TruSight™ Oncology 500

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TruSight™ Oncology 500 Assay (TSO 500)

	Target Specifications
Content	523 genes (1.94 Mb) Encompasses TruSight Tumor 170, MSK-IMPACT™ and FoundationOne®
Assay	TruSight Oncology with unique molecular identifiers (UMIs)
Target Performance	5% LOD for DNA variants >96% analytical sensitivity; >99.99% analytical specificity
Target Input	40 ng DNA from FFPE samples
Variants	Single nucleotide variants (SNVs), indels, tumor mutational burden (TMB), microsatellite instability (MSI)
Informatics	Local Docker-Based Variant Calling Software
Target Throughput	8 samples per NextSeq™ run
Output	Variant file with all passing, non-synonymous variants
Sequencing Instruments	NextSeq , NextSeq 550Dx

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Some targets covered by TSO500:

NTRK1, NTRK2, NTRK3 (pan-cancer) | MSI (pan-cancer)								
Lung	Melanoma	Colon	Ovarian	Breast	Gastric	Bladder	Myeloid	Sarcoma
AKT1 *ALK* *BRAF* *DDR2* *EGFR* *ERBB2* *FGFR1* *FGFR3* *KRAS* *MAP2K1* *MET* *NRAS* *PIK3CA* *PTEN* *RET* *TP53* TMB	*BRAF* *CTNNB1* *GNA11* *GNAQ* *KIT* *MAP2K1* *NF1* *NRAS* *PDGFRA* *PIK3CA* *PTEN* *TP53*	*AKT1* *BRAF* *HRAS* *KRAS* *MET* *MLH1* *MSH2* *MSH6* *NRAS* *PIK3CA* *PMS2* *PTEN* *SMAD4* *TP53*	*BRAF* *BRCA1* *BRCA2* *KRAS* *PDGFRA* *FOXL2* *TP53*	*AKT1* *AR* *BRCA1* *BRCA2* *ERBB2* *FGFR1* *FGFR2* *PIK3CA* *PTEN*	*BRAF* *KIT* *KRAS* *MET* *MLH1* *PDGFRA* *TP53*	*MSH6* *PMS2* *TSC1*	*ABL1* *ASXL1* *CALR* *CEBPA* *ETV6* *EZH2* *FLT3* *GATA2* *IDH1* *IDH2* *JAK2* *KIT* *MPL* *NPM1* *RUNX1* *SF3B1* *SRSF2* *TP53*	*ALK* *APC* *BRAF* *CDK4* *CTNNB1* *ETV6* *EWSR1* *FOXO1* *GLI1* *KIT* *MDM2* *MYOD1* *NAB2* *NF1* *PAX3* *PAX7* *PDGFRA* *PDGFRB* *SDHB* *SDHC* *SMARCB1* *TFE3* *WT1*

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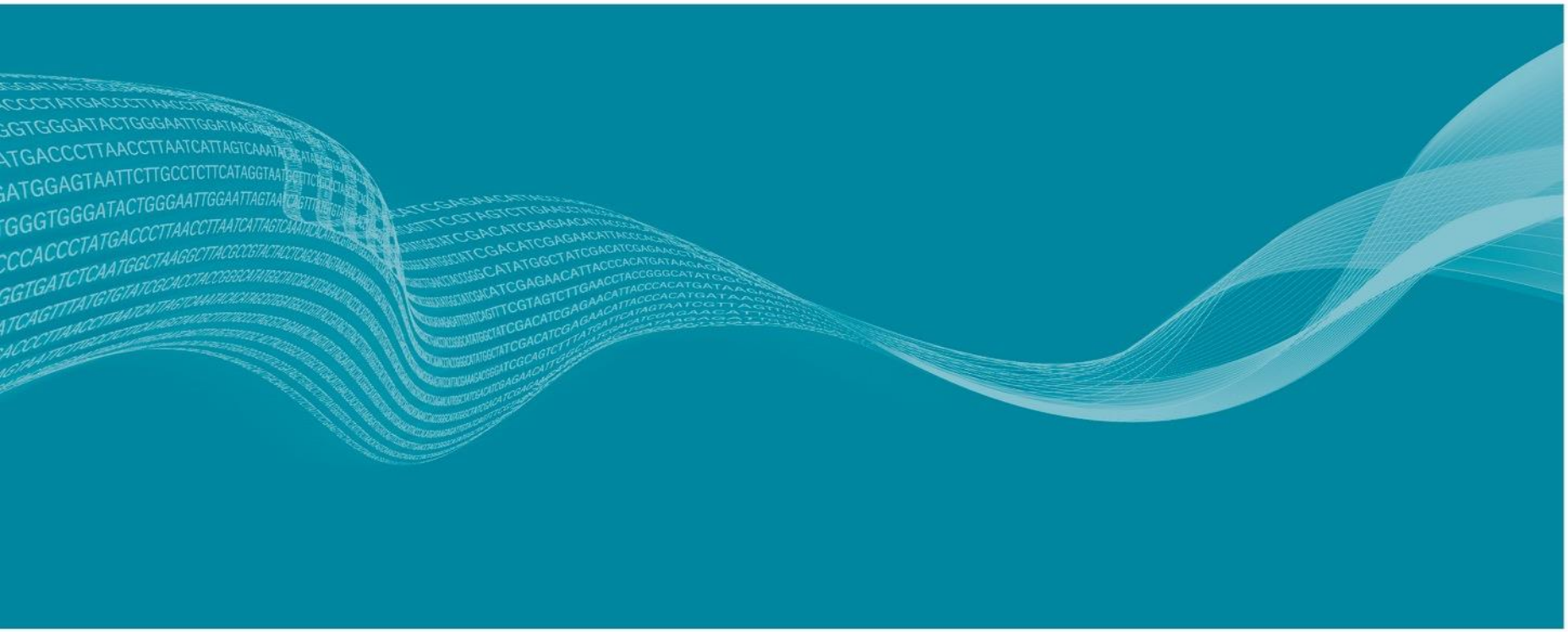
TSO500: Software TS Training overview

- **TSO 500 Assay**
  - Library Prep and Enrichment Workflow
  - Overview of library construction
- **Sequencing Recommendations**
- **Analysis: TSO 500 Local App**
  - Docker, Singularity container platforms
  - Field support model
  - Output folders and data metrics
  - Tumor Mutational Burden score
  - Microsatellite Instability score
  - TST 170 Local App for RNA Analysis
- **Other options and future plans**
- **Partnership with PierianDx**

TSO 500 Library Preparation: Quick review



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Library Prep Step 1: Fragment genomic DNA

