LymphoTrack®
IGH Assays

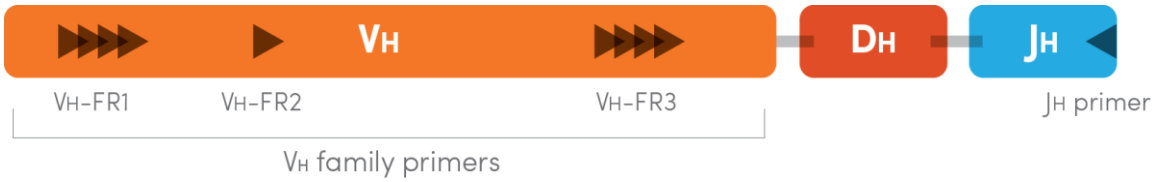
Instructions for Use

LymphoTrack® *IGH* (FR1, FR2, & FR3) Assays – MiSeq®

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

For Research Use Only. Not for use in diagnostic procedures.

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



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invivoscribe

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

Catalog #	Products	Quantity
REF 7-121-0009	LymphoTrack *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF 7-121-0039	LymphoTrack *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 reactions each
REF 7-121-0089	LymphoTrack *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF 7-121-0099	LymphoTrack *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 reactions each
REF 7-121-0109	LymphoTrack *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 reactions each
REF 7-121-0119	LymphoTrack *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 reactions each
REF 7-121-0129	LymphoTrack *IGH* FR1/2/3 Assay Kit A – MiSeq	8 indices per FR region – 5 reactions each
REF 7-121-0139	LymphoTrack *IGH* FR1/2/3 Assay Panel – MiSeq	24 indices per FR region – 5 reactions each

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1. Assay Uses

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 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.

2. Summary and Explanation of the Test

2.1. Background

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

Lymphoid cells are different from the other somatic cells in the body. During development, the antigen receptor genes in lymphoid cells undergo somatic gene rearrangements (Tonegawa S, *et al*., 1983). 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, all leukemias and lymphomas 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, 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 (Ghia P, *et al*., 2007).

Initially, clonal rearrangements were identified using Restriction Fragment, Southern Blot Hybridization (RF-SBH) techniques. However, these tests proved cumbersome and labor-intensive, they 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 (Trainor K.J., *et al*., 1990), and are considered the current gold standard method. PCR-based assays identify clonality on the basis of over-representation of amplified V_H - D_H - J_H (or

incomplete D_H - J_H products) gene 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.

2.2. Summary

The LymphoTrack *IGH* (FR1, FR2, & 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 products have two important and complementary uses: they provide critical information on the existence of clonality and identify sequence information required to track those clones in subsequent samples. The LymphoTrack *IGH* FR1 Assay additionally provides detailed sequence information on the degree of SHM.

Each single multiplex master mix for *IGH* targets one of the conserved 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 (Evans PA, *et al*., 2007).

Primers included in the master mixes are designed with Illumina adapters and up to 24 different indices. These assays allow for a one-step PCR reaction 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 24 samples per target to be analyzed in parallel in a single run.

The associated 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 (Cat#: 4-088-0008).

Note: For a more thorough explanation of the locus and the targeted sequencing strategy, please refer to (Miller J.E., 2013).

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 framework of the V_H regions and the conserved J_H regions of antigen receptor genes. These conserved regions, where primers target, lie on either side of an area where programmed genetic rearrangements occur during the maturation of all B and T lymphocytes. It is a result of these genetic rearrangements that different populations of the B and T lymphocytes arise.

The antigen receptor genes that undergo rearrangements are the immunoglobulin heavy chain (*IGH*) and light chains (*IGK* and *IGL*) in B cells, and the T cell receptor genes (*TRA*, *TRB*, *TRG*, *TRD*) in T cells. Each B and T cell has a single productive V - J rearrangement that is 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, reflecting the heterogeneous population, are generated. In some cases, where lymphocyte DNA is not present, no amplicons will be generated. For samples containing clonal populations, the yield is one or two prominent amplified products of the same length and sequence that

are detected with significant frequency of occurrence, within a diminished polyclonal background amplified at a lower frequency.

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 amplicons that are 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 massively parallel sequencing approaches, are often referred to as Next-Generation Sequencing (NGS). NGS technologies can use various combination strategies of template preparation, sequencing, imaging, and bioinformatics for genome alignment and assembly.

NGS technologies used in this product 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 emitted when fluorescently labeled nucleotides are added to the sequencing strand. 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

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, up to 24. 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. Later, the resulting sequences can be sorted by the bioinformatics software to identify 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 MiSeq kits such as

IGHV Leader, *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK* 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* and *TRG* amplicons together, the MiSeq v2 (500 cycle) or v3 (600 cycle) sequencing kit should be used. When multiplexing any of these amplicons together with *IGHV* Leader, the MiSeq v3 (600 cycle) sequencing kit should be used. If multiplexing only *IGH* FR3 and TRG amplicons together, which both have shorter amplicon sizes, MiSeq v2 (300 or 500 cycle) sequencing kits can be used, but the cycle settings must be adjusted in the sample sheet. For further instructions, please refer to Appendix A.

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 can generate 12-15 million reads. To determine the number of reads per sample, the total number of reads for the flow cell should be divided 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 - MiSeq can be used; however, this assay only targets a portion of the *IGHV* region. 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

For additional information related to how to reach a desired level of sensitivity for MRD studies using the LymphoTrack Assays, please refer to the technical bulletin: Study of MRD – Using LymphoTrack Assays – MiSeq. This technical bulletin can be found on the RUO LymphoTrack Instructions for Use CD (Part number 261406).

