LymphoTrack® Assays

Technical Training

MiSeq® and Ion S5/PGM™ Platforms

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LymphoTrack® - Workflow

LymphoTrack® Assays

MiSeq®

PGM™ S5™

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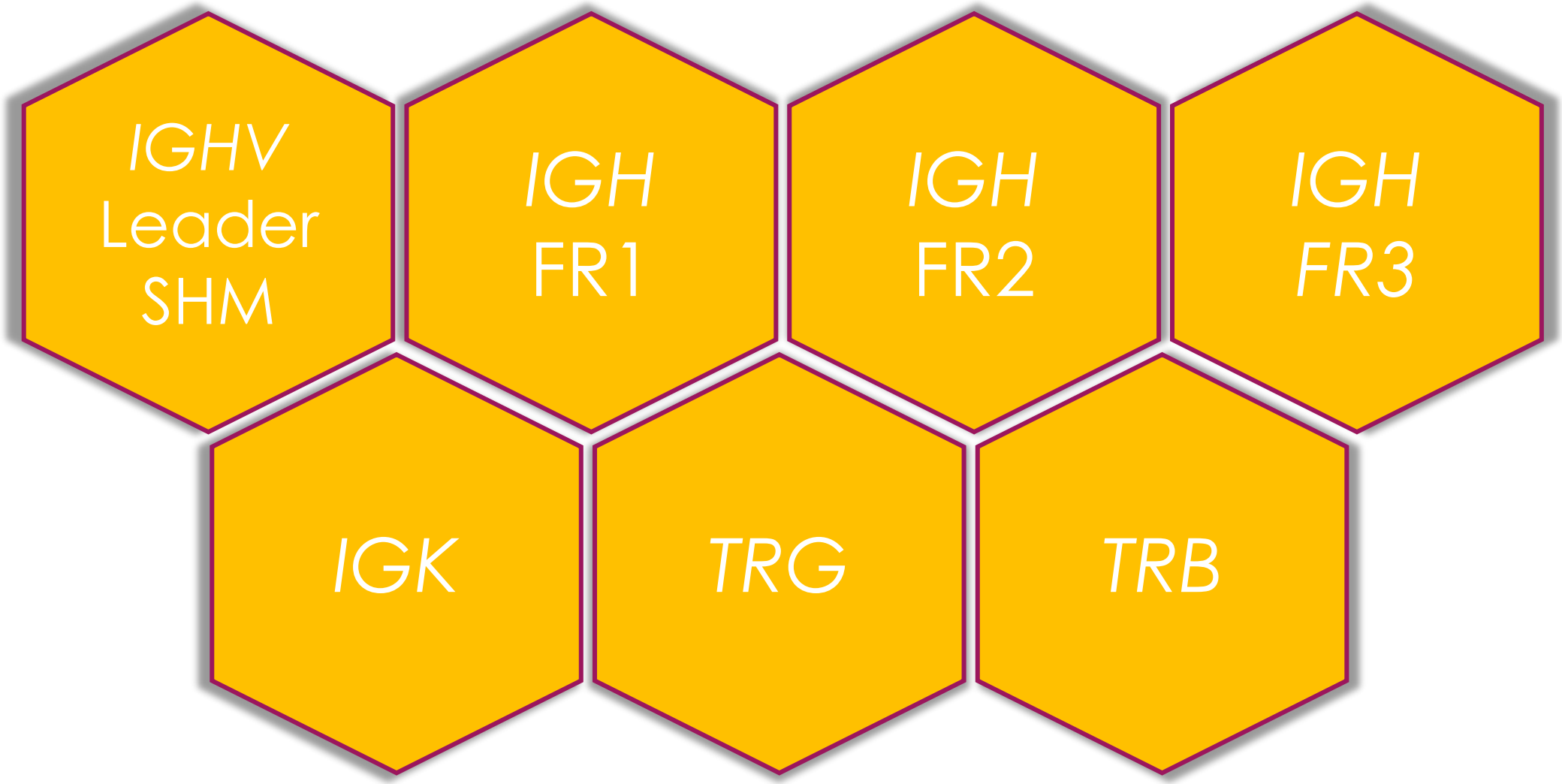
LymphoTrack® - MiSeq® Target Loci



LymphoTrack® - S5/PGM™ Target Loci

LymphoTrack® Assays

IGH FR1

IGH FR2

IGH *FR3*

IGK

TRG

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LymphoTrack® - Available Kits

LymphoTrack® Assays

RUO Products	MiSeq®		S5/PGM™ 12 indices (60 rxn)
	Kit A 8 indices (40 rxn)	Panel 24 indices (120 rxn)	
LymphoTrack® *IGHV* Leader Somatic Hypermutation Assay	✓	✓	
LymphoTrack® *IGH* FR1 Assay	✓	✓	✓
LymphoTrack® *IGH* FR2 Assay	✓	✓	✓
LymphoTrack® *IGH* FR3 Assay	✓	✓	✓
LymphoTrack® *IGH* FR1/2/3 Assay	✓	✓	✓
LymphoTrack® *IGK* Assay	✓	✓	✓
LymphoTrack® *TRG* Assay	✓	✓	✓
LymphoTrack® *TRB* Assay	✓	✓	

48 indices available for *IGH* FR1 MiSeq®

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LymphoTrack® Assays

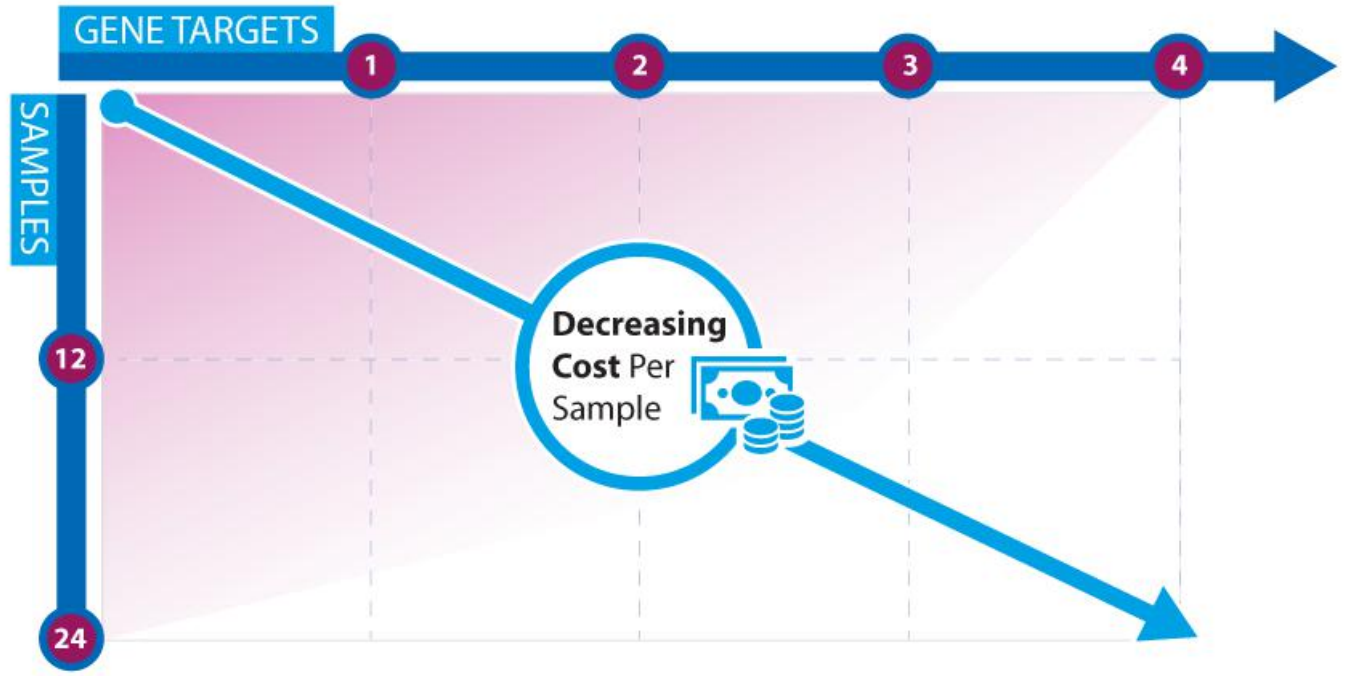
Experiment Planning

LymphoTrack® - Experiment Planning

LymphoTrack Assays

Key Factors

- How many samples?
- How many targets?
- Which targets?



