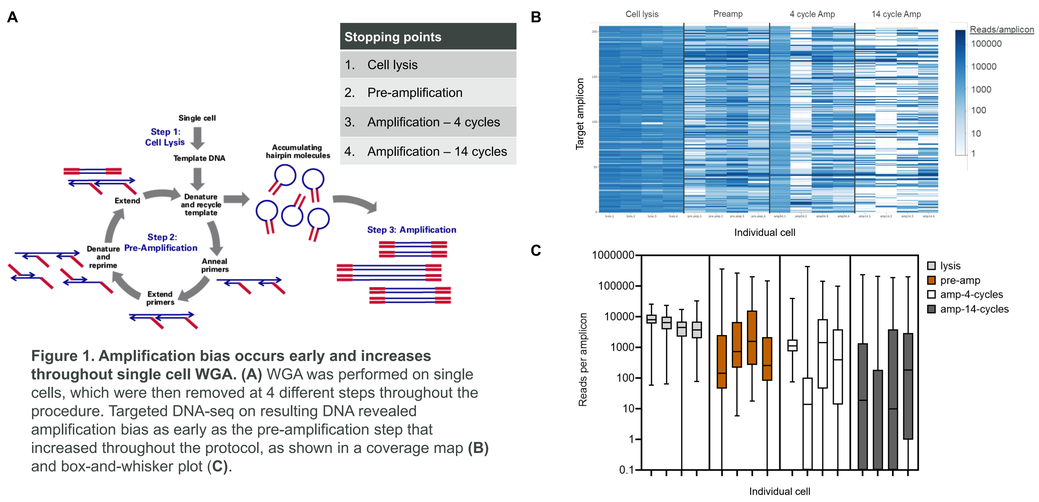


Figure 1. Amplification bias occurs early and increases throughout single cell WGA. (A) WGA was performed on single cells, which were then removed at 4 different steps throughout the procedure. Targeted DNA-seq on resulting DNA revealed amplification bias as early as the pre-amplification step that increased throughout the protocol, as shown in a coverage map **(B)** and box-and-whisker plot (**C**).

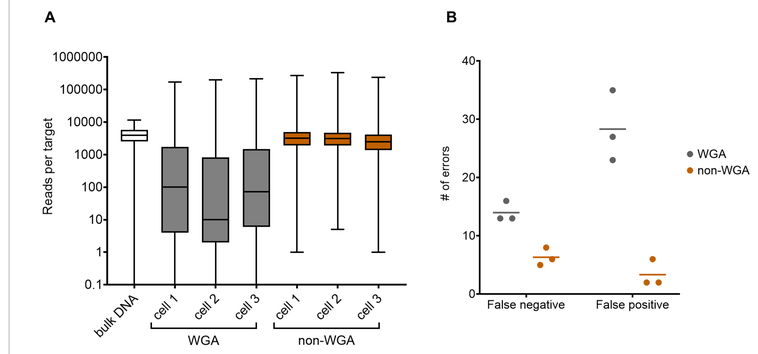


Figure 2. Single cell targeted DNA sequencing with CleanPlex OncoZoom. Single cell lysate is input as template into Paragon's CleanPlex OncoZoom, with modified primer concentration, PCR cycle number, and clean-up steps to compensate for low input DNA concentration. Using this non-WGA method vastly improves: **(A)** coverage uniformity, and incidence of **(B)** false negative and false positive errors, when compared to single cell WGA products.

A

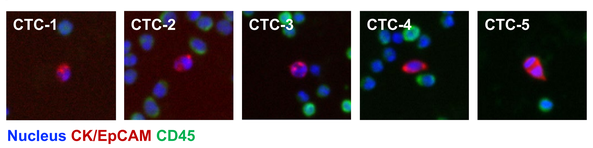
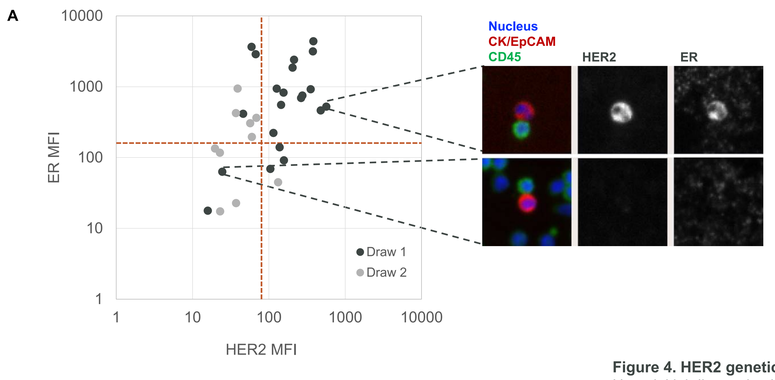


Figure 3. Detection of PIK3CA mutation in individual CTCs and matched cell-free DNA from a metastatic breast cancer patient. (A) Individual CTCs were isolated and used as template for single cell OncoZoom sequencing analysis, along with patient-matched WBCs and cell-free DNA. **(B)** PIK3CA E542K, a well-documented oncogenic mutation, was detected in 3 of 5 CTCs, as well as both cell-free DNA replicates.

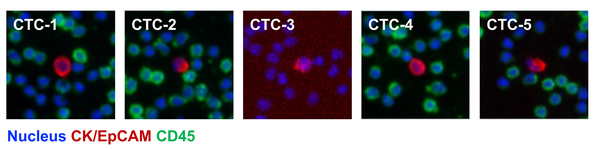
B

Sample	Co-retrieved WBCs	PIK3CA E542K VAF*
cfDNA-1	N/A	63%
cfDNA-2	N/A	63%
WBC-1	N/A	0%
WBC-2	N/A	0%
CTC-1	1	50%
CTC-2	4	0%
CTC-3	4	14%
CTC-4	5	13%
CTC-5	0	LC**

**VAF = variant allele frequency, i.e. % of reads containing the variant*
***LC = low coverage, insufficient to make call*



B



C

Sample	Co-retrieved WBCs	ERBB2 L755S VAF*
cfDNA-1	N/A	21%
cfDNA-2	N/A	25%
WBC-1	N/A	0%
WBC-2	N/A	0%
CTC-1	2	7%
CTC-2	4	19%
CTC-3	3	0%
CTC-4	5	0%
CTC-5	6	13%

**VAF = variant allele frequency, i.e. % of reads containing the variant*

Figure 4. HER2 genetic heterogeneity in metastatic breast cancer patient CTCs. Upon initial diagnosis, the patient presented with differential HER2 expression in mixed invasive ductal (HER2+) and lobular (HER2-) carcinoma. Patient blood was processed by AccuCyte and stained with RarePlex Enumeration or HER2/ER Panel Kits. **(A)** CTCs were identified and characterized by HER2 and ER expression. **(B)** Individual CTCs with unknown HER2/ER status were isolated and used as template for single cell OncoZoom sequencing analysis, along with patient-matched WBCs and cell-free DNA. **(C)** ERBB2 (HER2) L755S was detected in both cell-free DNA replicates, as well as in 3 of 5 CTCs, suggesting that there are two genetically distinct populations of CTCs disseminating from the two tumors. Research to correlate ERBB2 genotype with HER2/ER status in patient CTCs is ongoing.