

Instructions for Use

LymphoTrack[®] TRG Assay – MiSeq[™]

Identify and track clonal T-cell receptor gamma (*TRG*) 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 *TRG* gene locus:



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 72270019	LymphoTrack TRG Assay Kit A – MiSeq	8 indices – 5 reactions each
REF 72270009	LymphoTrack TRG Assay Panel – MiSeq	24 indices – 5 reactions each

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1. Assay Use

This Research Use Only assay identifies clonal *TRG* V γ -J γ rearrangements, the associated V γ -J γ region DNA sequences, and provides the frequency distribution of V γ region and J γ region segment utilization using the Illumina MiSeq platform.

2. Summary and Explanation of the Test

2.1. Background

The human T-cell receptor gamma (*TRG*, previously known as *TCRG*) gene locus on chromosome 7 (7q14) includes 14 V γ (variable region) genes (Group I, II, III, and IV), 5 J γ (joining region) gene segments, and 2 C γ (constant region) genes spread over 200 kilobases.

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 T-cell development genes encoding the *TRG* molecules are assembled from multiple polymorphic gene segments that undergo rearrangements and selection, generating V γ -J γ 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 *TRG* clonal rearrangements can be useful in the study of B- and T-cell malignancies.

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 Morley² and are considered the current gold standard method. These assays identify clonality on the basis of over-representation of amplified V-D-J (or incomplete D-J) products 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 that may differ in sequence but are the same size, nor can they detect multiple rearrangements that might lie beneath a single-sized peak, and are not designed to identify the specific V-J DNA sequence that is required to track clonal populations in subsequent analyses. This second limitation can be of particular importance, as once the unique clone-specific DNA sequence is identified, the sequence can be used in subsequent tests to track and follow the clonal cell populations.

2.2. Summary

This LymphoTrack *TRG* Assay – MiSeq represents a significant improvement over existing clonality assays using fragment analysis as it efficiently detects the majority of *TRG* gene rearrangements using a single multiplex master mix, and at the same time, identifies the DNA sequence specific for each clonal gene rearrangement. Therefore, this assay has two important and complementary uses: it both aids in the detection of initial clonal populations and identifies sequence information required to track those clones in subsequent samples.

Each single multiplex master mix for *TRG* targets the conserved regions within the V γ and the J γ regions described in lymphoid malignancies. Primers included in the master mixes are designed with Illumina adapters and up to 24 different indices. This 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, sold separately), onto one MiSeq flow cell allowing up to 24 samples per target to be analyzed in parallel in a single run.

The associated RUO LymphoTrack Software – MiSeq provides a simple and streamlined method of analysis and visualization of data.

Positive and negative controls for clonality are included in the kit.

Note: Please see references for a more thorough explanation of the locus and the targeted sequencing strategy.³

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 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 *TRG* 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 log₁₀ to generate a standard curve, which can then be used to calculate the concentration (pM) of the PCR amplicons derived from sample DNA. Calculating the concentration of PCR amplicons allows equal amplicon representation in the final pooled library that is loaded onto the MiSeq for sequencing.

3.4. Next-Generation Sequencing (NGS)

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

NGS technologies used in this assay rely on the amplification of genetic sequences using a series of consensus forward and reverse primers that include adapter and index tags. Amplicons generated with the LymphoTrack Master Mixes are quantified, pooled, and loaded onto a flow cell for sequencing with an Illumina MiSeq sequencing platform. Specifically, the amplified products in the library are hybridized to oligonucleotides on a flow cell and are amplified to form local clonal colonies (bridge amplification). Four types of reversible terminator bases (RT-bases) are added and the sequencing strand of DNA is extended one nucleotide at a time. To record the incorporation of nucleotides, a CCD camera takes an image of the light emitted as each RT-base is added, and then cleaved to allow incorporation of the next base. A terminal 3' blocker is added after each cycle of the sequencing process and any unincorporated nucleotides are removed prior to the addition of four new RT-bases.

