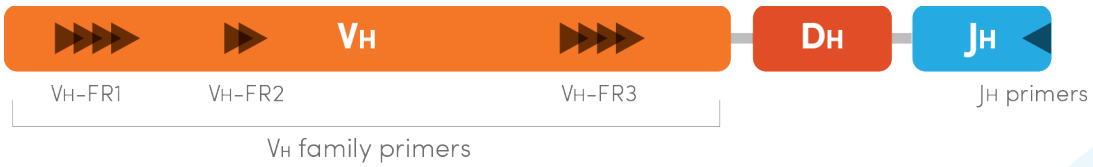
LymphoTrack®
IGH Assays

Instructions for Use
LymphoTrack® *IGH* (FR1/FR2/FR3) Assays – MiSeq™

Identify and track B-cell immunoglobulin heavy chain (*IGH*) gene rearrangements using next-generation sequencing with the Illumina® MiSeq.

RUO This assay is for Research Use Only. Not for use in Diagnostic Procedures.

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



invivoscribe®

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

Catalog #		Products	Quantity
REF	71210009	LymphoTrack *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF	71210039	LymphoTrack *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 reactions each
REF	71210149	LymphoTrack *IGH* FR1 Assay Panel B – MiSeq	24 indices – 5 reactions each
REF	71210089	LymphoTrack *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF	71210099	LymphoTrack *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 reactions each
REF	71210109	LymphoTrack *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF	71210119	LymphoTrack *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 reactions each
REF	71210129	LymphoTrack *IGH* FR1/2/3 Assay Kit A – MiSeq	8 indices per FR region– 5 reactions each
REF	71210139	LymphoTrack *IGH* FR1/2/3 Assay Panel – MiSeq	24 indices per FR region – 5 reactions each

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1. Assay Use

The **LymphoTrack *IGH* FR1 Assay – MiSeq** targets the conserved framework 1 (FR1) region within the V_H segments of the *IGH* gene to identify clonal *IGH* V_H–J_H rearrangements, the associated V_H–J_H region DNA sequences, provides the frequency distribution of V_H region and J_H region segment utilization, and the degree of somatic hypermutation (SHM) of rearranged genes using the Illumina MiSeq platform.

The **LymphoTrack *IGH* FR2 Assay – MiSeq** targets the conserved framework 2 (FR2) region within the V_H segments of the *IGH* gene to identify clonal *IGH* V_H–J_H rearrangements, the associated V_H–J_H region DNA sequences, and provides the frequency distribution of V_H region and J_H region segment utilization using the Illumina MiSeq platform.

The **LymphoTrack *IGH* FR3 Assay – MiSeq** targets the conserved framework 3 (FR3) region within the V_H segments of the *IGH* gene to identify clonal *IGH* V_H–J_H rearrangements, the associated V_H–J_H region DNA sequences, and provides the frequency distribution of V_H region and J_H region segment utilization using the Illumina MiSeq platform.

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 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

The LymphoTrack *IGH* (FR1, FR2 and FR3) Assays for 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, and at the same time, identify the DNA sequence specific for each clonal gene rearrangement. Therefore, these assays have three important and complementary uses: they provide critical information on the existence of clonality, identify sequence information required to track those clones in subsequent samples and provide 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]

Primers included in the master mixes are designed with Illumina adapters and up to 48 different indices. This method allows for a one-step PCR, and pooling of amplicons from several different samples and targets (generated with other LymphoTrack Assays for the Illumina MiSeq instrument) onto one MiSeq flow cell, allowing for up to 48 samples per target to be analyzed in parallel in a single run.

The associated RUO LymphoTrack Software for MiSeq provides a simple and streamlined method of analysis and visualization of 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. 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 the Agencourt® AMPure® XP system. This method utilizes 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 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 as each RT-base is added, and then cleaved to allow incorporation of the next base.

3.5. Multiplexing Amplicons

These products 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 48 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 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 the 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 18).

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 20-25 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. 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 *IGH* FR1 Assay 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.

3.7. Minimal Residual Disease Evaluation

The correlation of MRD status with overall survival rate was initially demonstrated for subjects with chronic lymphocytic leukemia using multi-parameter flow analysis.[6] However, flow-based methods have proven difficult to standardize outside individual centers. Therefore, flow cytometry is not a suitable technology for international standardization, as it is difficult to compare results from different centers. Fortunately, a number of investigators have described NGS-based approaches that have demonstrated success in detecting and monitoring MRD in Chronic Lymphocytic Leukemia (CLL), Acute Lymphoblastic Leukemia (ALL) and other lymphoid malignancies.[7,8]

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

Please refer to the MRD Software IFU (REF 280364) and the MRD Technical Bulletin (REF M-0031) for further details on how to use the Project Planner tool to successfully design an MRD experiment that meets or exceeds the required level of sensitivity and confidence.