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Cat#	Description	# of Indexed Master Mixes	Total Reactions
REF 7-121-0009	LymphoTrack® *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 7-121-0039	LymphoTrack® *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 7-121-0089	LymphoTrack® *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 7-121-0099	LymphoTrack® *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 7-121-0109	LymphoTrack® *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 7-121-0119	LymphoTrack® *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120
REF 7-121-0129	LymphoTrack® *IGH* FR1/2/3 Assay Kit A – MiSeq	(8+8+8) indices – 5 sequencing runs each	40+40+40
REF 7-121-0139	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 Components

Reagents	Reagent Components	Unit Quantity	7-121-0009 # of Units	7-121-0039 # of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR1 MiSeq 01	250 µL	1	1	-65 ºC to -85 ºC	N/A
	IGH FR1 MiSeq 02		1	1		
	IGH FR1 MiSeq 03		1	1		
	IGH FR1 MiSeq 04		1	1		
	IGH FR1 MiSeq 05		1	1		
	IGH FR1 MiSeq 06		1	1		
	IGH FR1 MiSeq 07		1	1		
	IGH FR1 MiSeq 08		1	1		
	IGH FR1 MiSeq 09		0	1		
	IGH FR1 MiSeq 10		0	1		
	IGH FR1 MiSeq 11		0	1		
	IGH FR1 MiSeq 12		0	1		
	IGH FR1 MiSeq 13		0	1		
	IGH FR1 MiSeq 14		0	1		
	IGH FR1 MiSeq 15		0	1		
	IGH FR1 MiSeq 16		0	1		
	IGH FR1 MiSeq 18		0	1		
	IGH FR1 MiSeq 19		0	1		
	IGH FR1 MiSeq 20		0	1		
	IGH FR1 MiSeq 21		0	1		
	IGH FR1 MiSeq 22		0	1		
	IGH FR1 MiSeq 23		0	1		
	IGH FR1 MiSeq 25		0	1		
	IGH FR1 MiSeq 27		0	1		
Positive Control DNA	*IGH* POS (+) (Cat#: 4-088-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA

Reagents	Reagent Components	Unit Quantity	7-121-0009 # of Units	7-121-0039 # of Units	Storage Temperature	Notes
Negative Control DNA	*IGH* NEG (-) (Cat#: 4-092-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	Tonsil DNA, highest sequence frequency can vary between lots

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

Table 3. LymphoTrack *IGH* FR2 Assay Kit Components

Reagents	Reagent Components	Unit Quantity	7-121-0089 # of Units	7-121-0099 # of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR2 MiSeq 01	250 µL	1	1	-65 ºC to -85 ºC	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 (+) (Cat#: 4-088-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA
Negative Control DNA	*IGH* NEG (-) (Cat#: 4-092-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	Tonsil DNA, highest sequence frequency can vary between lots

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

Table 4. LymphoTrack *IGH* FR3 Assay Kit Components

Reagents	Reagent Components	Unit Quantity	7-121-0109 # of Units	7-121-0119 # of Units	Storage Temperature	Notes
Master Mixes‡	*IGH* FR3 MiSeq 01	250 µL	1	1	-65 ºC to -85 ºC	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 (+) (Cat#: 4-088-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA
Negative Control DNA	*IGH* NEG (-) (Cat#: 4-092-0009)	45 µL	1	3	2 ºC to 8 ºC or -65 ºC to -85 ºC	Tonsil DNA, highest sequence frequency can vary between lots

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

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

FR1 Master Mixes‡	FR2 Master Mixes‡	FR3 Master Mixes‡	Unit Quantity	7-121-0129 # of Units per FR	7-121-0139 # 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	7-121-0129 # of Units	7-121-0139 # of Units
IGH POS (+) (Cat#: 4-088-0009)	*IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA	45 µL	2	6
IGH NEG (-) (Cat#: 4-092-0009)	Tonsil DNA, highest sequence frequency can vary between lots	45 µL	2	6

Note‡: The storage temperature for master mixes is -65 ºC to -85 ºC.
Note*: The storage temperature for controls is 2 ºC to 8 ºC or -65 ºC to -85 ºC.
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

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

- The assay kits should be used 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. Care should be taken to ensure use of correct thermocycler programs, 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.
- All laboratory procedures should be performed with standard personal protective equipment (gloves, laboratory coats and protective eye wear). Laboratory personnel should follow good laboratory practices and universal precautions when working with specimens. Specimens should be handled in approved biological safety containment facilities and opened only in certified biological safety cabinets. It is recommended that molecular biology grade water be used with the preparation of specimen DNA.
- Due to the high analytical sensitivity of this test, extreme care should be taken to avoid any contamination of reagents or amplification mixtures with samples, controls, or amplified materials. All reagents should be closely monitored 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.
- The work flow in the PCR laboratory should be uni-directional between the separate work areas: beginning with master mix preparation, moving 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.
- All pipettes, pipette tips, and any equipment used in a particular area should be dedicated to and not removed from that area of the laboratory.
- Non-disposable items must be decontaminated in 10% bleach and rinsed with distilled water two separate times before returning them to the starting areas.
- Sterile, disposable plasticware should be used whenever possible to avoid contamination.

4.3. Storage and Handling

- If the assay is not being used immediately, store at **-65 ºC to -85 ºC**.
- The optimum storage temperature for DNA controls is 2 ºC to 8 ºC, but DNA can also be stored at -65 ºC to -85 ºC.
- 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. The number of cycles should be limited to a maximum of five 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

5.1. Thermocycler

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

5.2. Magnetic Stand

- Use or Function: Purification of PCR products
- Suggested Instrument: Ambion® Magnetic Stand 96 (AM10027) or Agencourt SPRIPlate® 96 Ring Super Magnet Plate (Agencourt # A32782)
- 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:
  - Work with MiSeq Reagent Kit v2
  - Work 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.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. All specimens should be handled 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

Samples should be stored using a method that prevents degradation of DNA.

