LymphoTrack® Dx

IGHV Leader Somatic Hypermutation Assay

Instructions for Use

CE UK CA IVD

LymphoTrack® *IGHV* Leader Somatic Hypermutation Assay – MiSeq®

To identify and track clonal B cell immunoglobulin heavy chain *(IGH)* gene rearrangements using Next-Generation Sequencing (NGS) with the Illumina® MiSeq and to assess the extent of somatic hypermutation (SHM) in the variable (V) heavy chain gene sequence in chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) samples.

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

Schematic depiction of the *IGH* gene locus:

invivoscribe

Storage Conditions: **-85ºC to -65ºC**
(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

Catalog #		Products	Quantity
REF	91210059	LymphoTrack *IGHV* Leader Somatic Hypermutation Assay Kit A – MiSeq	8 indices – 5 reactions each
REF	91210069	LymphoTrack *IGHV* Leader Somatic Hypermutation Assay Panel – MiSeq	24 indices – 5 reactions each

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1. Intended Use

The LymphoTrack Dx *IGHV* Leader Somatic Hypermutation 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.

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 1,250 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 Morley[3] and are considered the current gold standard method. These 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 rearrangement 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

This LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay – MiSeq represents a significant improvement over existing clonality assays using fragment analysis as it efficiently detects the majority of *IGH* gene rearrangements using a single multiplex master mix and identifies the DNA sequence specific for each clonal gene rearrangement. Therefore, this assay has three important and complementary uses: it provides critical information in the existence of clonality, identifies sequence information required to track those clones in subsequent samples, and provides detailed sequence information required to calculate the degree of SHM.

Each single multiplex master mix targets the Leader (VHL) and the joining (J) gene regions of *IGH*. Primers included in the master mixes are designed with Illumina adapters (up to 24 different indices). This method 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) onto one MiSeq flow cell, allowing up to 24 samples 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 and a somatic hypermutation positive control are included in the kit.

Note: For a more thorough explanation of the locus and the targeted sequencing strategy, please refer to (Miller JE., 2013).[4]

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. These primers target the 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 which are detected with significant frequency 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 log10 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. A terminal 3' blocker is added after each cycle of the sequencing process and any unincorporated nucleotides are removed prior to the addition of four new RT-bases.

3.5. Multiplexing Amplicons

This assay was 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 MiSeq 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 the MiSeq v2 (300 or 500 cycle) sequencing kit 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 manufacturers other flow cells that utilize the same sequencing chemistry, but these 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

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

Catalog #	Description	# of Indexed Master Mixes	Total Reactions
REF 91210059	LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay Kit A - MiSeq	8 indices – 5 sequencing runs each	40
REF 91210069	LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay Panel - MiSeq	24 indices – 5 sequencing runs each	120

Table 2. Kit Components

Reagent	Reagent Components	Unit Quantity	91210059 # of Units	91210069 # of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* Leader MiSeq 01	250 µL	1	1	-85°C to -65°C	N/A
	IGH Leader MiSeq 02		1	1		
	IGH Leader MiSeq 03		1	1		
	IGH Leader MiSeq 04		1	1		
	IGH Leader MiSeq 05		1	1		
	IGH Leader MiSeq 06		1	1		
	IGH Leader MiSeq 07		1	1		
	IGH Leader MiSeq 08		1	1		
	IGH Leader MiSeq 09		0	1		
	IGH Leader MiSeq 10		0	1		
	IGH Leader MiSeq 11		0	1		
	IGH Leader MiSeq 12		0	1		
	IGH Leader MiSeq 13		0	1		
	IGH Leader MiSeq 14		0	1		
	IGH Leader MiSeq 15		0	1		
	IGH Leader MiSeq 16		0	1		
	IGH Leader MiSeq 18		0	1		
	IGH Leader MiSeq 19		0	1		
	IGH Leader MiSeq 20		0	1		
	IGH Leader MiSeq 21		0	1		
	IGH Leader MiSeq 22		0	1		
	IGH Leader MiSeq 23		0	1		
	IGH Leader MiSeq 25		0	1		
	IGH Leader MiSeq 27		0	1		
Positive Control DNA+	*IGH* SHM POS (+) (REF 40880008)	45 µL	1	3	2°C to 8°C or -85°C to -65°C	*IGH* V4-59_08 / *IGH* J4_02 DNA in tonsil DNA with ≥ 2% mutation rate compared to the germline sequence
	IGH POS (+) (REF 40880009)	45 µL	1	3		*IGH* V1-46_03 / *IGH* J4_02 DNA in tonsil DNA
Negative Control DNA	NGS NEG (-) (REF 40920018)	45 µL	1	3		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.
+Note: While the *IGH* Positive Control DNA is positive for clonality in the *IGH* locus, it is not positive for the presence of somatic hypermutation. The *IGH* SHM Positive Control DNA has been characterized as positive for somatic hypermutation in the *IGH* locus.