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog#	Description	# of Indexed Master Mixes	Total Reactions
REF 71210009	LymphoTrack *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 71210039	LymphoTrack *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 71210149	LymphoTrack *IGH* FR1 Assay Panel B – MiSeq	24 indices – 5 sequencing runs each	120
REF 71210089	LymphoTrack *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 71210099	LymphoTrack *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 71210109	LymphoTrack *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 71210119	LymphoTrack *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 71210129	LymphoTrack *IGH* FR1/2/3 Assay Kit A – MiSeq	(8+8+8) indices – 5 sequencing runs each	40+40+40
REF 71210139	LymphoTrack *IGH* FR1/2/3 Assay Panel – MiSeq	(24+24+24) indices – 5 sequencing runs each	120+120+120

Table 2. LymphoTrack *IGH* FR1 Assay Kit A – MiSeq Components (REF 71210009)

Reagents	Reagent Components	Unit Quantity	# of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR1 MiSeq 01	250 µL	1	-85°C to -65°C	N/A
	IGH FR1 MiSeq 02		1		
	IGH FR1 MiSeq 03		1		
	IGH FR1 MiSeq 04		1		
	IGH FR1 MiSeq 05		1		
	IGH FR1 MiSeq 06		1		
	IGH FR1 MiSeq 07		1		
	IGH FR1 MiSeq 08		1		
Positive Control DNA	*IGH* POS (+) (REF 40880009)	45 µL	1	2°C to 8°C or -85°C to -65°C	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA
Negative Control DNA	NGS NEG (-) (REF 40920018)	45 µL	1		Tonsil DNA, highest sequence frequency can vary between lots

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

Table 3. LymphoTrack *IGH* FR1 Assay Panel – MiSeq Components (REF 71210039)

Reagents	Reagent Components	Unit Quantity	# of Units[1]	Storage Temperature	Notes
Master Mixes‡	*IGH* FR1 MiSeq 01	250 µL	1	-85°C to -65°C	N/A
	IGH FR1 MiSeq 02		1		
	IGH FR1 MiSeq 03		1		
	IGH FR1 MiSeq 04		1		
	IGH FR1 MiSeq 05		1		
	IGH FR1 MiSeq 06		1		
	IGH FR1 MiSeq 07		1		
	IGH FR1 MiSeq 08		1		
	IGH FR1 MiSeq 09		1		
	IGH FR1 MiSeq 10		1		
	IGH FR1 MiSeq 11		1		
	IGH FR1 MiSeq 12		1		
	IGH FR1 MiSeq 13		1		
	IGH FR1 MiSeq 14		1		
	IGH FR1 MiSeq 15		1		
	IGH FR1 MiSeq 16		1		
	IGH FR1 MiSeq 18		1		
	IGH FR1 MiSeq 19		1		
	IGH FR1 MiSeq 20		1		
	IGH FR1 MiSeq 21		1		
	IGH FR1 MiSeq 22		1		
	IGH FR1 MiSeq 23		1		
	IGH FR1 MiSeq 25		1		
	IGH FR1 MiSeq 27		1		
Positive Control DNA	*IGH* POS (+) (REF 40880009)	45 µL	3	2°C to 8°C or -85°C to -65°C	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA
Negative Control DNA	NGS NEG (-) (REF 40920018)	45 µL	3		Tonsil DNA, highest sequence frequency can vary between lots

Note‡: There are no preservatives used in the manufacturing of these kits.
Note[1]: Indices 17, 24, and 26 are not used in this kit.

Table 4. LymphoTrack *IGH* FR1 Assay Panel B - MiSeq Components (REF 71210149)

Reagents	Reagent Components	Unit Quantity	# of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR1 MiSeq 17	250 µL	1	-85°C to -65°C	N/A
	IGH FR1 MiSeq 24		1		
	IGH FR1 MiSeq 26		1		
	IGH FR1 MiSeq 28		1		
	IGH FR1 MiSeq 29		1		
	IGH FR1 MiSeq 30		1		
	IGH FR1 MiSeq 31		1		
	IGH FR1 MiSeq 32		1		
	IGH FR1 MiSeq 33		1		
	IGH FR1 MiSeq 34		1		
	IGH FR1 MiSeq 35		1		
	IGH FR1 MiSeq 36		1		
	IGH FR1 MiSeq 37		1		
	IGH FR1 MiSeq 38		1		
	IGH FR1 MiSeq 39		1		
	IGH FR1 MiSeq 40		1		
	IGH FR1 MiSeq 41		1		
	IGH FR1 MiSeq 42		1		
	IGH FR1 MiSeq 43		1		
	IGH FR1 MiSeq 44		1		
	IGH FR1 MiSeq 45		1		
	IGH FR1 MiSeq 46		1		
	IGH FR1 MiSeq 47		1		
	IGH FR1 MiSeq 48		1		
Positive Control DNA	*IGH* POS (+) (REF 40880009)	45 µL	3	2°C to 8°C or -85°C to -65°C	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA
Negative Control DNA	NGS NEG (-) (REF 40920018)	45 µL	3		Tonsil DNA, highest sequence frequency can vary between lots

