TruSight Oncology Comprehensive HRD (DRAGEN)

illumina®

(EU) Package Insert

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Intended Use

TruSight™ Oncology Comprehensive HRD (DRAGEN) is a qualitative *in vitro* diagnostic test that uses targeted next-generation sequencing to enable assessment of Homologous Recombination Deficiency (HRD) using DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples from ovarian cancer patients using the Illumina® NextSeq™ 550Dx instrument. The test can be used to detect single nucleotide variants, insertions, deletions, and large rearrangements in BRCA1 and BRCA2 genes, and report a Genomic Instability Score (GIS).

The test is intended to be used as a companion diagnostic to identify cancer patients who may benefit from treatment with the targeted therapy listed in Table 1, in accordance with the approved therapeutic product labeling.

Table 1 Companion Diagnostic Indication

Tumor Type	Biomarker(s) Detected	Therapy
Ovarian cancer, including epithelial ovarian, fallopian tube, and primary peritoneal cancer	Homologous Recombination Deficiency (HRD) (defined as deleterious or suspected deleterious mutations in BRCA1 and BRCA2 genes and/or positive Genomic Instability Score)	LYNPARZA® (olaparib)

Summary and Explanation of the Assay

Clinical Description

Ovarian cancer has the highest mortality rate among the gynecological malignancies.[1] Ovarian cancer includes epithelial ovarian, fallopian tube, and primary peritoneal cancers, all of which share a common biological origin and are considered together for therapeutic purposes.[2,3] Approximately half of patients with high-grade serous and endometrioid ovarian cancer, which are applicable histologic sub-categories for consideration, carry genomic defects within the homologous recombination repair pathway that result in HRD.[4] The presence of HRD is associated with sensitivity to platinum-based chemotherapy[5] and clinical response to poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) inhibitors (PARPi), a class of targeted therapies for ovarian cancer,[6–12] resulting in improved progression-free and overall survival.[5,13,14] In accordance with findings from seminal clinical studies,[6,7,12,15] eligibility for PARPi treatment in ovarian cancer can be based on HRD positive status.[16] PAOLA-1 was a multicenter, Phase III, randomized, double-blind, placebo-controlled study

investigating the clinical efficacy of olaparib plus bevacizumab as a first-line maintenance therapy for patients with advanced ovarian cancer.[12] The TruSight Oncology Comprehensive (TSO Comprehensive) HRD (DRAGEN) assay was validated using samples from patients enrolled in the PAOLA-1 clinical trial.

Among various underlying mechanisms that impair the homologous recombination repair pathway, the best characterized are germline and/or somatic loss-of-function mutations in BRCA1 and BRCA2 genes.[4,13,17] Tests that evaluate genomic instability (eg, substitutions, deletions, copy number alterations, and structural rearrangements) assess for the presence of a genomic scar or mutational signature indicative of the presence of HRD regardless of underlying cause. GIS is a measurement of genomic instability that combines information on large-scale state transitions (LST), loss of heterozygosity (LOH), and telomeric-allelic imbalance (TAI). Available clinical trial data supports the clinical validity of GIS as a biomarker that identifies BRCA wild type ovarian cancer patients who gain significant benefits from PARPi therapy.[7,9,12,18]

TSO Comprehensive HRD (DRAGEN) is a qualitative next-generation sequencing (NGS) companion diagnostic (CDx) test that reports HRD status in ovarian cancer patients by detecting small DNA variants and exon-level deletions (referred to as large rearrangements [LR]) in the BRCA1 and BRCA2 genes, in combination with assessing GIS relative to a cut-off of 42.

For a list of regions in the BRCA1 and BRCA2 genes that are excluded from variant calling, refer to the *TruSight Oncology Comprehensive (DRAGEN) Block List (document # 200066147)* available from the Illumina support site.

Principles of Procedure

The TSO Comprehensive HRD (DRAGEN) assay is a distributed test that is performed manually using extracted nucleic acid as the input material. DNA extracted from FFPE tissue is used to prepare libraries, which are then enriched for HRD related genes and sequenced on the NextSeq 550Dx instrument.

The TSO Comprehensive HRD (DRAGEN) assay involves the following processes.

- **Library Preparation and Enrichment**—From FFPE tissue, 40 ng genomic DNA (gDNA) is sheared into small fragments. Universal adapters for sequencing are ligated onto the gDNA fragments. P5 and P7 adapter sequences are incorporated into each library to enable the capture of library fragments onto the surface of the flow cell during sequencing. The adapters include i5 and i7 index sequences to identify each individual sample and individual molecules with the use of Unique Molecular Identifiers (UMIs). The libraries are then enriched for the specific genes of interest using a capture-based method. Biotinylated probe sequences that span gene regions of interest targeted by the assay are hybridized to the libraries. The probes and hybridized targeted libraries are isolated from non-targeted libraries by capture with streptavidin magnetic particles. The targeted enriched libraries are washed and amplified. The quantity of each enriched library is then normalized using a bead-based method to ensure equal representation in the pooled libraries for sequencing.
- **Sequencing and Primary Analysis**—Normalized, enriched libraries are pooled and clustered onto a flow cell, and then sequenced using sequencing by synthesis (SBS) chemistry on the NextSeq 550Dx. SBS chemistry uses a reversible-terminator method to detect single, fluorescently labeled deoxynucleotide triphosphate (dNTP) bases as they are incorporated into growing DNA strands. During each sequencing

cycle, a single dNTP is added to the nucleic acid chain. The dNTP label serves as a terminator for polymerization. After each dNTP incorporation, the fluorescent dye is imaged to identify the base, and then cleaved to allow incorporation of the next nucleotide. Four reversible terminator-bound dNTPs (A, G, T, and C) are present as single, separate molecules. As a result, natural competition minimizes incorporation bias. During the primary analysis, base calls are made directly from signal intensity measurements during each sequencing cycle, resulting in base-by-base sequencing. A quality score is assigned to each base call.

- **Secondary Analysis**—The TSO Comprehensive (EU, DRAGEN) analysis module resides on the DRAGEN server paired with the NextSeq 550Dx instrument to facilitate TSO Comprehensive (EU, DRAGEN) run setup and to perform the secondary analysis of sequencing results. The TSO Comprehensive (EU, DRAGEN) analysis module performs analysis that includes validation of run processing and quality control, followed by demultiplexing, FASTQ file generation, alignment, and variant calling. Demultiplexing separates data from pooled libraries based on the unique sequence indexes that were added during the library preparation procedure. The software generates FASTQ intermediate files, which contain the sequencing reads with quality scores for each sample excluding reads from any clusters that did not pass filter. The sequencing reads are then aligned against a reference genome to identify a relationship between the sequences and are assigned a score based on regions of similarity. Aligned reads are written to files in BAM format. The assay software uses separate algorithms to call BRCA1 and BRCA2 small DNA variants, BRCA1 and BRCA2 large rearrangements, and GIS. Multiple outputs are generated by the analysis software module including sequencing metrics and Variant Call Format (VCF) files. VCF files contain information about variants found at specific positions in a reference genome. Sequencing metrics and individual output files are generated for each sample.
- **Tertiary Analysis**—Tertiary analysis, performed by the TSO Comprehensive (EU, DRAGEN) analysis module, consists of CDx calling and results report generation. The interpreted variant results are summarized in the TSO Comprehensive HRD (DRAGEN) results report. For details on secondary and tertiary analysis, refer to the *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*.

Limitations of the Procedure

For *in vitro* diagnostic use only.

- For prescription use only. The test must be ordered by a qualified medical professional in accordance with clinical laboratory regulations.
- Decisions on patient care and treatment must be based on the independent medical judgment of the treating physician, taking into consideration all applicable information concerning the patient's condition, such as patient and family history, physical examinations, information from other diagnostic tests, and patient preferences, in accordance with the standard of care in a given community.
- FFPE sample quality is highly variable. Quality can be impacted as follows.
  - Specimens that did not undergo standard fixation procedures might not generate extracted nucleic acids that meet the assay quality control requirements (*Quality Control* on page 63).
  - Somatic driver mutations may not be detected reliably if tumor content (by area) is less than 20%.

  - GIS may not be reliable if tumor content (by area) is less than 33%.
- Performance of TSO Comprehensive HRD (DRAGEN) in samples obtained from patients that have had organ or tissue transplantation has not been evaluated.
- Contamination detection can be impacted as follows.
  - In highly rearranged genomes with deletions and loss of heterozygosity, TSO Comprehensive (EU, DRAGEN) software can erroneously classify a DNA sample as contaminated (CONTAMINATION_SCORE > 1457).
  - Contamination during the procedure can result from nucleic acid from previous sample processing steps. Good laboratory practice and following all precautions and guidelines in this Package Insert helps to avoid cross-contamination between samples.
- A negative result does not rule out the presence of a mutation below the limits of detection (LoD) of the assay. Alterations at allele frequencies below the established LoD may not be detected consistently.
- The sensitivity for detection of small DNA variants can be impacted by:
  - Low complexity genomic context.
  - Increasing variant length.
- TSO Comprehensive HRD (DRAGEN) has been validated for detecting BRCA insertions and deletions up to 19 bp in length and BRCA large rearrangements that are exon-level or greater. Variants greater than 19 bp in length and alterations and events that are sub-exonic may not be detected by the assay but may impact HRD status. Appropriate clinicopathologic and laboratory correlations are advised.
- Exon-level deletions are the only large rearrangements reported by TSO Comprehensive HRD (DRAGEN). Exon-level insertions are not reported by the assay.

Product Components

The TSO Comprehensive HRD (DRAGEN) test consists of the following components:

- TruSight Oncology Comprehensive (DRAGEN) kit (Illumina catalog # 20140799)—The kit includes reagents with sufficient volume to generate 24 DNA libraries. This volume includes patient samples and controls. The kit contains the probe set used to detect BRCA mutations. Controls sold separately (refer to *Reagents Required, Not Provided* on page 13).

  ⚠ CAUTION

  RNA library preparation reagents are provided with the TSO Comprehensive (DRAGEN) kit but are not required for the TSO Comprehensive HRD (DRAGEN) assay.

- TruSight Oncology Comprehensive HRD (DRAGEN) kit (Illumina catalog # 20134650)—The kit includes reagents with sufficient volume to generate 24 DNA library enrichments. This volume includes patient samples and controls. The kit contains the probe set used for GIS determination.

- Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module (Illumina catalog # 20132935), which includes the following components:
  - TSO Comprehensive HRD (DRAGEN) Claims Packages
  - TSO Comprehensive HRD (DRAGEN) Software Suite
  - TSO Comprehensive HRD (DRAGEN) USB Kit

  An Illumina service representative installs the appropriate version of the TSO Comprehensive (EU, DRAGEN) analysis module and claims package on the DRAGEN server for the NextSeq 550Dx instrument. For information about the analysis module, refer to *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*.

Reagents

Reagents Provided

Illumina provides the following reagents.

TruSight Oncology Comprehensive (DRAGEN) kit (Illumina catalog # 20140799)

TruSight Oncology Comp Library Prep (Freeze), PN 20031118

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
End Repair A-tailing A (ERA1-A)	20031435	2	85 µl	Buffered aqueous solution containing T4 DNA polymerase and polynucleotide kinase	-25°C to -15°C
End Repair A-tailing B (ERA1-B)	20031436	2	210 µl	Buffered aqueous solution containing salts and nucleotides	-25°C to -15°C
Adaption Ligation Buffer 1 (ALB1)	20031437	2	1.73 ml	Buffered aqueous solution containing salts	-25°C to -15°C
DNA Ligase 3 (LIG3)	20031438	2	190 µl	Buffered aqueous solution containing ligase	-25°C to -15°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Short Universal Adapters 1 (SUA1)*	20031439	1	290 µl	Buffered aqueous solution containing universal sequencing oligonucleotides	-25°C to -15°C
UMI Adapters v1 (UMI)	20031496	1	290 µl	Buffered aqueous solution containing universal sequencing oligonucleotides	-25°C to -15°C
Stop Ligation Buffer (STL)	20031440	2	480 µl	Buffered aqueous solution containing salts	-25°C to -15°C
Enhanced PCR Mix (EPM)	20031441	2	550 µl	Buffered aqueous solution containing DNA polymerase and nucleotides	-25°C to -15°C

* Reagent provided but not required for TSO Comprehensive HRD (DRAGEN).

TruSight Oncology Comp Library Prep (Refrigerate), PN 20031119

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Resuspension Buffer (RSB)	20031444	1	12.4 ml	Buffered aqueous solution containing salts	2°C to 8°C
Sample Purification Beads (SPB)	20031442	2	6.11 ml	Aqueous solution containing magnetic beads	2°C to 8°C
TE Buffer (TEB)	20013443	1	10 ml	Tris EDTA solution	2°C to 8°C

TruSight Oncology Comp UP Index Primers, PN 20031120

Active ingredients: Buffered aqueous solution containing individually barcoded oligonucleotide primers.

⚠ CAUTION

Do not combine CPxx and UPxx index primers together in the same library.

Index Primer	Part Number	Quantity	Volume	i7 Index	i7 Sequence	i5 Index	i5 Sequence	Storage Temperature
UP01	20031445	1	24 µl	D702	TCCGGAGA	D503	AGGATAGG	-25°C to -15°C
UP02	20031446	1	24 µl	D707	CTGAAGCT	D504	TCAGAGCC	-25°C to -15°C
UP03	20031447	1	24 µl	D717	CGTAGCTC	D509	CATCCGAA	-25°C to -15°C
UP04	20031448	1	24 µl	D706	GAATTCGT	D510	TTATGAGT	-25°C to -15°C
UP05	20031449	1	24 µl	D712	AGCGATAG	D513	ACGAATAA	-25°C to -15°C
UP06	20031450	1	24 µl	D724	GCGATTAA	D515	GATCTGCT	-25°C to -15°C
UP07	20031451	1	24 µl	D705	ATTCAGAA	D501	AGGCTATA	-25°C to -15°C
UP08	20031452	1	24 µl	D713	GAATAATC	D502	GCCTCTAT	-25°C to -15°C
UP09	20031453	1	24 µl	D715	TTAATCAG	D505	CTTCGCCT	-25°C to -15°C
UP10	20031454	1	24 µl	D703	CGCTCATT	D506	TAAGATTA	-25°C to -15°C
UP11	20031455	1	24 µl	D710	TCCGCGAA	D517	AGTAAGTA	-25°C to -15°C
UP12	20031456	1	24 µl	D701	ATTACTCG	D518	GACTTCCT	-25°C to -15°C
UP13	20031457	1	24 µl	D716	ACTGCTTA	D511	AGAGGCGC	-25°C to -15°C
UP14	20031458	1	24 µl	D714	ATGCGGCT	D512	TAGCCGCG	-25°C to -15°C
UP15	20031459	1	24 µl	D718	GCCTCTCT	D514	TTCGTAGG	-25°C to -15°C
UP16	20031460	1	24 µl	D719	GCCGTAGG	D516	CGCTCCGC	-25°C to -15°C

TruSight Oncology Comp CP Index Primers, PN 20031126

Active ingredients: Buffered aqueous solution containing individually barcoded oligonucleotide primers.

⚠ CAUTION

Do not combine CPxx and UPxx index primers together in the same library.

Index Primer	Part Number	Quantity	Volume	i7 Index	Sequence	i5 Index	Sequence	Storage Temperature
CP01	20031461	1	20 µl	D721	CATCGAGG	D507	ACGTCCTG	-25°C to -15°C
CP02	20031462	1	20 µl	D723	CTCGACTG	D508	GTCAGTAC	-25°C to -15°C
CP03	20031463	1	20 µl	D709	CGGCTATG	D519	CCGTCGCC	-25°C to -15°C
CP04	20031464	1	20 µl	D711	TCTCGCGC	D520	GTCCGAGG	-25°C to -15°C
CP05	20031465	1	20 µl	D723	CTCGACTG	D507	ACGTCCTG	-25°C to -15°C
CP06	20031466	1	20 µl	D709	CGGCTATG	D507	ACGTCCTG	-25°C to -15°C

Index Primer	Part Number	Quantity	Volume	i7 Index	Sequence	i5 Index	Sequence	Storage Temperature
CP07	20031467	1	20 µl	D711	TCTCGCGC	D507	ACGTCCTG	-25°C to -15°C
CP08	20031468	1	20 µl	D721	CATCGAGG	D508	GTCAGTAC	-25°C to -15°C
CP09	20031469	1	20 µl	D709	CGGCTATG	D508	GTCAGTAC	-25°C to -15°C
CP10	20031470	1	20 µl	D711	TCTCGCGC	D508	GTCAGTAC	-25°C to -15°C
CP11	20031471	1	20 µl	D721	CATCGAGG	D519	CCGTCGCC	-25°C to -15°C
CP12	20031472	1	20 µl	D723	CTCGACTG	D519	CCGTCGCC	-25°C to -15°C
CP13	20031473	1	20 µl	D711	TCTCGCGC	D519	CCGTCGCC	-25°C to -15°C
CP14	20031474	1	20 µl	D721	CATCGAGG	D520	GTCCGAGG	-25°C to -15°C
CP15	20031475	1	20 µl	D723	CTCGACTG	D520	GTCCGAGG	-25°C to -15°C
CP16	20031476	1	20 µl	D709	CGGCTATG	D520	GTCCGAGG	-25°C to -15°C

TruSight Oncology Comp Enrichment (Refrigerate), PN 20031123

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Target Capture Buffer 1 (TCB1)	20031477	2	870 µl	Buffered aqueous solution containing formamide and salts	2°C to 8°C
Streptavidin Mag Beads (SMB)	20031478	2	7.78 ml	Buffered aqueous solution containing salts and solid phase paramagnetic beads covalently coated with streptavidin	2°C to 8°C
2N NaOH (HP3)	20031479	2	400 µl	Sodium hydroxide solution	2°C to 8°C
Elute Target Buffer 2 (ET2)	20031480	2	290 µl	Buffered aqueous solution	2°C to 8°C
Library Normalization Beads (LNB1)	20031481	1	1.04 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Library Normalization Wash 1 (LNW1)	20031482	2	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	2°C to 8°C
Library Normalization Storage Buffer 1 (LNS1)	20031483	2	3.5 ml	Buffered aqueous solution containing salts	2°C to 8°C
Resuspension Buffer (RSB)	20031444	1	12.4 ml	Buffered aqueous solution containing salts	2° C to 8°C
Sample Purification Beads (SPB)	20031442	2	6.11 ml	Aqueous solution containing magnetic beads	2°C to 8°C

TruSight Oncology Comp Enrichment (Freeze), PN 20031121

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Target Capture Additives 1 (TCA1)	20031486	2	521 µl	Buffered aqueous solution containing oligonucleotides	-25°C to -15°C
Enhanced Enrichment Wash (EEW)	20031487	1	50.4 ml	Buffered aqueous solution containing salts	-25°C to -15°C
Enrichment Solution 2 (EE2)	20031488	3	1.65 ml	Buffered aqueous solution containing detergent	-25°C to -15°C
Enhanced PCR Mix (EPM)	20031441	2	550 µl	Buffered aqueous solution containing DNA polymerase and nucleotides	-25°C to -15°C
PCR Primer Cocktail 3 (PPC3)	20031490	2	150 µl	Buffered aqueous solution containing P5 and P7 primers	-25°C to -15°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Library Normalization Additives 1 (LNA1)	20031491	1	4.6 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	-25°C to -15°C
PhiX (PX3 or PhiX)	20031492	1	10 µl	Buffered aqueous solution containing PhiX genomic DNA	-25°C to -15°C

TruSight Oncology Comp Content Set, PN 20031122

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Oncology RNA Probe Pool (OPR1)*	20031494	1	290 µl	Oligonucleotide probe pool	-25°C to -15°C
Oncology DNA Probe Pool 2 (OPD2)	20031495	1	290 µl	Oligonucleotide probe pool	-25°C to -15°C

* Reagent provided but not required for TSO Comprehensive HRD (DRAGEN).

TruSight Oncology Comp RNA Library Prep, PN 20031127

The following reagents are provided but not required for TSO Comprehensive HRD (DRAGEN).

