Development and Performance of SNAQ™-SEQ Spike-in standards in AML NGS Diagnostic and MRD testing

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BACKGROUND

The European Leukemia Net (ELN) criteria for AML risk assessment uses cytogenetic testing and molecular mutations for recurrent chromosomal aberrations as standard-of-care for risk stratification and treatment decisions in AML[1]. The prognosis and treatment of acute myeloid leukemia (AML) is largely defined by cytogenetics and molecular variants[1]. Accurate quantification of these mutations is important in risk determination and the evaluation of treatment response[2]. Standard clinical next generation sequencing (NGS) is impacted by pre-analytical factors, amplification and sequencing issues that often effect accurate assessment of reported variants. The existing test QC, such as QS20 or read depth, are indirect surrogates of sample performance, and positive external run control reference materials monitor ability to sequence cell line genomic DNA. What is needed is a simple direct QC that ensures the sensitivity and performance of each sample. We developed a "spike in" control to assist in accurate assessment of oncogenic and likely oncogenic variants in AML, allowing for the assessment and control of variations in each NGS sequencing sample independently.

HYPOTHESIS

Use of spike in standards can help in molecular assays for measurable residual disease (MRD) by serving as a robust internal control and allowing for more accurate VAFs.

METHODS

- **A panel of fourteen key hotspot targets in AML was identified to develop the internal standard (IS) spike-in controls.**
- **For MRD relevant VAF testing, a titration curve of 10 to 300 IS copies (Accukit Myeloid DNA IS, AccuGenomics Cat# 2023) were spiked into 120 ng of cell line DNA (Figure 2 shows results of 100-300 IS copies).**
- **To test the performance of IS as a spike in sensitivity control, 19 archival AML patient buffy coat DNA samples were spiked with 17 IS (Table 1).**
- **Samples were prepared into libraries using Oncomine Myeloid MRD protocol on the Ion Chef S5 then sequenced with Genestudio S5 (ThermoFisher).**
- **IS variants were added to the hotspot.bed file to ensure their reporting by the Ion Reporter Software, from which the resulting VCF were used to extract the IS VAF response (Figures 4, 5).**
- **The reproducibility of the method was tested in two labs (Figure 3).**

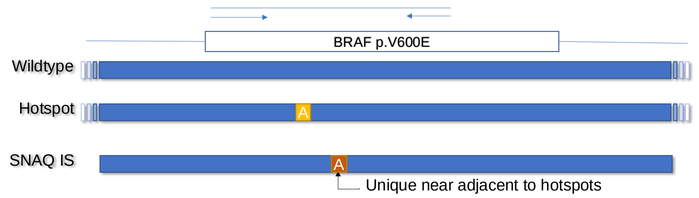


Figure 1: The concept and design of spike-in of AccuGenomics SNAQ™-SEQ internal standards (IS). IS feature unique variants that sit adjacent to hotspot base positions to allow their detection by existing bioinformatic pipelines

- SNAQ™ IS designed to biochemically covary with regions of interest
  - IS are spiked into every sample
  - IS are copurified and captured in library with sample template
  - IS are fragmented with sample or come pre-fragmented like cfDNA
  - SNAQ™ analysis performed on VCF
  - Unique synonymous variant to make bioinformatically identifiable
- IS variant placed near actionable pathogenic variant positions
  - **IS provide a per sample sensitivity control**
  - **IS standardize abundance measurements**

Table 1: AccuGenomics Internal Standards (IS) panel:

VARIANT	Gene	Mutation-nt	Mutation-aa
SNV	ABL1	c.942C>T	p.Ile315
	DNMT3A	c.2646G>A	p.Arg882
	FLT3	c.2501C>A	p.Arg834Leu
	IDH1	c.391C>T	p.Gly391Ser
	IDH2	c.512C>T	p.Gly171Asp
	JAK2	c.1853G>T	p.Cys618Phe
	KRAS	c.31C>T	p.Ala11Thr
	NRAS	c.441C>T	p.Lys147
	NRAS	c.180T>A	p.Gly60
	TET2	c.719C>T	p.Arg1262
	TP53	c.719C>T	p.Ser240Asn
INS	FLT3-short ITD	c.1808insA..28..T	p.Lys602_Trp603insPhe..Trp
	FLT3-long ITD	c.1741insC..124..T	p.Gln580_Val581insLys..Val
	NPM1	c.866_867incGTCT	p.Gln289Cfs*12
	NPM1	c.866_867incGCAT	p.Gln289Cfs*12
	NPM1	c.866_867incGCCT	p.Gln289Cfs*12
DEL	JAK2	c.1605_1610delGGTTGT	p.Met535_Val536delinsIle

RESULTS

Figure 2. AccuGenomics IS added to of background DNA The experiment demonstrates linear detection of the IS to low VAF levels (0.01%) in the contrived example of cell line DNA (GM12878). Total input sample DNA used is 120ng.