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

Table 5. LymphoTrack *IGH* FR2 Assay Kit Components

Reagents	Reagent Components	Unit Quantity	71210089 # of Units	71210099 # of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR2 MiSeq 01	250 µL	1	1	-85°C to -65°C	N/A
	IGH FR2 MiSeq 02		1	1		
	IGH FR2 MiSeq 03		1	1		
	IGH FR2 MiSeq 04		1	1		
	IGH FR2 MiSeq 05		1	1		
	IGH FR2 MiSeq 06		1	1		
	IGH FR2 MiSeq 07		1	1		
	IGH FR2 MiSeq 08		1	1		
	IGH FR2 MiSeq 09		0	1		
	IGH FR2 MiSeq 10		0	1		
	IGH FR2 MiSeq 11		0	1		
	IGH FR2 MiSeq 12		0	1		
	IGH FR2 MiSeq 13		0	1		
	IGH FR2 MiSeq 14		0	1		
	IGH FR2 MiSeq 15		0	1		
	IGH FR2 MiSeq 16		0	1		
	IGH FR2 MiSeq 18		0	1		
	IGH FR2 MiSeq 19		0	1		
	IGH FR2 MiSeq 20		0	1		
	IGH FR2 MiSeq 21		0	1		
	IGH FR2 MiSeq 22		0	1		
	IGH FR2 MiSeq 23		0	1		
	IGH FR2 MiSeq 25		0	1		
	IGH FR2 MiSeq 27		0	1		
Positive Control DNA	*IGH* POS (+) (REF 40880009)	45 µL	1	3	2°C to 8°C or -85°C to -65°C	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted 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.

Table 6. LymphoTrack *IGH* FR3 Assay Kit Components

Reagents	Reagent Components	Unit Quantity	71210109 # of Units	71210119 # 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 or -85°C to -65°C	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted 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[1]: Indices 17, 24, and 26 are not used in these kits.

Table 7. LymphoTrack *IGH* FR1/2/3 Assay Kit Components

FR1 Master Mixes[1]	FR2 Master Mixes[1]	FR3 Master Mixes[1]	Unit Quantity	71210129 # of Units per FR	71210139 # of Units per FR[‡]
IGH FR1 MiSeq 01	*IGH* FR2 MiSeq 01	*IGH* FR3 MiSeq 01	250 µL	1	1
IGH FR1 MiSeq 02	*IGH* FR2 MiSeq 02	*IGH* FR3 MiSeq 02		1	1
IGH FR1 MiSeq 03	*IGH* FR2 MiSeq 03	*IGH* FR3 MiSeq 03		1	1
IGH FR1 MiSeq 04	*IGH* FR2 MiSeq 04	*IGH* FR3 MiSeq 04		1	1
IGH FR1 MiSeq 05	*IGH* FR2 MiSeq 05	*IGH* FR3 MiSeq 05		1	1
IGH FR1 MiSeq 06	*IGH* FR2 MiSeq 06	*IGH* FR3 MiSeq 06		1	1
IGH FR1 MiSeq 07	*IGH* FR2 MiSeq 07	*IGH* FR3 MiSeq 07		1	1
IGH FR1 MiSeq 08	*IGH* FR2 MiSeq 08	*IGH* FR3 MiSeq 08		1	1
IGH FR1 MiSeq 09	*IGH* FR2 MiSeq 09	*IGH* FR3 MiSeq 09		0	1
IGH FR1 MiSeq 10	*IGH* FR2 MiSeq 10	*IGH* FR3 MiSeq 10		0	1
IGH FR1 MiSeq 11	*IGH* FR2 MiSeq 11	*IGH* FR3 MiSeq 11		0	1
IGH FR1 MiSeq 12	*IGH* FR2 MiSeq 12	*IGH* FR3 MiSeq 12		0	1
IGH FR1 MiSeq 13	*IGH* FR2 MiSeq 13	*IGH* FR3 MiSeq 13		0	1
IGH FR1 MiSeq 14	*IGH* FR2 MiSeq 14	*IGH* FR3 MiSeq 14		0	1
IGH FR1 MiSeq 15	*IGH* FR2 MiSeq 15	*IGH* FR3 MiSeq 15		0	1
IGH FR1 MiSeq 16	*IGH* FR2 MiSeq 16	*IGH* FR3 MiSeq 16		0	1
IGH FR1 MiSeq 18	*IGH* FR2 MiSeq 18	*IGH* FR3 MiSeq 18		0	1
IGH FR1 MiSeq 19	*IGH* FR2 MiSeq 19	*IGH* FR3 MiSeq 19		0	1
IGH FR1 MiSeq 20	*IGH* FR2 MiSeq 20	*IGH* FR3 MiSeq 20		0	1
IGH FR1 MiSeq 21	*IGH* FR2 MiSeq 21	*IGH* FR3 MiSeq 21		0	1
IGH FR1 MiSeq 22	*IGH* FR2 MiSeq 22	*IGH* FR3 MiSeq 22		0	1
IGH FR1 MiSeq 23	*IGH* FR2 MiSeq 23	*IGH* FR3 MiSeq 23		0	1
IGH FR1 MiSeq 25	*IGH* FR2 MiSeq 25	*IGH* FR3 MiSeq 25		0	1
IGH FR1 MiSeq 27	*IGH* FR2 MiSeq 27	*IGH* FR3 MiSeq 27		0	1

Control DNA[*]	Notes	Unit Quantity	71210129 # of Units	71210139 # of Units
IGH POS (+) (REF 40880009)	*IGH* V1-46_03 / *IGH* 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[1]: The storage temperature for master mixes is -65ºC to -85ºC.
Note[*]: The storage temperature for controls is 2ºC to 8ºC or -65ºC to -85ºC.
Note: There are no preservatives used in the manufacturing of these kits.
Note[‡]: Indices 17, 24, and 26 are not used in 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.

