ASXL1 p.G646Wfs*12 Detection Enabled and Integrated into a Rapid Automated Myeloid Variant Profiling Solution on Ion Torrent™ NGS Genexus™ System

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INTRODUCTION

Comprehensive gene variant profiling using next-generation sequencing (NGS) improved the research of relevant biomarkers for myeloid disorders. Previously, we described the capability of the Genexus™ System to deliver high quality Variant Calling (VC) results with the Oncomine™ Myeloid Assay GX v2 (OMAv2) in a 24h automated sample-to-report workflow with minimal hands-on-time.[1]

However, certain variants in low complexity genomic regions, like homopolymers, may pose challenges for sequencing and analysis, requiring additional testing and delaying delivery of variant results. One such mutation commonly observed in myeloid malignancies is *ASXL1* p.G646Wfs*12 c.1934dupG COSM34210 (Figure 1). Therefore, we explored general improvements to the GX OMAv2 including the ability to detect *ASXL1* c.1934dupG .

Figure 1. Schematic of *ASXL1* p.G646Wfs*12 c.1934dupG variant

A. ASXL1 wild-type (8G c.1927-1934)

5' ATC GGA GGG GGG GGT GGC CCG 3'
I G G G G G P

B. ASXL1 c.1934dupG (9G)

5' ATC GGA GGG GGG GGG TGG CCC 3'
I G G G G W P

The *ASXL1* c.1934dupG variant results from the insertion of an additional guanine next to an 8G homopolymer in the wild-type sequence (A). This insertion causes a frameshift and converts a glycine amino acid to a tryptophan (B). The variant is often challenging to detect due to the low complexity of this region of the gene.

MATERIALS AND METHODS

250+ BAM files were obtained with sequencing DNA from healthy blood donors, commercially available DNA analytical controls, and DNA research samples with known presence of *ASXL1* c.1934dupG. The dataset was used for fine-tuning of variant specific parameters in Torrent Variant Caller (TVC) algorithm to achieve >95% Sensitivity and >95% Specificity for *ASXL1* c.1934dupG at variant allele frequency (VAF) >10%.

To evaluate the effect of the new TVC parameters, we tested two Myeloid DNA analytical controls, Horizon Myeloid gDNA reference standard HD829 and Seraseq™ Myeloid DNA Mutation Mix (SCMM), containing *ASXL1* c.1934dupG, in multiple replicates (N≥20). The testing was performed in nucleic acid-to-report configuration and Genexus™ Software output was used for VC analysis.

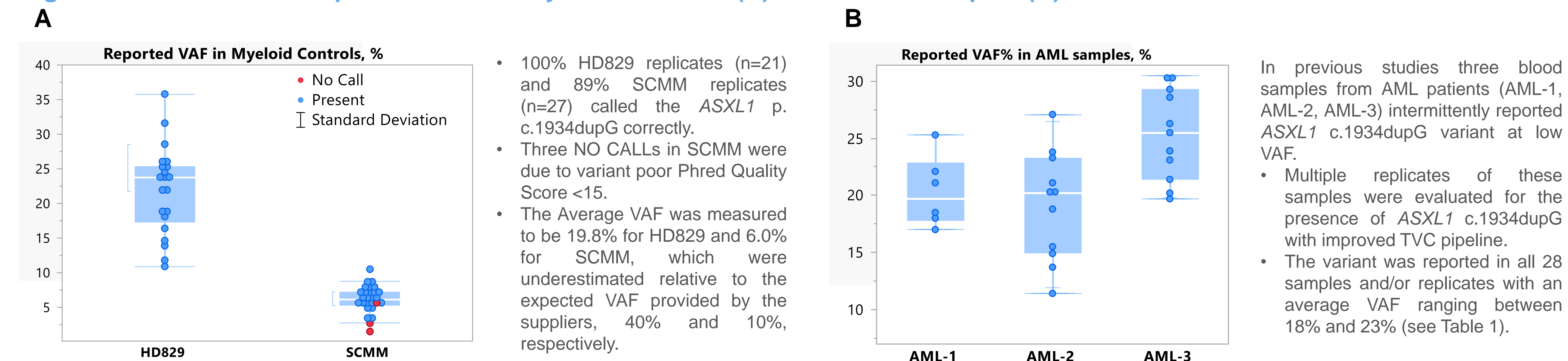
HD829 was mixed with variant negative samples, NA12878 or NA24385, to target *ASXL1* c.1934dupG VAF below 20%. DNA mixtures were sequenced with ≥5 replicates per VAF in the range 5-18%.