3.5. Multiplexing Amplicons

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

The second level of multiplexing originates from the ability of the accompanying software to sort sequencing data by both index and target. This allows amplicons generated with targeted primers (even those tagged with the same index) to be pooled together to generate the library to be sequenced on a single flow cell. An example would be to sequence a combination of products from several Invivoscribe LymphoTrack 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, 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 (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 can generate 12-15 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 these generate fewer reads. **When using these alternative flow cells, 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. 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.⁴ 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.^{5,6}

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.



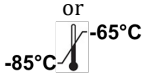
4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog #	Description	# of Indexed Master Mixes	Total Reactions
REF 72270019	LymphoTrack TRG Assay Kit A – MiSeq	8 indices – 5 sequencing runs each	40
REF 72270009	LymphoTrack TRG Assay Panel – MiSeq	24 indices – 5 sequencing runs each	120

Table 2. Kit Components

Reagent	Reagent Components	Unit Quantity	72270019 # of Units	72270009 # of Units	Storage Temperature	Notes				
Master Mixes[‡]	TRG MiSeq 01	225 µL	1	1		N/A				
	TRG MiSeq 02		1	1						
	TRG MiSeq 03		1	1						
	TRG MiSeq 04		1	1						
	TRG MiSeq 05		1	1						
	TRG MiSeq 06		1	1						
	TRG MiSeq 07		1	1						
	TRG MiSeq 08		1	1						
	TRG MiSeq 09		0	1						
	TRG MiSeq 10		0	1						
	TRG MiSeq 11		0	1						
	TRG MiSeq 12		0	1						
	TRG MiSeq 13		0	1						
	TRG MiSeq 14		0	1						
	TRG MiSeq 15		0	1						
	TRG MiSeq 16		0	1						
	TRG MiSeq 18		0	1						
	TRG MiSeq 19		0	1						
	TRG MiSeq 20		0	1						
	TRG MiSeq 21		0	1						
	TRG MiSeq 22		0	1						
	TRG MiSeq 23		0	1						
	TRG MiSeq 25		0	1						
	TRG MiSeq 27		0	1						
	Positive Control DNA		TRG POS (+) REF : 42270019)	45 µL			1	3		Vγ11/ Jγ 1/2 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.

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

4.3. Storage and Handling

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

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

5. Instruments

The specific instruments listed below are recommended based on the methods used to verify this 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: 15°C to 96°C
 - Minimum Ramping Speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.
- See section 7.4 *Amplification* for thermal cycler program.

5.2. Magnetic Stand

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

5.3. Real-time PCR Instrument

- Use or Function: Quantify purified PCR products
- Suggested Instrument: Applied Biosystems® 7500 Fast 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 7.11 *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. 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 μ L of sample DNA at a minimum concentration of 10 ng/ μ L).
- This assay tests extracted and purified genomic DNA. DNA must be quantified with a method specific for double-stranded DNA (dsDNA) and be free of PCR inhibitors.
- Resuspend DNA in an appropriate solution such as 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, prepared with molecular biology grade water) or molecular biology grade water alone.

6.4. Sample Storage

Store samples using a method that prevents degradation of DNA.

7. Assay Procedure

7.1. Materials Provided

See Table 2 for materials provided.

7.2. Materials Required (not provided)

Table 3. Materials Required (not provided)

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

7.3. Reagent Preparation

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

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

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

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

Table 4. Reaction Setup

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

7.4. Amplification

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

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

Table 5. 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 = $n \times 50 \mu\text{L}$ (n is number of samples to be purified).
- 7.5.3. Prepare a fresh stock (0.5 mL for each sample to be purified) of 80% ethanol using sterile water.