These will determine:

- # of indices needed (8 / 12 / 24)
- MiSeq® Kit/Cycles, Ion™ Chip
- Cost per target per sample

LymphoTrack® - MiSeq® Compatibility

LymphoTrack® Assays

LymphoTrack® Assay	Avg. Target Size incl. Target, Index & Adaptor (bp)	MiSeq® Reagent		
		Kit v2 (300 cycle)	Kit v2 (500 cycle)	Kit v3 (600 cycle)
IGHV Leader SHM	660	X	X	O
IGH FR1	450	X	O	O
IGH FR2	390	X	O	O
IGH FR3	260	O	O	O
IGK	410	X	O	O
TRG	300	O	O	O
TRB	400	X	O	O

MiSeq® Reagent Kit	Read Length	MCS Version	MiSeq® Run Time	Reads	%≥Q30
v2	2 x 151 bp	v2.6	~ 24 hours	up to 15 Million	> 80%
	2 x 251 bp	v2.6	~ 39 hours		> 75%
v3	2 x 301 bp	v2.6	~ 56 hours	up to 25 million	> 70%

LymphoTrack® - S5/PGM™ Compatibility

LymphoTrack® Assays

LymphoTrack® Assay	Avg. Target Size incl. Target, Index & Adaptor (bp)	Chef/OT2™ Reagent for S5/PGM™
IGH FR1	430	For Ion S5™ use: Ion 510, Ion 520, or Ion 530 – for Ion Chef™ or Ion 520 Chip or Ion 530 Chip - for OneTouch 2™ For Ion PGM™ use: PGM Hi-Q View
IGH FR2	370	
IGH FR3	240	
IGK	390	
TRG	280	

Platform	Template Prep.	Sequencing Kit	Chip	Reads
Ion S5™	Ion Chef™	Ion 510 – Chef Ion 520 – Chef Ion 530 – Chef	Ion 520 Chip	4 – 6 million
	OneTouch 2™	Ion 520 – OT2 Ion 530 – OT2	Ion 530 Chip	15 – 20 million
Ion PGM™	OneTouch 2™	PGM Hi-Q View – OT2	Ion 316 Chip kit v2 BC	2 – 3 million
			Ion 318 Chip Kit v2 BC	4 - 5.5 million

LymphoTrack® - Multiplexing

LymphoTrack® Assays

Single Assay Multiplexing

- Up to 12 (S5/PGM™) or 24 (MiSeq®) samples and controls can be multiplexed into one run
- Up to 48 indices available for *IGH* FR1 MiSeq®

Multi-Target Multiplexing

- The same sample can be tested across multiple LymphoTrack® assays in one run
- Each sample receives a unique index
- # of sequencing cycles must be sufficient for the largest amplicon in the multiplex

	1	2	3	4
A	Index 01 Sample A	Index 09 Sample I	Index 01 Sample A	Index 09 Sample I
B	Index 02 Sample B	Index 10 Sample J	Index 02 Sample B	Index 10 Sample J
C	Index 03 Sample C	Index 11 Sample K	Index 03 Sample C	Index 11 Sample K
D	Index 04 Sample D	Index 12 Sample L	Index 04 Sample D	Index 12 Sample L
E	Index 05 Sample E	Index 13 Sample M	Index 05 Sample E	Index 13 Sample M
F	Index 06 Sample F	Index 14 PosCtrl	Index 06 Sample F	Index 14 PosCtrl
G	Index 07 Sample G	Index 15 NegCtrl	Index 07 Sample G	Index 15 NegCtrl
H	Index 08 Sample H	Index 15 NTC	Index 08 Sample H	Index 15 NTC
	IGH FR1		*TRG*	

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LymphoTrack® - Example Calculation

LymphoTrack® Assays

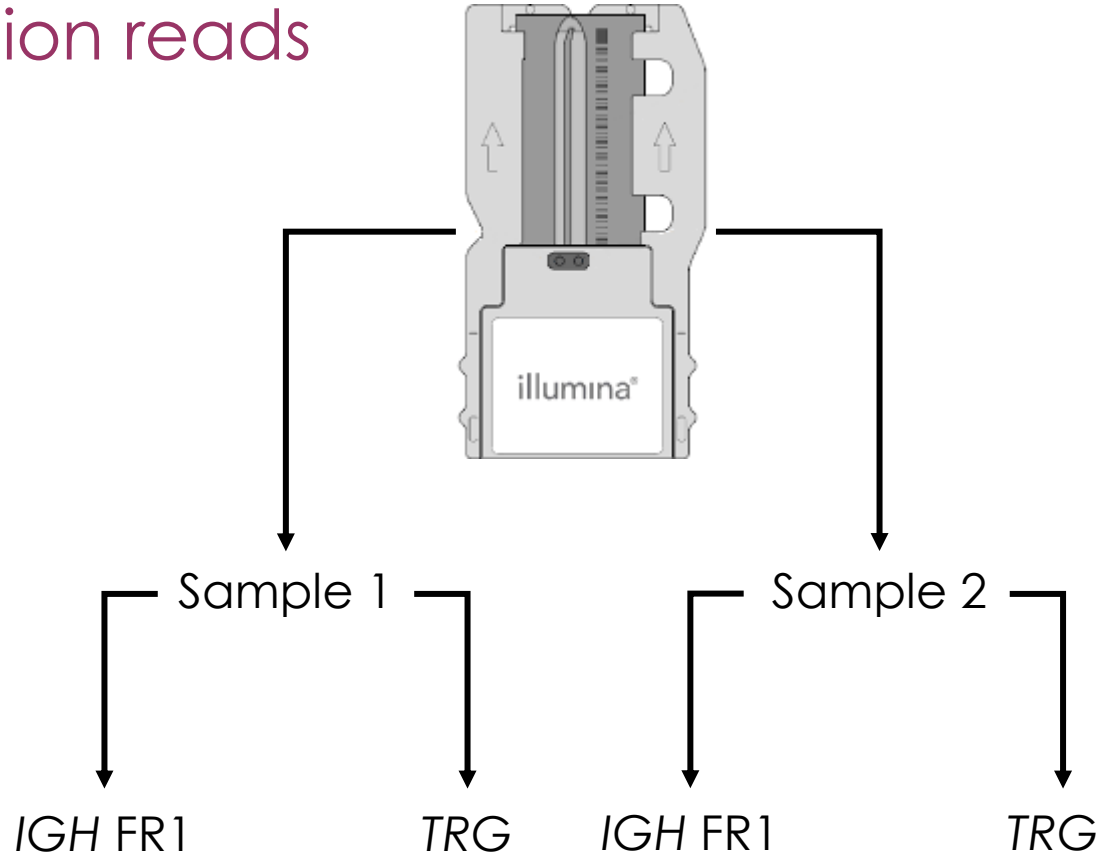
A MiSeq® v3 Flow Cell outputs >20 million reads

