Materials and Methods
Extracted DNAs from a combination of clinical samples (AML, MDS, MPN, and 7 normals, majority with prior known variants by Sangers sequencing or quantitative PCR) and cell line cultures were submitted to targeted, multiplex-PCR based sequencing for 54 genes (TruSight Myeloid, Illumina, Inc.). Diluted Jurkat DNA and NA12878 DNA served as positive and negative controls respectively. Somatic Variant Caller within MiSeq Reporter was used to detect variants, with annotation by Variant Studio (Illumina). Verification of filtered variants was done by Sangers sequencing (>20% AVF) or repeat NGS (<20% AVF). Sanger sequencing was performed for CEQPA (due to high GC content), KIT (exon 11), and CALR (exon 9). Fragment analysis was used to detect FLIT3-ITD.

Sample Performance Profile

To identify poor-performing amplicons out of the total of 573 amplicons, the % sample performance and reactions with greater than 500x coverage for each of the 573 amplicons were plotted as shown in Figure 2. Figure 3 is the same plot but shows in greater detail those amplicons for which less than 90% of samples have less than 500x coverage.

Amplicons that achieved >500X coverage in less than 60% of cases (<500x coverage are divided into the following categories: the CEBPA gene which will be sequenced independently due to its significant prognostic value in AML, amplicons exhibit little (<1%) or no evidence of mutation in the target region in the relevant assessed diseases will not be considered further for analysis, and amplicons with mutations in the diseases assessed with at least 100x coverage consistently, these will be analyzed further but with reduced limit of detection (20%).

NGS Accuracy

44 specimens each bearing known variants in genes tested in the NGS assay were used to establish the assay accuracy. The variants included both single nucleotide variants (SNV) and small rearrangements (indels) identified previously by Sanger’s sequencing or real-time QPCR. Only for the latter methodology was the AVF known, and permitted selection of specimens with AVF down to as low as 2%. Total number of variants previously known: 47

NGS Sensitivity

In order to achieve a sensitivity of 5%, dilutions of Jurkat DNA mixed with the Hapmap DNA at around the limit of detection were prepared. There were 12 variants within the target regions assessed in the current NGS panel that were found in the undiluted Jurkat cell line as previously reported in the COSMIC database. Based on the AVF detected for the 12 variants by NGS in a total of 8 runs, the average AVF and SD for each variant was calculated and a cutoff at 3% was established so variants present at 5% frequency can be reliably and consistently detected.

NGS Specificity

To facilitate disease identification and/or risk stratification in AML, MDS, and MPN, we have developed a multi-platform approach, permitting robust mutation analysis of 54 relevant genes. This approach is necessary due to limitations of variants observed in the NGS sequencing panel. In the future, optimization of amplicon design and data analysis pipeline will enable more robust assay based on NGS while other platforms can serve as secondary, independent validations of the NGS results.

Conclusions

Analytical specificity of the NGS Panel was assessed by the repeat sequencing of HapMap DNA in all runs and by sequencing normal samples with no known disease history. No new variants were identified and confirmed across multiple runs.

Reference and Acknowledgements

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* Behadel et al. JOM 11(1):71-76

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