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
First Strand Synthesis Mix (FSM)	20031431	1	260 µl	Buffered aqueous solution containing salts and nucleotides	-25°C to -15°C
Second Strand Mix (SSM)	20031432	1	720 µl	Buffered aqueous solution containing salts, DNA polymerase, RNase H, and nucleotides	-25°C to -15°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Elution Primer Frag Mix (EPH3)	20031433	1	250 µl	Buffered aqueous solution containing salts and random hexamers	-25°C to -15°C
Reverse Transcriptase (RVT)	20031434	1	70 µl	Buffered aqueous solution containing reverse transcriptase	-25°C to -15°C

TruSight Oncology Comprehensive HRD (DRAGEN) kit (Illumina catalog # 20134650)

TruSight Oncology Comp HRD Enrichment (Refrigerate), PN 20140116

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Target Capture Buffer 1 (TCB1)	20031477	1	870 µl	Buffered aqueous solution containing formamide and salts	2°C to 8°C
Streptavidin Mag Beads (SMB)	20031478	1	7.78 ml	Buffered aqueous solution containing salts and solid phase paramagnetic beads covalently coated with streptavidin	2°C to 8°C
2N NaOH (HP3)	20031479	1	400 µl	Sodium hydroxide solution	2°C to 8°C
Elute Target Buffer 2 (ET2)	20031480	1	290 µl	Buffered aqueous solution	2°C to 8°C
Library Normalization Beads (LNB1)	20031481	1	1.04 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Library Normalization Wash 1 (LNW1)	20031482	1	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	2°C to 8°C
Library Normalization Storage Buffer 1 (LNS1)	20031483	1	3.5 ml	Buffered aqueous solution containing salts	2°C to 8°C
Resuspension Buffer (RSB)	20031444	1	12.4 ml	Buffered aqueous solution containing salts	2° C to 8°C
Sample Purification Beads (SPB)	20031442	1	6.11 ml	Aqueous solution containing magnetic beads	2°C to 8°C

TruSight Oncology Comp HRD Enrichment (Freeze), PN 20140115

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Target Capture Additives 1 (TCA1)	20031486	1	521 µl	Buffered aqueous solution containing oligonucleotides	-25°C to -15°C
Enhanced Enrichment Wash (EEW)	20031487	1	50.4 ml	Buffered aqueous solution containing salts	-25°C to -15°C
Enrichment Solution 2 (EE2)	20031488	2	1.65 ml	Buffered aqueous solution containing detergent	-25°C to -15°C
Enhanced PCR Mix (EPM)	20031441	1	550 µl	Buffered aqueous solution containing DNA polymerase and nucleotides	-25°C to -15°C
PCR Primer Cocktail 3 (PPC3)	20031490	1	150 µl	Buffered aqueous solution containing P5 and P7 primers	-25°C to -15°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Library Normalization Additives 1 (LNA1)	20031491	1	4.6 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	-25°C to -15°C

TruSight Oncology Comp HRD Content Set, PN 20140117

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Oncology DNA Probe Pool 3 (OPD3)	20082795	1	290 µl	Oligonucleotide probe pool	-25°C to -15°C

Reagents Required, Not Provided

Pre-Amp Reagents

- DNA Extraction and Purification Reagents—Refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 20 for reagent requirements.
- DNA Quantification Reagents—Refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 20 for reagent requirements.
- TruSight Oncology Controls:
  - TruSight Oncology DNA Control v2 (Illumina catalog # 20090155)
- Ethanol (EtOH) 100% (200 proof), molecular biology grade
- RNase/DNase-free water

Post-Amp Reagents

- NextSeq 550Dx High-Output Reagent Kit v2.5 (300 cycles) (Illumina catalog # 20028871)
  - NextSeq 550Dx High Output Flow Cell Cartridge v2.5 (300 cycles)
  - NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles)
  - NextSeq 550Dx Buffer Cartridge v2 (300 cycles)
- EtOH 100% (200 proof), molecular biology grade
- RNase/DNase-free water

Reagent Storage and Handling

The following reagent boxes are shipped frozen. Store at -25°C to -15°C.

Box	Part Number	Lab Area
TruSight Oncology Comp Library Prep (Freeze)	20031118	Pre-amp
TruSight Oncology Comp UP Index Primers	20031120	Pre-amp
TruSight Oncology Comp CP Index Primers	20031126	Pre-amp
TruSight Oncology Comp Enrichment (Freeze)	20031121	Post-amp
TruSight Oncology Comp Content Set	20031122	Post-amp
TruSight Oncology Comp RNA Library Prep	20031127	Pre-amp
TruSight Oncology Comp HRD Enrichment (Freeze)	20140115	Post-amp
TruSight Oncology Comp HRD Content Set	20140117	Post-amp

The following reagent boxes are shipped on gel packs to maintain 0°C to 10°C. Store at 2°C to 8°C.

Box	Part Number	Lab Area
TruSight Oncology Comp Library Prep (Refrigerate)	20031119	Pre-amp
TruSight Oncology Comp Enrichment (Refrigerate)	20031123	Post-amp
TruSight Oncology Comp HRD Enrichment (Refrigerate)	20140116	Post-amp

⚠ CAUTION

Do not store reagents in a frost-free storage unit or in refrigerator door compartments.

⚠ CAUTION

Do not freeze reagents containing beads (LNB1, SPB, and SMB).

- Changes in the physical appearance of the reagents can indicate deterioration of the materials. If changes in the physical appearance occur (for example, changes in reagent color or cloudiness), do not use the reagents.
- ERA1-B and TCB1 can have product-related particulates. Follow the specific handling guidelines for each reagent.
- Stability of the TSO Comprehensive HRD (DRAGEN) assay has been evaluated and performance demonstrated for up to four uses of the kit. Reagents are stable when stored at the indicated temperatures until the specified expiration date listed on the box label.

Equipment and Materials

Equipment and Materials Required, Not Provided

Pre-Amp Equipment and Materials

Equipment	Supplier
Ultrasonicator with associated accessories Refer to *Ultrasonicator Configuration Settings for DNA Fragmentation* on page 18.	General lab supplier
Vortexer	General lab supplier
Microsample incubators (2) with inserts for 96-well MIDI plates (2)	General lab supplier
Microcentrifuge	General lab supplier
Plate centrifuge with the following specifications: • Compatible with 96-well microplates • Capable of 280 × g (+/- 10%)	General lab supplier
Plate shaker with the following specifications: • 2 mm orbit • Can shake at 1200 rpm and 1800 rpm	General lab supplier
Sealing wedge or roller	General lab supplier
Magnetic stand with the following specifications: • Designed for paramagnetic bead precipitation/separation • Magnets on the side of the stand (not on the bottom) • Compatible with 96-well MIDI plates	General lab supplier
Precision pipettes capable of accurately delivering volumes between 2 µl to 1000 µl with the following specifications: • Single- or multichannel pipette with increment of 0.02 µl • Single- or multichannel pipette with increment of 0.1 µl, 0.2 µl, or 0.5 µl • Single- or multichannel pipette with increment of 1 µl or 2 µl Pipettes must be calibrated regularly and accurate within 5% of stated volume.	General lab supplier
Pipette-aid	General lab supplier
Ice or cold block	General lab supplier
10 ml serological pipettes	General lab supplier

Equipment	Supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable • Suitable for skirted or semi-skirted PCR plates • Strong adhesive that withstands multiple temperature changes of -20°C to 100°C • DNase/RNase-free	General lab supplier
Microcentrifuge tubes with capacity of 1.7 ml, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
Compatible aerosol-resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859 or equivalent
96-well PCR plates compatible with thermal cycler, 0.2 ml (polypropylene wells)	General lab supplier

Post-Amp Equipment and Materials

Equipment	Supplier
NextSeq 550Dx Instrument	Illumina, catalog # 20005715
Plate centrifuge with the following specifications: • Compatible with 96-well microplates • Capable of 280 × g (+/- 10%)	General lab supplier
Thermal cycler with the following specifications: • Heated lid (100°C) • Encompass a 4°C to 99°C temperature range • ±0.25°C temperature accuracy • Compatible with 96-well PCR plates, 0.2 ml • Refer to *Thermal Cycler Ramp Rate* on page 19	General lab supplier
Vortexer	General lab supplier
Microsample incubator with insert for 96-well MIDI plates	General lab supplier

Equipment	Supplier
Dry heat block with the following specifications: • 25°C to 99°C temperature range • ±5°C temperature accuracy • Make sure that the microcentrifuge tubes are compatible with the heat block	General lab supplier
Plate shaker with the following specifications: • 2 mm orbit • Can shake at 1200 rpm and 1800 rpm	General lab supplier
Microcentrifuge	General lab supplier
Sealing wedge or roller	General lab supplier
Magnetic stand with the following specifications: • Designed for paramagnetic bead precipitation/separation • Magnets on the side of the stand (not on the bottom) • Compatible with 96-well MIDI plates	General lab supplier
Precision pipettes capable of accurately delivering volumes between 2 µl to 1000 µl with the following specifications: • Single- or multichannel pipette with increment of 0.02 µl • Single- or multichannel pipette with increment of 0.1 µl, 0.2 µl, or 0.5 µl • Single- or multichannel pipette with increment of 1 µl or 2 µl Pipettes must be calibrated regularly and accurate within 5% of stated volume.	General lab supplier
Pipette-aid	General lab supplier
10 ml serological pipettes	General lab supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable • Suitable for skirted or semi-skirted PCR plates • Strong adhesive that withstands multiple temperature changes of -20°C to 100°C • DNase/RNase-free	General lab supplier
Microcentrifuge tubes with capacity of 2 ml, nuclease-free	General lab supplier
Microcentrifuge tubes with capacity of 1.7 ml, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier

Equipment	Supplier
50 ml conical tubes	General lab supplier
Compatible aerosol-resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859 or equivalent
96-well PCR plates compatible with thermal cycler, 0.2 ml (polypropylene wells)	General lab supplier
Ice or cold block	General lab supplier

Ultrasonicator Configuration Settings for DNA Fragmentation

DNA fragmentation, or shearing, influences assay performance by determining the distribution of fragment size, which in turn affects sequencing coverage. Several focused-ultrasonication configurations were evaluated and optimized for the TSO Comprehensive HRD (DRAGEN) assay (Table 2).

- Shearing time was adjusted to maximize the MEDIAN_EXON_COVERAGE metric outlined in *Quality Control* on page 63. Shearing times (refer to Table 2) and MEDIAN_INSERT_SIZE results differed across configurations.
- Configurations 1–4 and 6 were tested with 8-strip glass tubes. Configuration 5 used a single glass tube. Tube volume capacities are shown in Table 2.
- Optimization of configurations 3–6 (smaller water bath volumes) used pulsing.
- Configurations 3–5 were sheared in smaller volume tubes. Tube volume capacities affect shearing parameters.
- Configuration 4 (line transducer, medium-size water bath volume, degassed water) required a long pulse delay time (40 seconds) to achieve similar MEDIAN_EXON_COVERAGE as configuration 1 and 2 at nominal 40 ng input.
- Optimal settings for configuration 3 resulted in a slightly larger fragment size distribution compared to the other configurations (MEDIAN_INSERT_SIZE was approximately 5–10 base pairs larger).
- Configurations 3 and 5 used non-degassed water and the smallest water bath volume.
- Configurations 3 and 5 needed increased DNA input (50 ng for configuration 3, 60 ng for configuration 5) to achieve similar MEDIAN_EXON_COVERAGE relative to the other 4 configurations, which used the nominal 40 ng input.
- Configurations 3 and 5 have more damage and/or denaturation and therefore a reduced effective mass of usable dsDNA molecules for library preparation.

Centrifuge the shearing tubes during the recovery process to make sure that the specified volume is retrieved as any loss of material can adversely affect performance.

Table 2 Focused-Ultrasonicator Configurations Evaluated

Parameter	Configuration					
	1	2	3	4	5	6
Transducer	Line	Point	Point	Line	Point	Line
Water bath volume	5 L	5 L	85 ml	500 ml	16 ml	1.7 L
Water degassed	Yes	Yes	No	Yes	No	Yes
Water chiller	Yes	Yes	Yes	Yes	Yes	Yes
Water bath temperature	7°C	7°C	12°C	12°C	20°C	10°C
Peak Incident Power (PIP)	450 W	175 W	50 W	350 W	50 W	450 W
% duty factor	30	10	30	25	20	25
Cycles per burst	200	200	1000	1000	1000	600
Pulsing (10-second bursts)	No	No	Yes	Yes	Yes	Yes
Pulsing delay time	N/A	N/A	10 s	40 s	10 s	10 s
Shearing time	250 s	280 s	200 s[1]	320 s[2]	200 s[1]	320 s[2]
Sample processing	1–8	1	1	1–8	1	1–8
Batch size	1–96	1–96	1–8	1–8	1	1–96
Glass 8-strip tube sample size	130 µl	130 µl	50 µl	50 µl	Single tube (50 µl)	130 µl (or 96 microTUBE Plate)
Dithering	N/A	N/A	N/A	3 mm Y @ 20 mm/s	N/A	1.5 mm Y @ 10 mm/s
DNA input equivalent (for median exon coverage)	40 ng	40 ng	50 ng	40 ng	60 ng	40 ng

[1] The shearing time of 200 seconds consists of 10-second bursts with 20 repeats.
[2] The shearing time of 320 seconds consists of 10-second bursts with 32 repeats.

Thermal Cycler Ramp Rate

Optimization of thermal cycler ramp rate is recommended. For example, a tested model was adjusted from a default (and maximum) ramp rate of 5°C/s to 3°C/s to obtain comparable results to other models with lower default ramp rates.

Specimen Collection, Transport, and Storage

Follow standard procedures when collecting, transporting, storing, and processing samples.

Sample Requirements

FFPE Tissue

The TSO Comprehensive HRD (DRAGEN) assay requires 40 ng DNA extracted from ovarian, fallopian tube, or primary peritoneal FFPE tissue. Tissue should be fixed using formalin fixative suitable for molecular analyses (for example, 10% neutral-buffered formalin). Tissue must not be decalcified. Before performing the TSO Comprehensive HRD (DRAGEN) assay, the tissue sample should be examined by a pathologist to make sure that it is appropriate for this test. A minimum of 20% tumor content (by area) is required to detect somatic driver mutations. A minimum of 33% tumor content (by area) is required to detect GIS.

For a high probability of extracting 40 ng DNA, the recommended tissue volume is ≥ 1.0 mm^3. This volume is equivalent to a cumulative viable tissue area of ≥ 200 mm^2 using 5 µm thick sections, or ≥ 100 mm^2 using 10 µm thick sections. Cumulative tissue area is the sum of the viable tissue area in all sections submitted for extraction. For example, a cumulative tissue area of 200 mm^2 may be obtained by extracting four 5 µm sections with 50 mm^2 tissue area each or five 10 µm sections with 20 mm^2 tissue area each. Tissue necrosis may decrease the nucleic acid yield. To minimize the possibility of false negative results, the tissue may be macrodissected to eliminate necrotic tissue and achieve a desirable viable tumor content.

Slide-mounted FFPE tissue can be stored for up to 28 days at room temperature.

Nucleic Acid Extraction, Quantification, and Storage

- Extract DNA from FFPE tissue samples using commercially available extraction kits. Differences in extraction kits can impact performance. Refer to *Nucleic Acid Extraction Kit Evaluation* on page 82.
- Store extracted stock nucleic acid following the instructions from the extraction kit manufacturer.
- Store extracted DNA for up to 28 days at -25°C to -15°C.
- To avoid changes in concentration over time, measure DNA within 28 days of starting library preparation. Quantify DNA using a fluorometric quantification method that uses nucleic acid binding dyes. Nucleic acid concentration should be the mean of at least three measurements.
- The assay requires 40 ng of each gDNA sample with a minimum extraction concentration of 3.33 ng/µl. Shearing requires a final volume of 52 µl (0.77 ng/µl) with a minimum of 40 µl TEB (provided) used as the diluent.

Library Storage

Store libraries in low bind PCR plates for 7 to 32 days, depending on the type of library (refer to Table 3).

Table 3 Library Storage Times

Library Type	Plate	Number of Days	Storage Temperature
Fragmented gDNA	LP PCR	≤ 7	-25°C to -15°C
Pre-enrichment	ALS PCR	≤ 30	-25°C to -15°C
Post-enrichment	ELU2 PCR	≤ 7	-25°C to -15°C
Post-enrichment PCR	PL PCR	≤ 30	-25°C to -15°C
Normalized	NL PCR	≤ 32	-25°C to -15°C

Warnings and Precautions

Safety

⚠ WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

- Some components of this assay contain formamide, an aliphatic amide that is a carcinogen and a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle used reagents as chemical waste and discard in accordance with the governmental safety standards for your region. For additional environmental, health, and safety information, refer to the SDS by searching the product code at support.illumina.com/sds.html. (Refer to *Reagents Provided* on page 5 for more information).
- Some components of this assay contain 2-mercaptoethanol, a reducing agent. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area and dispose of any containers and unused contents in accordance with applicable local governmental safety standards. For additional environmental, health, and safety information, refer to the SDS by searching the product code at support.illumina.com/sds.html. (Refer to *Reagents Provided* on page 5 for more information).
- Some components of this assay contain sodium hydroxide, a highly corrosive base. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle in accordance with Good Lab Practices and discard in

compliance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS by searching the product code at support.illumina.com/sds.html. (Refer to *Reagents Provided* on page 5 for more information).

- Some components of this assay (EPM) contain tetramethylammonium chloride, an acute toxicant, skin irritant, and marine pollutant. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Handle in accordance with Good Lab Practices and discard in compliance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS by searching the product code at support.illumina.com/sds.html. (Refer to *Reagents Provided* on page 5 for more information).
- Handle all specimens as if they are known to be infectious.
- Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- Immediately report any serious incidents related to this product to Illumina and the Competent Authorities of the member states in which the user and the patient are established.

Laboratory

- To prevent contamination, arrange the laboratory with a unidirectional workflow. Pre-amplification and post-amplification areas must have dedicated equipment and materials (for example, pipettes, pipette tips, vortexer, and centrifuge). To prevent amplification product or probe carryover, avoid returning to the pre-amplification area after entering the post-amplification area.
- Perform Index PCR and Enrichment steps in a post-amplification area to prevent amplification product carryover.
- The library preparation procedures require an RNase/DNase-free environment. Thoroughly decontaminate work areas with an RNase/DNase-inhibiting cleaner. Use plastics certified to be free of DNase, RNase, and human genomic DNA.
- For post-amplification procedures, clean work surfaces and equipment thoroughly before and after each procedure with a freshly made 0.5% sodium hypochlorite (NaOCl) solution. Allow the solution to contact surfaces for 10 minutes, and then thoroughly wipe clean with 70% ethyl or isopropyl alcohol.
- Use nuclease-free microcentrifuge tubes, plates, pipette tips, and reservoirs.
- Use calibrated equipment throughout the assay. Make sure to calibrate equipment to the speeds, temperatures, and volumes specified in this protocol.
- Use precision pipettes to ensure accurate reagent and sample delivery. Calibrate regularly according to manufacturer specifications.
- Use the following guidelines when using multichannel pipettes:
  - Pipette a minimum of ≥ 2 µl.

  - Make sure that barrier tips are well-fitting and appropriate for the multichannel pipette brand and model.
  - Affix tips with a rolling motion to make sure that all tips attach equally well.
  - Aspirate at a 90° angle, with equal volume levels of liquid across all tips.
  - Mix all components after delivery by pipetting the reaction mixture up and down.
  - After dispensing, make sure that liquid fully dispensed from every tip.
- Make sure to use equipment specified for the assay and to set programs as directed.
- Stated temperatures for the thermal cycler and the microsample incubator indicate reaction temperature, not necessarily the set temperature of the equipment.

Assay

- Avoid cross-contamination.
  - Follow proper laboratory practices when handling samples and reagents.
  - Use fresh consumable labware and fresh pipette tips between samples and between dispensing reagents.
  - Use aerosol resistant tips to reduce the risk of cross-contamination.
  - Use a unidirectional workflow when moving from pre-amplification to post-amplification areas.
  - Handle and open only one index primer at a time. Recap each index tube immediately after use. Extra caps are provided in the kit.
  - Change gloves often and if they come into contact with index primers or samples.
  - Remove unused index primer tubes from the working area.
  - Do not return reagents to stock tubes after use with a tube strip, trough, or reservoir.
  - Mix samples with a pipette and centrifuge the plate when indicated.
  - Use a microplate shaker. Do not vortex the plates.
- Do not interchange assay components from different reagent kit lots. Reagent kit lots are identified on the reagent kit box label and master lot sheet.
- Proper laboratory practices are required to prevent nucleases and PCR products from contaminating reagents, instrumentation, samples, and libraries. Nuclease and PCR product contamination can cause inaccurate and unreliable results.
- Proper plate type is required for optimal assay performance and storage. Make sure to follow plate transfer instructions in the *Instructions for Use* on page 32.
- Failure to follow the procedures as outlined can result in erroneous results, or a significant reduction in library quality.
- Unless a safe stopping point is specified in the *Instructions for Use* on page 32, proceed immediately to the next step.

