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Single cell mutation detection with multiplex PCR-based targeted enrichment sequencing

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Abstract

Genetic alteration is the driving factor in various biological processes such as developmental malformation, evolution, and cancer. Recent findings of genomic heterogeneity among ostensibly homogeneous cell populations such as tumor and neural cells demands genomic characterization at the individual cell level to better understand the underlying biology. In addition, single cell technologies make genomic analysis feasible for the characterization of rare cells such as circulating tumor cells, in-vitro fertilized embryos, and microbes. While copy number variation can be assessed by next generation sequencing (NGS) on whole genome scale at low read depth, single nucleotide variation (SNV) requires relatively higher coverage that often necessitates targeted enrichment or amplification to focus on sites of interest. Here a multiplex PCR-based targeted enrichment method was tested for mutation detection in single cells isolated from two colon cancer cell lines, Lovo and HT29. An NGS panel targeting colon cancer relevant genes was used to enrich DNA from whole genome amplified (WGA) single cell DNA followed by mutation detection with NGS. A similar experiment was carried out on DNA from bulk cells or bulk cell DNA which underwent WGA. Previously reported mutations such as KRAS (G13D) in LoVo and BRAF (V600E) in HT29 cells were successfully detected with this enrichment method on single cell DNA as well as on bulk DNA. These mutations were further confirmed by Pyrosequencing and a strikingly similar mutation frequency was observed between targeted NGS and Pyrosequencing in each sample. Interestingly mutation frequencies observed between bulk cell DNA and bulk cell WGA DNA were similar but varied greatly with respect to individual cells. Similar variations were observed on additional heterozygous SNV in single cell DNA as compared to bulk cell DNA. Whether the variation is due to tumor cell heterogeneity, selective WGA amplification, or both needs to be further defined. These results demonstrate that multiplex PCR targeted enrichment can be successfully applied for mutation detection on single cell amplified DNA, but multiple single-cell analysis is necessary for accurate interpretation of genomic alterations among heterogeneous cell populations.

Introduction

Individual cells with the same phenotype or morphology were once viewed as identical functional units within a tissue or organ. However, studies from recent single cell analyses revealed the uniqueness of each individual cell. Tumor genomes are highly heterogeneous and accumulate mutations at widely varied rates. Single-cell sequencing can deconvolute clonal variation as well as tracing the evolution and spread of disease, which would be difficult to reveal by bulk tumor sequencing. Recent advances in technologies for single cell isolation, whole genome amplification, and high-throughput targeted sequencing have made single-cell sequencing feasible.

Materials and methods

Single cells were isolated from colorectal cancer cell lines, HT29 and LoVo, using CellRaft™ Arrays (CellMicrosystems). The CellRaft™ Array consists of a high density of individual microwells (200 µm x 200 µm each) that isolate the cells and allow recovery by releasing individual carriers, or “rafts”, from each microwell using a piercing device under microscopic control. Released rafts carrying individual cells were transferred to PCR tubes for further processing using a magnetic wand.

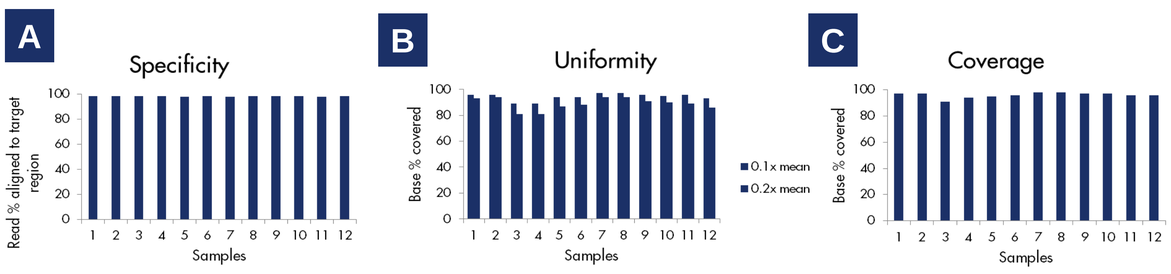
Genomic DNA was isolated and amplified from single cells using QIAGEN’s REPLI-g Single Cell Kit following manufacturer’s protocol. REPLI-g Single Cell Kit is based on Multiple Displacement Amplification (MDA) technology with typically negligible bias.

The Human Colorectal Cancer GeneRead DNAseq Targeted Panel V2 from QIAGEN was used to enrich the exonic sequences of 38 clinically relevant colorectal cancer genes (Figure 2) from single cell amplified DNA. Enriched and purified targeted regions were constructed into Illumina library using QIAGEN’s GeneRead Library Prep Kit. Samples were sequenced on MiSeq Sequencer according to manufacturer’s recommended procedure with median read depth around 1000x.