20 000 000 Reads

÷ 24 Samples

÷ 7 Targets

~120 000 Reads per Target, Per Sample



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LymphoTrack® Assays

Workflow Overview

LymphoTrack® - Workflow

LymphoTrack® Assays

All LymphoTrack® Assays follow the same **7 step procedure**

Multiplexed assays can be run side-by-side to **reduce laboratory time**

This simplified process allows even new NGS users to succeed with ease

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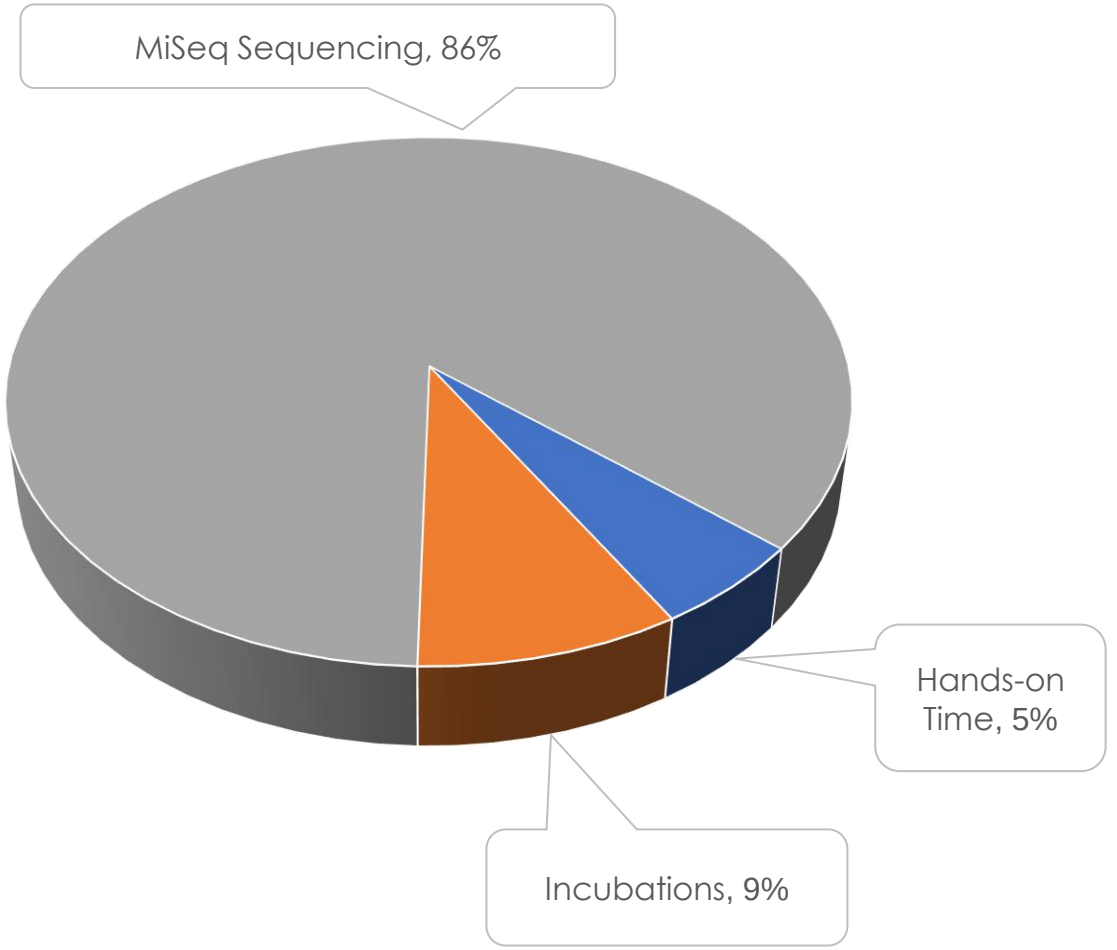
LymphoTrack® - MiSeq® Time

LymphoTrack® Assays

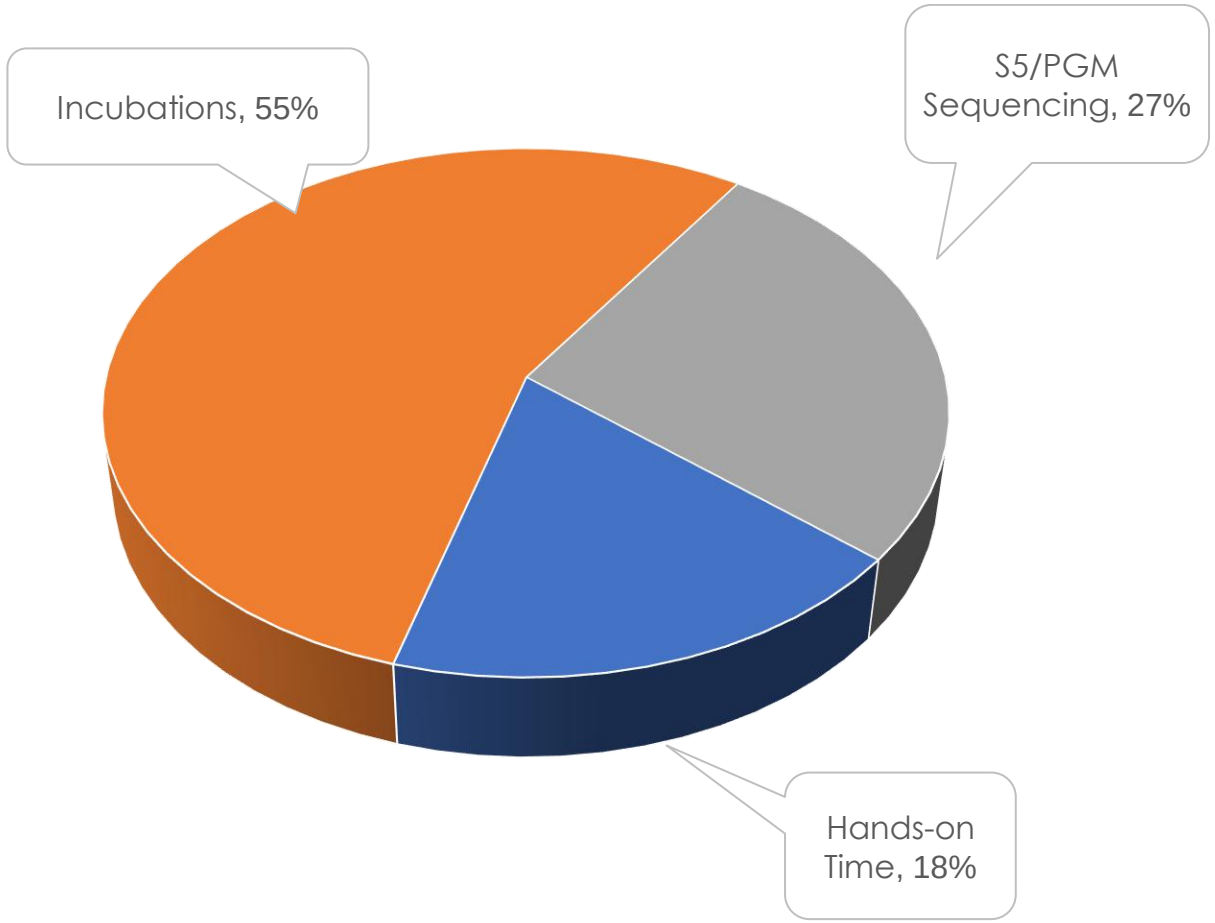
Step	Hands-On	Total Time
Prepare DNA	30 min	30 min
Run PCR	0 min	150 min
Purify Amplicons	30 min	45 min
Quantify	60 min	180 min
Dilute & Pool	30 min	30 min
Load Instrument	30 min	30 min
Sequencing	0 min	~48 hours
Total Time	**~3 hours**	**~56 hours**