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 plastic ware whenever possible to avoid contamination.

4.3. Storage and Handling

- If the assay is not being used immediately, store at -85ºC to -65ºC.
- The optimum storage temperature for DNA controls is 2ºC to 8ºC, but DNA can also be stored at -85ºC to -65ºC.
- 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: 15ºC to 96ºC
  - Minimum Ramping Speed: 0.8ºC/sec
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.
- See section 7.4 *Amplification* for the 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)
  - 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 the real-time PCR program.

5.4. Illumina MiSeq Instrument

- Use or Function: Sequence normalized DNA library
- Performance Characteristics and Specifications:
  - Compatible with MiSeq Reagent Kit v3*
- 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* for MiSeq parameters.

*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 specimens 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 for materials provided.

7.2. Materials Required (not provided)

Table 3. Materials Required (not provided)

Reagent/Material	Required or Recommended Reagents/Suppliers	Catalog #	Notes
DNA Polymerase	Roche: • EagleTaq™ DNA Polymerase or Invivoscribe: • 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: Agencourt AMPure XP	A63880	N/A
PCR Purification	Thermo Fisher Scientific: • Ambion Magnetic Stand 96 • DynaMag-96 Side Skirted Magnet or Beckman Coulter: • Agencourt SPRI Plate 96 Ring Super Magnet Plate or equivalent	AM10027 12027 or A32782	N/A
Amplicon & Library Quantification	KAPA Biosystems: KAPA Library Quantification Kit – Illumina	KK4824	N/A
MiSeq Run	Illumina: • MiSeq Reagent • v3 kit (600 cycles)	MS-102-3003	Standard flow cell
MiSeq Software	• 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 100, 200, 300, 400 and 600 ; 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 4. Reaction Setup

Reagent	Volume
Master Mix	45.0 µL
Taq DNA polymerase	0.2 µL
Sample or Control DNA	5.0 µL
Total Volume	**50.2 µL**

7.4. Amplification

7.4.1. Amplify the samples using the PCR program from Table 5.

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

Table 5. PCR Program

Step	Temperature	Time	Cycle
1	95°C	7 minutes	1
2	95°C	45 seconds	32x
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. Once the amplification program has completed, remove the amplified PCR plate from the thermal cycler. If not immediately continuing to the next steps, store 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 was 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 × 50 µL (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 the 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 image in Figure 1 illustrates the effectiveness of a typical purification (showing amplicons before and after purification).

