Study of MRD

Using LymphoTrack® Assays

Minimal Residual Disease (MRD) testing by Next-Generation Sequencing (NGS) enables objective tracking of multiple clonal sequences and is a proven tool in the management of hematologic malignancies.

The LymphoTrack® MRD Solution can be used to identify and track as little as one clonal cell in one million cells. The LymphoTrack® MRD Solution leverages NGS-based deep sequencing assays that detect virtually all clonal rearrangements within targeted immunoglobulin (Ig) or T-cell receptor (TCR) antigen receptor loci. This suggests that a tumor-specific biomarker target can be readily identified in all subjects. Once specific clonal rearrangements have been identified, the LymphoTrack MRD Solution utilizes the LymphoTrack Assays, LymphoTrack® Low Positive Controls, LymphoQuant® Internal Controls and LymphoTrack® MRD Software to objectively track and report these clonal sequences with a sensitivity only limited by DNA input.

LymphoTrack® Assay

1. ADVANTAGES OF NGS-MRD METHODOLOGIES

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 are difficult to standardize outside individual centers.[2] Fortunately, NGS methods have been proven to aid in the objective study of MRD, and kits using this technology are increasingly validated in global laboratories.

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] Moreover, both the National Comprehensive Cancer Network (NCCN) and the European LeukemiaNet (ELN) recognize the value of MRD testing. The NCCN guidelines now recommend MRD testing for several lymphoid cancers, including multiple myeloma, ALL, and CLL.[5,6,7,8] ELN guidelines recommend MRD testing following induction and consolidation courses to assess remission status and determine kinetics of disease response, and sequentially beyond consolidation to detect impending morphologic relapse.[9]

ADVANTAGES OF THE LYMPHOTRACK MRD SOLUTION INCLUDE THE ABILITY TO:

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

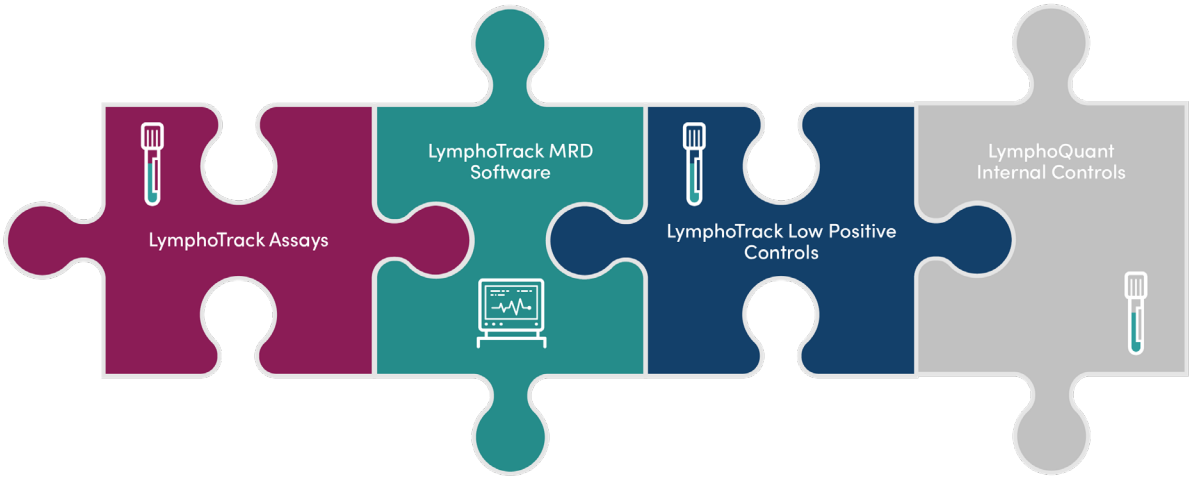
» Detect clones, newly emergent clones and sub-clones in follow-up samples.

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

» Consistently assess and track up to 5 clonal sequences over time.