Binding of Amplicons to Magnetic Particles:

- 7.5.4. Add 50 μ L of the aliquoted, **room temperature** Agencourt AMPure XP reagent to each sample to be purified.
- Mix by pipetting up and down 10 times.
 - The color of the mixture should appear homogenous after mixing.
 - Incubate 10 minutes at room temperature.
- 7.5.5. Place the mixed samples on 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 μ L, aspirate the cleared supernatant and discard.
- Use a P10 pipette (or equivalent multichannel pipette) set to 10 μ L to remove any excess supernatant.
 - Avoid removing any magnetic particles.

Washing:

- 7.5.7. Keeping the plate on the magnetic stand, add 200 μ L of 80% ethanol to each sample. Incubate for 30 seconds at room temperature.
- Using a P200 (or equivalent multichannel pipette) set to 195 μ L; aspirate the ethanol and discard.
 - Use a P10 pipette (or a multichannel pipette) set to 10 μ L 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 μ L 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 μ L 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 $^{\circ}$ C or proceed to the next step.

The gel image in Figure 1 illustrates the effectiveness of a typical purification (showing amplicons before and after purification).

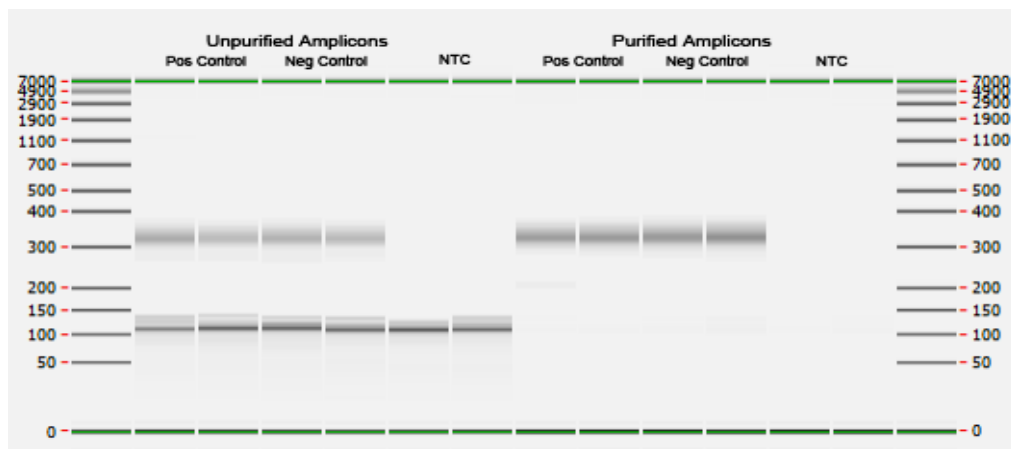


Figure 1: Example of a purification result for amplicons from the TRG MiSeq Master Mix. The image was generated by running unpurified and purified products on the LabChip GX.

7.6. Quantification of Amplicons

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

7.6.1. Dilution of amplicons

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

1: 4,000 Final:

- Step A:** 2 μ L of purified amplicon eluate + 198 μ L of Dilution Buffer A.
Mix well by pipetting up and down 10 times.
- Step B:** 5 μ L from Step A + 195 μ L of Dilution Buffer A.
Mix well by pipetting up and down 10 times.

7.6.2. Set up a qPCR run for amplicon quantification following Table 6 for each reaction (please refer to the KAPA library quantification kit instructions for more details):

Table 6. qPCR Setup

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

7.6.3. Follow the thermal program in Table 7 for qPCR.

Table 7. qPCR Program

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

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

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

If the Δ Ct \geq 4.0 for both controls, proceed to the next step. If the Δ Ct < 4.0 for either control, see section 12 *Troubleshooting Guide* for further instructions.

Note: Δ Ct for the *TRB* assay is set different than the other LymphoTrack assays:

$$\text{TRB } \Delta\text{Ct} \geq 3.0$$

$$\text{IGHV Leader, IGH FR1, IGH FR2, IGH FR3, IGK and TRG } \Delta\text{Ct} \geq 4.0$$

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

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

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

The average fragment length of amplicons generated using the *TRG* Assay is 300 bp (A = 300).
(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.**

- 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 6 for qPCR setup and Table 7 for the thermal cycler program.

