Study of MRD

Using LymphoTrack® Assays

invivoscribe®

Minimal Residual Disease (MRD) testing by Next-Generation Sequencing (NGS) is a proven tool in the development of hematologic management strategies.

The LymphoTrack® Assays are NGS-based deep sequencing assays that detect virtually all clonal rearrangements within targeted T-cell receptor (TCR) or immunoglobulin (Ig) antigen receptor loci. This performance suggests that a tumor-specific biomarker target can be readily identified in all subjects. Once a specific rearrangement (the clonotype) has been identified, LymphoTrack assays can be used to track these clonotype populations to a sensitivity as low as 10^{-6}.

MRD testing by NGS provides unparalleled sensitivity and specificity to detect the presence of residual disease and offers a number of advantages over alternatives such as flow cytometry and allele–specific oligonucleotide PCR.[1] While the correlation of MRD status with overall survival rate was initially demonstrated for subjects with Chronic Lymphocytic Leukemia (CLL) using multi-parameter flow analysis, flow-based methods have proven difficult to standardize outside individual centers.[2]

As it is difficult to compare results from different centers, flow is not a suitable technology for global standardization. Fortunately, a number of investigators have described NGS-based approaches that have demonstrated success in detecting and monitoring MRD in CLL, Acute Lymphoblastic Leukemia (ALL) and other lymphoid malignancies.[3,4] Advantages include the ability to:

» Offer concordant, objective and standardized testing worldwide by tracking sequence specific DNA targets.

» Detect clones and newly emergent clones and sub–clones in follow-up samples.

» Test at a level of sensitivity and confidence only limited by the DNA input amount interrogated and sequence read numbers.

» Examine sequence-specific Somatic Hypermutation (SHM), as well as B- and T-cell rearrangements, as prognostic markers.

References: (1) Blood 125:3501–08, 2015 and Blood 126:1045–47, 2015 (2) JCO 23(13):2884, 2005 (3) Leukemia 27:1659–1665, 2013 (4) Blood 120:5173–5180, 2012

When using LymphoTrack® Assays for MRD testing, Invivoscribe suggests use of 4 controls in each laboratory run: (1) a no template control (NTC), (2) LymphoTrack Low Positive Control, (3) LymphoQuant® Internal Control, and (4) a negative control should be considered for the longitudinal calibration of sampling cell numbers.

» **NTC:** The no template control (NTC) uses water in place of sample DNA in the PCR. Although the NTC requires use of a master mix (1 index) for the PCR, is not necessary to sequence this reaction.

» **Negative Control:** [NEG (–)] is provided in each LymphoTrack Assay kit. This template is devoid of Ig/TR clonotypes, and does not require further dilution prior to PCR set up.

» **Low Positive Control:** Designed specifically for MRD testing, the LymphoTrack Low Positive Controls are optimized to work in concert with the LymphoQuant Internal Controls. When run together as intended, these controls ensure that MRD levels of sensitivity are being confidently interrogated in other samples where the LymphoQuant Internal Control is being used.

*LymphoTrack kits include a positive control template [(POS (+)] that is not necessary for MRD testing. Rather, the following part numbers are sold separately and are necessary components to the preparation of the low positive control. Note that the control templates are unique for B- or T-cell assays.

LymphoQuant® Internal Control(s): B-cell or T-cell LymphoQuant Internal Control may be spiked into specimens to estimate the respective number of clonotype B-cell or T-cell equivalents and calculate the percent clonotype present. Addition of a LymphoQuant Internal Control to the specimen PCR facilitates clonotype tracking over time without additional sequencing run cost. Consistent use of a LymphoQuant Internal Control enables clinicians to objectively monitor the disease over time with a highly standardized, sensitive method.

Purchase control(s): Invivoscribe has developed both LymphoQuant® [spike-in] Internal Controls and LymphoTrack® Low Positive Controls. These respective B- and T-cell controls are available for purchase. Invivoscribe is also refining the now available LymphoTrack® MRD software to further facilitate MRD tracking.