LymphoTrack® - S5/PGM™ Time

LymphoTrack® Assays

Step	Hands-On	Total Time
Prepare DNA	30 min	30 min
Run PCR	0 min	150 min
Purify Amplicons	30 min	45 min
Quantify	15 min	45 min
Dilute & Pool	30 min	30 min
Load Instrument	OT2: 120 min Chef: 30 min	OT2: 7 hr Chef: 12 hr
Sequencing	30 min	~4 hours
Total Time	**~3-5 hours**	**~20-25 hours**



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Ion S5/PGM™ - Technical Differences

LymphoTrack® Assays

PGM™
S5™

LymphoTrack® Procedures are consistent across both platforms

Ion Chef™ helps automate template preparation

- Minimizes hands-on time
- Improves consistency of results

Ion S5™ is compatible with Ion OT2™, Chef™ & LymphoTrack® Assays

- Ability to generate longer amplicons
- Increased number of reads

Ion Chef™ & Ion S5™ provide a simple to use, automated NGS solution

- Cartridge-based reagents
- Consumable tracking with the automated RFID

Ion S5/PGM™ - Run Time Comparisons

LymphoTrack® Assays

PGM™ S5™

Step	Total Processing Time (Hours)		
	OT2/PGM	OT2/S5	Chef/S5
PCR	~2.5	~2.5	~2.5
AMPure XP Purification	~0.5	~0.5	~0.5
Quantification & Pooling	~1.0	~1.0	~1.0
Template Prep	~6.5*	~6.5*	~12.0*
Sequencing*	~4.0 – 8.0	~4.0	~4.0
Time-to-Result	**~14.5 – 18.5**	**~14.5**	**~20.0**

Sequencing time will vary depending on the chip in use
Analysis time will vary depending on instrument and chip type
*Hands-on time is significantly reduced for Ion Chef

Step 1 - Prepare DNA Samples

LymphoTrack® Assays

DNA Input

- Use an appropriate resuspension/ elution buffer such as 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- Quantify DNA with a method specific for double-stranded DNA (dsDNA) e.g. Qubit.
- Minimum input quantity is 50 ng of high-quality DNA in 5 µL volume (10 ng/µL)
- Invivoscribe Specimen Control Size Ladder can be used to confirm quality of DNA.

Step 2 - Run PCR

LymphoTrack® Assays

Simplified Setup

- Premade master mix: **just add enzyme and sample**
- Always include positive, negative, and non-template controls!

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

Step	Temperature	Time	Cycles
1	95 °C	07:00	1
2	95 °C	00:45	29x*
3	60 °C	00:45	
4	72 °C	01:30	
5	72 °C	10:00	1
6	15 °C	Hold	1

*32x for *IGHV* Leader

Step 3 - Purify Amplicons

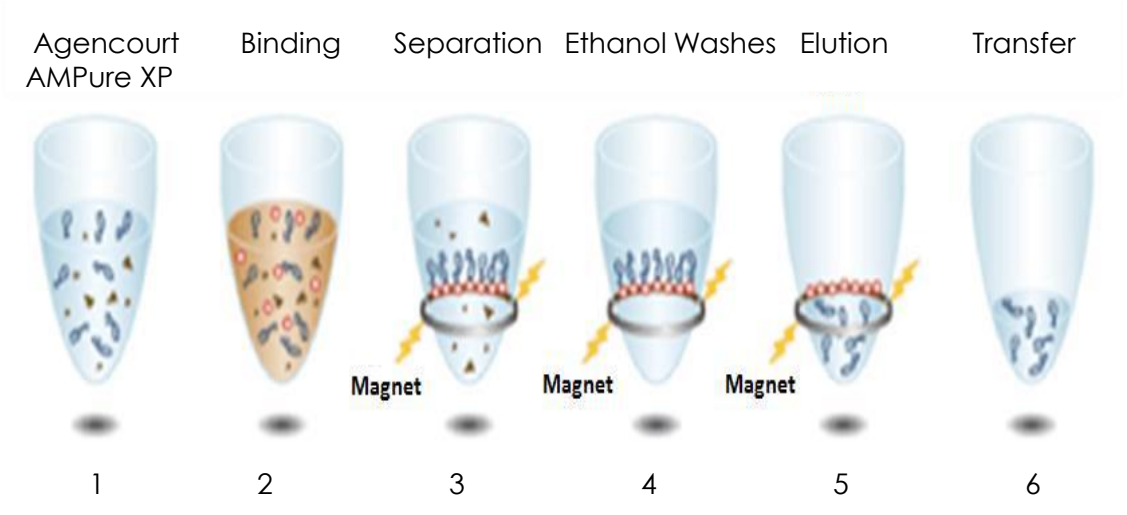
LymphoTrack® Assays

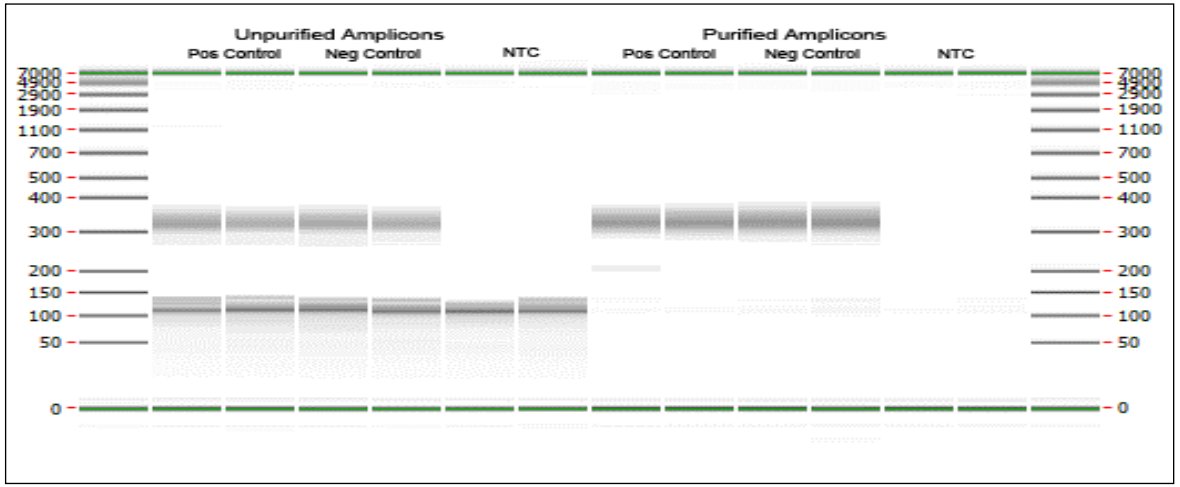
SPRI Bead Purification

- Removes excess primers, salts, and enzymes

Purification Procedure

- Add beads to each sample and incubate at room temperature
- Place on magnetic stand to separate beads from the supernatant
- Wash twice with 200 µL of fresh 70/80% Ethanol
- Air dry at room temperature
- Elute in 10mM Tris pH 8.0





Step 4 - Quantification

LymphoTrack® Assays

PCR Product Quantification (MiSeq®)

- Quantify PCR products using KAPA Library Quantification Kit for Illumina systems
- Compare Ct values of positive and negative controls to the NTC (ΔCt must be ≥ 4.0*)

PCR Product Quantification (S5/PGM™)

- Quantify PCR products using either 2100 BioAnalyzer or LabChip Gx systems

Library Pooling & Quantification

- Dilute samples to 4 nM in at least 10 µL and then pool them together to be equimolar
- Quantify library using the same quantification platform
- MiSeq®: Denature, and then dilute pool to 12-20 pM
- S5/PGM™: Dilute pool to 20 pM

