Targeted single cell DNA sequencing without prior whole genome amplification for mutational analysis of circulating tumor cells

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Background

RareCyte has developed platform 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 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. Amplicon-based next generation sequencing (abNGS) is a high-throughput method which enables genetic confirmation of malignancy and discovery of de novo pathogenic mutations. Here we present a method for performance of single cell abNGS on model CTCs without prior WGA using a commercially available pan-cancer hotspot panel.

Methods

A549 lung cancer cells as model CTCs (mCTCs) were spiked into whole blood, which was processed by AccuCyte® Sample Preparation System onto slides. After formalin fixation, multi-parameter immunofluorescence and automated imaging the CyteFinder® Imaging System were used to identify mCTCs – visualized as nucleated cells expressing epithelial markers (cytokeratin or EpCAM) and not expressing white blood cell markers. mCTCs were mechanically retrieved by CytePicker® into PCR tubes and either amplified by WGA (PicoPLEX®) or lysed in a PCR-compatible lysis buffer. WGA products or cell lysates were used as template for the AmpliSeq™ Cancer HotSpot Panel v2 for Illumina® library preparation; additional PCR cycles were added during target amplification to compensate for low DNA input in the non-WGA samples. Libraries were sequenced on an Illumina MiSeq and analyzed using the BaseSpace bioinformatics suite.

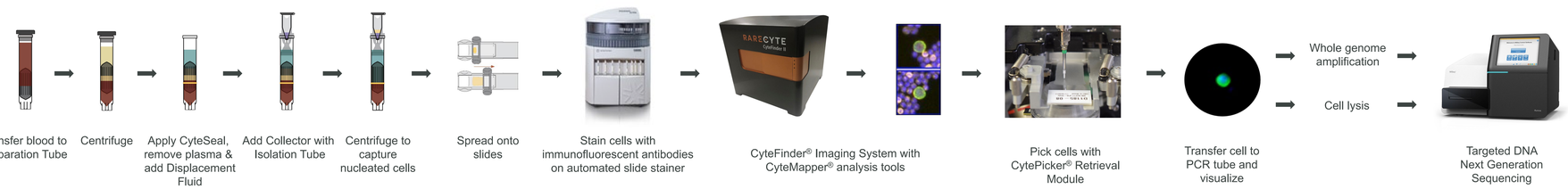
Results

Single mCTCs that underwent amplicon-based NGS library prep direct from cell lysate (non-WGA) displayed increased uniformity of coverage with decreased target dropout when compared to WGA cells. Median read depth increased 7-fold with the non-WGA method. On average, 8 of 15 variants present in bulk A549 genomic DNA were observed in single mCTCs sequenced after WGA, while 12 out of 15 were observed with the non-WGA method. Additionally, the false positive error frequency of non-WGA samples was < 5% of the WGA samples. The non-WGA method was applied to CTCs identified in blood from a prostate cancer patient and confirmed presence of PTEN and TP53 mutations identified by cell-free DNA analysis.

Conclusions

Amplicon-based targeted single-cell 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.

Sample preparation and analysis workflow



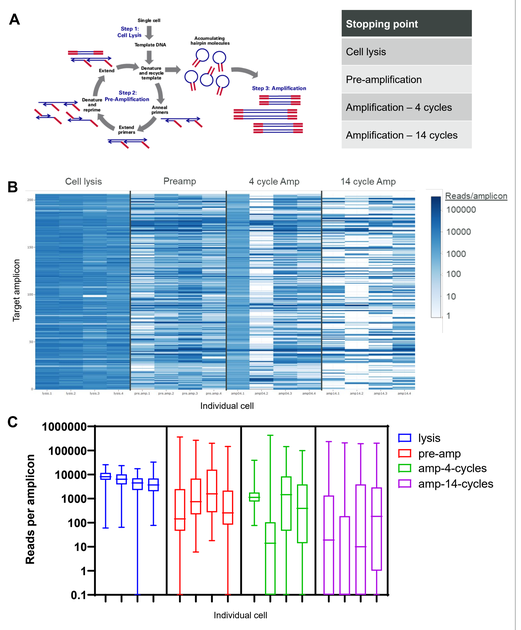


Figure 1. Amplification bias occurs early and increases throughout single cell WGA. (A) WGA was performed on single cells, which were then removed at 4 different steps throughout the procedure. Targeted DNA-seq on resulting DNA revealed amplification bias as early as the pre-amplification step that increased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