- 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 these tests, 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 **-65ºC to -85ºC**.
- The optimum storage temperature for DNA controls is 2ºC to 8ºC, but DNA can also be stored at -65ºC to -85ºC.
- 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 in below are recommended based on the methods used to verify the assay.

5.1. Thermal cycler

- Use or Function: Amplification of DNA samples
- Suggested Instrument: Veriti™ 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 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 Real-Time 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 Instrument

- Use or Function: Sequence normalized DNA library
- Performance Characteristics and Specifications:
  - Compatible with MiSeq Reagent Kit v2
  - Compatible with MiSeq Reagent Kit v3
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.
- See section 7.10 *MiSeq Flow Cell Loading,* 7.12 *MiSeq Sample Sheet Setup*, and 7.13 *MiSeq Run Start* for MiSeq parameters.

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 Tables 2 – 7 for materials provided in each kit.

7.2. Materials Required (not provided)

Table 8. Materials Required (not provided)

Reagent/Material	Required or Recommended Reagents/Suppliers	Catalog #	Notes
DNA Polymerase	Roche: • EagleTaq™ DNA Polymerase or Invivoscribe, Inc. • 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 SPRIPlate 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 v2 kit (500 cycles) or • v3 kit (600 cycles) When only sequencing IGH *FR3*: • MiSeq Reagent v2 kit (300 cycles) or • MiSeq Reagent v2 kit (500 cycles)	MS-102-2003 or MS-102-3003 MS-102-2002 or MS-102-2003	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 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 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 in the PCR thermal cycler.

Table 9. 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 10.

Please note the LymphoTrack *IGHV* Leader SHM Assay – MiSeq PCR program has different cycling conditions than the other LymphoTrack Assays.

Table 10. 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

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 the 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 verification 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.

AMPure XP reagent volume added to 50 µL of PCR product by assay:
TRB: 35 µL
IGHV Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK* and *TRG:* 50 µL

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.
- Incubate 10 minutes at room temperature.
- The color of the mixture should appear homogenous after mixing.

7.5.5. Place the mixed samples on an 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 images in Figure 1 - Figure 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.

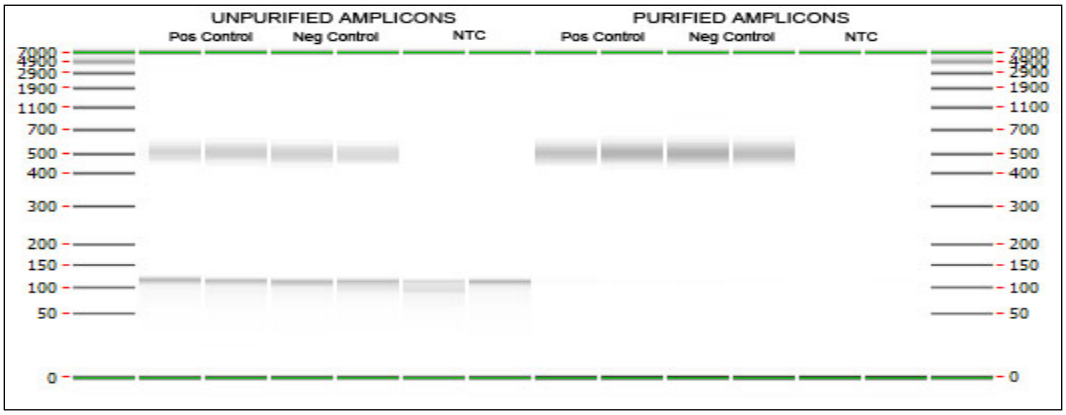

Figure 1: Example of a purification result for amplicons from the *IGH* FR1 MiSeq Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

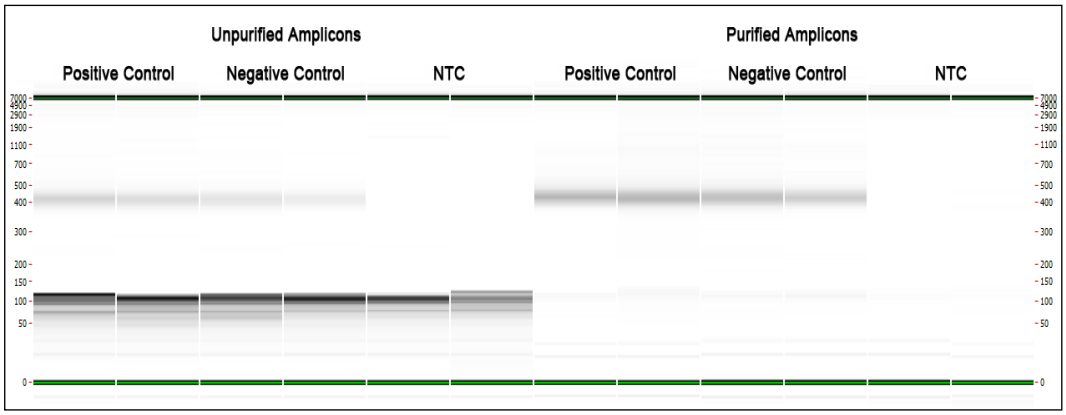

Figure 2: Example of a purification result for amplicons from the *IGH* FR2 MiSeq Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