* ΔCt must be ≥ 3.0 for *TRB* MiSeq Assay

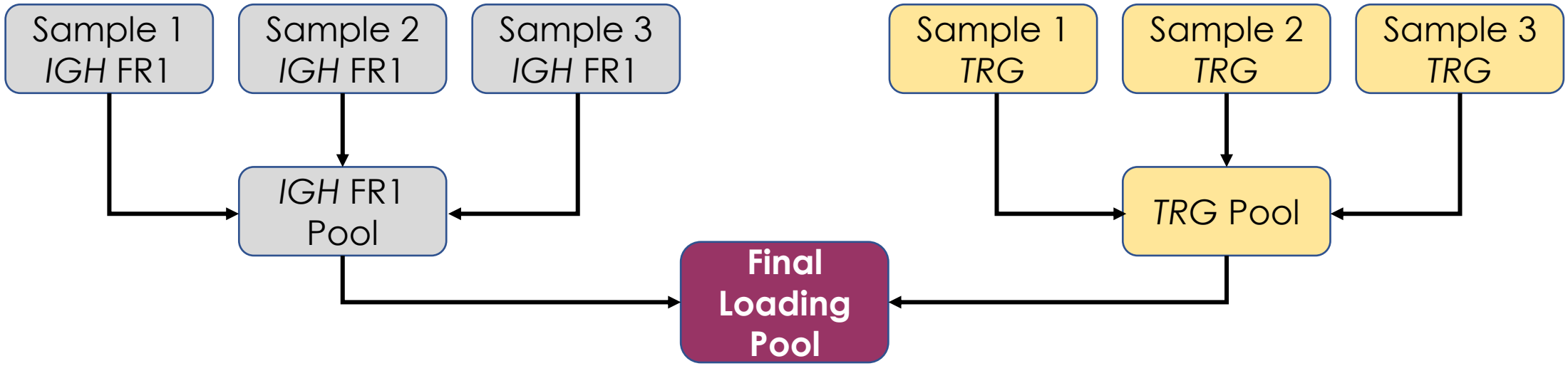
Optional - Target Multiplexing

LymphoTrack® Assays

Generate sample specific library pools & quantify

Use target size and pool concentrations to create an equimolar final loading pool

Best practice to quantify final loading pool for accurate loading



Step 5 - Load and Run MiSeq®

LymphoTrack® Assays

MiSeq®

Library Denaturation & Dilution

- The library pool is denatured into single strands using sodium hydroxide
- After incubation, the library is diluted to 40 pM

Reagent	Volume
4 nM Library	10 µL
0.2 N NaOH	10 µL
Incubate for 5 minutes at RT	
Cold HT1 Buffer	980 µL
Total (40 pM)	**1000 µL**

Loading Concentration

- One final dilution prepares the library for loading.
- The final loading concentration is dependent on the MiSeq® chemistry being used and the targets being sequenced
- Libraries should be loaded within an hour of denaturating & diluting

Reagent	Loading Concentration		
	12 pM	15 pM	20 pM
40 pM Library	300 µL	375 µL	500 µL
HT1 Buffer	700 µL	625 µL	500 µL
TOTAL	**1000 µL**	**1000 µL**	**1000 µL**

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Step 5 - Load and Run MiSeq®

LymphoTrack® Assays

MiSeq®

Loading

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

Sample Sheet

- Use Illumina Experiment Manager™ OR
- Use the provided SampleSheet.csv file and modify the template for your sample needs

Starting Your Run

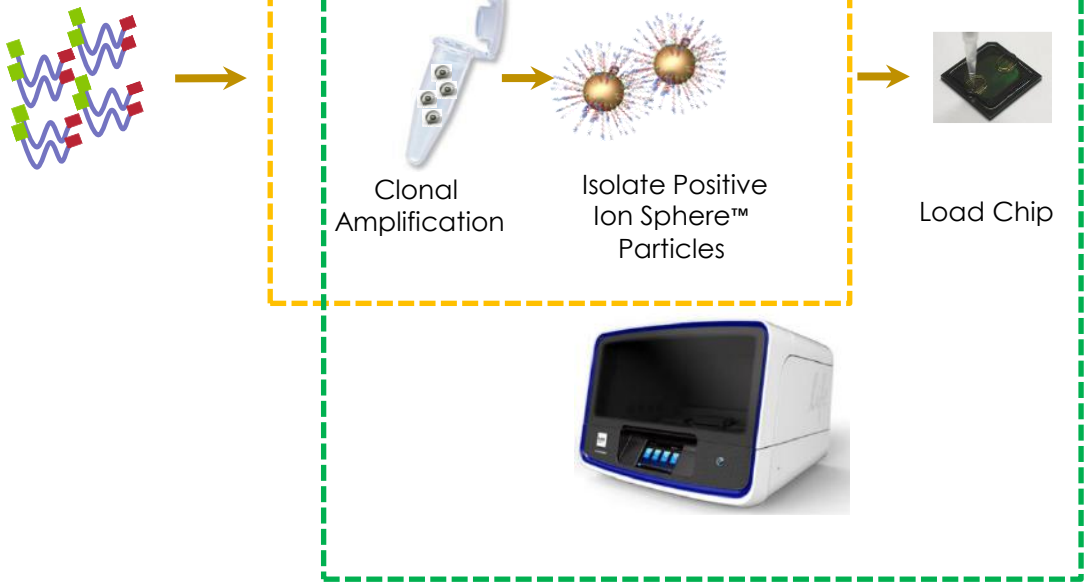
- Direct the MiSeq® to the saved sample sheet
- Start the instrument using the user interface

Step 5 - Load and Run S5/PGM™

LymphoTrack® Assays

PGM™
S5™

Ion OneTouch™ System



Ion OneTouch 2™

1. **Assemble amplification reaction 15 min. (15 min. hands-on)**
2. **Amplify, recover-Ion OneTouch™ 2 Instrument ~6-7 hours (20 min. hands-on)**
3. **Enrich – Ion OneTouch™ ES 35 min. (5 min. hands-on)**

Total Processing Time ~7-8 hours
Hands-On Time ~40 min.

Ion Chef™

1. **Ion Chef™ instrument setup (15 min. hands-on)**

Total Processing Time ~12 hours - Hands-On Time ~15 min.

Ion Chef™ System

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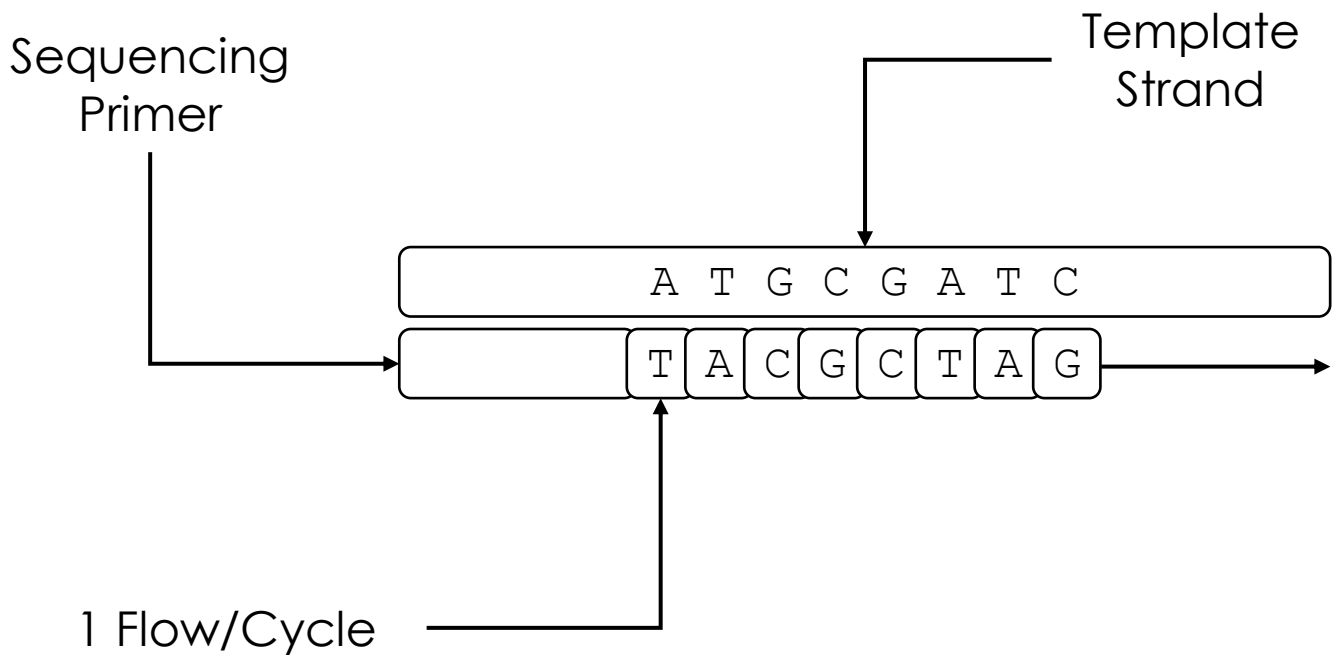
Sequencing by Synthesis