TABLE 1: CONTROLS - INQUIRE FOR PURCHASE

LYMPHOTRACK ASSAY	LYMPHOTRACK LOW POSITIVE CONTROL	LYMPHOQUANT INTERNAL CONTROL
IGHV Leader, *IGH* FR1/2/3, *IGK*	LymphoTrack® B-cell Low Positive Control Catalog #: 4-088-0098	LymphoQuant® B-cell Internal Control Catalog #: 4-088-0118
TRG, *TRB*	LymphoTrack® T-cell Low Positive Control Catalog #: 4-088-0108	LymphoQuant® T-cell Internal Control Catalog #: 4-088-0128

3 | Sample Preparation: DNA Qualification & Quantification

DNA templates subject to MRD tests must be free of PCR amplification inhibitors. Therefore, high-quality purified genomic DNA is always recommended. The Abs260/280 measurement of prepared DNA is reflective of sample purity and should be in the 1.8 - 2.0 range. Assessment of DNA concentration by a method specific for double-stranded DNA (dsDNA) is also necessary. Standard Pico Green protocols are appropriate, as are similar double-stranded DNA (dsDNA) binding fluorescent dye assays.

To ensure DNA inputs are not degraded and are suitable for qualitative assessment, samples may be tested with the Specimen Control Size Ladder master mix from Invivoscribe [catalog# 2-096-0021: ABI detection, catalog#: 2-096-0020: gel detection]. This master mix targets housekeeping genes for the amplification of 100, 200, 300, 400, and 600 base pair PCR products, and it was originally designed by the EuroClonality group as part of the BIOMED-2 concerted action (Leukemia 17:2257-2317, 2003). The successful qualification and combined quantification measurements described here are each recommended prior to use of a DNA template as input to a LymphoTrack MRD test.

4 | DNA Input Quantity

DNA input amounts are a critical factor of experimental design. Higher DNA inputs are suggested when performing MRD testing, because the overall cell equivalents interrogated determines the sensitivity of an MRD assay. When using the LymphoTrack assays, a maximum DNA input of 2 µg per PCR is recommended. When performing MRD testing, a total DNA input volume between 5 - 10 µL can be utilized. For example, if one adds 2 µL of a spike-in internal control the maximum volume of sample DNA input would be 8 µL.

Routine tracking of clones can be confidently achieved by detecting 1 clonal cell in a background of 10,000 cells with as little as 200 ng of input DNA **(Figure 1, Table 2)**. If lower sesitivity is desired, it is important to note that a human cell contains approximately 6.5 pg of DNA. Examples of sample set ups targeting sensitivities of 10^{-4}, 10^{-5} and 10^{-6} at a 95% confidence level can be found in **Table 2.** These examples include details on read depth requirements for reporting at various sensitivity levels. Alternatively, the LymphoTrack® MRD Software "Project Planner" tool may be used to determine the quantity of DNA required to achieve the desired confidence level for MRD testing **(see section 6)**.

TABLE 2. EXAMPLES OF DNA INPUT AND READ DEPTHS

95% CONFIDENCE OF A TRUE MRD NEGATIVE SAMPLE AT VARIOUS SENSITIVITY LEVELS			
SENSITIVITY	DNA PER REPLICATE	# REPLICATES	READ DEPTH PER REPLICATE
1×10^{-4}	200 ng	1 replicate of 200 ng	500,000
1×10^{-5}	700 ng*	5 replicates of 700 ng each	700,000
	2 µg*	2 replicates of 2 µg each	1,400,000

Note: A replicate is an independent PCR reaction with input DNA from the same subject.

1×10^{-6} PROVIDED FOR INFORMATIONAL PURPOSES ONLY			
SENSITIVITY	DNA PER REPLICATE	# REPLICATES	READ DEPTH PER REPLICATE
1×10^{-6}	2 µg*	Please contact Invivoscribe	2,100,000

*A DNA concentration step is often required to achieve the higher levels of DNA input.