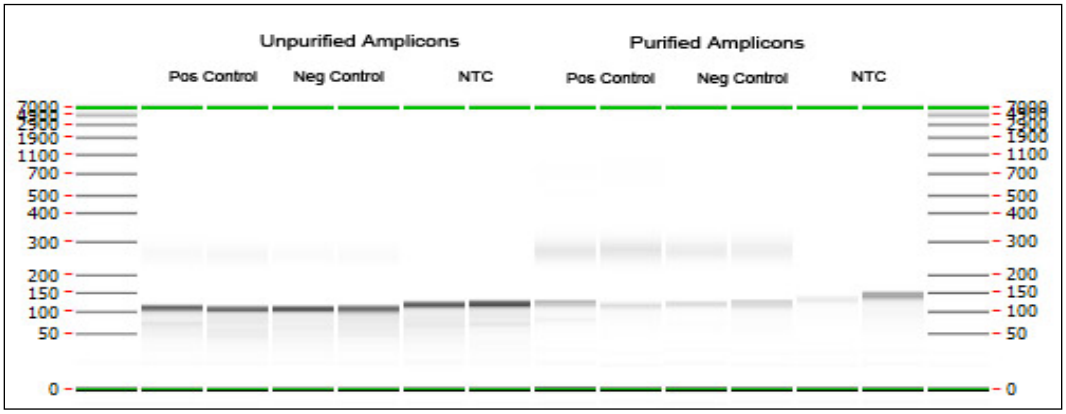

Figure 3: Example of a purification result for amplicons from the *IGH* FR3 MiSeq Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

7.6. Quantification of Amplicons

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

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 11 for each reaction (please refer to the KAPA library quantification kit instructions for more details):

Table 11. 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 12 for the thermal program for qPCR.

Table 12. 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 12 *Troubleshooting Guide* for further instructions.

Note: ΔCt for the *TRB* assay is set different than the other LymphoTrack assays:
TRB ΔCt ≥ 3.0
IGHV Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK* and *TRG* ΔCt ≥ 4.0

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

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

A= The average fragment length of amplicons generated by the assay:

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**).
(Note: Sequence length includes additional nucleotides that are necessary for sequencing).

The average fragment length of KAPA Illumina DNA Standard is 452 bp.

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.**

It is recommended that a separate pool is created for each LymphoTrack Assay and corresponding target (*IGH* FR1, FR2 or FR3). After final quantification of a pooled library for each target according to section 7.6, LymphoTrack Assays can be sequenced individually continuing with the instructions provided in section 7.8, or can be multiplexed together according to the instructions provided in Appendix A (section 18).

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 for 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 11 for qPCR setup and Table 12 for the thermal cycler program.

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

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

A= The average fragment length of amplicons generated by the assay:

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**).
(Note: Sequence length includes additional nucleotides that are necessary for sequencing).

The average fragment length of KAPA Illumina DNA Standard is 452 bp.

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 **12 - 20 pM for MiSeq reagent kit v3**. For multiplexing amplicons from different LymphoTrack Assays for the MiSeq into a single library, please refer to Appendix A: Building a Sequencing Library with Multiple NGS Targets.

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 13. 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 14. 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.

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:

For MiSeq Control Software (MCS v2.6 or later):
The concentration of the library DNA must be 12 pM for MiSeq reagent kit v2.
The concentration of the library DNA must be between 12 to 20 pM for MiSeq reagent kit v3.

- If the diluted ssDNA library concentration is 40 pM (the initial concentration was 4 nM), dilute to the desired MiSeq loading concentration using the examples shown in Table 15.

Table 15. Preparation of the Library for MiSeq Loading

Final Concentrations	12 pM	15 pM	20 pM
Denatured library	300 µL	375 µL	500 µL
HT1 Buffer	700 µL	625 µL	500 µL
Final NaOH Concentration (mM)	0.60 mM	0.75 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 (12 to 20 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]) with the LymphoTrack Kit A or Panel versions, select *TruSeq Nano DNA* for the Library Prep Kit and *TruSeq DNA Single Indexes Set A B* for the Index Kit. If using Panel B, use *ScriptSeqV2* or *ScriptSeq Complete* 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 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 **40-character limit** when choosing a naming convention.

Examples of Sample ID naming that can be used for tracking purposes are listed below:

- S1_FR1_FR2_FR3_IGK (one sample sequenced with multiple assays using the same index)
- S1_FR1_S4_Leader (multiple samples sequenced with multiple assays using the same index)
- Pool12_A012 (Pool12 refers to all samples/targets sequenced with index A012 and tracked elsewhere)

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 for tracking samples.