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

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

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

The average fragment length of amplicons generated using the **TRG** Assay is 300 bp (A = 300).

(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 between **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 (section 18).

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

Table 8. Library Denaturation

Reagent	Volume
Diluted library	10 µL
0.2 N NaOH	10 µL
Total Volume	20 µL

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

Table 9. HT1 Buffer Addition

Reagent	Volume
Denatured library	20 μL
HT1 Buffer	980 μL
Total Volume	1000 μL

- 7.10.5. Vortex briefly to mix, then pulse centrifuge the diluted and denatured library DNA solution.
- 7.10.6. Place the diluted and denatured library on ice until the next step.
- 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 (v2.6 or later):

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.

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

Table 10. Preparation of the Library for MiSeq Loading

Final Concentrations	12 pM	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). **Verify the final NaOH concentration is not more than 1.0 mM.**
- 7.10.8. Invert the final library 5 times to mix, and pulse centrifuge.
- 7.10.9. Place the final prepared library on ice until it is loaded onto the MiSeq Reagent Cartridge.

7.11. MiSeq Flow Cell Loading

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

7.12. MiSeq Sample Sheet Setup

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

Characters in the sample name:

- Create a unique name and identifier 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 up 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. This set of samples/targets should be given a unique identifier to include in the Sample ID field in the Sample Sheet. Please keep in mind that the Sample ID has a strict **40 character limit** when choosing a naming convention.

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 above 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 11. Sample Selection index names

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

7.13. MiSeq Run Start

Start the MiSeq run following the MiSeq Control Software Instructions. The approximate MiSeq run times are indicated in Table 12.

Table 12. MiSeq Run Times

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

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

8. Data Analysis

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

The MiSeq sequencing run of samples prepared with LymphoTrack *TRG* Assay provides FASTQ files that 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.

- *TRG* Positive Control top % reads $\geq 2.5\%$
- NGS Negative Control top % reads $< 1.0\%$
- MiSeq Run Validity
 - Q30 $> 80\%$ for v2 (2x151)
 - Q30 $> 75\%$ for v2 (2x251)
 - Q30 $> 70\%$ for v3 (2x301)

10. Limitations of Procedure

- This assay does 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.
- 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 TRG

Sample name: spikeddata_tcrq_S2_L001_001_combined

Total Read Count: 100000

IndexQ30: 99.83

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	CDR3 Sequence
1	GAAGACTAAGAACTTG	147	25000	Vg11	Jg1/2	25.00	25.00	not found
2	GGAATCAGTCGAGAAAA	142	20000	Vg8	Jg1/2	20.00	45.00	GCCACCTGGAAATTTA
3	TGGGTAAGACAAGCAAC	141	15000	Vg10	JgP1	15.00	60.00	GCTGCGTGGGATGGTTG
4	GAAGACTAAGAACTTG	147	12500	Vg11	JgP1	12.50	72.50	not found
5	TGGGTAAGACAAGCAAC	140	10000	Vg10	Jg1/2	10.00	82.50	not found
6	GAAGACTAAGAACTTG	137	5000	Vg11	JgP2	5.00	87.50	GCCTGCTGGAAAGCTGA
7	GGAATCAGTCGAGAAAA	132	4000	Vg8	JgP1	4.00	91.50	not found
8	GGAATCAGTCGAGAAAA	165	3000	Vg8	JgP2	3.00	94.50	GCCACC
9	GGAGTCAGTCCAGGGA	144	2000	Vg2	JgP1	2.00	96.50	not found
10	CGGCATTCCGTCAGGCA	155	1000	Vg9	Jg1/2	1.00	97.50	GCCTGTGGGAGGTATG

Figure 2: 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 TRG Assay - MiSeq and analyzed using the LymphoTrack Software - MiSeq (REF 75000009).

