

Instructions for Use



LymphoTrack[®] Dx IGH (FR1/FR2/FR3) Assays – MiSeq™

To identify and track B cell immunoglobulin heavy chain (*IGH*) gene rearrangements using Next-Generation Sequencing (NGS) with the Illumina[®] MiSeq.

IVD This assay is for *In Vitro* Diagnostic Use.

Schematic depiction of the *IGH* gene locus and the framework (FR) regions targeted:



Storage Conditions: -85°C to -65°C

(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

| Catalog # | Products | Quantity |
|---------------------|--|---|
| REF 91210009 | LymphoTrack Dx IGH FR1 Assay Kit A – MiSeq | 8 indices – 5 reactions each |
| REF 91210039 | LymphoTrack Dx IGH FR1 Assay Panel – MiSeq | 24 indices – 5 reactions each |
| REF 91210089 | LymphoTrack Dx IGH FR2 Assay Kit A – MiSeq | 8 indices – 5 reactions each |
| REF 91210099 | LymphoTrack Dx IGH FR2 Assay Panel – MiSeq | 24 indices – 5 reactions each |
| REF 91210109 | LymphoTrack Dx IGH FR3 Assay Kit A – MiSeq | 8 indices – 5 reactions each |
| REF 91210119 | LymphoTrack Dx IGH FR3 Assay Panel – MiSeq | 24 indices – 5 reactions each |
| REF 91210129 | LymphoTrack Dx IGH FR1/2/3 Assay Kit A – MiSeq | 8 indices per FR region – 5 reactions each |
| REF 91210139 | LymphoTrack Dx IGH FR1/2/3 Assay Panel – MiSeq | 24 indices per FR region – 5 reactions each |

Table of Contents

| | | |
|-------|---|----|
| 1. | INTENDED USE | 3 |
| 2. | SUMMARY AND EXPLANATION OF THE TEST | 3 |
| 2.1. | Background..... | 3 |
| 2.2. | Summary | 4 |
| 3. | PRINCIPLES OF THE PROCEDURE | 5 |
| 3.1. | Polymerase Chain Reaction (PCR) | 5 |
| 3.2. | Amplicon Purification | 5 |
| 3.3. | Amplicon Quantification..... | 5 |
| 3.4. | Next-Generation Sequencing (NGS) | 5 |
| 3.5. | Multiplexing Amplicons | 6 |
| 3.6. | <i>IGHV</i> Somatic Hypermutation (SHM) Evaluation..... | 6 |
| 4. | REAGENTS | 7 |
| 4.1. | Reagent Components | 7 |
| 4.2. | Warnings and Precautions..... | 11 |
| 4.3. | Storage and Handling | 11 |
| 5. | INSTRUMENTS | 12 |
| 5.1. | Thermal cycler | 12 |
| 5.2. | Magnetic Stand | 12 |
| 5.3. | Real-time PCR Instrument | 12 |
| 5.4. | Illumina MiSeq Dx Instrument* | 12 |
| 6. | SPECIMEN COLLECTION AND PREPARATION..... | 13 |
| 6.1. | Precautions..... | 13 |
| 6.2. | Interfering Substances..... | 13 |
| 6.3. | Specimen Requirements and Handling | 13 |
| 6.4. | Sample Storage..... | 13 |
| 7. | ASSAY PROCEDURE..... | 14 |
| 7.1. | Materials Provided | 14 |
| 7.2. | Materials Required (not provided)..... | 14 |
| 7.3. | Reagent Preparation | 15 |
| 7.4. | Amplification | 15 |
| 7.5. | AMPure XP Purification | 16 |
| 7.6. | Quantification of Amplicons..... | 18 |
| 7.7. | Pooling and Quantification of Library..... | 19 |
| 7.8. | Dilution of the Pooled Library | 19 |
| 7.9. | Setup qPCR for Library Quantification..... | 19 |
| 7.10. | Preparation of the Library for the MiSeq Sequencing Run | 19 |
| 7.11. | MiSeq Flow Cell Loading..... | 20 |
| 7.12. | MiSeq Sample Sheet Setup..... | 20 |
| 7.13. | MiSeq Run Start..... | 22 |
| 8. | DATA ANALYSIS | 22 |
| 9. | EXPECTED VALUES..... | 22 |
| 10. | LIMITATIONS OF PROCEDURE | 22 |
| 11. | INTERPRETATION AND REPORTING..... | 23 |
| 12. | SAMPLE DATA..... | 27 |
| 13. | PERFORMANCE CHARACTERISTICS | 28 |
| 14. | TROUBLESHOOTING GUIDE | 30 |
| 15. | TECHNICAL AND CUSTOMER SERVICE..... | 31 |
| 16. | REFERENCES..... | 31 |
| 17. | SYMBOLS | 31 |
| 18. | LEGAL NOTICE | 32 |
| 19. | LYMPHOTrack Dx <i>IGH</i> FR1/FR2/FR3 Assay - MiSeq: SINGLE PAGE GUIDE..... | 33 |
| 20. | APPENDIX A: BUILDING A SEQUENCING LIBRARY WITH MULTIPLE NGS TARGETS..... | 34 |

1. Intended Use

Intended Use (LymphoTrack Dx *IGH* FR1 Assay – MiSeq)

The LymphoTrack Dx *IGH* FR1 Assay for the Illumina MiSeq is an *in vitro* diagnostic product intended for next-generation sequencing (NGS) based determination of the frequency distribution of *IGH* gene rearrangements as well as the degree of somatic hypermutation of rearranged genes in patients suspected with having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders as well as providing an aid in determining disease prognosis.

Intended Use (LymphoTrack Dx *IGH* FR1/2/3 Assays – MiSeq)

The LymphoTrack Dx *IGH* FR1 Assay for the Illumina MiSeq is an *in vitro* diagnostic product intended for next-generation sequencing (NGS) based determination of the frequency distribution of *IGH* gene rearrangements as well as the degree of somatic hypermutation of rearranged genes in patients suspected with having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders as well as providing an aid in determining disease prognosis.

This LymphoTrack Dx *IGH* FR2 Assay is an *in vitro* diagnostic product intended for next-generation sequencing (NGS) for the Illumina MiSeq instrument. The assay will determine the frequency distribution of *IGH* V_H-J_H gene rearrangements in patients suspected with having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders.

The LymphoTrack Dx *IGH* FR3 Assay is an *in vitro* diagnostic product intended for next-generation sequencing (NGS) for the Illumina MiSeq instrument. The assay will determine the frequency distribution of *IGH* V_H-J_H gene rearrangements in patients suspected with having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders.

Data from all three framework regions is needed to determine evidence of clonality for a sample.

2. Summary and Explanation of the Test

2.1. Background

The immunoglobulin heavy chain (*IGH*) gene locus on chromosome 14 (14q32.3) includes 46-52 functional and 30 non-functional variable (V_H) gene segments, 27 functional diversity (D_H) gene segments and 6 functional joining (J_H) gene segments spread over 1250 kilobases. The V_H gene segments contain three conserved framework (FR) and two variable complementarity-determining regions (CDRs).

Lymphoid cells are different from the other somatic cells in the body; during development, the antigen receptor genes in lymphoid cells undergo somatic gene rearrangement.¹ For example, during B-cell development, genes encoding the *IGH* molecules are assembled from multiple polymorphic gene segments that undergo rearrangements and selection, generating V_H-D_H-J_H combinations that are unique in both length and sequence. Since leukemias and lymphomas originate from the malignant transformation of individual lymphoid cells, an individual's leukemic or lymphoma cells generally share one or more cell-specific or "clonal" antigen receptor gene rearrangements. Therefore, tests that detect *IGH* clonal rearrangements can be useful in the study of B- and T-cell malignancies.

In addition, immunoglobulin heavy chain variable region (*IGHV*) gene hypermutation status provides important prognostic information for patients with chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL). The presence of *IGHV* somatic hypermutation (SHM) is defined as greater or equal to 2% difference from the germline V_H gene sequence, whereas less than 2% difference is considered evidence of no somatic hypermutation. The status of somatic hypermutation for clone(s) has clinical relevance for B-CLL, as there is a clear distinction in the median survival of patients with and without somatic hypermutation. Hypermutation of the *IGHV* region is strongly predictive of a good prognosis while lack of mutation predicts a poor prognosis.²

Initially, clonal rearrangements were identified using Restriction Fragment, Southern Blot Hybridization (RF-SBH) techniques. However, these tests proved cumbersome, labor-intensive, required large amounts of DNA and were not suitable for analysis of many of the less diverse antigen receptor loci.

During the last several decades, the use of RF-SBH assays has been supplanted by PCR-based clonality tests developed by Alexander Morley³ and are considered the current gold standard method. These-based assays identify clonality on the basis of over-representation of amplified V_H-D_H-J_H (or incomplete D_H-J_H products) gene rearrangements following their separation using gel or capillary electrophoresis. Though sensitive and suitable for

testing small amounts of DNA, these assays cannot readily differentiate between clonal populations and multiple rearrangements that might lie beneath a single-sized peak and are not designed to identify the specific V_H – J_H DNA sequence that is required to track clonal populations in subsequent analyses. This second limitation can be of particular importance, as once the unique clone-specific DNA sequence is identified, the sequence can be used in subsequent tests to track and follow the clonal cell populations.

2.2. Summary

These LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays - MiSeq (sold separately and as a set) represent a significant improvement over existing clonality assays using fragment analysis as they efficiently detect *IGH* gene rearrangements using a single multiplex master mix and, at the same time, identify the DNA sequence specific for each clonal gene rearrangement. Therefore, these assays have two important and complementary uses: they provide critical information on the existence of clonality and identify sequence information required to track those clones in subsequent samples. The LymphoTrack Dx *IGH* FR1 Assay additionally provides detailed sequence information on the degree of SHM.

Each single multiplex master mix targets one of the conserved *IGH* framework regions (FR1, FR2, or FR3) within the V_H and the J_H regions described in lymphoid malignancies. Targeting all three framework regions significantly reduces the risk of not being able to detect the presence of clonality, as somatic hypermutations in the primer binding sites of the involved V_H gene segments can impede DNA amplification.⁴ **Data from all three framework regions is needed to determine evidence of clonality for a sample.**

Primers included in the master mixes are designed with Illumina adapters. This allows for a one-step PCR and pooling of amplicons from several different samples and targets (generated with other LymphoTrack Dx Assays for the Illumina MiSeq instrument, sold separately) onto one MiSeq flow cell, allowing up to 24 samples (including controls) per target to be analyzed in parallel in a single run.

The associated LymphoTrack Dx Software - MiSeq provides a simple and streamlined method of analysis and visualization of data. By following the guidelines provided in section 11 *Interpretation and Reporting*, the sample results summarized in the software can be easily interpreted for the presence or absence of clonality and somatic hypermutation. **Always interpret the results of molecular clonality tests in the context of clinical, histological and immunophenotypic data.**

Positive and negative controls for clonality are included in the kit. An additional positive control specific for somatic hypermutation is optional and can be purchased separately ([REF](#) 40880008).

Note: For a more thorough explanation of the locus and the targeted sequencing strategy, please refer to Miller J.E., 2013.⁵

3. Principles of the Procedure

3.1. Polymerase Chain Reaction (PCR)

PCR assays are routinely used for the identification of clonal B- and T-cell populations. These assays amplify the DNA between primers that target the conserved V and J regions of antigen receptor genes. The primers target these conserved regions and lie on either side of an area where programmed genetic rearrangements occur during the maturation of all B and T lymphocytes. Different populations of the B and T lymphocytes arise as a result of these genetic rearrangements.

The antigen receptor genes that undergo rearrangements are the immunoglobulin heavy chain (*IGH*) and light chain loci (*IGK* and *IGL*) in B cells and the T-cell receptor gene loci (*TRA*, *TRB*, *TRG*, and *TRD*) in T cells. Each B and T cell has one or two productive V – J rearrangements that are unique in both length and sequence. Therefore, when DNA from a normal or polyclonal population is amplified using DNA primers that flank the V– J region, amplicons that are unique in both sequence and length are generated, reflecting the heterogeneous population. In some cases, where lymphocyte DNA is absent, no amplicons will be generated. Samples containing *IGH* clonal populations yield one or two prominent amplified products of the same length and sequence that are detected with significant frequency of occurrence, within a diminished polyclonal background.

3.2. Amplicon Purification

PCR amplicons are purified to remove excess primers, nucleotides, salts and enzymes using solid-phase reversible immobilization (SPRI) paramagnetic bead technology for high-throughput purification of PCR amplicons. Using an optimized buffer, PCR products 100 bp or larger are selectively bound to paramagnetic beads while contaminants such as excess primers, primer dimers, salts and unincorporated dNTPs are washed away. Amplicons can then be eluted and separated from the paramagnetic beads resulting in a more purified PCR product for downstream analysis and amplicon quantification.

3.3. Amplicon Quantification

Purified amplicons are quantified using the KAPA™ Library Quantification Kits for Illumina platforms. Purified and diluted PCR amplicons and a set of six pre-diluted DNA standards are amplified by quantitative (qPCR) methods, using the KAPA SYBR® FAST qPCR Master Mix and primers. The primers in the KAPA kit target Illumina P5 and P7 flow cell adapter oligo sequences.