1

Fragment gDNA

Hands-on: 10 minutes
Total: 120 minutes
Reagents: TEB

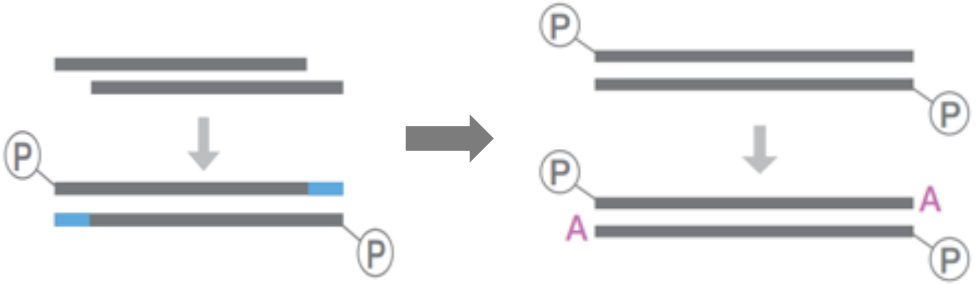
- **Covaris focused-ultrasonicator shears genomic DNA**
- **Generate 90 – 250 bp dsDNA fragments with 3’ and 5’ overhangs**

Library Prep Step 2: End Repair and A-Tailing

2

End Repair and A-Tailing

Hands-on: 10 minutes
Total: 70 minutes
Reagents: ERA1-A, ERA1-B



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Library Prep Step 3: Ligate Adapters to DNA fragments

Unique Molecular Identifiers

3

Ligate Adapters

Hands-on: 15 minutes
Total: 50 minutes
Reagents: ALB1, UMI v1, LIG3, STL

Library Prep Step 4: Ligation Clean Up

Purify DNA fragments with Sample Purification Beads

4

Ligation Clean Up

Hands-on: 40 minutes
Total: 50 minutes
Reagents: SPB, RSB, 80% EtOH

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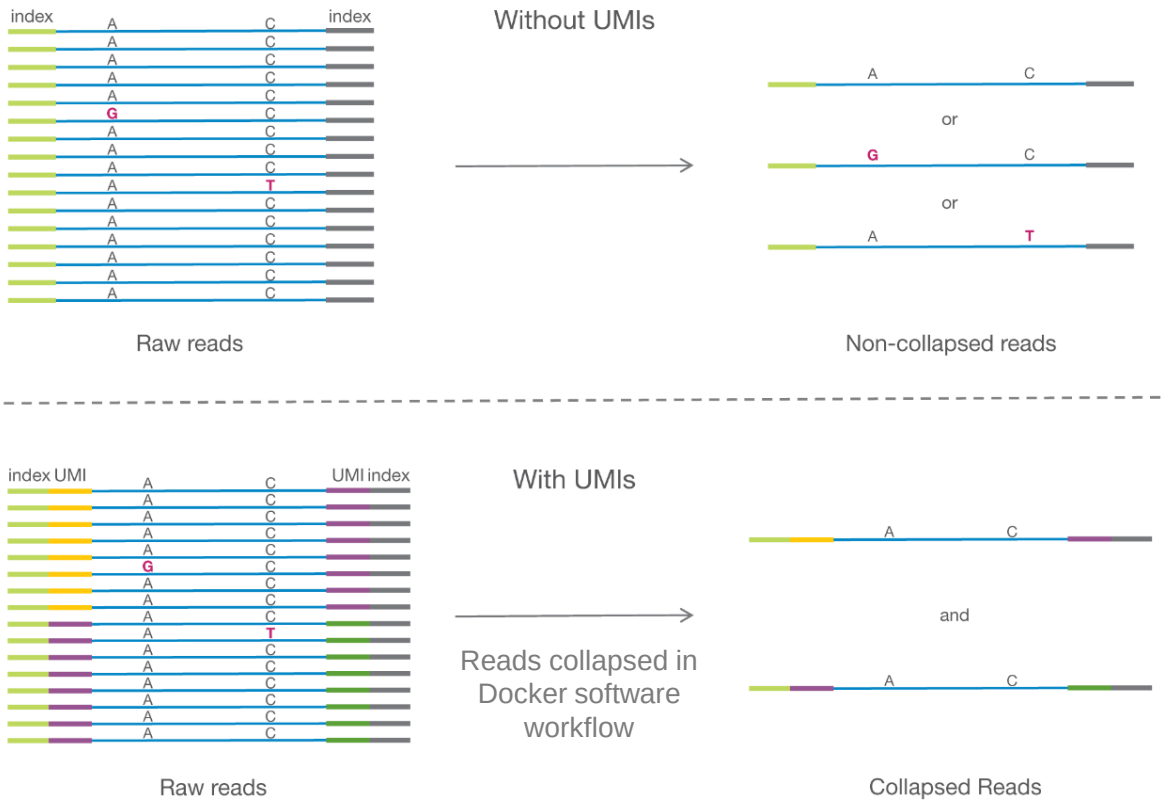
Unique Molecular Identifiers (UMIs)

- Every individual fragment is tagged for identification
- Reads derived from the same fragment have the same sequence
- UMIs enable Error Correction: distinction between errors and variants
- Reduce error rate to ≤ 0.007%



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Library Prep Step 5: Index PCR