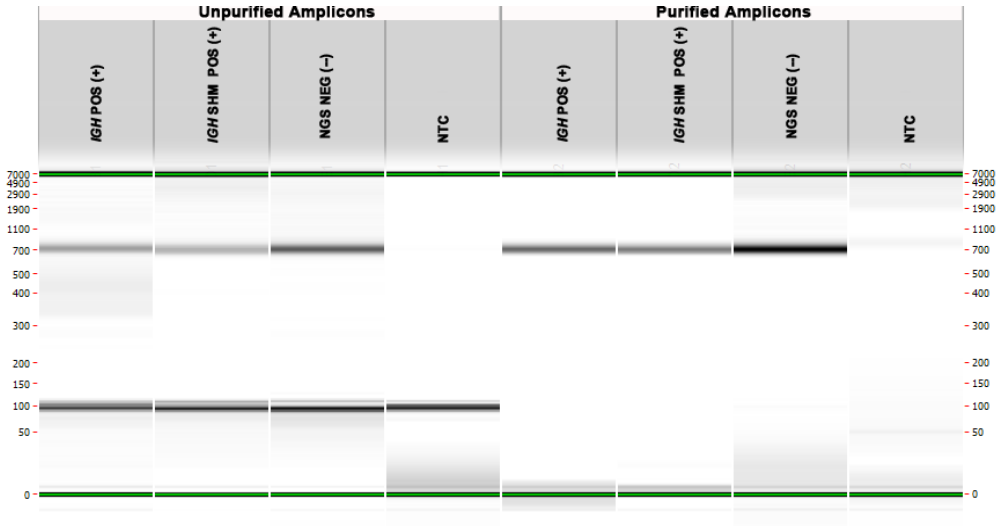


Figure 1: Example of a purification result for amplicons from the LymphoTrack Dx IGHV Leader Somatic Hypermutation Assay - MiSeq Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

7.6. Quantification of Amplicons

The following steps were performed during assay validation to quantify purified PCR amplicons generated from samples as well as 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 6 for each reaction (please refer to the KAPA library quantification kit instructions for more details):

Table 6. 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 7 for the thermal program for qPCR.

Table 7. 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 ΔCt values between the controls (positive and negative) and the NTC, using the following equation:

ΔCt = Ct (NTC) – Ct (Control)

If the ΔCt ≥ 4.0 for both controls, proceed to the next step. If the ΔCt < 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 ΔCt 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:

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

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

A = The average fragment length of amplicons generated using the ***IGHV* Leader** Assay = 660 bp (**A = 660**).
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 and obtaining high quality data in a sequencing run. **Quantification of the library by qPCR is strongly recommended.**

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.*, dilute each amplicon to 4 nM in a total volume of 10 µL using Dilution Buffer A as diluent.
- Combine 10 µL of each 4 nM amplicon.
- 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 6 for qPCR setup and Table 7 for the thermal cycler program.

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

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

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

A = The average fragment length of amplicons generated using the ***IGHV* Leader** Assay = 660 bp (**A = 660**).
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 - 20 pM for MiSeq reagent kit v3**. For multiplexing amplicons from different LymphoTrack Dx Assays for the MiSeq into a single library, please refer to *Appendix A: Building a Sequencing Library with Multiple NGS Targets* (section 20).

7.10.1. Determine the amount 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 8. Library Denaturation

Reagent	Volume
Diluted library	10 µL
0.2 N NaOH	10 µL
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 µL of pre-chilled HT1 buffer (provided in MiSeq Reagent kits) to the tube containing the denatured library DNA:

Table 9. HT1 Buffer Addition

Reagent	Volume
Denatured library	20 µL
HT1 Buffer	980 µL
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.

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 must be 12 - 20 pM for MiSeq reagent kit v3.

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 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 10. Preparation of the Library for MiSeq Loading

Final Concentrations	12 pM	20 pM
Denatured library	300 µL	500 µL
HT1 Buffer	700 µL	500 µL
Final NaOH Concentration (mM)	0.6 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 µL 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 for 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:

List each index in the Sample Sheet only one time; 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. This set of samples/targets should be given 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 **40-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 11. 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. The approximate MiSeq run times are indicated in Table 12.

Table 12. MiSeq Run Times

MiSeq Reagent Kit	Read Length	MCS Version	Total MiSeq Run Time
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 *IGHV* Leader Somatic Hypermutation Assay – MiSeq was 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. Assay Specifications

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

- *IGH* Positive Control top % reads ≥ 2.5%
- NGS Negative Control top % reads < 1.0%
- *IGH* SHM Positive Control top % reads ≥ 2.5%
- *IGH* SHM Positive Control mutation rate ≥ 2.0%
- MiSeq Run Validity %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.

10. Limitations of Procedure

- This assay does 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.