» Examine B- and T-cell clonal 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 | 5. *NCCN Clinical Practice Guidelines in Oncology: Multiple Myeloma.* Version 2.2020. | 6. *NCCN Clinical Practice Guidelines in Oncology: Acute Lymphoblastic Leukemia.* Version 2.2019. | 7. *NCCN Clinical Practice Guidelines in Oncology: Pediatric Acute Lymphoblastic Leukemia.* Version 1.2020. | 8. *NCCN Clinical Practice Guidelines in Oncology: Chronic Lymphocytic Leukemia.* Version 1.2020. | 9. Dohner et al. *Blood.* 2017 Jan 26; 129(4): 424–447. | 10. *Leukemia.* 17:2257-2317, 2003.



2. DESIGN OF EXPERIMENT – CONTROLS FOR MRD TESTS

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.

The LymphoTrack® MRD Software is designed to work with Invivoscribe's MRD controls to automate MRD analysis of up to 5 sequences simultaneously. It generates pdf reports which provide estimates of clonal cell equivalents and longitudinal graphs to visualize changes in clonal frequencies over time.

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, it 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.

LymphoTrack® Low Positive Control: Designed specifically for MRD testing, the LymphoTrack® Low Positive Controls are optimized to work in concert with the LymphoQuant® Internal Controls. B-Cell and T-Cell Low Positive Controls may be run in lieu of the LymphoTrack® Assay/Kit included positive controls to ensure that MRD levels of sensitivity are being confidently interrogated in samples.

LymphoQuant® Internal Control: The B-cell or T-cell LymphoQuant® Internal Control may be spiked into samples to estimate the respective number of clonal B- or T-cell equivalents and clonal frequencies. Addition of a LymphoQuant® Internal Control to the specimen PCR facilitates clonal 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.

Table 1: MRD Controls - For B- and T-Cell Assays

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.[10]

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. Note: The optimal sample for MRD assessment is the first pull or early pull of the bone marrow aspirate.

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 generally recommended. However, increasingly higher concentrations of DNA inputs have proven effective. As an example, DNA inputs up to 6.5 µg proved effective for 10^{-5} and 10^{-6} sensitivity when contrived DNA templates were spiked into background DNA. 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 LymphoQuant® Internal Control the maximum volume of sample DNA input would be 8 µL.

While routine tracking of clones can be 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)**, typically increased sensitivity is desired for MRD testing. If increased sensitivity is desired, it is important to note that a human cell contains approximately 6.5 pg of DNA and to consider the false negative risk in the sample tested. The LymphoTrack MRD Software Project Planner tool **(see section 7)** assists users as they determine the quantity of DNA required along with the read depth per replicate required to achieve the desired confidence level in a negative MRD result.

While the Project Planner is recommended for design of experiment, examples of sample set ups targeting sensitivities of 10^{-4}, 10^{-5} and 10^{-6} at a 95% confidence level are presented in **Table 2**. These examples include details on read depth and DNA input requirements for reporting at various sensitivity levels.

Table 2. Examples of DNA Input and Read Depths

95% CONFIDENCE OF A TRUE MRD NEGATIVE SAMPLE AT VARIOUS SENSITIVITY LEVELS		
Sensitivity	Total DNA Input	Total Read Depth
$1x10^{-4}$	200 ng	500,000
$1x10^{-5}$	2 ug	4,400,000
$1x10^{-6}$	20 µg	44,400,000

Note: Total **DNA Input** and **Total Read Depth** are divided across replicates tested, and a replicate is an independent PCR reaction with input DNA from the same subject. Read depth requirements assume 100% target cells. Read depth requirements may vary dependent on replicate numbers used to reach total DNA inputs.

Also, note: A DNA concentration step is often required to achieve the higher levels of DNA input.

