LymphoTrack® Dx
IGH Assays

# Instructions for Use

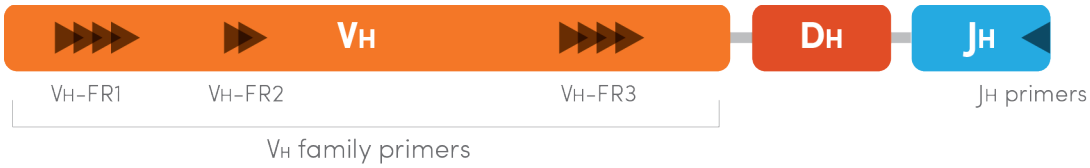
CE UK CA IVD

# LymphoTrack® Dx *IGH* (FR1/FR2/FR3) Assays – S5/PGM™

To identify and track B cell immunoglobulin heavy chain (*IGH*) gene rearrangements using next-generation sequencing with the Thermo Fisher Scientific® Ion S5™ or Ion PGM™.

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

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




invivoscribe®

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

| Catalog # | | Products | Quantity |
|---|---|---|---|
| REF | 91210007 | LymphoTrack Dx *IGH* FR1 Assay – S5/PGM | 12 indices – 5 reactions each |
| REF | 91210037 | LymphoTrack Dx *IGH* FR2 Assay – S5/PGM | 12 indices – 5 reactions each |
| REF | 91210047 | LymphoTrack Dx *IGH* FR3 Assay – S5/PGM | 12 indices – 5 reactions each |
| REF | 91210057 | LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM | 12 indices per target – 5 reactions each |

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# 1. Intended Use

**Intended Use (LymphoTrack Dx IGH FR1 Assay – S5/PGM)**
The LymphoTrack Dx *IGH* FR1 Assay – S5/PGM is an in vitro diagnostic product intended for next-generation sequencing (NGS) based determination of the frequency distribution of *IGH* gene rearrangements as well as the degree of somatic hypermutation (SHM) of rearranged genes in patients suspected of having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders as well as providing an aid in determining disease prognosis using the Thermo Fisher Scientific Ion S5 or Ion PGM platform.

**Intended Use (LymphoTrack Dx IGH FR1/2/3 Assay – S5/PGM)**
The LymphoTrack Dx *IGH* FR1 Assay – S5/PGM is an in vitro diagnostic product intended for next-generation sequencing (NGS), targeting the conserved framework 1 (FR1) region within the $V_H$ segments of the *IGH* gene to determine the frequency distribution of clonal *IGH* $V_H$ – $J_H$ rearrangements as well as the degree of somatic hypermutation (SHM) of rearranged genes in patients suspected of having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders as well as providing an aid in determining disease prognosis using the Thermo Fisher Scientific Ion S5 or Ion PGM platform.

This LymphoTrack Dx *IGH* FR2 Assay – S5/PGM is an in vitro diagnostic product intended for next-generation sequencing (NGS) for the Thermo Fisher Scientific Ion S5 and Ion PGM instruments. The assay will determine the frequency distribution of *IGH* $V_H$ - $J_H$ gene rearrangements in patients suspected of having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders using the Thermo Fisher Scientific Ion S5 or Ion PGM platform.

The LymphoTrack Dx *IGH* FR3 Assay – S5/PGM is an in vitro diagnostic product intended for Next Generation Sequencing (NGS) for the Thermo Fisher Scientific Ion PGM and Ion S5 instruments. The assay will determine the frequency distribution of *IGH* $V_H$--$J_H$ gene rearrangements in patients suspected of having lymphoproliferative disease. This assay aids in the identification of lymphoproliferative disorders using the Thermo Fisher Scientific Ion S5 or Ion PGM 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 1,250 kilobases. The $V_H$ gene segments contain three conserved framework (FR) and two variable complementarity-determining regions (CDRs).

Lymphoid cells are different from other somatic cells in the body; during development, the antigen receptor genes in lymphoid cells undergo somatic gene rearrangement (Tonegawa S., 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, an individual's leukemic or lymphocytic cells generally share one or more cell-specific or "clonal" antigen receptor gene rearrangements. Therefore, tests that detect IGH clonal rearrangements can be useful in the study of B- and T-cell malignancies.

In addition, immunoglobulin heavy chain variable region (IGHV) gene hypermutation status provides important prognostic information for patients with chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL). The presence of IGHV somatic hypermutation (SHM) is defined as greater or equal to 2% difference from the germline VH gene sequence, whereas less than 2% difference is considered evidence of no SHM. The status of SHM for clone(s) has clinical relevance for B-CLL, as there is a clear distinction in the median survival of patients with and without SHM. 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, labor-intensive, required large amounts of DNA and were not suitable for analysis of many of the less diverse antigen receptor loci.

During the last several decades, the use of RF-SBH assays has been supplanted by PCR-based clonality tests developed by Alexander Morley (Trainor K.J. *et al.,* 1990) and are considered the current gold-standard method. These assays

identify clonality on the basis of over-representation of amplified $V_H$–$D_H$–$J_H$ (or incomplete $D_H$–$J_H$ products) gene rearrangement following their separation using gel or capillary electrophoresis. Though sensitive and suitable for testing small amounts of DNA, these assays cannot readily differentiate between clonal populations and multiple rearrangements that might lie beneath a single-sized peak and are not designed to identify the specific $V_H$–$J_H$ DNA sequence that is required to track clonal populations in subsequent analyses.

## 2.2. Summary

The LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assay - Ion S5/PGM (sold separately and as a set) represent a significant improvement over existing clonality assays using fragment analysis, as they efficiently detect *IGH* gene rearrangements and at the same time identify the DNA sequence specific for each clonal gene rearrangement. Therefore, these assays have two important and complementary uses: they provide critical information on the existence of clonality and identify sequence information required to track those clones in subsequent samples. The LymphoTrack Dx *IGH* FR1 Assay additionally provides detailed sequence information on the degree of SHM.

Each single multiplex master mix targets one of the conserved *IGH* framework regions (FR1, FR2, or FR3) within the $V_H$ and the $J_H$ regions described in lymphoid malignancies. Targeting all three framework regions significantly reduces the risk of not being able to detect the presence of clonality, as somatic hypermutations in the primer binding sites of the involved $V_H$ gene segments can impede DNA amplification (Evans PA. *et al.,* 2007). **Data from all three framework regions is needed to determine evidence of clonality for a sample.**

Primers included in the master mixes are designed with Thermo Fisher Scientific adapters and 12 different indices. These assays allow for a one-step PCR and pooling of amplicons from several different samples and targets (generated with other LymphoTrack Dx Assays for the Ion S5 or Ion PGM) onto one Ion S5 or PGM chip, allowing up to 12 samples per target to be analyzed in parallel in a single sequencing run.

The associated LymphoTrack Dx Software – S5/PGM provides direct interpretation of the data generated from LymphoTrack Dx Assays via a simple and streamlined method of analysis and visualization. By following the guidelines provided in section 11 *: Interpretation* and Reporting*,* the sample results summarized in the software can be easily interpreted for the presence or absence of clonality and somatic hypermutation. **Always interpret the results of molecular clonality in the context of clinical, histological and immunophenotypic data.**

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

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

# 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 and J regions of antigen receptor genes. The primers target these conserved regions and lie on either side of an area where programmed genetic rearrangements occur during the maturation of all B and T lymphocytes. Different populations of 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 unique in both sequence and length are generated, reflecting the heterogeneous population. In some cases, where lymphocyte DNA is absent, no amplicons will be generated. Samples containing *IGH* clonal populations yield one or two prominent amplified products of the same length and sequence which are detected with significant frequency within a diminished polyclonal background.

## 3.2. Amplicon Purification

PCR amplicons are purified to remove excess primers, nucleotides, salts and enzymes using solid-phase reversible immobilization (SPRI) paramagnetic bead technology for high-throughput purification of PCR amplicons. Using an optimized buffer, PCR amplicons 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 utilizing capillary electrophoresis, which applies the principles of traditional gel electrophoresis to separate and quantify DNA on a chip based platform. Quantification is achieved by running a marker of known concentration alongside PCR amplicons and then extrapolating the concentration of the amplicons. Calculating the concentration of PCR amplicons allows equal amplicon representation in the final pooled library that is loaded onto the Ion S5 cartridge or Ion PGM chip 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 LymphoTrack Dx Master Mixes are quantified, pooled and loaded onto a chip for sequencing with the Thermo Fisher Scientific Ion S5 or Ion PGM platform. These platforms require the pooled library of DNA fragments to be bound to individual beads prior to sequencing, one unique sequence per bead. Once bound to the beads, the DNA fragments are amplified via emulsion PCR until they cover the surface of the bead. The beads are then loaded onto a semi-conductor chip, where each bead occupies an individual well and sequencing occurs.

Sequencing is conducted by flooding the chip with individual unincorporated nucleotides one base at a time (dATP, dCTP, dGTP, dTTP) and the sequencing instruments detect the addition of nucleotides when hydrogen ions are released during DNA polymerization causing a change in the pH of the wells, measured as a change in voltage. The voltage changes proportionally to the number of nucleotides added. After nucleotides are incorporated, unincorporated nucleotides are washed away and the process begins again with a new dNTP.

## 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 12. Each of these 12 indices can be considered to act 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 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 into a single the library and sequenced on a single sequencing chip. An example would be to sequence products from several Invivoscribe LymphoTrack Dx Assays together in the same run. However, it is important to obtain a sufficient number of reads, or depth of coverage, for valid interpretation of each sample.

Due to the capacity of the Thermo Fisher Scientific Ion PGM Ion 316™ Chip v2 BC, which generates 2-3 million reads, it recommended to multiplex no more than three different gene targets together, such as *IGH* FR1, *IGH* FR2 and *IGH* FR3 Up to five different gene targets can be multiplexed together on the Ion PGM Ion 318™ Chip v2 BC (4-5.5 million reads), Ion S5 Ion 520™ Chip (3-6 million reads) and Ion S5 Ion 530™ Chip (15-20 million reads).