- Store the assay reagents or components at the specified temperature in designated pre-amplification and post-amplification areas.
- Do not store reagents in a frost-free storage unit or in refrigerator door compartments.
- Do not freeze reagents containing beads (LNB1, SPB, and SMB).
- Do not use reagents that have been stored improperly.
- Do not deviate from the mixing and handling procedures specified for each reagent. Inadequate mixing or over-vortexing of reagents can result in failed sample results.
- ERA1-B and TCB1 can have product-related particulates. Follow handling guidelines for each specific reagent.
- Prepare fresh master mixes and discard the remaining volume after use.
- Always prepare fresh 80% ethanol with RNase/DNase-free water for wash steps. Ethanol can absorb water from the air, which might impact results. Dispose of 80% ethanol after use in accordance with local, state, and/or federal regulations.
- Transfer the specified volume of eluate. Transferring less than the specified volume of eluate during the elution steps may impact results.
- Use the following guidelines for ultrasonicators. Make sure to follow manufacturer instructions.
  - Load the gDNA into the ultrasonicator tube slowly to avoid creating bubbles. Excessive bubbles or an air gap in the shearing tube may lead to incomplete fragmentation.
  - Dispense into ultrasonicator tubes slowly and avoid splashing.
  - To avoid fluid displacement and loss of sample, do not insert the pipette tip to the bottom of the ultrasonicator tube when removing fragmented DNA.
- Do not pipette less than 2 µl sample input.
- Do not use a trough to dispense reagents for steps that require less than 10 µl material to be added to each sample well.
- Use a fine-tipped pipette when transferring fragmented gDNA samples from the ultrasonicator tubes to the Library Prep (LP) plate.
- Only use UMI adapters.
- Do not use SUA1 adapters.
- Assign different index primers to each library sample to identify each library when it is pooled for sequencing on a single flow cell.
- UPxx indexes or CPxx indexes can be used.
- Do not combine CPxx and UPxx index primers in the same library.
- Mismatches between the samples and index primers cause incorrect result reporting due to loss of positive sample identification. Enter sample IDs and assign indexes in the Illumina Connected Run TruSight Oncology Comprehensive (EU, DRAGEN) analysis module before beginning library preparation. Record sample IDs, indexing, and plate well orientation for reference during library preparation.

- Sequence a maximum of 16 DNA libraries per flow cell. Sequence a minimum of six libraries. Follow guidelines in *Number of Libraries and Selecting Indexes* on page 29.
- After the bind step in *Capture Targets One* on page 46 and *Capture Targets Two* on page 50, proceed immediately to the wash step to prevent bead pellets from drying.
- During wash steps, remove all 80% ethanol from the bottom of the wells. Residual ethanol can impact results.
- For optimal assay performance, follow the specified number of washes indicated in the *Instructions for Use* on page 32.
- During the *Normalize Libraries* on page 56 procedure, thoroughly resuspend the library bead pellet to achieve consistent cluster density on the flow cell.
- DNA libraries from the same sample are enriched with two different probe sets but use the same index and must be sequenced on the same flow cell.
- Positive controls and the no-template control (NTC) must be processed like a sample and split for enrichment with both probe sets.

Procedural Notes

- The TSO Comprehensive HRD (DRAGEN) workflow can be conducted according to the following schedule:
  - Day 1: DNA Fragmentation of gDNA samples, Library Preparation, and begin Overnight (First) Hybridization.
  - Day 2: Enrichment, Normalization of Enriched Libraries, and Loading of Libraries onto the NextSeq 550Dx instrument.

  If it is not possible to perform the TSO Comprehensive HRD (DRAGEN) workflow according to this schedule, several safe stopping points are specified throughout the protocol. Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.
- Libraries derived from DNA are split into separate wells for simultaneous enrichment with the OPD2 probe set and the OPD3 probe set. Positive controls and NTCs must follow the same procedure.
- Use the TruSight Oncology Comp Enrichment components (PN 20031123, PN 20031121) with OPD2 from the TruSight Oncology Comp Content Set (PN 20031122). Libraries enriched with OPD2 are referred to as DNA Libraries.
- Use the TruSight Oncology Comp HRD Enrichment components (PN 20140116, PN 20140115) with OPD3 from the TruSight Oncology Comp HRD Content Set (PN 20140117). Libraries enriched with OPD3 are referred to as DNA HRD libraries.
- Master mix preparation tables include volume overage to make sure that there is sufficient volume for the number of samples being processed.
- Use molecular-grade water that is free of nucleases.

- After reagent addition, rinse the tip by aspirating and dispensing one time into the appropriate well in the plate, unless otherwise specified in the procedure.
- Room temperature is defined as 15°C to 30°C.
- Reagents, samples, and libraries must be kept cold at certain steps in the Instructions for Use. This is defined as keeping on ice or equivalent.

Thermal Cycler Programs

- Program thermal cycler programs on post-amplification equipment before starting the protocol.
- Make sure that PCR plates fit snugly in the thermal cycler.
- Use plates recommended by the manufacturer of the thermal cycler.

Sealing and Unsealing the Plate

- Always seal plates with a new adhesive plate seal. Do not reuse seals.
- To seal the plate, securely apply the adhesive cover to the plate with a sealing wedge or roller.
- Always seal the 96-well plate with a new adhesive plate seal before the following steps in the protocol.
  - Plate shaking steps
  - Centrifugation steps
  - Thermal cycling steps
  - Hybridizations
  - Long-term storage
- Make sure that the edges and wells are sealed to reduce the risk of cross-contamination and evaporation.
- Place the plate on a flat surface before slowly removing the seal.
- Before unsealing, if any fluid or condensation is observed on the seal or side walls of the plate wells, centrifuge at 280 × g for 1 minute.
- Use adhesive plate seals that are effective at -20°C to 100°C, and suitable for skirted or semi-skirted PCR plates.

Equipment

- Make sure that laboratory personnel are familiar with manufacturer instructions for operating and maintaining all equipment before starting the assay.

Plate Type and Plate Transfers

- Proper plate type is required for optimal assay performance and storage.
- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the destination plate.

- Multichannel pipettes can be used when transferring samples between tube strips or plates.
- Use the following guidelines when shaking plates.
  - Use a plate shaker to shake plates. Do not vortex plates.
  - Shake PCR plates at 1200 rpm.
  - Shake MIDI plates at 1800 rpm.
  - Follow manufacturer instructions to make sure that the plate shaker holds the plate securely.

Centrifugation

- When instructions in the protocol indicate to centrifuge briefly, centrifuge at 280 × g for 1 minute.
- If liquid is observed on the seal or on the sides of a well, centrifuge plate at 280 × g for 1 minute.

Handling Reagents

- Tightly recap all reagent tubes immediately after use to limit evaporation and prevent contamination.
- Return reagents to the specified storage temperature when they are no longer needed for a procedure.
- Follow the reagent preparation that precedes each procedure section of the *Instructions for Use* on page 32.
- Make sure to prepare the required volume of master mix, elution mix, and 80% ethanol for the number of samples you process.
- Volumes provided in master mix and solution tables contain overage. Overage volume calculations are as follows.
  - Table 13
    - Volume of ERA1-B = (7.2 µl) x (number of libraries) x (1.20).
    - Volume of ERA1-A = (2.8 µl) x (number of libraries) x (1.20).
  - Table 23
    - Volume of EE2 = (20.9 µl) x (number of libraries) x (1.364).
    - Volume of HP3 = (1.1 µl) x (number of libraries) x (1.364).
  - Table 24
    - Volume of EE2 = (20.9 µl) x (number of libraries) x (1.364).
    - Volume of HP3 = (1.1 µl) x (number of libraries) x (1.364).
  - Table 30
    - Volume of LNA1 = (38.1 µl) x (number of libraries) x (2.0).
    - Volume of LNB1 = (6.9 µl) x (number of libraries) x (2.0).
  - Table 31
    - Volume of EE2 = (30.4 µl) x (number of libraries) x (1.25).
    - Volume of HP3 = (1.6 µl) x (number of libraries) x (1.25).

Adapter Sets

- Only use UMI adapters.
- SUA1 adapters are provided but not required for the TSO Comprehensive HRD (DRAGEN) assay.

Handling Beads

- Three types of beads are included in the TSO Comprehensive HRD (DRAGEN) assay (SPB, SMB, and LNB1). Make sure that the correct bead type is used during the procedure.
- Perform the correct number of washes for each bead type.
- Make sure that beads are at room temperature before use.
- Mix beads for 1 minute before use to ensure homogeneity.
- Use the following guidelines when mixing beads with a pipette:
  - Use a suitable pipette and tip size for the volume you mix.
  - Adjust the volume setting to approximately 50–75% of the sample volume.
  - Pipette slowly without releasing the plunger.
  - Avoid splashing and introducing bubbles.
  - Position the pipette tip above the pellet and dispense directly into the pellet to release beads from the well or tube.
  - Make sure that the bead pellet is fully in solution. The solution should look dark brown and have a homogeneous consistency.
  - Assess if a bead pellet is present. Carefully aspirate total bead solution of well in the tip and look at bottom of wells.
- If beads are aspirated into the pipette tips during magnetic separation steps, dispense the beads back to the plate well on the magnetic stand. Wait until the liquid is clear (approximately 2 minutes) before proceeding to the next step of the procedure.
- When washing beads:
  - Use the recommended magnetic stand for the plate.
  - Dispense liquid directly onto the bead pellet so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the procedure specifies to remove it.
  - Do not agitate the plate while on the magnetic stand.
  - While on the magnetic stand, do not disturb the bead pellet.
- When washing beads or removing supernatant, angle pipette tips at the bottom of the wells to avoid creating a vacuum and pulling solution into the pipette tip filters.

Number of Libraries and Selecting Indexes

Before run setup, plan the number of sample libraries and sample indexes for the sequencing run. The following sample number guidelines include positive controls but exclude NTCs. NTCs must be added to the planned run as an additional sample.

Index primers uniquely identify each sample so that libraries can be pooled together for sequencing on one flow cell. The indexes display on the Create Run screen during run setup on the Illumina Connected Run TruSight Oncology Comprehensive (EU, DRAGEN) analysis module. During library preparation, add the index primer to each sample library. ***Use a different index primer mix for each sample library.***

Indexed libraries from the same sample are split into two enrichments with two different probe sets. The resulting two enriched libraries share the same index and must be sequenced on the same flow cell.

Refer to Table 4 for the minimum and maximum libraries to sequence on one flow cell when sequencing.

Table 4 TSO Comprehensive HRD (DRAGEN) Sequencing

Library Type	Enrichment	Minimum*	Maximum*
DNA	OPD2	3	8
DNA HRD	OPD3	3	8

* NTCs do not contribute to the plexity.

For optimal reagent usage when sequencing on the NextSeq 550Dx instrument, sequence 8 DNA (OPD2) libraries *and* 8 DNA HRD (OPD3) libraries per flow cell.

Make sure that the index primers that you use with samples match the indexes you select for analysis with the Illumina Connected Run TruSight Oncology Comprehensive (EU, DRAGEN) analysis module. ***Mismatches cause incorrect result reporting due to loss of positive sample identification.***

There are two types of indexes (UPxx and CPxx) in the TSO Comprehensive HRD (DRAGEN) assay.

When sequencing the minimum number of libraries (3 DNA libraries and 3 DNA HRD libraries), the following requirements apply:

- Do not use CPxx index sets.
- One of the following UPxx index sets is required to provide sufficient diversity:
  - UP01, UP02, and UP03
  - UP04, UP05, and UP06
  - UP07, UP08, and UP09
  - UP10, UP11, and UP12

  For example, the first library is assigned UP01, the second library UP02, and the third library UP03.

TruSight Oncology DNA Control v2

TSO Comprehensive HRD (DRAGEN) requires the TruSight Oncology DNA Control v2 as a positive control. Include the TruSight Oncology DNA Control v2 for each DNA sequencing run within a given library preparation event. A unique positive control is prepared for each planned sequencing run. Refer to the TruSight Oncology DNA Control v2 (EU) Package Insert for more information.

The TruSight Oncology DNA Control v2 input amount is 2 µl.

Include the NTC in each library preparation event. The NTC is sequenced repeatedly within one library preparation event. Follow these guidelines for the positive control and the NTC:

- Prepare libraries from positive controls and NTCs identically to samples. The positive control and the NTC must be split for enrichment with both probe sets identically to samples.
- Use TEB for the DNA NTC.
- The positive controls are included in the maximum library requirement.
- The NTCs are not included in the minimum library requirement.
- Use UP indexes for the NTC when sequencing the minimum number of libraries.
- As the NTC is sequenced repeatedly, the index selected for this control cannot be repeated in the library preparation event.

The following tables show example plate layouts for library preparation workflow (Figure 1). Each numbered column represents a single sequencing run. The NTC is sequenced for each column or set of columns.

Table 5 Library Preparation Plate of a Single Run Including Six Patient Samples

	1	2	3	4	5	6
A	Pos DNA Control	empty	empty	empty	empty	empty
B	DNA 1	empty	empty	empty	empty	empty
C	DNA 2	empty	empty	empty	empty	empty
D	DNA 3	empty	empty	empty	empty	empty
E	DNA 4	empty	empty	empty	empty	empty
F	DNA 5	empty	empty	empty	empty	empty
G	DNA 6	empty	empty	empty	empty	empty
H	DNA NTC	empty	empty	empty	empty	empty

Table 6 Library Preparation Plate of Three Runs Including 20 Patient Samples

	1	2	3	4	5	6	7
A	Pos DNA Control	Pos DNA Control	Pos DNA Control	empty	empty	empty	empty
B	DNA 1	DNA 7	DNA 14	empty	empty	empty	empty
C	DNA 2	DNA 8	DNA 15	empty	empty	empty	empty
D	DNA 3	DNA 9	DNA 16	empty	empty	empty	empty
E	DNA 4	DNA 10	DNA 17	empty	empty	empty	empty
F	DNA 5	DNA 11	DNA 18	empty	empty	empty	empty
G	DNA 6	DNA 12	DNA 19	empty	empty	empty	empty
H	DNA NTC	DNA 13	DNA 20	empty	empty	empty	empty

Instructions for Use

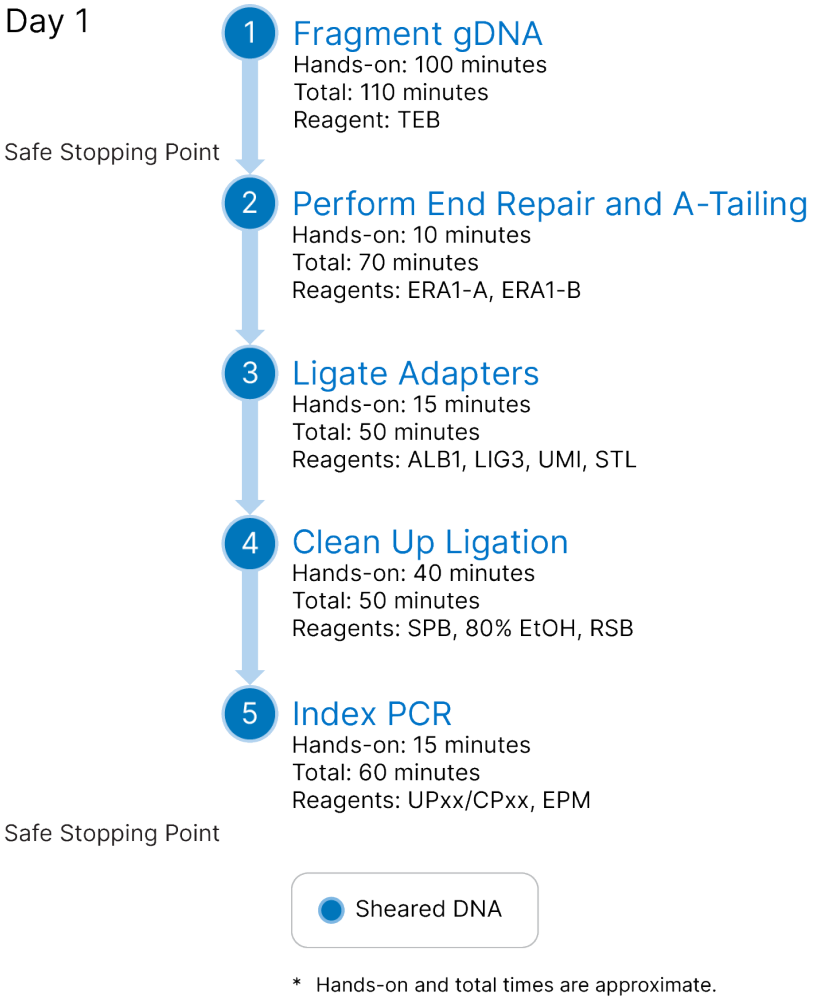
An overview of the TSO Comprehensive HRD (DRAGEN) workflow is shown in Figure 1 and Figure 2.

Library Prep Workflow

Figure 1 illustrates the library prep workflow for TSO Comprehensive HRD (DRAGEN). Positive controls and NTCs are processed identically to samples. Safe stopping points are marked between steps.

Before starting the protocol, enter run and sample information into the Illumina Connected Run TruSight Oncology Comprehensive (EU, DRAGEN) analysis module. Refer to the *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*.

Figure 1 TSO Comprehensive HRD (DRAGEN) Workflow (Part 1)

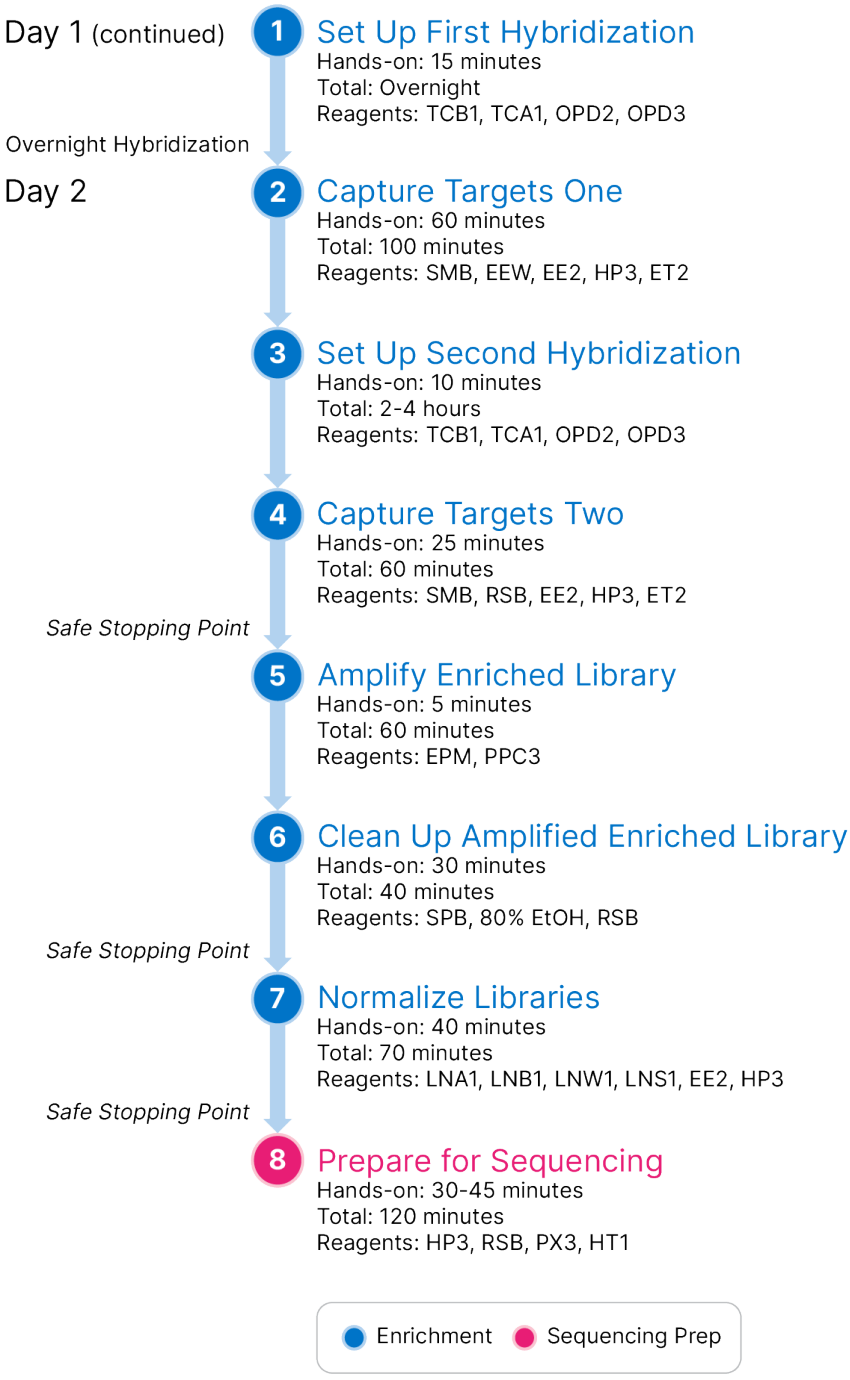


* Hands-on and total times are approximate.