Relationship Between DNA Input, Read Frequency, & Level of Confidence

Legend:
- 10^{-4} – 200ng – 1rep
- 10^{-5} – 700ng – 5 reps
- 10^{-5} – 2000ng – 2 reps
- 10^{-6} – 2000ng – 18 reps

Y-axis: % Confidence of negative
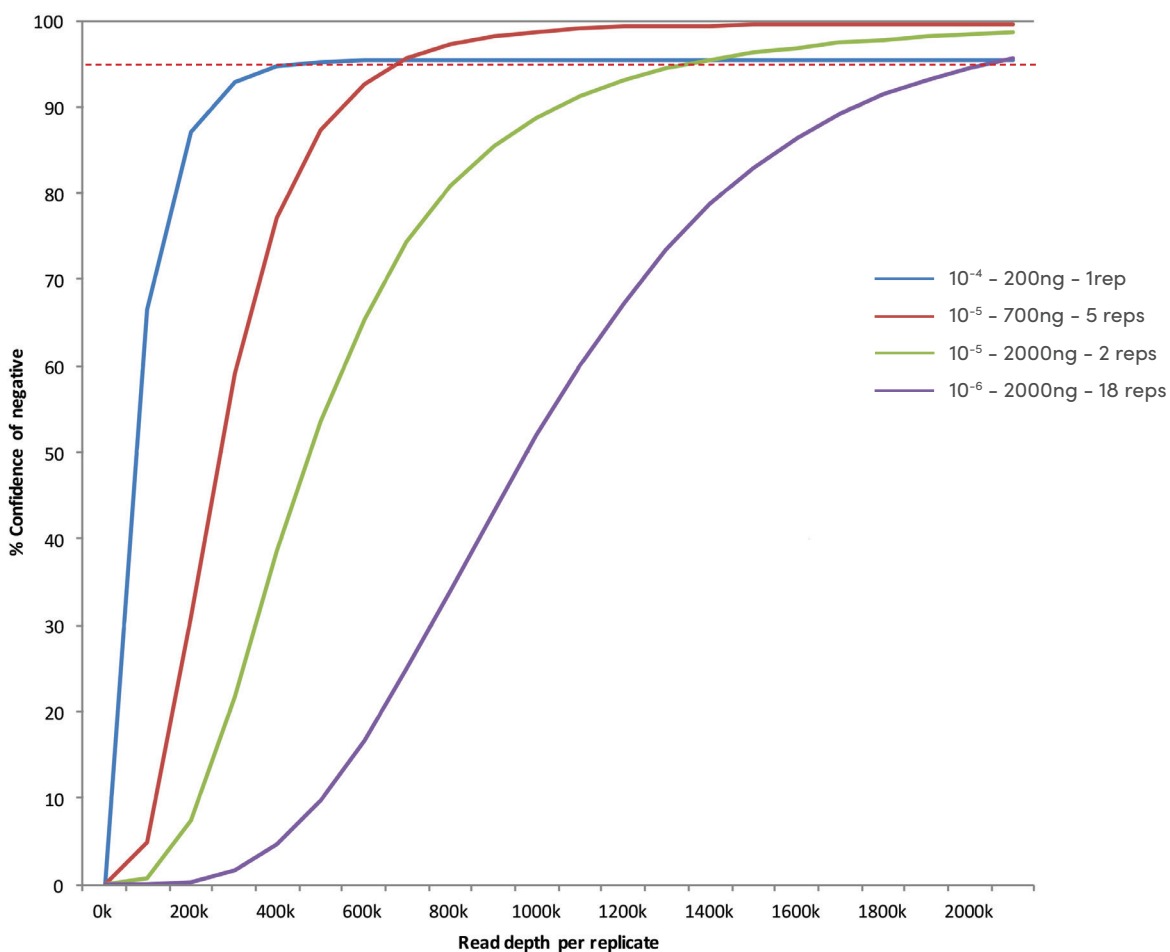X-axis: Read depth per replicate

FIGURE 1. 95% CONFIDENCE OF A TRUE MRD NEGATIVE SAMPLE AT VARIOUS SENSITIVITY LEVELS

The level of confidence for detecting a clonotype (detected with at least 5 reads) at various DNA input quantities and replicates as a function of the number of sequencing reads obtained. The red-dotted line indicates the 95% confidence level. The confidence levels for detecting clonotype sequences depicted in **Figure 1** were calculated using a statistical model. This model does not incorporate PCR bias and, consequently, the calculated confidence levels are theoretical and not empirically determined.