Amplify DNA and add indexed adapters for sample multiplexing

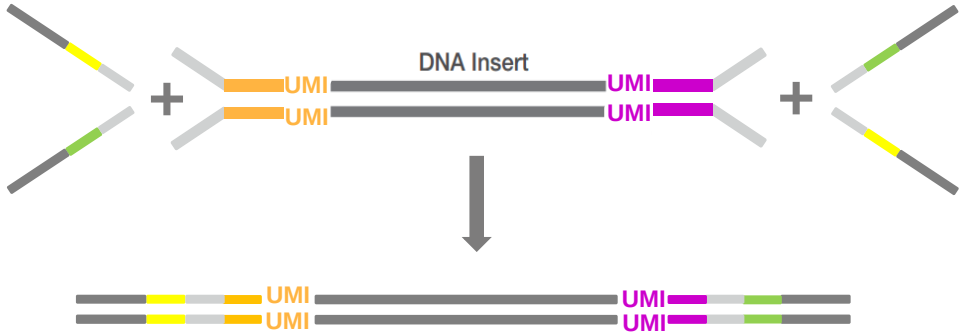
5

Index PCR

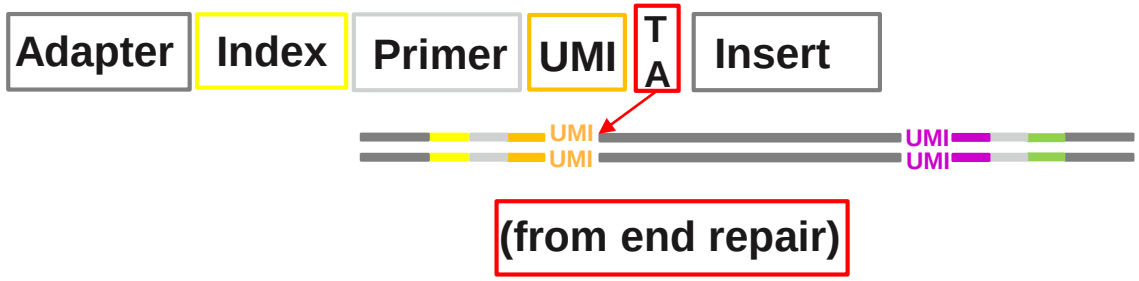
Hands-on: 15 minutes

Total: 60 minutes

Reagents: EPM, UPXX



Library Prep Step 5: Final Library



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TSO 500 Enrichment

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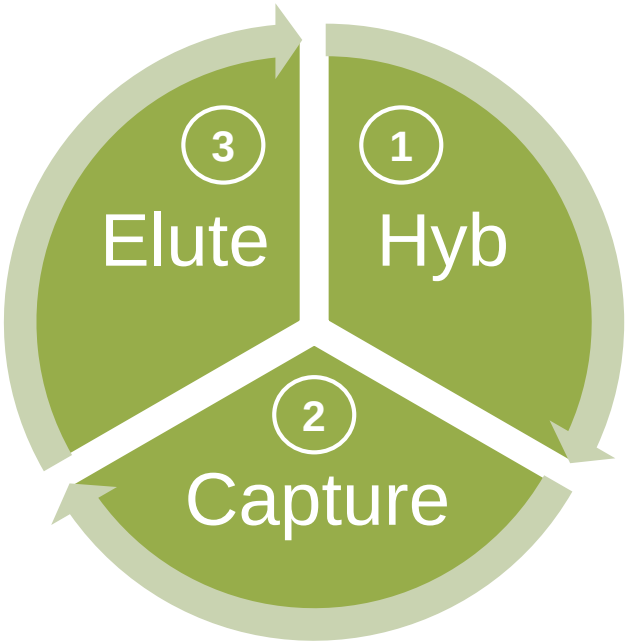
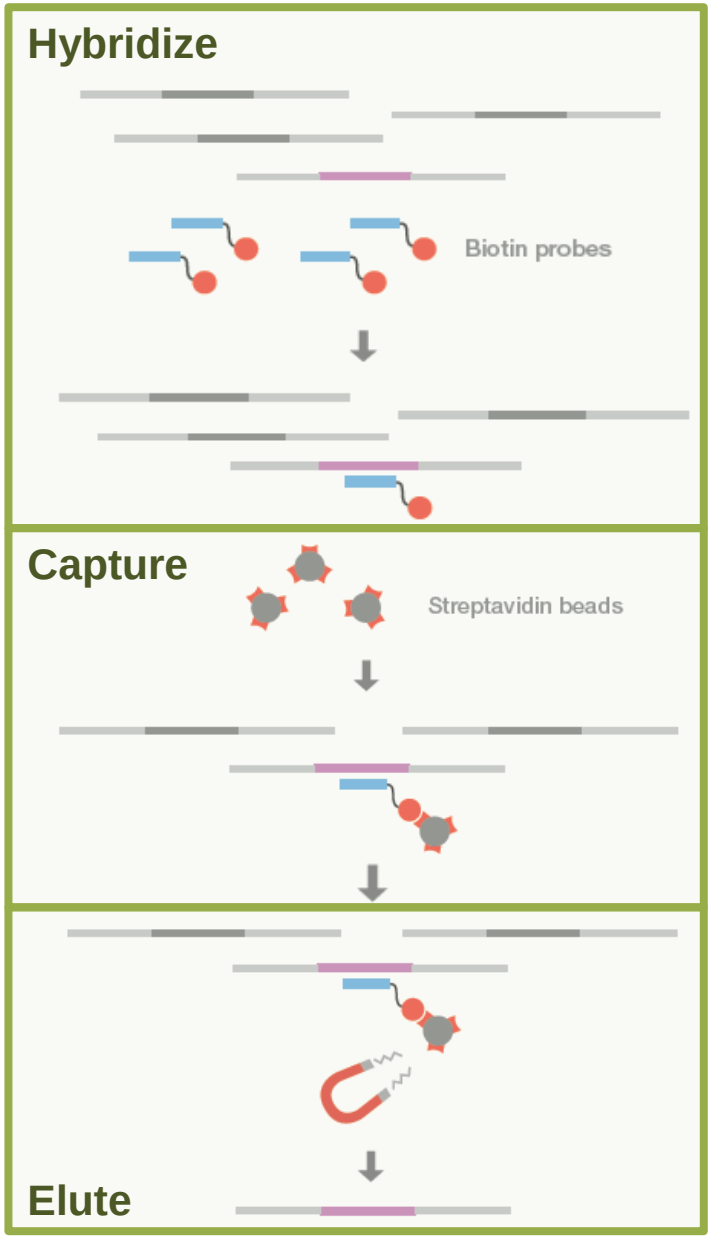
Enrichment

- **TSO 500 target probes capture and enrich library fragments that contain targeted regions**





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TruSight Oncology 500 Kits

DNA-only or DNA+RNA reagents

- **TSO 500 DNA Kit (**20028213**)**
  - Library Prep and Enrichment Reagents
  - No NextSeq core reagents
  - 16 indexes, 48 samples
- **TSO 500 DNA Kit, plus NextSeq kit (**20028214**)**
  - Library Prep and Enrichment Reagents
  - 16 indexes, 48 samples
- **TSO 500 DNA/RNA Kit (**20028215**)**
  - Library Prep and Enrichment Reagents
  - No NextSeq core reagents
  - 16 indexes, 24 samples★
- **TSO 500 DNA/RNA Kit, plus NextSeq kit (**20028216**)**
  - Library Prep and Enrichment Reagents
  - 16 indexes, 24 samples★

★
24 samples →
24 DNA libraries +
24 RNA libraries

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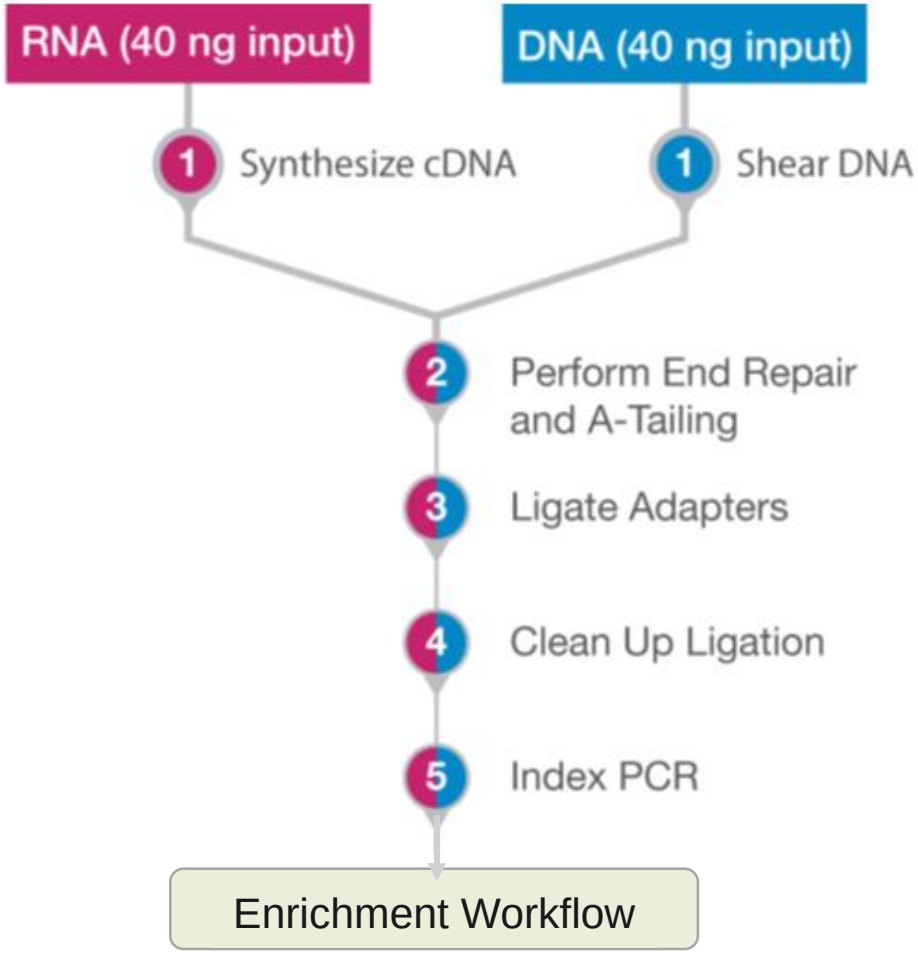
TSO 500 DNA + TST 170 RNA Workflow Overview