Enrichment Workflow

Figure 2 illustrates the enrichment workflow. Safe stopping points are marked between steps.

Figure 2 TSO Comprehensive HRD (DRAGEN) Workflow (Part 2)



Program Thermal Cyclers

Before starting the assay, save the following programs on post-amplification thermal cyclers.

Table 7 Post-amplification Thermal Cycler Programs

Procedural Step	Program Name	Lid Temperature	Reaction Volume	Thermal Cycler Parameters

Index PCR	I-PCR	100°C	50 µl	• 98°C for 30 seconds
				• 15 cycles of:
				  • 98°C for 10 seconds
				  • 60°C for 30 seconds
				  • 72°C for 30 seconds
				• 72°C for 5 minutes
				• Hold at 10°C

Perform First Hybridization	HYB1	100°C	50 µl	• 95°C for 10 minutes • 85°C for 2 min 30 seconds • 75°C for 2 min 30 seconds • 65°C for 2 min 30 seconds • Hold at 57°C for 8 to 24 hours
Perform Second Hybridization	HYB2	100°C	50 µl	• 95°C for 10 minutes • 85°C for 2 min 30 seconds • 75°C for 2 min 30 seconds • 65°C for 2 min 30 seconds • Hold at 57°C for 1.5 to 4 hours

Amplify Enriched Library	EL-PCR	100°C	50 µl	• 98°C for 30 seconds
				• 18 cycles of:
				  • 98°C for 10 seconds
				  • 60°C for 30 seconds
				  • 72°C for 30 seconds
				• 72°C for 5 minutes
				• Hold at 10°C

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

⚠ CAUTION

All procedures in the workflow require an RNase/DNase-free environment.

1. Thoroughly decontaminate work areas with an RNase/DNase-inhibiting cleaner.
2. Follow manufacturer instructions to set up the ultrasonicator.
3. Remove DNA controls from storage.

4. Remove the reagent tube from the box and follow thaw instructions.

Table 8 TruSight Oncology Comp Library Prep (Refrigerate) (PN 20031119)

Reagent	Storage	Thaw Instructions	Protocol Step
TEB	2°C to 8°C	Bring to room temperature.	Fragment gDNA

Fragment gDNA

This process fragments gDNA and generates dsDNA fragments with 3' or 5' overhangs.

Preparation

1. Follow recommendations in *Nucleic Acid Extraction, Quantification, and Storage* on page 20 to quantify samples.
2. Prepare TEB—Invert or vortex to mix.

Procedure

Prepare the Plate

1. Label a new 96-well MIDI plate LP (Library Preparation).
   If you are stopping at the *SAFE STOPPING POINT* on page 36, use a PCR plate.
2. Cover and set aside for use in *Transfer Fragmented DNA* on page 36.

Dilute gDNA

1. Thaw gDNA samples and DNA controls at room temperature.
2. Pipette each gDNA sample 10 times to mix.
3. Centrifuge tube briefly to collect droplets.
4. Invert or vortex TEB to mix.
5. Use TEB to prepare each gDNA sample in a final volume of 52 µl. Refer to the following table for input amounts and minimum concentrations based on sample type.
   - Assay requires a minimum extraction concentration to allow for at least 40 µl TEB of the 52 µl volume.
   - For DNA control, use 2 µl in a final volume of 52 µl.
   - To prevent sample loss, do not pipette less than 2 µl sample into this dilution.

Sample Type	Input Amount (ng)	Minimum Concentration (ng/µl)
FFPE	40	3.33
Control	40	20

Fragment

1. Add 52 µl of each gDNA sample into a separate well of the ultrasonicator tube.

   ⚠ CAUTION

   Load the gDNA into the tube slowly, making sure that there are no air gaps at the bottom of the tube. For more information, refer to *Assay* on page 23 and manufacturer instructions.

2. Record the orientation of the strip.
3. Fragment gDNA into fragments with the ultrasonicator.

Transfer Fragmented DNA

1. Make sure that sample plate layout and indexes for each sample match the run you select for analysis with the TSO Comprehensive (EU, DRAGEN) analysis module.
2. Follow ultrasonicator manufacturer instructions to recover the sample.
   For some ultrasonicator tube types, centrifugation is required to consolidate the sample in the tube.
3. For each fragmented gDNA sample, use a pipette with fine tips to perform three transfers of 16.7 µl into an empty well of the LP plate.
4. Apply adhesive plate seal to the LP plate.

SAFE STOPPING POINT

If you are stopping, apply an adhesive plate seal to the LP PCR plate and centrifuge at 280 × g for 1 minute. Store at -25°C to -15°C for up to 7 days.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

Make sure that post-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 34.

1. Prepare an ice bucket or equivalent.
2. Remove the reagent tubes from the box and follow thaw instructions.

Table 9 TruSight Oncology Comp Library Prep (Freeze) Box (PN 20031118)

Reagent	Storage	Thaw Instructions	Protocol Step
ERA1-A	-25°C to -15°C	Keep cold.	Perform End Repair and A-Tailing
ERA1-B	-25°C to -15°C	Thaw to room temperature.	Perform End Repair and A-Tailing
ALB1	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters
LIG3	-25°C to -15°C	Keep cold.	Ligate Adapters

Reagent	Storage	Thaw Instructions	Protocol Step
UMI (white cap)	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters
STL	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters
EPM	-25°C to -15°C	Keep cold.	Index PCR

Table 10 TruSight Oncology Comp Library Prep (Refrigerate) Box (PN 20031119)

Reagent	Storage	Thaw Instructions	Protocol Step
SPB (light green label)	2°C to 8°C	Bring to room temperature for 30 minutes.	Clean Up Ligation
RSB	2°C to 8°C	Bring to room temperature.	Clean Up Ligation

Table 11 TruSight Oncology Comp UP Index Primers Box (PN 20031120)

Reagent	Storage	Thaw Instructions	Protocol Step
UPxx	-25°C to -15°C	Thaw the appropriate index primer tubes to room temperature.	Index PCR

Table 12 TruSight Oncology Comp CP Index Primers Box (PN 20031126)

Reagent	Storage	Thaw Instructions	Protocol Step
CPxx	-25°C to -15°C	Thaw the appropriate index primer tubes to room temperature.	Index PCR

Perform End Repair and A-Tailing

This process repairs the overhangs resulting from fragmentation into ends with overhanging A-tails using an End Repair A-Tailing master mix (ERA1).

The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. The 3' ends are A-tailed during this reaction to prevent them from ligating to each other during the adapter ligation reaction.

Preparation

1. Preheat two microsample incubators with MIDI heat block inserts as follows.
   - Preheat a microsample incubator to 30°C.
   - Preheat a microsample incubator to 72°C.
2. Prepare the following reagents.
   - ERA1-A—Centrifuge briefly, and then pipette to mix. Keep cold.

- ERA1-B—Vortex to mix, and then centrifuge briefly.
  Inspect for precipitates. If present, warm the tube to 37°C, and then pipette to mix until precipitates dissolve.

3. Prepare ERA1 master mix in a microcentrifuge tube.

Table 13 ERA1 Master Mix*

Master Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries
ERA1-B	35 µl	69 µl	138 µl	207 µl
ERA1-A	13.5 µl	27 µl	54 µl	81 µl

* This table includes volume overage. Refer to *Handling Reagents* on page 27 for calculations.

4. Pipette slowly 10 times to ensure homogeneity, and then centrifuge briefly. Keep ERA1 master mix cold.
5. To prepare the plate, select one of the following options:
   - If samples are in a MIDI plate, prepare as follows.
     a. Relabel the MIDI plate LP2 (Library Preparation 2).
     b. If some samples are in separate MIDI plates, move all samples to separate wells of the same MIDI plate according to the plate layout.
   - If the plate is frozen, prepare as follows.
     a. Thaw the LP PCR plate to room temperature.
     b. Centrifuge the plate at 280 × g for 1 minute.
     c. Pipette 10 times to mix.
     d. Label a new 96-well MIDI plate LP2 (Library Preparation 2).
     e. Transfer the entire 50 µl each sample from the LP PCR plate to the corresponding well of the LP2 MIDI plate.
     f. Discard LP PCR plate.

Procedure

1. Add 10 µl ERA1 master mix to each sample well in the LP2 MIDI plate.
2. Discard remaining ERA1 master mix.
3. Apply adhesive plate seal to the LP2 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
4. Shake at 1800 rpm for 2 minutes.
5. Incubate in the preheated microsample incubator at 30°C for 30 minutes.
6. Immediately transfer to a second, preheated microsample incubator.
7. Incubate at 72°C for 20 minutes.
8. Keep the LP2 MIDI plate cold for 5 minutes.

Ligate Adapters

This process ligates adapters to the ends of the gDNA fragments.

Preparation

⚠ CAUTION

Only use UMI adapters. SUA1 adapters are provided but are not required for the TSO Comprehensive HRD (DRAGEN) assay.

1. Prepare the following reagents.
   - ALB1—Vortex to mix for a minimum of 10 seconds, and then centrifuge briefly.
   - LIG3—Centrifuge briefly, and then pipette to mix. Keep cold.
   - UMI—Vortex to mix for a minimum of 10 seconds, and then centrifuge briefly.
   - STL—Set aside for use in the procedure.

Procedure

1. Remove the LP2 MIDI plate from ice or equivalent.
2. Add 60 µl ALB1 to each sample well of the LP2 MIDI plate. ALB1 is a viscous solution. Pipette slowly to minimize bubble formation.
3. Add 5 µl LIG3 to each sample well.
4. Add 10 µl UMI (white cap) to each sample well.
5. Apply adhesive plate seal to the LP2 MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 2 minutes.
7. Incubate at room temperature for 30 minutes.
8. Vortex STL to mix, and then centrifuge briefly.
9. Add 5 µl STL to each sample well of the LP2 MIDI plate.
10. Apply adhesive plate seal to the LP2 MIDI plate.
    Seal edges and wells completely to prevent evaporation.
11. Shake at 1800 rpm for 2 minutes.

Clean Up Ligation

This process uses SPB to purify the adapter-ligated gDNA fragments and removes unwanted products. The beads are washed twice with fresh 80% ethanol. The adapter-ligated samples are eluted with RSB.

Preparation

1. Prepare the following reagents.
   - SPB—Make sure that beads are at room temperature for 30 minutes.
   - RSB—Set aside for use in the procedure.
2. Prepare fresh 80% EtOH in a 15 ml or 50 ml conical tube.

   Table 14 Prepare Fresh 80% Ethanol

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries
100% EtOH, pure	2 ml	4 ml	8 ml	12 ml
RNase/DNase-free water	500 µl	1 ml	2 ml	3 ml

3. Vortex fresh 80% EtOH to mix.
4. Set out the magnet.

Procedure

Bind

1. Vortex SPB for 1 minute to resuspend beads.
2. Immediately add 112 µl SPB to each sample well in the LP2 MIDI plate.
   If using a trough to dispense SPB, include a 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SPB has been added to each sample well.
3. Apply adhesive plate seal to the LP2 MIDI plate.
   Seal edges and wells completely.
4. Shake at 1800 rpm for 2 minutes.
5. Incubate at room temperature for 5 minutes.
6. Place the LP2 MIDI plate on the magnetic stand for 10 minutes.
7. Without disturbing the bead pellet, use a pipette set at 200 µl to remove and discard all supernatant from each sample well.

Wash

1. Wash beads as follows.
   a. Keep the LP2 MIDI plate on the magnetic stand and add 200 µl fresh 80% EtOH to each sample well.
   b. Wait 30 seconds.
   c. Without disturbing the bead pellet, use a pipette set at 200 µl to remove and discard all supernatant from each sample well.
2. Wash beads a **second** time.
3. Use a pipette with fine tips to remove residual EtOH from each well.
4. Discard unused 80% EtOH.

Elute

1. Remove the LP2 MIDI plate from the magnetic stand.
2. Invert or vortex RSB to mix.
3. Add 27.5 µl RSB to each sample well.
4. Apply adhesive plate seal to the LP2 MIDI plate.
   Seal edges and wells completely.
5. Shake at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 2 minutes.
7. Place the LP2 MIDI plate on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate LS (Library Samples).
9. Transfer 25 µl each eluate from the LP2 MIDI plate to the corresponding well of the LS PCR plate.
10. Discard the empty LP2 MIDI plate.

Index PCR

In this step, library fragments are amplified using primers that add index sequences for sample multiplexing. The resulting product contains the complete library of DNA fragments flanked by adapters required for cluster generation.

Preparation

1. Prepare the following reagents.
   - EPM—Keep cold.
   - UPxx—Vortex to mix and centrifuge briefly.
   - CPxx—Vortex to mix and centrifuge briefly.

2. Make sure that indexes for each sample match the run planned on the TSO Comprehensive (EU, DRAGEN) analysis module during run setup. Make sure to follow instructions regarding index selection in *Number of Libraries and Selecting Indexes* on page 29.

   ⚠ CAUTION

   Mismatches between the samples and indexing primers cause incorrect result reporting due to loss of positive sample identification.

Procedure

1. Add 5 µl of the appropriate index primer (UPxx or CPxx) to the corresponding sample well in the LS PCR plate according to the indexes selected.

   ⚠ CAUTION

   Handle and open only one index primer tube at a time. Recap each index tube with a new cap immediately after use. Do not combine index primers together.

2. Vortex EPM to mix for 5 seconds, and then centrifuge briefly.
3. Add 20 µl EPM to each sample well.
4. Apply adhesive plate seal to the LS PCR plate.
   Seal edges and wells completely to prevent evaporation.
5. Shake at 1200 rpm for 1 minute.
6. Return pre-amplification reagents to storage.

   ⚠ CAUTION

   Perform all subsequent steps in a post-amplification area to prevent amplification product carryover.

7. Centrifuge the LS PCR plate at 280 × g for 1 minute.
8. Place on the preprogrammed post-amplification thermal cycler and run the I-PCR program.
   Refer to *Program Thermal Cyclers* on page 34.
   If continuing with *Set Up First Hybridization* on page 43, follow the thaw instructions for reagents in the Prepare Protocol Steps.
9. After the I-PCR program completes, centrifuge the LS PCR plate at 280 × g for 1 minute.
10. Relabel the plate ALS (Amplified Library Samples).

SAFE STOPPING POINT

If you are stopping, store ALS PCR plate at -25°C to -15°C for up to 30 days.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the overnight hybridization.

1. Make sure that post-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 34.
2. Remove the reagent tubes from the box and follow thaw instructions.

Table 15 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123) in addition to TruSight Oncology Comp HRD Enrichment (Refrigerate) Box (PN 20140116)

Reagent	Storage	Thaw Instructions	Protocol Step
TCB1	2°C to 8°C	Bring to room temperature.	Set Up First Hybridization

Table 16 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121) in addition to TruSight Oncology Comp HRD Enrichment (Freeze) Box (PN 20140115)

Reagent	Storage	Thaw Instructions	Protocol Step
TCA1	-25°C to -15°C	Thaw to room temperature.	Set Up First Hybridization

Table 17 TruSight Oncology Comp Content Set Box (PN 20031122)

Reagent	Storage	Thaw Instructions	Protocol Step
OPD2 (white cap)	-25°C to -15°C	Thaw to room temperature.	Set Up First Hybridization

Table 18 TruSight Oncology Comp HRD Content Set Box (PN 20140117)

Reagent	Storage	Thaw Instructions	Protocol Step
OPD3 (blue cap)	-25°C to -15°C	Thaw to room temperature.	Set Up First Hybridization

Set Up First Hybridization

During this process, two pools of oligos (OPD2 and OPD3) hybridize to gDNA libraries prepared in *Index PCR* on page 41. Enrichment of targeted regions requires two hybridization steps. In the first hybridization, oligos hybridize to gDNA libraries overnight (8 hours to 24 hours).

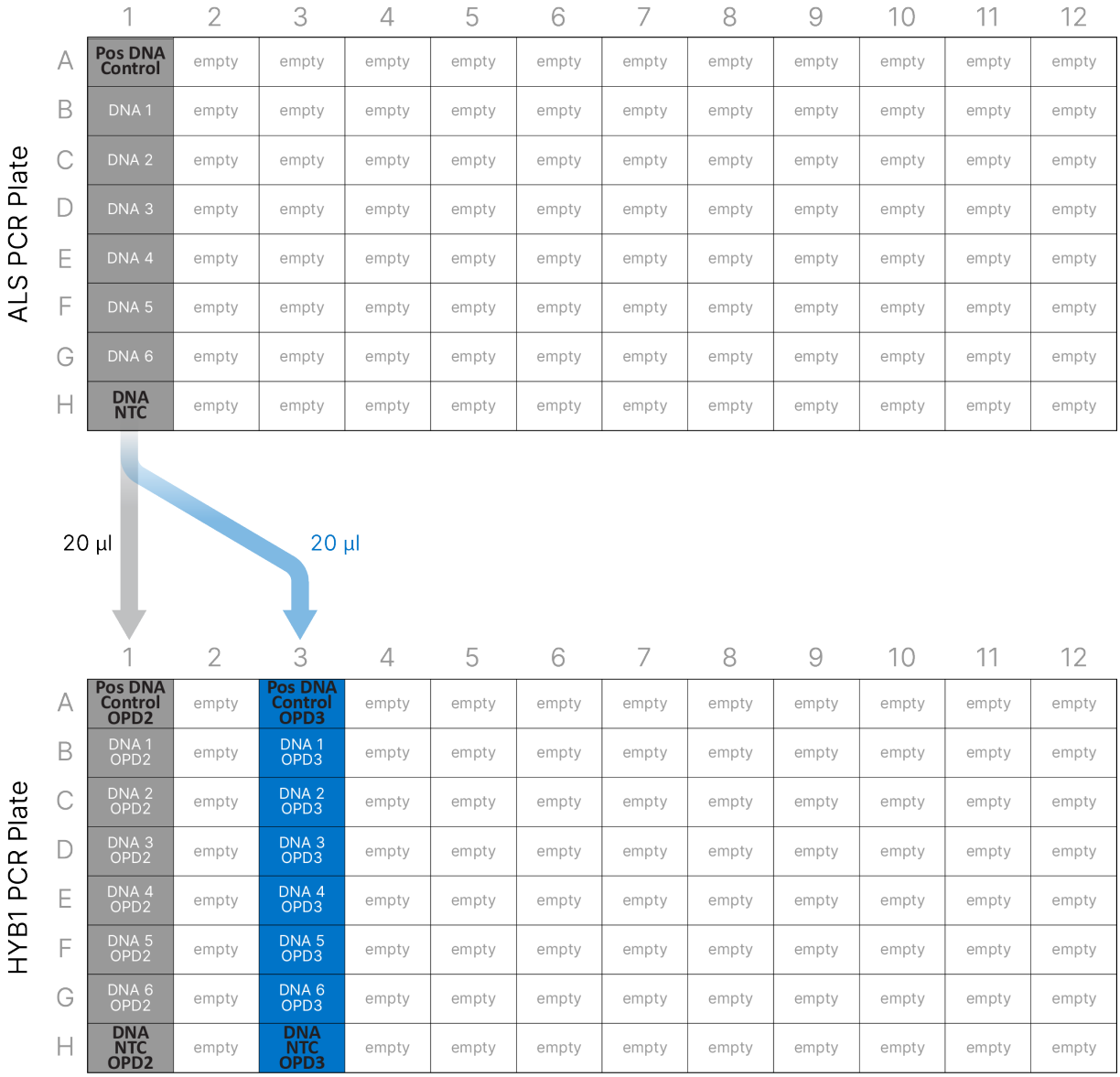
Preparation

1. Prepare the following reagents.
   - TCB1—Warm the tube at 37°C for 5 minutes. Vortex to mix for 10 seconds, and then centrifuge briefly.
   - TCA1—Vortex to mix, and then centrifuge briefly.
   - OPD2—Vortex to mix, and then centrifuge briefly.
   - OPD3—Vortex to mix, and then centrifuge briefly.
2. If the ALS PCR plate was stored, thaw to room temperature and centrifuge at 280 × g for 1 minute. Pipette to mix.
3. Label a new 96-well PCR plate HYB1 (Hybridization 1).

Procedure

1. Transfer 20 µl each gDNA library from the ALS PCR plate to the corresponding well in the HYB1 PCR plate for enrichment with OPD2 probes. Include positive controls and NTCs for enrichment with OPD2 probes.
2. Transfer an additional 20 µl gDNA library from the ALS PCR plate to a second well in the HYB1 PCR plate for enrichment with OPD3 probes, as shown in Figure 3. Include positive controls and NTCs for enrichment with OPD3 probes.