5 | Design of Experiment: Sample Batching (Indices) & Assay Multiplexing

LymphoTrack Assays are available for use with the MiSeq®, Ion S5™ and Ion PGM™ sequencing platforms. The LymphoTrack Assays are designed to provide the end user efficient and flexible workflow options. Users can design cost-effective MRD runs by multiplexing assays and batching samples, while keeping in mind the NGS flow cell capacity and the desired MRD sensitivity.

Assay Multiplexing: The full LymphoTrack Clonality Suite consists of 7 independent assays each targeting one of the following respective loci (*TRG, TRB, IGH* FR1/2/3, *IGHV* Leader, and *IGK*). These assays were specifically designed so that up to 7 clonality targets can be multiplexed together in a single LymphoTrack MiSeq® run.

Sample Batching: LymphoTrack one-step PCR incorporates a molecular barcode (index) onto each amplicon. By design, a unique index is used for each specimen sample. The sample barcodes are read during sequencing and used by the LymphoTrack Software to de-multiplex samples.

» LymphoTrack Assay kits for the MiSeq® are provided with up to 24 different indices (up to 48 with *IGH* FR1). Thus, when tracking clonotype sequences, these panels allow 22 different subject samples to be run along with 2 external controls on a single flow cell (or up to 46 different FR1 subject samples).

» LymphoTrack Assay kits for the Ion S5/PGM™ are provided with 12 different indices, thus allowing 10 different subject samples to be run along with 2 external controls on a single sequencing chip.
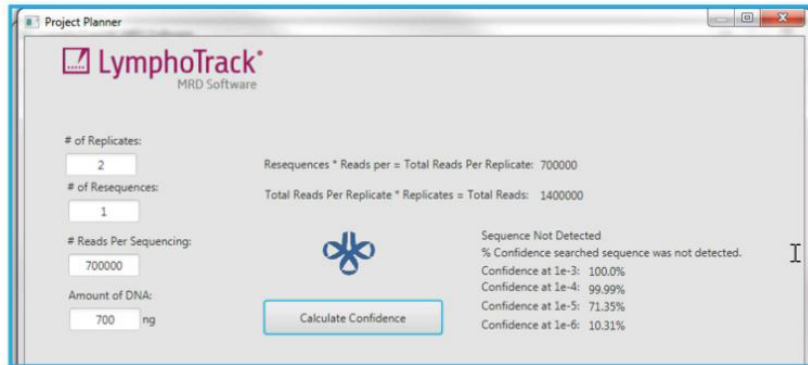
6 | LymphoTrack® MRD Software

The LymphoTrack MRD Software facilitates the longitudinal assessment of clonal populations by providing multiple functionalities to the user including project planning features, and automated bioinformatics applications. Together these features enable simultaneous tracking of 2 clonotype sequences in follow-up samples.

Project Planner Tool
This application provides design of experiment features including a confidence interval calculator. The software embedded calculator adds sample replicate count, resequencing count, read depth, and DNA input amount to determine the confidence level of a true MRD negative sample from 10^{-3} to 10^{-6} **(Figure 2a)**. This tool ensures experimental design meets the user-defined specification for combined sensitivity and confidence for MRD monitoring.

Automated Bioinformatics Data Analysis

The LymphoTrack MRD software facilitates tracking of up to 2 defined population clonotypes as well as the degree of mismatches (0, 1, 2). It further calculates clonotype read frequency, analyzes multiple sample and/or resequencing replicates, and reports the level of confidence when the clonotype is not present at the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} levels. Examples of MRD Software input **(Figure 2b)** and output **(Figures 2c and 2d)** are displayed below.