The average Ct score for the pre-diluted DNA Standards are plotted against \log_{10} to generate a standard curve, which can then be used to calculate the concentration (pM) of the PCR amplicons derived from sample DNA. Calculating the concentration of PCR amplicons allows equal amplicon representation in the final pooled library that is loaded onto the MiSeq for sequencing.

3.4. Next-Generation Sequencing (NGS)

Sanger sequencing methods represent the most popular in a range of ‘first-generation’ nucleic acid sequencing technologies. Newer methods, which leverage tremendously parallel sequencing approaches, are often referred to as NGS. These technologies can use various combination strategies of template preparation, sequencing, imaging and bioinformatics for genome alignment and assembly.

NGS technologies used in this assay rely on the amplification of genetic sequences using a series of consensus forward and reverse primers that include adapter and index tags. Amplicons generated with the LymphoTrack Dx Master Mixes are quantified, pooled and loaded onto a flow cell for sequencing with an Illumina MiSeq sequencing platform. Specifically, the amplified products in the library are hybridized to oligonucleotides on a flow cell and are amplified to form local clonal colonies (bridge amplification). Four types of reversible terminator bases (RT-bases) are added and the sequencing strand of DNA is extended one nucleotide at a time. To record the incorporation of nucleotides, a CCD camera takes an image of the light emitted as each RT-base is added, and then cleaved to allow incorporation of the next base.

3.5. Multiplexing Amplicons

These assays were designed to allow for two different levels of multiplexing in order to reduce costs and time for laboratories. The first level of multiplexing originates from the multiple indices that are provided with the assays. Each of these 24 indices acts as a unique barcode that allows amplicons from individual samples to be pooled together after PCR amplification to generate the sequencing library. The resulting sequences are sorted by the bioinformatics software which identifies those that originated from an individual sample.

The second level of multiplexing originates from the ability of the accompanying software to sort sequencing data by both index and target. This allows amplicons generated with targeted primers (even those tagged with the same index) to be pooled together to generate the library to be sequenced on a single flow cell. An example would be to sequence a combination of products from several Invivoscribe LymphoTrack Dx Assay kits for the MiSeq such as *IGHV* Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRB* and *TRG* together. **When multiplexing amplicons of different gene targets it is important to use the appropriate sequencing chemistry. The number of sequencing cycles must be sufficient to sequence the largest amplicon in the multiplex.** For example, when multiplexing a combination of *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRB* and *TRG* amplicons together, use the MiSeq v2 (500 cycle) sequencing kit for up to 4 targets or v3 (600 cycle) sequencing kit for up to 7 targets. When multiplexing any of these amplicons together with *IGHV* Leader, use the MiSeq v3 (600 cycle) sequencing kit. If multiplexing only *IGH* FR3 and *TRG* amplicons together, which both have shorter amplicon sizes, use MiSeq v2 (300 or 500 cycle) sequencing kits and adjust the cycle settings in the sample sheet. For further instructions, please refer to *Appendix A: Building a Sequencing Library with Multiple NGS Targets* (section 20).

The number of samples that can be multiplexed onto a single flow cell is also dependent on the flow cell that is utilized. Illumina's standard flow cells (MiSeq v3) can generate 22-25 million reads. To determine the number of reads per sample, divide the total number of reads for the flow cell by the number of samples that will be multiplexed and the number of reads for each sample will be sufficient for valid interpretation. For more information refer to section 11 *Interpretation and Reporting*. Illumina also manufactures other flow cells that utilize the same sequencing chemistry, but generate fewer reads. When using these alternative flow cells one must consider that fewer total reads either means less depth per sample or fewer samples can be run on the flow cell to achieve the same depth per sample.

3.6. *IGHV* Somatic Hypermutation (SHM) Evaluation

For evaluation of the somatic hypermutation rate of the *IGHV* region, the LymphoTrack Dx *IGH* FR1 – Master Mixes can be used; however, this only targets a portion of the *IGHV* region as the sequence upstream of the primer binding site will not be assessed. When analyzing the somatic hypermutation status of samples, the bioinformatics software will provide the mutation rate based upon the percent mismatch of the clonal amplicons as compared to germline reference genes, a prediction of whether the protein translation would be in or out of frame, a prediction of whether mutations or gene rearrangements result in a pre-mature stop codon and the percentage of V_H gene coverage for the region targeted by the assay.

4. Reagents

4.1. Reagent Components

Table 1. Available Kits














| Cat# | Description | # of Indexed Master Mixes | Total Reactions |
|--|---|---|-----------------|
|  91210009 | LymphoTrack Dx <i>IGH</i> FR1 Assay Kit A – MiSeq | 8 indices – 5 sequencing runs each | 40 |
|  91210039 | LymphoTrack Dx <i>IGH</i> FR1 Assay Panel – MiSeq | 24 indices – 5 sequencing runs each | 120 |
|  91210089 | LymphoTrack Dx <i>IGH</i> FR2 Assay Kit A – MiSeq | 8 indices – 5 sequencing runs each | 40 |
|  91210099 | LymphoTrack Dx <i>IGH</i> FR2 Assay Panel – MiSeq | 24 indices – 5 sequencing runs each | 120 |
|  91210109 | LymphoTrack Dx <i>IGH</i> FR3 Assay Kit A – MiSeq | 8 indices – 5 sequencing runs each | 40 |
|  91210119 | LymphoTrack Dx <i>IGH</i> FR3 Assay Panel – MiSeq | 24 indices – 5 sequencing runs each | 120 |
|  91210129 | LymphoTrack Dx <i>IGH</i> FR1/2/3 Assay Kit A – MiSeq | (8+8+8) indices – 5 sequencing runs each | 40+40+40 |
|  91210139 | LymphoTrack Dx <i>IGH</i> FR1/2/3 Assay Panel – MiSeq | (24+24+24) indices – 5 sequencing runs each | 120+120+120 |




Table 2. LymphoTrack Dx *IGH* FR1 Assay Kit Components

| Reagent | Reagent Components | Unit Quantity | 91210009 # of Units | 91210039 # of Units | Storage Temperature | Notes |
|---------------------------------|--|---------------|------------------------|------------------------|---|--|
| Master Mixes[†] | <i>IGH</i> FR1 MiSeq 01 | 250 µL | 1 | 1 |  | N/A |
| | <i>IGH</i> FR1 MiSeq 02 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 03 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 04 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 05 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 06 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 07 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 08 | | 1 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 09 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 10 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 11 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 12 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 13 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 14 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 15 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 16 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 18 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 19 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 20 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 21 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 22 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 23 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 25 | | 0 | 1 | | |
| | <i>IGH</i> FR1 MiSeq 27 | | 0 | 1 | | |
| Positive Control DNA | <i>IGH</i> POS (+)  40880009 | 45 µL | 1 | 3 |  | <i>IGH</i> V1-46_03 / <i>IGH</i> J4_02 DNA diluted in tonsil DNA |
| Negative Control DNA | NGS NEG (-)  40920018 | 45 µL | 1 | 3 | or  | Tonsil DNA, highest sequence frequency can vary between lots |

Note: There are no preservatives used in the manufacturing of these kits.

†Note: Indices 17, 24, and 26 are not used in these kits.




Table 3. LymphoTrack Dx *IGH* FR2 Assay Kit Components

| Reagents | Reagent Components | Unit Quantity | 91210089 # of Units | 91210099 # of Units | Storage Temperature | Notes |
|-----------------------------|--------------------------------------|---------------|------------------------|------------------------|---|--|
| Master Mixes† | <i>IGH</i> FR2 MiSeq 01 | 250 µL | 1 | 1 |  -85°C to -65°C | N/A |
| | <i>IGH</i> FR2 MiSeq 02 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 03 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 04 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 05 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 06 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 07 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 08 | | 1 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 09 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 10 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 11 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 12 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 13 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 14 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 15 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 16 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 18 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 19 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 20 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 21 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 22 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 23 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 25 | | 0 | 1 | | |
| | <i>IGH</i> FR2 MiSeq 27 | | 0 | 1 | | |
| Positive Control DNA | <i>IGH</i> POS (+) (REF 40880009) | 45 µL | 1 | 3 |  2°C to 8°C | <i>IGH</i> V1-46_03 / <i>IGH</i> J4_02 DNA diluted in tonsil DNA |
| Negative Control DNA | NGS NEG (-) (REF 40920018) | 45 µL | 1 | 3 | or  -85°C to -65°C | Tonsil DNA, highest sequence frequency can vary between lots |

Note: There are no preservatives used in the manufacturing of these kits.

†Note: Indices 17, 24, and 26 are not used in these kits.

Table 4. LymphoTrack Dx IGH FR3 Assay Kit Components

| Reagents | Reagent Components | Unit Quantity | 91210109 # of Units | 91210119 # of Units | Storage Temperature | Notes |
|---------------------------------|-------------------------------|---------------|------------------------|------------------------|--|--|
| Master Mixes[‡] | IGH FR3 MiSeq 01 | 250 µL | 1 | 1 |  -85°C to -65°C | N/A |
| | IGH FR3 MiSeq 02 | | 1 | 1 | | |
| | IGH FR3 MiSeq 03 | | 1 | 1 | | |
| | IGH FR3 MiSeq 04 | | 1 | 1 | | |
| | IGH FR3 MiSeq 05 | | 1 | 1 | | |
| | IGH FR3 MiSeq 06 | | 1 | 1 | | |
| | IGH FR3 MiSeq 07 | | 1 | 1 | | |
| | IGH FR3 MiSeq 08 | | 1 | 1 | | |
| | IGH FR3 MiSeq 09 | | 0 | 1 | | |
| | IGH FR3 MiSeq 10 | | 0 | 1 | | |
| | IGH FR3 MiSeq 11 | | 0 | 1 | | |
| | IGH FR3 MiSeq 12 | | 0 | 1 | | |
| | IGH FR3 MiSeq 13 | | 0 | 1 | | |
| | IGH FR3 MiSeq 14 | | 0 | 1 | | |
| | IGH FR3 MiSeq 15 | | 0 | 1 | | |
| | IGH FR3 MiSeq 16 | | 0 | 1 | | |
| | IGH FR3 MiSeq 18 | | 0 | 1 | | |
| | IGH FR3 MiSeq 19 | | 0 | 1 | | |
| | IGH FR3 MiSeq 20 | | 0 | 1 | | |
| | IGH FR3 MiSeq 21 | | 0 | 1 | | |
| | IGH FR3 MiSeq 22 | | 0 | 1 | | |
| | IGH FR3 MiSeq 23 | | 0 | 1 | | |
| | IGH FR3 MiSeq 25 | | 0 | 1 | | |
| | IGH FR3 MiSeq 27 | | 0 | 1 | | |
| Positive Control DNA | IGH POS (+) (REF 40880009) | 45 µL | 1 | 3 |  2°C to 8°C | IGH V1-46_03 / IGH J4_02 DNA diluted in tonsil DNA |
| Negative Control DNA | NGS NEG (-) (REF 40920018) | 45 µL | 1 | 3 | or  -85°C to -65°C | Tonsil DNA, highest sequence frequency can vary between lots |

Note: There are no preservatives used in the manufacturing of these kits.

‡Note: Indices 17, 24, and 26 are not used in these kits.

Table 5. LymphoTrack Dx *IGH* FR1/2/3 Assay Kit Components

| FR1 Master Mixes [‡] | FR2 Master Mixes [‡] | FR3 Master Mixes [‡] | Unit Quantity | 91210129 # of Units per FR | 91210139 # of Units per FR | |
|---|-------------------------------|---|---------------|-------------------------------|-------------------------------|------------------------|
| <i>IGH</i> FR1 MiSeq 01 | <i>IGH</i> FR2 MiSeq 01 | <i>IGH</i> FR3 MiSeq 01 | 250 µL | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 02 | <i>IGH</i> FR2 MiSeq 02 | <i>IGH</i> FR3 MiSeq 02 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 03 | <i>IGH</i> FR2 MiSeq 03 | <i>IGH</i> FR3 MiSeq 03 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 04 | <i>IGH</i> FR2 MiSeq 04 | <i>IGH</i> FR3 MiSeq 04 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 05 | <i>IGH</i> FR2 MiSeq 05 | <i>IGH</i> FR3 MiSeq 05 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 06 | <i>IGH</i> FR2 MiSeq 06 | <i>IGH</i> FR3 MiSeq 06 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 07 | <i>IGH</i> FR2 MiSeq 07 | <i>IGH</i> FR3 MiSeq 07 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 08 | <i>IGH</i> FR2 MiSeq 08 | <i>IGH</i> FR3 MiSeq 08 | | 1 | 1 | |
| <i>IGH</i> FR1 MiSeq 09 | <i>IGH</i> FR2 MiSeq 09 | <i>IGH</i> FR3 MiSeq 09 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 10 | <i>IGH</i> FR2 MiSeq 10 | <i>IGH</i> FR3 MiSeq 10 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 11 | <i>IGH</i> FR2 MiSeq 11 | <i>IGH</i> FR3 MiSeq 11 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 12 | <i>IGH</i> FR2 MiSeq 12 | <i>IGH</i> FR3 MiSeq 12 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 13 | <i>IGH</i> FR2 MiSeq 13 | <i>IGH</i> FR3 MiSeq 13 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 14 | <i>IGH</i> FR2 MiSeq 14 | <i>IGH</i> FR3 MiSeq 14 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 15 | <i>IGH</i> FR2 MiSeq 15 | <i>IGH</i> FR3 MiSeq 15 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 16 | <i>IGH</i> FR2 MiSeq 16 | <i>IGH</i> FR3 MiSeq 16 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 18 | <i>IGH</i> FR2 MiSeq 18 | <i>IGH</i> FR3 MiSeq 18 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 19 | <i>IGH</i> FR2 MiSeq 19 | <i>IGH</i> FR3 MiSeq 19 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 20 | <i>IGH</i> FR2 MiSeq 20 | <i>IGH</i> FR3 MiSeq 20 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 21 | <i>IGH</i> FR2 MiSeq 21 | <i>IGH</i> FR3 MiSeq 21 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 22 | <i>IGH</i> FR2 MiSeq 22 | <i>IGH</i> FR3 MiSeq 22 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 23 | <i>IGH</i> FR2 MiSeq 23 | <i>IGH</i> FR3 MiSeq 23 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 25 | <i>IGH</i> FR2 MiSeq 25 | <i>IGH</i> FR3 MiSeq 25 | | 0 | 1 | |
| <i>IGH</i> FR1 MiSeq 27 | <i>IGH</i> FR2 MiSeq 27 | <i>IGH</i> FR3 MiSeq 27 | | 0 | 1 | |
| Control DNA [*] | | Notes | | Unit Quantity | 91210129 # of Units | 91210139 # of Units |
| <i>IGH</i> POS (+) (REF 40880009) | | <i>IGH</i> V1-46_03 / <i>IGH</i> J4_02 DNA diluted in tonsil DNA | | 45 µL | 2 | 6 |
| NGS NEG (-) (REF 40920018) | | Tonsil DNA, highest sequence frequency can vary between lots | | 45 µL | 2 | 6 |