7. Assay Procedure

7.1. Materials Provided

See Tables 2 - 5 for materials provided in each kit.

7.2. Materials Required But Not Provided

Table 6. Materials Not Provided

Reagent/Material	Required or Recommended Reagents/Suppliers	Catalog #	Notes
DNA Polymerase	Roche: EagleTaq™ DNA Polymerase Or Applied Biosystems: AmpliTaq Gold® DNA Polymerase or Equivalent	05206944190 Or N808-0241	5 U/µL
Molecular Biology Grade Water	N/A	N/A	Water should be sterile and free of DNases and RNases.
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 (5 mL for 100 samples)	A63880	N/A
PCR Purification	Thermo Fisher Scientific: Ambion Magnetic Stand 96 or Beckman Coulter: Agencourt SPRIPlate 96 Ring Super Magnet Plate	AM10027 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 v2 kit (500 cycles) should be used.	MS-102-2003 Or MS-102-3003 MS-102-2002 or MS-102-2003	N/A
MiSeq Software	MiSeq Control Software v2.3 or v2.5	N/A	Illumina Experiment Manager v.1.4 or higher
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 (Cat#: 2-096-0021 for ABI detection or Cat#: 2-096-0020 for gel detection). The Specimen Control Size Ladder targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400, and 600 basepairs. This is especially important for DNA from challenging specimens, *e.g.*, FFPE tissue.

The **positive and negative controls should always be used** for each target to ensure the assay has been performed correctly.

A **no template control (NTC) should always be set up** for each target 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 of the Master Mix and one Master Mix per sample). Each run should include two controls (one positive and one negative), and one NTC. For the NTC, use molecular biology grade water as template instead of DNA.

7.3.3. Add 0.2 µL of AmpliTaq Gold or EagleTaq DNA polymerase (Taq at 5 U/µL per 50 µL total PCR reaction volume) 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 take it to the PCR thermocycler.

Table 7. Reaction Setup

Reagent	Volume
Master Mix	45 µL
AmpliTaq Gold or EagleTaq DNA polymerase	0.2 µL
Sample or Control DNA	5 µL
Total Volume	50.2 µL

7.4. Amplification

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

Table 8. PCR Program

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

7.4.2. Remove the amplification plate from the thermocycler. If not immediately proceeding to the next steps, amplified PCR products can be stored at 4 °C for 1 day.

7.5. AMPure XP Purification

Purify the PCR products from samples, positive and negative controls, and no template controls using the Agencourt AMPure XP PCR Purification system.

Preparation:

7.5.1. Prepare a fresh stock (0.5 mL for each sample to be purified) of 80% ethanol using sterile water. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.

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)

Binding of Amplicons to Magnetic Particles:

7.5.3. 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.

7.5.4. 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 7.5.9 below.)**

7.5.5. 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:

7.5.6. 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.

7.5.7. Repeat Step 7.5.6.

7.5.8. With the plate still on the magnetic stand, allow the magnetic particles to air-dry for 5 minutes.

Elution:

7.5.9. 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.

7.5.10. Incubate at room temperature for 2 minutes.

7.5.11. Place the plate on the magnetic stand for 5 minutes or until the supernatant has cleared.

7.5.12. 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.

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

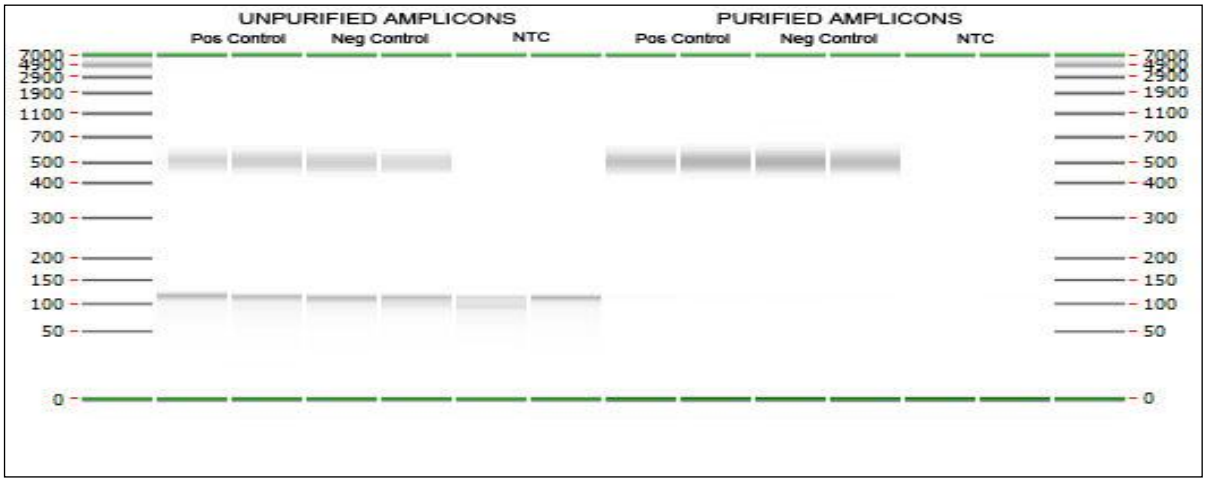

Figure 1: Example of a purification result for amplicons from the *IGH* FR1 Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

