**Introduction**

Targeted deep sequencing in clinical applications is hindered by the necessity of using formalin-fixed paraffin embedded (FFPE) specimens, which are characterized by their contamination with normal cells, minute amount of tissue, and DNA damage. The use of genomic DNA extracted from FFPE sample into NGS assays is challenging. Numerous FFPE samples present low tumor content leading to a loss of confidence in the detection of variants due to the presence of false positive calls at the same low frequencies as the true variants. The validation of those variants by an alternative sequencing platforms is recommended, but is often impossible due to insufficient sample availability and cost. Here we show how the analysis of digitally-sorted, pure cell subpopulations yields to all and only true-positive actionable somatic variants, independent of the sequencing platform used.

**Methods**

All FFPE samples from 2 lung and 1 ovarian cancer patients were dissociated to single cell suspensions. Using the DEPArray™ digital sorter, 78-300 cells were recovered per sample, corresponding to 0.5-2ng DNA from FFPE pure tumor and normal stromal cell subpopulations as distinguished by Keratin/Vimentin immunofluorescence and DNA content. After lysis, libraries from cell recoveries, as well as unsorted samples, were prepared for IonTorrent™ PGM and Illumina MiSeq sequencing, using Amplicseq™ Cancer Hotspot Panel v2 and Swift Biosciences Accel-Amplicon™ 56G Oncology Panel, respectively. Both PCR-based target enrichment kits perform well with small amounts of DNA as required by minute clinical specimens.

We then carried out a comparative analysis of the variant calls obtained by both sequencers, retaining only the variants covered by both panels.

**Results**

Using the variant interpretation from DEPArray™ sorted cells (tumor vs. stroma), we clearly identified 4 true-positive non-synonymous somatic variants in TP53 and STK11 genes. Also, 3 out of those 4 variants are annotated by the COSMIC database and were detected by IonTorrent sequencing platforms (100% concordance and specificity). However, by using the variant interpretation from unsorted samples only, we observed a sharp decrease in the specificity. Only 4 variants out of 24 (16%) and 4 variants out of 23 (17%) showed a partial correct interpretation for the PGM and MiSeq assay, respectively. The sensitivity of both assays is excellent, but the level of confidence in the calls is greatly affected by the increase of false positive somatic heterozygous misinterpretations of actual germline variants. Out of a total of 25 variants detected in the unsorted population, only 3 discordant variants were found between the 2 assays (88% concordance). Since those 3 variants were not detected in the sorted populations, we suspect that they are false positive results from PCR artifacts or from the damage inflicted to the DNA during the sample fixation. The variant interpretation of unsorted variants versus matched normal improved specificity of true-positive non-synonymous somatic variants to 4/5 (80%) for PGM and 4/4 (100%) for MiSeq. In addition, digital sorting revealed 8 cases of Loss-of-Heterozygosity (LoH) and 4 events of copy-gain otherwise undetectable in unsorted samples (alone or vs normal). All 3 FFPE samples had relatively high tumor cell content (40-60%) and true positive variants were also detected in the unsorted population. However the specificity was dramatically improved in sorted cells. We predict the use of DEPArray™ sorted cells from low tumor content FFPE will be a determinant factor to detect variants with a high confidence level.

**Conclusions**

Our data showed that i) both AmpliSeq™/PGM and Accel-Amplicon™/MiSeq provide consistent results on low DNA inputs (0.5-2ng), ii) comparison with matched normal is mandatory for correct interpretation of actionable somatic mutations, iii) only sorting cells by DEPArray™ guarantees the utmost accuracy required by clinical actionability of NGS results, including LOH events.

**References**

[Medoro 2011] Use of the DEPArray™ platform to detect, isolate, and molecularly characterize pure tumor cells from peripheral blood samples.