- Extract RNA and process in parallel with DNA
- Detect fusions and splice variants from 55 genes



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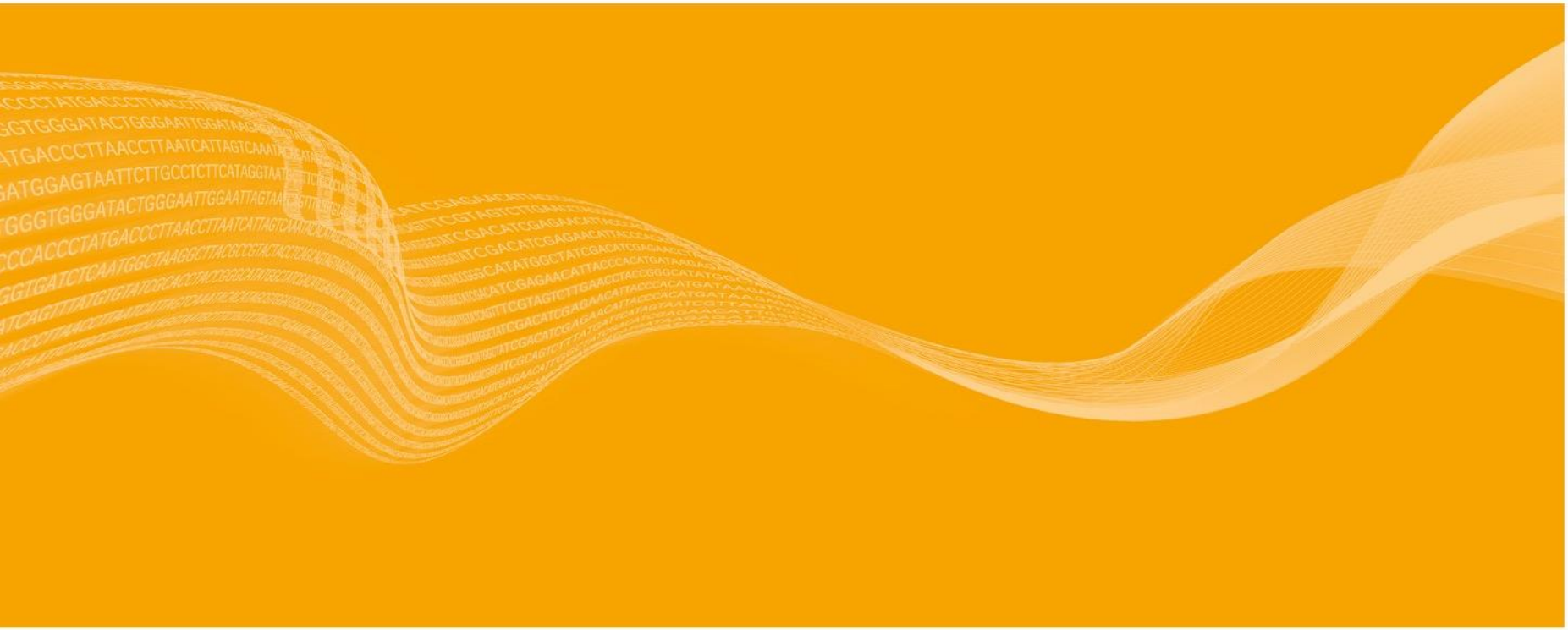
TSO 500 Sequencing



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Recommended Sequencing Configuration

- Compatible platforms:
  - **NextSeq 500/550**
  - **NextSeq 550Dx (RUO Mode)**
  - ***Other platforms have not been tested and are not supported***
  - ***Possible NovaSeq support in future***
- 2 x 101 Run in Standalone mode
  - **BaseSpace uploads not supported**
- Paired-End (required), Dual Index (required)
- Minimum of 3 libraries for sufficient diversity
  - UPXX index primer sets for low-plexity pooling provided in TSO 500 Reference Guide
- 8 samples maximum for optimal coverage
  - 8 DNA libraries + 8 RNA libraries
  - 80M PE reads per library

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TruSight Oncology 500 Analysis

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TSO 500 Local App Agenda

- **TSO 500 Local App**
  - Overview
  - Installation
  - Launching analysis
- **Output folders and data metrics**
  - Tumor Mutational Burden score
  - Microsatellite Instability score
- **TST 170 RNA Analysis**

TSO 500 Local App

Overview

- **A software package that reports:**
  - Single Nucleotide Variants (SNVs)
  - Small Insertions and Deletions (indels)
  - Tumor Mutational Burden score (TMB)
  - Microsatellite Instability score (MSI)

- **Analyzes BCL files from a NextSeq:TSO 500 run folder**
  - NextSeq 500/550 or NextSeq 550Dx (RUO Mode)
  - Requires Sample Sheet **(on support site)**
  - FASTQ analysis workflow available
- **Output files:**
  - Variant Call Files (VCFs)
  - Metrics Report
  - Biomarker Reports with TMB score and MSI score

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TSO 500 Local App: Docker or Singularity

- **What is Docker?**

  An open platform for easily developing and running software

  - **Docker engine** is installed on a Linux server (CentOS)
  - Applications are packaged in **containers** and run on Docker
  - Uses a command line interface