It is important to use the appropriate sequencing chemistry when multiplexing amplicons of different gene targets. The number of sequencing cycles must be sufficient to sequence the largest amplicon in the multiplex. Two or more sequencing libraries generated from the same LymphoTrack Dx gene target master mixes (*e.g*., two *IGH* FR1 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.

## 3.6. *IGHV* Somatic Hypermutation (SHM) Evaluation

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

# 4. Reagents

## 4.1. Reagent Components

**Table 1**. Available Kits

| Cat # | Description | # of Indexed Master Mixes | Total Reactions |
|---|---|---|---|
| REF 91210007 | LymphoTrack Dx *IGH* FR1 Assay – S5/PGM | 12 indices<br>5 sequencing runs each | 60 |
| REF 91210037 | LymphoTrack Dx *IGH* FR2 Assay – S5/PGM | 12 indices<br>5 sequencing runs each | 60 |
| REF 91210047 | LymphoTrack Dx *IGH* FR3 Assay – S5/PGM | 12 indices<br>5 sequencing runs each | 60 |
| REF 91210057 | LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM | 12 indices<br>5 sequencing runs each | 60+60+60 |

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

| Reagents | Reagent Components | Index Number | Unit Quantity | 91210007 # of Units | Storage Temperature | Notes |
|---|---|---|---|---|---|---|
| **Master Mixes‡** | *IGH* FR1 S5/PGM 01 | IonXpress_001 | 250 µL | 1 | -85°C to -65°C | N/A |
| | *IGH* FR1 S5/PGM 02 | IonXpress_002 | | 1 | | |
| | *IGH* FR1 S5/PGM 03 | IonXpress_003 | | 1 | | |
| | *IGH* FR1 S5/PGM 04 | IonXpress_004 | | 1 | | |
| | *IGH* FR1 S5/PGM 07 | IonXpress_007 | | 1 | | |
| | *IGH* FR1 S5/PGM 08 | IonXpress_008 | | 1 | | |
| | *IGH* FR1 S5/PGM 09 | IonXpress_009 | | 1 | | |
| | *IGH* FR1 S5/PGM 10 | IonXpress_010 | | 1 | | |
| | *IGH* FR1 S5/PGM 11 | IonXpress_011 | | 1 | | |
| | *IGH* FR1 S5/PGM 12 | IonXpress_012 | | 1 | | |
| | *IGH* FR1 S5/PGM 13 | IonXpress_013 | | 1 | | |
| | *IGH* FR1 S5/PGM 14 | IonXpress_014 | | 1 | | |
| **Positive Control DNA** | *IGH* POS (+) (REF 40880009) | N/A | 45 µL | 2 | 2°C to 8°C<br>or<br>-85°C to -65°C | *IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA |
| **Negative Control DNA** | NGS NEG (-) (REF 40920018) | N/A | 45 µL | 2 | | Tonsil DNA, highest sequence frequency can vary between lots |

**Note:** There are no preservatives used in the manufacturing of these kits.
**Note‡:** IonXpress Indices 5 and 6 are not used in this kit.

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

| Reagents | Reagent Components | Index Number | Unit Quantity | 91210037 # of Units | Storage Temperature | Notes |
|---|---|---|---|---|---|---|
| **Master Mixes‡** | *IGH* FR2 S5/PGM 01 | IonXpress_001 | 250 µL | 1 | -85°C to -65°C | N/A |
| | *IGH* FR2 S5/PGM 02 | IonXpress_002 | | 1 | | |
| | *IGH* FR2 S5/PGM 03 | IonXpress_003 | | 1 | | |
| | *IGH* FR2 S5/PGM 04 | IonXpress_004 | | 1 | | |
| | *IGH* FR2 S5/PGM 07 | IonXpress_007 | | 1 | | |
| | *IGH* FR2 S5/PGM 08 | IonXpress_008 | | 1 | | |
| | *IGH* FR2 S5/PGM 09 | IonXpress_009 | | 1 | | |
| | *IGH* FR2 S5/PGM 10 | IonXpress_010 | | 1 | | |
| | *IGH* FR2 S5/PGM 11 | IonXpress_011 | | 1 | | |
| | *IGH* FR2 S5/PGM 12 | IonXpress_012 | | 1 | | |
| | *IGH* FR2 S5/PGM 13 | IonXpress_013 | | 1 | | |
| | *IGH* FR2 S5/PGM 14 | IonXpress_014 | | 1 | | |
| **Positive Control DNA** | *IGH* POS (+) (REF 40880009) | N/A | 45 µL | 2 | 2°C to 8°C or -85°C to -65°C | *IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA |
| **Negative Control DNA** | NGS NEG (-) (REF 40920018) | N/A | 45 µL | 2 | | Tonsil DNA, highest sequence frequency can vary between lots |

**Note:** There are no preservatives used in the manufacturing of these kits.
**Note‡:** IonXpress Indices 5 and 6 are not used in this kit.

**Table 4.** LymphoTrack Dx *IGH* FR3 Assay Kit Components

| Reagents | Reagent Components | Index Number | Unit Quantity | 91210047 # of Units | Storage Temperature | Notes |
|---|---|---|---|---|---|---|
| **Master Mixes‡** | *IGH* FR3 S5/PGM 01 | IonXpress_001 | 250 µL | 1 | -85°C to -65°C | N/A |
| | *IGH* FR3 S5/PGM 02 | IonXpress_002 | | 1 | | |
| | *IGH* FR3 S5/PGM 03 | IonXpress_003 | | 1 | | |
| | *IGH* FR3 S5/PGM 04 | IonXpress_004 | | 1 | | |
| | *IGH* FR3 S5/PGM 07 | IonXpress_007 | | 1 | | |
| | *IGH* FR3 S5/PGM 08 | IonXpress_008 | | 1 | | |
| | *IGH* FR3 S5/PGM 09 | IonXpress_009 | | 1 | | |
| | *IGH* FR3 S5/PGM 10 | IonXpress_010 | | 1 | | |
| | *IGH* FR3 S5/PGM 11 | IonXpress_011 | | 1 | | |
| | *IGH* FR3 S5/PGM 12 | IonXpress_012 | | 1 | | |
| | *IGH* FR3 S5/PGM 13 | IonXpress_013 | | 1 | | |
| | *IGH* FR3 S5/PGM 14 | IonXpress_014 | | 1 | | |
| **Positive Control DNA** | *IGH* POS (+) (REF 40880009) | N/A | 45 µL | 2 | 2°C to 8°C or -85°C to -65°C | *IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA |
| **Negative Control DNA** | NGS NEG (-) (REF 40920018) | N/A | 45 µL | 2 | | Tonsil DNA, highest sequence frequency can vary between lots |

**Note:** There are no preservatives used in the manufacturing of these kits.
**Note‡:** IonXpress Indices 5 and 6 are not used in this kit.

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

| FR1 Master Mixes‡ | # of Units | FR2 Master Mixes‡ | # of Units | FR3 Master Mixes‡ | # of Units | Index Number | Unit Quantity |
|---|---|---|---|---|---|---|---|
| *IGH* FR1 S5/PGM 01 | 1 | *IGH* FR2 S5/PGM 01 | 1 | *IGH* FR3 S5/PGM 01 | 1 | IonXpress_001 | 250 µL |
| *IGH* FR1 S5/PGM 02 | 1 | *IGH* FR2 S5/PGM 02 | 1 | *IGH* FR3 S5/PGM 02 | 1 | IonXpress_002 | |
| *IGH* FR1 S5/PGM 03 | 1 | *IGH* FR2 S5/PGM 03 | 1 | *IGH* FR3 S5/PGM 03 | 1 | IonXpress_003 | |
| *IGH* FR1 S5/PGM 04 | 1 | *IGH* FR2 S5/PGM 04 | 1 | *IGH* FR3 S5/PGM 04 | 1 | IonXpress_004 | |
| *IGH* FR1 S5/PGM 07 | 1 | *IGH* FR2 S5/PGM 07 | 1 | *IGH* FR3 S5/PGM 07 | 1 | IonXpress_007 | |
| *IGH* FR1 S5/PGM 08 | 1 | *IGH* FR2 S5/PGM 08 | 1 | *IGH* FR3 S5/PGM 08 | 1 | IonXpress_008 | |
| *IGH* FR1 S5/PGM 09 | 1 | *IGH* FR2 S5/PGM 09 | 1 | *IGH* FR3 S5/PGM 09 | 1 | IonXpress_009 | |
| *IGH* FR1 S5/PGM 10 | 1 | *IGH* FR2 S5/PGM 10 | 1 | *IGH* FR3 S5/PGM 10 | 1 | IonXpress_010 | |
| *IGH* FR1 S5/PGM 11 | 1 | *IGH* FR2 S5/PGM 11 | 1 | *IGH* FR3 S5/PGM 11 | 1 | IonXpress_011 | |
| *IGH* FR1 S5/PGM 12 | 1 | *IGH* FR2 S5/PGM 12 | 1 | *IGH* FR3 S5/PGM 12 | 1 | IonXpress_012 | |
| *IGH* FR1 S5/PGM 13 | 1 | *IGH* FR2 S5/PGM 13 | 1 | *IGH* FR3 S5/PGM 13 | 1 | IonXpress_013 | |
| *IGH* FR1 S5/PGM 14 | 1 | *IGH* FR2 S5/PGM 14 | 1 | *IGH* FR3 S5/PGM 14 | 1 | IonXpress_014 | |

| Reagents | Reagent Components | Notes | # of Units | Unit Quantity |
|---|---|---|---|---|
| **Positive Control DNA*** | *IGH* POS (+) (REF 40880009) | *IGH* V1-46_03 / *IGH* J4_02 DNA diluted in tonsil DNA | 4 | 45 µL |
| **Negative Control DNA*** | NGS NEG (-) (REF 40920018) | Tonsil DNA, highest sequence frequency can vary between lots | 4 | 45 µL |

**Note‡:** The storage temperature for master mixes is -85ºC to -65ºC.
**Note*:** The storage temperature for controls is 2ºC to 8ºC or -85ºC to -65ºC.
**Note:** There are no preservatives used in the manufacturing of these kits.
**Note‡:** IonXpress Indices 5 and 6 are not used in this kit.