5. ESTABLISHING CONFIDENCE IN A TRUE NEGATIVE MRD RESULT

THE RELATIONSHIP BETWEEN DNA INPUT, READ DEPTH, AND CONFIDENCE LEVEL MUST BE CONSIDERED IN MRD STUDIES.

The Project Planner tool in the LymphoTrack MRD Software can be used to create an experimental design that allows a user to optimize confidence in the result using the available sample and read depth. This tool allows for user-defined permutations to DNA input, read depth, and/or replicates; performing calculations based on these permutations to determine confidence levels at various sensitivity levels. These numerical outputs can be used to generate confidence models as shown in **Figure 1**.

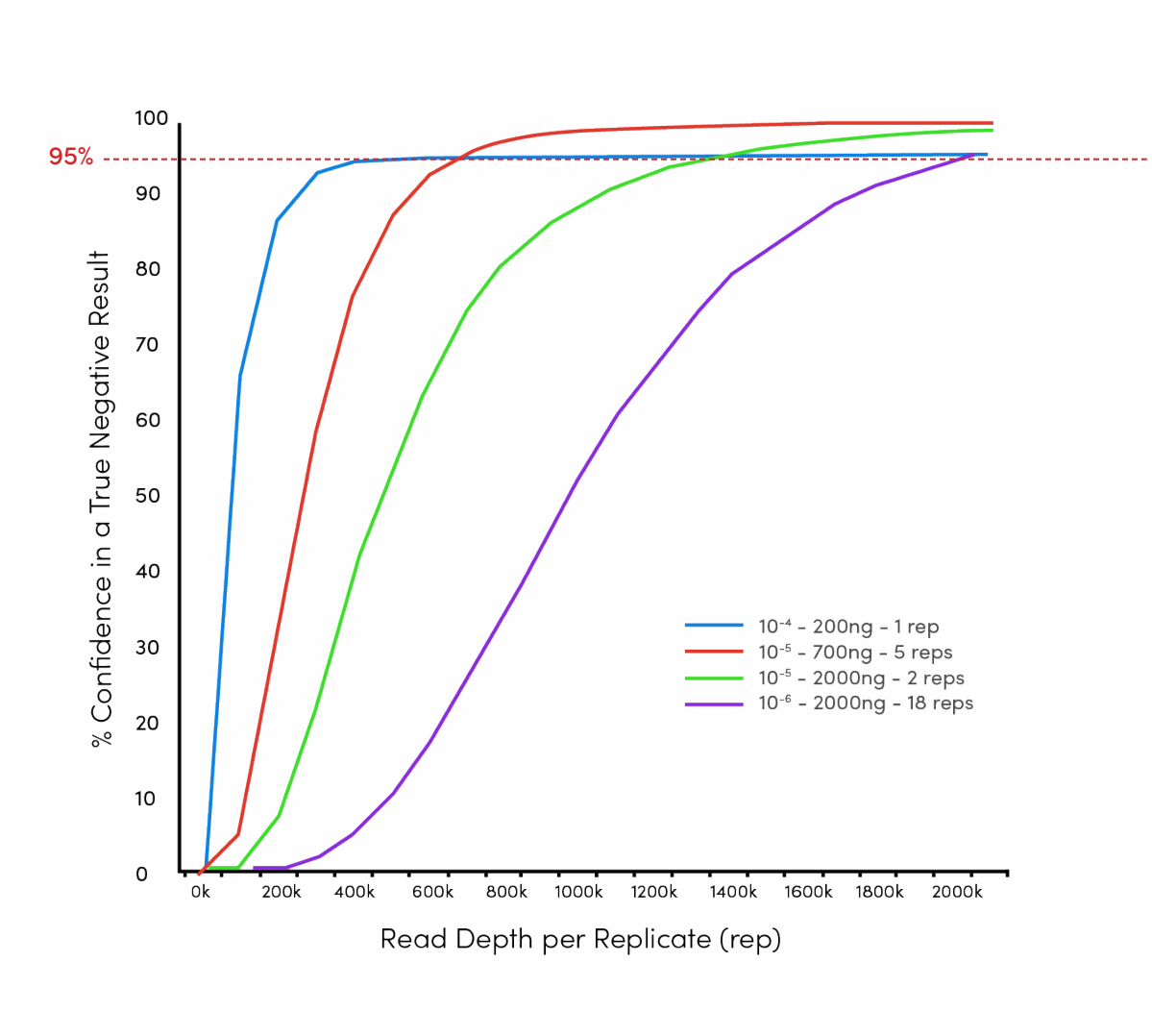


Figure 1: 95% Confidence of a True MRD Negative Sample at Various Sensitivity Levels

This model dataset demonstrates the relationship between DNA input quantities, read depth, and confidence. In this statistical model, the level of confidence that a clonal sequence was not detected (with at least 5 reads) is shown at various DNA input quantities and replicates as a function of the number of sequencing reads obtained. This model does not incorporate PCR bias and, consequently, the calculated confidence levels are theoretical and not empirically determined.

CONFIDENTLY REPORT TRUE NEGATIVE RESULTS WITH 10^{-6} SENSITIVITY

Increased DNA inputs are required to achieve greater sensitivity in MRD testing due to the probability that a measured specimen will contain a subject's cancer cells. **Figure 2** explains in detail the cell sampling required to reach 10^{-6} sensitivity with 95% confidence.

HOW MANY CELLS MUST BE TESTED TO REACH 1X10^{-6} SENSITIVITY WITH >95% CONFIDENCE THAT IT IS A TRUE NEGATIVE RESULT?