ASXL1 c.1934dupG variant detection was also evaluated in Acute Myeloid Leukemia (AML) research blood samples, herein referred as AML-1, AML-2, and AML-3. Sanger sequencing was used as an orthogonal method to confirm the variant presence. OMAv2 gene specific primers targeting amplicon with *ASXL1* c.1934dupG variant were 5' and 3' tailed with an M13 universal sequences to make them compatible with Applied Biosystems™ Dye Primer sequencing. VAFs from the Sanger sequencing data were estimated using QSVanalyser.

Key *ASXL1* variant in low complexity region detected by Oncomine™ Myeloid Assay GX v2

RESULTS

Figure 2. *ASXL1* c.1934dupG detection in Myeloid Controls (A) and in AML samples (B)



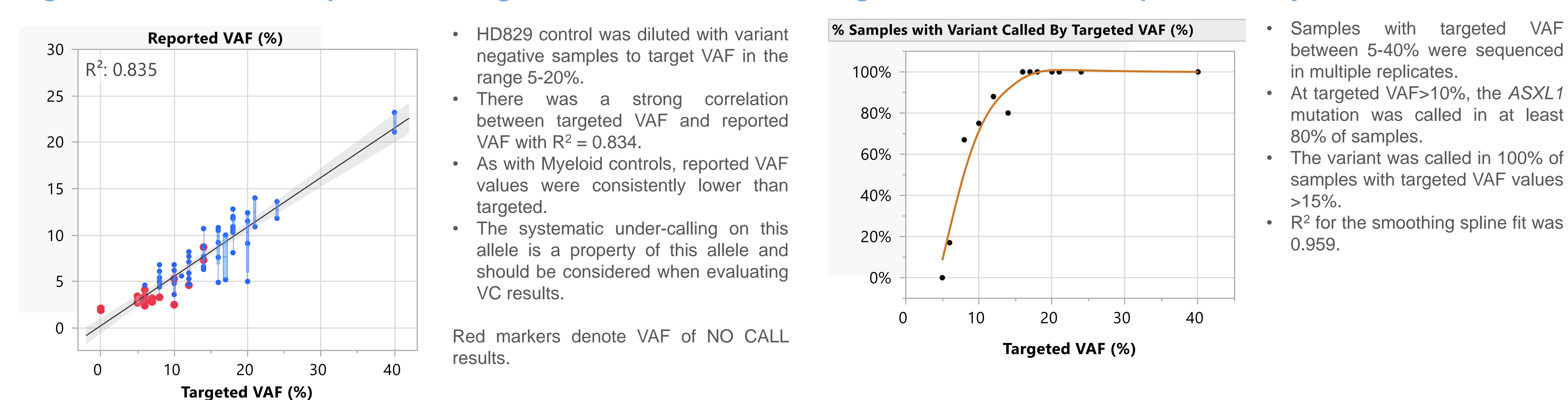
- 100% HD829 replicates (n=21) and 89% SCMM replicates (n=27) called the *ASXL1* p. c.1934dupG correctly.
- Three NO CALLs in SCMM were due to variant poor Phred Quality Score <15.
- The Average VAF was measured to be 19.8% for HD829 and 6.0% for SCMM, which were underestimated relative to the expected VAF provided by the suppliers, 40% and 10%, respectively.

In previous studies three blood samples from AML patients (AML-1, AML-2, AML-3) intermittently reported *ASXL1* c.1934dupG variant at low VAF.

- Multiple replicates of these samples were evaluated for the presence of *ASXL1* c.1934dupG with improved TVC pipeline.
- The variant was reported in all 28 samples and/or replicates with an average VAF ranging between 18% and 23% (see Table 1).

