invivoscribe®

Performance Comparison of Invivoscribe's LymphoTrack® Assays Between Complete Genomics DNBSEQ-G99 and Illumina® MiSeq™ NGS Platforms

INTRODUCTION

Next-generation sequencing (NGS) technology is rapidly transforming the paradigm of how gene rearrangement information can be used in the study of hematological malignancies. While traditional gene rearrangement assays, such as PCR-based techniques, have limitations with samples containing low tumor burden and require expert data interpretation, NGS-based methods effectively address these limitations while offering superior sensitivity and specificity. The high resolution of NGS allows the meticulous differentiation between clonal and polyclonal T-cell receptor (TCR) or immunoglobulin (Ig) gene rearrangements.

This translates to a heightened ability to detect measurable residual disease (MRD), with potential clinical applications including identification of early relapse, refractory disease and has the potential to optimize treatment strategies. NGS can uncover minute traces of malignant cells lingering after treatment, providing a significant advantage over traditional methods that may miss these low-burden populations. The National Comprehensive Cancer Network (NCCN) guidelines recommend MRD testing for several lymphoid cancers, including multiple myeloma (MM), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL).[1,2,3,4,5,6]

Over the past decade, Invivoscribe has pioneered the use of NGS to identify gene rearrangements and track residual disease over time. Invivoscribe offers seven different LymphoTrack Assays using the Illumina MiSeq™ NGS platform, which can detect target-specific sequences that aid in the detection and monitoring of hematological malignancies. NGS technology has made significant progress in recent years, with advancements in read depth, data generation, and sequencing accuracy; there is a demand to adapt existing NGS assays to higher-throughput platforms such as the Complete Genomics DNBSEQ-G99 (called G99 hereafter) NGS platform. Here we present a performance comparison between the MiSeq and G99 platforms using identical libraries generated by these seven LymphoTrack Assays.

Invivoscribe NGS Assays Overview

Clonality NGS Assays for Lymphoproliferative Disorders

All of Invivoscribe's LymphoTrack Assays for MiSeq represent a significant improvement over existing clonality assays that apply fragment analysis because of the enhanced detection efficiency for gene rearrangements and ability to identify the DNA sequence specific for each clonal gene rearrangement. Therefore, these assays have three important and complementary uses:

1. Provides critical information on the existence of clonality.
2. Identifies sequence information required to track clones in subsequent samples.
3. Supplies detailed sequence information on the degree of somatic hypermutation (*IGH* and IGHV Leader specific).

invivoscribe®

LymphoTrack Assays are distributable kits that include assay controls as well as master mixes containing primers designed with Illumina adapters, up to 24 different indices. This design allows for a single step PCR, followed by amplicon purification and pooling several different samples and targets when assays are multiplexed together. Combining multiple assays on a single flow cell allows each target to be analyzed in parallel from one sequencing run. Additionally, the associated bioinformatics analysis, a pipeline version of LymphoTrack Software, provides a simple and streamlined method for analysis and visualization of data.

NGS Instrument Overview

Illumina MiSeq™

The Illumina MiSeq is a benchtop instrument that uses 4-channel sequencing by synthesis (SBS) technology. It is designed for targeted sequencing applications and is recognized for its high accuracy and relatively low throughput, making it suitable for small to medium-sized laboratories.

Complete Genomics DNBSEQ-G99

The Complete Genomics G99 was recently introduced to the market and utilizes rolling circle replication (RCR) to create billions of DNA nanoballs (DNBs) generated from the original DNA circle in a single tube. The sequencing technology allows competitive data generation speed with mid-range throughput and the ability to run two flow cells independently.

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

MATERIALS AND METHODS

Clonality Assay

Contrived samples were prepared using DNA isolated from clonal (positive) and polyclonal (negative) cell lines to create panels for each assay. The evaluated assays target *IGH* V_H-J_H rearrangements (IGHV Leader, FR1, FR2 and FR3), immunoglobulin light chain (*IGK*), T-cell receptor gamma (*TRG*), and T-cell receptor beta (*TRB*) genes.