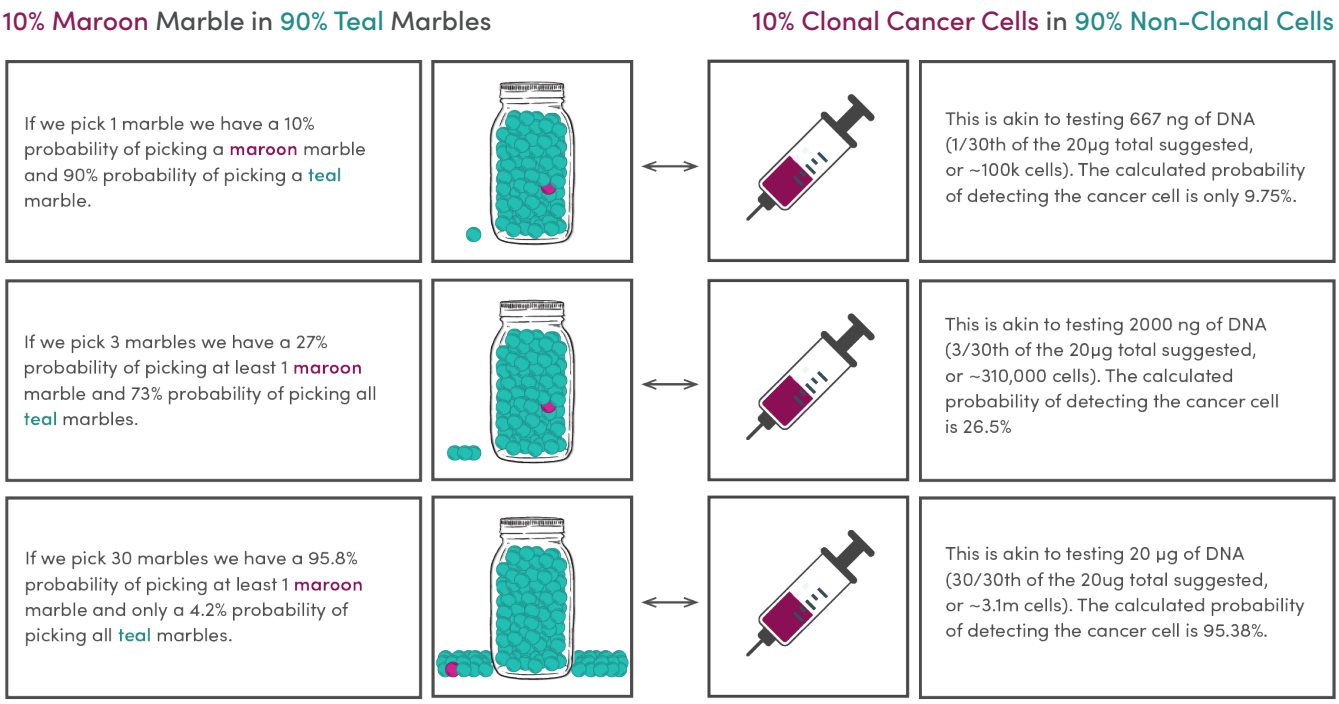


Figure 2: 20 µg DNA input minimum must be tested to establish a true negative result with 1x10^{-6} sensitivity (>95% confidence).

In this example, the maroon marble(s) found in the teal marble background represents the rare clonal cancer cell(s) in a subject's otherwise non-clonal cell background. The 3 scenarios presented here demonstrate that laboratories must test 20 µg of DNA for 95% confidence that they can find 1 rare clonal cell in 1 million cells (1x10^{-6} sensitivity). It is important to note that the probability is entirely dependent on the assumed proportion of maroon marbles (or clonal cells). If we KNOW there should be 10% maroon marbles, then picking 30 teal marbles tells us we are 95.8% sure that the jar does not have 10% maroon marbles. This is the same logic that is used for confidence in a negative MRD signal using 20 µg of DNA. The actual proportion might be less than 1x10^{-6} sensitivity, but we are 95% sure it is not equal to or higher than 1x10^{-6}.

6. DESIGN OF EXPERIMENT: SAMPLE BATCHING AND 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 remaining mindful of 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 (*IGHV* Leader, *IGH* FR1/FR2/FR3, *IGK*, *TRG* and *TRB*). These assays were specifically designed so that up to 7 clonality targets can be multiplexed together in a single LymphoTrack® MiSeq® run. Up to 5 independent targets (*IGH* FR1/FR2/FR3, *IGK*, and *TRG*) can be multiplexed together if using the LymphoTrack Assays for Ion S5™ or Ion PGM™.

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

» LymphoTrack® Assay or Panel kits for the MiSeq® are provided with up to 24 different indices (up to 48 with *IGH* FR1). Thus, when tracking clonal 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.