## 4.2. Warnings and Precautions

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

- IVD This product is for In Vitro Diagnostic Use.
- Use the assay kit as a system. Do not substitute other manufacturers' reagents. Dilution, reducing amplification reactions, or other deviations from this protocol may affect the performance of this test and/or nullify any limited sublicense that 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.
- Adherence to the protocol will assure optimal performance and reproducibility. Ensure correct thermal cycler programs are used, as other programs may provide inaccurate/faulty data, such as false-positive and false-negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- Perform all laboratory procedures with standard personal protective equipment (gloves, laboratory coats and protective eye wear). Follow good laboratory practices and universal precautions when working with specimens. Do not pipette by mouth. Do not eat, drink, or smoke in laboratory work areas. Wash hands thoroughly after handling specimens and assay reagents. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets. Use molecular biology grade water for the preparation of specimen DNA.
- Due to the high analytical sensitivity of these tests, use extreme care to avoid any contamination of reagents or amplification mixtures with samples, controls, or amplified materials. Use fresh, aerosol-resistant pipette tips between samples and between dispensing reagents. Closely monitor all reagents for signs of contamination (e.g., negative controls giving positive signals). Discard any reagents suspected of contamination.
- To minimize contamination wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to setting up PCR.
- Follow uni-directional work flow between separate work areas in the PCR laboratory : begin with master mix preparation, move to specimen preparation, then to amplification and finally to detection. Autoclaving does not eliminate DNA contamination. Perform pre- and post-PCR steps in separate spaces. Avoid taking paper and other materials from post-PCR into the pre-PCR space.
- Dedicate all pipettes, pipette tips and any equipment used in a particular area to that area of the laboratory.
- Decontaminate non-disposable items with 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

- Store the assay at **-85°C to -65°C** until ready to use.
- The optimum storage temperature for DNA controls is 2°C to 8°C, but DNA can also be stored at -85°C to -65°C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that 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 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

The instruments listed in Table 6 are recommended to use with the following validated platform combinations for the LymphoTrack *IGH* (FR1/FR2/FR3) Assays – S5/PGM library preparation and sequencing:

- Ion Chef™ and Ion S5
- Ion OneTouch 2™ (OT2) and Ion S5
- Ion OT2 and Ion PGM

**Table 6.** Recommended Instruments.

| Instrument Function | Validated Platform Combination with Recommended Instrument / Specifications | | |
|---|---|---|---|
| | **Ion Chef and Ion S5** | **Ion OT2 and Ion S5** | **Ion OT2 and Ion PGM** |
| **Amplification of DNA samples** | **Veriti™ Thermal Cycler* or equivalent**<br>Minimum Thermal Range: 15ºC to 96ºC<br>Minimum Ramping Speed: 0.8ºC / sec<br><br>See section 7.4. *Amplification* for thermal cycler program. | | |
| **Purification of PCR products** | **Ambion® Magnetic Stand 96*** (REF AM10027),<br>**Agencourt SPRIPlate® 96 Ring Super Magnet Plate*** (REF A32782),<br>**Thermo Fisher Scientific DynaMag™-96 Side Skirted Magnet*** (REF 12027) **or equivalent**<br><br>See section 7.5. *AMPure XP Purification* for PCR product purification methods. | | |
| **Quantification of purified PCR products** | **Agilent 2100 Bioanalyzer* or Perkin Elmer LabChip GX***<br><br>See section 7.6. *Quantification of Amplicons* for further details. | | |
| **Template Preparation** | **Thermo Fisher Scientific Ion Chef System***<br><br>See section 7.9. *Template Preparation and Sequencing using the Ion Chef and Ion S5* for further details | **Thermo Fisher Scientific Ion OT2 System***<br><br>See section 7.10. *Template Preparation and Sequencing using the Ion OT2 and Ion S5* for further details. | **Thermo Fisher Scientific Ion OT2 System***<br><br>See section 7.11. *Template Preparation and Sequencing using the Ion OT2 and Ion PGM* for further details. |
| **Sequencing** | **Thermo Fisher Scientific Ion S5 Instrument***<br><br>See sections 7.9. *Template Preparation and Sequencing using the Ion Chef and Ion S5* and 7.10. *Template Preparation and Sequencing using the Ion OT2 and Ion S5* for further details. | | **Thermo Fisher Scientific Ion PGM Instrument***<br><br>See section 7.11. *Template Preparation and Sequencing using the Ion OT2 and Ion PGM* for further details. |

**Note:** Follow manufacturer's installation, operation, calibration and maintenance procedures.

*Warning: These are not CE-marked products

# 6. Specimen Collection and Preparation

## 6.1. Precautions

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

## 6.2. Interfering Substances

The following substances are known to interfere with PCR:

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

## 6.3. Specimen Requirements and Handling

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

## 6.4. Sample Storage

Store samples using a method that prevents degradation of DNA.

# 7. Assay Procedure

## 7.1. Materials Provided

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

## 7.2. Materials Required (not provided)

**Table 7.** Materials Required (not provided)

| Reagent/Material | Required and/or Recommended Reagents/Suppliers | Catalog # | Notes |
|---|---|---|---|
| **DNA Polymerase** | Roche:<br>• EagleTaq™ DNA Polymerase<br>Invivoscribe, Inc.<br>• FalconTaq DNA Polymserase<br>or equivalent | 05206944190<br><br>60970130 | 5 U/µL |
| **Molecular Biology Grade Water** | N/A | N/A | DNase / RNase free |
| **18 MΩ water** | N/A | N/A | Required for PGM run |
| **Calibrated Pipettes** | N/A | N/A | Must be able to accurately measure volumes between 0.2 µL and 1000 µL |
| **Vortex Mixer** | N/A | N/A | N/A |
| **PCR Plates or Tubes** | N/A | N/A | N/A |
| **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:<br>• Agencourt AMPure XP | A63880 | N/A |
| **SHM Positive Control** | Invivoscribe, Inc. | 40880008 | Optional for SHM testing |
| **Amplicon & Library Quantification** | Agilent Technologies:<br>• Agilent DNA 1000 Kit<br>or<br>Perkin Elmer:<br>• HT DNA 1K/12K/Hi Sens Labchip &<br>• HT DNA HiSens Reagents | 5067-1504<br>or<br><br>760517 &<br>CLS760672 | N/A |
| **Ion S5 Sequencing** | Thermo Fisher Scientific:<br>• Ion 520 & Ion 530 Kit – OT2<br>or<br>• Ion 510 & Ion 520 & Ion 530 Kit – Chef | A27751<br>or<br>A34019 | N/A |
| | Thermo Fisher Scientific:<br>• Ion 520 Chip Kit<br>or<br>• Ion 530 Chip Kit | A27761<br>or<br>A27764 | N/A |
| | Thermo Fisher Scientific:<br>• Ion PGM Hi-Q View OT2 Kit | A29900 | N/A |

**Table 7.** Materials Required (not provided)

| Reagent/Material | Required and/or Recommended Reagents/Suppliers | Catalog # | Notes |
|---|---|---|---|
| **Ion PGM Sequencing** | Thermo Fisher Scientific:<br>• Ion PGM Enrichment Beads | 4478525 | N/A |
| | Thermo Fisher Scientific:<br>• Ion PGM Hi-Q View Sequencing Kit<br>• & Ion PGM Wash 2 Bottle Kit | A30044<br>& A25591 | N/A |
| | Thermo Fisher Scientific:<br>• Ion 316 Chip Kit v2 BC<br>or<br>• Ion 318 Chip Kit v2 BC | 4488149<br>or<br>4488146 | N/A |
| **The Torrent Suite™ Software for Ion PGM System or Ion S5 System** | Version 5.0.4 or 5.2.2 for PGM*<br>or<br>Version 5.6 for S5* | N/A | N/A |

***Note:** These software versions were used for assay validation on the specified instruments.

## 7.3. Reagent Preparation

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

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

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

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 every 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, briefly centrifuge and place in the PCR thermal cycler.

**Table 8.** Reaction Setup

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

## 7.4. Amplification

7.4.1. Amplify the samples using the PCR program from Table 9.

**Table 9.** 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 validation using the Agencourt AMPure XP PCR Purification system.

**Preparation:**

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

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 × 90 µL (n is number of samples to be purified).

7.5.3. Prepare a fresh stock (0.5 mL for each sample to be purified) of 70% ethanol using sterile water.

**Binding of Amplicons to Magnetic Particles:**

7.5.4. Add 90 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 5 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 135 µL, aspirate the clear supernatant and discard.

- Use a P10 pipette (or equivalent multichannel pipette) set to 10 µL to remove any excess supernatant.
- Avoid removing any magnetic particles.

**Washing:**

7.5.7. Keeping the plate on the magnetic stand, add 200 µL of 70% ethanol to each sample. Incubate for 30 seconds at room temperature.

- Using a P200 (or equivalent multichannel pipette) set to 195 µL; aspirate the ethanol and discard.
- Use a P10 pipette (or a multichannel pipette) set to 10 µL to remove excess ethanol.
- Avoid removing any magnetic particles.

7.5.8. Repeat step 7.5.7 for a total of two washes.

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

**Elution:**

7.5.10. Remove the plate from the magnetic stand. Add 40 µL of 1X TE 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 at least 5 minutes or until the supernatant has cleared.

7.5.13. Transfer 35 µL of the eluate to a fresh plate and seal with cap strips. Label the plate and briefly centrifuge to ensure the supernatant has completely settled to the bottom of the well.

- Store at -20 °C or proceed to the next step.