Figure 3. Correlation of reported and targeted VAF

Figure 4. *ASXL1* c.1934dupG sensitivity as a function of VAF

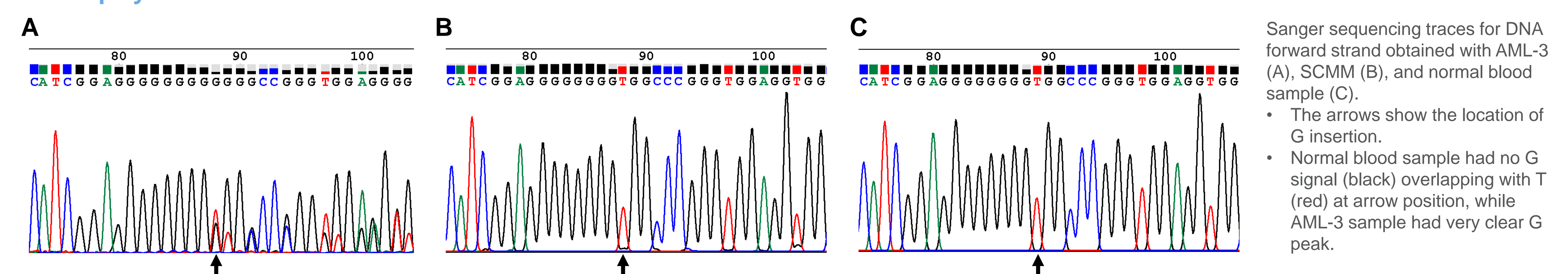


- HD829 control was diluted with variant negative samples to target VAF in the range 5-20%.
- There was a strong correlation between targeted VAF and reported VAF with $R^2 = 0.834$.
- As with Myeloid controls, reported VAF values were consistently lower than targeted.
- The systematic under-calling on this allele is a property of this allele and should be considered when evaluating VC results.

Red markers denote VAF of NO CALL results.

- Samples with targeted VAF between 5-40% were sequenced in multiple replicates.
- At targeted VAF>10%, the *ASXL1* mutation was called in at least 80% of samples.
- The variant was called in 100% of samples with targeted VAF values >15%.
- R^2 for the smoothing spline fit was 0.959.

Figure 5. Sanger sequencing confirms *ASXL1* c.1934dupG presence in AML samples but lacks sensitivity to call variant in Seraseq Myeloid DNA Mutation Mix



Sanger sequencing traces for DNA forward strand obtained with AML-3 (A), SCMM (B), and normal blood sample (C).

- The arrows show the location of G insertion.
- Normal blood sample had no G signal (black) overlapping with T (red) at arrow position, while AML-3 sample had very clear G peak.

Table 1. VAF in AML samples estimated with QSVanalyser and reported by Genexus

Sample	Forward strand (%)	Reverse strand (%)	Reported by Genexus
AML-1	37.8	23.8	20.3 (n=6)
AML-2	39.1	20.2	19.1 (n=11)
AML-3	40.4	25.1	25.3 (n=11)
SCMM	7.1	NA	6.5 (n=24)

- VAF values in Sanger sequencing results were estimated to be between 37-40% in the forward DNA strand and 20-25% on the reverse strand.
- For SCMM VAF was estimated to be ~7% with DNA forward strand. DNA reverse strand sequencing data were too noisy to estimate VAF.
- Note the systematic difference between forward and reverse strand VAF.

False Positive (FP) rate and Specificity evaluation

FP rate was estimated using sequencing results from 550+ variant negative samples. Five FP were reported yielding FP rate below 1% and Specificity >99%. The average FP VAF was 4.4%. Yannakou, C.K., Jones, K., McBean, M. et al.[2] discuss the limitation of different detection technologies, including Sanger sequencing, fragment analysis, and NGS.

CONCLUSIONS

Variant calling improvements were made in the Oncomine™ Myeloid Assay GX v2 to enable robust detection of challenging variants in low complexity regions including *ASXL1* c.1934dupG. The capability to accurately report *ASXL1* c.1934dupG within OMAv2 is important to provide complete results. Our data support an LOD of 15% VAF for the detection of this variant with the improved analysis workflow. Using the Genexus™ automated NGS sequencing system, the OMAv2 assay enables detection of key Myeloid variants with a 24hr turn-around-time.

REFERENCES

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2. Yannakou, C.K., Jones, K., McBean, M. et al. ASXL1 c.1934dup;p.Gly646Trpfs*12—a true somatic alteration requiring a new approach. Blood Cancer Journal 7, 656 (2017)

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