A direct amplicon-based targeted sequencing assay for mutation analysis of single circulating tumor cells and correlation with circulating tumor DNA

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POSTER 5363

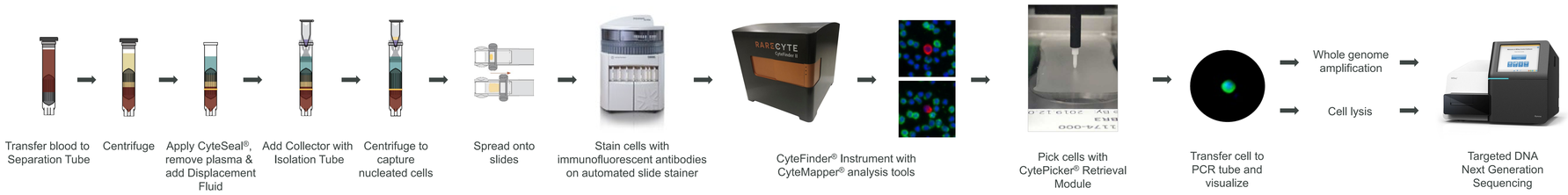
Abstract

There is increasing interest in sequence analysis of circulating tumor cells (CTCs) to identify actionable mutations that may complement circulating tumor DNA (ctDNA) findings. Because the amount of DNA present in a single cell is miniscule (~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 replication errors that can lead to false positive results. Targeted sequencing is an alternative approach that does not require WGA. We modified the CleanPlex® OncoZoom® Cancer Hotspot Panel protocol to perform targeted sequencing of single CTCs isolated by the RareCyte platform using A549 cell line spike-in and patient samples. We also used the CleanPlex panel to evaluate ctDNA from plasma isolated by blood collection tubes from two manufacturers (RareCyte and Streck). In both cases we compared OncoZoom results with and without prior WGA. Increased uniformity of coverage with decreased target dropout was observed when CleanPlex NGS libraries were prepared directly from cell lysates without 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 without WGA in single model CTCs, 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; false negative error frequency was 2-fold lower. 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 present at similar allelic frequencies in plasma isolated from RareCyte and Streck cell-free DNA BCT tubes. This approach shows promise for cell-based liquid biopsy diagnostic applications.

Conclusions

- Single-cell CleanPlex amplicon-based sequencing without prior WGA resulted in libraries with more complete and consistent coverage and reduced error frequencies, enabling efficient and accurate assessment of somatic mutations in CTCs.
- Median read depth increased 48-fold compared to the WGA method.
- False positive errors were reduced 8-fold; false negative errors were reduced 2-fold.
- Targeted sequencing of CTCs and cfDNA from metastatic breast cancer patients identified clinically significant PIK3CA and ERBB2 mutations. The mutations were present at similar allelic frequencies in plasma from RareCyte and Streck tubes.

Sample preparation and analysis workflow



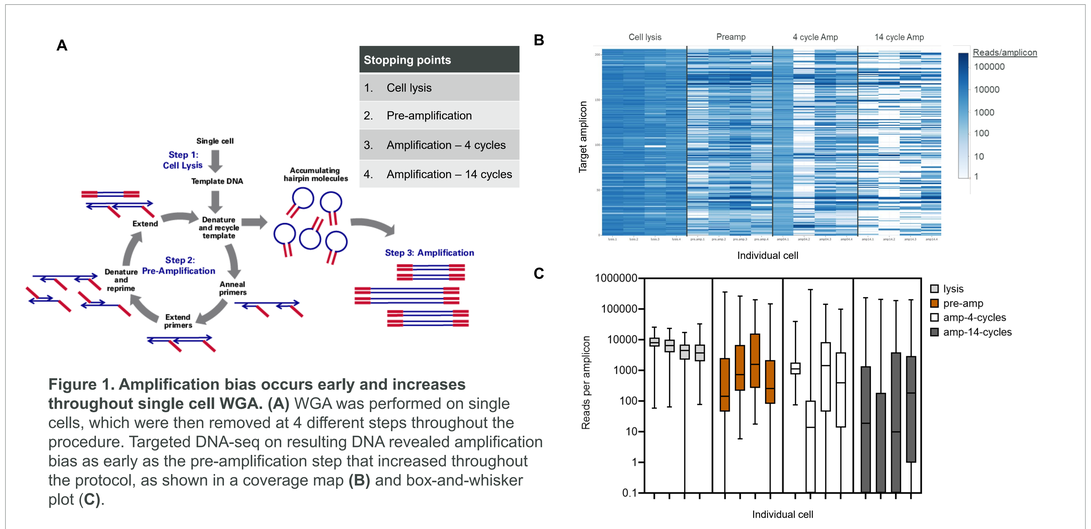


Figure 1. Amplification bias occurs early and increases throughout single cell WGA. (A) WGA was performed on single cells, which were then removed at 4 different steps throughout the procedure. Targeted DNA-seq on resulting DNA revealed amplification bias as early as the pre-amplification step that increased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

