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Comparison of Complete Genomics DNBSEQ-G99 Sequencer and Illumina® MiSeq™ using Invivoscribe's *FLT3* ITD MRD Assay and *NPM1* MRD Assay

INTRODUCTION

Next-generation sequencing (NGS) is reshaping the paradigm of oncology, transforming how genetic mutations are identified and integrated into patient treatment and management. Unlike traditional PCR-based assays, which struggle with detecting mutations in low tumor burden samples and demand expert interpretation, NGS offers superior sensitivity and specificity, enabling more accurate identification of rare variants and a broader spectrum of genetic alterations. This technology advancement is rapidly revolutionizing prognostic and diagnostic capabilities, facilitating personalized treatment strategies, and enhancing clinical decision-making in cancer care.

The high specificity and resolution of NGS allows the identification of genomic aberrations associated with *FLT3* internal tandem duplications (ITD) and *NPM1* mutations. Deep sequencing can uncover minute traces of malignant cells lingering after treatment, providing a significant advantage over traditional methods that might miss these low-burden populations. This heightened ability to detect measurable residual disease (MRD) has potential clinical applications including prognostication, early identification of relapse, monitoring disease evolution, as well as optimizing and personalizing treatment strategies. Global recognition of this clinical value has caused NGS-based testing to be added to several clinical guidelines including NCCN[1] and ELN.[2]

Benefits of using NGS for MRD detection include:

- Higher Sensitivity and Specificity: This allows for the detection of low-frequency mutations, enhancing diagnostic accuracy and the ability to monitor disease progression.[3]
- Objective and Quantitative Analysis: NGS eliminates the subjectivity of other methods and enables precise monitoring of disease burden over time, which is crucial for MRD assessment, guiding treatment decisions, and predicting relapse.[3]

Over the past decade, NGS testing has become more affordable, and is now often used to identify mutations and track residual disease over time. The need to detect and monitor low burden disease has led to the development of assays for use with various NGS platforms. Today, the use of NGS technology for hematological disease research has become widely adopted, driving advancements for increased read depth, superior sequencing accuracy, streamlined data generation, faster sequencing time and higher sample throughput. These technological improvements have created an increased demand to adapt existing NGS assays for higher-throughput platforms, such as the DNBSEQ-G99 System (called G99 hereafter) from Complete Genomics.

Invivoscribe NGS Assays Overview

NGS Assays for MRD Detection of Myeloid Diseases

Invivoscribe's suite of NGS products includes two pivotal Research Use Only (RUO) MRD NGS assays.

- *FLT3* ITD MRD Assay (RUO): targets the FMS-like tyrosine kinase 3 (*FLT3*) gene which encodes a receptor tyrosine kinase that is normally expressed on many cell types, including hematologic stem cells. Mutation of the *FLT3* receptor, by internal tandem duplication of the juxtamembrane domain, causes constitutive activation of the *FLT3* receptor. *FLT3* ITD mutations are present in about 25% of individuals with Acute Myeloid Leukemia (AML) and are characterized by an increased risk of relapse.[4]

- *NPM1* MRD Assay (RUO): targets the Nucleophosmin (*NPM1*) gene which encodes for a protein involved in cellular activities that may relate to proliferative and growth-suppressive roles in the cell. As one of the most frequently mutated genes in AML, *NPM1* gene mutations occur in about one-third of the cases of primary AML. Of the individuals with an *NPM1* mutation at diagnosis, roughly 50% relapse during the first 3 years, notably those with a concurrent *FLT3* ITD mutation.[5]

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Recent research suggests the presence of *FLT3* ITD and *NPM1* mutations can be indicative of a disease outcome. In a study evaluating the persistence of residual *FLT3* ITD and *NPM1* mutations among subjects with AML in first remission prior to allogeneic cell transplant, those with an allele fraction ≥0.01% trended towards an elevated risk of relapse and mortality compared to those without these mutations.[6]

Invivoscribe's *FLT3* ITD MRD and *NPM1* MRD Assays for MiSeq include 24 uniquely indexed master mixes, allowing multiple samples and targets to be sequenced on the same flow cell for cost efficiency. Assay interpretation and data analysis of MiSeq output is completed with either the *FLT3* ITD MRD Software or *NPM1* MRD Software in under an hour, providing precise sequence annotations and objective results.