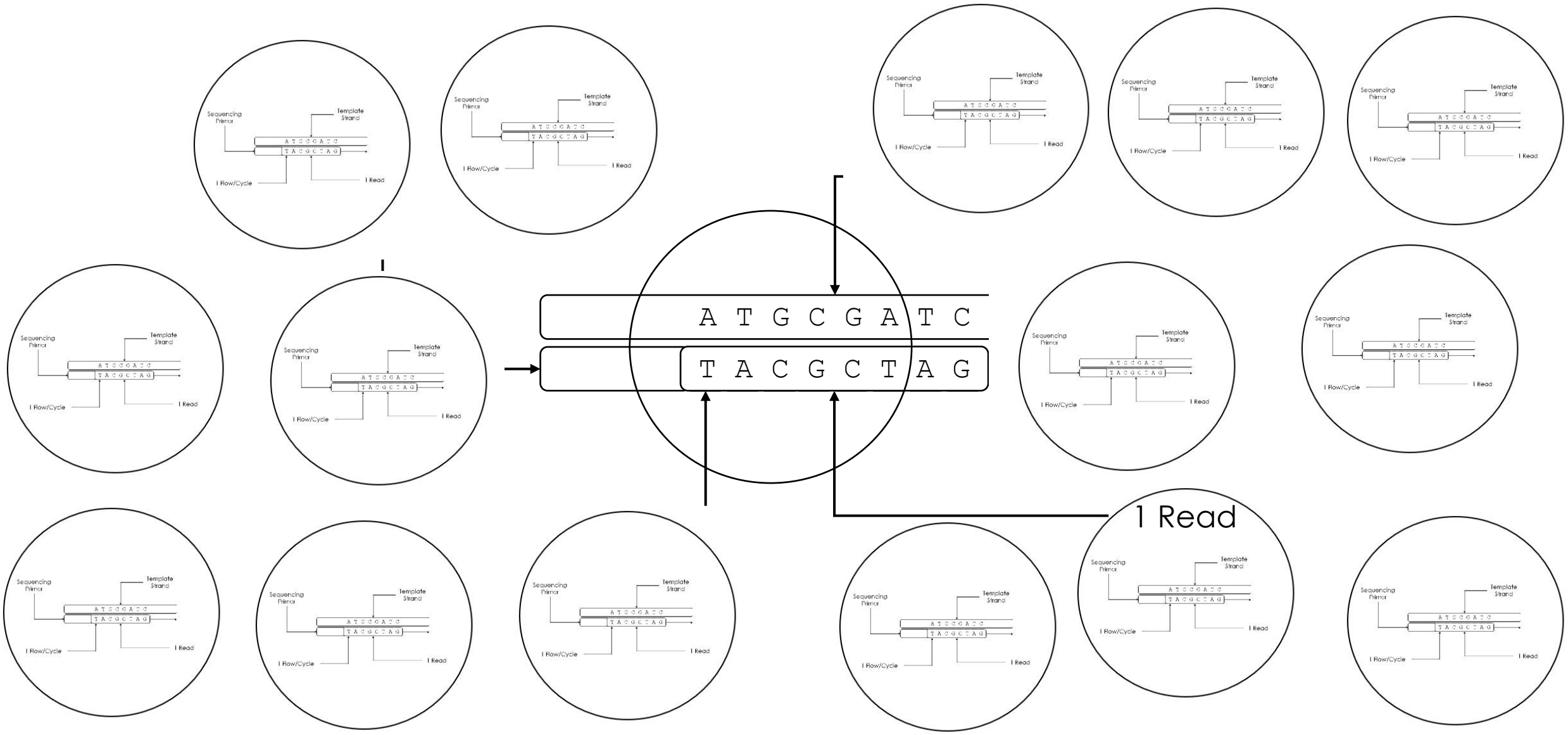
A quick look at what is happening inside the sequencer