Figure 3 TSO Comprehensive HRD (DRAGEN) First Hybridization Example Plate Layout



3. Apply adhesive plate seal to the ALS PCR plate and set aside.
   Seal edges and wells completely to prevent evaporation.
4. Inspect TCB1 for precipitates. If present, warm the tube again and vortex the tube until the crystals dissolve.

5. Add 15 µl TCB1 to each library well in the HYB1 PCR plate.
6. Add 10 µl TCA1 to each library well in the HYB1 PCR plate.
7. Add probes.
   Do ***not*** combine different types of probes together. Add only one probe set per well.
   - **[DNA library wells]**—Add 5 µl OPD2 (white cap) to each library derived from DNA.
   - **[DNA HRD library wells]**—Add 5 µl OPD3 (blue cap) to each library derived from DNA for HRD enrichment.
8. Apply adhesive plate seal to the HYB1 PCR plate.
   Seal edges and wells completely to prevent evaporation.
9. Shake at 1200 rpm for 2 minutes.
10. Place on the thermal cycler and run the HYB1 program.
    Refer to *Program Thermal Cyclers* on page 34.
11. Hybridize at 57°C for a minimum of 8 hours to a maximum of 24 hours.
12. Return hybridization reagents to storage.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

At the beginning of day 2, remove the reagent tubes from the box and follow thaw instructions.

Table 19 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123) in addition to TruSight Oncology Comp HRD Enrichment (Refrigerate) Box (PN 20140116)

Reagent	Storage	Thaw Instructions	Protocol Step
SMB (dark blue label)	2°C to 8°C	Bring to room temperature for 30 minutes.	Capture Targets One Capture Targets Two
ET2	2°C to 8°C	Bring to room temperature.	Capture Targets One Capture Targets Two
HP3	2°C to 8°C	Bring to room temperature.	Capture Targets One Capture Targets Two Normalize Libraries
TCB1	2°C to 8°C	Bring to room temperature.	Set Up Second Hybridization
RSB	2°C to 8°C	Bring to room temperature.	Capture Targets Two Clean Up Amplified Enriched Library

Table 20 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121) in addition to TruSight Oncology Comp HRD Enrichment (Freeze) Box (PN 20140115)

Reagent	Storage	Thaw Instructions	Protocol Step
EE2	-25°C to -15°C	Thaw to room temperature.	Capture Targets One Capture Targets Two Normalize Libraries
EEW	-25°C to -15°C	Thaw to room temperature.	Capture Targets One
TCA1	-25°C to -15°C	Thaw to room temperature.	Set Up Second Hybridization

Table 21 TruSight Oncology Comp Content Set Box (PN 20031122)

Reagent	Storage	Thaw Instructions	Protocol Step
OPD2 (white cap)	-25°C to -15°C	Thaw to room temperature.	Set Up Second Hybridization

Table 22 TruSight Oncology Comp HRD Content Box (PN 20140117)

Reagent	Storage	Thaw Instructions	Protocol Step
OPD3 (blue cap)	-25°C to -15°C	Thaw to room temperature.	Set Up Second Hybridization

Capture Targets One

This step uses SMB to capture probes hybridized to the targeted regions of interest. The beads are washed three times with EEW. The enriched libraries are eluted using fresh EE2 + HP3 elution mix and neutralized with ET2.

Preparation

1. Preheat a microsample incubator with a MIDI heat block insert to 57°C.
2. Prepare the following reagents.
   - EEW—Vortex to mix for 1 minute.
   - EE2—Vortex to mix, and then centrifuge briefly.
   - HP3—Vortex to mix, and then centrifuge briefly.
   - SMB—Make sure that beads are at room temperature for 30 minutes.
     Make sure to use **SMB**, not SPB, for this procedure.
   - ET2—Set aside for use in the procedure.
3. Prepare fresh EE2 + HP3 elution mix in a microcentrifuge tube.

Table 23 EE2 + HP3 Elution Mix for Capture Targets One*

Elution Mix Component	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	228 µl	456 µl	684 µl	1368 µl
HP3	12 µl	24 µl	36 µl	72 µl

* This table includes volume overage. Refer to *Handling Reagents* on page 27 for calculations.

4. Vortex EE2 + HP3 elution mix, and then centrifuge briefly. Set aside for the *Elute* on page 48 step.
5. Label a new 96-well MIDI plate CAP1 (Capture 1).
6. Set out the magnet.

Procedure

Bind

1. Remove the HYB1 PCR plate from the thermal cycler.
2. Centrifuge the HYB1 PCR plate at 280 × g for 1 minute.
3. Vortex SMB for 1 minute to resuspend beads.
   If precipitate or the bead pellet is present, make sure to reach room temperature, pipette up and down to release the pellet, and then vortex to resuspend.
4. Immediately add 150 µl SMB to each library well of the CAP1 MIDI plate.
   If using a trough to dispense SMB, include a 1.05 overage factor when aliquoting to allow sufficient material per sample.
5. After adding SMB to each sample well, discard any remaining material.
6. Set pipette to 50 µl and transfer entire volume of each library from the HYB1 PCR plate to the corresponding well in the CAP1 MIDI plate.
7. Discard the empty HYB1 PCR plate.
8. Apply adhesive plate seal to the CAP1 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
9. Shake at 1800 rpm for 2 minutes.
10. Incubate in the preheated microsample incubator at 57°C for 25 minutes.
11. Place the CAP1 MIDI plate on a magnetic stand for 2 minutes.
12. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

⚠ CAUTION

Proceed immediately to the next step (*Wash* on page 48). Do not allow the bead pellet to sit for an extended amount of time without liquid present.

Wash

1. Wash beads as follows.
   a. Remove the CAP1 MIDI plate from the magnetic stand.
   b. Add 200 µl EEW to each well.
   c. Use a pipette set to 150 µl and pipette a minimum of 10 times to mix. Make sure that all beads are resuspended.
      Make sure that no bead pellets are present by carefully aspirating total bead solution of well into the tip. Visually inspect the bottom of each well. If a bead pellet is present, angle pipette tip towards pellet during wash steps to dislodge pellet. Make sure that the bead pellet is fully immersed in solution. The solution should look dark brown and have a homogenous consistency.
   d. Apply adhesive plate seal to the CAP1 MIDI plate.
      Seal edges and wells completely to prevent evaporation.
   e. Shake at 1800 rpm for 4 minutes.
   f. Incubate in a microsample incubator at 57°C for 5 minutes.
   g. Place the CAP1 MIDI plate on a magnetic stand for 2 minutes.
   h. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.
2. Wash beads a **second** time.
3. Wash beads a **third** time.
4. Use a pipette with fine tips to remove residual EEW from each well.

Elute

1. Remove the CAP1 MIDI plate from the magnetic stand.
2. Vortex fresh EE2 + HP3 Elution Mix, and then centrifuge briefly.
3. Carefully add 17 µl EE2 + HP3 Elution Mix to each library well in the CAP1 MIDI plate.
4. Discard remaining EE2 + HP3 Elution Mix.
5. Apply adhesive plate seal to the CAP1 MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 2 minutes.
7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate ELU1 (Elution 1).
9. Vortex ET2 to mix, and then centrifuge briefly.
10. Add 5 µl ET2 to each corresponding library well in the new ELU1 PCR plate.
11. Carefully transfer 15 µl eluate from each library well of the CAP1 MIDI plate to the corresponding well in the ELU1 PCR plate.
12. Discard empty CAP1 MIDI plate.

13. Apply adhesive plate seal to the ELU1 PCR plate.
    Seal edges and wells completely to prevent evaporation.
14. Shake at 1200 rpm for 2 minutes.
15. Return EEW to storage.

Set Up Second Hybridization

This step binds targeted regions of the enriched gDNA libraries with capture probes a second time. The second hybridization ensures high specificity of the captured regions. To ensure optimal enrichment of libraries, perform the second hybridization step at 57°C for a minimum of 1.5 hours to a maximum of 4 hours.

Preparation

1. Prepare the following reagents.
   - TCB1—Warm the tube at 37°C for 5 minutes. Vortex to mix for 10 seconds, and then centrifuge briefly.
   - TCA1—Vortex to mix, and then centrifuge briefly.
   - OPD2—Vortex to mix, and then centrifuge briefly.
   - OPD3—Vortex to mix, and then centrifuge briefly.

Procedure

1. Inspect TCB1 for precipitates. If present, warm the tube again and vortex until crystals dissolve.
2. Add 15 µl TCB1 to each library well in the ELU1 PCR plate.
3. Add 10 µl TCA1 to each library well.
4. Add the same probe used during the first hybridization to each well. Add only one probe set per well.
   Do *not* combine different types of probes together.
   - **[DNA library wells]**—Add 5 µl OPD2 (white cap) to each library derived from DNA.
   - **[DNA HRD library wells]**—Add 5 µl OPD3 (blue cap) to each library derived from DNA for HRD enrichment.
5. Apply adhesive plate seal to the ELU1 PCR plate.
   Seal edges and wells completely to prevent evaporation.
6. Shake at 1200 rpm for 2 minutes.
7. Place on a thermal cycler and run the HYB2 program.
   Refer to *Program Thermal Cyclers* on page 34.
8. Hybridize at 57°C for a minimum of 1.5 hours to a maximum of 4 hours.
9. Return hybridization reagents to storage.

Capture Targets Two

This step uses SMB to capture probes hybridized to the targeted regions of interest. The beads are washed one time with RSB. The enriched libraries are eluted using fresh EE2 + HP3 elution mix and neutralized with ET2.

Preparation

1. Preheat a microsample incubator with MIDI heat block insert to 57°C.
2. Prepare the following reagents.
   - EE2—Vortex to mix, and then centrifuge briefly.
   - HP3—Vortex to mix, and then centrifuge briefly.
   - SMB—Make sure that beads are at room temperature for 30 minutes.
     Make sure to use **SMB**, not SPB, for this procedure.
   - RSB—Set aside for use in the procedure.
   - ET2—Set aside for use in the procedure.
3. Prepare fresh EE2 + HP3 elution mix in a microcentrifuge tube.

   Table 24 EE2 + HP3 Elution Mix for Capture Targets Two*

Elution Mix Component	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	228 µl	456 µl	684 µl	1368 µl
HP3	12 µl	24 µl	36 µl	72 µl

   * This table includes volume overage. Refer to *Handling Reagents* on page 27 for calculations.
4. Vortex to mix, and then centrifuge briefly. Set aside for the *Elute* on page 51 step.
5. Label a new 96-well MIDI plate CAP2 (Capture 2).
6. Set out the magnet.

Procedure

Bind

1. Remove the ELU1 PCR plate from the thermal cycler.
2. Centrifuge ELU1 PCR plate at 280 × g for 1 minute.
3. Vortex SMB for 1 minute to resuspend beads.
4. Immediately add 150 µl SMB to each library well of the CAP2 MIDI plate.
   If using a trough to dispense SMB, include a 1.05 overage factor when aliquoting to allow sufficient material per sample.
5. After adding SMB to each sample well, discard any remaining material.
6. Set pipette to 50 µl and transfer entire volume of each library from the ELU1 PCR plate to the corresponding well of the CAP2 MIDI plate.

7. Discard the empty ELU1 PCR plate.
8. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
9. Shake at 1800 rpm for 2 minutes.
10. Incubate in a microsample incubator at 57°C for 25 minutes.
    If continuing with *Amplify Enriched Library* on page 52, follow thaw instructions for reagents in the Prepare for Protocol section.
11. Place on a magnetic stand for 2 minutes.
12. Keep the CAP2 MIDI plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

    ⚠ CAUTION

    Proceed immediately to the next step (*Wash* on page 51). Do not allow the bead pellet to sit for an extended amount of time without liquid present.

Wash

1. Remove the CAP2 MIDI plate from the magnetic stand.
2. Invert or vortex RSB to mix.
3. Add 200 µl RSB to each well.
4. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely.
5. Shake at 1800 rpm for 4 minutes.
6. Place the plate on the magnetic stand for 2 minutes.
7. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.
8. Use a pipette with fine tips to remove residual RSB from each well.

Elute

1. Remove the CAP2 MIDI plate from the magnetic stand.
2. Vortex fresh EE2 + HP3 Elution Mix, and then centrifuge briefly.
3. Add 22 µl EE2 + HP3 Elution Mix to each library well in the CAP2 MIDI plate.
4. Discard remaining EE2 + HP3 Elution Mix.
5. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 2 minutes.
7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate ELU2 (Elution 2).

9. Vortex ET2 to mix, and then centrifuge briefly.
10. Add 5 µl ET2 to each corresponding library well in the new ELU2 PCR plate.
11. Carefully transfer 20 µl eluate from each library well of the CAP2 MIDI plate to the corresponding well in the ELU2 PCR plate.
12. Discard empty CAP2 MIDI plate.
13. Apply adhesive plate seal to the ELU2 PCR plate.
    Seal edges and wells completely to prevent evaporation.
14. Shake at 1200 rpm for 2 minutes.
15. Return SMB, EE2, HP3, RSB, and ET2 to storage.

SAFE STOPPING POINT

If you are stopping, centrifuge ELU2 PCR plate at 280 × g for 1 minute and store at -25°C to -15°C for up to 7 days.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Prepare an ice bucket or equivalent.
2. Remove the reagent tubes from the box and follow thaw instructions.

Table 25 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121) in addition to TruSight Oncology Comp HRD Enrichment (Freeze) Box (PN 20140115)

Reagent	Storage	Thaw Instructions	Protocol Step
PPC3	-25°C to -15°C	Thaw to room temperature.	Amplify Enriched Library
EPM	-25°C to -15°C	Keep cold.	Amplify Enriched Library

Table 26 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123) in addition to TruSight Oncology Comp HRD Enrichment (Refrigerate) Box (PN 20140116)

Reagent	Storage	Thaw Instructions	Protocol Step
SPB (light green label)	2°C to 8°C	Bring to room temperature for 30 minutes.	Clean Up Amplified Enriched Library
RSB	2°C to 8°C	Bring to room temperature.	Clean Up Amplified Enriched Library Prepare for Sequencing

Amplify Enriched Library

This step uses primers to amplify enriched libraries.

Preparation

1. If the ELU2 plate was stored, thaw to room temperature and then centrifuge at 280 × g for 1 minute.

Procedure

1. Vortex PPC3 to mix, and then centrifuge briefly.
2. Add 5 µl PPC3 to each library well of the ELU2 PCR plate.
3. Vortex EPM to mix for 5 seconds, and then centrifuge briefly.
4. Add 20 µl EPM to each library well.
5. Apply adhesive plate seal to the ELU2 PCR plate.
   Seal edges and wells completely to prevent evaporation.
6. Shake at 1200 rpm for 2 minutes.
7. Place on a thermal cycler and run the EL-PCR program.
   Refer to *Program Thermal Cyclers* on page 34.
   If continuing with *Normalize Libraries* on page 56, follow the thaw instructions in the Prepare for Protocol section.
8. Return PPC3 and EPM to storage.

Clean Up Amplified Enriched Library

This step uses SPB to purify the enriched libraries from unwanted reaction components. The beads are washed twice with fresh 80% ethanol. The libraries are eluted with RSB.

Preparation

1. Prepare the following reagents.
   - SPB—Make sure that beads are at room temperature for 30 minutes. Make sure to use ***SPB***, not SMB, for this procedure.
   - RSB—Set aside for use in the procedure.
2. Prepare fresh 80% ethanol in a 15 ml or 50 ml conical tube.

   Table 27 Prepare Fresh 80% Ethanol

Reagent	8 Libraries	16 Libraries	24 Libraries	48 Libraries
100% EtOH, pure	4 ml	8 ml	12 ml	24 ml
RNase/DNase-free water	1 ml	2 ml	3 ml	6 ml

3. Vortex fresh 80% EtOH to mix.
4. Label a new 96-well MIDI plate BIND2 (Clean Up Binding).
5. Set out the magnet.

Procedure

Bind

1. Remove the ELU2 PCR plate from the thermal cycler.
2. Centrifuge the ELU2 PCR plate at 280 × g for 1 minute.
3. Vortex SPB for 1 minute to resuspend the beads.
4. Immediately add 110 µl SPB to each library well of the BIND2 MIDI plate.
   If using a trough to dispense SPB, include a 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SPB has been added to each sample well.
5. Transfer 50 µl each library from the ELU2 PCR plate to the corresponding well of the BIND2 MIDI plate.
6. Discard empty ELU2 PCR plate.
7. Apply adhesive plate seal to the BIND2 MIDI plate.
   Seal edges and wells completely.
8. Shake at 1800 rpm for 2 minutes.
9. Incubate at room temperature for 5 minutes.
10. Place the BIND2 MIDI plate on magnetic stand for 5 minutes.
11. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

Wash

1. Wash beads as follows.
   a. Keep the BIND2 MIDI plate on magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.
2. Wash beads a **second** time.
3. Use a pipette with fine tips to remove residual EtOH from each well.
4. Discard unused 80% EtOH.

Elute

1. Remove the BIND2 MIDI plate from the magnetic stand.
2. Invert or vortex to mix RSB.
3. Add 32 µl RSB to each library well.
4. Apply adhesive plate seal to the BIND2 MIDI plate.
   Seal edges and wells completely.
5. Shake at 1800 rpm for 2 minutes.

6. Incubate at room temperature for 2 minutes.
7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate PL (Purified Libraries).
9. Transfer 30 µl each eluate from the BIND2 MIDI plate to the corresponding well of the PL PCR plate.
10. Discard the empty BIND2 MIDI plate.
11. Apply adhesive plate seal to the PL PCR plate.
12. Return SPB and RSB to storage.

SAFE STOPPING POINT

If you are stopping, centrifuge the PL PCR plate at 280 × g for 1 minute and store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagent tubes from the box and follow thaw instructions.

Table 28 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121) in addition to TruSight Oncology Comp HRD Enrichment (Freeze) Box (PN 20140115)

Reagent	Storage	Thaw Instructions	Protocol Step
LNA1	-25°C to -15°C	Thaw to room temperature.	Normalize Libraries
EE2	-25°C to -15°C	Thaw to room temperature.	Normalize Libraries

Table 29 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123) in addition to TruSight Oncology Comp HRD Enrichment (Refrigerate) Box (PN 20140116)

Reagent	Storage	Thaw Instructions	Protocol Step
LNB1	2°C to 8°C	Bring to room temperature for 30 minutes.	Normalize Libraries
HP3	2°C to 8°C	Bring to room temperature.	Normalize Libraries Prepare for Sequencing
LNW1	2°C to 8°C	Bring to room temperature.	Normalize Libraries
LNS1	2°C to 8°C	Bring to room temperature.	Normalize Libraries

2. If you are continuing the same day with *Prepare for Sequencing* on page 59, follow the thaw instructions in the Prepare for Protocol section.

Normalize Libraries

This process uses LNB1 plus additives (LNA1) to normalize the quantity of each library to ensure a uniform library representation in the pooled libraries. The beads are washed twice with LNW1. The libraries are eluted with fresh EE2 + HP3 elution mix and neutralized with LNS1.

Preparation

1. Prepare the following reagents.
   - LNB1—Make sure that the beads are at room temperature for 30 minutes.
   - LNA1—Vortex to mix.
   - EE2—Vortex to mix, and then centrifuge briefly.
   - HP3—Vortex to mix, and then centrifuge briefly.
   - LNW1—Vortex to mix. Set aside for use in procedure.
   - LNS1—Vortex to mix. Set aside for use in the procedure.
2. Pulse vortex LNB1 for 1 minute to resuspend beads.
   Invert LNB1 tube to make sure that all beads are resuspended.
3. Repeat pulse vortexing for 1 minute if a pellet is observed.
4. Use a pipette set at 800 µl to pipette LNB1 up and down 10 times to ensure resuspension.
5. Immediately prepare fresh LNA1 + LNB1 Master Mix in a conical tube.

   ⚠ CAUTION

   Completely resuspend the LNB1 bead pellet at the bottom of the tube to prevent inconsistent cluster density.

   Table 30 LNA1 + LNB1 Master Mix*

Master Mix Component	8 Libraries	16 Libraries	24 Libraries	48 Libraries
LNA1	610 µl	1219 µl	1829 µl	3658 µl
LNB1	110 µl	221 µl	331 µl	662 µl

   * This table includes volume overage. Refer to *Handling Reagents* on page 27 for calculations.
6. Vortex LNA1 + LNB1 master mix. Set aside for the *Bind* on page 57 step.
7. Prepare fresh EE2 + HP3 Elution Mix in a microcentrifuge tube.