11. Interpretation and Reporting

Use the *Merged Read Summary* report to identify the top merged read sequences and their frequencies prior to clonality determination using the criteria listed below. Refer to section 8 *Data Analysis* 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 in making 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.

Table 13. Clonality 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

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

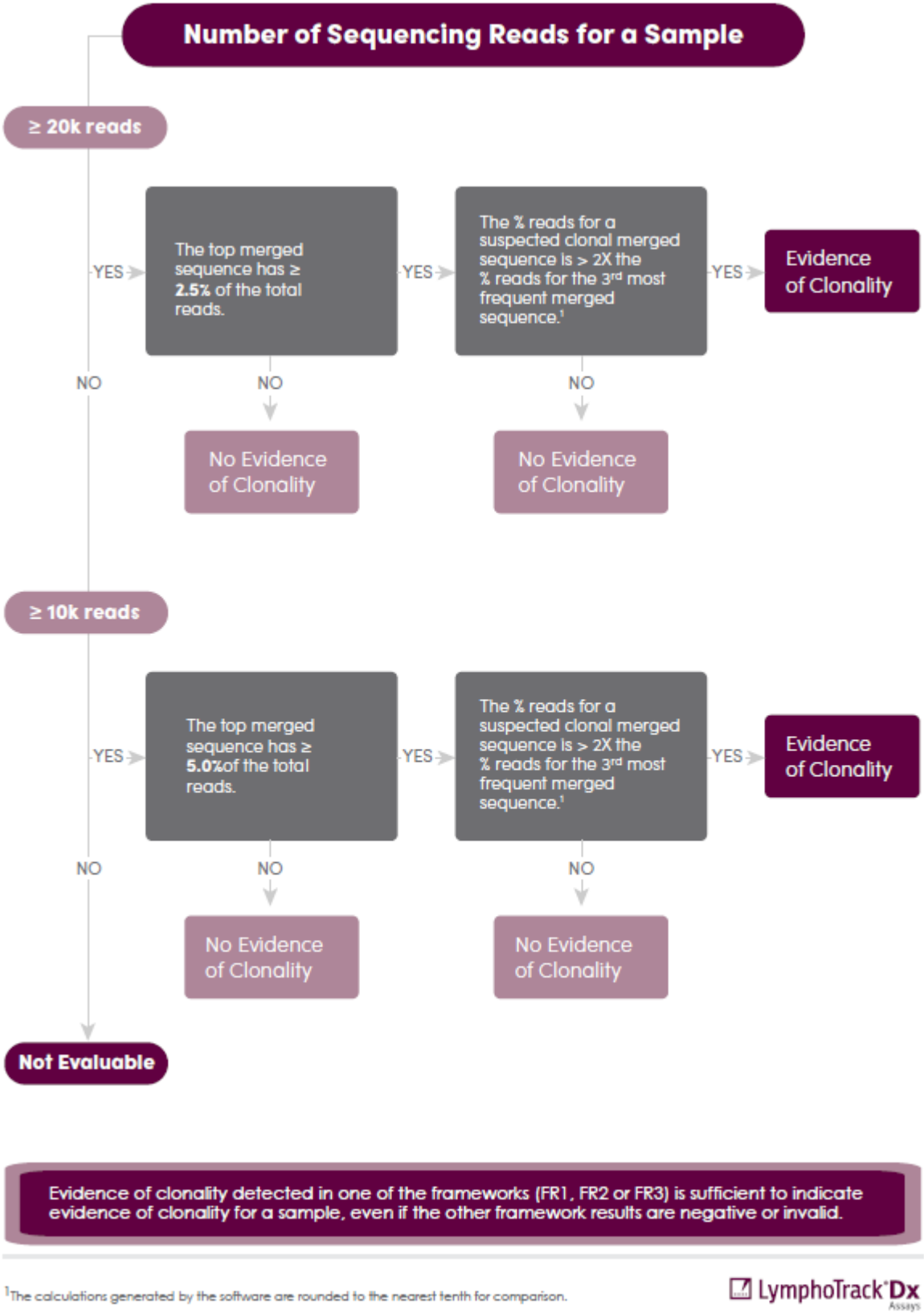


Figure 2: Clonality Interpretation of data based on criteria from Table 13.