NGS Instrument Overview

Illumina MiSeq™

The Illumina MiSeq™ is a benchtop sequencer designed for low-mid throughput targeted sequencing applications. The MiSeq utilizes double stranded (dsDNA) libraries and a method called sequencing by synthesis (SBS) where individual bases are identified one at a time as they are incorporated into a growing DNA strand during a synthesis reaction. Since each base being labeled with a fluorescent dye is identified during the sequencing process; essentially, the sequence is "read" as the DNA is built, base by base, enabling high-throughput parallel sequencing of millions of DNA fragments simultaneously. It has been widely adopted due to its accuracy and relatively low cost, making it suitable for research applications in small to medium sized laboratories.

Complete Genomics DNBSEQ-G99

The G99 is a fast sequencer offering low-to mid-range throughput and the ability to run two flow cells concurrently on the same platform. In contrast to Illumina sequencing platforms which use dsDNA libraries, the Complete Genomics G99 is a new NGS platform, that utilizes DNBSEQ™ technology -a process of rolling circle replication (RCR) to create billions of DNBs in a single tube where DNBs are generated from the original DNA circle to provide efficient and accurate sequencing. Thus, each copy is created from the original DNA, minimizing amplification errors often produced by PCR.[7]

To facilitate the conversion from linear dsDNA libraries made for Illumina platforms into circular single stranded DNA (ssDNA) libraries compatible with the G99 sequencing platform, Complete Genomics offers a Universal Library Conversion Kit (App-A), which employs adapter-conversion PCR to add specific adapters to the primers.

Table 1:

Sequencer	Illumina MiSeq	Complete Genomics DNBSEQ-G99
Sequencing Depth	~50 million paired-end reads (2x300 bp)	~80 million paired-end reads (2x300 bp)
Accuracy	Base calling accuracy >99.9%	Base calling accuracy >99.99%
Turnaround Time	~ 56 hours for 2x300 bp run	~ 30 hours for 2x300 bp run
Cost-Effectiveness	Instrument and reagent cost economical for moderate sample processing volume labs	Instrument and reagent cost lower than MiSeq

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MATERIALS AND METHODS

Positive DNA from cell lines and clinical samples were contrived using mutation negative DNA as a background to create panels for each assay. Clinical *FLT3* ITD and *NPM1* samples were diluted to 5.0E-05. (*NPM1* cell line samples were diluted to 1.0E-05.) These dilution levels mimic MRD levels of *FLT3* ITD and *NPM1* mutations of the *FLT3* and *NPM1* genes. The workflow for the NGS MRD assays on MiSeq is shown in Figure 1 and for G99 using existing libraries in Figure 2:

Two pooled libraries were generated:

Library A: comprised of *FLT3* ITD MRD Assay samples
Library B: comprised of *NPM1* MRD Assay samples

Libraries A (*FLT3* ITD) and B (*NPM1*) were sequenced separately, using the MiSeq Reagent Kit v3[A] at 2x300 cycles on the MiSeq with a loading concentration of 14 pM and 12.5% PhiX.

The same library A and B went through library conversion using CG's App-A conversion kit[B] per manufacturer instruction. These DNBs were applied to a high-density patterned flow cell with a loading concentration of approximately 38 ng/µL. The Standard Library Reagent-TP v5.0C was spiked at 30%. Sequencing was performed on the G99 using the APP-D PE300 SM+ reagent kit.[D]

FASTQ files from the MiSeq were analyzed using the *FLT3* ITD MRD Software (v1.2) and *NPM1* MRD Software (v1.1.1). FASTQ files from the G99 were analyzed using an in-house developed bioinformatics pipeline. Detected variant read frequencies (VRFs) from each of the target specific mutations were compared between the NGS platforms.

