The Ion AmpliSeq Cancer Panel 2.0 (Ion Torrent) allows for targeted sequencing of 180 regions of interest in 46 cancer-related genes. We used well-characterized cancer cell lines harboring clinically relevant variants as positive controls and FFPE specimens NA12878 and NA12940 as wild-type control samples. A total of 57 FFPE samples of a variety of cancer types were analyzed in a blinded fashion to evaluate the diagnostic sensitivity and specificity. With no gold standard available as a reference method to detect mutations with comparable sensitivity, we analyzed the same sets of samples with both the Ion AmpliSeq and the Illumina TruSeq Amplicon Cancer Panel for comparison. These two cancer panels use distinct library preparation methods, as well as different sequencing chemistries and analysis pipelines. They share largely overlapping regions of interest and have comparable sensitivity of minor allele detection. Variants detected by both panels were considered as true positive variants. While most variants can be calls submitted for Ion and Illumina, variants that were only covered by one panel but not the other were confirmed by a third method.

Analytical Validation

To confirm the precision of the detection frequency, we performed an analysis using Ion Torrent™ Fluorescence in situ hybridization (FISH). In total, 80 of 85 expected mutations were reproducible in duplicate and triplicate runs. Of the three non-reproducible variants, two were low-frequency (frequencies close to 2%), and one was a high-frequency variant in a homopolymer region. The precision of variant frequency detection was determined using 36 mutations in 6 samples with triplicates or duplicates (Figure 1). In general, the CV is between 0.02%, showing inverse correlation with variant frequency.

Materials and Methods

Genomic DNA (10ng) was amplified using Ion AmpliSeq™ Primer Pools (v1.1). The resulting amplicons were treated with full-length T4 DNA polymerase to partially digest primer sequences and ligation to Ion sequencing adapters. For sample multiplexing, libraries were barcoded using the Ion Xpress™ Barcode Adapters 1-3. The ligated amplicons were purified by AmPure XP and quantified by qPCR using Ion library quantitation kit. Quantified libraries were qualified with Caliper LCM (optional) and amplified on IonOneTouch using the OneTouch template preparation v2 kit and sequenced on PGM using Ion PGM 2.01 sequencing kit. Sequence alignment was performed by Torrent Suite 2.2 (ref. 2). and variants were called by TS 2.2 variant caller. In addition to reporting hotspot variants, we report all variants at frequency ≥2% with potential impact on protein.

Results

Analytical Sensitivity: Equimolar mixture of gDNA from six cell lines (DG41, HS252, HSF35, HSF37, HSF25, and HSF25A23) was diluted into NA12878 gDNA with final concentrations of 1%, 5%, 10%, and 20% for cancer cell line DNA (Table 1). The LOD for single-base substitutions and indels was determined to be 2.5%.

Analytical Accuracy: Among 10 samples with known mutations (cell line DNA and FFPE), 80 of 85 expected mutations were reproducible in duplicate and triplicate runs. Of the three non-reproducible variants, two were low-frequency (frequencies close to 2%), and one was a high-frequency variant in a homopolymer region. The precision of variant frequency detection was determined using 36 mutations in 6 samples with triplicates or duplicates (Figure 1). In general, the CV is between 0.02%, showing inverse correlation with variant frequency.

Conclusions

• Our validation demonstrates that the Ion AmpliSeq Cancer Panel combined with MolecularMD’s proprietary analysis pipeline provides a robust and accurate test to profile clinically relevant gene variants with analytical sensitivity of 2.0% minor allele frequency.

• In addition to reporting hotspot mutations, we also report other variants that would impact protein (all except synonymous and in-frame mutations). This diagnostic sensitivity and specificity is calculated based on a blinded study of 37 FFPE samples is 97% and 98%, respectively.

• In this study, we developed a unique cross-validation strategy using two different NGS platforms. We also established a rapid and accurate restriction endonuclease-based fragment analysis method for confirming variants at frequencies below 10%.

• Our validation study covers 301 unique mutations in 23 genes, with 7 indels, 1 nonsense and 8 missense mutations.

• The Ion AmpliSeq Cancer Panel combined with MolecularMD’s proprietary analysis pipeline has excellent sensitivity for single base substitutions with only one mutation (FRKCA ES590) missed due to the pseudogene interference. This can be resolved by manual review of Bam files in IGV for this genomic locus in the future.

• One weakness of this cancer panel in its current version is the inability to detect single base indels in homopolymer regions. However, it tolerates other indels fairly well; indels ranging from 1-2 bp were successfully detected in this Ion.

• Ion AmpliSeq is a cancer panel well-suited for small clinical samples with only 20ng DNA input. There was a zero failure rate for 41 FFPE samples that we tested.

• We are currently exploring methods to improve the variant calling for indels in homopolymer regions by using TS 3.0 and other pipelines.

References

1. Life Technologies; Ion AmpliSeq™ Library Kit 2.0 manual.
2. Life Technologies; Torrent Suite 2.0 manual.

For Further Information

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