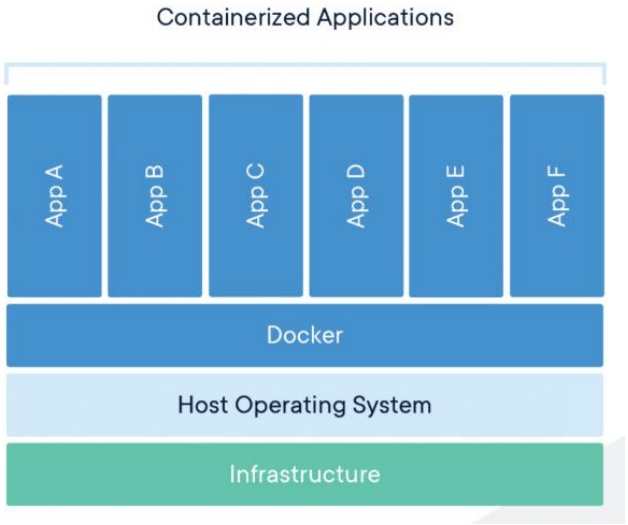
- **What is Singularity?**

  Another open platform option for TSO 500

- **What is a Container?**

  A standardized unit of software

  - A standalone, executable software package
  - Includes everything needed to run the app
  - Depends on elements of the OS



Source: www.docker.com/resources/what-container

TSO 500 Local App Installation

- **How to access the TSO 500 local app?**
  - Install Docker or Singularity on CentOS 7.3
  - Download TSO 500 app bundle with HTTP client (Curl, Wget)
    - Container Resources folder includes manifest, sample sheet template, and hg19 genome
    - Illumina Technical Support will provide link to customer by email
  - Install TSO 500 per User Guide instructions

- **User Requirements**
  - Basic user knowledge of:
    - Linux operating system
    - Docker software container platform
  - Confirm OS meets computing requirements in User Guide
    - ILMN can recommend server if needed

TSO 500 Local App: Launching the Analysis

- Instructions and command line entries provided in User Guide
- One analysis at a time recommended
  - Wait for any running TSO 500 container to complete before launching new analysis
- Start software with TruSight_Oncology_500.sh script on command line as described in User Guide

Resource:
TruSight Oncology 500 Local App User Guide

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TSO 500 Local App: Required Inputs

Docker analysis from BCL files

Paths in command line must be absolute paths:

- Path to the TruSight Oncology 500 Resources folder
- Path to an empty output folder for analysis results
  - User must create this folder first
- Path to the sequencer run data folder -OR- a FASTQ folder
- Path to SampleSheet.csv file if not located in run folder
  - Example on TSO 500 Support page
  - Must be named “SampleSheet.csv”
  - Specific instructions provided in Local app User Guide

Resource:
TruSight Oncology 500 Local App User Guide

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TSO 500 SampleSheet.csv

[Header]								
IEMFileVersion	4							
Investigator Name	User Name							
Experiment Name	Experiment							
Date	10/3/2018							
Workflow	GenerateFASTQ ★							
Application	NextSeq FASTQ Only							
Assay								
Description								
Chemistry	Default							
[Reads]								
101								
101								
[Settings]								
Adapter	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA							
AdapterRead2	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT							
Read1UMILength	7 ★							
Read2UMILength	7 ★							
Read1StartFromCycle	9 ★							
Read2StartFromCycle	9 ★							
[Data]								
Sample_ID ★	Sample_Name	Sample_Plate	Sample_Well	Index_ID	index	I7_Index_	index2	I5_Index_ID
Test_Sample_UP01				UP01	TCCGGAGA	D702	AGGATAGG	D503
Test_Sample_UP02				UP02	CTGAAGCT	D707	TCAGAGCC	D504
Test_Sample_UP03				UP03	CGTAGCTC	D717	CATCCGAA	D509
Test_Sample_UP04				UP04	GAATTCGT	D706	TTATGAGT	D510
Test_Sample_UP05				UP05	AGCGATAG	D712	ACGAATAA	D513
Test_Sample_UP06				UP06	GCGATTAA	D724	GATCTGCT	D515
Test_Sample_UP07				UP07	ATTCAGAA	D705	AGGCTATA	D501
Test_Sample_UP08				UP08	GAATAATC	D713	GCCTCTAT	D502

Valid Sample Sheet examples:

- Included in TSO 500 local app Resources folder
- Provided on TSO 500 support page

- Name “SampleSheet.csv”
- Add to run folder or provide path

Base 8 is from end repair, skipped for analysis

Delete unused indexes from template

Sample_Name:
Blank or identical to Sample_ID

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TSO 500 Local App: Launch Analysis

Example launch from run folder with BCL files

At command prompt enter:

{APP_PATH}/{Version}/TruSight_Oncology_500.sh --user=$UID --remove \

--analysisFolder /full/path/to/output/analysisFolder \

--resourcesFolder /{RESOURCE_FOLDER}/resources \

--runFolder /full/path/to/runFolder

Variables:

- APP_PATH: /scratch
- Version: 1.3.0
- /full/path/to/output/analysisFolder : /scratch/tso500_180914_NB501433_0065_AH3CVYBGX9
- RESOURCE_FOLDER: /scratch/1.3.0/
- /full/path/to/runFolder: /scratch/180914_NB501433_0065_AH3CVYBGX9

Complete example:

/scratch/1.3.0/TruSight_Oncology_500.sh --user=$UID --remove --analysisFolder /scratch/tso500_180914_NB501433_0065_AH3CVYBGX9 --resourcesFolder /scratch/1.3.0/resources --runFolder /scratch/180914_NB501433_0065_AH3CVYBGX9

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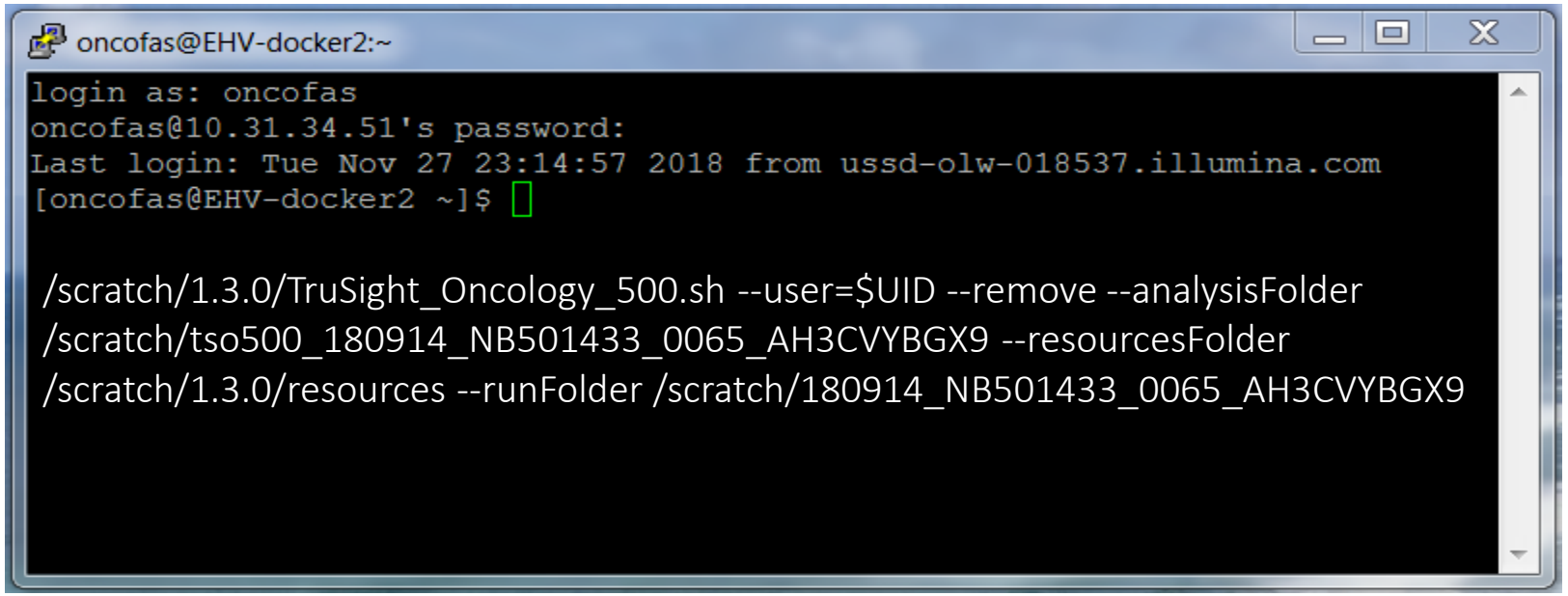
TSO 500 Local App: Required Inputs