***Note:** The storage temperature for master mixes is -85°C to -65°C.

***Note:** The storage temperature for controls is 2°C to 8°C or -85°C to -65°C.

Note: There are no preservatives used in the manufacturing of these kits.

4.2. Warnings and Precautions



Please read the Instructions for Use carefully prior to starting the assay procedure and follow each step closely.

- **IVD** This product is for *in vitro* Diagnostic Use.
- Use the assay kit as a system; do not substitute other manufacturers' reagents. Dilution, reducing amplification reactions, or other deviations from this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of these kits.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Close adherence to the protocol will assure optimal performance and reproducibility. Ensure correct thermal cycler programs are used, as other programs may provide inaccurate/faulty data, such as false-positive and false-negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- Perform all laboratory procedures with standard personal protective equipment (gloves, laboratory coats and protective eye wear). Follow good laboratory practices and universal precautions when working with specimens. Do not pipette by mouth. Do not eat, drink, or smoke in laboratory work areas. Wash hands thoroughly after handling specimens and assay reagents. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets.
- Use molecular biology grade water for the preparation of specimen DNA.
- Due to the high analytical sensitivity of this test, use extreme care to avoid any contamination of reagents or amplification mixtures with samples, controls, or amplified materials. Use fresh, aerosol-resistant pipette tips between samples and between dispensing reagents. Closely monitor all reagents for signs of contamination (e.g., negative controls giving positive signals). Discard any reagents suspected of contamination.
- To minimize contamination wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to setting up PCR.
- Follow uni-directional work flow in the PCR laboratory between the separate work areas: begin with master mix preparation, move to specimen preparation, then to amplification and finally to detection. Autoclaving does not eliminate DNA contamination. Do not bring amplified DNA into the areas designated for master mixes or specimen preparation.
- Dedicate all pipettes, pipette tips and any equipment used in a particular area to that area of the laboratory.
- Decontaminate non-disposable items in 10% bleach and rinse with distilled water two separate times before returning them to the starting areas.
- Use sterile, disposable plasticware whenever possible to avoid contamination.

4.3. Storage and Handling

- If the assay is not being used immediately, store at **-85°C to -65°C**.
- The optimum storage temperature for DNA controls is 2°C to 8°C, but DNA can also be stored at -85°C to -65°C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure they are completely resuspended.
- Due to high salt concentrations, PCR master mixes are sensitive to freeze/thaw cycles. Limit the number of cycles to a maximum of four times.

If you have any questions, please contact the Invivoscribe technical staff. We would be happy to help you determine your optimal storage needs.

5. Instruments

The specific instruments listed below are recommended based on the methods used to validate this assay.

5.1. Thermal cycler

- Use or Function: Amplification of DNA samples
- Suggested Instrument: Veriti™ Dx Thermal Cycler or equivalent
- Performance Characteristics and Specifications:
 - Minimum Thermal Range: 15°C to 96°C
 - Minimum Ramping Speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.4 *Amplification* for thermal cycler program.

5.2. Magnetic Stand

- Use or Function: Purification of PCR products
- Suggested Instrument:
 - Ambion® Magnetic Stand 96* (REF AM10027)
 - Agencourt SPRIPlate® 96 Ring Super Magnet Plate* (REF A32782) or
 - Thermo Fisher Scientific DynaMag™-96 Side Skirted Magnet* (REF 12027) or equivalent
- Performance Characteristics and Specifications:
 - Precipitate paramagnetic beads
- See section 7.5 *AMPure XP Purification* for PCR product purification methods.

5.3. Real-time PCR Instrument

- Use or Function: Quantify purified PCR products
- Suggested Instrument: Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument or equivalent
- Performance Characteristics and Specifications:
 - Can detect SYBR Green wavelength
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.6 *Quantification of Amplicons* for real-time PCR program.

5.4. Illumina MiSeq Dx Instrument*

- Use or Function: Sequence normalized DNA library
- Performance Characteristics and Specifications:
 - Compatible with MiSeq Reagent Kit v2*
 - Compatible with MiSeq Reagent Kit v3* when multiplexing
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.11 *MiSeq Flow Cell Loading*, 7.12 *MiSeq Sample Sheet Setup*, and 7.13 *MiSeq Run Start*.

*Warning: These are not CE-marked products.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle all in accordance with your institute's Bloodborne Pathogen program and/or Biosafety Level 2.

6.2. Interfering Substances

The following substances are known to interfere with PCR:

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)
- Heparin

6.3. Specimen Requirements and Handling

- The minimum input quantity is 50 ng of high-quality DNA (5 µL of sample DNA at a minimum concentration of 10 ng/µL).
- This assay tests extracted and purified genomic DNA. DNA must be quantified with a method specific for double-stranded DNA (dsDNA) and be free of PCR inhibitors.
- Resuspend DNA in an appropriate solution such as 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, prepared with molecular biology grade water) or molecular biology grade water alone.

6.4. Sample Storage

Store samples using a method that prevents degradation of DNA.

7. Assay Procedure

7.1. Materials Provided

See Table 2 – Table 5 for materials provided.

7.2. Materials Required (not provided)

Table 6. Materials Required (not provided)

| Reagent/Material | Required or Recommended Reagents/Suppliers | Catalog # | Notes |
|--|---|--|---|
| DNA Polymerase | Roche: <ul style="list-style-type: none"> EagleTaq™ DNA Polymerase or Invivoscribe: <ul style="list-style-type: none"> FalconTaq DNA Polymerase or equivalent | 05206944190 or 60970130 | 5 U/μL |
| Molecular Biology Grade Water | N/A | N/A | DNase / RNase free |
| Calibrated Pipettes | N/A | N/A | Must be able to accurately measure volumes between 0.2 μL and 1000 μL |
| PCR Plates or Tubes | N/A | N/A | DNase / RNase / PCR inhibitor-free |
| Filter Barrier Pipette Tips | N/A | N/A | Sterile, RNase/DNase/Pyrogen-free |
| Microcentrifuge Tubes | N/A | N/A | Sterile |
| PCR Purification Kit | Beckman Coulter, Inc: <ul style="list-style-type: none"> Agencourt AMPure XP | A63880 | N/A |
| PCR Purification | Thermo Fisher Scientific: <ul style="list-style-type: none"> Ambion Magnetic Stand 96 DynaMag-96 Side Skirted Magnet or Beckman Coulter: <ul style="list-style-type: none"> Agencourt SPRI Plate, 96 Ring Super Magnet Plate or equivalent | AM10027 12027 or A32782 | N/A |
| Amplicon & Library Quantification | KAPA Biosystems: <ul style="list-style-type: none"> KAPA Library Quantification Kit – Illumina | KK4824 | N/A |
| MiSeq Run | Illumina: <ul style="list-style-type: none"> MiSeq Reagent v2 kit (500 cycles) or v3 kit (600 cycles) when multiplexing When only sequencing <i>IGH</i> FR3: MiSeq Reagent v2 kit (300 cycles) or v2 kit (500 cycles) | MS-102-2003 or MS-102-3003 MS-102-2002 or MS-102-2003 | Standard flow cell |
| MiSeq Software | <ul style="list-style-type: none"> MiSeq Control Software v2.6 or later Local Run Manager v2.0 or later | N/A | N/A |
| Dilution Buffer A | N/A | N/A | Prepare a 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20 solution |

7.3. Reagent Preparation

To ensure DNA samples contain no PCR inhibitors and are of sufficient quality and quantity to generate a valid result samples may be tested with the Specimen Control Size Ladder Master Mix from Invivoscribe ([REF](#) 20960021 for ABI detection or [REF](#) 20960020 for gel detection). The Specimen Control Size Ladder targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400 and 600 bp; sizing may vary +/- 5 bp due to size standard and/or instrument differences. Verifying the DNA integrity is especially important for challenging specimens e.g., FFPE tissue.

Always use the positive and negative controls to ensure the assay has been performed correctly.

Always set-up a no template control (NTC) to check for contamination during the PCR set-up process.

- 7.3.1. Using gloved hands, remove the Master Mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix followed by a very brief centrifugation.
- 7.3.2. In a containment hood or dead air box, pipette 45 µL from each Master Mix tube into a clean PCR plate (one well for each Master Mix and one Master Mix per sample).
 - Include two controls in each run (one positive and one negative) as well as one NTC.
 - For the NTC, use molecular biology grade water as template instead of DNA.
- 7.3.3. Add 0.2 µL of Taq DNA polymerase (@5 U/µL) to each well containing aliquoted Master Mixes.
- 7.3.4. Add 5 µL of sample DNA (at a minimum concentration of 10 ng/µL), control DNA, or molecular biology grade water (NTC) to the individual wells containing the respective Master Mix reactions.
 - Pipette up and down 5-10 times to mix.
 - Seal the plate and place it in the PCR thermal cycler.

Table 7. Reaction Setup

| Reagent | Volume |
|-----------------------|----------------|
| Master Mix | 45 µL |
| Taq DNA polymerase | 0.2 µL |
| Sample or Control DNA | 5 µL |
| Total Volume | 50.2 µL |

7.4. Amplification

- 7.4.1. Amplify the samples using the PCR program from Table 8.

If multiplexing multiple targets, please refer to Appendix A (section 20) for other LymphoTrack Dx Assay - MiSeq thermal cycling conditions.

Table 8. PCR Program

| Step | Temperature | Time | Cycle |
|------|-------------|------------|-------|
| 1 | 95°C | 7 minutes | 1 |
| 2 | 95°C | 45 seconds | 29x |
| 3 | 60°C | 45 seconds | |
| 4 | 72°C | 90 seconds | |
| 5 | 72°C | 10 minutes | 1 |
| 6 | 15°C | ∞ | 1 |

Note: Set the heated lid to 105°C and reaction volume to 50 µL.

- 7.4.2. Remove the amplification plate from the thermal cycler. If not immediately continuing to the next steps, store the amplified PCR products at 4°C for 1 day.

7.5. AMPure XP Purification

Purification of the PCR products from samples, positive and negative controls and no template controls were performed during assay validation using the Agencourt AMPure XP PCR Purification system.

Preparation:

- 7.5.1. Remove the AMPure XP reagent from storage and allow it to equilibrate to room temperature before use. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.

If multiplexing multiple targets, please refer to Appendix A (section 20) for AMPure XP reagent volumes used in other LymphoTrack Dx Assay - MiSeq PCR products.

- 7.5.2. Transfer the appropriate volume of Agencourt AMPure XP reagent needed for the plate to a new 2 mL tube to minimize the risk of contamination by pipette tips.
 - The required volume of Agencourt AMPure XP reagent = $n \times 50 \mu\text{L}$ (n is number of samples to be purified)
- 7.5.3. Prepare a fresh stock (0.5 mL for each sample to be purified) of 80% ethanol using sterile water.

Binding of Amplicons to Magnetic Particles:

- 7.5.4. Add 50 μL of the aliquoted, **room temperature** Agencourt AMPure XP reagent to each sample to be purified.
 - Mix by pipetting up and down 10 times.
 - The color of the mixture should appear homogenous after mixing.
 - Incubate 10 minutes at room temperature.
- 7.5.5. Place the mixed samples on a DynaMag-96 Side Skirted or Ambion Magnetic Stand-96 and incubate at room temperature for 5 minutes to allow the magnetic particles to separate from the solution.
 - Keep the plate on the magnetic stand at all times during this procedure, until step 7.5.10 below.
- 7.5.6. Using a P200 (or equivalent multichannel pipette) set to 95 μL , aspirate the cleared supernatant and discard.
 - Use a P10 pipette (or equivalent multichannel pipette) set to 10 μL to remove any excess supernatant.
 - Avoid removing any magnetic particles.