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

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

Table 16. Sample Selection index names

LymphoTrack Assay – MiSeq PCR Master Mix Index	Index Sequence	LymphoTrack Assay Kit or Panel	TruSeq DNA Single Indexes Set A B (LRM "Index Kit")	ScriptSeqV2 / ScriptSeq Complete (LRM "Index Kit")
id01	ATCACG	Kit A / Panel	AR001	A001
id02	CGATGT	Kit A / Panel	AR002	A002
id03	TTAGGC	Kit A / Panel	AR003	A003
id04	TGACCA	Kit A / Panel	AR004	A004
id05	ACAGTG	Kit A / Panel	AR005	A005
id06	GCCAAT	Kit A / Panel	AR006	A006
id07	CAGATC	Kit A / Panel	AR007	A007
id08	ACTTGA	Kit A / Panel	AR008	A008
id09	GATCAG	Panel	AR009	A009
id10	TAGCTT	Panel	AR010	A010
id11	GGCTAC	Panel	AR011	A011
id12	CTTGTA	Panel	AR012	A012
id13	AGTCAA	Panel	AR013	A013
id14	AGTTCC	Panel	AR014	A014
id15	ATGTCA	Panel	AR015	A015
id16	CCGTCC	Panel	AR016	A016
id18	GTCCGC	Panel	AR018	A018
id19	GTGAAA	Panel	AR019	A019
id20	GTGGCC	Panel	AR020	A020
id21	GTTTCG	Panel	AR021	A021
id22	CGTACG	Panel	AR022	A022
id23	GAGTGG	Panel	AR023	A023
id25	ACTGAT	Panel	AR025	A025
id27	ATTCCT	Panel	AR027	A027

Table 16. Sample Selection index names

LymphoTrack Assay – MiSeq PCR Master Mix Index	Index Sequence	LymphoTrack Assay Kit or Panel	TruSeq DNA Single Indexes Set A B (LRM "Index Kit")	ScriptSeqV2 / ScriptSeq Complete (LRM "Index Kit")
id17	GTAGAG	Panel B	N/A	A017
id24	GGTAGC	Panel B	N/A	A024
id26	ATGAGC	Panel B	N/A	A026
id28	CAAAAG	Panel B	N/A	A028
id29	CAACTA	Panel B	N/A	A029
id30	CACCGG	Panel B	N/A	A030
id31	CACGAT	Panel B	N/A	A031
id32	CACTCA	Panel B	N/A	A032
id33	CAGGCG	Panel B	N/A	A033
id34	CATGGC	Panel B	N/A	A034
id35	CATTTT	Panel B	N/A	A035
id36	CCAACA	Panel B	N/A	A036
id37	CGGAAT	Panel B	N/A	A037
id38	CTAGCT	Panel B	N/A	A038
id39	CTATAC	Panel B	N/A	A039
id40	CTCAGA	Panel B	N/A	A040
id41	GACGAC	Panel B	N/A	A041
id42	TAATCG	Panel B	N/A	A042
id43	TACAGC	Panel B	N/A	A043
id44	TATAAT	Panel B	N/A	A044
id45	TCATTC	Panel B	N/A	A045
id46	TCCCGA	Panel B	N/A	A046
id47	TCGAAG	Panel B	N/A	A047
id48	TCGGCA	Panel B	N/A	A048

7.13. MiSeq Run Start

Start the MiSeq run following the MiSeq Control Software Instructions. The approximate MiSeq run times are indicated in Table 17.

Table 17. MiSeq Run Times for LymphoTrack

MiSeq Reagent Kit	Read Length	MCS Version	Total MiSeq Run Time
v2	2x151 bp (FR3 only)	2.6 or later	~24 hours
	2x251 bp	2.6 or later	~ 39 hours
v3	2x301 bp	2.6 or later	~ 56 hours

8. Data Analysis

The LymphoTrack *IGH* (FR1/FR2/FR3) Assays – MiSeq were designed to produce sequencing data that can be analyzed using the RUO LymphoTrack Software – MiSeq package provided on the associated CD (REF 75000009), which is included with the assay purchase. This CD includes detailed instructions for installation and use of the software package.

Samples prepared with LymphoTrack *IGH* (FR1, FR2 and/or FR3) Assays can be easily processed into fully analyzed data using the LymphoTrack Software - MiSeq.

9. Expected Values

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 (REF 40880008, can be purchased separately) top % reads ≥ 2.5%
- MiSeq Run Validity*
    - Q30 > 80% for v2 (2x151)
    - Q30 >75% for v2 (2x251)
    - Q30 > 70% for v3 (2x301)

*Q30 from all analytical validations has met the above criteria from Illumina MiSeq Q30 specification.

However, Q30 score may vary depending on sample quality. If Q30 falls below Illumina Q30 specification, check the index Q30 value from the LymphoTrack Report after data analysis by the LymphoTrack Software - MiSeq. If an index Q30 score on the LymphoTrack Report does not meet the Illumina specification, consider that index to be invalid.)

10. Limitations of Procedure

- These assays do not identify 100% of clonal cell populations.
- 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.
- The LymphoTrack *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 Research Use Only LymphoTrack *IGHV* Leader Somatic Hypermutation Assay for the Illumina MiSeq (REF 71210059 or REF 71210069) can be purchased separately if complete *IGHV* sequencing is required.
- 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.