Launch analysis from BCL files



- Analysis can also be started from FASTQs, assuming FASTQs have been generated with appropriate UMI settings (directions in user guide).

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TSO 500 Local App Output

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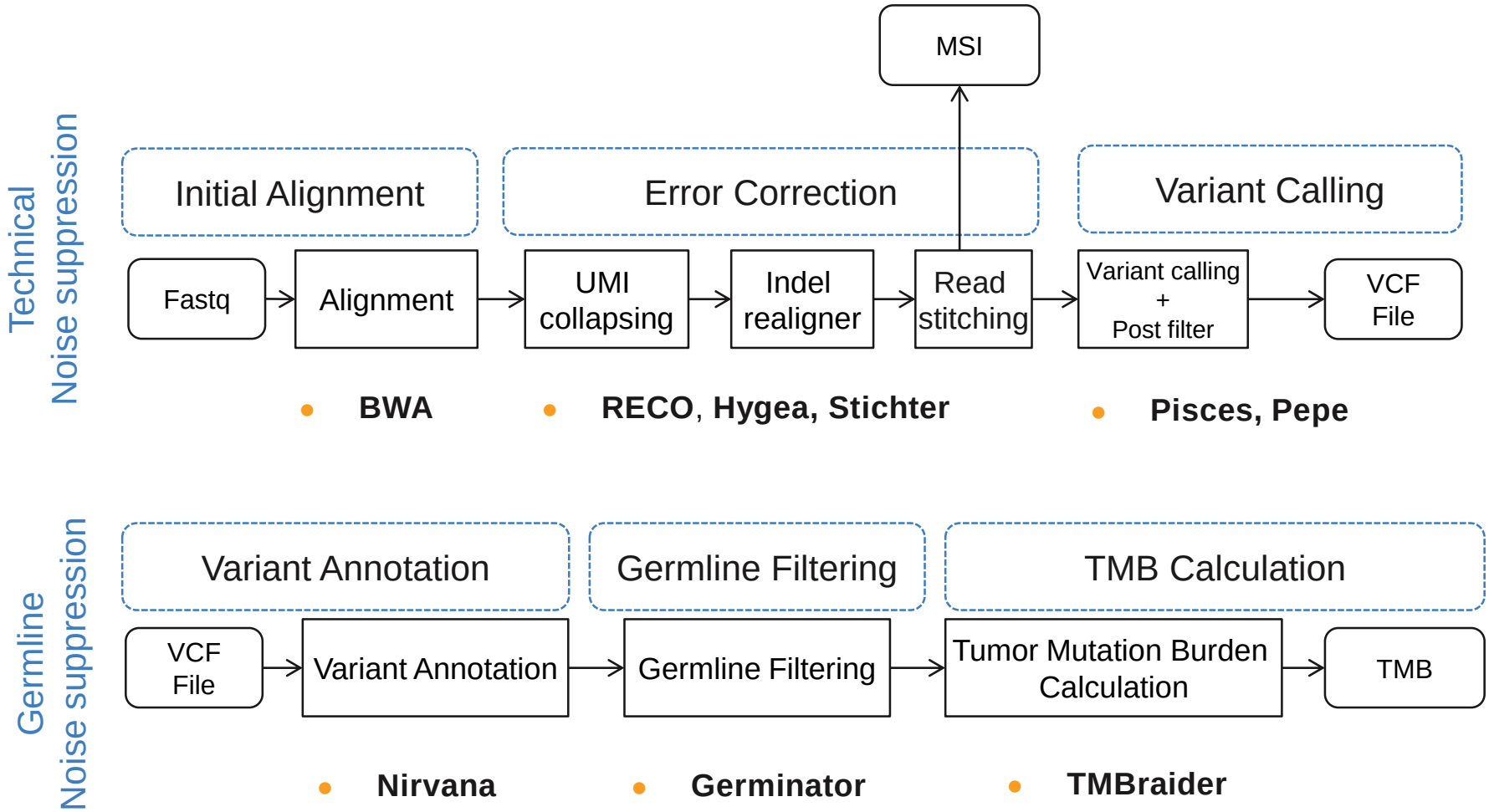
TSO500 Analysis workflow



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TSO 500 Local App Analysis Output Folder

- Logs_Intermediates
  - Annotation
  - CollapsedReads
  - Contamination
  - CvAnnotation
  - CvDnaAlignment
  - DnaAlignment
  - DnaQCMetrics
  - DnaRealignment
  - FastqGeneration
    - Sample1
      - Sample1_S1_L001_R1_001.fastq.gz
      - Sample1_S1_L001_R2_001.fastq.gz
      - Sample1_S1_L002_R1_001.fastq.gz
      - Sample1_S1_L002_R2_001.fastq.gz
      - Sample1_S1_L003_R1_001.fastq.gz
      - Sample1_S1_L003_R2_001.fastq.gz
      - Sample1_S1_L004_R1_001.fastq.gz
      - Sample1_S1_L004_R2_001.fastq.gz
  - FastqValidation
  - HypothesisCaller
  - IndelRealignment
  - MarkDuplicates
  - MetricsAggregation
  - Msi
  - ResourceVerification
  - RunQC
  - SampleSheetValidation
  - SmallVariantFilter
  - StitchedReads
  - StitchedReads
  - Tmb
  - VariantCaller
  - TruSight-Oncology-500-pipeline-<datetime>.log
- Results
  - <sample-id>_BiomarkerReport.txt
  - MetricsReport.tsv

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Results Folder: Metrics Report

Metrics Report

Combined key analysis metrics in a .tsv file

LSL and USL guidelines provided per sample metric

Header
Report date, time, version

Run Metrics
% Reads Passing Filter and
% Base Calls ≥ Q30

DNA Library QC Metrics
Contamination Score and P Value;
QC metrics for TMB and MSI

