ABSTRACT AND INTRODUCTION

Tumor analysis is complicated by sample heterogeneity, whereas single-cell isolation methods such as the High-Definition Circulating Tumor Cell (HD-CTC) assay can extract specific sub-populations. Next-generation sequencing technologies have had limited applicability for genetic characterization of single cells due to high DNA input requirements. Therefore, genetic profiling of single cells is usually done using single-cell PCR. Multiplexed targeted amplification (4,000-16,000-plank) using AmpliSeq™ Cancer Panels enables interrogation of thousands of cancer hotspot mutations from as little as 30 ng of DNA (~100 cells). Multiplexed targeted methodology was used to profile genetic alterations in 1-100 cells isolated using a variety of methods: FACSorting, LCM, and HD-CTC capture. We describe two methods for constructing sequencing libraries: (i) Single tube Direct cell lysis without prior genomic DNA isolation; (ii) whole-genome amplification (WGA). The Direct method achieved >98% on-target reads, 92% coverage at 100X, and 84% uniformity from 10-25 cells. Using WGA, we find 96% on-target reads, 100% coverage at 10X, and 90% with single cells. Average uniformity for whole-genome libraries compared to only control wells was <20% (data not shown). Carrier agent addition improved coverage uniformity and reduced variation, with the effect more striking for 1.9 HeLa cells. In Known MAFs mutation present at 0.5 frequency in HeLa cell line was detected in all samples. Averaging results from 8 single cells improved accuracy of variant detection. Aliquot droplet was further reduced by adding carrier agent to the lysate buffer.

RESULTS

Figure 3. AmpliSeq™ Libraries from 1-20 FACSorting-sorted cells

Figure 4. AmpliSeq™ CHPo libraries were generated using the Direct Lysis method from FFPE SbB1 cells. Coverage uniformly >80% was achieved with fixed single cells (>170,000 amplicons detected from single cell), all interrogating ~2000 COSMIC mutations.

Procedure: Lysis buffer-0.5% carrier agent was added directly to LCM cap containing SbB1 cells (1,100 and 100 cells). Cells were isolated from FFPE tissue. LCM caps (CaptSure®) were used with the LCM ExtractSure™ Device to reduced extraction volume to 10µL.

Figure 5. Circulating tumor cell isolation for single-cell analysis

High-Definition Circulating Tumor Cell (HD-CTC) Isolation Method

Table 1. Samples Used in HD-CTC Study

Figure 6. AmpliSeq™ analysis of CTCs, Tumor, and Normal Tissue

A. Histology Analysis of HCC464 tumor
B. Histology Analysis of HCC464 CTCs

Figure 7. Circulating tumor cell isolation for single-cell analysis

D. Variant detection in tumor (CCP panel) and 6 CTPs (CHPv2 panel)

HCC Tumor (42 variants detected)

C TCTs (3-9 detected per CTC)

Table 1. Mutations and their corresponding CTCs

A selection of the genes for which variants were identified in the bulk HCC tumor and CTCs collected from peripheral blood. These mutations were detected after removing variants present in the corresponding germline sample (white blood cells), and filtering out those with a p-value > 0.0001, and less than 50 reads per amplicon.

SUMMARY

Ion AmpliSeq™ libraries can be prepared directly from cells without gDNA isolation using either the Direct Amplification or Whole Genome Amplification Methods

Multiple single-cell isolation techniques are compatible with AmpliSeq™ library preparation: FACSorting, LCM, CTC-isolation and micromanipulation of live and fixed cells

Initial feasibility study demonstrated ability to detect cancer-relevant mutations in Circulating Tumor Cells derived from archived samples using AmpliSeq™ Cancer Panels

ACKNOWLEDGEMENTS

The authors thank Shirley Chu and Kristin Schmidt for providing the LCM cell samples.

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Sophia Rozenthal

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Cancer Hotspot Panel v2

10ng DNA, Tube: 2800 COSMIC mutations, 207 amplicons 50 oncogenes and tumor suppressor genes, Comprehensive Cancer Panel

Comprehensive Cancer Panel

40ng DNA, 4 tubes, 6,000 mutations, 16,000 amplicons ~90 oncogenes and tumor suppressor genes

AmpliSeq Library Construction

WGA Method: Whole Genome Amplification was performed using the WGA kit (Sigma). WGA DNA was quantitated on the Qubit (Life Technologies) and 10ng was used in the standard AmpliSeq™ workflow with the Ion AmpliSeq™ Comprehensive Cancer Panel (CCP), Direct lysis method.

CTCs were lysed at room temperature (RT) in Single Cell Lysys Buffer (Ambion Cell Lysys Kit, Life Technologies) modified with 0.5% carrier agent to reduce DNA loss and increase recovery, followed by 2min, RT to Stop solution. The lysates were used directly for multiplex PCR with the Cancer Hotspot Panel v2 (CHPv2) primer pool, using modified PEGs (adjusted for input amount). Library quantification was performed with the Ion Pico Assay Kit.

PGE Sequencing: One Ion 318™ chip was used to sequence each CEP Library, and up to 10 pooled, barcoded CHPv2 libraries. Data analysis was performed using Torrent Suite™ and Ion Reporter™ workflows.

A. Single-day workflow using an Ion 318™ chip

Library Preparation

B. AmpliSeq Library Construction

C. WGA Method: Whole Genome Amplification

D. Direct lysis method

Figure 8. Ion AmpliSeq™ Technology

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