Sequencing by Synthesis



Sequencing by Synthesis

LymphoTrack® Assays

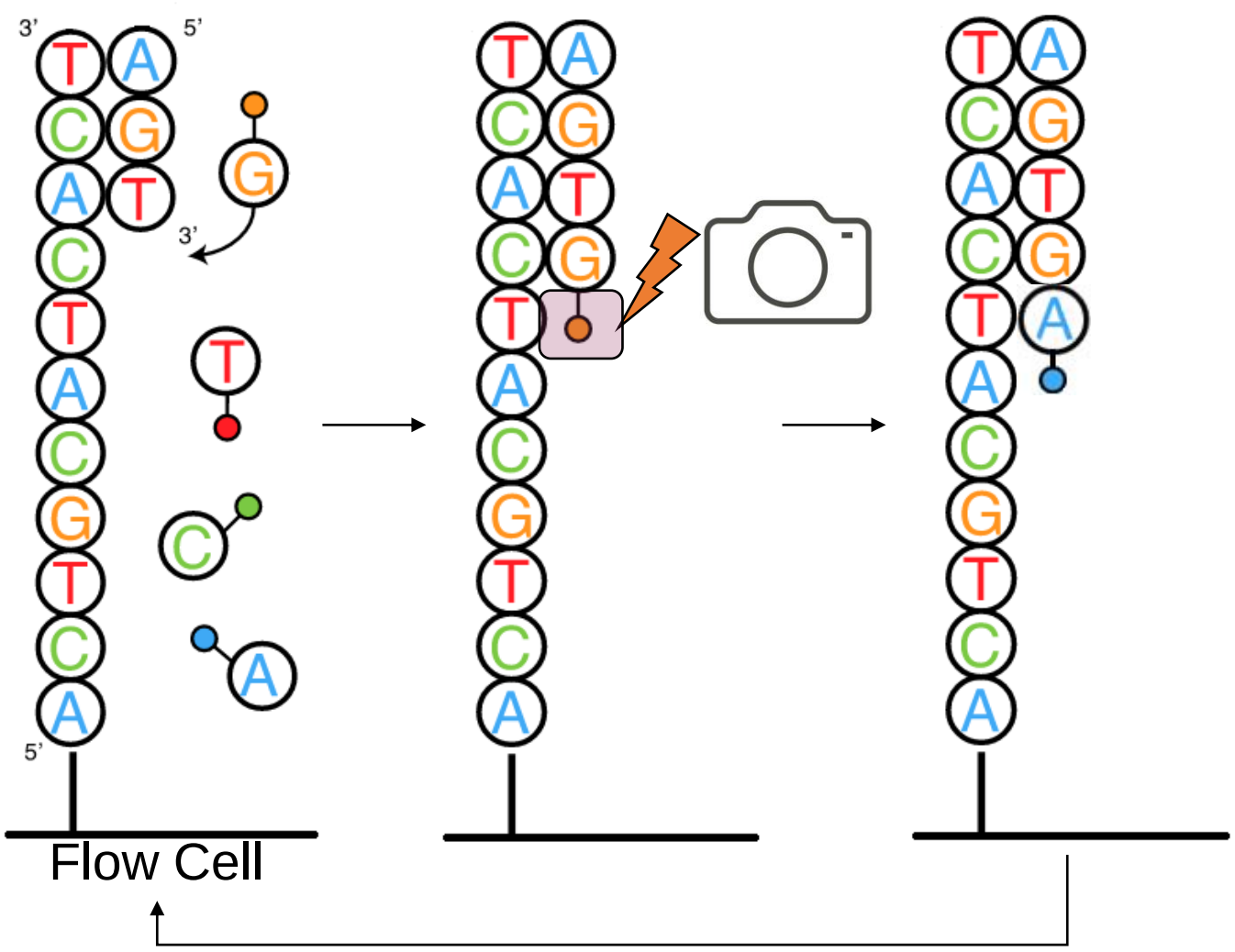


MiSeq® Base Calling

LymphoTrack® Assays

Fluorescence Detection

- Nucleotides with fluorophores are used to extend from the sequencing primer
- The added base is excited with light and the resulting fluorescence is imaged by a camera
- The color that is emitted allows the instrument to determine which base was added
- The dye and terminator are removed and the process is repeated for the next cycle/base



https://binf.snipcademy.com/lessons/ngs-techniques/illumina-solexa

Reads & Depth

LymphoTrack® Assays

Reads

- When a single strand of DNA is sequenced, this is considered one '**read**'
- During PCR, many copies of each starting DNA strand were generated
- Copies of a normal rearrangement can be sequenced, producing **multiple reads with the same sequence**
- When a very large number of reads have the same sequence, it likely means there were more than one of the original strand
- This is the **basis for clonality testing by NGS**: an **overabundance in reads** of one sequence when compared to a background of relatively low prevalence (polyclonal) reads

https://binf.snipcademy.com/lessons/ngs-techniques/illumina-solexa

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LymphoTrack® Assays

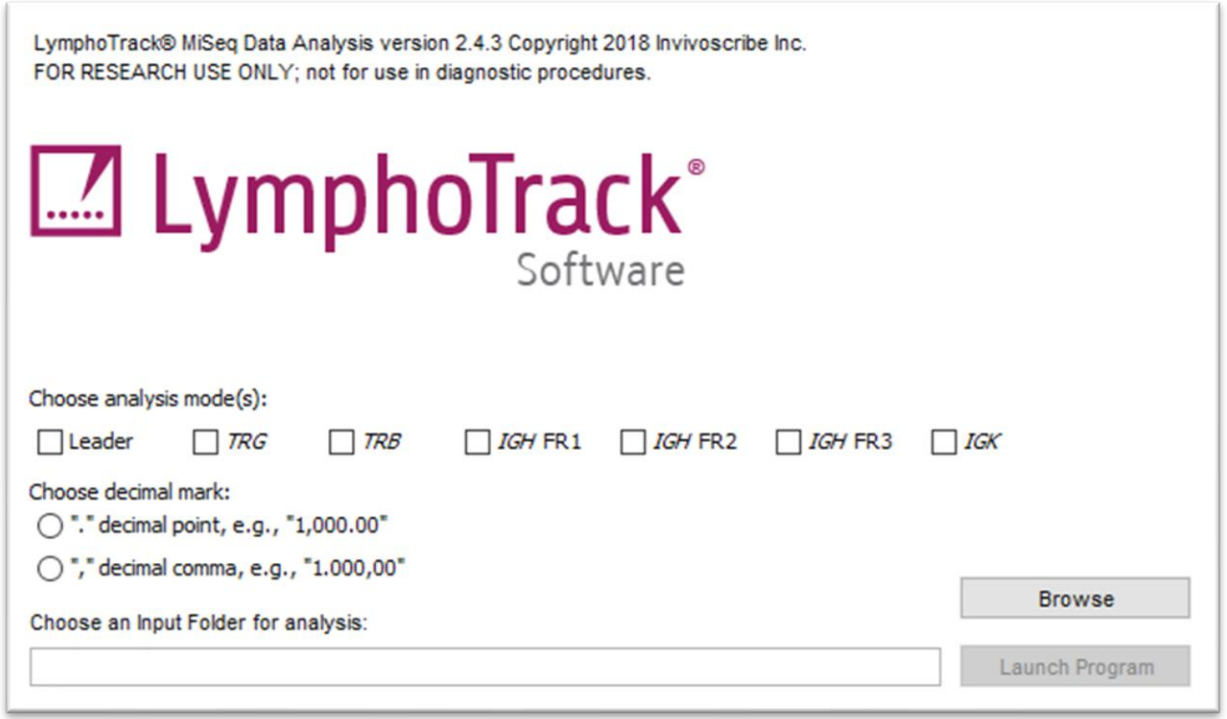
Workflow Overview (continued)

Step 6 - Included Bioinformatics

LymphoTrack® Assays

Data Analysis

- Transfer the appropriate files to a local folder (not on the Instrument)
- Launch LymphoTrack® Software
- Select targets, decimal format, and location of .fastq files
- Select 'Launch Program'



Step 6 - Included Bioinformatics

LymphoTrack® Assays

PDF reports are automatically generated per sample and target

Data is presented numerically to allow for **objective interpretation**

Graphs are provided to allow for visualization of results

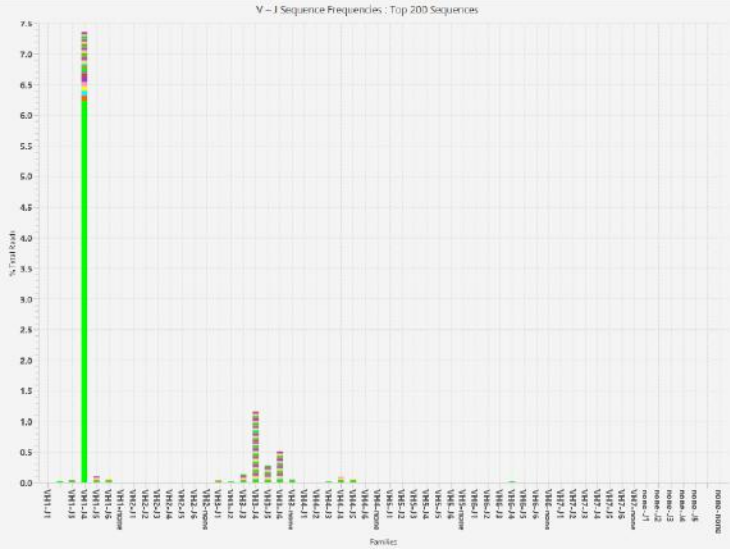
Total Read Count: 673441

IndexQ30: 89.45

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	49206	IGHV1-46_03	IGHJ4_02	7.31	7.31	0.00	Y	Y	100.00	not found
2	GCCTCTGGATTCA	300	181	IGHV3-35_01	IGHJ6_02	0.03	7.33	3.96	N	N	99.12	not found
3	ACCTCTGCAATCA	159	172	IGHV3-21_04	IGHJ6_02	0.03	7.36	1.32	n/a	N	22.03	not found
4	ACCTCTGGATTTC	272	171	IGHV3-74_02	IGHJ4_02	0.03	7.38	6.67	Y	Y	88.44	not found
5	GCGTCTGGAATCA	166	160	IGHV3-30_02	none	0.02	7.41	5.73	n/a	N	72.25	not found



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Step 7 - Analyze

LymphoTrack® Assays

MiSeq® Validity Specs

- MiSeq® instrument run validity specifications are dependent on the MiSeq® kit chemistry and the number of sequencing cycles