LymphoTrack MRD Specimen Report

This PDF report summarizes clonotype call details, *i.e.* if a clonotypes was detected or not detected, the number of replicates, the total DNA input amount, and the total reads analyzed. When a clonotype of interest is not detected, the call confidence is reported for sensitivities of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}.
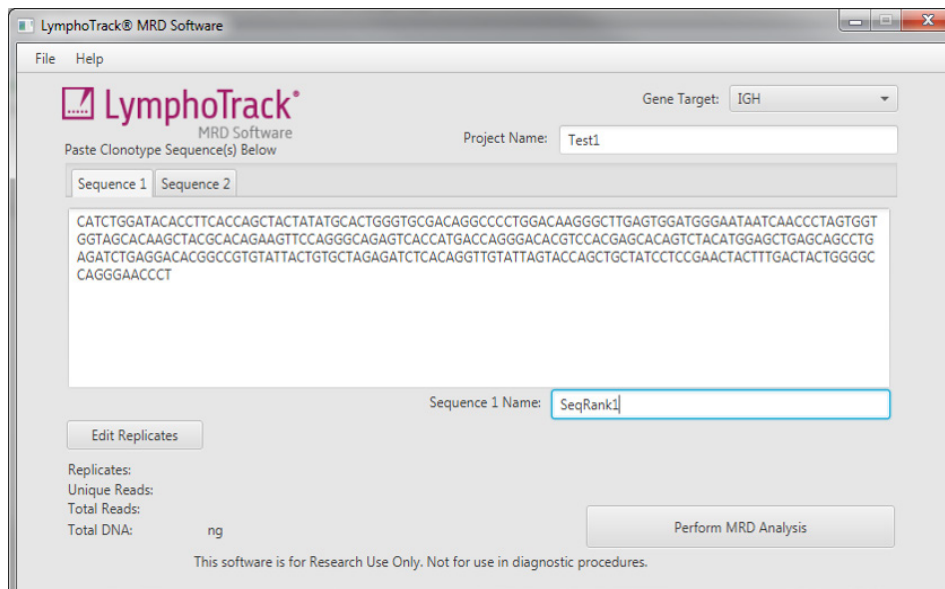
FIGURE 2b: MRD SOFTWARE INPUT SCREEN EXAMPLE