11. Sample Data

For Research Use Only. Not for use in Diagnostic Procedures.

LymphoTrack Report for assay IGH_FR1

Sample name: spikeddata_igh_S1_L001_001_combined

Total Read Count: 100000

IndexQ30: 98.9

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	25000	IGHV1-46_03	IGHJ4_02	25.00	25.00	0.00	Y	Y	100.00	not found
2	GCCTCTGGATTCA	272	20000	IGHV3-11_01	IGHJ4_02	20.00	45.00	10.57	Y	Y	98.24	GCGCGAGGTCAC
3	GCCTCTGGATTCA	300	15000	IGHV3-7_03	IGHJ6_02	15.00	60.00	0.00	N	N	99.56	not found
4	GCCTCTGGATTCA	284	12500	IGHV3-23_04	IGHJ1_01	12.50	72.50	8.37	Y	Y	100.00	GCGAAAGATGGTC
5	GCCTCTGGATTCA	217	10000	IGHV3-7_03	IGHJ4_02	10.00	82.50	0.88	n/a	N	71.81	not found
6	GCGTCTGGATTCA	278	5000	IGHV3-33_01	IGHJ6_02	5.00	87.50	0.00	Y	Y	98.68	not found
7	GCCTCTGGATTCA	281	4000	IGHV3-9_01	IGHJ6_02	4.00	91.50	4.37	Y	Y	98.69	GCAAAAGGCCAAG
8	GCCTCTGGATTCA	272	3000	IGHV3-48_01	IGHJ4_02	3.00	94.50	0.00	Y	Y	100.00	not found
9	GCCTCTGGATTCA	293	2000	IGHV3-15_02	IGHJ6_02	2.00	96.50	1.72	Y	Y	100.00	not found
10	GCCTCTGGATTCA	263	1000	IGHV3-23_04	IGHJ6_02	1.00	97.50	0.00	Y	Y	98.24	GCGAGGGACAGA

Figure 4: This table, generated via the LymphoTrack 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 *IGH* FR1 Assay –MiSeq and analyzed using the LymphoTrack Software – MiSeq (REF 75000009).

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LymphoTrack Report for assay IGH_FR2

Sample name: spike_combo_S3_L001_001_combined

Total Read Count: 50004

IndexQ30: 97.1

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	TGGTGGACAAGGC	246	12500	IGHV1-46_03	IGHJ4_02	25.00	25.00	N/A	Y	Y	100.00	GCTAGAGATCTCA
2	ATCATCGAGAGGC	183	10000	IGHV6-1_02	IGHJ4_02	20.00	45.00	N/A	N	N	57.47	not found
3	TGGTGGACAAGGC	219	7500	IGHV1-2_03	IGHJ4_02	15.00	60.00	N/A	Y	Y	100.00	not found
4	CGGGCGGGAAAG	223	6250	IGHV5-51_01	IGHJ4_02	12.50	72.49	N/A	Y	Y	100.00	GCGAGACATCGGT
5	TGGTGGACAAGGC	225	5000	IGHV1-69_09	IGHJ4_02	10.00	82.49	N/A	Y	Y	99.42	GCGACTTTTGCCA
6	TGGTGGACAAGGC	234	2500	IGHV1-46_02	IGHJ4_02	5.00	87.49	N/A	Y	Y	100.00	GCGAGAGATGAGC
7	TGGTGGACAAGGC	228	2000	IGHV1-46_03	IGHJ6_02	4.00	91.49	N/A	Y	Y	97.66	not found
8	CGGGCGGGAAAG	235	1500	IGHV5-51_03	IGHJ4_02	3.00	94.49	N/A	Y	Y	100.00	GCGAGGGTTTATT
9	TGGTGGACAAGGC	228	1000	IGHV1-18_01	IGHJ4_02	2.00	96.49	N/A	n/a	N	92.40	not found
10	TGGACAAGGGCTT	225	500	IGHV7-4-1_02	IGHJ4_02	1.00	97.49	N/A	Y	Y	98.85	GCGAGAAGAAGGC

Figure 5: This table, generated via the LymphoTrack 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 *IGH* FR2 Assay –MiSeq and analyzed using the LymphoTrack Software – MiSeq (REF 75000009).

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LymphoTrack Report for assay IGH_FR3