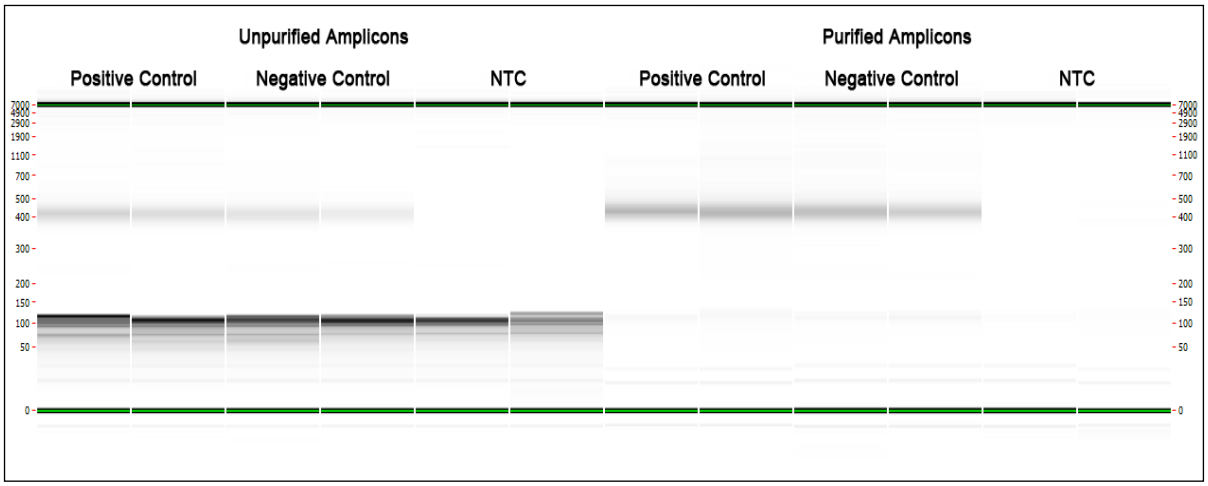

Figure 2: Example of a purification result for amplicons from the *IGH* FR2 Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

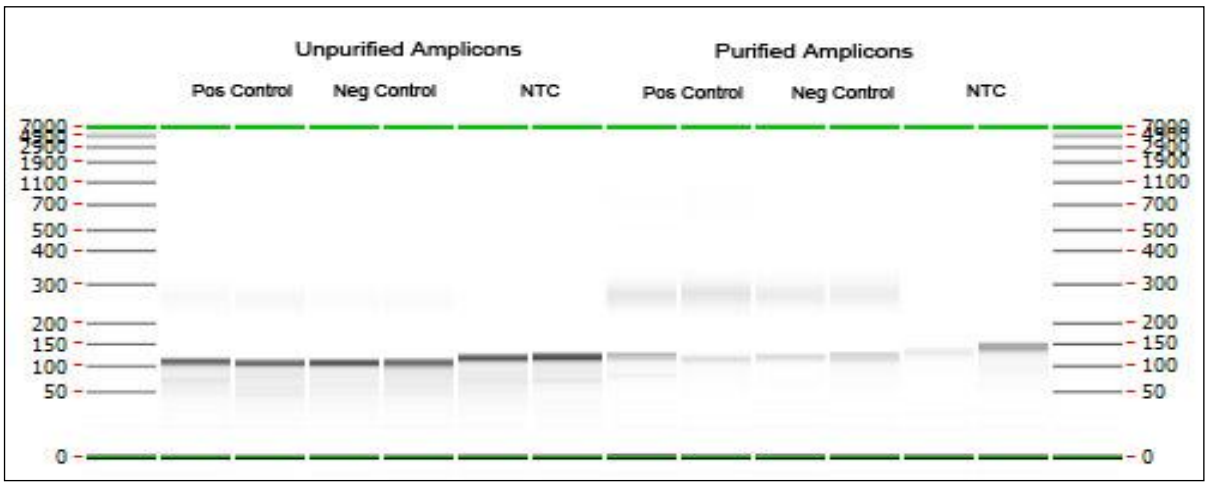

Figure 3: Example of a purification result for amplicons from the *IGH* FR3 Master Mixes. The image was generated by running unpurified and purified products on the LabChip GX.

7.6. Quantification of Amplicons

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

7.6.1. Dilution of amplicons

(1: 4,000 Final):

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

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

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

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

Table 9. qPCR Setup

Reagent	Volume
PCR-grade water	3.6 µL
KAPA SYBR FAST qPCR Master Mix containing Primer Premix	12.0 µL
ROX	0.4 µL
Diluted amplicons or Standard (1-6)	4.0 µL
Total Volume	20 µL

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

Table 10. qPCR Program

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

7.6.4. Use the data from the qPCR run to determine the concentration of amplicons by calculating the ΔCt values between each sample and the NTC from the qPCR run, using the following equation (Typically the ΔCt values should be greater than 4.0):

$$\Delta Ct = Ct\ (NTC) - Ct\ (Sample)$$

7.6.5. Use the following equation to determine the concentration for each sample:

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

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 for obtaining high-quality data in a sequencing run. **Quantification of the library by qPCR is strongly recommended.**

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

7.7.1. Based on the amplicon concentration calculated from the qPCR results, add an equal amount of amplicons (with the exception of NTC, which can be excluded), *e.g.*, combine 4 nM of each amplicon (generated from the same LymphoTrack Assay) in a total volume of 10 µL using Dilution Buffer A as diluent. For the 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 9 for qPCR setup and Table 10 for the thermal program.

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

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

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 should be **12 pM for MiSeq reagent kit v2** and **12 - 20 pM for MiSeq reagent kit v3** with MiSeq Control Software (MCS v2.3, v2.5, or v2.6). For multiplexing amplicons from different LymphoTrack MiSeq Assays into a single library, please refer to Appendix A.

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 denaturating sample DNA and for optimal cluster generation on the MiSeq.
- Add 10 µL of 0.2 N NaOH to the diluted library (10 µL) prepared in the previous step.