After clonality is determined, samples can be evaluated for evidence of somatic hypermutation (SHM). The SHM interpretation criteria listed below are suggestions for using immunoglobulin gene sequence analysis for CLL prognostication based on current literature.[5]

For SHM interpretation, the top two merged sequences should 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 3 for corresponding flow chart).

Table 14. Suggested SHM Interpretation Criteria

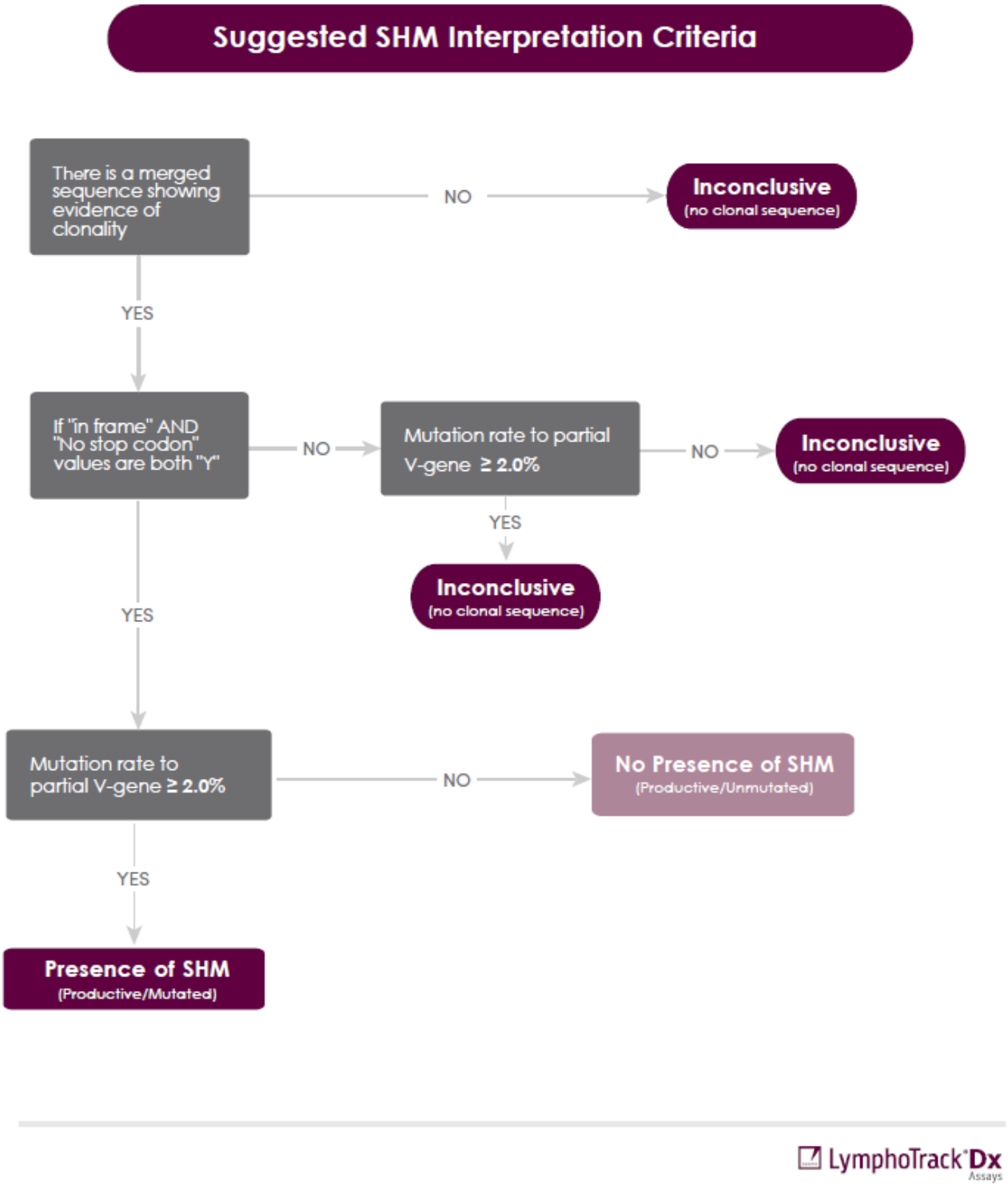
Criterion 1	Criterion 2	Criterion 4	Result
There is a merged sequence showing evidence of clonality.	If "**In-frame**" AND "**No stop codon**" values are both "Y"	Mutation rate to partial V-gene **≥ 2.0%**	**PRESENCE OF SHM** (Productive/Mutated)
		Mutation rate to partial V-gene **< 2.0%**	**No presence of SHM** (Productive/Unmutated)
	If either (or both) "**In-frame**" OR "**No stop codon**" value is "N"	Mutation rate to partial V-gene **≥ 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 the SHM result is "inconclusive" due to no merged sequences showing evidence of clonality, the SHM status can be further assessed by running the sample with the LymphoTrack Dx *IGH* FR1 Assay – MiSeq.