7. LYMPHOTRACK® MRD SOFTWARE

The LymphoTrack® MRD Software seamlessly automates control and sample analyses. The powerful bioinformatics software facilitates the longitudinal assessment of up to 5 clonal populations by providing multiple functionalities to the user for project planning, data analyses and reporting. Project planner features assist the user in design of experiment and project management with a built in "project" feature to save and load "projects" as additional time points are tested. When Invivoscribe® LymphoTrack® MRD controls are used, the software automates MRD tracking, reports clonal counts and frequencies, and provides multiple graphs to visualize changes in clonal frequencies over time. **Refer to the LymphoTrack® MRD Software Technical Bulletin for additional details.**

PROJECT PLANNER TOOL

This application provides design of experiment features including a confidence interval calculator. The software embedded calculator considers sample replicate count, resequencing count, read depth, and DNA input amount to determine the confidence level of a true MRD negative sample. This tool also allows the user the ability to customize the desired levels of confidence to ensure the experimental design meets the user-defined specification for combined sensitivity and confidence for MRD monitoring.

AUTOMATED BIOINFORMATICS DATA ANALYSES

The LymphoTrack® MRD software facilitates tracking of up to 5 defined clonal sequences as well as the degree of mismatches (0, 1, 2). It further calculates clonal read frequency, analyzes multiple samples and/or resequencing replicates, and reports the level of confidence when the clonal sequence is not detected at various levels.