Table 11. Library denaturation

Reagent	Volume
Diluted library	10 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 12. 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 you are ready to proceed.

7.10.7. Prepare the library to be loaded on the MiSeq.

For MiSeq Control Software (MCS v2.3, 2.5, or 2.6):
The concentration of the library DNA should be 12 pM for MiSeq reagent kit v2
The concentration of the library DNA should be 12 to 20 pM for MiSeq reagent kit v3

- For the 4 nM libraries, dilute the denatured ssDNA to the desired concentration using the following examples:

Table 13. 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	0.75	1.0

- For the less than 4 nM libraries, dilute the denatured DNA to 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 you are ready to load it onto the MiSeq Reagent Cartridge.

7.11. MiSeq Flow Cell Loading

7.11.1. Load 600 µL of the Final Prepared Library onto a MiSeq Reagent Cartridge.

7.12. MiSeq Sample Sheet Setup

Set up a MiSeq Sample Sheet Using the Illumina Experiment Manager (This can be downloaded from www.illumina.com).

Characters in the sample name:

Give each sample a unique name or identifier when naming samples.
If you are running samples in duplicate for example, you can name duplicates Sample1a and Sample1b.
Please make sure to only use these characters when setting up the Sample Sheet (A-Z, a-z, 0-9, ., _,-,) and no more than one consecutive space is permitted.

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 gets incorporated into the FASTQ file).

It is recommended to 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 20 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)

Note: Failure to provide unique names to samples that will be run together on the same flow cell will result in only one sample being analyzed by the LymphoTrack Software - MiSeq during the analysis process.

7.12.1. Open Illumina Experiment Manager v1.4 or higher version.

7.12.2. Select '**Create Sample Sheet**'.

7.12.3. In the *Instrument Selection* Page, Click '**MiSeq**', then '**Next**'.

7.12.4. In the *MiSeq Workflow Selection* Page, Select '**Other**' for *Category*, '**FASTQ only**' for *Application*, then '**Next**'.

7.12.5. In the Workflow Parameters Page,

- Type in the bardcode in the *MiSeq Reagent Cartridge Barcode* box

7.12.6. Select '**TruSeq LT**' in the *Assay* Box

- Select '**1**' for the *Index Reads*
- Fill in the information for the Experiment Name, Investigator Name, Description and Date
- Select '**Paired End**' for *Read Type*
- Select '**251**' for *Cycles Read 1*
- Select '**251**' for *Cycles Read 2*

When running the ***IGH* FR3** assay by itself, or multiplexing only the ***IGH* FR3** and ***TRG*** assays together, please use the cycle settings listed below due to these targets' shorter amplicon sizes:

- Select '**151**' for Cycles Read 1
- Select '**151**' for Cycles Read 2

- Make sure '**Use Adaptor Trimming**' is checked
- Make sure '**Use Adaptor Trimming Read 2'** is checked
- Select '**Next**'

7.12.7. In the Sample selection page,

- Uncheck '**Maximize**'
- Select '**New Plate**' for the *Sample Plate*

7.12.8. In the Popped-up *Assay Parameters* page,

- Fill in the information for the *Unique Plate Name* Box
- Select '**1**' for *Index Reads*
- Select '**Next**'

7.12.9. In the Popped-up *Plate Samples* page,

- Fill in the information for the *Sample ID* column
- Select '**A0XX**' for *Index1 (I7)* column (XX should be correlated to the Master Mix id, *e.g.*, if Master Mix id is '**01**', select '**A001**')
- Select '**Finish**'
- Save the file
- Select '**Select All**' on the *Sample Plate*
- Select '**Add Selected Samples =>**' on the *Sample Plate*
- Select '**Finish**'

Important! Adaptor sequences are not recognized by the LymphoTrack Software – MiSeq.
☑ Adaptor trimming must be selected when creating the sample sheet.

7.12.10. Save the file. Make sure the file name is the MiSeq Reagent Cartridge barcode that is on the Reagent Cartridge for the current MiSeq run, *e.g.*, MS200xxxx-500v2. (This .csv file will be used for the MiSeq run).

- Copy this file to MiSeq Control Software sample sheet folder.

7.13. MiSeq Run Start

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

Table 14. MiSeq Run Times

MiSeq Reagent Kit	Read Length	MCS Version	Total MiSeq Run Time
v2	2x250 bp	2.3, 2.5, or 2.6	~ 39 hours
v3	2x300 bp	2.3	~ 65 hours
	2x300 bp	2.5 or 2.6	~ 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 LymphoTrack Software – MiSeq v2.1.1 package provided on the associated CD (Cat#: 7-500-0009). **This CD includes detailed instructions for installation and use of the software package, as well as two essential tools for analysis:**

- LymphoTrack Data Analysis (run with Microsoft Windows®)
- LymphoTrack Data Visualization (run with Microsoft Excel®)

The MiSeq sequencing run of samples prepared with LymphoTrack *IGH* (FR1, FR2, and/or FR3) Assays provides FASTQ files that can be easily processed into fully analyzed data using the LymphoTrack Data Analysis – MiSeq Application.

Characters in pathname and file name:

1) Spaces in the pathname for the data files or software (pathnames include file folders and file names) should be avoided and more than one consecutive space is not permitted.
2) It is important that the file names only contain the following characters (A-Z, a-z, 0-9, ., _ (underscore), - (hyphen)). If the software encounters a character not within this set, it may fail. Please make sure to only use these characters when setting up the Sample Sheet.