Sequencing data were analyzed using QIAGEN’s GeneRead Targeted Exon Enrichment Panel Data Analysis Web Portal. High-quality variants were identified with standard data analysis workflow. CLC Genomics Workbench from QIAGEN was also used to characterize low-frequency variants.

Selected variants were verified using QIAGEN's PyroMark Q24 Pyrosequencing Assays following the manufacturer’s protocols.

Outstanding sequencing metrics



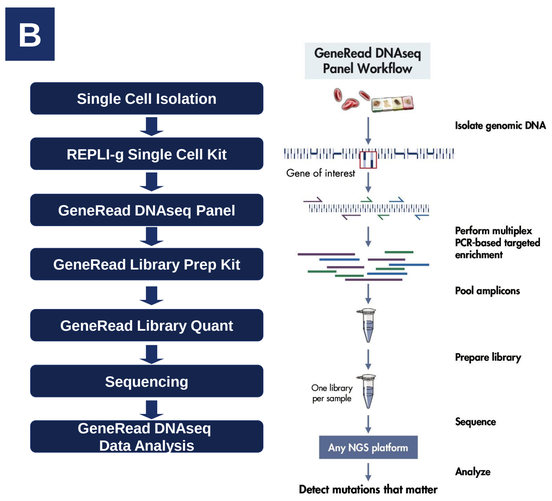
Basic sequencing metrics of bulk and single cell samples. Outstanding specificity, uniformity and coverage were achieved on sequencing results of all samples. Samples 1–6 were LoVo cells and 7–12 were HT29 cells. Samples 1 and 7 were DNA from bulk cells. Samples 2 and 8 were DNA from bulk cells that underwent WGA. The rest of samples were DNA from individual cells. (A) Specificity is calculated as percentage of reads mapped to targeted region of interest out of total number of reads per run. (B) Coverage uniformity is measured as percentage of bases in the region of interest covered at least 0.1x or 0.2x mean coverage depth. (C) Coverage was calculated as percentage of bases covered at 100x read depth.

GeneRead DNAseq Colorectal Cancer Panel V2 Workflow

A

Target size	38 genes, 183 Kb					
% coding region covered	98.7% (experimental coverage)					
Multiplexing	~488 amplicons per pool, 4 pools					
Average amplicon size	150 bp (100–180 bp)					
Input amount	40ng per sample (10 ng per pool)					
Processing time	3 hrs					
NGS platform	MiSeq					
Target gene list	ACVR1B	BRAF	EP300	MAP2K4	MSH6	SLC9A9
	AKT1	CASP8	ERBB2	MAP7	MYO1B	SMAD2
	APC	CDC27	FBXW7	MIER3	NRAS	SMAD4
	ATM	CTNNB1	FZD3	MLH1	PIK3CA	TCERG1
	ATP6V0D2	DCC	GPC6	MSH2	PIK3R1	TCF7L2
	BAX	DMD	KRAS	MSH3	PTPN12	TGFBR2
	TP53	WBSCR17				

B



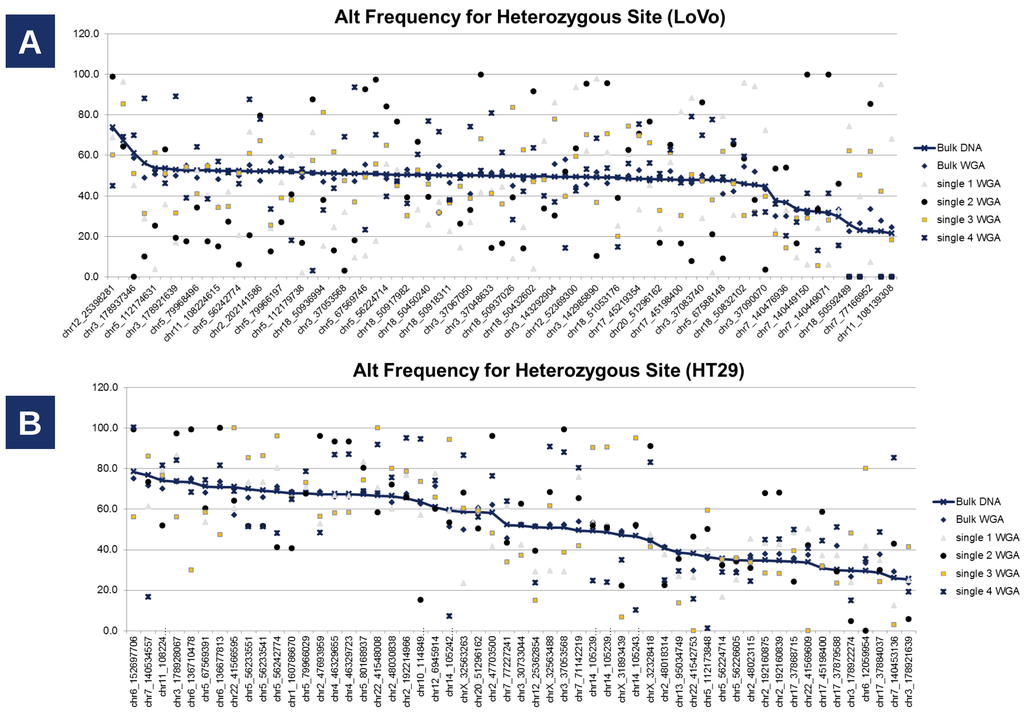
Single cell sequencing workflow with GeneRead DNAseq Targeted Panel V2 System. (A) Information on the Human Colorectal Cancer GeneRead DNAseq Targeted Panel V2. (B) A complete sample-to-insight workflow for single cell sequencing with GeneRead DNAseq Targeted Panel V2 system and various NGS solutions from QIAGEN.