LYMPHOTRACK® MRD SUMMARY REPORT

The MRD Summary Report provides the status of all the clonal sequences being tracked. Also, longitudinal graphs of clonal frequencies for each sequence are automatically generated if the Invivoscribe LymphoQuant® Internal Control is used (graphical representation shown in **Figure 3**).
Sample findings are detailed as follows for the most recent time point.

» Specimen Details: (a) Sample Type, (b) Total DNA input amount, (c) Collection Date / Time Point

» MRD Result (Detected/Not Detected)

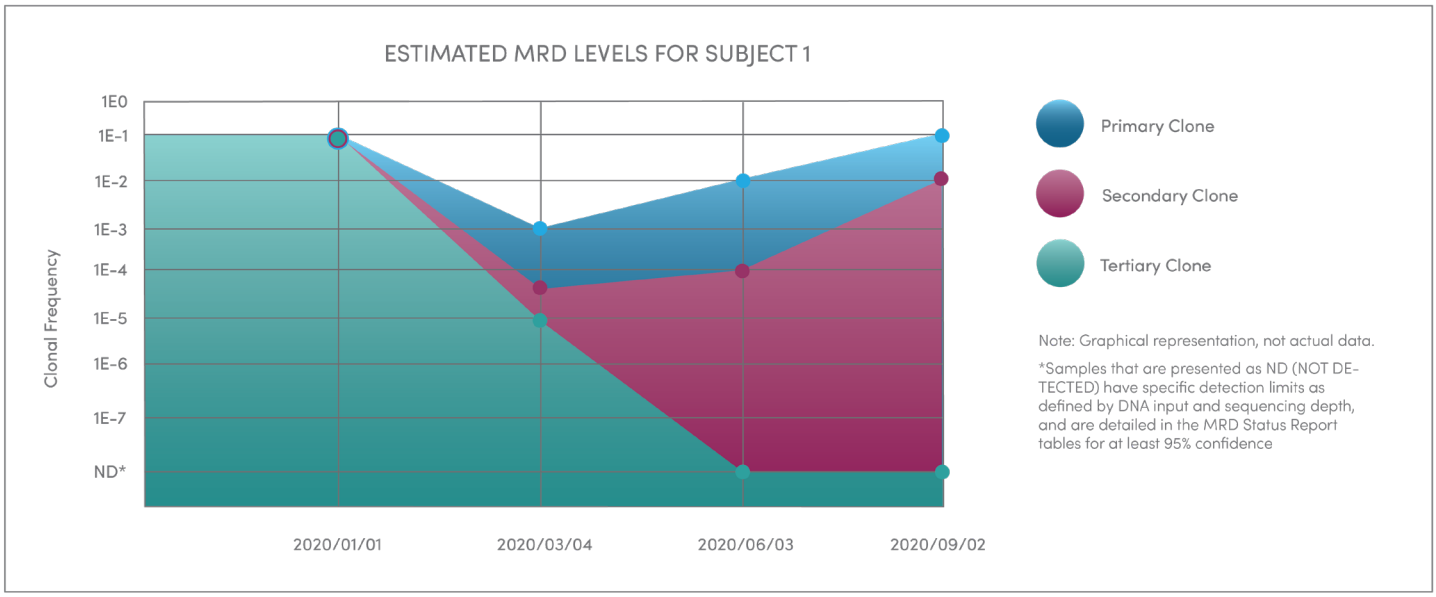
» Clonal Frequency* (If Detected) or % Confidence (If Not Detected)

» Estimated Clonal Cell Equivalents*

» Estimated Clonal Cell Equivalents per 1 Million Total Nucleated Cells*

*LymphoQuant Internal Control must be used in order for the software to estimate these values.