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

Table 15. 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**



Note: If there are two merged sequences for a sample that both show evidence of clonality, evaluate each using Table 15 to determine the final SHM result for that sample.

Figure 3: Suggested Somatic Hypermutation (SHM) Interpretation of data based on criteria from Table 14.

12. Sample Data

LymphoTrack Dx Report for assay LEADER

Sample name: Leader_SHM_positive_S23_L001_001_combined

Total Read Count: 474947

IndexQ30: 87.88

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	TTCTCGTGGTGGC	455	50248	IGHV4-59_08	IGHJ4_02	10.58	10.58	11.26	Y	Y	98.63	GCGAGACGGAGC
2	CTGCTACTGACTG	319	192	IGHV2-70_10	IGHJ4_02	0.04	10.62	4.32	n/a	N	35.55	not found
3	CTGCTGCTGACCA	466	175	IGHV2-5_01	IGHJ5_01	0.04	10.66	6.62	Y	Y	100.00	GCACACAGACCG
4	CTGCTGCTGACCA	457	162	IGHV2-5_05	IGHJ6_02	0.03	10.69	2.99	Y	Y	99.67	GCACACAGATACT
5	CTGCTGCTGACCA	474	154	IGHV2-5_05	IGHJ4_02	0.03	10.72	3.99	Y	Y	99.67	GCACACAGATACT
6	CTGCTGCTGACCA	454	150	IGHV2-5_10	IGHJ5_02	0.03	10.76	11.78	Y	Y	98.99	GCATATGGTGTAA
7	CTGCTGCTGACCA	469	139	IGHV2-5_01	IGHJ4_02	0.03	10.78	1.32	Y	Y	97.68	GCACTCGCGACAC
8	CTCGCCCTCCTCC	466	139	IGHV5-51_01	IGHJ4_02	0.03	10.81	7.09	Y	Y	99.32	GCGAGATACTATT
9	CTGCTACTGACTG	490	137	IGHV2-70_10	IGHJ3_02	0.03	10.84	0.66	Y	Y	99.34	GCACGGATTCCTG
10	CTGCTGCTGACCA	478	135	IGHV2-5_10	IGHJ6_02	0.03	10.87	3.70	Y	Y	98.99	GCATACACTTGTT

Figure 4: 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 nucleotides (nts) different. Sequences were generated using the **LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay – MiSeq** and analyzed using the LymphoTrack Dx Software – MiSeq (REF 95000009).

Please note: when the LymphoTrack Dx Software – MiSeq is unable to assign the V-gene or J-gene, the In-frame result will report *N/A*. If the software is unable to determine In-frame, it will not be able to make an accurate assessment for No stop codon. All open reading frames will be checked for a stop codon and will most likely find one (No stop codon = N). Be sure to use other resources to determine productivity if the V or J gene is labeled *none*.

13. Performance Characteristics

The somatic hypermutation status of clinical samples was evaluated using LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay - MiSeq and Sanger sequencing of *IGH* FR1 PCR amplicons. The results were compared and the concordance (or overall percent agreement), positive percent agreement (PPA), and negative percent agreement (NPA) were: 98% (44/45 cases), 100%, and 94%, respectively.