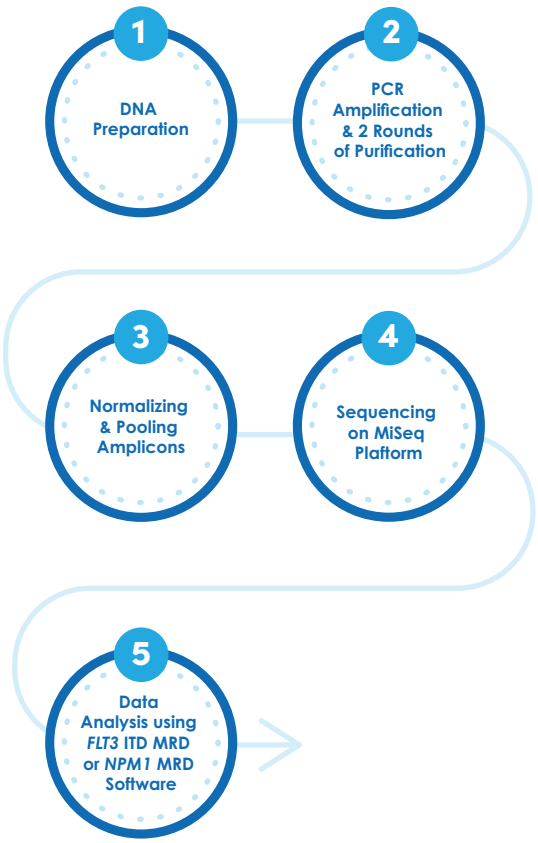

Figure 1: Invivoscribe's AML MRD NGS Assay Workflow on MiSeq

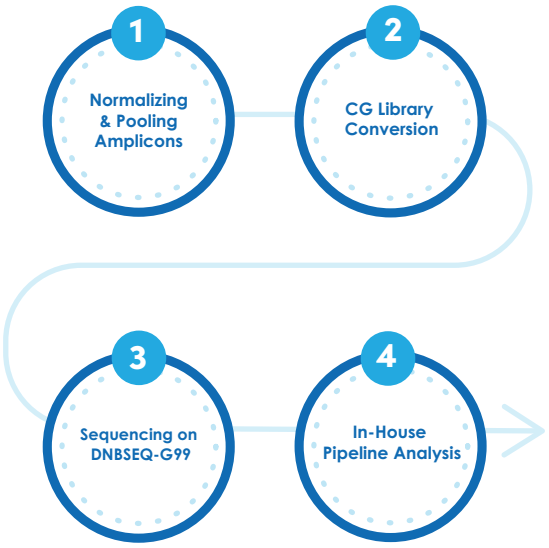

Figure 2: DNBSEQ-G99 NGS Assay Workflow Starting with Existing Libraries

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RESULTS

MRD Assays

Separate libraries were generated for each mutation target (*FLT3* ITD and *NPM1*) and sequenced individually on the MiSeq to ensure a suitable read depth was achieved. The MiSeq generated 19 million pass filter (PF) reads with the *FLT3* ITD MRD Assay library, and 14 million PF reads with the *NPM1* MRD Assay library.

The same libraries were then combined and sequenced on the G99 post library conversion resulting in 6-fold more PF reads (110.6 million), as compared to the two MiSeq runs. The results generated from both the instruments were compared and the relative R^2 of the VRF correlation for each were greater than 0.99. Each assay's relative R^2 values are listed in Table 2.

Table 2: MRD Assay Results Correlation between Instruments

Assay	Correlation (R^2)	Key Run Metrics	
		MiSeq	G99 (2x300 bp)
FLT3 ITD MRD	0.997	PF: 19.2 million Q30: 89.28% Sequencing time: ~55 hours	PF: 110.6 million Q30: 93.47% Sequencing time: ~32 hours
NPM1 MRD	0.999	PF: 14.1 million Q30: 89.94% Sequencing time: ~55 hours	

VRF comparison between the instruments is depicted in Figures 3 and 4.