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

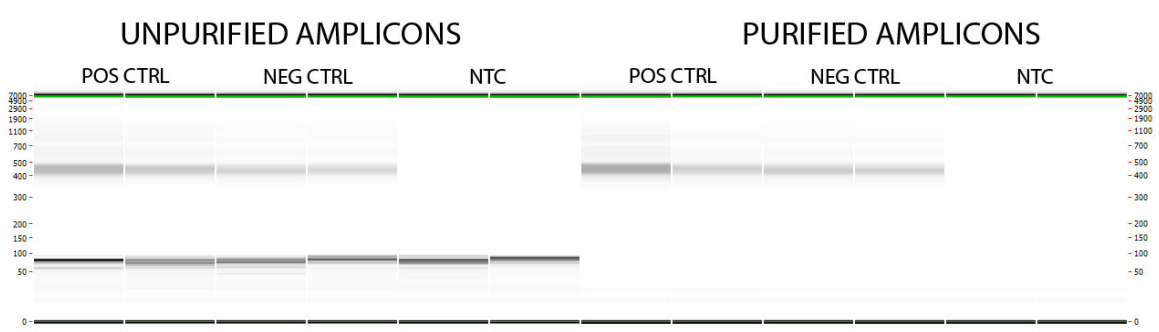



**Figure 1:** Example of a purification result for amplicons from the **LymphoTrack Dx *IGH* FR1 Master Mix**. This image was generated by running unpurified and purified products on the LabChip GX.

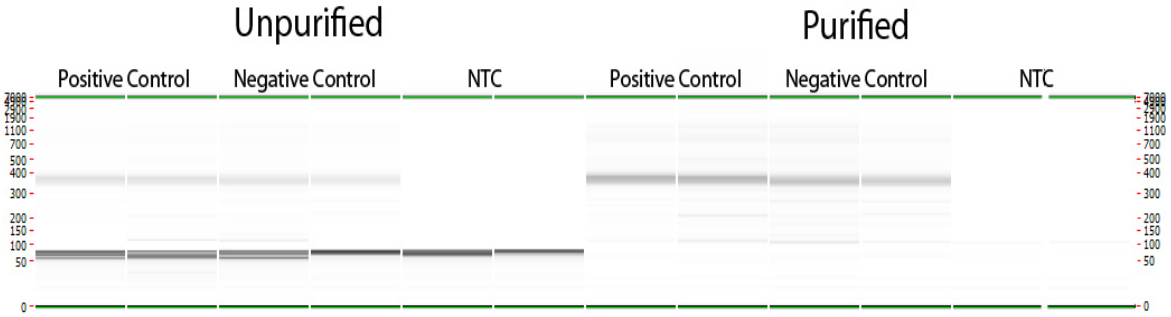



**Figure 2**: Example of a purification result for amplicons from the **LymphoTrack Dx *IGH* FR2 Master Mix**. This image was generated by running unpurified and purified products on the LabChip GX.

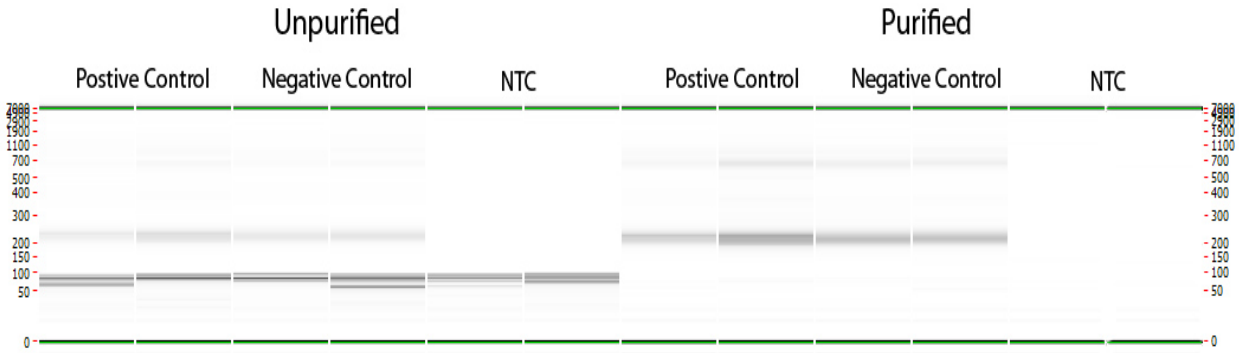


**Figure 3**: Example of a purification result for amplicons from the **LymphoTrack Dx *IGH* FR3 Master Mix**. This image was generated by running unpurified and purified products on the LabChip GX.

## 7.6. Quantification of Amplicons

The following steps were performed during assay validation to analyze data generated from samples, as well as positive, negative and no template controls using either the Agilent 2100 Bioanalyzer, starting with step 7.6.1 or the Perkin Elmer LabChip GX, starting at step 7.6.3.

If quantifying purified PCR amplicons from different LymphoTrack Dx Assays, be sure to analyze each target separately (including the different frameworks) due to the different size ranges of each assay target.

### Agilent 2100 Bioanalyzer Quantification

Prepare an Agilent DNA 1000 Chip for use (please refer to the Agilent DNA 1000 kit instructions for more details).

**Result Interpretation:**

7.6.1. The electropherogram of the ladder well is expected to resemble the electropherogram in Figure 4. Major features of a successful run are:

- 13 peaks for the DNA 1000 ladder
- All peaks are well resolved
- Flat baseline
- Correct identification of both markers

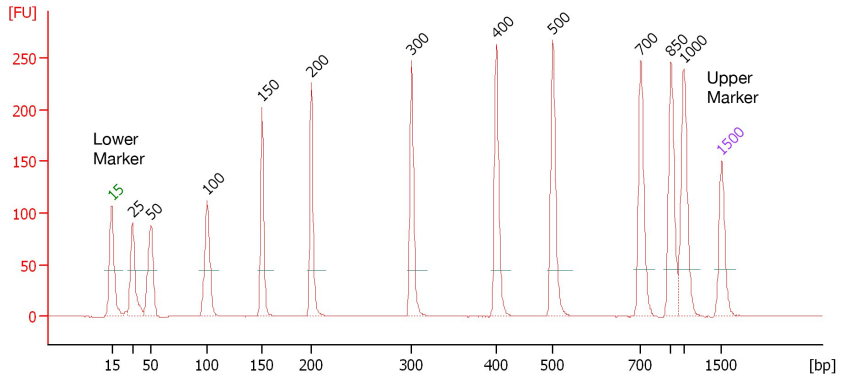



**Figure 4**: Example of an electropherogram of the ladder well showing all 13 peaks.

7.6.2. Determine the molar concentration (nmol/L) of each sample using the Bioanalyzer software. If necessary use manual integration to place the entire range of library fragments within a single peak.

### Perkin Elmer LabChip GX Quantification

Prepare the LabChip GX Chip for use (please refer to the Perkin Elmer LabChip GX instructions for more details).

**Result Interpretation:**

7.6.3. By default, each time an experiment is run, the data file (**.gxd*) is saved in a new folder (named with the current date) that can be accessed by the Data folder shortcut on the Desktop.

7.6.4. Transfer the folder containing the data file (**.gxd*) to the computer.

7.6.5. Open the LabChip GX software, go to the menu bar and select **File → Import Data** file to open the transferred data file (**.gxd*).

7.6.6. In the upper left hand corner of the screen is a plate diagram. Select the wells used in the experiment to display the associated data in the data tables below. The wells will appear blue when selected.

7.6.7. Go to the menu bar and select **Analysis → Analysis Settings**.

- Select the **Smear Analysis** tab and add the applicable information from Table 10 - Table 12.
- Once all information has been edited, click on the **Apply** button

**Table 10.** Settings for the Smear Analysis with *IGH* FR1

| Start Size (BP) | End Size (BP) | Color | Name | Property Displayed in Well Table | Apply to Wells |
|---|---|---|---|---|---|
| 350 | 600 | Red | Region [350-600] | Size at Maximum [BP] | <all> |
| 350 | 600 | Red | Region [350-600] | Molarity (nmol/L) | <all> |

**Table 11.** Settings for the Smear Analysis with *IGH* FR2

| Start Size (BP) | End Size (BP) | Color | Name | Property Displayed in Well Table | Apply to Wells |
|---|---|---|---|---|---|
| 300 | 500 | Red | Region [300-500] | Size at Maximum [BP] | <all> |
| 300 | 500 | Red | Region [300-500] | Molarity (nmol/L) | <all> |

**Table 12.** Settings for the Smear Analysis with *IGH* FR3

| Start Size (BP) | End Size (BP) | Color | Name | Property Displayed in Well Table | Apply to Wells |
|---|---|---|---|---|---|
| 150 | 300 | Red | Region [150-300] | Size at Maximum [BP] | <all> |
| 150 | 300 | Red | Region [150-300] | Molarity (nmol/L) | <all> |

7.6.8. Back at the main screen, go to the menu bar and select **File → Export**.

7.6.9. Check the *Well Table* in the pop-up window (entitled LabChip GX – Export).

7.6.10. Click **OK** to export a **.csv* file.

7.6.11. Calculate undiluted amplicon concentration by multiplying the dilution factor (50) with the concentration given from the LabChip GX (nmol/L).