The data output can then be visualized in Microsoft Excel using the LymphoTrack Visualization target-specific application. The following reports are generated:

- *Read Summary*
- *Merged Read Summary**
- *VJ Sequence Frequency Graph*
- *VJ Usage*
- *VJ Usage Percent Graph*
- *VJ Usage Raw Graph*
- *VJ Sequence Frequency*
- *Unique Reads*

Merged Read Summary. Due to the amplification and sequencing error rates found within NGS technology, it is recommended to merge reads and their frequencies prior to clonality determination. This report has the same format as the *Read Summary,* but only includes the top 10 reads from the *Read Summary* merged with reads in the top 500 sequences. A read will merge with another if they are only 1 or 2 nucleotides different from each other. The read with the higher count maintains its sequence identity, while the read with the lower count is added to the higher one.

9. Expected Values

- *IGH* Positive Control top % reads ≥ 2.5%
- *IGH* Negative Control top % reads < 1.0%
- *IGH* SHM Positive Control (Cat#: 4-088-0008, can be purchased separately) top % reads ≥ 2.5%
- MiSeq Run Validity Q30 ≥ 80% for v2 (2x150); Q30 ≥ 75% for v2 (2x250); Q30 ≥ 70% for v3 (2x300)

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. The Research Use Only LymphoTrack *IGHV* Leader Somatic Hypermutation Assay for the Illumina MiSeq (Cat#: 7-121-0059 or 7-121-0069) can be purchased separately if complete *IGHV* sequencing is required.

11. Sample Data

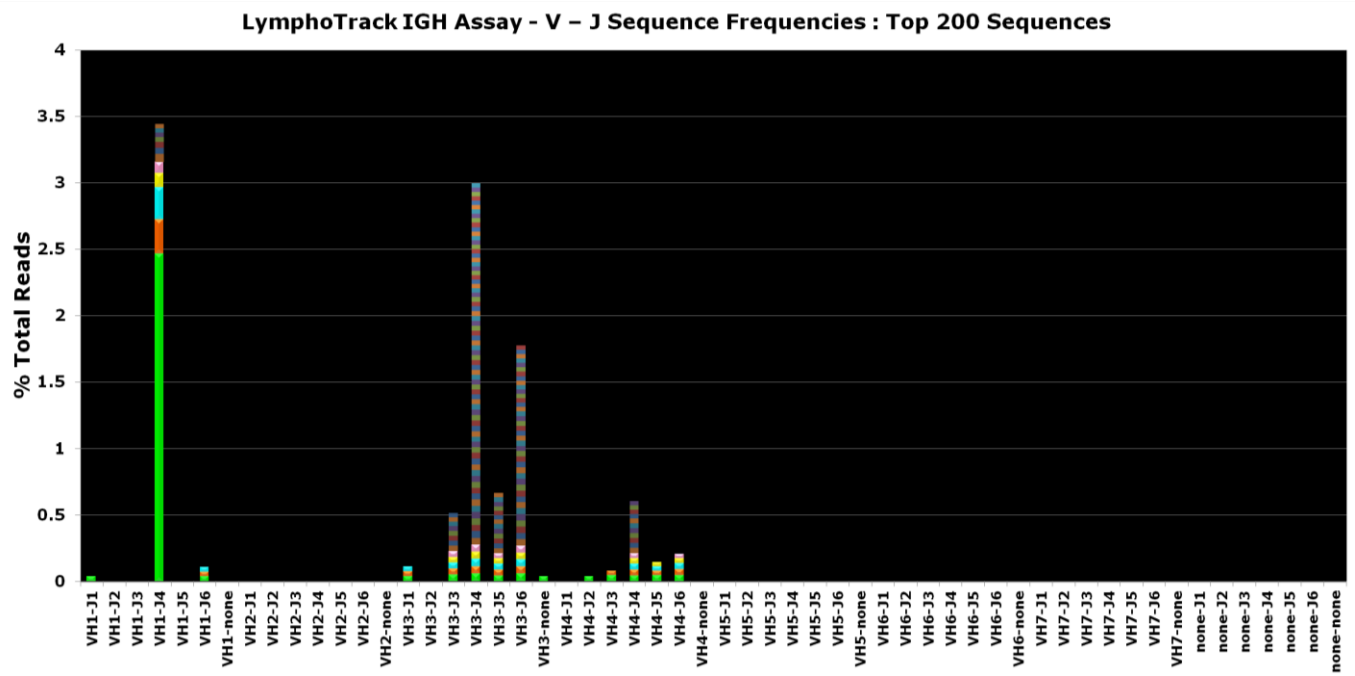

Figure 4: An example of a positive sample showing evidence for clonality. Sequences were generated using the **LymphoTrack *IGH* FR1 Assay – MiSeq.** The percentage of total reads is shown on the y-axis, and each V-J rearrangement is represented along the x-axis. Each color within the bars of the histogram represents a unique DNA sequence. FASTQ files were analyzed by the LymphoTrack Software – MiSeq (Cat#: 7-500-0009) package. The graph title specifies the gene targeted but does not include the framework. This information can be located in the Read Summary tab within the data file location. The data output folder specifies the gene and framework region analyzed by the software.