FIGURE 2c: MRD SOFTWARE OUTPUT SCREEN EXAMPLE – SEQUENCE OR MUTANT DETECTED

The PDF report and screen will read "Sequence Detected" if the read count is greater than 5 reads. The software includes additional information regarding read counts that may be used for longitudinal sample calibration when an internal spike-in control is utilized.
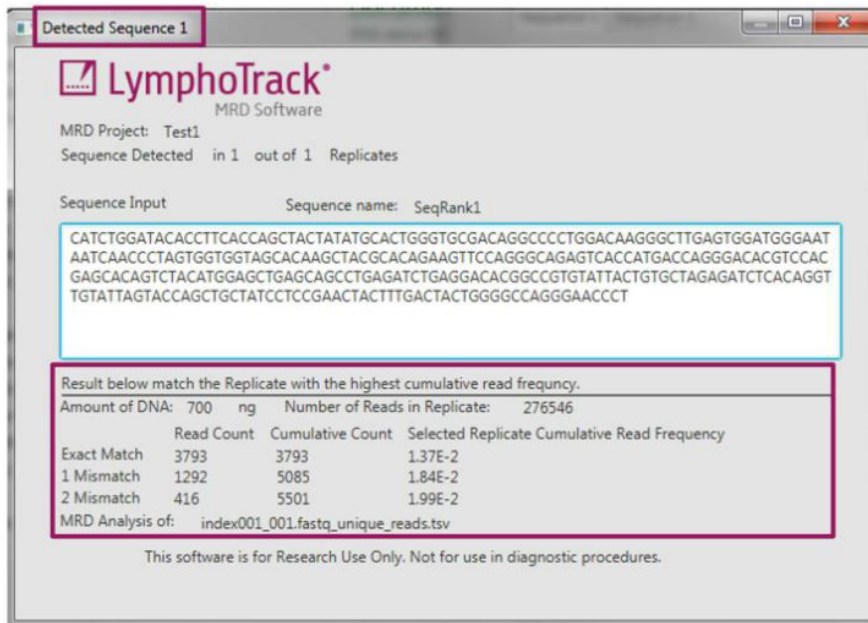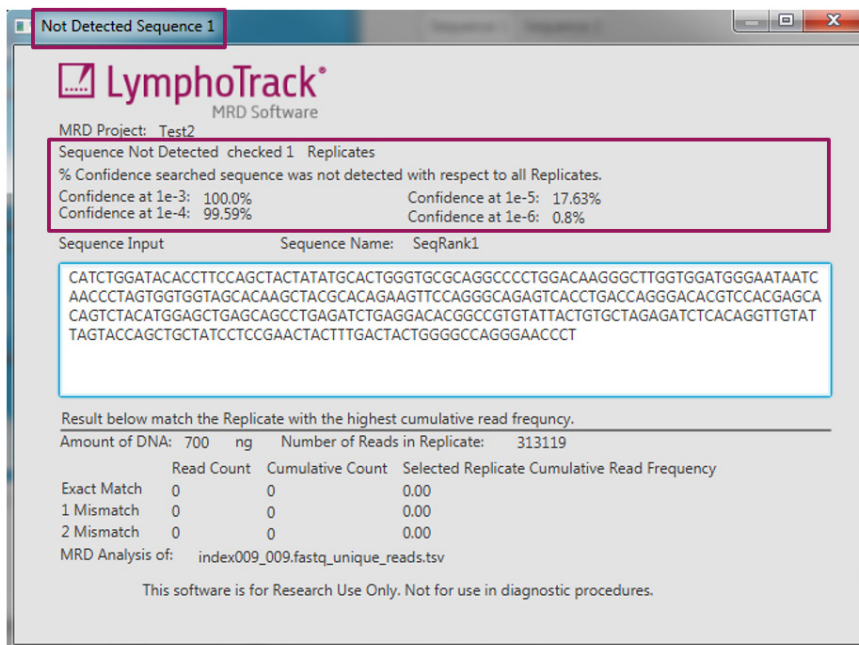

FIGURE 2d: MRD SOFTWARE OUTPUT SCREEN EXAMPLE – SEQUENCE NOT DETECTED

For sequences "Not Detected" by the MRD Software the % confidence is reported. When the sequence is not detected by the MRD software, the % confidence for this call is reported at various confidence levels as low as 10^{-6}.

7 | Target Specific Considerations

When tracking multiple clonotype sequences, it is important to consider the type of gene rearrangement that is being tracked. There are multiple rearrangements that can be found in the negative control leading to a false positive.

Immunoglobulin heavy chain (*IGH*): A clonotype sequence identified by the LymphoTrack software can be tracked and interpreted by the MRD software as "Detected" or "Not Detected."

Immunoglobulin kappa chain (*IGK*): There are three common rearrangements that are not suitable for MRD analysis due to the high frequency in which they occur. As a result, any clonotype sequence that is listed below should not be used for minimal residual disease analysis:

1. Intron-Kdel

2. V3D-20 with any J or Kdel

3. V3-11 with any J or Kdel

T-cell receptor gamma (*TRG*): When tracking *TRG* gene rearrangements it should be noted that these receptors are composed of fewer genes and as a result, are less diverse than *IGH*. Rearrangements of the same sequence in 2 cells can occur at a higher prevalence, and can therefore lead to a higher probability of false positive MRD results. Exact matches for the clonotype sequence support the probability of a true positive result. Furthermore, only bi-allelic samples in which both clonotype sequences can be detected should be evaluated for MRD.