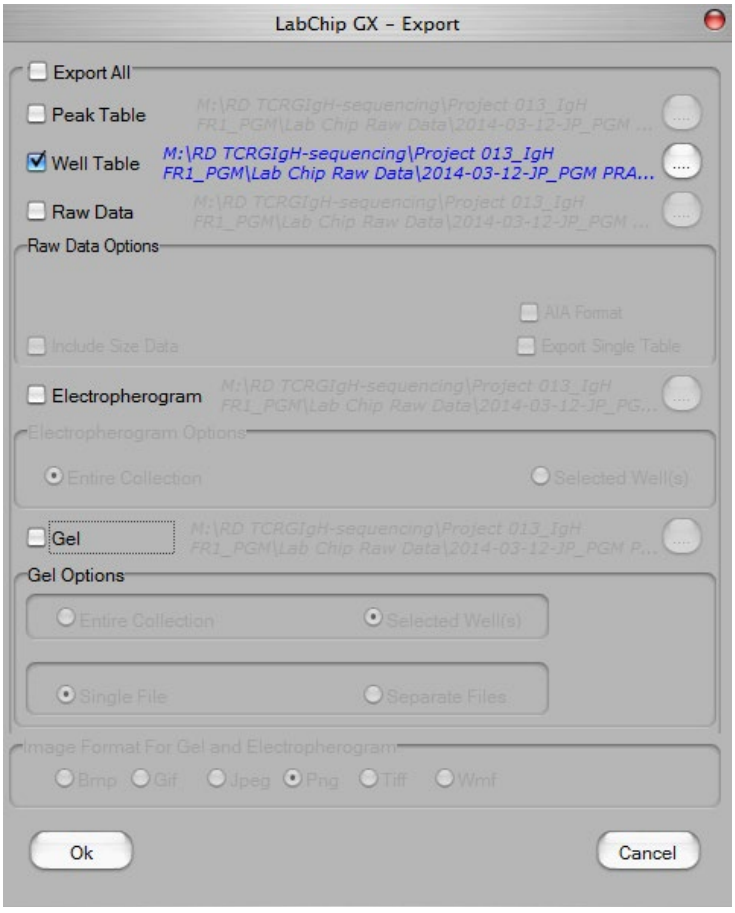



**Figure 5:** Example of LabChip GX – Export pop up window.

## 7.7. Pooling and Quantification of Library

The quantity of library DNA loaded into the Ion S5 or Ion PGM emulsion PCR is critical for generating high-quality data in a sequencing run.

Amplicons generated from one or multiple LymphoTrack Dx Assays can be pooled together into one library for sequencing using the instructions listed below.

7.7.1. Based on the amplicon concentration calculated by the Bioanalyzer or LabChip GX, add an **equal amount of amplicons** (with the exception of the NTC sample).

- *e.g.*, combine 10 µL of amplicons at 4 nM each in a tube; using 1X TE buffer as a diluent.
- Vortex the library tube for 5-15 seconds and then centrifuge for 3-5 seconds.

If there are any samples with concentrations considerably lower or higher than 4 nM adjust the sample/TE buffer volumes added to the library, ensuring that equal amount of amplicons per sample are pipetted.

## 7.8. Dilution of the Pooled Library

7.8.1. Determine the template dilution factor that gives a final concentration of ~20 pM (~12 x $10^6$ molecules per µL) using the following formula:

**Template Dilution Factor = (Library Concentration in pM) / 20 pM**

**Example:**
The library concentration is 4 nM (4000 pM)
Template Dilution Factor = 4000 pM / 20 pM = 200

Thus, 1 µL of library mixed with 199 µL of 1X TE buffer or the Nuclease-free water provided in the Ion PGM Hi-Q View OT2 Kit (1:200 dilution) yields approximately 20 pM (~12 x $10^6$ molecules per µL).

Use the diluted library within 48 hours of preparation.

## 7.9. Template Preparation and Sequencing using the Ion Chef and Ion S5

Prepare and enrich template-positive Ion Sphere Particles followed by sequencing on the Ion S5 adhering to the following Thermo Fisher Scientific User Guide:

- Ion 510 & Ion 520 & Ion 530 Kit – Chef (REF MAN0016854)

7.9.1. Create planned run following section 7.12.

All steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions.

## 7.10. Template Preparation and Sequencing using the Ion OT2 and Ion S5

Prepare and enrich template-positive Ion Sphere Particles using the OT2 followed by sequencing with the Ion S5, adhering to the following Thermo Fisher Scientific User Guide:

- Ion 520 & Ion 530 Kit – OT2 (REF MAN0010844)

7.10.1. Create planned run following section 7.12.

All steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions.

## 7.11. Template Preparation and Sequencing using the Ion OT2 and Ion PGM

Prepare and enrich template-positive Ion Sphere Particles using the OT2 followed by sequencing with the Ion PGM, adhering to the following Thermo Fisher Scientific User Guides:

- Ion PGM Hi-Q View OT2 Kit (REF MAN0014579)
- Ion PGM Hi-Q View Sequencing Kit (REF MAN0014583)

All steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.

**Note:** **Do not use** the Ion PGM Calibration Standard.

7.11.1. Prepare template using the Ion OT2

- Select **PGM: Ion PGM Hi-Q View OT2 Kit - 400** from the drop-down menu

7.11.2. Sequence the library using the Ion PGM

- Follow section 7.11.3 for Chip Loading
- Create planned run following section 7.12.

7.11.3. Ion PGM Chip Loading

Use the chip loading instructions below for optimal chip loading.

7.11.3.1. Following *Chip Check*, prepare the chip for loading following the manufacturer's instructions.

7.11.3.2. After dialing the pipette down to load the ISPs (~30 µL) into the chip at a rate of ~1 µL per second, transfer the chip in the bucket to the minifuge with the chip tab pointing in (toward the center of the minifuge).

- Use 30 second minifuge spins with the chip tab pointing in and then turned to point out.

7.11.3.3. Firmly tap the point of the chip tab on the bench top 2-3 times. To avoid the generation of bubbles, do not pipet the sample out and then back into the chip.

7.11.3.4. Tilt the chip at a 45° angle and slowly remove as much liquid as possible from the loading port by dialing up the pipette. Discard the liquid.

7.11.3.5. If some liquid remains in the chip, perform a 5-second quick spin with the chip-tab pointing out and remove and discard any additional liquid. Do not spin the chip upside-down.

7.11.3.6. If some liquid remains in the chip after the quick spin, lightly and rapidly tap the point of the chip tab against the benchtop a few times and remove and discard any collected liquid. Do not flush the chip. Immediately proceed to selecting the *Planned Run* and *Performing the Run* (section 7.12).

**Note:** Validations were performed following the Thermo Fisher Scientific User Guides listed in section 16: *References* along with the weighted bucket chip loading procedure. After the sequencing primer annealing step, the reactions remained in the thermal cycler at 15°C instead of at room temperature.

If using Torrent Suite Software v5.2.2 or v5.6, follow the procedure in Appendix A to verify the configuration of the *FileExporter* plugin and load the custom barcodes using the *LymphoTrack_IonXpress.csv* file included on the provided CD (REF 95000007). If using TSS v5.0.4, please continue to step 7.12.

## 7.12. Create a Planned Run.

7.12.1. Create a Planned Run for **Ion S5** or **PGM**. Log into the Torrent Browser for the Torrent Server connected to the system.

7.12.2. Click the **Plan** tab and then click on **Generic Sequencing** under *Templates,* then select **Plan New Run** on the top right.

7.12.3. In the *Plan Run Wizard,* review each screen and make each selection following Table 13.

7.12.4. Select the *Planned Run* and perform the run.

**CAUTION!** If using TSS v5.2.2or v5.6 please contact Thermo Fisher Tech Support for assistance with uploading barcodes.

**Characters in file name:**

Give each sample a unique name or identifier when naming samples. If duplicate samples are run, a similar name can be used (*i.e.,* Sample1a and Sample1b).

Failure to provide unique names to samples that will be run together on the same chip will result in multiple sample results being combined by the LymphoTrack Dx Software – S5/PGM during the analysis process.

It is important that the filenames only contain the following characters (A-Z, a-z, 0-9, ., _ (underscore), - (hyphen)).

If the software encounters a character not within this set or more than one consecutive space, it may fail.

**Sample name when multiplexing:**

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

It is recommended to keep track of all samples and targets in an Ion S5 or Ion PGM run that are sequenced using the same index. This set of samples/targets should be given a unique identifier to include in the Sample Name field in the Planned Run.

Examples of Sample Names 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_TRG (multiple samples sequenced with multiple assays using the same index)
- Pool02_IX002 (Pool 02 refers to all samples/targets sequenced with IonXpress_002 and tracked elsewhere)

**Table 13.** Run Plan Wizard settings by platform combination.

| Setting | Validated Platform Combination | | |
|---|---|---|---|
| | **Ion Chef and Ion S5** | **OT2 and Ion S5** | **OT2 and Ion PGM** |
| **Ion Reporter** | Ion Reporter – None<br>Sample Grouping – Blank | Ion Reporter – None<br>Sample Grouping – Blank | Ion Reporter – None<br>Sample Grouping – Blank |
| **(Research) Application** | Application – DNA<br>Target Technique – Other | Application – DNA<br>Target Technique – Other | Application – DNA<br>Target Technique – Other |
| **Kits** | Sample Preparation Kit – leave blank | Sample Preparation Kit – leave blank | Sample Preparation Kit – leave blank |
| | Library Kit Type – leave blank | Library Kit Type – leave blank | Library Kit Type – leave blank |
| | Template Kit:<br>▪ Select Instrument: Chef<br>▪ Ion 510 & Ion 520 & Ion 530 Kit – Chef | Template Kit:<br>▪ Select Instrument: OneTouch<br>▪ Ion 520 & Ion 530 Kit – OT2 | Template Kit:<br>▪ Select Instrument: OneTouch<br>▪ Ion PGM Hi-Q View OT2 Kit - 400 |
| | Sequencing Kit<br>▪ Ion 510 & Ion 520 & Ion 530 Kit – Chef | Sequencing Kit<br>▪ Ion 520 & Ion 530 Kit – OT2 | Sequencing Kit<br>▪ Ion PGM Hi-Q View Sequencing Kit |
| | Templating Size – 400bp | Templating Size – 400bp | n/a |
| | Flows – 850 | Flows – 850 | Flows – 850 |
| | Control Sequence – leave blank | Control Sequence – leave blank | Control Sequence – leave blank |
| | Chip Type:<br>▪ Ion 520 Chip or<br>▪ Ion 530 Chip | Chip Type:<br>▪ Ion 520 Chip or<br>▪ Ion 530 Chip | Chip Type:<br>▪ Ion 316 Chip v2 BC or<br>▪ Ion 318 Chip v2 BC |
| | Barcode Set – IonXpress or LymphoTrack_IonXpress[3] | Barcode Set – IonXpress or LymphoTrack_IonXpress[3] | Barcode Set – IonXpress or LymphoTrack_IonXpress[3] |
| **Plug-ins** | Select FileExporter[1,2] | Select FileExporter[1,2] | Select FileExporter[1,2] |
| **Projects** | Select the appropriate Project folder for saving the run or add a new project | Select the appropriate Project folder for saving the run or add a new project | Select the appropriate Project folder for saving the run or add a new project |
| **Template Name** | Enter a Template Name and add any notes | Enter a Template Name and add any notes | Enter a Template Name and add any notes |

