Analysis of *TP53* variants in Bone Marrow Aspirates With TruSight® Tumor 15

Exploring the use of next-generation sequencing (NGS) for variant detection in bone marrow aspirates.

Introduction

TruSight Tumor 15 uses NGS to assess genes that are frequently mutated in solid tumors. The targeted sequencing assay was optimized to work with highly fragmented and degraded DNA in formalin-fixed, paraffin-embedded (FFPE) samples. However, the abundance of these variants in hematological neoplasms prompted the exploration of using TruSight Tumor 15 for analyzing cancer-related genes in bone marrow aspirates.

For example, Tumor Protein P53 (*TP53*) is a gene frequently associated with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[1] This application note demonstrates variant analysis of the *TP53* gene from bone marrow aspirates using the standard workflow of the TruSight Tumor 15 assay. Four individual studies were performed to evaluate the potential of bone marrow aspirate analysis, including:

- evaluating whether sample processing methods impact sequencing results
- evaluating whether interfering substances impact sequencing results
- establishing preliminary values for limit of blank
- establishing preliminary values for limit of detection

Methods

Four individual studies were performed with bone marrow aspirate samples collected and deidentified by the NeoGenomics Clinical Department.[2] DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN), and 200 µl of the extracted DNA was used for library preparation with the TruSight Tumor 15 Library Prep Kit as described in the TruSight Tumor 15 Reference Guide.[3] Sequencing was performed on the MiSeq™Dx System in Research Use Only (RUO) mode. The number of samples varied for each study, but a *TP53* positive control sample, containing a p.Val272Met mutation at 80% variant allele frequency (VAF), was included in each sequencing run.

The sample processing study examined three methods of harvesting blood cells prior to DNA extraction: direct extraction (none), enrichment by ficoll gradient, and red blood cell (RBC) lysis. For library preparation, 200 µl sample volume was used. For the interfering substances study, the *TP53* variant positive control sample was diluted to ~5% VAF and then aliquoted into seven tubes. Each tube was spiked with one of six potentially interfering substances and two separate libraries were made from each sample and sequenced. For VAF analysis, samples were initially analyzed and preliminary VAF calculations were made for each sample. Preliminary estimates were used to dilute each sample with wild-type DNA to approximate 5% VAF. Each diluted sample was aliquoted and reanalyzed with the TruSight Tumor 15 assay.

Results

Sample Processing Study

Seven bone marrow aspirate samples were divided for three conditions (no treatment, ficoll gradient, and RBC lysis) prior to DNA extraction. Equal volumes were then used for library prep and sequencing. Both RBC lysis and ficoll gradient methods yielded significantly fewer cells (Table 1) and lower DNA concentrations (Figure 1) than untreated samples. Lower yields were expected because these methods remove undesired cell types. However, all three methods yielded enough DNA to meet the minimum concentration (≥ 2 ng/µl) required for library preparation. When the same DNA samples were used for library preparation and sequencing, similar sequencing metrics were observed from all three methods. For example, percentages of on-target reads were similar for all seven samples (Figure 2). In sample SP5, similar VAF measurements of two detected *TP53* variants were also demonstrated by all three methods (Figure 3).

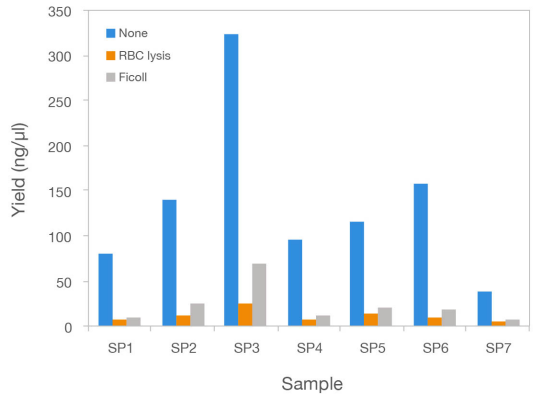

Figure 1: DNA Yield From Three Sample-Processing Methods—Seven bone marrow aspirate samples were divided for three conditions and DNA was extracted with the QIAamp DNA Blood Mini Kit. Minimum input for library preparation is 2 ng/µl.

Table 1: Cell Yield From Three Sample-Processing Methods

Processing Method	Average Yield (Cells/µl)
None	24,700
RBC lysis	1228
Ficoll	2642

Average yield is shown from seven samples.