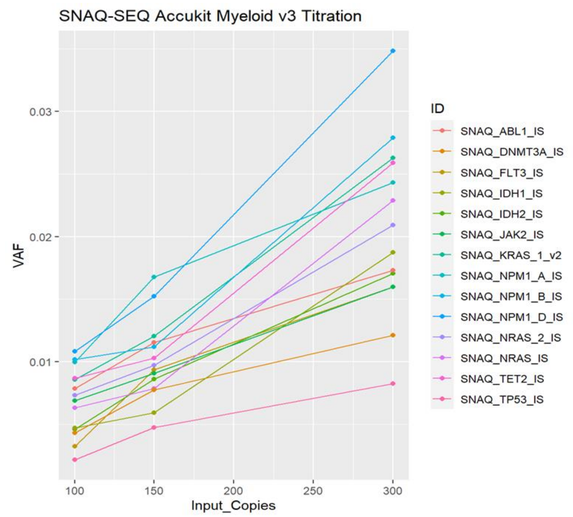
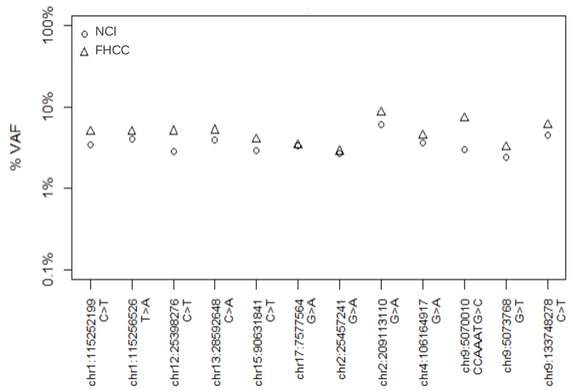
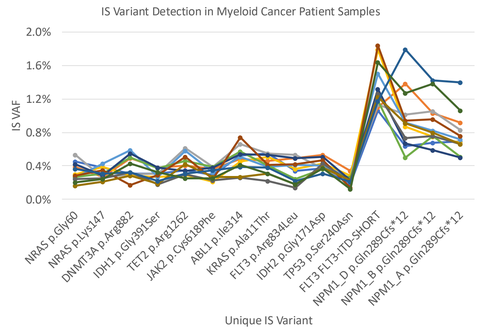


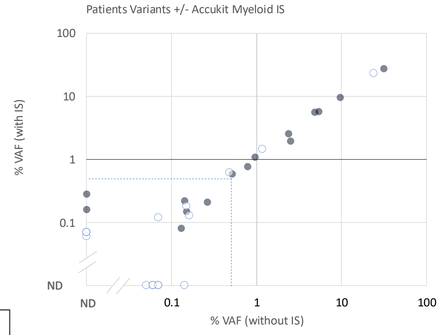
Figure 3. The performance of IS are comparable across labs. Both labs showed detection of the IS near the expected value of 4% across all twelve IS targets. This demonstrates the robust performance of the SNAQ™-SEQ IS.



- Two labs tested IS (Fred Hutch, MoCha/Fredericks NIH)
- Labs mixed 26 ng DNA + 300 copies IS mixture
- NGS via Thermofisher Oncomine Myeloid Assay v2 detected using Genexus™ Software
- Near adjacent hotspots extracted from VCF
- All 12 variants were detected at near expected VAF at method LoD
- **Demonstration of using an internal limit control to standardize inter laboratory performance**

Figure 4. IS detection is accurate after spike-in into AML samples. This experiment demonstrates the addition of IS spike-ins in a background of AML DNA at expected does not effect IS amplification and detection at expected values





- 150 copies IS mixture added to twelve 120 ng AML patient DNA samples, sequenced using the Thermofisher Oncomine Myeloid MRD test
- All unique IS variants detected at low levels (expected value 0.4%)
- High IS indel VAF indicates test method over-estimation

Figure 5. IS spike-ins do not affect or bias detection of AML targets at expected VAF. This experiment demonstrates the converse of Figure X, above: the introduction of the IS does not affect the amplification and detection of the AML targets, as the AML target with and without IS spike-ins are the same.

- 150 copies IS mixture added to twelve 120 ng AML patient DNA samples, sequenced using the Thermofisher Oncomine Myeloid MRD test
- All AML samples also sequenced ***without*** IS spike-ins
- Y axis VAF of AML samples with IS spike-ins when tested by Oncomine Myeloid MRD test
- X axis VAF of AML samples without spike-ins when tested by Oncomine Myeloid MRD test
- Hash-boxed variants were below the Oncomine Myeloid MRD test limit of detection
- All patient sample variants were either previously identified by TwinStrand sequencing (closed circles), or detected during the Oncomine Myeloid MRD testing

CONCLUSIONS

1. Two laboratories have demonstrated interlab reproducibility with the Oncomine Myeloid MRD protocol on the Ion Chef S5/Genestudio S5 system.
2. The Oncomine Myeloid MRD protocol has a LOD of 0.05% without added IS mixture an using the recommended sample input of 120ng DNA.
3. Spike-in IS can reproducibly be detected at levels consistent with MRD detection (<0.1%)
4. The use of IS in the context of AML samples
   A. Does not affect IS detection and quantification
   B. Does not affect AML target detection and quantification

REFERENCES

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2. Simone E. Dekker et al., Using Measurable Residual Disease to Optimize Management of AML, ALL, and Chronic Myeloid Leukemia. Am Soc Clin Oncol Educ Book 43, e390010(2023). DOI:10.1200/EDBK_390010

ACKNOWLEDGEMENT

We would like to thank the 1690 repository, patients and clinical care teams for enabling this research.