**Table 13.** Run Plan Wizard settings by platform combination.

| Setting | Validated Platform Combination | | |
|---|---|---|---|
| | **Ion Chef and Ion S5** | **OT2 and Ion S5** | **OT2 and Ion PGM** |
| **Reference** | Leave all sections blank | Leave all sections blank | Leave all sections blank |
| **Monitoring** | Bead Loading (%) ≤ 30<br>Key Signal (1-100) ≤ 30<br>Usable Sequence (%) ≤ 30 | Bead Loading (%) ≤ 30<br>Key Signal (1-100) ≤ 30<br>Usable Sequence (%) ≤ 30 | Bead Loading (%) ≤ 30<br>Key Signal (1-100) ≤ 30<br>Usable Sequence (%) ≤ 30 |

**Note[1]**: The Torrent Suite™ Software version 5.0.4 PlugIn *FileExporter* may not generate FASTQ Files. Contact Thermo Fisher Tech Support for further assistance if a "DOCSTRING ERROR" message is received.
**Note[2]:** Avoid long file names as they may interfere with the PlugIn *FileExporter*.
**Note[3]:** If using TSS v5.2 or v5.6, please refer to the *LymphoTrack_IonXpress.csv* file located in the provided software CD (REF 95000007)

# 8. Data Analysis

The LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – S5/PGM were designed to produce sequencing data that can be analyzed using the LymphoTrack Dx Software – S5/PGM package provided on the associated CD (REF 95000007). **This CD includes detailed instructions for installation and use of the software.**

Samples prepared with the LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – S5/PGM provides FASTQ files that can be easily processed into fully analyzed data using the LymphoTrack Dx Data Analysis application.

**Characters in pathname and file name:**

1) Avoid spaces in the pathname for the data files or software (pathnames include file folders and file names); more than one consecutive space is not permitted.

2) It is important that the filenames only contain the following characters (A-Z, a-z, 0-9, _ (underscore), - (hyphen)).

If the software encounters a character not within this set or more than one consecutive space in the file name, the LymphoTrack Dx Software – S5/PGM may fail. Please make sure to only use these characters when setting up the planned run.

# 9. Assay Specifications

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

- S5 and PGM Run Validity
  - Loading > 50%,
  - Enrichment > 50%
  - Clonal > 50%
- *IGH* Positive Control: top % reads ≥ 2.5%
- NGS Negative Control: top % reads < 1.0%
- *IGH* SHM Positive Control (REF 40880008, can be purchased separately) top % reads ≥ 2.5%

# 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.
- Always interpret the results of molecular clonality tests in the context of clinical, histological and immunophenotypic data.

# 10.

# 11. Interpretation and Reporting

Use the *Merged Read Summary* report to identify the top merged read sequences and their frequencies prior to clonality determination using the criteria listed below. Refer to section 8: *Data Analysis* section for more information on the *Merged Read Summary* report. There are some clonal processes that may result in the detection of two or more clones. Examples of this include a dominant population with a small sub-clonal population or when multiple lymphoproliferative disorders are present. It is especially important that these cases are interpreted within their clinical context.

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

**Table 14.** Interpretation Criteria

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

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

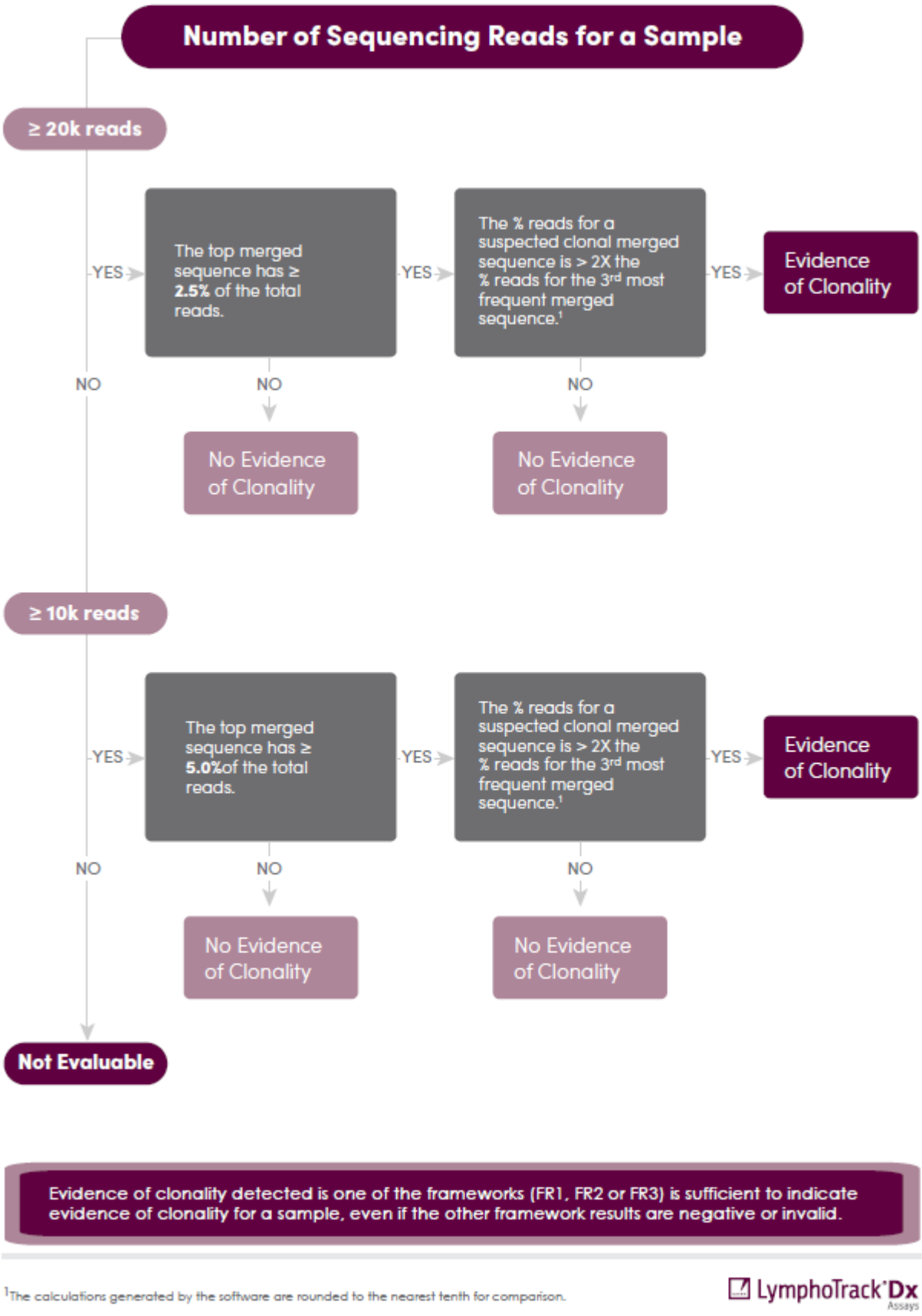



**Figure 6**: Interpretation of data based on Criteria from Table 14.

After clonality is determined, samples can be evaluated using the LymphoTrack Dx *IGH* FR1 Assay – S5/PGM for evidence of somatic hypermutation (SHM) if needed. The SHM interpretation criteria listed below are suggestions for using immunoglobulin gene sequence analysis for CLL prognostication based on current literature (Langerak *et al.,* 2011).

For SHM interpretation, evaluate both the top two merged sequences for evidence of clonality using the clonality interpretation provided in the previous section. Each merged sequence showing evidence of clonality can then be evaluated using the SHM criteria listed below (see Figure 7 for corresponding flow chart). Interpret rearrangements with mutational status at or near the 2% cutoff with caution for the purpose of prognosis. The *IGH* FR1 forward primers are not located at the immediate 5' end of the *IGHV* region. Any mutations in nucleotides upstream of the primer binding site will not be included in the calculated mutation rate.