   Table 31 EE2 + HP3 Elution Mix for Normalize Libraries*

Elution Mix Component	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	304 µl	608 µl	912 µl	1824 µl
HP3	16 µl	32 µl	48 µl	96 µl

   * This table includes volume overage. Refer to *Handling Reagents* on page 27 for calculations.

8. Vortex fresh elution mix, and then centrifuge briefly. Set aside for the *Elute* on page 58 step.
9. If the PL PCR plate was stored, thaw to room temperature, centrifuge at 280 × g for 1 minute. Pipette to mix.
10. Label a new 96-well MIDI plate BBN (Bead-Based Normalization).
11. Set out the magnet.

Procedure

Bind

1. Vortex LNA1 + LNB1 master mix.
2. Immediately add 45 µl LNA1 + LNB1 Master Mix to each library well of the BBN MIDI plate.
3. Discard remaining LNA1 + LNB1 master mix.
4. Add 20 µl of each library from the PL PCR plate to the corresponding well of the BBN MIDI plate.
5. Apply adhesive plate seal to the BBN MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 30 minutes.
7. Apply adhesive plate seal to the PL PCR plate and return to storage.
8. Place the BBN MIDI plate on a magnetic stand for 2 minutes.
9. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

Wash

1. Wash beads as follows.
   a. Remove the BBN MIDI plate from the magnetic stand.
   b. Add 45 µl LNW1 to each library well.
   c. Apply adhesive plate seal to the BBN MIDI plate.
      Seal edges and wells completely.
   d. Shake at 1800 rpm for 5 minutes.
   e. Place the BBN MIDI plate on a magnetic stand for 2 minutes.
   f. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.
2. Wash beads a ***second*** time.
3. Use a pipette with fine tips to remove residual supernatant from each well.

Elute

1. Remove the BBN MIDI plate from the magnetic stand.
2. Vortex fresh EE2 + HP3 Elution Mix, and then centrifuge briefly.
3. Add 32 µl EE2 + HP3 solution to each library well of the BBN MIDI plate.
4. Discard remaining elution mix.
5. Apply adhesive plate seal to the BBN MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 2 minutes.
7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate NL (Normalized Libraries).
9. Carefully transfer 30 µl eluate from each library well of the BBN MIDI plate to the corresponding well of the NL PCR plate.

   ⚠ CAUTION

   If beads are aspirated into the pipette tips, dispense the beads back onto the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes) before proceeding to the next step of the procedure.

10. Discard the empty BBN MIDI plate.
11. Vortex LNS1 to mix.
12. Add 30 µl LNS1 to each library well in the new NL PCR plate.
13. Pipette to mix five times.
14. Apply adhesive plate seal to the NL PCR plate.
    Seal edges and wells completely.
15. Return LNB1, LNA1, EE2, LNW1, and LNS1 to storage.

SAFE STOPPING POINT

If you are stopping, centrifuge NL PCR plate at 280 × g for 1 minute and store at -25°C to -15°C for up to 32 days.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to sequencing.

Start the preparation of sequencing consumables from the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) (PN 20028871) at least an hour before use.

1. Remove Library Dilution Buffer (HT1) from -25°C to -15°C storage. Thaw to room temperature. After thawing, keep cold.
2. Follow preparation instructions in the *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)* for other consumables in the kit.

- NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles)
- NextSeq 550Dx Buffer Cartridge v2 (300 cycles)
- NextSeq 550Dx High Output Flow Cell Cartridge v2.5 (300 cycles)

3. Remove the reagent tubes from the box and follow thaw instructions.

Table 32 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

Reagent	Storage	Thaw Instructions	Protocol Step
PhiX Control	-25°C to -15°C	Thaw to room temperature. After thawing, keep cold.	Prepare for Sequencing

Table 33 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123) in addition to TruSight Oncology Comp HRD Enrichment (Refrigerate) Box (PN 20140116)

Reagent	Storage	Thaw Instructions	Protocol Step
HP3	2°C to 8°C	Bring to room temperature.	Prepare for Sequencing
RSB (pink label)	2°C to 8°C	Bring to room temperature.	Prepare for Sequencing

Prepare for Sequencing

Each DNA sequencing run should include a positive control and an NTC. The NTC is sequenced repeatedly as needed so that each run contains an NTC. Each run includes a separate positive control.

Preparation

1. Review the guidelines for *Number of Libraries and Selecting Indexes* on page 29.
2. Label a microcentrifuge tube dHP3 (diluted HP3).
3. Label a microcentrifuge tube dPhiX (diluted PhiX).
4. Preheat a heat block to 96°C for microcentrifuge tubes.
5. Prepare an ice bucket or equivalent.

Dilute and Denature PhiX Control

1. Vortex HP3 to mix, and then centrifuge briefly.
2. Combine the following volumes in the dHP3 microcentrifuge tube.
   - 10 µl HP3
   - 190 µl RNase/DNase-free water
3. Vortex dHP3 to mix, and then centrifuge briefly.
4. Invert or vortex RSB to mix.
5. Vortex PhiX control to mix, and then centrifuge briefly.

6. Combine the following volumes in the dPhiX microcentrifuge tube.
   - 8 µl RSB
   - 2 µl PhiX control
7. Add 10 µl dHP3 to the dPhiX tube.
8. Discard the dHP3 tube.
9. Vortex the dPhiX tube to mix, and then centrifuge briefly.
10. Incubate dPhiX at room temperature for 5 minutes to denature.
11. Vortex HT1 to mix.
12. Immediately add 980 µl prechilled HT1 to dPhiX.
13. Vortex to mix, and then centrifuge briefly.
14. Keep dPhiX cold until use in the preparation for the second dilution.
    The final concentration is 20 pM dPhiX.
15. Return PhiX, HP3, and RSB to storage.

Pool and Denature Libraries

1. If the NL PCR plate was stored, thaw to room temperature, and then centrifuge the plate at 280 × g for 1 minute.
2. Using a multichannel pipette set at 30 µl, gently pipette-mix the libraries in the NL PCR plate 5 times.
   Use fresh tips for each library.

   ⚠ CAUTION

   Make sure to mix libraries well for optimal performance.
3. Label a microcentrifuge tube PDL (Pooled DNA Libraries).
4. Label a microcentrifuge tube PHL (Pooled HRD Libraries).
5. Transfer 10 µl of each normalized OPD2 enriched DNA library from the NL plate to the PDL tube.
   Do not pool two libraries with the same index primer.
6. Transfer 10 µl of each normalized OPD3 enriched DNA HRD library from the NL plate to the PHL tube.
   Do not pool two libraries with the same index primer.
7. Apply adhesive plate seal to the NL PCR plate.
   Seal edges and wells completely.
8. Vortex PDL and PHL tubes to mix.
9. Centrifuge PDL and PHL tubes briefly.
10. Incubate PDL and PHL tubes in a heat block at 96°C for 2 minutes.
11. Place PDL and PHL tubes on ice for 5 minutes.
12. Vortex PDL and PHL tubes to mix, and then centrifuge briefly.
13. Return PDL and PHL tubes to ice.

Prepare First Dilution

1. Label a microcentrifuge tube DIL1 (Dilution 1).
2. Transfer 20 µl PDL to the empty DIL1 tube.
3. Discard the PDL tube.
4. Add 5 µl PHL to DIL1.
5. Add 475 µl prechilled HT1 to the DIL1 tube (1:20 dilution).
6. Vortex DIL1 tube to mix, and then centrifuge briefly.
7. Discard the PHL tube.
8. **[Troubleshooting]** After sequencing, if run-level or systemic library QC failures associated with bead-based normalization are observed, proceed to *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 61.

Prepare Second Dilution

1. Label a 2.0 ml microcentrifuge tube DIL2 (Dilution 2).
2. Transfer 40 µl DIL1 to the empty DIL2 tube.
3. Discard the DIL1 tube.
4. Add 1660 µl prechilled HT1 to the DIL2 tube (1:850 dilution).
5. Vortex prepared 20 pM dPhiX to mix, and then centrifuge briefly.
6. Add 2.5 µl prepared 20 pM dPhiX to the DIL2 tube.
7. Vortex to mix, and then centrifuge briefly.
8. Load 1300 µl DIL2 to the thawed NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles).
   For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.
9. Discard the DIL2 tube.
10. Centrifuge NL PCR plate at 280 × g for 1 minute, and store at -25°C to -15°C for up to 32 days.
11. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Troubleshooting Workflow: Prepare Adjusted Second Dilution

Use this troubleshooting workflow when, after sequencing, run-level or systemic library QC failures associated with bead-based normalization are observed (refer to *Troubleshooting* on page 66 for more information). Cluster densities outside the range of 170–220 K/mm² should not be adjusted unless associated with a QC failure. After completing the ***Prepare First Dilution*** step, prepare a second dilution using adjusted DIL1 and HT1 volumes.

NOTE The adjusted second dilution is applied on a per library preparation basis. After high or low cluster density causes an invalid sequencing run or an invalid library QC, respectively, subsequent runs from the same library preparation can be adjusted using the same cluster density value. Runs from a new library preparation follow the standard workflow.

1. Use Table 34 to find the cluster density range (K/mm²) inclusive of the value observed in the sequencing run. Note the corresponding adjusted volume of DIL1 (µl) and HT1 (µl).
   The combined reagent volume is always 1700 µl, matching the nominal dilution volume.

Table 34 Volume Adjustments Based on Cluster Density

Cluster Density Range (K/mm²)	Volume DIL1 (µl)	Volume HT1 (µl)
≤ 50	199	1501
51–70	169	1531
71–90	123	1577
91–110	95	1605
111–130	76	1624
131–150	63	1637
151–169	53	1647
170–220	40	1660
221–240	31	1669
241–260	28	1672
261–280	25	1675
281–300	22	1678
≥ 300	21	1679

2. Label a 2.0 ml microcentrifuge tube TDIL2 (Troubleshooting Dilution 2).
3. Transfer the volume of DIL1 from Table 34 to the empty TDIL2 tube.
4. Discard the DIL1 tube.
5. Add the volume of prechilled HT1 from Table 34 to the TDIL2 tube.
6. Vortex prepared 20 pM dPhiX to mix, and then centrifuge briefly.
7. Add 2.5 µl prepared 20 pM dPhiX to the TDIL2 tube.
8. Vortex to mix, and then centrifuge briefly.
9. Load 1300 µl TDIL2 into the thawed NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles).
   For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.
10. Discard the TDIL2 tube.
11. Centrifuge the NL PCR plate at 280 × g for 1 minute, and then store at -25°C to -15°C for up to 32 days.

12. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Interpretation of Results

The sequencing results from the TSO Comprehensive HRD (DRAGEN) assay are reported for each sample individually in a PDF report and a JSON report. A Low Depth Report (`LowDepthReport.tsv`) is also generated at the sample level.

At the run level, the following output files are generated:

- `ControlOutput.tsv`
- `MetricsOutput.tsv`

Only variants that pass quality control appear in the PDF and JSON reports.

For detailed analysis information, refer to the *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*.

Companion Diagnostic Results

For the CDx intended use, there are three possible results:

- **Positive**—A variant or biomarker is detected (CDx).
- **Not detected**—No variants or biomarkers associated with the CDx intended use are detected in the sample. The cancer type selected for the sample is appropriate for the CDx.
- **No result**—A determination of a variant or biomarker status is not possible for one or more of the following reasons:
  - The sequencing run failed quality control specifications.
  - The library failed required quality control specifications. For QC categories that did pass, no companion diagnostic variants or biomarkers were detected.
  - The library failed required NTC and positive control specifications.

All CDx results are reported in the Companion Diagnostic Results section of the JSON report. Only positive results are listed in the Companion Diagnostic Results section of the PDF report.

Quality Control

Sequencing run, library, and control validity is determined automatically and reported by the TSO Comprehensive (EU, DRAGEN) analysis module. Refer to Table 35 and Table 36 for the QC metrics and specifications applied for sequencing run, library, and controls. If the positive control or NTC does not meet specifications, the software automatically invalidates patient samples based on control sample results. For detailed analysis information, refer to *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*. The TSO Comprehensive HRD (DRAGEN)

report, which is available in PDF and JSON formats, summarizes quality control results. The report files are in the analysis folder. Refer to *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)* for the location of the analysis folder (contains PDF and JSON reports) and the run folder.

Invalid sequencing runs and libraries should be repeated. For more information on repeating sequencing runs or tests of libraries, refer to *Troubleshooting* on page 66. Perform additional quality control measures per the laboratory's quality management system in accordance with local, state, and/or federal regulations or accreditation requirements.

For nucleic acid quantification information and minimum input material requirements, refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 20.

Table 35 TSO Comprehensive HRD (DRAGEN) Report Result QC Metrics

Output Type	Metric	Specification	Description	Impact of Specification Failure*
Sequencing Run	PCT_PF_READS (%)	≥ 80.0	Percentage of reads passing filter (PF).	Sequencing run invalidated. No results reported for any sample in the run.
	PCT_Q30_R1 (%)	≥ 80.0	Average percent of base calls with quality score of Q30 or higher for Read 1.	
	PCT_Q30_R2 (%)	≥ 80.0	Average percent of base calls with quality score of Q30 or higher for Read 2.	

Output Type	Metric	Specification	Description	Impact of Specification Failure*
DNA Libraries	CONTAMINATION_ SCORE	≤ 1457	The contamination score based on variant allele frequency (VAF) distribution of SNPs.	No DNA results reported.
	MEDIAN_INSERT_ SIZE (bp)	≥ 70	The median fragment length in the sample	No BRCA small DNA variant results reported.
	MEDIAN_EXON_ COVERAGE (count)	≥ 150	Median exon fragment coverage across all exon bases.	
	PCT_EXON_50X (%)	≥ 90.0	Percent exon bases with 50X fragment coverage.	
	PCT_CHIMERIC_ READS (%)	≤ 8	Percent of chimeric reads.	
	GENE_SCALED_ MAD (count)	0 ≤ x ≤ 0.134	The median of absolute deviations from the median of the normalized count of each CNV target region.	No BRCA large rearrangement results reported.
	MEDIAN_BIN_ COUNT_CNV_ TARGET (count)	≥ 1.0	The median raw bin count per CNV target.	
	PCT_ TARGET_ HRD_50X (%)	≥ 50.0	Percent HRD probes with 50X fragment coverage.	No GIS result reported.
	EXCESSIVE_ TF (count)	≤ 0	Error mode metric indicating whether tumor fraction is excessive and tumor and normal signals can no longer be differentiated for calculating GIS.	

* Successful results show PASS.

Table 36 TSO Comprehensive HRD (DRAGEN) Report Result Control Metrics

Output Type	Metric	Specification	Impact of Specification Failure*
DNA Positive Control	Small DNA Variant Sensitivity	51 of 54 specified variants detected	The software automatically invalidates patient samples based on control sample results. No BRCA small DNA variant results reported.
	Gene amplification Sensitivity	3 of 3 specified variants detected	The software automatically invalidates patient samples based on control sample results. No BRCA large rearrangement results reported.
	PCT_ TARGET_ HRD_50X (%)	≥ 50	The software automatically invalidates patient samples based on control sample results. No GIS result reported.
DNA No-Template Control	MEDIAN_ EXON_ COVERAGE	≤ 8	The software automatically invalidates patient samples based on control sample results. No BRCA small DNA variant and BRCA large rearrangement results reported.
	MEDIAN_ TARGET_ COVERAGE	≤ 2	The software automatically invalidates patient samples based on control sample results. No GIS result reported.

* Successful results show PASS.

Troubleshooting

Use Table 37 to troubleshoot issues in the workflow. If a sequencing run or library preparation for a sample fails two times, additional troubleshooting may be necessary. Contact Illumina Technical Support.

Before taking the recommended troubleshooting actions, consider the two general categories of causes for libraries not passing quality control (QC) specifications. When DNA libraries do not pass QC, the underlying cause is typically either ***sample- or well-specific*** or ***systemic*** to the workflow.

Sample- or well-specific errors can result from handling errors or inherent sample quality. Systemic errors can arise from use, equipment, reagents, or reagent handling and can affect multiple libraries. While systemic errors are most likely when all libraries fail QC, variability in sample quality can result in systemic errors affecting only a subset of libraries or a single library.

Analyze the following metrics to identify the likely source of the invalid outcome.

- Inspect the total PF reads (expanded metric) for each library from the `MetricsOutput.tsv`. Similar values indicate a systemic issue, whereas low total PF reads for one or a few samples, including the samples not passing library quality control, suggests a sample or well issue.

- Inspect the total PF reads for the DNA positive control in `<run folder>/<analysis folder>/Logs_Intermediates/DnaQCMetrics/<control ID>/<control ID-DNA.aligned.metrics.json>` and compare the metric to historic values. A reduction in this metric for a positive control might be indicative of a systemic issue.

If a sequencing run does not pass run quality control specifications, the cause is a systemic error.

Table 37 TSO Comprehensive HRD (DRAGEN) Troubleshooting

Observation	Possible Cause	Recommended Action
Sequencing run does not pass run quality control specifications.	• Pooling error • Dilution error • Issues with sequencing consumables preparation (for example, not thawed adequately, condensation/debris on flow cell)	Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 59.
	Bead-based normalization: high cluster density	If a listed error is suspected, correct the issue and resequence the libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 59. If none of the other listed errors are suspected, the concentration of beads from the LNB1 reagent tube may have caused high cluster density. This could be due to insufficient bead mixing. Find the cluster density value from `RunCompletionStatus.xml` in the run folder. The optimal cluster density range is 170–220 K/mm², but higher values can still produce runs that pass QC specifications. If the cluster density is above the optimal range and the sequencing run did not pass QC specifications, the libraries from the Normalized Libraries (NL) PCR plate can be resequenced with and adjustment to the second dilution. Repeat *Prepare for Sequencing* on page 59 and follow the instructions for troubleshooting.

Observation	Possible Cause	Recommended Action
Error with report generation or general instrument error (network error, errors loading/unloading reagents, etc.).	Software or instrument issue.	Refer to *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)* for help with report generation. Contact Illumina Technical Support for additional help.
DNA library does not pass quality control specifications.	Requirements for sample input were not met.	Ensure appropriate sample input and repeat library preparation from the Fragment gDNA step. Refer to *Sample Requirements* on page 20 and *Nucleic Acid Extraction, Quantification, and Storage* on page 20.
	Use or equipment error in the assay workflow.	Repeat library preparation from one of the following steps depending on where suspected use or equipment error occurred. If unknown, or other errors occurred, contact Illumina Technical Support to troubleshoot your run. • Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 59. • Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 35.

Observation	Possible Cause	Recommended Action
DNA library does not pass quality control specifications (continued).	CONTAMINATION_SCORE criteria are not met.	Review Warnings and Precautions for information on avoiding cross contamination. Review plate layout and library indexing to make sure that samples of the same index were not sequenced together. For impacted samples, start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 35. Contamination may have occurred during sample extraction. It may be necessary to repeat extraction to make sure that the sample is free from contamination.
	Sample may be overly fragmented or have nucleic acid damage that impacts the ability to generate sufficient unique libraries.	Review *Ultrasonicator Configuration Settings for DNA Fragmentation* on page 18 and ultrasonicator manufacturer settings for use and operation (including water level and tube type). Ensure appropriate sample input into the assay. Refer to *Sample Requirements* on page 20 and *Nucleic Acid Extraction, Quantification, and Storage* on page 20. A new sample extraction and/or repeating the Fragment gDNA step may be necessary if the sample is overly fragmented or damaged.
	• Pooling error. • Dilution error. • Incomplete heat denaturation of PDL/PHL.	Rule out DNA-related causes listed above (eg, sample input, sample quality, equipment). Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 59.

Observation	Possible Cause	Recommended Action
DNA library does not pass quality control specifications (continued).	Bead-based normalization (low cluster density).	If none of the other possible causes for library QC failure are suspected, the concentration of beads from the LNB1 reagent tube may have caused low cluster density due to insufficient mixing. Find and record the cluster density value from `RunCompletionStatus.xml` in the run folder. If the cluster density is below 170 K/mm² and a systemic bead-based normalization is suspected (including evidence from total PF reads for the positive controls), resequence libraries from the Normalized Libraries (NL) PCR plate and follow the instructions for an adjusted second dilution. Refer to *Prepare for Sequencing* on page 59 and *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 61.
	Error in library preparation workflow during or prior to index PCR step.	Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 35.
	• Incorrect use of enrichment probes. • Error in library preparation workflow during or after first hybridization step.	A systemic probe pool error (eg, swapping probe pools) might result in normal total passing filter reads, but median exon coverage fail QC. Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 35.