Expanded QC Metrics
% Read Enrichment,
Target Coverage metrics

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MetricsReport.tsv

TruSight Oncology 500 - Metrics Report					
For Research Use Only. Not for use in diagnostic procedures					
[Header]					
Report Date	1/4/2019				
Report Time	22:22:11				
Pipeline Version	1.3.1.3				
[Run Metrics]					
Metric (UOM)	LSL Guideline	USL Guideline	Value		
PCT_PF_READS (%)	80	NA	90.2		
PCT_Q30_R1 (%)	80	NA	90.4		
PCT_Q30_R2 (%)	80	NA	87.3		
[DNA Library QC Metrics]					
Metric (UOM)	LSL Guideline	USL Guideline	CCL-DNA	FFPE-Lung-DNA	HD753-DNA
CONTAMINATION_SCORE (NA)	0	3106	223	7535	30409
CONTAMINATION_P_VALUE (NA)	0	0.049	1	0	0.416
[DNA Library QC Metrics for Small Variant Calling and TMB]					
Metric (UOM)	LSL Guideline	USL Guideline	CCL-DNA	FFPE-Lung-DNA	HD753-DNA
MEDIAN_INSERT_SIZE (bp)	70	NA	104	108	118
MEDIAN_EXON_COVERAGE (Count)	150	NA	390	483	602
PCT_EXON_50X (%)	90	100	98.6	99.3	99
[DNA Library QC Metrics for MSI]					
Metric (UOM)	LSL Guideline	USL Guideline	CCL-DNA	FFPE-Lung-DNA	HD753-DNA
USABLE_MSI_SITES (Count)	40	NA	102	120	125

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MetricsReport.tsv – Contamination Detection

Contamination Detection
SNPs in VCFs analyzed to detect foreign DNA contamination.
Evaluate each sample using **both** Contamination Score and Contamination P value.

[DNA Library QC Metrics]			No Contam	CN Events	CONTAMINATED
Metric (UOM)	LSL Guideline	USL Guideline	CCL-DNA	FFPE-Lung-DNA	HD753-DNA
CONTAMINATION_SCORE (NA)	0	3106	223	7535	30409
CONTAMINATION_P_VALUE (NA)	0	0.049	1	0	0.416

CONTAMINATION_SCORE:
- Evaluate common SNPS with expected allele frequencies of 0%, 50%, or 100%
- Frequencies shift when sample is contaminated
- Score is likelihood that observed allele frequency is due to mixing DNA from different samples

CONTAMINATION_P_VALUE:
- Measures the uniformity of low frequency SNPs across the genome
- The probability that a truly contaminated sample has a non-uniform SNP distribution is very small (unless there have been copy number events)
- Measures certainty of copy number events

MetricsReport.tsv continued

[DNA Expanded Metrics]					
Metric (UOM)	LSL Guideline	USL Guideline	CCL-DNA	FFPE-Lung-DNA	HD753-DNA
TOTAL_PF_READS (Count)	NA	NA	89277228	92653732	90620250
MEAN_FAMILY_SIZE (Count)	NA	NA	4.1	3.2	2.9
MEDIAN_TARGET_COVERAGE (Count)	NA	NA	372	425	580
PCT_CHIMERIC_READS (%)	NA	NA	0.1	0.8	0.1
PCT_EXON_100X (%)	NA	NA	96.8	98.3	98.3
PCT_READ_ENRICHMENT (%)	NA	NA	87.6	84.8	85.3
PCT_USABLE_UMI_READS (%)	NA	NA	99.7	99.8	99.8
MEAN_TARGET_COVERAGE (Count)	NA	NA	376	523.5	578
PCT_ALIGNED_READS (%)	NA	NA	95.3	95.6	95.5
PCT_CONTAMINATION_EST (%)	NA	NA	0	0.1	0.2
PCT_PF_UQ_READS (%)	NA	NA	100	100	100
PCT_TARGET_0.4X_MEAN (%)	NA	NA	91.1	82.8	93.1
PCT_TARGET_100X (%)	NA	NA	95.2	97.2	97.3
PCT_TARGET_250X (%)	NA	NA	77.1	75.1	92.4

Results Folder: Biomarker Report

Biomarker Report

Final Report per sample that reports TMB and MSI metrics in a .txt file

Header
Sample ID, Date, and Time of Analysis

Sample Status
Analysis steps completed, failed, or skipped

Tumor Mutational Burden (TMB)
Total TMB and values used in calculation

Microsatellite Instability (MSI)
Percent Unstable Microsatellite Sites

SampleID_BiomarkerReport.txt

TruSight Oncology 500 - Biomarker Report			
For Research Use Only. Not for use in diagnostic procedures			
For QC Status and Information, reference the MetricsReport.tsv contained in the Results folder.			
[HEADER]			
Sample ID:	FFPE-Lung-DNA		
Report Date:	1/4/2019		
Report Time:	22:22:10		
Pipeline Version:	1.3.1.3		
[SAMPLE STATUS]			
Completed all Steps:	TRUE		
Failed Steps:	NA		
Steps not Executed:	NA		
[TMB]			
Total TMB:	4.708		
Nonsynonymous TMB:	1.569		
Coding Region Size in Megabases:	1.275		
Number of Passing Eligible Variants:	6		
Number of Passing Eligible Nonsynonymous Variants:	2		
[MSI]			
Usable MSI Sites:	108		
Total Microsatellite Sites Unstable:	5		
Percent Unstable Sites:	4.63		

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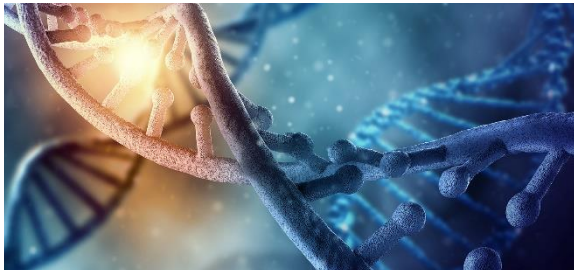
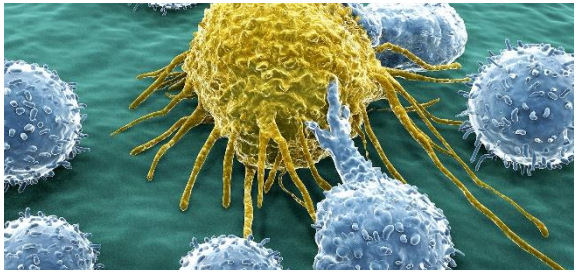
Tumor Mutational Burden (TMB)

Correlation to immunotherapy outcome

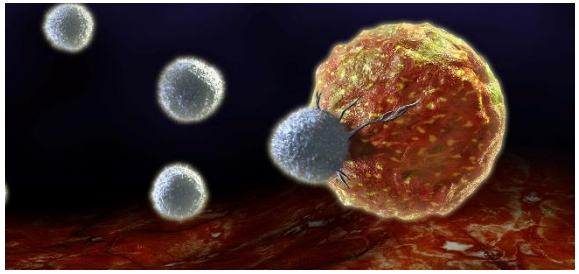
Not every tumor responds well to immunotherapy.
High *Tumor Mutational Burden* has been linked to better responses



Tumor Cells with high TMB…[1,2]



…may have high neoantigen load…[1,2]



…which can lead to increased immunotherapy response.[2-5]

TUMOR MUTATIONAL BURDEN (TMB):

- The number of nonsynonymous mutations carried by tumor cells
- TMB is emerging as a potential predictive biomarker to better segment patient populations for immunotherapies treatment [6]

1. Schumacher TN, Schreiber RD. Science. 2015; 348(6230): 69-74.
2. Kim JM, Chren DS. Ann.Oncol. 2016; 27(8):1492-1504.
3. Liontos M et al. Ann Transl Med. 2016; 4(14): 264.
4. Sharma P, Allison JP. Science. 2015; 348 (6230): 56-61.
5: Giannakis M et al. Cell Rep. 2016; 15(4): 857-865.
6. Chalmers ZR et al. Genome Med. 2017; 9(1):34.