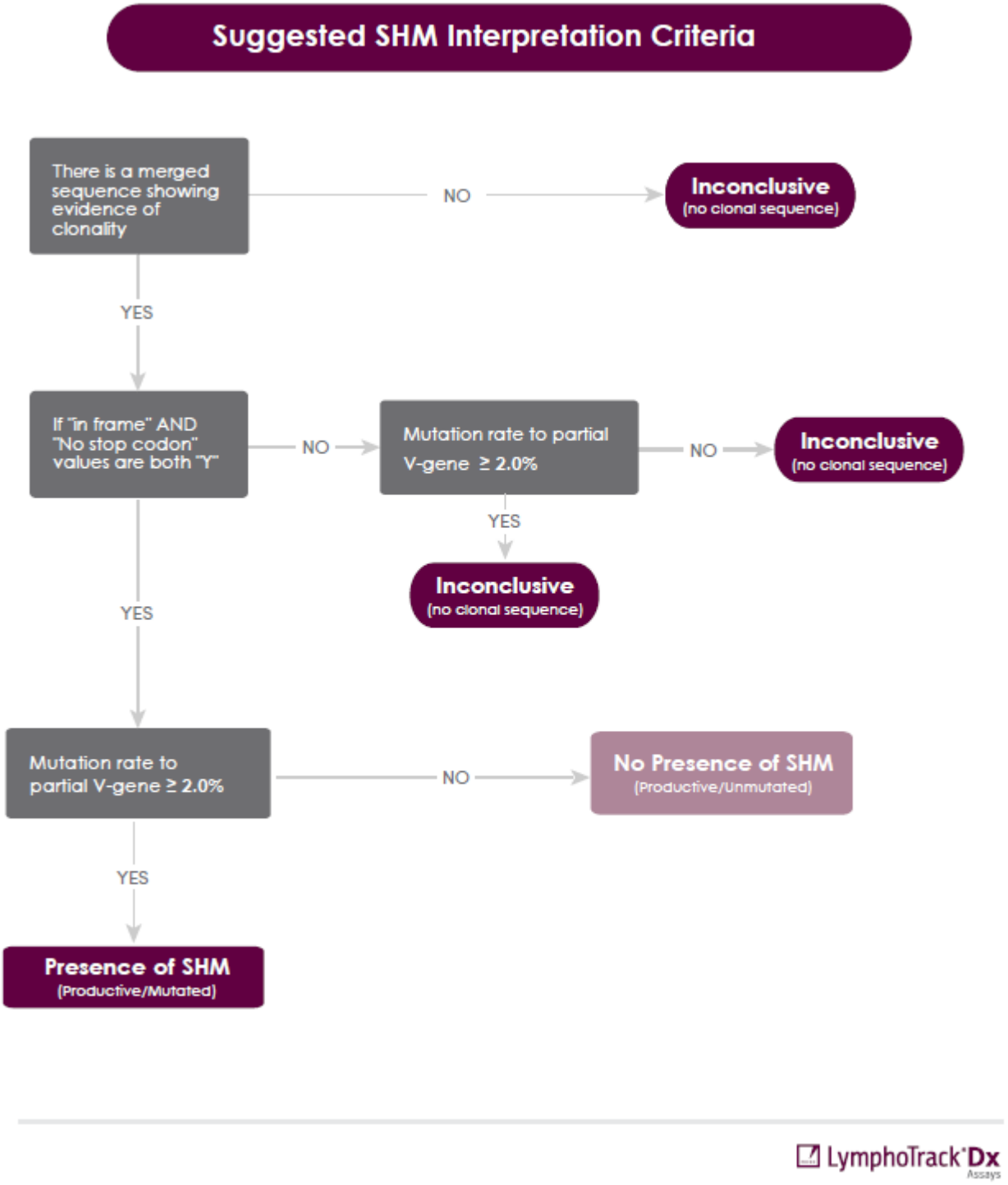
**Table 15.** Suggested SHM Interpretation Criteria

| Criterion 1 | Criterion 2 | Criterion 4 | Result |
|---|---|---|---|
| There is a merged sequence showing evidence of clonality. | If "**In-frame**" AND "**No stop codon**" values are both "**Y**" | Mutation rate to partial V-gene **≥ 2.0%** | **PRESENCE OF SHM** (Productive/Mutated) |
| | | Mutation rate to partial V-gene **< 2.0%** | **No presence of SHM** (Productive/Unmutated) |
| | If either (or both) "**In-frame**" OR "**No stop codon**" value is "**N**" | Mutation rate to partial V-gene **≥ 2.0%** | **Inconclusive** (Unproductive/Mutated) |
| | | Mutation rate to partial V-gene **< 2.0%** | **Inconclusive** (Unproductive/Unmutated) |
| There are no merged sequences showing evidence of clonality. | N/A | N/A | **Inconclusive** (no clonal sequence) |

If there are two merged sequences for a sample that both show evidence of clonality, evaluate each using Table 16 to determine the final SHM result for that sample.

**Table 16.** Suggested Double Rearrangement SHM Interpretation Criteria

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




**Note:** If there are two merged sequences for a sample that both show evidence of clonality, evaluate each using Table 16 to determine the final SHM result for that sample.

**Figure 7**: Suggested Somatic Hypermutation (SHM) Interpretation of data based on criteria from Table 15.

# 12. Sample Data

LymphoTrack Dx Report for assay IGH_FR1

Sample name: index001_001

Total Read Count: 271857

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

| Rank | Sequence | Length | Merge count | V-gene | J-gene | % total reads | Cumulative % | Mutation rate to partial V-gene (%) | In-frame (Y/N) | No Stop codon (Y/N) | V-coverage | CDR3 Seq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CATCTGGATACAC | 295 | 28770 | IGHV1-46_03 | IGHJ4_02 | 10.58 | 10.58 | 0.00 | Y | Y | 100.00 | GCTAGAGATCTCA |
| 2 | GCCTCTGGATTCA | 116 | 146 | IGHV3-66_03 | IGHJ4_02 | 0.05 | 10.64 | 0.45 | N | N | 14.73 | not found |
| 3 | TATTGTCTCTGGTC | 279 | 130 | IGHV4-31_03 | none | 0.05 | 10.68 | 19.66 | n/a | N | 97.86 | GCGAGACATGGTA |
| 4 | GCCTCTGGATTCA | 292 | 102 | IGHV3-23_04 | IGHJ6_02 | 0.04 | 10.72 | 2.64 | N | N | 99.12 | not found |
| 5 | CGCTGTCTATGGT | 188 | 101 | IGHV4-34_12 | IGHJ6_02 | 0.04 | 10.76 | 1.33 | n/a | N | 34.07 | not found |
| 6 | TTTCTGGATGTAC | 170 | 96 | IGHV5-51_05 | IGHJ5_02 | 0.04 | 10.79 | 0.48 | n/a | N | 20.57 | not found |
| 7 | CACCTTCTCTGGG | 177 | 86 | IGHV2-5_10 | none | 0.03 | 10.83 | 4.05 | n/a | N | 70.04 | not found |
| 8 | TGTCATCTCCGGG | 279 | 78 | IGHV6-1_02 | IGHJ6_02 | 0.03 | 10.85 | 11.20 | Y | Y | 98.34 | not found |
| 9 | CTTCTGGATACAC | 307 | 75 | IGHV1-8_01 | IGHJ6_02 | 0.03 | 10.88 | 0.00 | Y | N | 99.56 | not found |
| 10 | CTTCTGGTTACAC | 280 | 74 | IGHV1-18_01 | IGHJ4_02 | 0.03 | 10.91 | 0.00 | Y | Y | 99.56 | GCGAGAGTGGATA |

**Figure 8**: This table, generated via the LymphoTrack Reporter, 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 one or two bp different. Sequences were generated using the LymphoTrack Dx *IGH* FR1 Assay – S5/PGM and analyzed using the LymphoTrack Dx Software – S5/PGM (REF 95000007).

# 13. Performance Characteristics

The LymphoTrack Dx *IGH* FR1 Assay – S5/PGM results were compared to clinical diagnosis and the concordance (or the overall percent agreement), positive percent agreement (PPA) and negative percent agreement (NPA) of 93% (27/29 cases), 88% and 100%, respectively.

**Table 17.** Comparison Between LymphoTrack Dx *IGH* FR1 Assay – S5/PGM and the Clinical Diagnosis

| | | Clinical Diagnosis | |
|---|---|---|---|
| | | Clonal | Non-Clonal |
| **LymphoTrack Dx *IGH* FR1 Assay – S5/PGM** | **Clonal** | 15 | 2 |
| | **Non-Clonal** | 0 | 12 |

The analytical performance of the LymphoTrack Dx *IGH* FR1 Assay – S5/PGM was evaluated by testing spiked clonal cell line DNA into tonsil DNA at different dilutions. The Limit of Detection (LoD) was observed at 5% DNA dilution. The highest % reads from tonsil DNA was < 1.0%. The linear regression $R^2$ was > 0.99 for a range of 0 to 10% DNA dilution. The coefficient of variation (CV%) across 8 runs (per instrument, Ion S5 and Ion PGM) from 2 operators, 2 reagents lots and 2 instruments was less than 10% when testing 5% and 10% DNA dilutions.

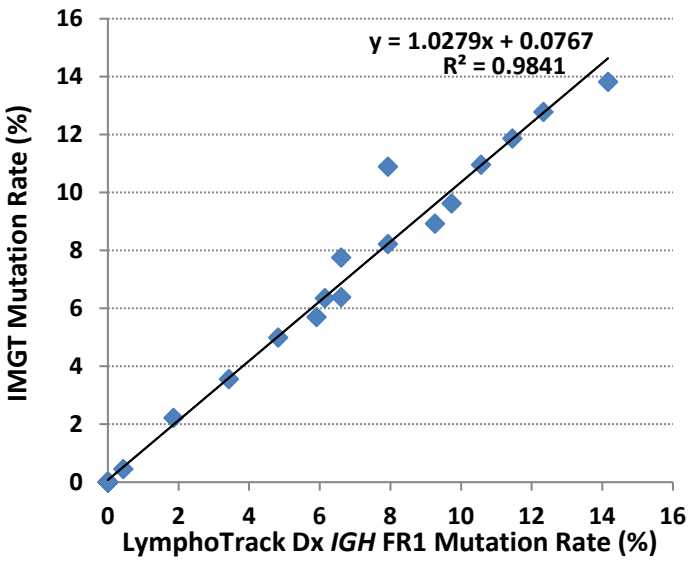



**Figure 9**: Comparison of the somatic hypermutation (SHM) rate for 27 samples determined by LymphoTrack Dx *IGH* FR1 Assay – S5/PGM analyzed either with the LymphoTrack Dx Software – S5/PGM or using IMGT analysis.