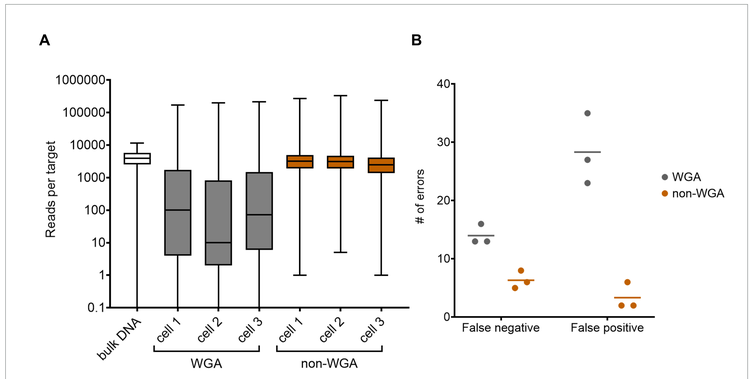


Figure 2. Single cell targeted DNA sequencing with CleanPlex OncoZoom. Single cell lysate is input as template into Paragon's CleanPlex OncoZoom, with modified primer concentration, PCR cycle number, and clean-up steps to compensate for low input DNA concentration. Using this non-WGA method vastly improves: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

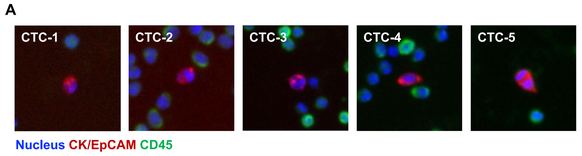
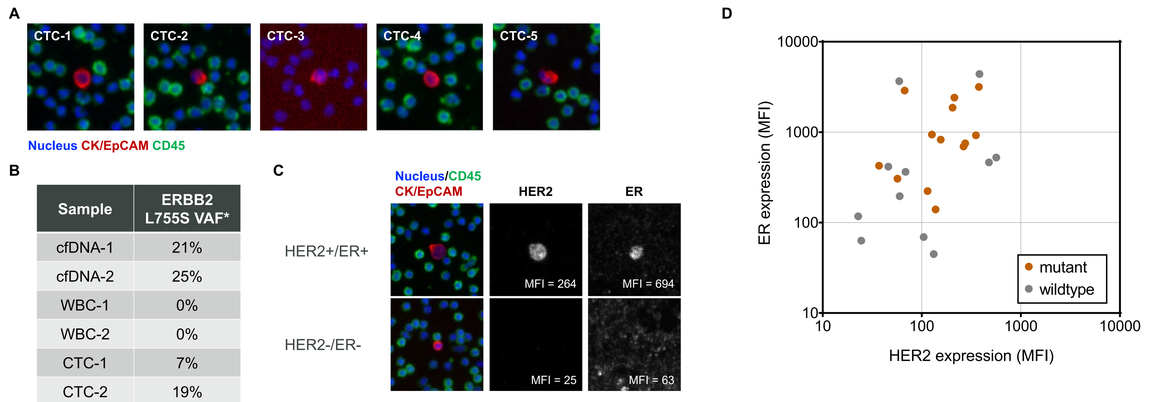


Figure 3. Detection of PIK3CA mutation in individual CTCs and matched cell-free DNA from a metastatic breast cancer patient. (A) Individual CTCs were isolated and used as template for single cell OncoZoom sequencing analysis, along with patient-matched WBCs and cell-free DNA isolated from RareCyte BCT (cfDNA-1) or Streck Cell-Free DNA BCT (cfDNA-2). **(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	PIK3CA E542K VAF*
cfDNA-1	63%
cfDNA-2	63%
WBC-1	0%
WBC-2	0%
CTC-1	50%
CTC-2	0%
CTC-3	14%
CTC-4	13%
CTC-5	LC**

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



B

Sample	ERBB2 L755S VAF*
cfDNA-1	21%
cfDNA-2	25%
WBC-1	0%
WBC-2	0%
CTC-1	7%
CTC-2	19%
CTC-3	0%
CTC-4	0%
CTC-5	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 CTC Enumeration or HER2/ER Panel Kits. **(A)** 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 isolated from RareCyte BCT (cfDNA-1) or Streck Cell-Free DNA BCT (cfDNA-2). **(B)** ERBB2 (HER2) L755S, a mutation that confers resistance to common anti-HER2 therapies, was detected in both cell-free DNA replicates, as well as in 3 of 5 CTCs. Additional CTCs were identified from another blood draw and characterized by HER2 and ER expression, with representative cells shown in **(C).** **(D)** Each CTC was retrieved and used as template for OncoZoom sequencing, and then assessed for presence of the ERBB2 L755S mutation.