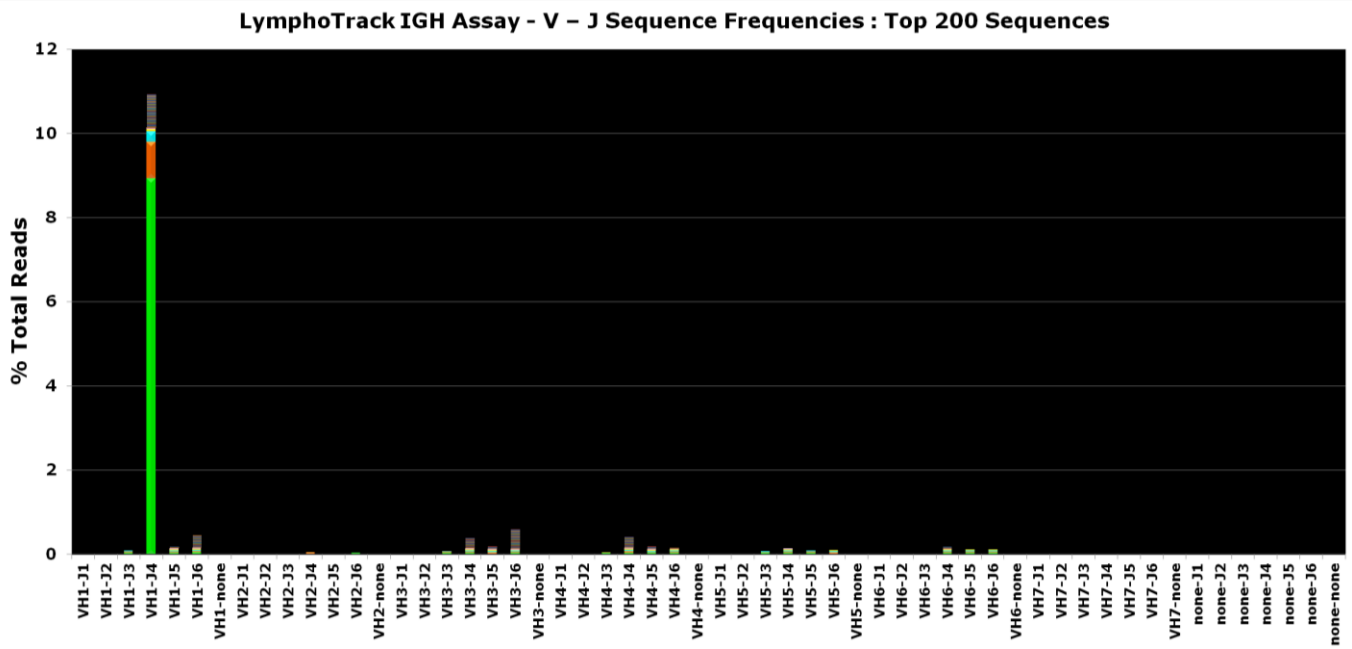

Figure 5: An example of a positive sample showing evidence for clonality. Sequences were generated using the **LymphoTrack *IGH* FR2 Assay – MiSeq**. The percentage of total reads is shown on the y-axis, and each V-J rearrangement is represented along the x-axis. Each color within the bars of the histogram represents a unique DNA sequence. FASTQ files were analyzed by the LymphoTrack Software – MiSeq (Cat#: 7-500-0009) package. The graph title specifies the gene targeted but does not include the framework. This information can be located in the Read Summary tab within the data file location. The data output folder specifies the gene and framework region analyzed by the software.

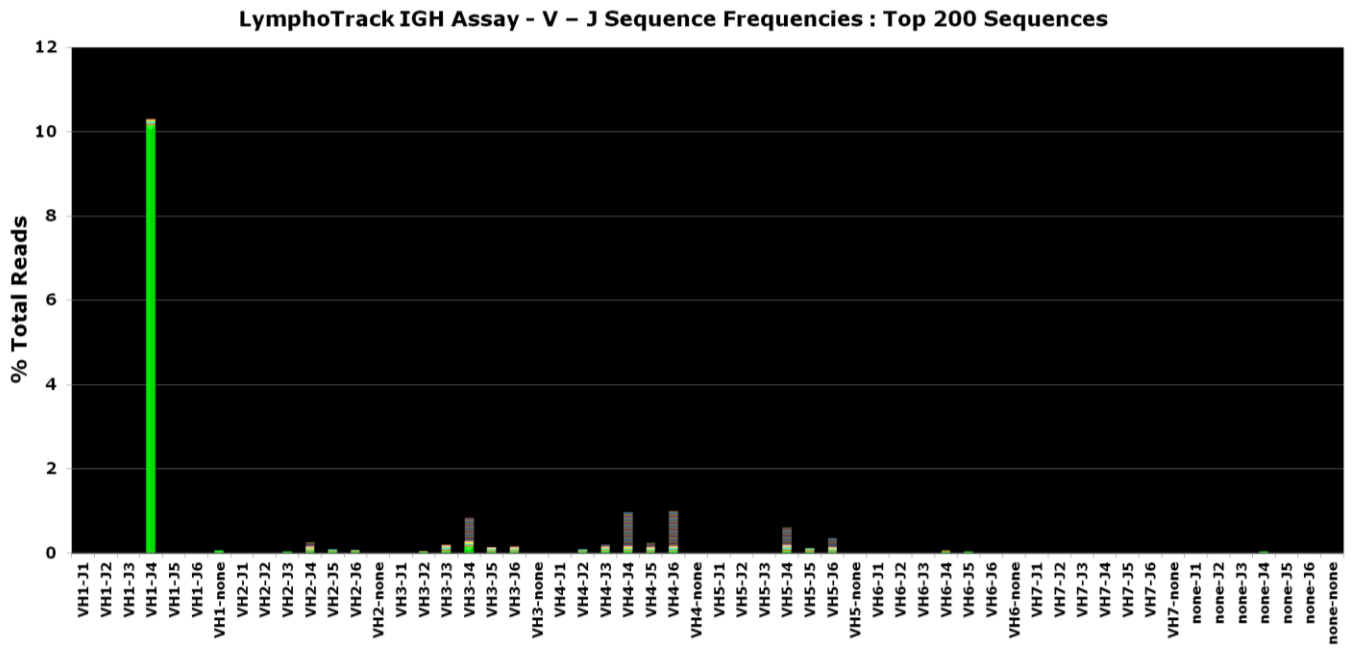

Figure 6: An example of a positive sample showing evidence for clonality. Sequences were generated using the **LymphoTrack *IGH* FR3 Assay – MiSeq**. The percentage of total reads is shown on the y-axis, and each V-J rearrangement is represented along the x-axis. Each color within the bars of the histogram represents a unique DNA sequence. FASTQ files were analyzed by the LymphoTrack Software – MiSeq (Cat#: 7-500-0009) package. The graph title specifies the gene targeted but does not include the framework. This information can be located in the Read Summary tab within the data file location. The data output folder specifies the gene and framework region analyzed by the software.