12. Troubleshooting Guide

Table 13. Troubleshooting Guide

Occurs During	Error	Action
Sample and reagent preparation	Sample DNA quantity is less than 50 ng by a dsDNA-based method	Do not test the sample
Sample and reagent preparation	Sample DNA integrity is low	Test the sample using the Specimen Control Size Ladder available from Invivoscribe (REF: 20960021 for ABI detection or REF: 20960020 for gel detection)
Quantification of amplicons using the KAPA library quantification kit	$\Delta Ct < 4.0$	Check the standard curve in qPCR. Check for contamination and repeat the KAPA qPCR for all samples and controls. If $\Delta Ct < 4.0$ again, redo the PCR and qPCR for all samples and controls.
Library creation by amplicon quantification and pooling	Amplicon concentration is less than 1 nM	Check the standard curve in qPCR and repeat PCR if less than 1 nM
MiSeq run set up	Sample sheet not found	Refer to Illumina troubleshooting Or call Illumina Tech Support +1-800-809-4566
	Improperly formatted sample sheet	
	Fluidics check failed	
	Disk space low	
	Empty waste bottle	
	Network disconnected	
	RFID Failure	
MiSeq run	Q30 < 80% for v2 (2x151) Q30 < 75% for v2 (2x251) Q30 < 70% for v3 (2x301)	Call Invivoscribe Tech Support +1-858-224-6600
CD installation	LymphoTrack 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 from 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 TRG Assay – MiSeq. We appreciate your business. We are happy to assist you with understanding this assay, and will provide ongoing technical assistance Monday through Friday to keep the assays performing efficiently in your laboratory.

Contact Information




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




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

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 - LymphoTrack Software – MiSeq Package Instructions for Use ( 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.

	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, 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 TRG 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 μ L of Master Mix into individual wells of a PCR plate. One well for each Master Mix and one Master Mix per sample, positive, negative, or no template controls.
- 17.3. Add 0.2 μ L Taq DNA polymerase (@ 5 U/ μ L) to each Master Mix.
- 17.4. Add 5 μ L of sample DNA (at a minimum concentration of 10 ng/ μ L) and 5 μ L of control samples to wells containing the respective Master Mix reactions, and pipette up and down 5-10 times to mix.
- 17.5. Add 5 μ L of molecular biology grade water to the well containing the respective Master Mix for no template control, and pipette up and down 5-10 times to mix.
- 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 μ L of particles to each 50 μ L reaction; elute DNA in 25 μ L 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 v2.6 or later).
- 17.12. Load 600 μ L of denatured and diluted library onto 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 14 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 14 Cycle Settings and Reagent Kits for a Multi-Targeted MiSeq Run

Multiplexing Targets	Sample Sheet Settings	MiSeq Reagent Kit	Catalog #
Only <i>IGH</i> FR3 and <i>TRG</i> together	151 cycles Read 1 151 cycles Read 2	v2 kit (300 cycle) or v2 kit (500 cycle)	MS-102-2002 or MS-102-2003
Any combination of these targets together: <i>IGH</i> FR1, <i>IGH</i> FR2, <i>IGH</i> FR3, <i>IGK</i> , <i>TRB</i> and <i>TRG</i>	251 cycles Read 1 251 cycles Read 2	v2 kit (500 cycle) up to 4 targets or v3 kit (600 cycle)	MS-102-2003 or MS-102-3003
When combining any of the assays together with <i>IGHV</i> 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*). U, V, W, X, Y, and Z are volumes in μL .

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

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

Table 15. Calculation of Individual Library Inputs to Generate a Multi-Targeted Sequencing Library for the MiSeq Run

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

18.3. Denature the combined libraries to 2 nM.

- Add reagents according to Table 16 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 15, mix the applicable libraries first, then add 18 μL to the denaturing reaction as shown in Table 16.

Table 16. Library Denaturation

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

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

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 17. Vortex briefly to mix and pulse centrifuge the sample.

Table 17. 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.