The libraries constructed from the LymphoTrack Assays are linear double-stranded DNA (dsDNA). However, the Complete Genomics DNBSEQ platform requires circular, single-stranded DNA libraries (ssDNA). To bridge this gap, a library conversion kit was used to convert the linear dsDNA libraries into circular ssDNA libraries (DNBSEQ Universal Library Conversion Kit [App-A][B]).

The workflow for the LymphoTrack Assays using both sequencing platforms is shown in Figure 1.

Following amplification, each target was purified, quantified, and normalized according to the product IFU. These normalized amplicons were then used to create a seven-combo library by pooling equimolar quantities of each target: IGHV Leader, *IGH* FR1/2/3, *IGK, TRG*, and *TRB*. This seven-combo library was then sequenced on the Illumina MiSeq and Complete Genomics G99 instruments. Sequencing parameters for the MiSeq included a library loading concentration of 18pM, using MiSeq Reagent kit v3[A] at 2x300bp cycles. To sequence the same seven-combo library using the Complete Genomics G99 platform, the library was converted from dsDNA to circular ssDNA using the DNBSEQ Universal Library Conversion Kit (App-A)[B], which denatures and circularizes the resulting ssDNA, then applies rolling circle replication (RCR) to generate DNBs for each sample. These DNBs were loaded at a concentration of ~37 ng/µL onto a high-density patterned flow cell, then sequenced using the DNBSEQ-G99 High throughput sequencing set (App-D FCL PE300)[C] at 2x300bp.

Data analysis for MiSeq FASTQ files was performed using the a pipeline version of LymphoTrack Software. Data analysis for the G99 FASTQ files was performed using an in-house bioinformatics pipeline. The top % reads generated by each target were compared between the two different platforms.