Washing:

- 7.5.7. Keeping the plate on a magnetic stand, add 200 μL of 80% ethanol to each sample. Incubate for 30 seconds at room temperature.
 - Using a P200 (or equivalent multichannel pipette) set to 195 μL ; aspirate the ethanol and discard.
 - Use a P10 pipette (or a multichannel pipette) set to 10 μL to remove excess ethanol.
 - Avoid removing any magnetic particles.
- 7.5.8. Repeat step 7.5.7 for a total of two washes.
- 7.5.9. With the plate still on the magnetic stand, allow the magnetic particles to air-dry for 5 minutes.

Elution:

- 7.5.10. Remove the plate from the magnetic stand. Add 25 μL of 10 mM Tris-HCl, pH 8.0 buffer.
 - Mix by pipetting until homogeneous.
 - Make sure all magnetic particles are in solution.
- 7.5.11. Incubate at room temperature for 2 minutes.
- 7.5.12. Place the plate on the magnetic stand for 5 minutes or until the supernatant has cleared.
- 7.5.13. Transfer 22 μL of the eluate to a fresh plate. Seal with cap strips. Label the plate and briefly centrifuge to ensure the supernatant has completely settled to the bottom of the well. Store at -20°C or proceed to the next step.

The gel images in Figures 1 - 3 illustrate the effectiveness of a typical purification (showing amplicons before and after purification) using *IGH* FR1, *IGH* FR2 and *IGH* FR3 MiSeq Master Mixes respectively.

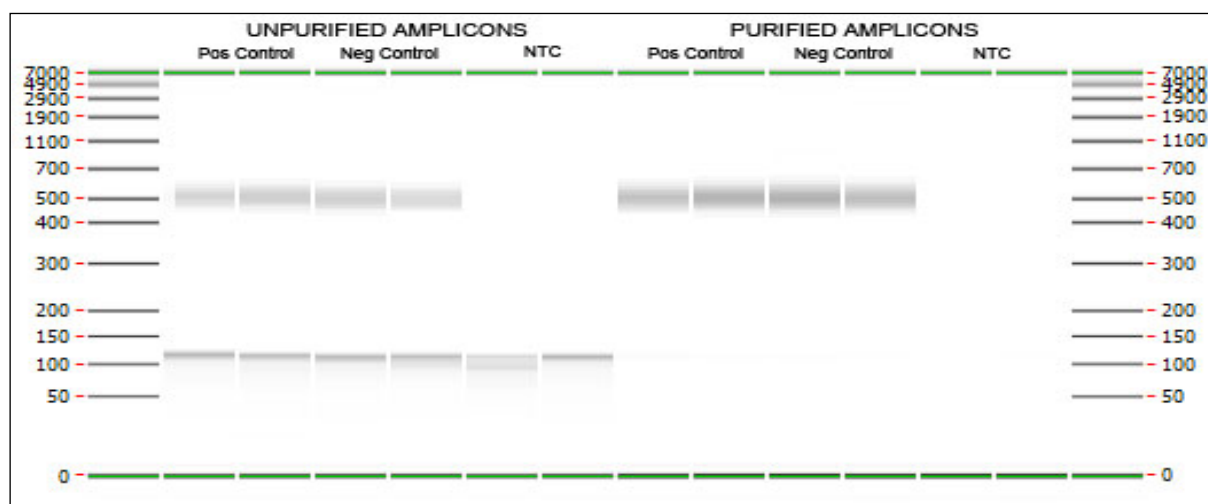


Figure 1 Example of a purification result for amplicons from the **LymphoTrack Dx IGH FR1 MiSeq Master Mixes**. The image was generated by running unpurified and purified products on the LabChip GX.

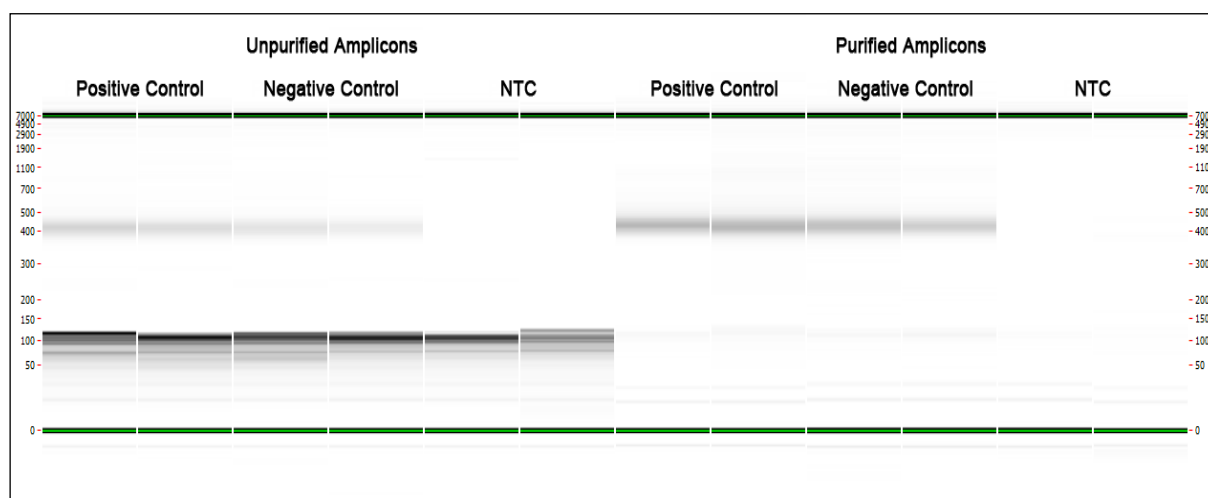


Figure 2 Example of a purification result for amplicons from the **LymphoTrack Dx IGH FR2 MiSeq Master Mixes**. The image was generated by running unpurified and purified products on the LabChip GX.

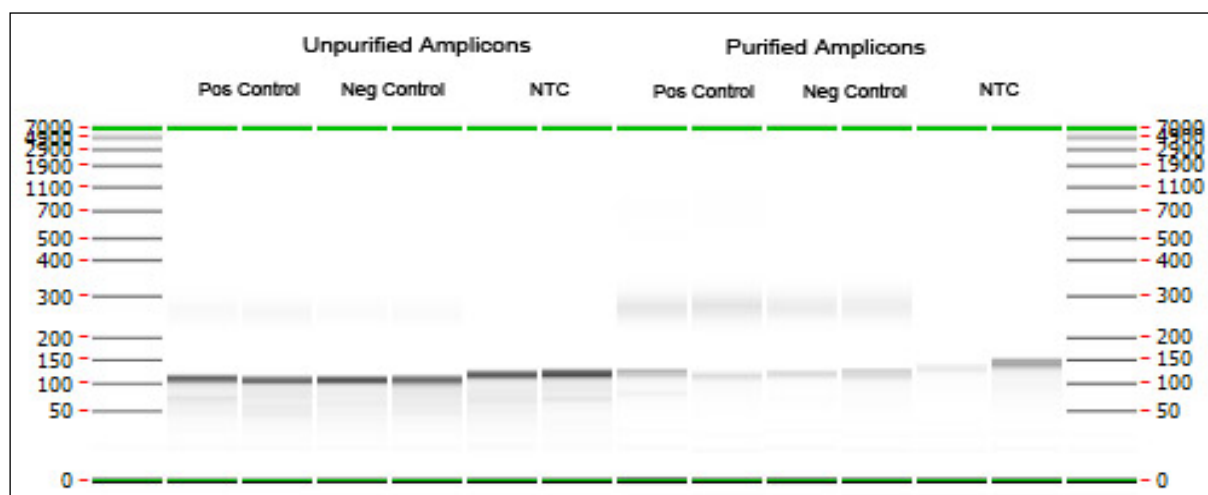


Figure 3: Example of a purification result for amplicons from the **LymphoTrack Dx IGH FR3 MiSeq Master Mixes**. The image was generated by running unpurified and purified products on the LabChip GX.

7.6. Quantification of Amplicons

The following steps were performed during assay validation to quantify purified PCR amplicons generated from samples, positive, negative and no template controls using the KAPA library quantification kit (KAPA Biosystems).

7.6.1. Dilution of amplicons

Dilution Buffer A below refers to: 10 mM Tris-HCl, pH 8.0 + 0.05% Tween 20

1: 4,000 Final:

Step A: 2 µL of purified amplicon eluate + 198 µL of Dilution Buffer A.
Mix well by pipetting up and down 10 times.

Step B: 5 µL from Step A + 195 µL of Dilution Buffer A.
Mix well by pipetting up and down 10 times.

7.6.2. Set up a qPCR run for amplicon quantification following Table 9 for each reaction (please refer to the KAPA library quantification kit instructions for more details):

Table 9. qPCR Setup

| Reagent | Volume |
|---|----------------|
| PCR-grade water | 3.6 µL |
| KAPA SYBR FAST qPCR Master Mix containing Primer Premix | 12.0 µL |
| ROX | 0.4 µL |
| Diluted amplicons or Standard (1-6) | 4.0 µL |
| Total Volume | 20.0 µL |

7.6.3. Follow Table 10 for the thermal program for qPCR.

Table 10. qPCR Program

| Step | Temperature | Time | Cycle |
|------|-------------|--------------------------------|-------|
| 1 | 95 °C | 5 minutes | 1 |
| 2 | 95 °C | 30 seconds | 35x |
| | 60 °C | 45 seconds (plate read) | |

7.6.4. Use the data from the qPCR run to check for contamination by calculating the ΔC_t values between the controls (positive and negative) and the NTC, using the following equation:

$$\Delta C_t = C_t (\text{NTC}) - C_t (\text{Control})$$

If the $\Delta C_t \geq 4.0$ for both controls, proceed to the next step. If the $\Delta C_t < 4.0$ for either control, see section 14 *Troubleshooting Guide* for further instructions.

If multiplexing multiple targets, please refer to Appendix A (section 20) for the qualifying ΔC_t value for other LymphoTrack Dx Assay - MiSeq PCR products.

7.6.5. Use the data from the qPCR run to determine the concentration of amplicon for each sample using the following equation:

$$\text{Concentration of undiluted amplicon (nM)} = \frac{452 \times \text{Avg Conc. (pM) Calculated by qPCR}}{A} \times 4$$

452 represents the average fragment length (bp) of the KAPA Illumina DNA Standard.

The average fragment length of amplicons generated using the **IGH FR1** Assay is 450 bp (**A = 450**).

The average fragment length of amplicons generated using the **IGH FR2** Assay is 390 bp (**A = 390**).

The average fragment length of amplicons generated using the **IGH FR3** Assay is 260 bp (**A = 260**).

Sequence length includes additional nucleotides that are necessary for sequencing.

Note: If multiplexing multiple targets, please refer to Appendix A (section 20) for the average fragment length of amplicons generated for other LymphoTrack Dx Assay - MiSeq

7.7. Pooling and Quantification of Library

The quantity of library DNA loaded onto the MiSeq flow cell is critical for generating optimal cluster density on the flow cell and for obtaining high-quality data in a sequencing run. **Quantification of the library by qPCR is strongly recommended.**

It is recommended that a separate pool is created for each LymphoTrack Dx Assay and corresponding target (*IGH* FR1, FR2, or FR3). After final quantification of a pooled library for each target according to section 7.9, LymphoTrack Dx Assays can be sequenced individually continuing with the instructions provided in section 7.10, or can be multiplexed together according to the instructions provided in Appendix A.

- 7.7.1. Based on the amplicon concentration calculated from the qPCR results, add an equal amount of amplicons (with the exception of NTC, which can be excluded).
 - e.g., combine 4 nM of each amplicon in a total volume of 10 µL using Dilution Buffer A as diluent.
 - For samples that have < 4 nM concentration, add the maximum amount of sample possible (10 µL) and do not add any Dilution Buffer A to that sample.
- 7.7.2. Gently vortex to mix followed by a brief centrifugation.

7.8. Dilution of the Pooled Library

1:1,000 Final:

Step A: 2 µL of pooled library + 198 µL of Dilution Buffer A.
Mix well by pipetting up and down 10 times.

Step B: 20 µL from Step A + 180 µL of Dilution Buffer A.
Mix well by pipetting up and down 10 times.

7.9. Setup qPCR for Library Quantification

Refer to Table 9 for qPCR setup and Table 10 for the thermal cycler program.

- 7.9.1. Determine the concentration of the pooled library from the qPCR results.

$$\text{Concentration of undiluted amplicon (nM)} = \frac{452 \times \text{Avg Conc. (pM) Calculated by qPCR}}{A}$$

452 represents the average fragment length (bp) of the KAPA Illumina DNA Standard.

The average fragment length of amplicons generated using the *IGH* FR1 Assay is 450 bp (**A = 450**).

The average fragment length of amplicons generated using the *IGH* FR2 Assay is 390 bp (**A = 390**).

The average fragment length of amplicons generated using the *IGH* FR3 Assay is 260 bp (**A = 260**).

Sequence length includes additional nucleotides that are necessary for sequencing.