Table 16. Comparison between LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay – MiSeq and Sanger Sequencing for Somatic Hypermutation Status

		Sanger Sequencing	
		Presence of SHM	No Presence of SHM
LymphoTrack Dx *IGHV* Leader Assay - MiSeq	Presence of SHM	28	1
	No Presence of SHM	0	16

The analytical performance of the LymphoTrack Dx *IGHV* Leader Somatic Hypermutation 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 a 5% DNA dilution. The highest % reads from tonsil DNA was < 1%. The linear regression R^2 was > 0.99 for a range of 0 to 10% DNA dilution. The coefficient of variation (CV%) across 8 runs from 2 operators, 2 reagent lots and 2 instruments was less than 20% when testing 5% and 10% DNA dilutions.

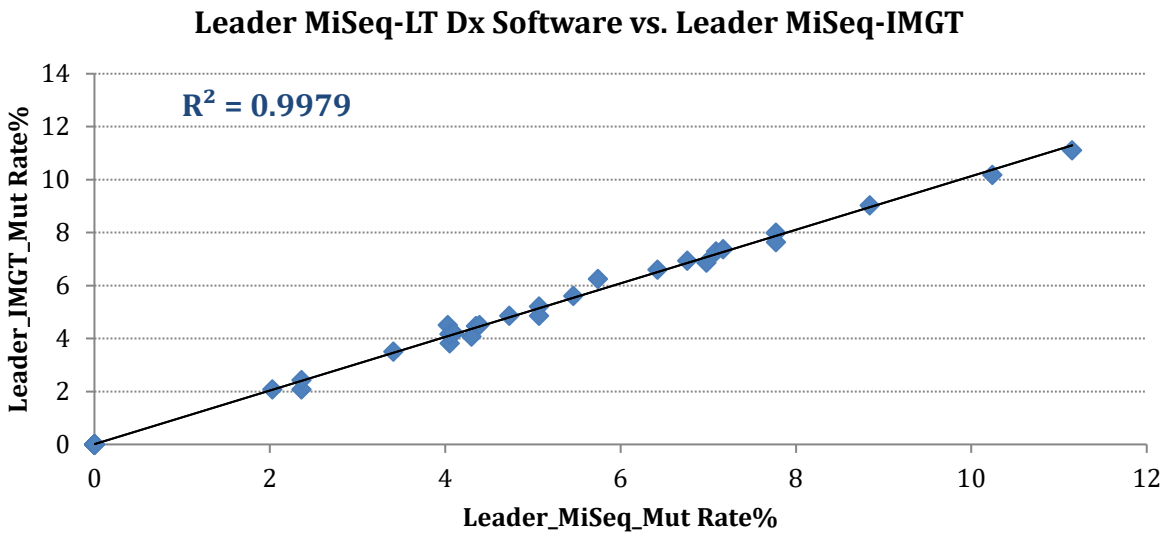


Figure 5: Comparison of the somatic hypermutation (SHM) rate for 45 CLL samples determined by LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay - MiSeq and analyzed either with the LymphoTrack Dx Software - MiSeq or using the ImMunoGeneTics® (IMGT) analysis.

14. Troubleshooting Guide

Table 17. 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	ΔCt < 4.0 ΔCt = Ct (NTC) – Ct (Control)	Check the standard curve in qPCR. Check for contamination and repeat the KAPA qPCR for all samples and controls. If ΔCt < 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 < 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 does not meet the Illumina specification, consider that index to be invalid.

15. Technical and Customer Service

Thank you for purchasing our LymphoTrack Dx *IGHV* Leader Somatic Hypermutation Assay – MiSeq. We appreciate your business. We are happy to assist you with understanding this assay, and will provide ongoing technical assistance Monday through Friday to keep the assays performing efficiently in your laboratory.