Figure 3: Estimated MRD Levels



NOTE: Graphical representation only

LYMPHOTRACK® MRD SAMPLE REPORT

The MRD Sample Report provides a summary table for the latest time point for all clonal sequences. Details are included for each sequence analyzed including the Clonal Frequency if DETECTED and the % Confidence if NOT DETECTED. Detailed information applicable to analysis is summarized for the most recent time point as follows:

» Clonal Sequence(s) Detected or Not Detected

» Number of PCR Replicates

» Total Read Number Analyzed

» Cumulative Target Read Number

» LymphoQuant® Internal Control Read Number

» When a clonal sequence of interest is Not Detected, the call confidence is reported for various sensitivities.

8. ASSAY PERFORMANCE

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.

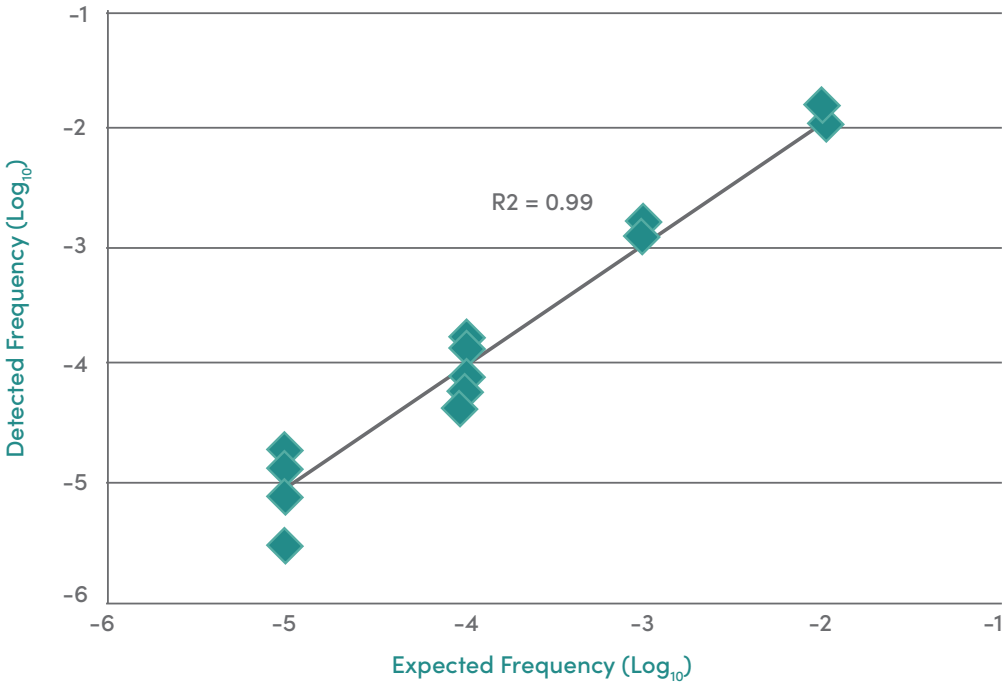
MATERIALS & METHOD

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 clonal 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 converts percent reads into clonal frequencies if LymphoQuant® Internal Control is used.

RESULTS & CONCLUSION

Excellent linearity was observed across the 10^{-2} to 10^{-5} dilution series **(Figure 4)**. Correlation of observed versus expected frequencies was further demonstrated for each sample tested. Total read count per sample tested ranged from 253,295 to 663,625.

Figure 4: Dilution Series Demonstrates Linearity of LymphoTrack® Assays



LYMPHOTRACK® MRD CONTROLS

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

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

M-0036 Rev 01 October 2023

invivoscribe®
www.invivoscribe.com

Invivoscribe, Inc.
Tel +1 858.224.6600 | Fax +1 858.224.6601
sales@invivoscribe.com

Invivoscribe, SARL
Tel +33 (0) 4.42.01.78.10 | Fax +33 (0) 4.88.56.22.89
sales-eu@invivoscribe.com