Note: If multiplexing multiple targets, please refer to Appendix A (section 20) for the average fragment length of amplicons generated for other LymphoTrack Dx Assay - MiSeq

7.10. Preparation of the Library for the MiSeq Sequencing Run

At the end of this section, the concentration of the library DNA will be **12 pM for MiSeq reagent kit v2** and **20 pM for MiSeq reagent kit v3** when multiplexing. For multiplexing amplicons from different LymphoTrack Dx Assays for the MiSeq into a single library, please refer to Appendix A.

- 7.10.1. Determine the concentration of library to be prepared based on the concentration of the pooled library from the qPCR results and dilute if necessary:
 - If the library is more than 4 nM, dilute the library to 4 nM in a final volume of 10 µL using Dilution Buffer A.
 - If the library is less than 4 nM, use 10 µL of the library directly for the next step.
- 7.10.2. Use the following instructions to denature the library DNA.
 - Prepare a fresh solution of 0.2 N NaOH. A fresh solution is essential for completely denaturing sample DNA and for optimal cluster generation on the MiSeq.
 - Add 10 µL of 0.2 N NaOH to the diluted library (10 µL) prepared in the previous step.

Table 11. Library Denaturation

| Reagent | Volume |
|---------------------|-----------------------------|
| Diluted library | 10 μ L |
| 0.2 N NaOH | 10 μ L |
| Total Volume | 20 μL |

- 7.10.3. Vortex briefly to mix the solution, and then briefly centrifuge to ensure all the solution has settled to the bottom of the tube. Incubate for 5 minutes at room temperature to denature the dsDNA library into single-stranded DNA (ssDNA).
- 7.10.4. Add 980 μ L of pre-chilled HT1 buffer (provided in MiSeq Reagent kits) to the tube containing the denatured library DNA:

Table 12. HT1 Buffer Addition

| Reagent | Volume |
|---------------------|-------------------------------|
| Denatured library | 20 μ L |
| HT1 Buffer | 980 μ L |
| Total Volume | 1000 μL |

- 7.10.5. Vortex briefly to mix, then pulse centrifuge the diluted and denatured library DNA solution.
- 7.10.6. Place the diluted and denatured library on ice until the next step.
- 7.10.7. Remove the diluted ssDNA library from ice and use the following instructions to dilute the library further in preparation for loading onto the MiSeq

If multiplexing multiple targets, please refer to Appendix A (section 20) for loading concentration and MiSeq reagent kit.

For MiSeq Control Software (MCS v2.6 or later):

The concentration of the library DNA is 12 pM for MiSeq reagent kit v2.

The concentration of the library DNA is 20 pM for MiSeq reagent kit v3 when multiplexing.

- If the diluted ssDNA library concentration is 40 pM (the initial concentration was 4 nM) dilute to the desired MiSeq loading concentration using the following examples:

Table 13. Preparation of the Library for MiSeq Loading

| Final Concentrations | 12 pM | 20 pM |
|-------------------------------|-------------|-------------|
| Denatured library | 300 μ L | 500 μ L |
| HT1 Buffer | 700 μ L | 500 μ L |
| Final NaOH Concentration (mM) | 0.60 mM | 1.0 mM |

- If the diluted ssDNA library concentration is less than 40 pM (the initial concentration was less than 4 nM), dilute the denatured DNA appropriately to the desired MiSeq loading concentration (*e.g.* 12 pM). **Be sure that the final NaOH concentration is not more than 1.0 mM.**
- 7.10.8. Invert the final library 5 times to mix and pulse centrifuge.
- 7.10.9. Place the final prepared library on ice until it is loaded onto the MiSeq Reagent Cartridge.

7.11. MiSeq Flow Cell Loading

Load 600 μ L of the Final Prepared Library onto a MiSeq Reagent Cartridge.

7.12. MiSeq Sample Sheet Setup

Please refer to the latest Illumina documentation for Sample Sheet creation. Upload the sample sheet to the MiSeq instrument. If using Illumina-associated software (such as Local Run Manager [LRM]), select *TruSeq Nano DNA* for the Library Prep Kit and *TruSeq DNA Single Indexes Set A B* for the Index Kit.

Characters in the sample name:

- Create a unique name and identifier when naming each sample . If duplicate samples are run, a similar name can be used (*i.e.*, Sample1a and Sample1b).
- Failure to provide unique names to samples sequenced on the same flow cell will result in only one sample being analyzed by the LymphoTrack Dx Software – MiSeq during the analysis process.
- Use only alphanumeric and hyphen characters (A-Z, a-z, 0-9, -, ., _) when preparing the Sample Sheet.

Sample name when multiplexing:

Each index can only be listed in the Sample Sheet once; therefore, any necessary tracking information for samples sequenced with multiple targets using the same index must be included within one Sample ID field (which is incorporated into the FASTQ file name).

Keep track of all samples and targets in a MiSeq run that are sequenced using the same index. Give this set of samples/targets a unique identifier to include in the Sample ID field in the Sample Sheet. Please keep in mind that the Sample ID has a strict **20-character limit** when choosing a naming convention.



The *Sample Name* field in the *Sample Sheet* is incorporated by default into the FASTQ file name instead of the *Sample ID* when information is entered into this field. Please leave this field blank or copy the information that was entered for the *Sample ID*. If alternative information is entered into the *Sample Name* field, please make sure that it includes a unique identifier and conforms to the recommendations above for tracking samples.

Important!

Adapter sequences are not recognized by the LymphoTrack Dx Software – MiSeq.

- ☒ If using Illumina-associated software (such as Local Run Manager [LRM]), **adapter trimming must be selected when creating the sample sheet.**

Table 14. Indices used with LymphoTrack Dx Assay master mixes

| LymphoTrack Dx Assay – MiSeq PCR master mix index | Index Sequence | TruSeq DNA Single Indexes Set A B (LRM “Index Kit”) |
|--|-------------------|--|
| id01 | ATCACG | AR001 |
| id02 | CGATGT | AR002 |
| id03 | TTAGGC | AR003 |
| id04 | TGACCA | AR004 |
| id05 | ACAGTG | AR005 |
| id06 | GCCAAT | AR006 |
| id07 | CAGATC | AR007 |
| id08 | ACTTGA | AR008 |
| id09 | GATCAG | AR009 |
| id10 | TAGCTT | AR010 |
| id11 | GGCTAC | AR011 |
| id12 | CTTGTA | AR012 |
| id13 | AGTCAA | AR013 |
| id14 | AGTTCC | AR014 |
| id15 | ATGTCA | AR015 |
| id16 | CCGTCC | AR016 |
| id18 | GTCCGC | AR018 |
| id19 | GTGAAA | AR019 |
| id20 | GTGGCC | AR020 |
| id21 | GTTTCG | AR021 |
| id22 | CGTACG | AR022 |
| id23 | GAGTGG | AR023 |
| id25 | ACTGAT | AR025 |
| id27 | ATTCCT | AR027 |

7.13. MiSeq Run Start

Start the MiSeq run following the MiSeq Control Software Instructions on the MiSeq. The approximate MiSeq run times are indicated in the following table.

Table 15. MiSeq Run Times

| MiSeq Reagent Kit | Read Length | MCS Version | Total MiSeq Run Time |
|-------------------|---------------------|---------------|----------------------|
| v2 | 2x151 bp (FR3 only) | v2.6 or later | ~ 24 hours |
| | 2x251 bp | v2.6 or later | ~ 39 hours |
| v3 | 2x301 bp | v2.6 or later | ~ 56 hours |

Note: Using a kit with fewer cycles will not be sufficient to generate read lengths required for this assay.

8. Data Analysis

The LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – MiSeq were designed to produce sequencing data that can be analyzed using the LymphoTrack Dx Software – MiSeq package provided on the associated CD (REF 95000009), which is included with your order. This CD includes detailed instructions for installation and instruction of the software package.

9. Expected Values

The calculations generated by the software are rounded to the nearest tenth to determine assay result.

- *IGH* Positive Control top % reads $\geq 2.5\%$
- NGS Negative Control top % reads $< 1.0\%$
- *IGH* SHM Positive Control (REF 40880008, can be purchased separately) top % reads $\geq 2.5\%$
- MiSeq Run Validity
 - %Q30 $> 80\%$ for v2 (2x151)
 - %Q30 $> 75\%$ for v2 (2x251)
 - %Q30 $> 70\%$ for v3 (2x301)

***Note:** Q30 from all analytical validations has met the above criteria from Illumina MiSeq Q30 specification. However, the Q30 score may vary depending on sample quality. If Q30 falls below Illumina Q30 specification, check the index Q30 value from the LymphoTrack Dx Report after data analysis by the LymphoTrack Dx Software - MiSeq. If an index Q30 score on the LymphoTrack Dx Report does not meet the Illumina specification, consider that index to be invalid.

MiSeq v2 chemistry has been validated for this single-target assay. MiSeq v3 chemistry has been validated for assay multiplexing.

10. Limitations of Procedure

- These assays do not identify 100% of clonal cell populations.
- A higher level of variance at or near the analytical limit of detection (LOD) is inherent to most technologies; including, but not limited to next generation sequencing. Follow-up testing is suggested when a result presents near the assay analytical LOD.
- PCR-based assays are subject to interference by degradation of DNA or inhibition of PCR amplification due to heparin or other agents that might be present in the analyzed sample.
- Always interpret the results of molecular clonality tests in the context of clinical, histological and immunophenotypic data.
- The LymphoTrack Dx *IGH* FR1 Assay – MiSeq only targets a portion of the *IGHV* region, as the sequence upstream of the primer binding site will not be assessed. The LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay for the Illumina MiSeq (REF 91210059 or REF 91210069) can be purchased separately if complete *IGHV* sequencing is required.

11. Interpretation and Reporting

The *Merged Read Summary* report can be used to identify the top merged read sequences and their frequencies prior to clonality determination using the criteria listed below. Refer to the *Data Analysis* section for more information on the *Merged Read Summary* report. There are some clonal processes that may result in the detection of two or more clones. Examples of this include a dominant population with a small sub-clonal population or when multiple lymphoproliferative disorders are present. It is especially important that these cases are interpreted within their clinical context.

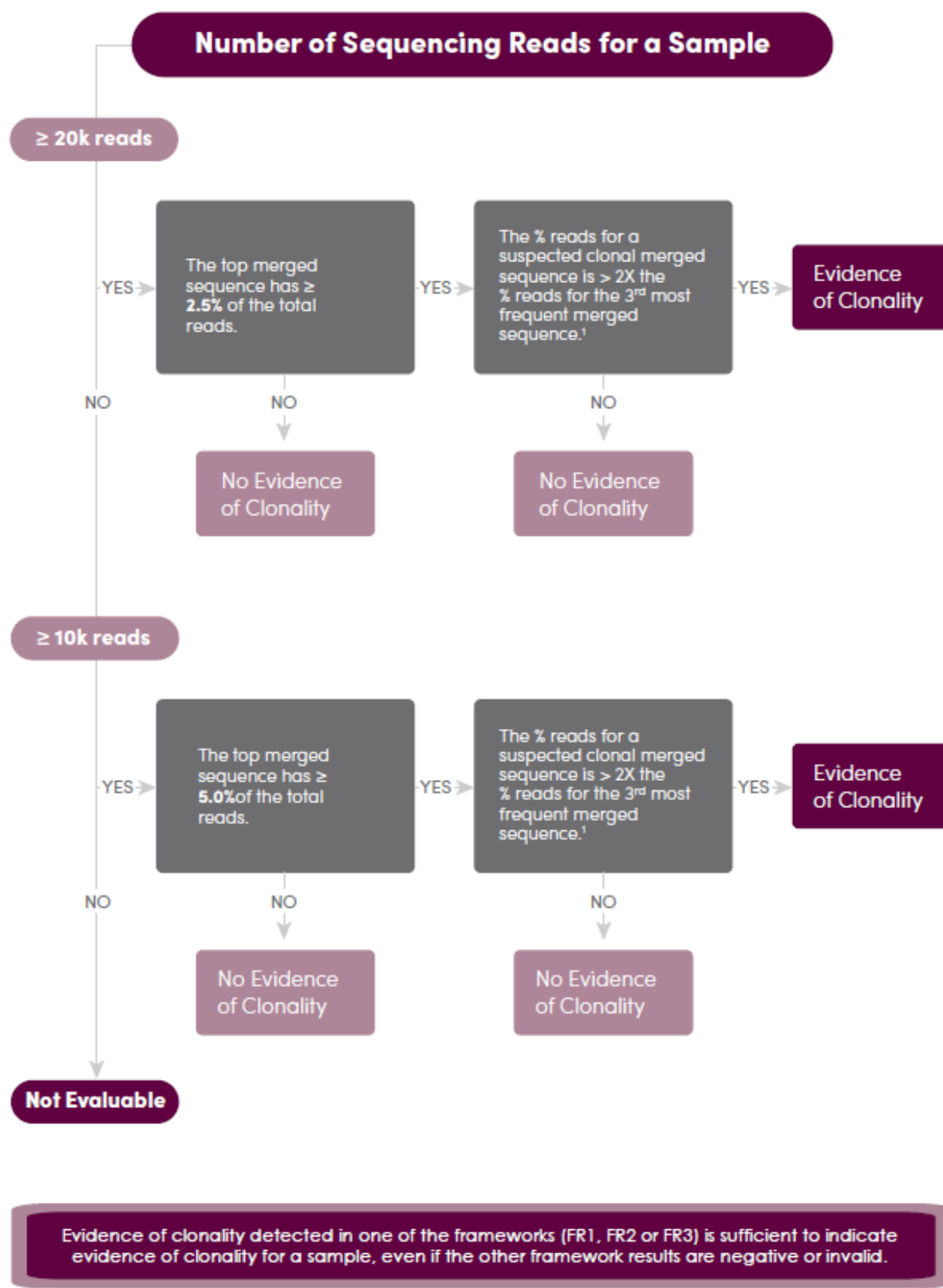
* Be cautious when making an interpretation if “none” is present for V, D and/or J gene in suspected clonal reads. “None” is assigned when the alignment does not meet the minimum quality threshold due to poor alignment.