Contact Information

Invivoscribe, Inc
10222 Barnes Canyon Road | Building 1 | San Diego | California 92121-2711 | USA
Phone: +1 858 224-6600 | Fax: +1 858 224-6601 | Business Hours: 7:00AM - 5:00 PM PST/PDT
Technical Service: support@invivoscribe.com | Customer Service: sales@invivoscribe.com | Website: www.invivoscribe.com

16. References

1. Tonegawa, S. (1983). Somatic Generation of Antibody Diversity. *Nature* 302, 575–581.
2. Ghia, P. *et al.,* (2007). ERIC recommendations on *IGHV* gene mutational status in chronic lymphocytic leukemia. *Leukemia* 21, 1–3.
3. Trainor, KJ. *et al.,* (1990). Monoclonality in B-lymphoproliferative disorders detected at the DNA level. *Blood* 75, 2220–2222.
4. Miller, JE. (2013). Principle of Immunoglobulin and T Cell Receptor Gene Rearrangement. In Cheng, L., Zhang, D., Eble, JN. (Eds), *Molecular Genetic Pathology* (2nd Ed., sections 30.2.7.13 and 30.2.7.18). New York, USA: Springer Science & Business Media.
5. 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.

- 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	CE	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 *IGHV* Leader Somatic Hypermutation 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 µL 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 µL Taq DNA polymerase (@5 U/µL) to each Master Mix.

19.4. Add 5 µL of sample DNA at a minimum concentration of 10 ng/µL and 5 µL 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 µL 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	32x
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 µL of particles to each 50 µL reaction; elute DNA in 25 µL 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 - 20 pM for MiSeq reagent kit v3 (MCS v2.6 or later).

19.12. Load 600 µL 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 18, below, summarizes these procedural differences. For full instructions, please see the Instructions For Use for the respective LymphoTrack Dx Assay – MiSeq.

Table 18. Cycle Settings and Reagent Kits for a Single-Target MiSeq Run

Procedure Step	Description	LymphoTrack Dx Assay - MiSeq						
		IGHV Leader	*IGH* FR1	*IGH* FR2	*IGH* FR3	*IGK*	*TRG*	*TRB*
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 19. Cycle Settings and Reagent Kits for a Multi-Targeted MiSeq Run

Multiplexing Targets	Sample Sheet Settings	MiSeq Reagent Kit	Loading Concentration	Illumina Catalog #
Only *IGH* FR3 and *TRG* together	151 cycles Read 1 151 cycles Read 2	v2 kit (300 cycle) or v2 kit (500 cycle) or v3 kit (600 cycle)	12 pM (v2) or 20 pM (v3)	MS-102-2002 or MS-102-2003
Any combination of these targets together: *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRB* and *TRG*	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 *IGHV* 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 *IGHV* Leader library concentration (nM)]

U = 40 fmole / [n x *IGH* FR1 library concentration (nM)]

V = 40 fmole / [n x *IGH* FR2 library concentration (nM)]

W = 40 fmole / [n x *IGH* FR3 library concentration (nM)]

X = 40 fmole / [n x *IGK* library concentration (nM)]

Y = 40 fmole / [n x *TRG* library concentration (nM)]

Z = 40 fmole / [n x *TRB* library concentration (nM)]

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

Table 20. 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
IGHV Leader	2.3	T	2.5	2.9	3.5	4.3		
IGH FR1	1.5	U	3.8	4.4	5.3	6.7	8.9	
IGH FR2	4	V	1.4	1.7	2	2.5	3.3	
IGH FR3	2.1	W	2.7	3.2	3.8	4.8	6.4	
IGK	3.5	X	1.6	1.9	2.3			5.7
TRG	2.6	Y	2.2	2.6				7.7
TRB	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 21 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 20, mix the applicable libraries first, then add 18 µL to the denaturing reaction as shown in the following table.

Table 21. Library Denaturation

Reagent	Volume (µL)
IGHV Leader Library	T
IGH FR1 Library	U
IGH FR2 Library	V
IGH FR3 Library	W
IGK Library	X
TRG Library	Y
TRB 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 22. Vortex briefly to mix and pulse centrifuge the sample.

Table 22. 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.