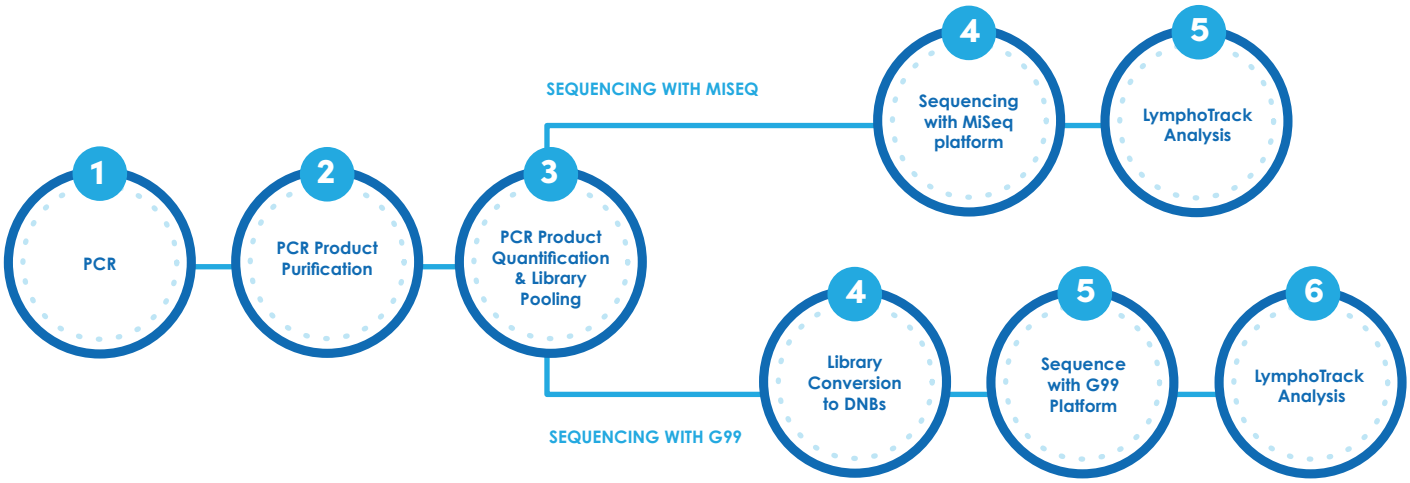

Figure 1: LymphoTrack Workflow with both platforms

RESULTS

Clonality Assays

The seven-combo library generated 36 million pass filter (PF) reads from the MiSeq run and 95 million (PF) reads using the G99 (2.6x more reads). After analyzing the FASTQ files from each instrument using a pipeline version of LymphoTrack Software, the top % reads were compared, demonstrating coefficient R^2 values > 0.99 for five of seven targets (IGHV Leader, *IGH* FR1, *IGH* FR2, *IGK* and *TRB*) as listed in Table 3 and depicted in Figure 2. As indicated below, the *IGH* FR3 and *TRG* targets, which are also the smallest amplicons in the library, performed poorly using the G99 instrument with 2x300bp after library conversion.

invivoscribe®

Table 2: Top % Reads Results Correlation between the Instruments for a Seven Combo Run

Assay Target	Correlation (R^2)	Key Run Metrics MiSeq (2x300bp)	Key Run Metrics G99 (2x300bp)
IGHV Leader	0.997	**Total Reads:** 36 million **Q30:** 77.02% **Sequencing time:** ~55 hours	**Total Reads:** 95 million **Q30:** 95.56% **Sequencing time:** ~32 hours
IGH FR1	0.997		
IGH FR2	0.997		
IGH FR3	0.006		
IGK	0.997		
TRB	0.991		
TRG	0.827		

DISCUSSION

Data generated using the seven LymphoTrack targets indicated disparity among the sequencing results of the two smallest target amplicons (*IGH* FR3 and *TRG*), in which the G99 sequencing quality was too poor to perform the subsequent analysis.

It has been observed that for small amplicons, adapter trimming is necessary to remove what was sequenced beyond the insert (e.g. Illumina adapter). Without trimming, the sequenced read may contain two shorter sequences, further reducing the read count. In the case of *IGH* FR3, the gene is too small to be effectively sequenced with DNBSEQ-G99 using PE300 long cycles. Even before analysis, very few raw FASTQ reads were produced. The total count for *IGH* FR3 was significantly lower than other targets (with fewer than 20,000 reads available), so even adapter trimming would likely have minimal impact. Here are possible explanations:

1. Library Preparation Bias: Short amplicons may be underrepresented due to PCR amplification or adapter ligation biases, allowing longer fragments to dominate in a mixed insert pool.

2. Sequencing Chemistry: The G99 sequencing chemistry could be optimized for longer reads, resulting in less efficient sequencing of shorter amplicons.

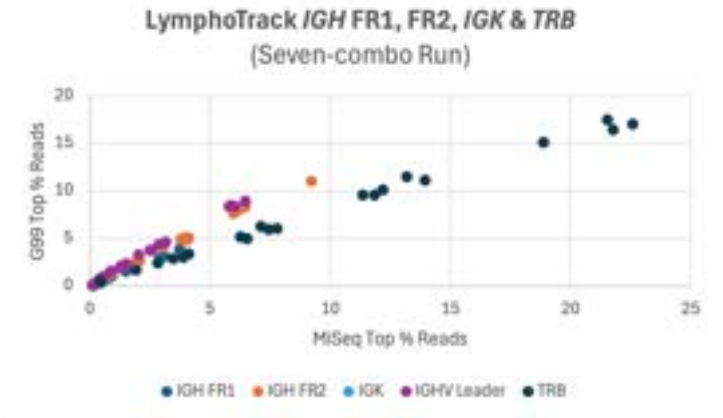

Figure 2: IGHV Leader, IGH FR1, IGH FR2, IGK and TRB Comparison

Note: IGH FR3 and TRG not displayed as there was poor correlation between G99 and MiSeq platforms

Thus, further investigation aimed to assess G99 performance of these smaller target amplicons using an optimal sequencing chemistry was performed on two additional libraries:

- A six-combo library containing LymphoTrack targets *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK, TRB* and *TRG* was prepared according to the associated instructions. The pooled library was converted from dsDNA to circular ssDNA to generate DNBs, which were then loaded at a concentration of 37 ng/µL onto a high-density patterned flow cell and sequenced using the DNBSEQ-G99 High throughput sequencing set (App-D FCL PE300)[C] at 2x250bp.

invivoscribe®

A two-combo library containing the smallest LymphoTrack amplicon targets *IGH* FR3 and *TRG* was prepared according to the associated instructions, and the pooled library was converted from dsDNA to circular ssDNA. The DNBs were then loaded at a concentration of 37 ng/µL onto a high-density patterned flow cell and sequenced using the DNBSEQ-G99 High throughput sequencing set (App-C FCL PE150)[D] at 2x150bp.