12. Troubleshooting Guide

Table 15. 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 (Cat#: 2-096-0021 for ABI detection or Cat#: 2-096-0020 for gel detection)
Quantification of amplicons using the KAPA library quantification kit	ΔCt=Ct (NTC) – Ct (Sample) < 4.0	Check the standard curve in qPCR Check for contamination and repeat the PCR and the KAPA qPCR
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 (2x150) Q30 < 75% for v2 (2x250) Q30 < 70% for v3 (2x300)	Call Illumina Tech Support +1-800-809-4566
CD installation	LymphoTrack Software does not install properly	Call Invivoscribe Tech Support +1-858-224-6602
Data analysis	LymphoTrack Software stops running	Call Invivoscribe Tech Support +1-858-224-6602
Data analysis	Excel Macro cannot be executed	Call Invivoscribe Tech Support +1-858-224-6602
Data analysis	No clonal sequence is detected for the Positive Control	Call Invivoscribe Tech Support +1-858-224-6602
No Template Control (NTC)	NTC shows amplification after PCR	Repeat the assay

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.

Manufacturer

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- Invivoscribe LymphoTrack Software - MiSeq Package Instructions for Use (Cat# 7-500-0009)
- 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 now used in labeling for Invivoscribe NGS diagnostic products.

	Catalog Number		Expiration Date
	Reagent Volume		Authorized Representative in the European Community
	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 Technologies, Inc. (IVS). United States Patent No. 7,785,783, United States Patent 8859748 B2 and other pending applications originating from the United States Patent Application Number 10/531,106, European Patent Number EP 1549764B1 and other pending patent applications originating from European Patent Application Numbers 03756746.8 and 047326551.9 (16 countries), Japanese Patent Number JP04708029B2, Japanese Patent Application Number 2006-529437, Brazil Patent Application Number PI0410283.5, Canadian Patent Application Number 2525122, Indian Patent Application Number 5792/DELNP/2005, Mexican Patent Application Number PA/a/2005/012102, Chinese Patent Application Number 200480016603.5, and Korean Patent Application Number 10-2005-7021561.

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 Technologies, Inc., expressly or by implication.

17. Single Page Flow Chart

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 of the Master Mixes and one Master Mix per sample, positive, negative, or no template controls.

17.3. Add 0.2 µL AmpliTaq Gold or EagleTaq DNA polymerase (Taq @ 5 U/µL per 50 µL total PCR reaction volume) to each of the Master Mixes.

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 thermocycler 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	'forever'	1

17.7. Remove the amplification plate from the thermocycler.

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 v2.3, 2.5, or 2.6).

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

17.13. Set up a MiSeq sample sheet using the Illumina Experiment Manager (This can be downloaded from www.illumina.com)

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* and *TRG* Assays together into a single sequencing library. Please refer to the following table to determine the cycle settings and Illumina MiSeq Reagent kits to be used with different combinations of targets.

Table 16. 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 MS-102-2003
Any other combination of these targets together: *IGH* FR1, *IGH* FR2, *IGH* FR3, *IGK*, *TRG*	251 cycles Read 1 251 cycles Read 2	v2 kit (500 cycle) or v3 kit (600 cycle)	MS-102-2003 MS-102-3003
When combining any of the above assays together with: *IGHV* Leader	301 cycles Read 1 301 cycles Read 2	v3 kit (600 cycle)	MS-102-2003

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

18.2. Determine the amount of each library to be denatured

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

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

Note: The 40 fmole value corresponds to the 20 µL of 2 nM at the end of step 18.3.

Table 17. 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
Assay Name	Concentration (nM)		n=6	n=5	n=3	n=2
IGHV Leader	2.3	U	2.9	3.5		
IGH FR1	1.5	V	4.4	5.3	8.9	
IGH FR2	4.0	W	1.7	2.0	3.3	
IGH FR3	2.1	X	3.2	3.8	6.3	
IGK	3.5	Y	1.9	2.3		5.7
TRG	2.6	Z	2.6			7.7
		U+V+W+X+Y+Z	16.7	16.9	18.5	13.4

18.3. Denature the combined libraries to 2 nM.

- Add reagents according to Table 18 based on the amount determined from the previous step.

If U+V+W+X+Y+Z >18, such as in case C from Table 17, mix the applicable libraries first, then add 18 µL to the denaturing reaction as shown in the following table.

Table 18. Library denaturation

Reagent	Volume (µL)
IGHV Leader Library	U
IGH FR1 Library	V
IGH FR2 Library	W
IGH FR3 Library	X
IGK Library	Y
TRG Library	Z
1N NaOH	2
10 mM Tris-HCl pH 8.0, 0.05% Tween 20	18 – (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.

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 v2.3, v2.5, or v2.6) by following the table below. Vortex briefly to mix and pulse centrifuge the sample.

Table 19. Preparation of the combo library for MiSeq loading

Reagent	Volume (µL)	
	12 pM	20 pM
40 pM library	300	500
Chilled HT1 Buffer	700	500
Total	**1000**	**1000**

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