Observation	Possible Cause	Recommended Action
Positive Control Failure.	Requirements for sample input for the positive control were not met.	Ensure appropriate input into the assay. Review plate layout and ensure appropriate reagents (probes, indexes) are in appropriate wells. Ensure positive control sample stored according to label. For all samples that share the positive control, repeat library preparation from one of the following steps depending on where suspected use or equipment error occurred. If unknown, or other errors occurred, contact Illumina Technical Support to troubleshoot your run. • Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 59. • Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 35.
	Use or equipment error in the assay workflow.	
NTC Failure.	Cross contamination occurred or contamination of work area.	Review Warnings and Precautions section for information on decontaminating work areas and avoiding cross contamination. Review plate layout and library indexing to make sure that samples of the same index were not sequenced together. Repeat library preparation from the beginning of the workflow for all libraries that share No-Template Control.
	Incorrect indexing of library.	

Observation	Possible Cause	Recommended Action
Positive and/or negative controls in the sequencing run were analyzed as non-Control samples.	Incorrect assignment of Specimen Type during sequencing run setup.	Requeue analysis with controls correctly identified as instructed in the Analysis Module Workflow Guide (refer to *Illumina Connected Run TruSight Oncology Comprehensive HRD (EU, DRAGEN) Analysis Module Workflow Guide (document # 200080937)*).

Performance Characteristics

TSO Comprehensive HRD (DRAGEN) is an assay that enables assessment of HRD using DNA extracted from FFPE tumor tissue samples from ovarian cancer patients. It uses two NGS panels. One panel is used for detection of deleterious and suspected deleterious alterations in BRCA1 and BRCA2 genes, including small DNA variants and large rearrangements. A second NGS panel is used to measure genomic instability which is reported as a GIS. BRCA-positive is defined as detection of a deleterious or suspected deleterious alteration in either BRCA1 or BRCA2 genes. GIS-positive is defined as GIS ≥ 42. HRD-positive is defined as BRCA-positive and/or GIS-positive.

FFPE samples with specific CDx positive variants were included in the studies.

Two-sided 95% confidence intervals (CI) are calculated using the Wilson Score Method unless stated otherwise.

Table 38 provides definitions of metrics calculated in various studies.

Table 38 Metrics Definitions

Term	Definition
Positive Percent Agreement (PPA)	The percentage of comparator method positive results in which TSO Comprehensive HRD (DRAGEN) is positive.
Negative Percent Agreement (NPA)	The percentage of comparator method negative results in which TSO Comprehensive HRD (DRAGEN) is negative.
Percent Positive Call (PPC)	Percentage of observations that are positive for a target among observations expected to be positive for the target.
Percent Negative Call (PNC)	Percentage of observations that are negative for a target among observations expected to be negative for the target.
n/N	The number of positive or negative observations (n) divided by the total observations (N) to calculate PPC or PNC, respectively. The values of n and N can be at different levels (eg, variant or gene) or combined across a group (eg, SNVs).
Standard Deviation (SD)	A measure of the amount of variation of the values of a variable about its mean.
Percent Coefficient of Variation (%CV)	Standard deviation divided by the mean as a percentage.

Accuracy

The accuracy of the TSO Comprehensive HRD (DRAGEN) assay for detecting HRD in patients with ovarian cancer was demonstrated by assessing concordance of HRD status, GIS status, and BRCA status results between the TSO Comprehensive HRD (DRAGEN) assay and a validated NGS-based comparator assay.

Accuracy was estimated relative to the orthogonal method; PPA, NPA, and the associated two-sided 95% Wilson Score CI were calculated.

There were 175 ovarian cancer FFPE DNA samples tested with the TSO Comprehensive HRD (DRAGEN) assay and the orthogonal method. One sample had invalid TSO Comprehensive HRD (DRAGEN) results, and another sample had invalid comparator assay results. The evaluable dataset for GIS and HRD status was 173 samples. The evaluable dataset for BRCA status was 169 due to exclusion of four additional samples with BRCA variants outside the measuring range of the TSO Comprehensive HRD (DRAGEN) assay. The agreement analyses based on valid test results are shown in Table 39, Table 40, and Table 41.

Table 39 Concordance Between the TSO Comprehensive HRD (DRAGEN) Assay and Comparator Method for Detection of HRD Status

		Comparator Method Result		
		HRD Positive	**HRD Negative**	**Total**
TSO Comprehensive HRD (DRAGEN) Assay Result	HRD Positive	97	6	103
	HRD Negative	6	64	70
	Total	103	70	173
Agreement Statistics	PPA% (n/N; 95% CI)	94.2% (97/103; 87.9%, 97.3%)		
	NPA% (n/N; 95% CI)	91.4% (64/70; 82.5%, 96.0%)		

Table 40 Concordance Between the TSO Comprehensive HRD (DRAGEN) Assay and Comparator Method for Detection of GIS Status

		Comparator Method Result		
		GIS Positive	**GIS Negative**	**Total**
TSO Comprehensive HRD (DRAGEN) Assay Result	GIS Positive	95	3	98
	GIS Negative	6	69	75
	Total	101	72	173
Agreement Statistics	PPA% (n/N; 95% CI)	94.1% (95/101; 87.6%, 97.2%)		
	NPA% (n/N; 95% CI)	95.8% (69/72; 88.5%, 98.6%)		

Table 41 Concordance Between the TSO Comprehensive HRD (DRAGEN) Assay and Comparator Method for Detection of BRCA Status

		Comparator Method Result		
		BRCA Positive	**BRCA Negative**	**Total**
TSO Comprehensive HRD (DRAGEN) Assay Result	BRCA Positive	40	8	48
	BRCA Negative	2	119	121
	Total	42	127	169
Agreement Statistics	PPA% (n/N; 95% CI)	95.2% (40/42; 84.2%, 98.7%)		
	NPA% (n/N; 95% CI)	93.7% (119/127; 88.1%, 96.8%)		

Reproducibility

The reproducibility and repeatability of the TSO Comprehensive HRD (DRAGEN) assay was evaluated across three external testing sites with two operators per site, two within-run replicates, three lots, and three non-consecutive days. At each of the three sites, two operators independently conducted testing on separate NextSeq 550Dx instruments. The testing was conducted with a 27-member reproducibility panel containing DNA extracted from FFPE ovarian tissue specimens with known BRCA1 and BRCA2 mutations and/or GIS.

Of the 27 panel members, 16 panel members were targeted for small DNA variants in BRCA genes that include: SNVs, variants in homopolymer regions ≥ 5 bp, insertions and deletions < 10 bp, and insertions and deletions ≥ 10 bp. For the BRCA LR variant type, two panel members were used for BRCA LR variants < 3 exons and one panel member for the BRCA LR variants ≥ 3 exons. For GIS, three panel members represented High GIS (+) status, three panel members represented GIS (-), two panel members represented Low GIS (+), and one of the panel members already representing the LR variants < 3 exons level also represented one Low GIS (+) member. At each site, each operator tested the panel members over the course of three non-consecutive days, generating six observations per target per panel member. From all three sites, 36 observations were generated per panel member (three sites × two operators/instruments × three days x two within-run replicates).

PPC and PNC for targeted panel members were determined. Analyses were performed to estimate PPC and PNC (with associated 95% CIs) by combining the results from six operators across three sites. For each targeted small DNA variant and LR variant, TSO Comprehensive HRD (DRAGEN) assay observations in panel members expected to be negative for the targeted variant were combined to calculate PNC. TSO Comprehensive HRD (DRAGEN) assay observations in GIS (-) panel members were combined to calculate PNC for GIS (-) panel members.

Small DNA Variants in BRCA

PPC values for both high-level (2-3×C95) and low-level (1-1.5×C95) BRCA small DNA variant panel members were all 100% (Table 42). PNC values for the BRCA small DNA variant panel members were also all 100% (Table 43).

Table 42 PPC for Targeted BRCA Small DNA Variants by Variant Level and Variant Class

Variant Level	Variant Class	Targeted Variant	Mean VAF	PPC (%) (n/N)	95% CI
High	SNV	BRCA1 p.(A1708E)	0.129	100 (36/36)	(90.4, 100)
		BRCA1 c.80+1G>A	0.121	100 (36/36)	(90.4, 100)
	Variants in homopolymers ≥ 5 bp	BRCA2 p.(E2981Rfs*37)	0.248	100 (34/34)	(89.8, 100)
		BRCA1 p.(R1726Kfs*3)	0.239	100 (36/36)	(90.4, 100)
	Insertion or deletion (< 10 bp)	BRCA1 p.(A1279Hfs*28)	0.166	100 (36/36)	(90.4, 100)
		BRCA1 p.(Q1756Pfs*74)	0.153	100 (36/36)	(90.4, 100)
	Insertion or deletion (≥ 10 bp)	BRCA1 p.(C994Sfs*7)	0.189	100 (36/36)	(90.4, 100)
		BRCA2 p.(E1811Kfs*3)	0.141	100 (33/33)	(89.6, 100)
Low	SNV	BRCA1 p.(A1708E)	0.087	100 (36/36)	(90.4, 100)
		BRCA1 c.80+1G>A	0.087	100 (36/36)	(90.4, 100)
	Variants in homopolymers ≥ 5 bp	BRCA2 p.(E2981Rfs*37)	0.147	100 (36/36)	(90.4, 100)
		BRCA1 p.(R1726Kfs*3)	0.142	100 (36/36)	(90.4, 100)
	Insertion or deletion (< 10 bp)	BRCA1 p.(A1279Hfs*28)	0.121	100 (36/36)	(90.4, 100)
		BRCA1 p.(Q1756Pfs*74)	0.122	100 (35/35)	(90.1, 100)
	Insertion or deletion (≥ 10 bp)	BRCA1 p.(C994Sfs*7)	0.109	100 (35/35)	(90.1, 100)
		BRCA2 p.(E1811Kfs*3)	0.111	100 (36/36)	(90.4, 100)

Table 43 PNC for Targeted BRCA Small DNA Variants by Variant Class

Variant Class	Targeted Variant	PNC (%) (n/N)	95% CI
SNV	BRCA1 p.(A1708E)	100 (585/585)	(99.3, 100)
	BRCA1 c.80+1G>A	100 (557/557)	(99.3, 100)
Variants in homopolymers ≥ 5 bp	BRCA2 p.(E2981Rfs*37)	100 (566/566)	(99.3, 100)
	BRCA1 p.(R1726Kfs*3)	100 (492/492)	(99.2, 100)
Insertion or deletion (< 10 bp)	BRCA1 p.(A1279Hfs*28)	100 (627/627)	(99.4, 100)
	BRCA1 p.(Q1756Pfs*74)	100 (632/632)	(99.4, 100)
Insertion or deletion (≥ 10 bp)	BRCA1 p.(C994Sfs*7)	100 (584/584)	(99.3, 100)
	BRCA2 p.(E1811Kfs*3)	100 (634/634)	(99.4, 100)

Table 44 shows the variance components analysis by variation source for high- and low-levels for each BRCA small DNA variant class. The mean VAF across the targeted high-level BRCA small DNA variants ranged from 0.121 to 0.248, and targeted low-level BRCA small DNA variants ranged from 0.087 to 0.147. The total SD

across the targeted BRCA small DNA variants ranged from 0.019 (11.4% CV) to 0.041 (28.7% CV) for high-level BRCA small DNA variants, and from 0.016 (18.9% CV) to 0.043 (30.1% CV) for low-level BRCA small DNA variants.

Table 44 Variance Component Analysis of VAF in Targeted BRCA Small DNA Variants

Variant Level	Variant Class	Targeted Variant	N	Mean VAF	Site SD (%CV)	Operator/ instrument SD (%CV)	Day SD (%CV)	Lot SD (%CV)	Repeatability SD (%CV)	Total SD (%CV)
High	SNV	BRCA1 p.(A1708E)	36	0.129	0.000 (0.0)	0.000 (0.0)	0.007 (5.5)	0.000 (0.0)	0.020 (15.4)	0.021 (16.3)
		BRCA1 c.80+1G>A	36	0.121	0.007 (5.5)	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.023 (19.2)	0.024 (19.9)
	Homopolymer variants ≥ 5 bp	BRCA2 p.(E2981Rfs*37)	34	0.248	0.000 (0.0)	0.011 (4.3)	0.008 (3.3)	0.000 (0.0)	0.025 (10.1)	0.029 (11.5)
		BRCA1 p.(R1726Kfs*3)	36	0.239	0.000 (0.0)	0.000 (0.0)	0.006 (2.5)	0.000 (0.0)	0.041 (17.0)	0.041 (17.1)
	Insertion or deletion < 10 bp	BRCA1 p.(A1279Hfs*28)	36	0.166	0.006 (3.7)	0.005 (3.2)	0.000 (0.0)	0.000 (0.0)	0.017 (10.3)	0.019 (11.4)
		BRCA1 p.(Q1756Pfs*74)	36	0.153	0.008 (5.1)	0.000 (0.0)	0.013 (8.5)	0.000 (0.0)	0.020 (12.9)	0.025 (16.3)
	Insertion or deletion ≥ 10 bp	BRCA1 p.(C994Sfs*7)	36	0.189	0.000 (0.0)	0.000 (0.0)	0.006 (3.3)	0.015 (8.0)	0.033 (17.5)	0.037 (19.6)
		BRCA2 p.(E1811Kfs*3)	33	0.141	0.000 (0.0)	0.013 (9.5)	0.000 (0.0)	0.000 (0.0)	0.038 (27.1)	0.041 (28.7)
Low	SNV	BRCA1 p.(A1708E)	36	0.087	0.000 (0.0)	0.000 (0.0)	0.003 (3.0)	0.000 (0.0)	0.016 (18.7)	0.016 (18.9)
		BRCA1 c.80+1G>A	36	0.087	0.003 (3.7)	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.019 (22.0)	0.019 (22.3)
	Homopolymer variants ≥ 5 bp	BRCA2 p.(E2981Rfs*37)	36	0.147	0.000 (0.0)	0.003 (2.0)	0.000 (0.0)	0.000 (0.0)	0.021 (14.0)	0.021 (14.1)
		BRCA1 p.(R1726Kfs*3)	36	0.142	0.011 (8.0)	0.000 (0.0)	0.010 (7.2)	0.015 (10.7)	0.037 (26.0)	0.043 (30.1)
	Insertion or deletion < 10 bp	BRCA1 p.(A1279Hfs*28)	36	0.121	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.004 (3.7)	0.017 (14.0)	0.017 (14.5)
		BRCA1 p.(Q1756Pfs*74)	35	0.122	0.004 (3.4)	0.007 (5.6)	0.000 (0.0)	0.006 (5.0)	0.021 (16.9)	0.023 (18.8)
	Insertion or deletion ≥ 10bp	BRCA1 p.(C994Sfs*7)	35	0.109	0.000 (0.0)	0.000 (0.0)	0.007 (6.5)	0.006 (5.5)	0.026 (24.3)	0.028 (25.8)
		BRCA2 p.(E1811Kfs*3)	36	0.111	0.000 (0.0)	0.006 (5.7)	0.000 (0.0)	0.006 (5.7)	0.029 (26.0)	0.030 (27.2)

Large Rearrangement Variants in BRCA

PPC values for the targeted BRCA LRs were 100% (29/29) for BRCA2 LR ≥ 3 exons, 97.2% (35/36) for BRCA2 LR < 3 exons, and 94.3% (33/35) for BRCA1 LR < 3 exons (Table 45). PNC values for the targeted BRCA LRs were 98.9% (630/637) for BRCA1, and 100% (607/607) for BRCA2 (Table 46).

Table 45 PPC for Targeted BRCA LRs by Variant Class

Variant Class	Targeted Variant	Mean VAF	PPC (%) (n/N)	95% CI*
LR ≥ 3 exons	BRCA2 LOSS	0.448	100 (29/29)	(88.3, 100)
LR < 3 exons	BRCA2 LOSS	0.602	97.2 (35/36)	(85.8, 99.5)
	BRCA1 LOSS	0.680	94.3 (33/35)	(81.4, 98.4)

Table 46 PNC for Targeted BRCA LRs

Targeted Variant	PNC (%) (n/N)	95% CI*
BRCA1 LOSS	98.9 (630/637)	(97.7, 99.5)
BRCA2 LOSS	100 (607/607)	(99.4, 100)

Table 47 shows the variance component analysis by variation source for each BRCA LR class. The mean VAF for the LR ≥ 3 exons variant was 0.448 with a total SD of 0.026 (5.7% CV). The mean VAF for the LR < 3 exons variants ranged from 0.602 to 0.680, and the total SD ranged from 0.038 (6.3% CV) to 0.079 (11.6% CV).

Table 47 Variance Component Analysis of VAF in Targeted BRCA LRs

Variant Class	Targeted Variant	N	Mean VAF	Site SD (%CV)	Operator / Instrument SD (%CV)	Day SD (%CV)	Lot SD (%CV)	Repeatability SD (%CV)	Total SD (%CV)
LR ≥ 3 exons	BRCA2 LOSS	29	0.448	0.000 (0.0)	0.005 (1.1)	0.013 (2.9)	0.000 (0.0)	0.022 (4.8)	0.026 (5.7)
LR < 3 exons	BRCA2 LOSS	36	0.602	0.000 (0.0)	0.000 (0.0)	0.015 (2.5)	0.000 (0.0)	0.035 (5.8)	0.038 (6.3)
	BRCA1 LOSS	35	0.680	0.000 (0.0)	0.000 (0.0)	0.048 (7.1)	0.000 (0.0)	0.062 (9.2)	0.079 (11.6)

Genomic Instability Score (GIS)

PPC values were 100% for High GIS (+) panel members. Low GIS (+) panel members had PPC values ranging from 94.4% to 100% (Table 48). PNC values were 100% across all GIS (-) panel members (Table 49).

Table 48 PPC for GIS by Targeted GIS Status and Panel Member

Targeted GIS Status	Targeted GIS	Mean GIS	PPC (%) (n/N)	95% CI*
High GIS (+)	69.8	70.2	100 (36/36)	(90.4, 100)
	59.5	60.1	100 (36/36)	(90.4, 100)
	63.7	61.7	100 (36/36)	(90.4, 100)
Low GIS (+)	47.2	46.3	94.4 (34/36)	(81.9, 98.5)
	52.7	53.1	100 (36/36)	(90.4, 100)
	53.0	52.5	100 (36/36)	(90.4, 100)

Table 49 PNC for GIS Negative Targeted Panel Members

Targeted GIS Status	Targeted GIS	Mean GIS	PNC (%) (n/N)	95% CI*
GIS (-)	24.5	23.3	100 (36/36)	(90.4, 100)
	19.2	18.2	100 (36/36)	(90.4, 100)
	19.5	21.0	100 (36/36)	(90.4, 100)

Table 50 shows the variance components analysis by variation source for each targeted High GIS (+), Low GIS (+), and GIS (-) panel member. The mean GIS across the GIS targeted panel members ranged from 60.1 to 70.2 for High GIS (+), from 46.3 to 53.1 for Low GIS (+), and from 18.2 to 23.3 for GIS (-). The total SD across the targeted GIS panel members ranged from 2.5 (3.5% CV) to 3.2 (5.2% CV) for High GIS (+), from 1.5 (2.9% CV) to 3.8 (7.2% CV) for Low GIS (+), and from 2.5 (11.7% CV) to 3.3 (14.1% CV) for GIS (-) panel members.

Table 50 Variance Component Analysis of GIS in Targeted Panel Members

Targeted GIS Status	Targeted GIS	N	Mean GIS	Site SD (%CV)	Operator / Instrument SD (%CV)	Day SD (%CV)	Lot SD (%CV)	Repeatability SD (%CV)	Total SD (%CV)
High GIS (+)	69.8	36	70.2	0.0 (0.0)	1.2 (1.7)	0.0 (0.0)	0.0 (0.0)	2.2 (3.1)	2.5 (3.5)
	59.5	36	60.1	0.0 (0.0)	0.0 (0.0)	1.0 (1.7)	0.0 (0.0)	2.7 (4.4)	2.8 (4.7)
	63.7	36	61.7	0.0 (0.0)	0.0 (0.0)	0.9 (1.5)	0.0 (0.0)	3.1 (5.0)	3.2 (5.2)
Low GIS (+)	47.2	36	46.3	0.0 (0.0)	1.7 (3.7)	0.0 (0.0)	0.7 (1.6)	2.8 (6.1)	3.4 (7.3)
	52.7	36	53.1	0.9 (1.7)	1.2 (2.3)	0.0 (0.0)	0.4 (0.8)	3.5 (6.5)	3.8 (7.2)
	53.0	36	52.5	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.5 (2.9)	1.5 (2.9)
GIS (-)	24.5	36	23.3	0.9 (3.7)	1.6 (6.9)	1.6 (7.0)	0.9 (3.7)	2.0 (8.7)	3.3 (14.1)
	19.2	36	18.2	0.7 (3.7)	0.0 (0.0)	0.6 (3.5)	0.1 (0.4)	2.4 (13.4)	2.6 (14.4)
	19.5	36	21.0	0.0 (0.0)	0.0 (0.0)	0.6 (3.0)	1.3 (6.3)	2.0 (9.4)	2.5 (11.7)

Limit of Blank

The percentage of false positives (out of the total expected negatives) was assessed by replicate testing of FFPE benign adjacent ovarian tissue. The tissue was expected to be GIS negative and not contain deleterious or suspected deleterious BRCA1 and BRCA2 mutations (small DNA variants and LRs). Seven benign ovarian FFPE DNA samples were run in duplicate with two operators across three days for each of the two reagent lots in 8-plex format. A subset of samples was re-pooled and re-sequenced in a 3-plex format to evaluate false positives with several multiplex configurations supported by this device. In total, there were 168 possible observations. The percentage of false positives was calculated at the position level for BRCA small DNA variants (approximately 3 million positions), at the gene level for BRCA LRs, and at the sample level for GIS. The percentage of false positives per variant type is shown in Table 51.