100% concordance between variants detected with GeneRead DNAseq Targeted Panel V2 and PyroMark Assays

No.	Sample	Mutation	Mutation rate (%)	
			Colon cancer panel	Pyrosequencing
1	gLoVo-3	KRAS p.G13D	74	71.37
2	WGA LoVo-3	KRAS p.G13D	73.3	67.82
3	Single cell LoVo-3 WGA	KRAS p.G13D	67.7	67.22
4	Single cell LoVo-3 WGA	KRAS p.G13D	98.8	93.4
5	Single cell LoVo-3 WGA	KRAS p.G13D	59.2	55.41
6	Single cell LoVo-3 WGA	KRAS p.G13D	44.5	41.73
7	gHT29	BRAF p.V600E	24.9	23.93
8	WGA HT29	BRAF p.V600E	28.8	28.09
9	Single cell HT29 WGA	BRAF p.V600E	12.2	13.12
10	Single cell HT29 WGA	BRAF p.V600E	43.6	45.03
11	Single cell HT29 WGA	BRAF p.V600E	3% reads	3.56
12	Single cell HT29 WGA	BRAF p.V600E	85.2	84.56

Known variants in LoVo cells (KRAS p.G13D) and HT29 cells (BRAF p.V600E) were detected by NGS with GeneRead DNAseq Colorectal Cancer Panel V2 and verified by PyroMark assays. For each sample, similar mutation frequency was identified by both methods, suggesting faithful characterization of variant frequency by GeneRead DNAseq Targeted Panels V2. Although similar variant rates were detected between bulk cell and bulk cell that underwent WGA samples, mutation frequency among different single cell samples showed variation, especially in HT29 cells.

Frequency of heterozygous sites



Allele frequency of heterrozygous sites between bulk cell and single cell samples. All heterozygous SNPs identified by Low Frequency Variant Detection of CLC Genomics Workbench within the targeted region of GeneRead Colorectal Cancer Panel V2 in either LoVo (A) or HT29 (B) cells are shown. The majority of heterozygous SNP frequency in bulk cell samples was around 50% (as expected) and consistent heterozygous SNP frequency was also observed between bulk cell DNA and bulk cell DNA that underwent WGA. In contrast, allele frequency of heterozygous sites varied between single cell and bulk cell samples as well as among individual single cell samples. This variation of heterozygous SNP frequency reflects the heterogeneous nature of cancer cells.

Conclusions

DNA from single cell samples prepared by REPLI-g whole genome amplification on single cells isolated with CellRaft technology was suitable for next generation sequencing analyses.

A multiplex PCR-based targeted enrichment method was successfully applied on whole genome amplified single cell DNA for next generation sequencing on selected regions of the genome.

Mutations detected by the GeneRead DNAseq Colorectal Cancer Panel V2 were verified by PyroMark assays. Variant frequency between these two sequencing methods was strikingly consistent, indicating accurate identification of variant frequency with the GeneRead DNAseq Targeted Panel V2.

Both cancer cell heterogeneity and technical variation associated with the single cell method could cause different allele frequency of heterozygous sites among individual single cells. The MDA-based REPLI-g Single Cell Kit typically has negligible bias, suggesting biological variation here. Further studies are required to better characterize and distinguish biological variation from technical noise.

The GeneRead DNAseq Targeted Panel V2 combined with REPLI-g whole genome amplification and CellRaft single cell isolation technology provide a verified, complete workflow for targeted sequencing of regions of interest in single cell DNA samples.

The applications presented here are for research use only. Not for use in diagnostic procedures.

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