**Table 18.** Somatic Hypermutation (SHM) Testing Method Comparison Between LymphoTrack Dx Software – S5/PGM and the Control Method (IMGT)

| | | IMGT | |
|---|---|---|---|
| | | **Mutated** | **Unmutated** |
| **LymphoTrack Dx Software – S5/PGM** | **Mutated** | 14 | 0 |
| | **Unmutated** | 1 | 12 |

Running the LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM improved identification of clonal events compared to running the LymphoTrack Dx *IGH* FR1 Assay – S5/PGM alone. The LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM and traditional capillary electrophoresis assay (IdentiClone® *IGH* Gene Clonality Assay (Tubes A+B+C) – ABI detection, REF 91010061) were compared and the concordance (or the overall percent agreement), positive percent agreement (PPA) and negative percent agreement (NPA) were: 98% (40/41 cases), 100% and 95%, respectively.

**Table 19.** Comparison Between LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM and IdentiClone *IGH* Gene Clonality Assay – ABI

| | | IdentiClone *IGH* Gene Clonality Assay – ABI | |
|---|---|---|---|
| | | **Clonal** | **Non-Clonal** |
| **LymphoTrack Dx *IGH* FR1/2/3 Assay – S5/PGM** | **Clonal** | 22 | 0 |
| | **Non-Clonal** | 1 | 18 |

# 14. Troubleshooting Guide

**Table 20.** Troubleshooting

| 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) |
| Library creation by amplicon quantification and pooling | Amplicon concentration is less than 1 nM | Check the Bioanalyzer or LabChip GX ladder and repeat PCR if less than 1 nM. |
| Template preparation and S5 or PGM initialization | N/A | Call Thermo Fisher Scientific Tech Support +1-800-831-6844 |
| CD installation | LymphoTrack Dx Software does not install properly | Call Invivoscribe Tech Support +1-858-224-6600 |
| Data analysis | LymphoTrack Dx Software stops running | Call Invivoscribe Tech Support +1-858-224-6600 |
| Data analysis | Excel Macro cannot be executed | 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 amplicons after PCR | Repeat the assay |

# 15. Technical and Customer Service

Thank you for purchasing our LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – S5/PGM. 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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10222 Barnes Canyon Road | Building 1 | San Diego | California 92121-2711 | USA
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Technical Service: support@invivoscribe.com | Customer Service: sales@invivoscribe.com | Website: www.invivoscribe.com

# 16. References


- Tonegawa, S. (1983). Somatic Generation of Antibody Diversity. Nature 302:575-581.
- Ghia, P. *et al.,* (2007). ERIC recommendations on *IGHV* gene mutational status analysis in chronic lymphocytic leukemia. *Leukemia* 21, 1–3.
- Trainor KJ, *et al.,* (1990). Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood 75:2220-2222.
- Miller, J.E. (2013). Principle of Immunoglobulin and T Cell Receptor Gene Rearrangement. In Cheng, L., Zhang, D., Eble, J. N. (Eds), *Molecular Genetic Pathology* (2nd Ed., sections 30.2.7.13 and 30.2.7.18). New York, USA: Springer Science & Business Media.
- LymphoTrack Dx Software – S5/PGM package Instructions for Use (REF 95000007)
- User Guide: Ion 510 & Ion 520 & Ion 530 Kit – Chef (REF Man0016854, Rev. C.0)
- User Guide: Ion 520 & Ion 530 Kit – OT2 (REF Man0010844, Rev. D.0)
- User Guide: Ion PGM Hi-Q View OT2 Kit (REF Man0014579 Rev A.0)
- User Guide: Ion PGM Hi-Q View Sequencing Kit (REF Man0014583, Rev A.0)
- Agilent DNA 1000 Kit Guide
- LabChip GX/GX II User Manual
- HT DNA High Sensitivity LabChip Kit LabChip GX/GXII User Guide
- http://www.thermofisher.com
- http://ioncommunity.thermofisher.com
- http://www.agilent.com
- http://www.perkinelmer.com


# 17. Symbols

The following symbols are used in Invivoscribe NGS diagnostic product labeling.

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

# 18. Legal Notice

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

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.

# 19. LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – S5: Single Page Guide

19.1. Using gloved hands, remove the Master Mixes from the freezer. Use a different indexed Master Mix for each sample and controls. Allow the Master Mix tubes to thaw; then gently vortex to mix.

19.2. In a containment hood or dead air box pipette 45 µL of Master Mix into individual wells of a PCR plate for the samples, positive, negative, and no template controls (one well for each indexed Master Mix).

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

19.4. Add 5 µL sample DNA (at a minimum concentration of 10 ng/µL) and 5 µL of control samples to wells containing the respective Master Mix reactions, and pipette up and down 5-10 times to mix.

19.5. Add 5 µL molecular biology grade water to the well containing the respective Master Mix for no template control, and pipette up and down 5-10 times to mix.

19.6. Seal the plate and amplify target DNA using the standard thermal cycler program:

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

19.7. Remove the amplification plate from the thermal cycler.

19.8. Purify the PCR products using the Agencourt AMPure XP PCR Purification system. Add 90 µL of particles to each 50 µL reaction then elute purified DNA in 40 µL TE buffer.

19.9. Quantify amplicons with an appropriate method (*e.g.,* Agilent 2100 Bioanalyzer or LabChip GX**)**.

19.10. Based on the quantification, combine an equal amount of each amplicon in a tube (do not include the no template control); use TE buffer to make up a total volume of 10 µL per Master Mix. Gently vortex to mix followed by a brief centrifugation.

19.11. Dilute the library to 20 pM with Nuclease-free water.

19.12. Perform emulsion PCR to prepare the template by using either the Ion Chef or the Ion OT2 paired with the Ion ES instruments.

a. Using the Ion Chef instrument with the Ion 510 & Ion 520 & Ion 530 Kit – Chef or
b. Using the Ion OneTouch instrument with Ion 520 & Ion 530 Kit – OT2

19.13. Initialize the Ion S5 and load the Ion 520 or Ion 530 chip with the ISPs.

19.14. Create a Planned Run using the Torrent Browser.

19.15. Start the Ion S5 run.

19.16. Analyze and visualize the acquired data using the associated LymphoTrack Software – S5/PGM package.

# 20. LymphoTrack Dx *IGH* (FR1/FR2/FR3) Assays – PGM: Single Page Guide

20.1. Using gloved hands, remove the Master Mixes from the freezer. Use a different indexed Master Mix for each sample and controls. Allow the Master Mix tubes to thaw; then gently vortex to mix.

20.2. In a containment hood or dead air box pipette 45 µL of Master Mix into individual wells of a PCR plate for the samples, positive, negative and no template controls (one well for each indexed Master Mix).

20.3. Add 0.2 µL Taq DNA polymerase (@5 U/µL) to each of the Master Mixes.

20.4. Add 5 µL 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.

20.5. Add 5 µL 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.

20.6. Seal the plate and amplify target DNA using the standard 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 |

20.7. Remove the amplification plate from the thermal cycler.

20.8. Purify the PCR products using the Agencourt AMPure XP PCR Purification system. Add 90 µL of particles to each 50 µL reaction then elute purified DNA in 40 µL TE buffer.

20.9. Quantify amplicons with an appropriate method (*e.g.,* Agilent 2100 Bioanalyzer or LabChip GX**)**.

20.10. Based on the quantification, combine an equal amount of each amplicon in a tube (do not include the no template control); use TE buffer to make up a total volume of 10 µL per Master Mix. Gently vortex to mix followed by a brief centrifugation.

20.11. Dilute the library to 20 pM with 1X TE buffer or the Nuclease-free water provided with the Hi-Q View OT2 Kit.

20.12. Using the Ion OneTouch 2 instrument with the Ion PGM Hi-Q View OT2 Kit, perform an emulsion PCR to create template-positive ion sphere particles (ISPs).

20.13. Enrich the template-positive ISPs with the Ion OneTouch ES.

20.14. Initialize the Ion PGM and load the Ion 316 Chip v2 BC (or Ion 318 Chip v2 BC) with the ISPs.

20.15. Create a Planned Run using the Torrent Browser.

20.16. Start the Ion PGM run.

20.17. Analyze and visualize the acquired data using the associated LymphoTrack Dx Software – S5/PGM package.

# 21. Appendix A: Configure the Plugin *FileExporter* and Load Custom Barcodes

If using Torrent Suite Software v5.2.2 or v5.6, verify the configuration of the *FileExporter* plugin and load the custom barcodes using the *LymphoTrack_IonXpress.csv* file included on the provided software CD (REF 95000007).

## 21.1. Verify the configuration of plugin *FileExporter*.

21.1.1. Log in to Torrent Suite Software as admin.

21.1.2. Confirm the correct plugin configuration for *FileExporter*.

- From the gear icon ( ⚙ ) on the *Torrent Suite Server Home Screen* and select **Plugins** from the dropdown menu.
- Find the *FileExporter* plugin and click on the gear icon, then select **Configure** (the configuration window will open).
- Under **File > Options**: check the FASTQ boxes.
- For *Archive Type*, select **Zip**.
- Select the preferred *Naming Option*.
- Save the configuration.

21.1.3. Verify the **Change Plugin Configuration** for the *FileExporter* plugin.

- Click on the gear icon ( ⚙ ) and select **Configure**.
- Scroll down and click **Admin Interface**.
- Scroll down and select **Plugins** on the left hand side, then select **FileExporter**.
- Verify the sections for *Status* and *Userinputfields* display '**{ }**', if not, delete the contents until '**{ }**' is displayed. Do not change any other parameters (not mentioned here).
- Select **Save**.

## 21.2. Load Custom barcodes.

21.2.1. Log on to Torrent Suite Software as admin.

- Select the gear icon ( ⚙ ) the select **References** from the dropdown menu.
- Select Barcodes then select Add new DNA Barcodes.
- Select **Choose File** and upload the *LymphoTrack IonXpress.csv* file
- Name *Barcode set name* (*e.g.* LymphoTrack_IonXpress) and click **Upload**
- This name of barcodes will be used for all LymphoTrack assays in the run set up.
- Verify the newly added barcodes are shown in the **Barcodes** menu.

21.2.2. Proceed to step 7.12. *Create a Planned Run*.