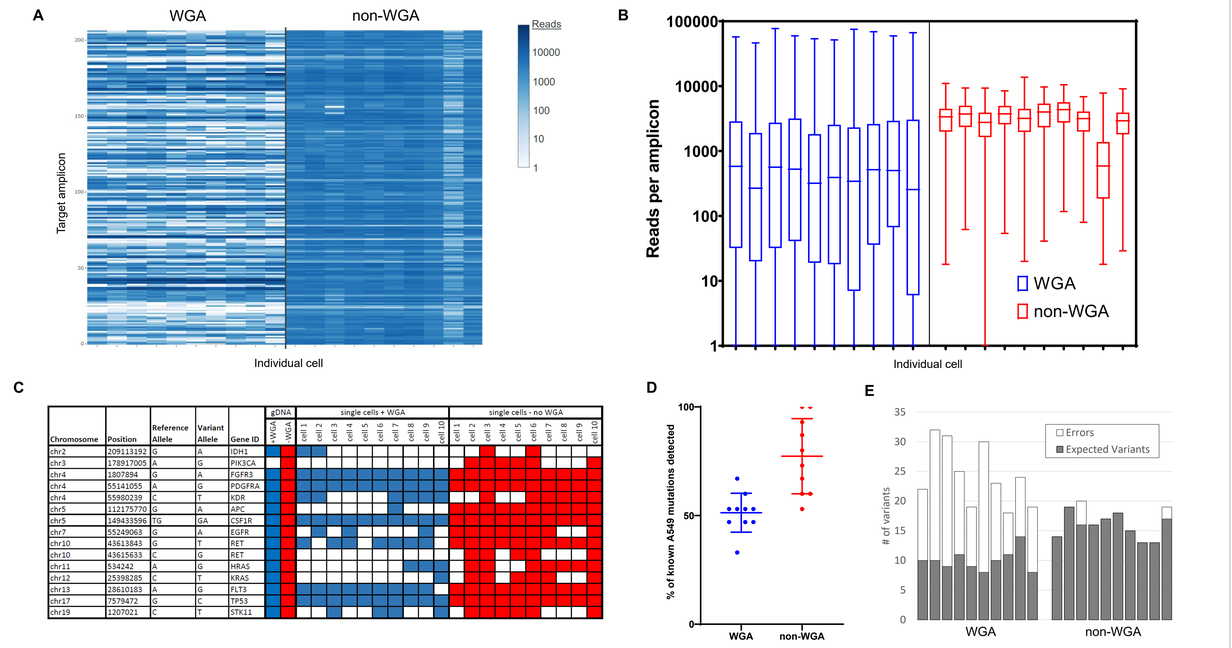
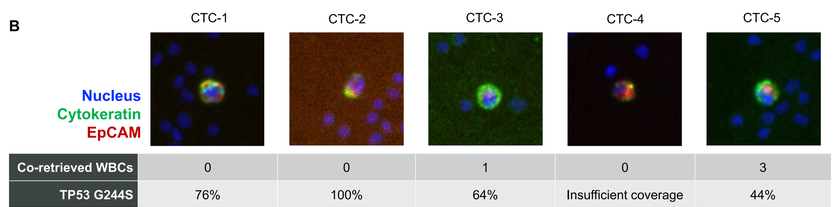


Figure 2. Single cell targeted DNA-seq without WGA enhances coverage and improves sensitivity and specificity of variant detection. Single A549 cells were prepared with WGA or non-WGA methods, followed by targeted DNA-seq. **(A)** Coverage maps and **(B)** box-and-whisker plots demonstrated an increased median of reads per amplicon, as well as improved evenness of coverage with the non-WGA methodology. **(C)** Known A549 mutations within the sequencing panel were detected at higher frequencies in non-WGA-prepared cells (red boxes) than in WGA-prepared cells (blue boxes). **(D)** On average, sensitivity was 51% in WGA-prepared cells and 77% in non-WGA-prepared cells. **(E)** False positive variants were detected at a much higher frequency in WGA-prepared cells, likely due to polymerase errors that occurred during the WGA process.

Figure 3. Detection of patient tumor-specific mutations in individual CTCs. (A) Guardant360 cell-free DNA sequencing in a prostate adenocarcinoma patient identified somatic mutations in TP53 and PTEN. **(B)** Individual CTCs (as well as white blood cells for germline controls) were isolated from patient blood, followed by targeted DNA sequencing using a non-WGA methodology. The TP53 and PTEN mutations were both detected in 4 of 5 CTCs (at the indicated percentage of reads at that locus), and 0 of 3 WBC samples, demonstrating clinical application of the non-WGA method.



	CTC-1	CTC-2	CTC-3	CTC-4	CTC-5
Co-retrieved WBCs	0	0	1	0	3
TP53 G244S	76%	100%	64%	Insufficient coverage	44%
PTEN deletion	51%	62%	16%	Insufficient coverage	35%