Evaluate the data from all three framework regions to determine evidence of clonality for a sample. Evidence of clonality detected in one of the frameworks (FR1, FR2, or FR3) is sufficient to indicate evidence of clonality for a sample, even if the other framework results are negative or invalid.

Table 16. Interpretation Criteria

| Criterion 1 | Criterion 2 | Criterion 3 | Result |
|--|---|--|-----------------------------------|
| The total number of reads for each sample is ≥ 20,000 . | The top merged sequence has ≥ 2.5% of the total reads. | The % reads for a suspected clonal merged sequence is > 2X the % reads for the 3rd most frequent merged sequence. ¹ | EVIDENCE OF CLONALITY DETECTED |
| | | The % reads for a suspected clonal merged sequence is ≤ 2X the % reads for the 3rd most frequent merged sequence. ¹ | No evidence of clonality detected |
| The total number of reads for each sample is ≥ 10,000 and < 20,000 . | The top merged sequence has ≥ 5.0% of the total reads. | The % reads for a suspected clonal merged sequence is > 2X the % reads for the 3rd most frequent merged sequence. ¹ | EVIDENCE OF CLONALITY DETECTED |
| | | The % reads for a suspected clonal merged sequence is ≤ 2X the % reads for the 3rd most frequent merged sequence. ¹ | No evidence of clonality detected |
| The total number of reads for each sample is < 10,000 . | N/A | N/A | Not evaluable |

¹Software calculations are rounded to the nearest tenth for comparison.



¹ The calculations generated by the software are rounded to the nearest tenth for comparison.

Figure 4: Interpretation of data based on criteria from Table 16.

After clonality is determined, samples can be evaluated using the LymphoTrack Dx *IGH* FR1 Assay – MiSeq for evidence of somatic hypermutation (SHM) if needed. The SHM interpretation criteria listed below are suggestions for using immunoglobulin gene sequence analysis for CLL prognostication based on current literature.⁶

For SHM interpretation, the top two merged sequences can both be evaluated for evidence of clonality using the clonality interpretation provided in the previous section. Each merged sequence showing evidence of clonality can be evaluated using the SHM criteria listed below (see Figure 5 for corresponding flow chart). If the SHM mutational status is at or near the 2% cutoff, run the sample with the LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay – MiSeq which targets the complete *IGHV* gene sequence.

Table 17. Suggested SHM Interpretation Criteria

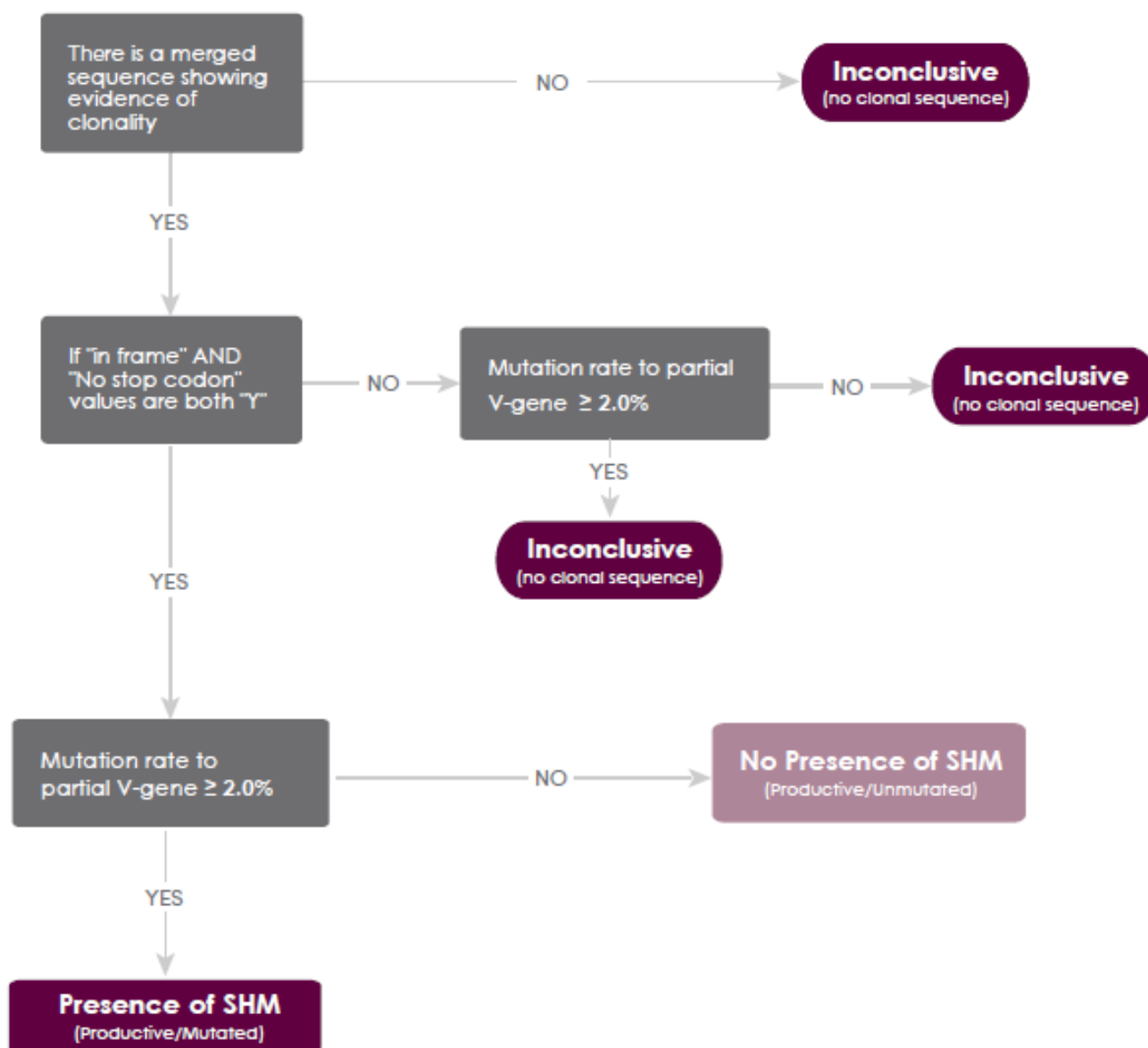
| Criterion 1 | Criterion 2 | Criterion 3 | Result |
|--|--|--|---|
| There is a merged sequence showing evidence of clonality. | If "In-frame" AND "No stop codon" values are both "Y" | Mutation rate to partial V-gene $\geq 2.0\%$ | PRESENCE OF SHM (Productive/Mutated) |
| | | Mutation rate to partial V-gene $< 2.0\%$ | No presence of SHM (Productive/Unmutated) |
| | If either (or both) "In-frame" OR "No stop codon" value is "N" | Mutation rate to partial V-gene $\geq 2.0\%$ | Inconclusive (Unproductive/Mutated) |
| | | Mutation rate to partial V-gene $< 2.0\%$ | Inconclusive (Unproductive/Unmutated) |
| There are no merged sequences showing evidence of clonality. | N/A | N/A | Inconclusive (no clonal sequence) |

If there are two merged sequences for a sample that both show evidence of clonality, evaluate each using the table below to determine the final SHM result for that sample.

Table 18. Suggested Double Rearrangement SHM Interpretation Criteria

| Criterion 1 | Clonal Sequence A | Clonal Sequence B | Result |
|-----------------------|---|---|---------------------------|
| Double Rearrangements | PRESENCE OF SHM (Productive/Mutated) | PRESENCE OF SHM (Productive/Mutated) | PRESENCE OF SHM |
| | PRESENCE OF SHM (Productive/Mutated) | No presence of SHM (Productive/Unmutated) | Inconclusive |
| | PRESENCE OF SHM (Productive/Mutated) | Inconclusive (Unproductive/Mutated) | PRESENCE OF SHM |
| | PRESENCE OF SHM (Productive/Mutated) | Inconclusive (Unproductive/Unmutated) | PRESENCE OF SHM |
| | No presence of SHM (Productive/Unmutated) | No presence of SHM (Productive/Unmutated) | No presence of SHM |
| | No presence of SHM (Productive/Unmutated) | Inconclusive (Unproductive/Mutated) | Inconclusive |
| | No presence of SHM (Productive/Unmutated) | Inconclusive (Unproductive/Unmutated) | No presence of SHM |
| | Any Inconclusive | Any Inconclusive | Inconclusive |

Suggested SHM Interpretation Criteria



Note: If there are two merged sequences for a sample that both show evidence of clonality, evaluate each using Table 17 to determine the final SHM result for that sample.

Figure 5: Suggested Somatic Hypermutation (SHM) Interpretation of data based on criteria from Table 18.

12. Sample Data

LymphoTrack Dx Report for assay IGH_FR1

Sample name: FR1_positive_S23_L001_001_combined

Total Read Count: 460347

IndexQ30: 92.15

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

| Rank | Sequence | Length | Merge count | V-gene | J-gene | % total reads | Cumulative % | Mutation rate to partial V-gene (%) | In-frame (Y/N) | No Stop codon (Y/N) | V-coverage | CDR3 Seq |
|------|----------------|--------|-------------|-------------|----------|---------------|--------------|-------------------------------------|----------------|---------------------|------------|---------------|
| 1 | CATCTGGATACAC | 295 | 31266 | IGHV1-46_03 | IGHJ4_02 | 6.79 | 6.79 | 0.00 | Y | Y | 100.00 | not found |
| 2 | GTCTCTGGATTCA | 216 | 138 | IGHV3-48_03 | IGHJ4_02 | 0.03 | 6.82 | 0.00 | n/a | N | 35.24 | GCGACAGACCGG |
| 3 | GCCTCTGGATTCA | 272 | 136 | IGHV3-74_01 | IGHJ4_02 | 0.03 | 6.85 | 1.32 | Y | Y | 99.56 | not found |
| 4 | GCCCTCTGGATTCA | 231 | 130 | IGHV3-23_04 | IGHJ5_02 | 0.03 | 6.88 | 1.32 | n/a | N | 40.97 | ATCTGCAAATGAA |
| 5 | GCCTCTGGATTCA | 272 | 130 | IGHV3-48_01 | IGHJ4_02 | 0.03 | 6.91 | 0.44 | Y | Y | 98.68 | not found |
| 6 | GCGTCTGGATTCA | 266 | 128 | IGHV3-33_01 | none | 0.03 | 6.94 | 0.00 | n/a | N | 99.12 | not found |
| 7 | GCGTCTGGATTCA | 272 | 128 | IGHV3-33_01 | IGHJ4_02 | 0.03 | 6.96 | 0.00 | Y | Y | 100.00 | not found |
| 8 | GCCTCTGGATTCA | 293 | 124 | IGHV3-21_02 | IGHJ6_02 | 0.03 | 6.99 | 0.00 | Y | Y | 98.24 | not found |
| 9 | GCCTCTGGATTCA | 272 | 124 | IGHV3-74_03 | IGHJ4_02 | 0.03 | 7.02 | 3.08 | Y | Y | 98.68 | not found |
| 10 | GCCTCTGGATTCA | 281 | 124 | IGHV3-23_04 | IGHJ6_02 | 0.03 | 7.04 | 0.00 | Y | Y | 99.56 | GCGAAAGGCCAA |

Figure 6: This table, generated via the LymphoTrack Dx Software - MiSeq, shows the top 10 reads from the read summary merged with the top 500 reads; a read will merge with another if they are only 1 or 2 base pair (bp) different. Sequences were generated using the **LymphoTrack Dx IGH FR1 Assay – MiSeq** and analyzed using the LymphoTrack Dx Software – MiSeq (REF 95000009).