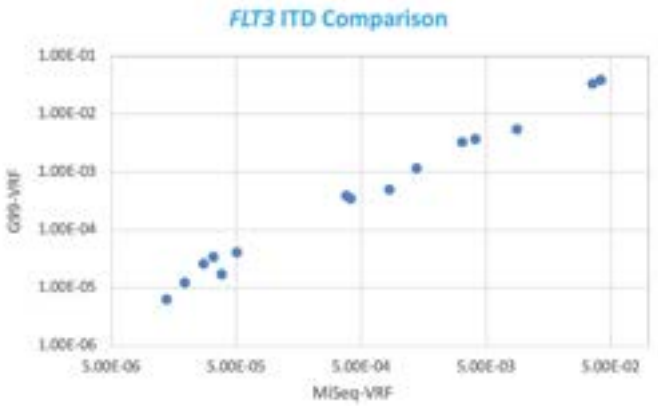

Figure 3: FLT3 ITD MRD Assay VRF Comparison

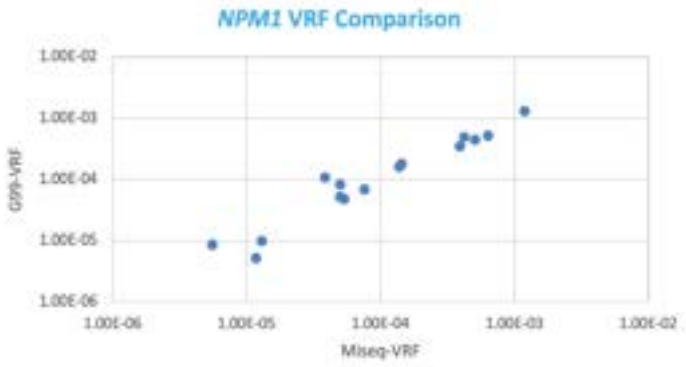

Figure 4: NPM1 MRD Assay VRF Comparison

CONCLUSION

Invivoscribe's *FLT3* ITD and *NPM1* MRD Assays demonstrated high concordance between the MiSeq and G99 platforms with a limit of detection at 5.0E-05. These results demonstrate that Invivoscribe's RUO *FLT3* ITD and *NPM1* MRD Assays perform similarly on both the sequencing platforms and suggest that these assays can be utilized with Complete Genomics' App-A conversion kit and the DNBSEQ-G99.

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Furthermore, the G99 offers a significant advantage cost, faster turn around time (TAT) and higher throughput. During this performance comparison study, the G99 generated a substantially greater number of reads compared to the MiSeq (up to 6-fold) which translates to:

- Comparable Sensitivity: Detection of low-frequency mutations is particularly important for MRD studies where identification of minute traces of residual disease is critical.
- Enhanced Confidence in Results: Deeper sequencing translates to a higher number of reads available for analysis which provides greater confidence in negative MRD results, reducing the risk of false negatives and delayed detection of relapse.
- Improved Accuracy and Efficiency: The G99 demonstrated faster run times and better Q30 scores (quality metric used to indicate that there is a 1 in 1,000 chance (or 0.1%) of an incorrect base call) compared to the MiSeq when used with the MRD assays. These improvements enable laboratories to accurately process more samples with faster TAT.

ABOUT INVIVOSCRIBE

Invivoscribe is a global, vertically integrated biotechnology company dedicated to Improving Lives with Precision Diagnostics®. For nearly thirty years, Invivoscribe has improved the quality of healthcare worldwide by providing high quality standardized research reagents, tests, and bioinformatics tools to advance the field of precision medicine. Invivoscribe has a successful track record of partnerships with global pharmaceutical companies interested in developing and commercializing companion diagnostics and provides expertise in both regulatory and laboratory services. Providing distributable reagents and kits, as well as clinical trial services through its globally located clinical lab subsidiaries (LabPMM), Invivoscribe is an ideal partner from proof of concept through commercialization.

To learn more about how you can use Invivoscribe's assays on Complete Genomics DNBSEQ-G99 sequencer please email inquiry@invivoscribe.com. To learn more about Invivoscribe and LabPMM®, visit us online at invivoscribe.com or call us at +1.858.224.6600.

Invivoscribe's RUO MRD Assays and Software for Myeloid Disease*

Catalog #	Product Description	Quantity
14120019	*FLT3* ITD MRD Assay	96 Reactions
14160019	*NPM1* MRD Assay	96 Reactions
S100005	*FLT3* ITD MRD Software – MiSeq	1 software package
S100004	*NPM1* MRD Software - MiSeq	1 software package

**Software compatibility with G99 is planned.*

Illumina and Complete Genomics Reagents

Vendor	Description	Catalog #
[A]Illumina	MiSeq Reagent Kit v3	MS-102-3003
[B]Complete Genomics	DNBSEQ Universal Library Conversion Kit (App-A)	100-00041-55
[C]Complete Genomics	Standard Library Reagent-TP v5.0	940-001278-00
[D]Complete Genomics	DNBSEQ-G99 High throughput sequencing set (App-D FCL PE300)	940-001717-00

References
1. NCCN Clinical Practice Guidelines in Oncology: Acute Myeloid Leukemia. Version 4. 2023. | 2. Dohner et al. *Blood.* 2017 Jan 26; 129(4): 424–447. | 3. Ho et al. *J Mol Diag.* 2021 May;23(7): 805–815. | 4. Daver et al. Nature. Leukemia. 2019 33, 299 -312. | 5. Kronke et al. *Blood.* 2013 Jul 4; 122 (1): 100–108. | 6. Dillon et al. *JAMA.* 2023 Mar 7; 329(9):745–755. | 7. High-throughput Sequencing Set DNBSEQ-G99RS versión 3.0 (6500066ce3188).