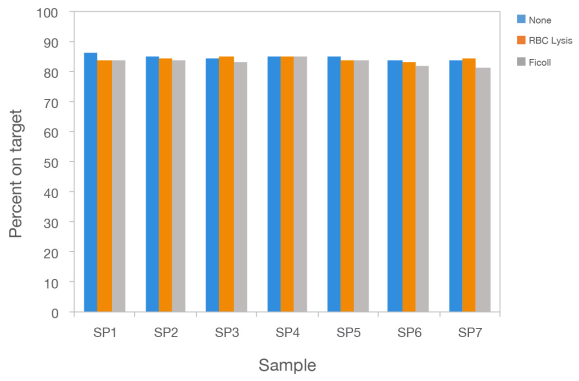

Figure 2: Percent of On-Target Reads From Three Sample-Processing Methods—Seven bone marrow aspirate samples were split for three conditions. Following DNA extraction, 200 µl sample was used for library prep. Three sequencing runs were performed for each sample including a positive control.

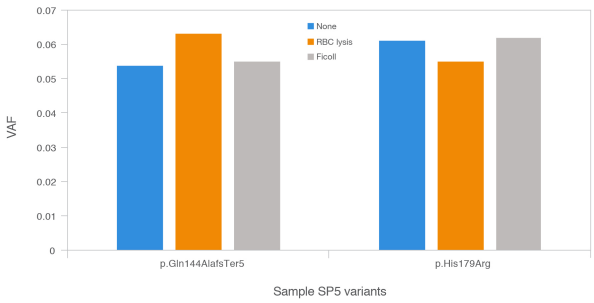

Figure 3: VAF Measurement in Sample SP5 From Three Sample-Processing Methods—Seven bone marrow aspirate samples were split for three conditions. Following DNA extraction, 200 µl sample volume was used for library prep. Similar VAFs were observed in two variants detected in sample SP5.

Interfering Substances Study

The purpose of this study was to determine the extent of potential interference of endogenous or contaminating substances in bone marrow aspirate analysis. Control *TP53* variant DNA was diluted to ~5% VAF, aliquoted, and spiked with six interfering substances (Table 2) before library preparation. Both ethylenediaminetetraacetic acid (EDTA) and hemoglobin appeared to interfere with the PCR amplification step resulting in the low library concentrations. After 1:5 dilution of hemoglobin and 1:1000 dilution of EDTA, all samples passed library prep performance specifications, and two TruSight Tumor 15 libraries were sequenced per substance.

All spiked samples were reported to harbor the variant with VAF values ranging from 2.6–4.5%, suggesting these substances do not interfere with the TruSight Tumor 15 assay (Table 2). The *TP53* p.Val272Met variant was not called in one of the bilirubin samples because of detection below the variant-calling threshold, but a manual review of the BAM files confirmed detection of the variant. No pattern of reduced coverage, or bases on target, was observed resulting from the presence of a specific substance with the DNA.

Table 2: Variant Detection in the Presence of Interfering Substances

Interfering Substance	Substance Concentration	Library Prep	Sequencing Run	VAF Measurement (Two Runs)
Undiluted control	NA	Pass	Pass	0.773,0.789
Diluted control	NA	Pass	Pass	0.037,0.031
Bilirubin (conjugated)	342 µM	Pass	Pass	ND[a],0.037
Hemoglobin	2 g/l	Fail	NA	NA
Hemoglobin	2 mg/l	Pass	Pass	0.035,0.042
Intralipid	37 mM	Pass	Pass	0.045,0.032
EDTA	3.6 mg/ml	Fail	NA	NA
EDTA	0.72 mg/ml	Pass	Pass	0.045,0.026
Heparin	30 USP units/ml	Pass	Pass	0.049,0.041
Ethanol	5% vol/vol	Pass	Pass	0.028,0.042

Abbreviations: NA (not applicable), EDTA (ethylenediaminetetraacetic acid), USP (United States Pharmacopeia)

a. ND = Not detected using TruSight Tumor 15 App for analysis. The TruSight Tumor 15 App does not make calls for variants at very low VAF. When the same data was analyzed with VariantStudio 2.2, VAF was called at 0.022.

Limit of Blank Study

The purpose of this study was to characterize the background *TP53* signal in DNA isolated from bone marrow. Two samples were previously evaluated and determined to be variant-negative. DNA from these samples was aliquoted and used to generate 21 libraries. Three sequencing runs were performed with seven experimental samples and a positive control sample in each run. Each run resulted in detection of *TP53* variants in the positive controls (VAF values of 0.784, 0.779, and 0.781), but no *TP53* mutations were detected in each of the 21 experimental samples. These results indicate no *TP53* variant background in the bone marrow aspirates using the TruSight Tumor 15 assay.