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TSO 500 Output: TMB Score

Number of eligible somatic variants per Mb

Variant calling

BCL > FASTQ Alignment → Read collapsing Realignment Stitching → Variant calling → Small Variant Filtering → VCF files

TMB Calculations

$$TMB = \frac{N}{M}$$

N = Total eligible variants*

- Coding region
- Somatic variants only
- VAF ≥ 5%
- No hotspots
- No MNVs
- ≥ 50X coverage

*Listed in tmb.tsv file

÷

M = Total coding region ≥ 50X coverage

The term describes mutations occurring at a chromosomal region, which is more susceptible to genetic damage/change than average sequences, or contains sequences that are more lik
by spontaneous mutations, such as CpG dinucleotides.

Biomarker Report

```
[TMB]
Total TMB: 298.989
Nonsynonymous TMB:    237.618 TMB
Coding Region Size in Megabases: 1.271 M
Number of Passing Eligible Variants:  380
Number of Passing Eligible Nonsynonymous Variants:    302 N
```


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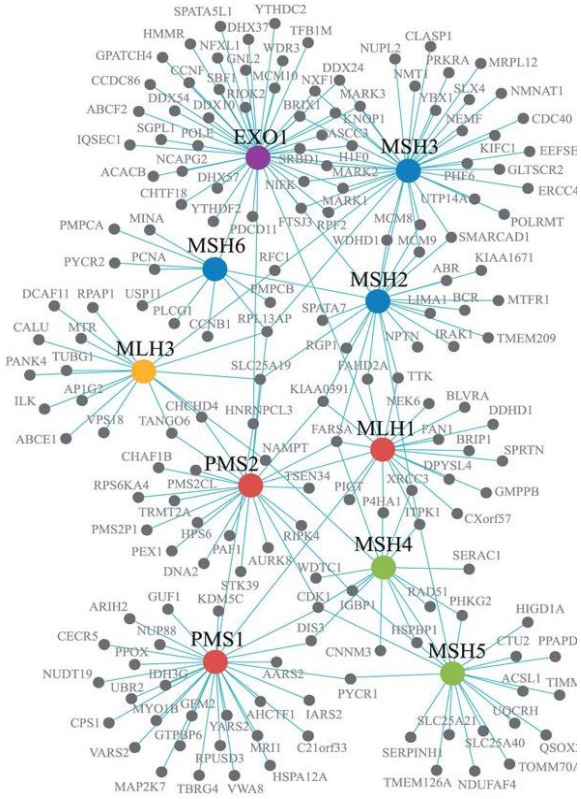
Microsatellite Instability (MSI)

FDA-approved biomarker for immunotherapy



Normal Microsatellite Allele:

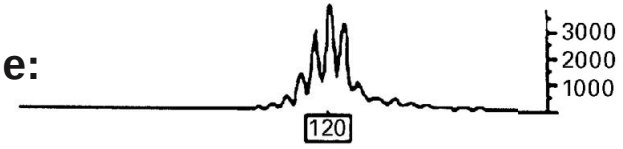
GGTA CA CA CA CA CA CA CA TTGC

Tumor MSI Alleles:

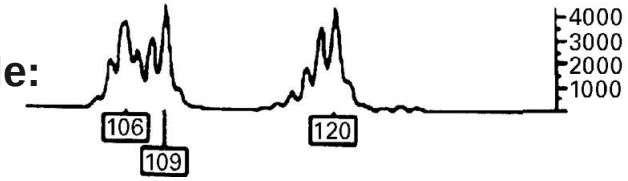
GGTA CA CA CA CA CA TTGC

GGTA CA CA CA CA CA CA CA CA CA CA TTGC

Normal Microsatellite Allele:



Tumor w/ MSI Allele:



Mismatch Repair Pathways

- Mismatch Detection
- Messaging/Signaling/ Repair Activation
- Repair
- Cell cycle arrest / Apoptosis activation
- Defects drive Microsatellite Instability

Microsatellite

- Genomic region of repeated sequence
- Repeating motifs of ~1–9 bp
- Repeat ~5–50X
- All alleles shown in green are examples of microsatellites

Microsatellite Instability

- Indicator of MMR pathway deficiency
- Traditionally analyzed by PCR or IHC
- Can analyze more loci by NGS than PCR

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TSO 500 Output: MSI Score

FASTQ Workflow

BCL > FASTQ
Alignment

Read collapsing
Realignment
Stitching

StitchedReads.bam

MSI Status (Hubble)

Unstable MSI sites

÷

Total Assessed MSI sites

=

MSI Score

≥ 20% MSI-High
< 20% MS-Stable

Biomarker Report

```
[MSI]
Usable MSI Sites:      102
Total Microsatellite Sites Unstable:   82
Percent Unstable Sites:     80.390
```


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TST 170 RNA Analysis

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TSO 170 RNA Analysis options

- **TruSight Tumor 170 Local App**
  - A Docker or Singularity based software package
  - Requires separate SampleSheet.csv (RNA libraries only)
  - Template available on TruSight Tumor 170 support page

- **BaseSpace Sequence Hub TST 170 app**

- **Sequencing requirements for RNA analysis**
  - Minimum of 16 M reads (8 M sequencing clusters) recommended
  - Paired-end samples with the same read lengths required

TSO 170 RNA Analysis output

- **Fusions (Manta): {SampleID}_Fusions.csv**
  - All candidate fusions identified by the RNA analysis pipeline
- **Splice variants (STAR): {SampleID}_SpliceVariants.vcf**
  - All candidate splice variants identified by the RNA analysis pipeline
  - Annotated by Nirvana
- **High Confidence Variants: {Sample_ID}_HighConfidenceVariants.csv**
  - Fusions passing filter with at least one gene of the pair in the 55-gene target list
  - High confidence splice variants targeted by the panel (total of 3 genes) passing filter
- **Published Fusions: {SampleID}_PublishedFusions.csv**
  - List of published fusions (Mitelman Database) targeted by TST 170 panel

Resources:
TruSight Tumor 170 Local App User Guide

Questions

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