The six-combo library generated 113 million reads and coefficient R^2 values of the top % reads were all greater than 0.99 for each target (*IGH* FR1, FR2, FR3, *IGK, TRB* and *TRG*) as listed in Table 4 and depicted in Figure 3.

Table 3: Top % Reads Results Correlation between the Instruments for a Six Combo Run

Assay Target	Correlation (R^2)	Key Run Metrics G99 (2x250bp)
IGH FR1	0.998	**Total Reads:** 113 million **Q30:** 93.69% **Sequencing time:** ~25 hours
IGH FR2	0.998	
IGH FR3	0.989	
IGK	0.997	
TRG	0.994	
TRB	0.990	

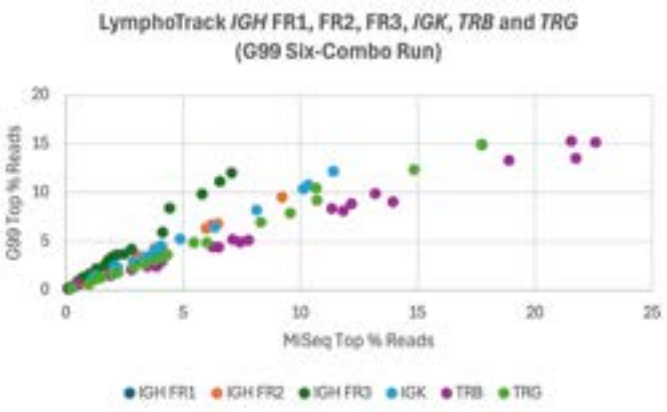

Figure 3: IGH FR1, IGH FR2, IGH FR3 and IGK Comparison between MiSeq and G99 (Six-Combo Run)

The two-combo library G99 sequencing results generated 136 million reads, indicating excellent correlation between the two targets with R^2 value of ≥0.99 for these 2 targets (*IGH* FR3 and *TRG*) as listed in Table 5 and depicted in Figure 4.

Table 4: Top % Reads Results Correlation between the Instruments for G99 Two-Combo Run

Assay Target	Correlation (R2)	Key Run Metrics G99 (2x150bp)
IGH FR3	0.990	**Total Reads:** 136 million **Q30:** 95.65% **Sequencing time:** ~12 hours
TRG	0.997	

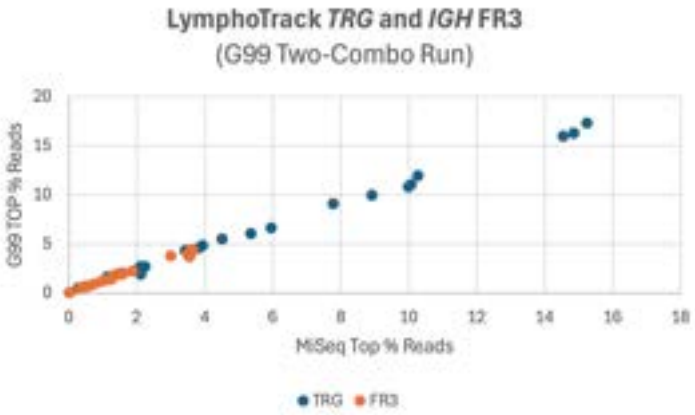

Figure 4: TRG and IGH FR3 Comparison

CONCLUSION

Invivoscribe' s NGS assays demonstrate high concordance between the Illumina's MiSeq and Complete Genomics' DNBSEQ-G99 platform for clonality detection, demonstrating highly correlated results (R^2 > 0.99) and robust detection of gene rearrangements and somatic hypermutation for all targets except *IGH* FR3 and *TRG* which are smaller targets and had incompatibility at longer sequencing length of 2x300. Table 7 indicates the recommended sequencing length for the different LymphoTrack Assay target combinations.