Table 51 False Positives by Variant Type

Variant Type	False Positives*
BRCA Small DNA Variants	0% (0/3,076,657)
BRCA LR	0% (0/384)
GIS	0% (0/192)

* Denominators are totals of 8-plex and 3-plex runs.

Limit of Detection

The Limit of Detection (LoD) of BRCA small DNA variants (SNVs, insertions or deletions ≥ 10 bp, insertions or deletions < 10 bp, variants in homopolymer regions ≥ 5 bp), BRCA LRs < 3 exons and ≥ 3 exons, and GIS were determined in cancer FFPE DNA samples. The LoD is the lowest analyte value that can be detected consistently (95% hit rate or a type II error of 5%). The LoD for GIS was defined as the lowest tumor content that achieves ≥ 95% GIS detection and for BRCA small DNA variants and LRs as variant allele frequency (VAF). Ovarian cancer FFPE DNA samples were used for BRCA small DNA variants, GIS, and BRCA LR ≥ 3 exons. A breast cancer FFPE DNA sample was used for BRCA LR < 3 exons due to the unavailability of ovarian cancer FFPE tissue. Samples were diluted to multiple test levels, with 12 observations for each test level per reagent lot per variant, generated by multiple operators on three non-consecutive days using two reagent lots. The larger LoD across the two reagent lots was taken as the limit of detection for the variant (Table 52).

Table 52 Limit of Detection for BRCA small DNA Variants, BRCA LRs, GIS

Variant Type	Variant Class	C95
BRCA Small DNA Variants	SNV	0.05 VAF
	Insertions or Deletions ≥ 10 bp	0.06 VAF
	Insertions or Deletions < 10 bp	0.07 VAF
	Variants in homopolymer region ≥ 5 bp	0.07 VAF
BRCA Large Rearrangements	LR < 3 Exons	0.61 VAF
	LR ≥ 3 Exons	0.46 VAF
GIS	GIS	33% tumor content

Interfering Substances

The impact of potential endogenous and exogenous substances on the performance of the TSO Comprehensive HRD (DRAGEN) assay was evaluated with the endogenous substance (hemoglobin) that was spiked into samples during the nucleic acid extraction process, as well as exogenous substances (ethanol, Proteinase K, wash buffer, paraffin, xylene, and excess index primers) that were spiked into the purified nucleic acid before library preparation. Four replicates of the sample libraries were generated by one or more operators. For all substances 10 ovarian cancer FFPE samples were used for analysis of BRCA small DNA variants, BRCA LRs, and GIS. The effect of necrosis was also assessed on a different set of three ovarian cancer FFPE samples with a range of necrotic tissue (10%-20%). A macro-dissected no necrosis control was included for each necrosis sample. Four replicates of DNA libraries and their controls were processed together. For all interferents, sample replicates per substance were compared to their respective control replicates. Hemoglobin (4 mg/ml), ethanol (5%), Proteinase K (2x of representative extraction method concentration), wash buffer (2.5%), paraffin (25% of the homogenized digested tissue), xylene (0.0001%), index primers (30% excess) do not interfere with the detection of BRCA small DNA variants, BRCA LRs, and GIS. BRCA small DNA variant VAF

levels and GIS were unaffected by the presence of any of the tested interferents. The presence of necrotic tissue up to 20% did not interfere with BRCA small DNA variants and GIS. BRCA LRs were not evaluated for necrosis due to the unavailability of a necrotic ovarian cancer FFPE sample harboring a BRCA LR variant.

Nucleic Acid Input Titration Guardbanding

TSO Comprehensive HRD (DRAGEN) requires a fixed input of 40 ng of DNA sample. The performance of the assay was evaluated using nine DNA samples extracted from ovarian FFPE tissue to establish tolerances for nucleic acid input variation that could lead to assay failure or negatively impact output of the assay. Each FFPE sample contained one or more BRCA small DNA variants, BRCA LRs, or GIS and was tested at five input levels (20 ng, 30 ng, 40 ng [nominal], 60 ng, and 100 ng) in triplicate. Results demonstrated that BRCA small DNA variant detection, BRCA LR calling, and GIS status were not affected by difference in input amount.

The results also showed that nucleic acid inputs two-fold below the nominal condition had an increased number of QC failures leading to fewer samples evaluated for variant calling. At the 20 ng input level a library validity rate of 55.5% was obtained due to failures in percent exon coverage at 50X (PCT_EXON_50X) and median exon coverage (MEDIAN_EXON_COVERAGE). BRCA small DNA variant VAF and GIS were not affected at the 20 ng input level.

Cross-Contamination

The cross-contamination study was conducted to evaluate if false positive results were due to well-to-well contamination during sample library preparation or run-to-run contamination between consecutive sequencing runs. Analysis was performed for BRCA small DNA variants, BRCA LR and GIS. Libraries were prepared from characterized FFPE ovarian cancer samples in a checkerboard layout with alternating samples to evaluate well-to-well contamination, and with alternating indexes to evaluate sequencing run-to-run contamination when sequenced consecutively on the same NextSeq 550Dx instrument. The cross-contamination study showed 0.8% contamination rate (2/252 contamination events) observed by examining the detected variants in each sample. No contamination events were found by analyzing recipient libraries based on variant calling for BRCA small DNA variants and BRCA LR. The two contamination events observed were false positives for GIS in GIS recipient samples due to intrinsic GIS variation and the GIS of these two samples being close to the decision threshold.

The study demonstrated that TSO Comprehensive HRD (DRAGEN) assay is expected to have a low occurrence of cross-contamination from well-to-well or run-to-run. These results together with the contamination metrics in the software mitigate the risk of false variant results due to sample contamination.

Nucleic Acid Extraction Kit Evaluation

Three commercially available DNA extraction kits were evaluated with TSO Comprehensive HRD (DRAGEN). The three extraction kits differed in their deparaffinization agent and nucleic acid binding steps (Table 53). Kit 3 was the predominant extraction kit used to determine TSO Comprehensive HRD (DRAGEN) performance.

Five ovarian FFPE samples were subjected to nucleic acid extraction (three kits x two operators x three days x two replicates) to obtain DNA (36 total observations). Qualitative analysis showed that there was no discernible difference in variant calling for any of the variant types (BRCA small DNA variants, BRCA LRs, GIS), as all variant observations had PPC values > 97%. Quantitative analysis showed no significant difference associated with kits for BRCA small DNA variant VAF and GIS.

Table 53 Kit Characteristics

Kit	Kit Name	Deparaffinization Agent	Nucleic Acid Binding
1	Qiagen QIAamp DSP DNA FFPE Tissue	Xylene	Column
2	Beckman Coulter FormaPure XL DNA	Mineral Oil	Magnetic beads
3	Qiagen AllPrep DNA/RNA FFPE	Proprietary Deparaffinization Solution	Column

Reagent Stability

Real-Time Stability

Real-time stability was evaluated using six FFPE ovarian cancer tissue samples. Three reagent lots were tested across six time points spaced apart with similar increments (3 months, 6 months, 9 months, 13 months, 19 months, and 25 months). Two operators performed library preparation events for each lot, including three replicates of each sample, for a total of six replicates per lot. For targeted BRCA small DNA variants aggregate PPC was 100%, for targeted BRCA LR aggregate PPC was ≥ 92%, and for GIS aggregate PPC was ≥ 83%. In addition, there were no significant differences associated with time for GIS or BRCA small DNA variant VAF.

Kit In-Use Stability

Kit in-use stability of the TSO Comprehensive HRD (DRAGEN) assay kit was evaluated under standard use conditions over the course of the shelf life to support multiple kit uses. The frozen components of the reagent kit were subjected to multiple freeze/thaws and refrigerated reagents were brought up to room temperature and tested to support up to four uses of the kit. All quality control criteria were met for all freeze-thaw cycles and time points tested. Variant calling was assessed for BRCA small DNA variants, BRCA LRs, and GIS. The results support up to four freeze/thaws of the kit. Additionally, one reagent kit was used to prepare a total of 24 DNA samples by one operator demonstrating that there is sufficient reagent volume for 24 DNA and 24 HRD libraries.

Transport Stability

Kit transport stability of the TSO Comprehensive HRD (DRAGEN) assay was evaluated using nine ovarian cancer FFPE samples to show functionality of the TSO Comprehensive HRD (DRAGEN) reagent kits after exposure to simulated distribution conditions. Eight ovarian cancer FFPE samples were used to show functionality after

reagent kit exposure to simulated international summer and winter temperature profiles. Functional testing was performed post-exposure using the above-mentioned samples and consisted of six to 12 replicates of each sample per condition. The physical attributes of the kits were also observed and found to be maintained after distribution for all test cases. Functional testing concluded that shipping did not negatively impact variant calling for BRCA small DNA variants (PPC ≥ 96%), BRCA LRs (PPC ≥ 94%), and GIS (PPC = 100%), relative to the control condition.

Sample Stability

Nucleic Acid Stability

The stability of nucleic acids (DNA) and their associated quantitation for use with the TSO Comprehensive HRD (DRAGEN) assay was evaluated using ovarian cancer FFPE tissue samples. FFPE blocks were sectioned and DNA was extracted. Extracted nucleic acid was thoroughly mixed, quantified, checked for nucleic acid quality and aliquoted into two sets of single-use tubes to be frozen for two time points: T0 (control) and T1 (to support 28 days). All extracted DNA was stored at -25°C to -15°C until being tested at T0 or T1 with multiple replicates and operators. T1 was compared to T0 for BRCA small DNA variants, BRCA LRs, and GIS. The data indicate that nucleic acids and their associated quantitation for use with the TSO Comprehensive HRD (DRAGEN) assay are stable for up to 28 days when stored at the recommended temperatures (-25°C to -15°C).

Library Stability

The stability of libraries utilizing each of the five safe stopping points for the assay (refer to Table 54) was evaluated using multiple ovarian cancer FFPE tissue samples and one breast cancer FFPE tissue sample. Control libraries (T0, no stopping points) were sequenced immediately at the end of the workflow. Aliquots from the same libraries were held at the stopping points at -25°C to -15°C for the time (T1) to support the days listed in Table 54. T1 was compared to T0 for BRCA small DNA variants, BRCA LRs, and GIS. The data indicate that libraries generated from the TSO Comprehensive HRD (DRAGEN) assay are stable in accordance with the Instructions for Use.

Table 54 Library Storage Times

Library Type	Plate	Number of Days
Fragmented gDNA	LP PCR	≤ 7
Pre-enrichment	ALS PCR	≤ 30
Post-enrichment	ELU2 PCR	≤ 7
Post-enrichment PCR	PL PCR	≤ 30
Normalized	NL PCR	≤ 32

Slide-Mounted FFPE Tissue Stability

The stability of slide-mounted FFPE tissue for use with the TSO Comprehensive HRD (DRAGEN) assay was evaluated by sectioning FFPE blocks (5 µm sections) from ten ovarian cancer FFPE tissue samples, mounted on slides, followed by storage at room temperature (22°C) for two time points: T0 (control) and T1 (to support 28 days). Nucleic acid material was quantified and then processed through the TSO Comprehensive HRD (DRAGEN) assay within 24 hours for each time point. At each time point multiple replicates per sample were tested by multiple operators. T1 was compared to T0 for BRCA small DNA variants, BRCA LRs, and GIS. Variant calling was assessed. The data indicate that slide-mounted FFPE tissue for use with TSO Comprehensive HRD (DRAGEN) is stable at room temperature for up to 28 days.

HRD Clinical Performance Bridging Study

To validate the TSO Comprehensive HRD (DRAGEN) assay as a CDx for the selection of patients with ovarian cancer for treatment with LYNPARZA (olaparib), residual samples from patients enrolled in the PAOLA-1 clinical trial (NCT02477644) were tested to support a Clinical Bridging Study.

The study was conducted to assess the concordance of HRD status between the TSO Comprehensive HRD (DRAGEN) assay and the clinical trial assay (CTA) used in PAOLA-1, and to assess the clinical efficacy of olaparib in the HRD-positive patients identified by the TSO Comprehensive HRD (DRAGEN) assay. PAOLA-1 was a multicenter, Phase III, randomized, double-blind, placebo-controlled study investigating the clinical efficacy of olaparib plus bevacizumab as a first-line maintenance therapy for patients with advanced ovarian cancer. The HRD status of patients was determined post-randomization before the primary analysis using the CTA. The primary endpoint was progression-free survival (PFS), defined as time from randomization to progression determined by investigator assessment using modified RECIST 1.1, or death. Out of the 806 patients randomized in the PAOLA-1 study, 399 residual samples were available for testing with TSO Comprehensive HRD (DRAGEN). The demographics of subjects with available TSO Comprehensive HRD (DRAGEN) results were similar to those in the PAOLA-1 study. Likewise, participant characteristics were generally similar between those who were HRD-positive with TSO Comprehensive HRD (DRAGEN) versus those who were HRD-positive by the CTA.

Concordance Results

Of the 399 samples available for testing with TSO Comprehensive HRD (DRAGEN), 382 yielded an evaluable result. The 17 non-evaluable samples did not yield a result due to failures of either test quality control (N=12) or pathology review (N=5) (assay invalid rate of 3% [12/394]). A total of 365 enrolled patients had samples with both evaluable CTA and TSO Comprehensive HRD (DRAGEN) results. Agreement of TSO Comprehensive HRD (DRAGEN) results relative to CTA results among samples with valid results are shown in Table 55.

Table 55 Concordance Between TSO Comprehensive HRD (DRAGEN) and CTA for Detection of HRD

		CTA Result		
		HRD Positive	**HRD Negative**	**Total**
TSO Comprehensive HRD (DRAGEN) Assay Result	HRD Positive	204	12	216
	HRD Negative	5	144	149
	Total	209	156	365
Agreement Statistics	PPA% (n/N; 95% CI*)	97.6% (204/209; 94.5%, 99.2%)		
	NPA% (n/N; 95% CI*)	92.3% (144/156; 86.9%, 96.0%)		
	OPA% (n/N; 95% CI*)	95.3% (348/365; 92.6%, 97.3%)		

* CI based on the exact binomial method (Clopper and Pearson, 1934).

Clinical Efficacy Results

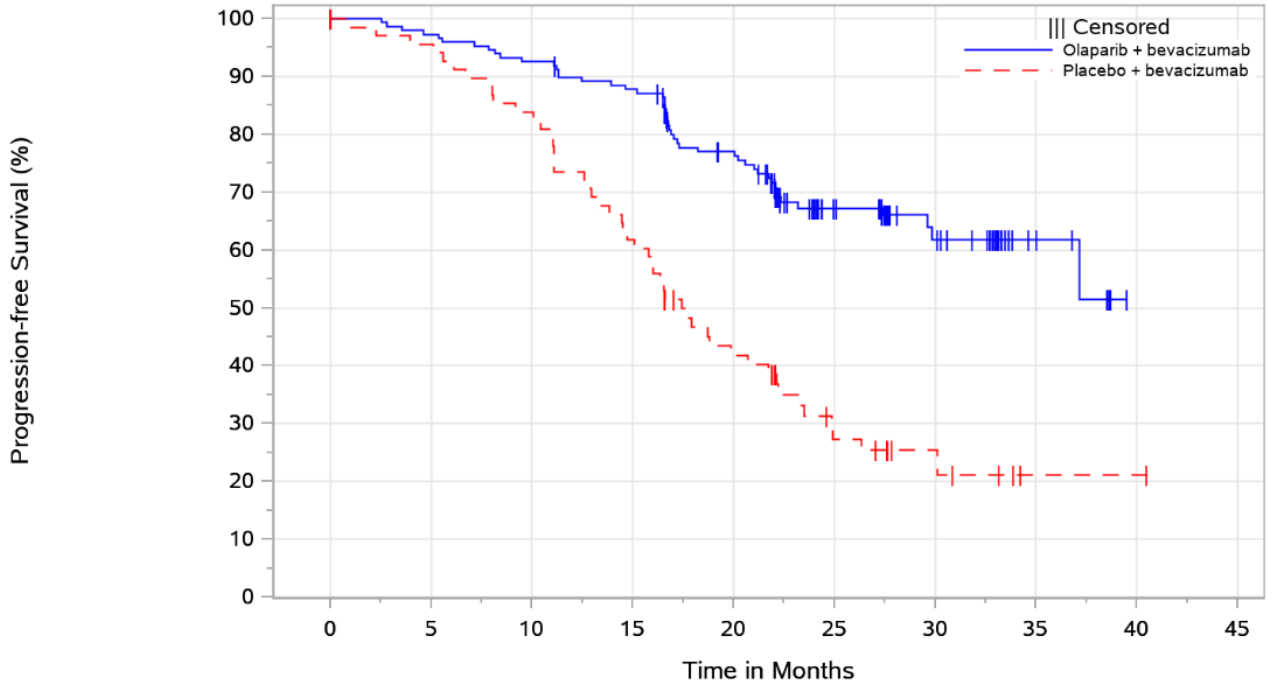
The clinical performance of TSO Comprehensive HRD (DRAGEN) was established by determination of clinical efficacy in the HRD-positive population. PFS hazard ratios (HR) for the olaparib vs placebo treatment groups were calculated using an unadjusted Cox proportional hazards model. Table 56 shows the PFS hazard ratios in the CTA and TSO Comprehensive HRD (DRAGEN) HRD-positive participants. Figure 4 shows the Kaplan-Meier curves for PFS in TSO Comprehensive HRD (DRAGEN) positive participants. In CTA and TSO Comprehensive HRD (DRAGEN) HRD-positive populations, the PFS hazard ratio was 0.33 (95% CI: 0.25, 0.45) and 0.32 (95% CI: 0.22, 0.48), respectively, and represented a 67% and 68% reduction in the risk of progression or death for participants in the olaparib plus bevacizumab group compared to the placebo plus bevacizumab group. A sensitivity analysis addressing the efficacy of imputed missing CDx results demonstrated that the PFS hazard ratios in the imputed HRD-positive populations were consistent with the primary analysis estimates.

Table 56 Clinical Efficacy in the CTA and TSO Comprehensive HRD (DRAGEN) Assay

		HRD Positive	
		Olaparib +bev	**Placebo +bev**
CTA	N	225	132
	PFS[1] (95% CI)[2]	37.2 (36.0, NR[4])	17.7 (15.8, 19.9)
	PFS HR (95% CI)[3]	0.33 (0.25, 0.45)	
TSO Comprehensive HRD (DRAGEN)	N	149	71
	PFS[1] (95% CI)[2]	NR[4] (37.2, NR[4])	17.4 (14.7, 22.1)
	PFS HR (95% CI)[3]	0.32 (0.22, 0.48)	

[1] PFS median in months.
[2] From product-limit (Kaplan-Meier) method for censored data.
[3] Based on unstratified Cox regression model with treatment as covariate.
[4] NR = Not Reached

Figure 4 Kaplan-Meier Curve - PFS in HRD-positive participants identified by TSO Comprehensive HRD (DRAGEN)



At Risk

Olaparib + bevacizumab	149	144	137	129	101	60	29	8	0	0
Placebo + bevacizumab	71	65	57	42	26	14	6	1	1	0

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Revision History

Document	Date	Description of Change
Document # 200080934 v00	May 2026	Initial release.

Patents and Trademarks

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Product Labeling

For a complete reference of symbols that appear on product packaging and labeling, refer to the symbol key at support.illumina.com on the *Documentation* tab for your kit.

A Summary of Safety and Performance (SSP) is located at https://ec.europa.eu/tools/eudamed, after launch of the European Database on Medical Devices (Eudamed). It is linked to the Basic UDI-DI (0081627002TSOHRDDGRK).