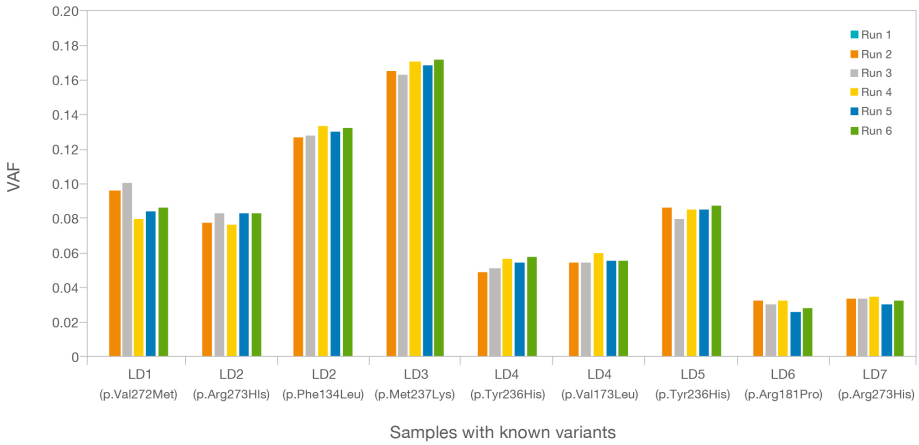

Figure 4: VAF Measurement in Samples with Known *TP53* Variants—Variants in seven bone marrow aspirate samples were measured by NeoGenomics Laboratories to determine VAF values. Then the same samples were diluted to approximately %VAF and analyzed with TruSight Tumor 15. Samples LD2 and LD4 had two *TP53* variants each, so the dilution was based on the lower-frequency variant in each sample.

Limit of Detection Study

The limit of detection study was performed to confirm that TruSight Tumor 15 detects *TP53* mutations from bone marrow aspirates at the same level of sensitivity (5% VAF) expected for analysis of FFPE tissue samples. Seven samples were previously evaluated and determined to be positive for *TP53* variants. DNA from these samples was diluted to approximately 5% VAF. Two libraries were generated from each DNA sample and each library was sequenced three times.

The expected *TP53* variants were detected in each of the seven evaluated samples. Reproducibility of the assay was demonstrated by similar VAF values from six independent sequencing runs (Figure 4). The coefficient of variation for all sample libraries was < 10%, except the second library from sample LD6 (10.66%), which was detected at 2.9% VAF, below the normal 5% threshold. The results from these samples indicate that the TruSight Tumor 15 assay is capable of detecting *TP53* variants from bone marrow at 5% VAF.

Conclusions

TruSight Tumor 15 has been used successfully with various tissue types and with DNA samples of varying quality. This study demonstrates the feasibility of using TruSight Tumor 15 to detect *TP53* mutations in bone marrow aspirate. Two commonly used cell enrichment methods (ficoll gradient and RBC lysis) resulted in lower DNA yields than direct extraction, but neither method impacted library preparation and sequencing results. No interference from common blood-related substances was observed, although hemoglobin and EDTA required dilution prior to the amplification step in library preparation.

Sequencing metrics and analysis results were consistent with established performance specification of TruSight Tumor 15. No background *TP53* variant signals (false negatives) were detected in variant-negative samples. The conservative VAF limit of detection of 5% was observed, with successful detection as low as 3% in some samples, indicating that bone marrow aspirates do not pose any known challenges with this assay. Although TruSight Tumor 15 was optimized to work with FFPE tissue samples, it can also be used for tissue types that would ideally be harvested by different methods, such as blood and bone marrow.

Learn More

To learn more about the TruSight Tumor 15 assay, visit www.illumina.com/products/trusight-tumor-15-gene.html

References

1. Ballya C, Adèsa L, Renneville A, et al. Prognostic value of TP53 gene mutations in myelodysplastic syndromes and acute myeloid leukemia treated with azacitidine. *Leuk Res.* 2014;38(7):751-755.
2. NeoGenomics. neogenomics.com/ Accessed September 27, 2017.
3. Illumina (2016) TruSight Tumor 15 Reference Guide. (support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/trusight/trusight-tumor-15-reference-guide-1000000001245-05.pdf).

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