S5/PGM™ Run Validity Specs

- Loading > 50%
- Enrichment > 50%
- Clonal > 50%

Assay Specifications

- Positive Control top % Reads ≥ 2.5%
- Negative Control top % Reads < 1.0%
- If all validity criteria are met, move forward to interpretation & reporting

Step 7 – Analyze

LymphoTrack Assays

Top 10 Merged Read Summary

- The Read Summary tab only shows the top 10 reads after merging with the top 500 reads that differ in 1 or 2 nucleotides

Note: This analysis is also possible with *IGH FR1*

LymphoTrack Report for assay LEADER

Sample name: Leader_positive_S23_L001_001_combined

Total Read Count: 474947

IndexQ30: 87.88

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop codon (Y/N)	V-coverage	CDR3 Seq
1	TTCTCGTGGTGGC	455	50248	IGHV4-59_08	IGHJ4_02	10.58	10.58	11.26	Y	Y	98.63	GCGAGACGGAGGC
2	CTGCTACTGACTG	319	192	IGHV2-70_10	IGHJ4_02	0.04	10.62	4.32	n/a	N	35.55	not found
3	CTGCTGCTGACCA	466	175	IGHV2-5_01	IGHJ5_01	0.04	10.66	6.62	Y	Y	100.00	GCACACAGACCGC
4	CTGCTGCTGACCA	457	162	IGHV2-5_05	IGHJ6_02	0.03	10.69	2.99	Y	Y	99.67	GCACACAGATACT
5	CTGCTGCTGACCA	474	154	IGHV2-5_05	IGHJ4_02	0.03	10.72	3.99	Y	Y	99.67	GCACACAGATACT
6	CTGCTGCTGACCA	454	150	IGHV2-5_10	IGHJ5_02	0.03	10.76	11.78	Y	Y	98.99	GCATATGGTGTAA
7	CTGCTGCTGACCA	469	139	IGHV2-5_01	IGHJ4_02	0.03	10.78	1.32	Y	Y	97.68	GCACTCGCGACAC
8	CTCGCCCTCCTCC	466	139	IGHV5-51_01	IGHJ4_02	0.03	10.81	7.09	Y	Y	99.32	GCGAGATACTATT
9	CTGCTACTGACTG	490	137	IGHV2-70_10	IGHJ3_02	0.03	10.84	0.66	Y	Y	99.34	GCACGGATTCCTG
10	CTGCTGCTGACCA	478	135	IGHV2-5_10	IGHJ6_02	0.03	10.87	3.70	Y	Y	98.99	GCATACACTTGTT

Step 7 – Analyze

LymphoTrack Assays

LymphoTrack Report for assay LEADER

Sample name: Leader_positive_S23_L001_001_combined

Total Read Count: 474947

IndexQ30: 87.88

Caution: Do not edit fields and save.

Top 10 Merged Read Summary

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop codon (Y/N)	V-coverage	CDR3 Seq
1	TTCTCGTGGTGGC	455	50248	IGHV4-59_08	IGHJ4_02	10.58	10.58	11.26	Y	Y	98.63	GCGAGACGGAGC
2	CTGCTACTGACTG	319	192	IGHV2-70_10	IGHJ4_02	0.04	10.62	4.32	n/a	N	35.55	not found
3	CTGCTGCTGACCA	466	175	IGHV2-5_01	IGHJ5_01	0.04	10.66	6.62	Y	Y	100.00	GCACACAGACCG
4	CTGCTGCTGACCA	457	162	IGHV2-5_05	IGHJ6_02	0.03	10.69	2.99	Y	Y	99.67	GCACACAGATACT
5	CTGCTGCTGACCA	474	154	IGHV2-5_05	IGHJ4_02	0.03	10.72	3.99	Y	Y	99.67	GCACACAGATACT
6	CTGCTGCTGACCA	454	150	IGHV2-5_10	IGHJ5_02	0.03	10.76	11.78	Y	Y	98.99	GCATATGGTGTAA
7	CTGCTGCTGACCA	469	139	IGHV2-5_01	IGHJ4_02	0.03	10.78	1.32	Y	Y	97.68	GCACTCGCGACAC
8	CTCGCCCTCCTCC	466	139	IGHV5-51_01	IGHJ4_02	0.03	10.81	7.09	Y	Y	99.32	GCGAGATACTATT
9	CTGCTACTGACTG	490	137	IGHV2-70_10	IGHJ3_02	0.03	10.84	0.66	Y	Y	99.34	GCACGGATTCCTG
10	CTGCTGCTGACCA	478	135	IGHV2-5_10	IGHJ6_02	0.03	10.87	3.70	Y	Y	98.99	GCATACACTTGTT

Expected Values *IGHV* SHM

- *IGH* Positive Control top % reads ≥ 2.5%
- NGS Negative Control top % reads < 1.0%
- *IGH* SHM Positive Control (4-088-0008, can be purchased separately) top % reads ≥ 2.5%
- *IGH* SHM Positive Control mutation rate ≥ 2.0%
- MiSeq Run Validity Q30 > 70% for v3 (2x301)

Optional - Raw Data

LymphoTrack® Assays

Merged Top 10 File

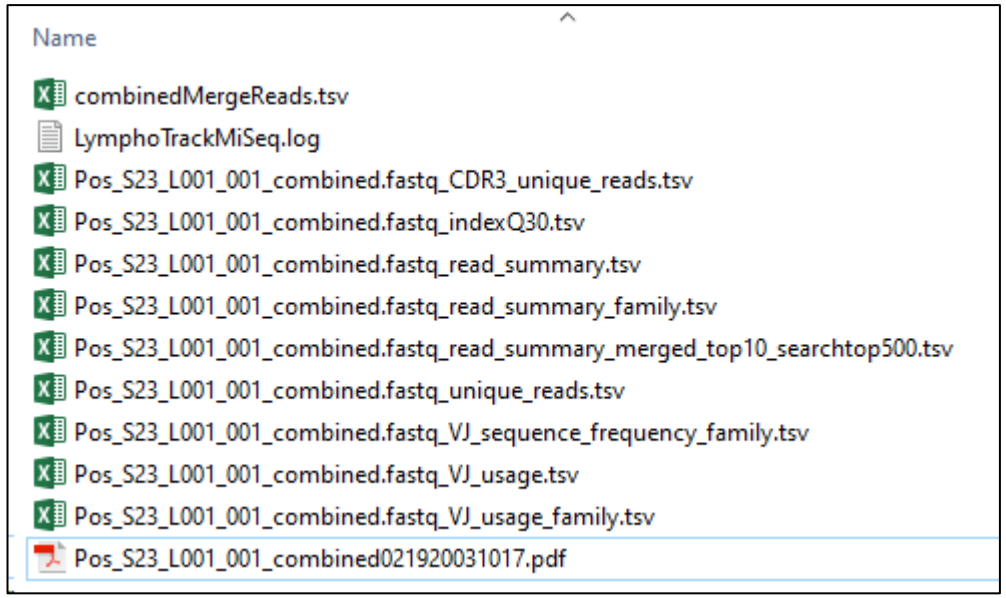
- 10 most prevalent merged sequences
- Top 500 reads are scanned for having up to 2 mismatches to the top 10 sequences to account for PCR & Sequencing error

Read Summary File

- Top 200 unmerged reads
- Aligned to identified V and J genes

Unique Reads File

- All unique reads identified in the fastq file. Can be useful for troubleshooting



Take Home Message

LymphoTrack® Assays

All LymphoTrack® assays follow **one simple workflow**

NGS can seem overwhelming – **LymphoTrack® simplifies the process** by reducing steps, labor costs, and analysis time

Multiplexing targets into one run is a great way to **reduce cost per target**

Included bioinformatics package **streamlines analysis** and provides flexible reporting methods