LymphoTrack Dx Report for assay IGH_FR2

Sample name: FR2_positive_S22_L001_001_combined

Total Read Count: 94189

IndexQ30: 94.08

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

| Rank | Sequence | Length | Merge count | V-gene | J-gene | % total reads | Cumulative % | Mutation rate to partial V-gene (%) | In-frame (Y/N) | No Stop codon (Y/N) | V-coverage | CDR3 Seq |
|------|----------------|--------|-------------|-------------|----------|---------------|--------------|-------------------------------------|----------------|---------------------|------------|---------------|
| 1 | CGGGAAAGGCCTC | 216 | 65 | IGHV5-51_03 | IGHJ4_02 | 0.07 | 0.07 | N/A | Y | Y | 100.00 | GCGAGGTCTCGG |
| 2 | CGGGAAAGGCCTC | 225 | 65 | IGHV5-a_03 | IGHJ4_02 | 0.07 | 0.14 | N/A | Y | Y | 100.00 | GCGAGACAGGGT |
| 3 | CGGGAAAGGCCTC | 225 | 64 | IGHV5-51_01 | IGHJ4_02 | 0.07 | 0.21 | N/A | Y | Y | 100.00 | GCGAGACGGGGA |
| 4 | CGGGAAAGGCCTC | 234 | 59 | IGHV5-a_03 | IGHJ6_02 | 0.06 | 0.27 | N/A | Y | Y | 100.00 | GCGACCCGGTTTC |
| 5 | CGGGAAAGGCCTC | 231 | 58 | IGHV5-51_01 | IGHJ5_02 | 0.06 | 0.33 | N/A | Y | Y | 100.00 | GCGAGACGCTCA |
| 6 | CGGGAAAGGCCTC | 222 | 57 | IGHV5-a_03 | IGHJ5_02 | 0.06 | 0.39 | N/A | Y | Y | 100.00 | GCGAGAGGGGCT |
| 7 | CGGGAAAGGCCTC | 222 | 56 | IGHV5-a_03 | IGHJ5_02 | 0.06 | 0.45 | N/A | Y | Y | 100.00 | GCGAGAACGGTA |
| 8 | CGGGAAAGGCCTC | 228 | 55 | IGHV5-51_01 | IGHJ2_01 | 0.06 | 0.51 | N/A | Y | Y | 100.00 | GCGAGACGGAAT |
| 9 | CGGGAAAGGCCTC | 240 | 54 | IGHV5-51_01 | IGHJ5_02 | 0.06 | 0.57 | N/A | Y | Y | 100.00 | GCGAGACTAGGG |
| 10 | AGGGAAAGGGACTC | 198 | 54 | IGHV4-61_08 | IGHJ6_01 | 0.06 | 0.62 | N/A | Y | Y | 97.08 | not found |

Figure 7: This table, generated via the LymphoTrack Dx Software – MiSeq, shows the top 10 reads from the read summary merged with the top 500 reads; a read will merge with another if they are only 1 or 2 base pair (bp) different. Sequences were generated using the **LymphoTrack Dx IGH FR2 Assay – MiSeq** and analyzed using the LymphoTrack Dx Software – MiSeq (REF 95000009).

LymphoTrack Dx Report for assay IGH_FR3

Sample name: FR3_positive_S23_L001_001_combined

Total Read Count: 467001

IndexQ30: 95.62

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

| Rank | Sequence | Length | Merge count | V-gene | J-gene | % total reads | Cumulative % | Mutation rate to partial V-gene (%) | In-frame (Y/N) | No Stop codon (Y/N) | V-coverage | CDR3 Seq |
|------|---------------|--------|-------------|-------------|----------|---------------|--------------|-------------------------------------|----------------|---------------------|------------|---------------|
| 1 | TCTGAGGACACGC | 104 | 60764 | IGHV1-46_03 | IGHJ4_02 | 13.01 | 13.01 | N/A | Y | Y | 100.00 | GCTAGAGATCTCA |
| 2 | ACACGGCCGTGT | 97 | 1632 | IGHV3-72_01 | IGHJ4_02 | 0.35 | 13.36 | N/A | Y | Y | 71.79 | GCTAGAGATCTCA |
| 3 | ACACGGCCGTTTA | 76 | 462 | IGHV4-59_10 | IGHJ3_02 | 0.10 | 13.46 | N/A | Y | Y | 100.00 | GCGAGATCGTTCT |
| 4 | GTGGACACAGCC | 95 | 343 | IGHV2-70_12 | IGHJ3_02 | 0.07 | 13.53 | N/A | Y | Y | 100.00 | GCACACAGACTAC |
| 5 | GTGGACACAGCC | 77 | 338 | IGHV2-26_01 | IGHJ3_02 | 0.07 | 13.61 | N/A | Y | Y | 100.00 | GCACGATTTCCT |
| 6 | ACACGGCTGTGTA | 82 | 256 | IGHV4-39_02 | IGHJ4_02 | 0.05 | 13.66 | N/A | Y | Y | 96.55 | GCGAGAGAACTAA |
| 7 | GTGGACACAGCC | 80 | 255 | IGHV2-70_11 | IGHJ4_02 | 0.05 | 13.72 | N/A | Y | Y | 100.00 | GCACGGATGACCC |
| 8 | GTGGACACAGCC | 77 | 252 | IGHV2-70_12 | IGHJ4_02 | 0.05 | 13.77 | N/A | Y | Y | 100.00 | GCACACAGACTGC |
| 9 | GTGGACACAGCC | 80 | 246 | IGHV2-70_12 | IGHJ4_02 | 0.05 | 13.82 | N/A | Y | Y | 100.00 | GCACACAGACGG |
| 10 | GTGGACACAGCC | 86 | 237 | IGHV2-26_01 | IGHJ3_02 | 0.05 | 13.87 | N/A | Y | Y | 100.00 | GCACGGATGGTCC |

Figure 8: This table, generated via the LymphoTrack Dx Software – MiSeq, shows the top 10 reads from the read summary merged with the top 500 reads; a read will merge with another if they are only 1 or 2 base pair (bp) different. Sequences were generated using the LymphoTrack Dx IGH FR3 Assay – MiSeq and analyzed using the LymphoTrack Dx Software – MiSeq (REF 95000009).

13. Performance Characteristics

The LymphoTrack Dx IGH FR1 Assay - MiSeq and traditional capillary electrophoresis assay (IdentiClone® IGH Gene Clonality Assay (Tubes A+B+C) – ABI detection, REF 91010061) were compared and the concordance (or overall percent agreement), positive percent agreement (PPA) and negative percent agreement (NPA) were: 92% (60/65 cases), 93% and 89%, respectively.

Table 19. Comparison between LymphoTrack Dx IGH Assay – MiSeq and IdentiClone IGH Gene Clonality Assay – ABI

| | | IdentiClone IGH Gene Clonality Assay - ABI | |
|--------------------------------------|------------|--|------------|
| | | Clonal | Non-Clonal |
| LymphoTrack Dx IGH FR1 Assay - MiSeq | Clonal | 52 | 1 |
| | Non-Clonal | 4 | 8 |

The analytical performance of the LymphoTrack Dx IGH FR1 Assay – MiSeq was evaluated by testing spiked clonal cell line DNA into tonsil DNA at different dilutions. The Limit of Detection (LoD) was observed at 5% DNA dilution. The highest % reads from tonsil DNA was < 0.1%. The linear regression R^2 was > 0.97 for a range of 0 to 10% DNA dilution. The coefficient of variation (CV%) across 8 runs from 2 operators, 2 reagents lots and 2 instruments was less than 20% when testing 5% and 10% DNA dilutions.

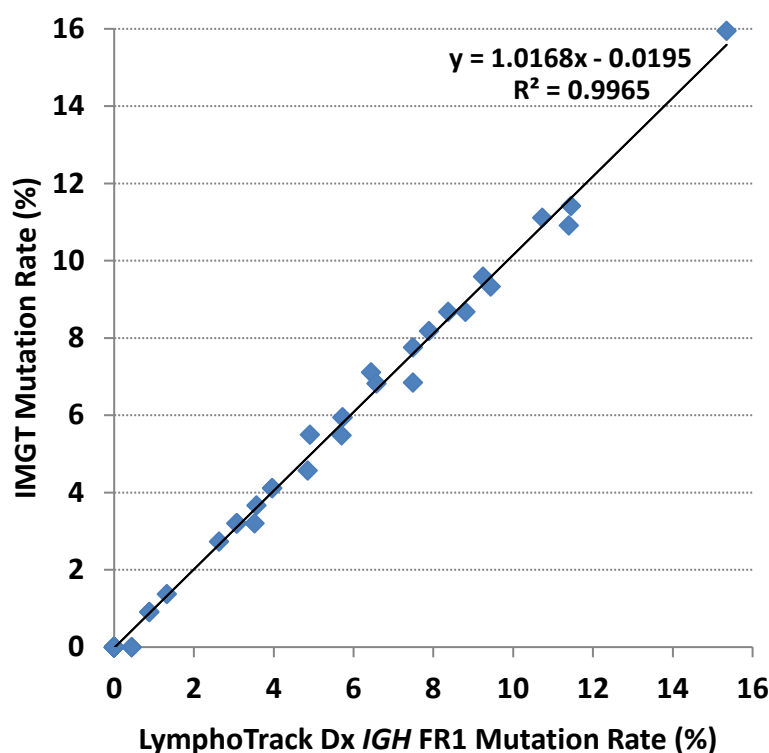


Figure 9: Comparison of the somatic hypermutation (SHM) rate for 51 CLL samples determined by LymphoTrack Dx *IGH* FR1 Assay – MiSeq and analyzed either with the LymphoTrack Dx Software – MiSeq or using IMGT analysis.

Table 20. Somatic Hypermutation (SHM) Testing Method Comparison between LymphoTrack Dx Software - MiSeq and the Control Method (IMGT).

| | | IMGT | |
|---------------------------------|-----------|---------|-----------|
| | | Mutated | Unmutated |
| LymphoTrack Dx Software - MiSeq | Mutated | 26 | 0 |
| | Unmutated | 0 | 25 |

Running the LymphoTrack Dx *IGH* FR1/2/3 Assay – MiSeq improved identification of clonal events compared to running the LymphoTrack Dx *IGH* FR1 Assay – MiSeq alone. The LymphoTrack Dx *IGH* FR1/2/3 Assay - MiSeq and traditional capillary electrophoresis assay (IdentiClone® *IGH* Gene Clonality Assay (Tubes A+B+C) – ABI Fluorescence Detection, [REF](#) 91010061) were compared and the concordance (or overall percent agreement), positive percent agreement (PPA) and negative percent agreement (NPA) were: 95% (42/44 cases), 96% and 95%, respectively.

Table 21. Comparison between LymphoTrack Dx *IGH* FR1/FR2/FR3 Assay – MiSeq and IdentiClone *IGH* Gene Clonality Assay – ABI Fluorescence Detection (control method)

| | | IdentiClone <i>IGH</i> Gene Clonality Assay – ABI Fluorescence Detection | |
|---|------------|--|------------|
| | | Clonal | Non-Clonal |
| LymphoTrack Dx <i>IGH</i> FR1/2/3 Assay - MiSeq | Clonal | 24 | 1 |
| | Non-Clonal | 1 | 18 |

14. Troubleshooting Guide

Table 22. Troubleshooting Guide

| Occurs During | Error | Action |
|---|--|--|
| Sample and reagent preparation | Sample DNA quantity is less than 50 ng by a dsDNA-based method | Do not test the sample |
| Sample and reagent preparation | Sample DNA integrity is low | Test the sample using the Specimen Control Size Ladder available from Invivoscribe (REF 20960021 for ABI detection or REF 20960020 for gel detection) |
| Quantification of amplicons using the KAPA library quantification kit | $\Delta Ct < 4.0$ $\Delta Ct = Ct (NTC) - Ct (Control)$ | Check the standard curve in qPCR. Check for contamination and repeat the KAPA qPCR for all samples and controls. If $\Delta Ct < 4.0$ again, redo the PCR and qPCR for all samples and controls. |
| Library creation by amplicon quantification and pooling | Amplicon concentration is less than 1 nM | Check the standard curve in qPCR and repeat PCR if less than 1 nM |
| MiSeq run set up | Sample sheet not found | Refer to Illumina troubleshooting Or call Illumina Tech Support +1-800-809-4566 |
| | Improperly formatted sample sheet | |
| | Fluidics check failed | |
| | Disk space low | |
| | Empty waste bottle | |
| | Network disconnected | |
| | RFID Failure | |
| MiSeq run | %Q30 < 80% for v2 (2x151)* %Q30 < 75% for v2 (2x251)* %Q30 < 70% for v3 (2x301)* | Call Invivoscribe Tech Support +1-858-224-6600 |
| CD installation | LymphoTrack Dx Software does not install properly | Call Invivoscribe Tech Support +1-858-224-6600 |
| Data analysis | LymphoTrack Dx Software stops running | Call Invivoscribe Tech Support +1-858-224-6600 |
| Data analysis | No clonal sequence is detected for the Positive Control | Call Invivoscribe Tech Support +1-858-224-6600 |
| No Template Control (NTC) | NTC shows amplification after PCR | Repeat the assay |

* Q30 from all analytical validations has met the above criteria from Illumina MiSeq Q30 specification. However, the Q30 score may vary depending on sample quality. If Q30 falls below Illumina Q30 specification, check the index Q30 value from the LymphoTrack Dx Report after data analysis by the LymphoTrack Dx Software - MiSeq. If an index Q30 score on the LymphoTrack Dx Report also does not meet the Illumina specification, consider that index to be invalid.

15. Technical and Customer Service

Thank you for purchasing our LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays - MiSeq. We appreciate your business. We are happy to assist you with understanding these assays and will provide ongoing technical assistance Monday through Friday to keep the assays performing efficiently in your laboratory.