T-cell receptor beta (*TRB*): When tracking *TRB* gene rearrangements, please note that D-J rearrangements are less suitable for tracking MRD due to the high frequency in which they occur.

8 | Mitigate Sequencing Contamination Risk

In any high-throughput sequencing technology that utilizes sample barcodes such as the MiSeq®, Ion S5™, and Ion PGM™, the high number of clonotype sequences present in an initial sample may contaminate subsequent samples if some precautions are not taken. To mitigate the risk of sequencing artifacts, Invivoscribe provides the following guidelines.

For *MiSeq*®:

- » Conduct an Illumina® 'Template Line Wash' with bleach after each MiSeq® run.

- » Avoid running subsequent timepoints immediately after a MiSeq® run that contained the initial sample.

For *MiSeq*® or S5/PGM™:

- » Test follow up samples separately from the initial clonal sample.

- » Use different indices for the initial sample and follow up samples (*e.g.*, index 1 for identifying the clonotype sequence and index 2 for a follow up sample from the same subject). Alternatively, unrelated samples may be run between the initial sample and follow up runs on the same instrument as long as a template line wash with bleach is conducted.

- » Avoid running known high positives with follow-up samples screened at high-read depths on the same chip or flow cell.

To demonstrate the linearity, accuracy and Limit of Detection (LOD) of LymphoTrack Assays for MRD testing, a B-cell line of known rearrangement and sequence was diluted and subjected to LymphoTrack MRD testing.

Materials & Method

DNA from a B-cell line with a known V_H-J_H rearrangement was serially diluted into a background of tonsil DNA (abundance of T- and B- cells) to generate clonotype frequencies ranging from 10^{-2} to 10^{-5}. Input DNA quantity was adjusted to 700 ng per dilution point, then sequenced using a LymphoTrack Assay. In all cases, samples were tested across the LymphoTrack *IGH* FR1 Assay for the MiSeq® or S5/PGM™ with bioinformatics analysis performed by the LymphoTrack MRD Software. This software automates detection of known clonal sequences and further reports clonotype frequency.

Results & Conclusion

Excellent linearity was observed across the 10^{-2} to 10^{-5} dilution series **(Figure 3)**. Correlation of observed versus expected frequencies was further demonstrated for each sample tested **(Figure 3)**. Total read count per sample tested ranged from 253,295 to 663,625.

FIGURE 3: DILUTION SERIES DEMONSTRATES LINEARITY OF LYMPHOTRACK® ASSAYS.
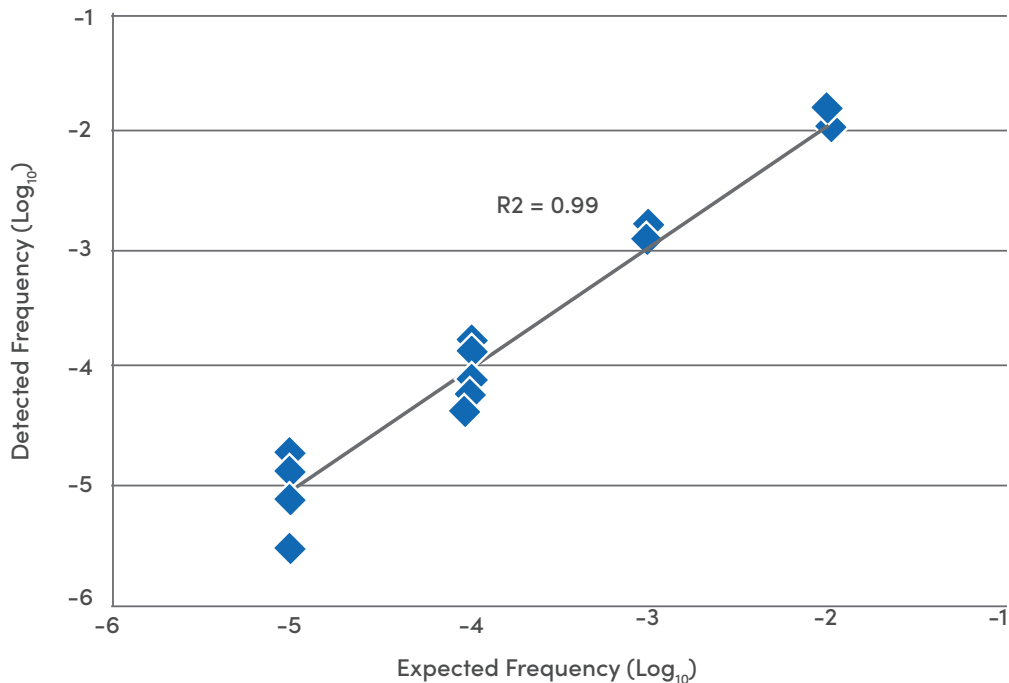
LymphoTrack MRD Controls