This study not only establishes compatibility between Invivoscribe's LymphoTrack Assays and the Complete Genomics DNBSEQ-G99, but also presents DNBSEQ-G99's potential cost efficiencies with decreased processing time and significantly improved sequencing quality.

invivoscribe®

Table 5: Target Combination Recommendation of G99 Sequencing Length Compatibility for LymphoTrack Assay targets

Combined Targets	G99 Recommended Sequencing Length
IGHV Leader, *IGH* FR1, *IGH* FR2, *IGK, TRB*	2x300bp
IGH FR1, *IGH* FR2, *IGH* FR3, *IGK, TRB, TRG*	2x250bp
IGH FR3, *TRG*	2x150bp

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.

Invivoscribe's NGS Assays and Software for use with MiSeq*

Catalog #	Product Description	Quantity
40880098	LymphoTrack B-cell Low Positive Control	5 reactions
40880108	LymphoTrack T-cell Low Positive Control	5 reactions
40880118	LymphoQuant B-cell Internal Control	120 reactions
40880128	LymphoQuant T-cell Internal Control	120 reactions
71210009	LymphoTrack *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210039	LymphoTrack *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 reactions each
71210089	LymphoTrack *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210099	LymphoTrack *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 reactions each
71210109	LymphoTrack *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210119	LymphoTrack *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 reactions each
71210129	LymphoTrack *IGH* FR1/2/3 Assay Kit A – MiSeq	8 indices per FR region– 5 reactions each
71210139	LymphoTrack *IGH* FR1/2/3 Assay Panel – MiSeq	24 indices per FR region – 5 reactions each
71210059	LymphoTrack IGHV Leader Somatic Hypermutation Assay Kit A – MiSeq	8 indices – 5 reactions each
71210069	LymphoTrack IGHV Leader Somatic Hypermutation Assay Panel – MiSeq	24 indices – 5 reactions each
71220009	LymphoTrack *IGK* Assay Kit A – MiSeq	8 indices – 5 sequencing reactions each
71220019	LymphoTrack *IGK* Assay Panel – MiSeq	24 indices – 5 sequencing reactions each
72250009	LymphoTrack *TRB* Assay Kit A – MiSeq	8 indices – 5 reactions each

invivoscribe®

Invivoscribe's NGS Assays and Software for use with MiSeq* CONTINUED

Catalog #	Product Description	Quantity
72250019	LymphoTrack *TRB* Assay Panel – MiSeq	24 indices – 5 reactions each
72270019	LymphoTrack *TRG* Assay Kit A – MiSeq	8 indices – 5 reactions each
72270009	LymphoTrack *TRG* Assay Panel – MiSeq	24 indices – 5 reactions each
75000009	LymphoTrack Software - MiSeq	1 software package
S100003	LymphoTrack Enterprise Software	1 software package

**Software compatibility with G99 is planned.*

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.**

Illumina and Complete Genomics Reagents

Manufacturer	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	DNBSEQ-G99 High throughput sequencing set (App-D FCL PE300)	940-001717-00
[D]Complete Genomics	DNBSEQ-G99 High throughput sequencing set (App-C FCL PE150)	940-000907-00

References 1. Ho et al. *J Mol Diag.* 2021 May;23(7): 805–815. | 2. Arcila et al. *J Mol Diag.* 2019 March;21(2): 330–342. | 3. NCCN Clinical Practice Guidelines in Oncology: Multiple Myeloma. Version 2.2020. | 4. NCCN Clinical Practice Guidelines in Oncology: Acute Lymphoblastic Leukemia. Version 2.2019. | 5. NCCN Clinical Practice Guidelines in Oncology: Pediatric Acute Lymphoblastic Leukemia. Version 1.2020. | 6. NCCN Clinical Practice Guidelines in Oncology: Chronic Lymphocytic Leukemia. Version 1.2020. | 7. High-throughput Sequencing Set DNBSEQ-G99RS versión 3.0 (6500066ce3188).