Contact Information



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Technical Service: support@invivoscribe.com | Customer Service: sales@invivoscribe.com | Website: www.invivoscribe.com

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 6. Langerak, AW. et al., (2011) Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. *Leukemia* 25, 979–984.
- Invivoscribe LymphoTrack Dx Software - MiSeq Package Instructions for Use (**REF** 95000009)
 - <https://www.beckmancoulter.com>
 - <http://www.illumina.com>
 - <http://www.invitrogen.com>
 - <http://www.kapabiosystems.com>
 - <http://www.thermofisher.com>

17. Symbols

The following symbols are used in labeling for Invivoscribe NGS diagnostic products.

| | | | |
|---------------|---------------------------------|---------------|---|
| REF | Catalog Number | | Expiration Date |
| VOL | Reagent Volume | EC REP | Authorized Representative in the European Community |
| LOT | Lot Number | | Consult Instructions for Use |
| | Storage Conditions | IVD | For In Vitro Diagnostic Use |
| UDI | Unique Device Identifier | | Manufacturer |
| UK CA | UK Conformity Assessed | UKRP | UK Responsible Person |
| CH REP | Swiss Authorized Representative | | European Conformity |
| | Software Application | | |

18. Legal Notice

This product is covered by one or more of the following patents and patent applications owned by or exclusively licensed to Invivoscribe, Inc. (IVS). United States Patent Number 7,785,783, United States Patent Number 8,859,748 (together with divisional application claims relating to the same original application), European Patent Number EP 1549764B1 (validated in 16 countries and augmented by related European Patents Numbered EP2418287A3 and EP 2460889A3), Japanese Patent Number JP04708029B2, Japanese Patent Application Number 2006-529437, Brazil Patent Application Number PI0410283.5, Canadian Patent Number CA2525122, Indian Patent Number IN243620, Mexican Patent Number MX286493, Chinese Patent Number CN1806051 and Korean Patent Number 101215194.

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19. LymphoTrack Dx *IGH* FR1/FR2/FR3 Assay - MiSeq: Single Page Guide

- 19.1. Using gloved hands, remove the Master Mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
- 19.2. In a containment hood or dead air box, pipette 45 μ L of Master Mix into individual wells of a PCR plate. One well for each Master Mix and one Master Mix per sample, positive, negative or no template controls.
- 19.3. Add 0.2 μ L Taq DNA polymerase (@5 U/ μ L) to each Master Mix.
- 19.4. Add 5 μ L of sample DNA (at a minimum concentration of 10 ng/ μ L) and 5 μ L of control samples to wells containing the respective Master Mix reactions and pipette up and down 5-10 times to mix.
- 19.5. Add 5 μ L of molecular biology grade water to the well containing the respective Master Mix for no template control and pipette up and down 5-10 times to mix.
- 19.6. Amplify target DNA using the following thermal cycler program:

| Step | Temperature | Time | Cycle |
|------|-------------|------------|-------|
| 1 | 95°C | 7 minutes | 1 |
| 2 | 95°C | 45 seconds | 29x |
| 3 | 60°C | 45 seconds | |
| 4 | 72°C | 90 seconds | |
| 5 | 72°C | 10 minutes | 1 |
| 6 | 15°C | ∞ | 1 |

- 19.7. Remove the amplification plate from the thermal cycler.
- 19.8. Purify the PCR products using the Agencourt AMPure XP PCR Purification system. Add 50 μ L of particles to each 50 μ L reaction; elute DNA in 25 μ L eluate.
- 19.9. Quantify amplicons using the KAPA library quantification kit according to the kit instructions. Dilute amplicons 1:4,000 before proceeding to qPCR.
- 19.10. Pool equal amounts of amplicons from samples (do not include the no template control), dilute 1:1,000 and quantify the library using the KAPA library quantification kit.
- 19.11. Denature and dilute the library to 12 pM for MiSeq reagent kit v2 and 20 pM for MiSeq reagent kit v3 when multiplexing (MCS v2.6 or later).
- 19.12. Load 600 μ L of denatured and diluted library to the MiSeq Reagent Cartridge.
- 19.13. Set up a MiSeq sample sheet, and upload the sample sheet to the instrument (if necessary).
- 19.14. Start the MiSeq run.
- 19.15. Analyze and visualize the acquired data using the associated LymphoTrack Dx Software – MiSeq package.

20. Appendix A: Building a Sequencing Library with Multiple NGS Targets

When running multiple targets using different LymphoTrack Dx Assay - MiSeq in parallel, it is important to note the procedural differences between each assay. For example, the *IGHV* Leader assay utilizes 32 PCR cycles and should be placed on a separate thermal cycling run than other LymphoTrack Dx Assays which utilize only 29 PCR cycles. Table 23 below summarizes these procedural differences. For full instructions, please see the Instructions For Use for the respective LymphoTrack Dx Assay – MiSeq.

Table 23. Cycle Settings and Reagent Kits for a Single-Target MiSeq Run

| Procedure Step | Description | LymphoTrack Dx Assay - MiSeq | | | | | | |
|-----------------|--|------------------------------|-------------------|-------------------|----------------------|-------------------|----------------------|---------------------|
| | | <i>IGHV</i> Leader | <i>IGH</i> FR1 | <i>IGH</i> FR2 | <i>IGH</i> FR3 | <i>IGK</i> | <i>TRG</i> | <i>TRB</i> |
| 7.4.1 | Number of PCR Cycles | 32 | 29 | 29 | 29 | 29 | 29 | 29 |
| 7.5.1 | Volume of AMPure XP Reagent (Ratio) | 50 µL (1:1 ratio) | 50 µL (1:1 ratio) | 50 µL (1:1 ratio) | 50 µL (1:1 ratio) | 50 µL (1:1 ratio) | 50 µL (1:1 ratio) | 35 µL (0.7:1 ratio) |
| 7.6.4 | Contamination Check ΔCt Value = Ct (NTC) – Ct (Control) | ΔCt ≥ 4.0 | ΔCt ≥ 4.0 | ΔCt ≥ 4.0 | ΔCt ≥ 4.0 | ΔCt ≥ 4.0 | ΔCt ≥ 4.0 | ΔCt ≥ 3.0 |
| 7.6.5 and 7.9.1 | A (Average Fragment Length) | 660 bp | 450 bp | 390 bp | 260 bp | 410 bp | 300 bp | 400 bp |
| 7.10.7 | Loading Concentration | 12 – 20 pM | 12 pM | 12 pM | 12 pM | 8 pM | 12 pM | 12 pM |
| | MiSeq Reagent Kit for Single-Target Sequencing* | v3 (600) | v2 (500) | v2 (500) | v2 (300) v2 (500) | v2 (500) | v2 (300) v2 (500) | v2 (500) |
| 7.12 | Sample Sheet Settings for: Cycles Read 1* Cycles Read 2* | 301 | 251 | 251 | 151 | 251 | 151 | 251 |

***Note:** MiSeq v2 chemistry has been validated for these single-target assays. MiSeq v3 chemistry has been validated for *IGHV* Leader and assay multiplexing.

Two or more sequencing libraries generated from the same LymphoTrack gene target master mixes (e.g., two *TRG* sequencing libraries, either from the same or different kit lots) can also be multiplexed together into a single sequencing library as long as each index for that master mix is only included once per sequencing run. Please refer to the following table to determine the cycle settings and Illumina MiSeq Reagent kits to be used with different combinations of targets. It is recommended to use MiSeq reagent kit v3 when sequencing all seven targets together in order to achieve enough reads per sample.

Table 24. Cycle Settings and Reagent Kits for a Multi-Targeted MiSeq Run

| Multiplexing Targets | Sample Sheet Settings | MiSeq Reagent Kit | Loading Concentration | Catalog # |
|---|--|---|--------------------------------|----------------------------------|
| Only <i>IGH</i> FR3 and <i>TRG</i> together | 151 cycles Read 1 151 cycles Read 2 | v2 kit (300 cycle) or v2 kit (500 cycle) | 12 pM (v2) or 20 pM (v3) | MS-102-2002 or MS-102-2003 |
| Any combination of these targets together: <i>IGH</i> FR1, <i>IGH</i> FR2, <i>IGH</i> FR3, <i>IGK</i> , <i>TRB</i> and <i>TRG</i> | 251 cycles Read 1 251 cycles Read 2 | v2 kit (500 cycle) up to 4 targets or v3 kit (600 cycle) | 12 pM (v2) or 20 pM (v3) | MS-102-2003 or MS-102-3003 |
| When combining any of the assays with <i>IGHV</i> Leader | 301 cycles Read 1 301 cycles Read 2 | v3 kit (600 cycle) | 20 pM (v3) | MS-102-3003 |

20.1. Determine the concentration of each individual library (e.g., *IGHV* Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRB* and *TRG*).

20.2. Determine the amount of each library to be denatured

In the table below, Case A, B, C, D, E and F are different examples of assay multiplexing (e.g., Case A is a multiplex of *IGHV* Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRB* and *TRG*). T, U, V, W, X, Y and Z are volumes in μL .

| | | |
|---|---|--|
| n | = | number of targets being loaded to a MiSeq cartridge |
| T | = | 40 fmole / [n x <i>IGHV</i> Leader library concentration (nM)] |
| U | = | 40 fmole / [n x <i>IGH</i> FR1 library concentration (nM)] |
| V | = | 40 fmole / [n x <i>IGH</i> FR2 library concentration (nM)] |
| W | = | 40 fmole / [n x <i>IGH</i> FR3 library concentration (nM)] |
| X | = | 40 fmole / [n x <i>IGK</i> library concentration (nM)] |
| Y | = | 40 fmole / [n x <i>TRG</i> library concentration (nM)] |
| Z | = | 40 fmole / [n x <i>TRB</i> library concentration (nM)] |

Note: The 40 fmole value corresponds to the 20 μL of 2 nM at the end of step 20.3.

Table 25. Calculation of Individual Library Inputs to Generate a Multi-Targeted Sequencing Library for the MiSeq Run

| Library | | Individual Library Volume (μL) | | | | | | |
|--------------------|--------------------|---|---------------|---------------|---------------|---------------|---------------|---------------|
| Assay Name | Concentration (nM) | | Case A n=7 | Case B n=6 | Case C n=5 | Case D n=4 | Case E n=3 | Case F n=2 |
| <i>IGHV</i> Leader | 2.3 | T | 2.5 | 2.9 | 3.5 | 4.3 | | |
| <i>IGH</i> FR1 | 1.5 | U | 3.8 | 4.4 | 5.3 | 6.7 | 8.9 | |
| <i>IGH</i> FR2 | 4 | V | 1.4 | 1.7 | 2 | 2.5 | 3.3 | |
| <i>IGH</i> FR3 | 2.1 | W | 2.7 | 3.2 | 3.8 | 4.8 | 6.4 | |
| <i>IGK</i> | 3.5 | X | 1.6 | 1.9 | 2.3 | | | 5.7 |
| <i>TRG</i> | 2.6 | Y | 2.2 | 2.6 | | | | 7.7 |
| <i>TRB</i> | 2 | Z | 2.9 | | | | | |
| | | T+U+V+W+X+Y+Z | 17.1 | 16.7 | 16.9 | 18.3 | 18.6 | 13.4 |

20.3. Denature the combined libraries to 2 nM.

- Add reagents according to Table 26 based on the amount determined in the previous step.

If T+U+V+W+X+Y+Z >18, such as in case D and E from Table 25, mix the applicable libraries first, then add 18 μL to the denaturing reaction as shown in the following table.

Table 26. Library Denaturation

| Reagent | Volume (μL) |
|---------------------------------------|----------------------|
| <i>IGHV</i> Leader Library | T |
| <i>IGH</i> FR1 Library | U |
| <i>IGH</i> FR2 Library | V |
| <i>IGH</i> FR3 Library | W |
| <i>IGK</i> Library | X |
| <i>TRG</i> Library | Y |
| <i>TRB</i> Library | Z |
| 1N NaOH | 2 |
| 10 mM Tris-HCl pH 8.0, 0.05% Tween 20 | 18 – (T+U+V+W+X+Y+Z) |
| Total | 20 |

Vortex briefly to mix the solution, and then briefly centrifuge to ensure all the solution has settled to the bottom of the tube. Incubate for 5 minutes at room temperature to denature the combined library DNA into single strands.

20.4. Dilute the denatured library to 40 pM.

Add 980 μL of pre-chilled HT1 buffer (provided in MiSeq Reagent kit) to the tube containing the 20 μL denatured library DNA. Vortex briefly to mix and pulse centrifuge the sample.

20.5. Prepare the denatured library for loading onto the MiSeq.

Dilute the library to 12 pM for MiSeq reagent kit v2 and 20 pM for MiSeq reagent kit v3 when multiplexing (MCS v2.6 or later) by following Table 27. Vortex briefly to mix and pulse centrifuge the sample.

Table 27. Preparation of the Combo Library for MiSeq Loading

| Reagent | Volume | |
|--------------------|----------------|----------------|
| | 12 pM | 20 pM |
| 40 pM library | 300 μL | 500 μL |
| Chilled HT1 Buffer | 700 μL | 500 μL |
| Total | 1000 μL | 1000 μL |

20.6. Load 600 μL of the combined denatured library from the previous step onto a MiSeq Reagent Cartridge.

20.7. Set up a MiSeq sample sheet, and upload the sample sheet to the instrument (if necessary).

20.8. Start the MiSeq run.

20.9. Analyze and visualize the acquired data using the associated LymphoTrack Dx Software – MiSeq package.