4-088-0108	LymphoTrack® T-cell Low Positive Control	*TRG & TRB* Compatible
4-088-0128	LymphoQuant® T-cell Internal Control	*TRG & TRB* Compatible
4-088-0098	LymphoTrack® B-cell Low Positive Control	*IGHV* Leader, *IGH* FR1/2/3, & *IGK* Compatible
4-088-0118	LymphoQuant® B-cell Internal Control	*IGHV* Leader, *IGH* FR1/2/3, & *IGK* Compatible

Associated LymphoTrack Products

Catalog #	Products	Quantity
7-121-0057	LymphoTrack® *IGH* FR1/2/3 Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-121-0007	LymphoTrack® *IGH* FR1 Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-121-0037	LymphoTrack® *IGH* FR2 Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-121-0047	LymphoTrack® *IGH* FR3 Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-122-0007	LymphoTrack® *IGK* Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-227-0007	LymphoTrack® *TRG* Assay – S5/PGM™	Indices 12 (5 sequencing reactions each)
7-121-0129	LymphoTrack® *IGH* FR1/2/3 Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-121-0139	LymphoTrack® *IGH* FR1/2/3 Assay Panel – MiSeq®	Indices 24 (5 sequencing reactions each)
7-121-0009	LymphoTrack® *IGH* FR1 Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-121-0039	LymphoTrack® *IGH* FR1 Assay Panel – MiSeq®	Indices 24 (5 sequencing reactions each)
7-121-0149	LymphoTrack® *IGH* FR1 Assay Panel B – MiSeq®	Indices 25 - 48 (5 sequencing reactions each)
7-121-0089	LymphoTrack® *IGH* FR2 Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-121-0099	LymphoTrack® *IGH* FR2 Assay Panel- MiSeq®	Indices 24 (5 sequencing reactions each)
7-121-0109	LymphoTrack® *IGH* FR3 Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-121-0119	LymphoTrack® *IGH* FR3 Assay Panel- MiSeq®	Indices 24 (5 sequencing reactions each)
7-122-0009	LymphoTrack® *IGK* Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-122-0019	LymphoTrack® *IGK* Assay Panel – MiSeq®	Indices 24 (5 sequencing reactions each)
7-225-0009	LymphoTrack® *TRB* Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-225-0019	LymphoTrack® *TRB* Assay Panel – MiSeq®	Indices 24 (5 sequencing reactions each)
7-227-0019	LymphoTrack® *TRG* Assay Kit A – MiSeq®	Indices 8 (5 sequencing reactions each)
7-227-0009	LymphoTrack® *TRG* Assay Panel – MiSeq	Indices 24 (5 sequencing reactions each)

Bioinformatics Software

7-500-0009	LymphoTrack® Software – MiSeq®	1 CD complimentary with purchase
7-500-0007	LymphoTrack® Software – S5/PGM™	1 CD complimentary with purchase
7-500-0008	LymphoTrack® MRD Software	1 CD complimentary with purchase

These products are sold FOR RESEARCH USE ONLY. Not for use in diagnostic procedures

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