Sample name: spike_combo_S3_L001_001_combined

Total Read Count: 100008

IndexQ30: 97.1

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	CGGCCGTGTATTA	94	12500	IGHV3-72_01	IGHJ4_02	12.50	12.50	N/A	Y	Y	64.10	GCTAGAGATCTCA
2	GTATTACTGTGCT	87	10000	IGHV3/OR15-7_05	IGHJ4_02	10.00	22.50	N/A	Y	Y	40.00	GCTAGAGATCTCA
3	ATCAGGCCGAATA	85	7500	IGHV4/OR15-8_03	IGHJ4_02	7.50	30.00	N/A	Y	Y	100.00	GCGAGAGTGGAAC
4	ACACGAGACACGC	86	6250	IGHV4-39_02	IGHJ4_02	6.25	36.25	N/A	Y	Y	93.10	GCGAGCAGGGGA
5	GTGGACACAGGCA	77	5000	IGHV2-70_12	IGHJ3_02	5.00	41.25	N/A	Y	Y	100.00	GCACACGTCATAA
6	ACACGGCCGTCTA	79	2500	IGHV4-61_06	IGHJ4_02	2.50	43.75	N/A	Y	Y	100.00	GCCAGAACAGTGC
7	GTGGAGCTGTGTA	65	2000	IGHV2-70_13	IGHJ4_02	2.00	45.75	N/A	n/a	N	68.97	not found
8	ACACGGCTGTGTA	79	1500	IGHV4-39_01	IGHJ4_02	1.50	47.25	N/A	Y	Y	96.55	GCGAGACATAGCT
9	ACACGGCTGTGTA	112	1000	IGHV4-39_02	IGHJ6_03	1.00	48.25	N/A	Y	Y	96.55	not found
10	GTGGACACAGCCA	101	500	IGHV2-70_12	IGHJ4_02	0.50	48.75	N/A	Y	Y	100.00	GCACACATCGCGC

Figure 6: This table, generated via the LymphoTrack 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 *IGH* FR3 Assay –MiSeq and analyzed using the LymphoTrack Software – MiSeq (REF 75000009).

12. Troubleshooting Guide

Table 18. 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	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 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 Software does not install properly	Call Invivoscribe Tech Support +1-858-224-6600
Data analysis	LymphoTrack 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, Q30 score may vary depending on sample quality. If Q30 falls below Illumina Q30 specification, check the index Q30 value from the LymphoTrack Report after data analysis by the LymphoTrack Software - MiSeq. If an index Q30 score on the LymphoTrack Report does not meet the Illumina specification, consider that index to be invalid

.

13. Technical and Customer Service

Thank you for purchasing our LymphoTrack *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

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

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2. Ghia, P. *et al.* (2007). ERIC recommendations on *IGHV* gene mutational status in chronic lymphocytic leukemia. *Leukemia* 21, 1–3.
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6. Montserrat, E. (2005). Treatment of Chronic Lymphocytic Leukemia: Achieving Minimal Residual Disease–Negative Status As a Goal. *Journal of Clinical Oncology*, 23(13), pp. 2884–2885.
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8. Campana, D. *et al.*, (2012). Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. *Blood* 120:5173-5180.

- LymphoTrack Software – MiSeq Package Instructions for Use (REF 75000009)
- https://www.beckmancoulter.com
- http://www.illumina.com
- http://www.invitrogen.com
- http://www.kapabiosystems.com
- http://www.thermofisher.com

15. Symbols

The following symbols are used in Invivoscribe NGS product labeling.

REF	Catalog Number		Expiration Date
VOL	Reagent Volume	EC REP	Authorized Representative in the European Community
LOT	Lot Number		Manufacturer
	Storage Conditions		Consult Instructions for Use

16. 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.

Use of this product may require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). Any necessary license to practice amplification methods or to use reagents, amplification enzymes or equipment covered by third party patents is the responsibility of the user and no such license is granted by Invivoscribe, Inc., expressly or by implication.

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

17.1. Using gloved hands, remove the Master Mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.

17.2. 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.

17.3. Add 0.2 µL Taq DNA polymerase (@5 U/µL) to each Master Mix.

17.4. 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.

17.5. 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.

17.6. 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

17.7. Remove the amplification plate from the thermal cycler.

17.8. 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.

17.9. Quantify amplicons using the KAPA library quantification kit according to the kit instructions. Dilute amplicons 1:4,000 before proceeding to qPCR.

17.10. 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.

17.11. Denature and dilute the library to 12 pM for MiSeq reagent kit v2 and 12 - 20 pM for MiSeq reagent kit v3 (MCS 2.6 or later).

17.12. Load 600 µL of denatured and diluted library on the MiSeq Reagent Cartridge.

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

17.14. Start the MiSeq run.

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

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

This appendix contains instructions for multiplexing any combination of LymphoTrack *IGHV* Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK, TRB* and *TRG* Assays together into a single sequencing library. 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 Table 19 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 7 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	Catalog #
Only *IGH* FR3 and *TRG* together	151 cycles Read 1 151 cycles Read 2	v2 kit (300 cycle) or v2 kit (500 cycle)	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)	MS-102-2003 or MS-102-3003
When combining any of the assays together with: *IGHV* Leader	301 cycles Read 1 301 cycles Read 2	v3 kit (600 cycle)	MS-102-3003

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

18.2. 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* 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 18.3.

Table 20. Calculation of Individual Library Inputs to Generate a Multi-Targeted Sequencing Library for the MiSeq Run

Library		Individual Library Volume (µL)						
			Case A	Case B	Case C	Case D	Case E	Case F
Assay Name	Concentration (nM)		n=7	n=6	n=5	n=4	n=3	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

18.3. Denature the combined libraries to 2 nM.

- Add reagents according to Table 21 based on the amount determined from 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 Table 21.

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.

18.4. 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.

18.5. 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 (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**

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