TruSight Oncology Comprehensive (EU)

illumina®

Package Insert

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Intended Use

TruSight Oncology Comprehensive (EU) is an *in vitro* diagnostic test that uses targeted next-generation sequencing to detect variants in 517 genes using nucleic acids extracted from formalin-fixed, paraffin embedded (FFPE) tumor tissue samples from cancer patients with solid malignant neoplasms using the Illumina® NextSeq™ 550Dx instrument. The test can be used to detect single nucleotide variants, multi-nucleotide variants, insertions, deletions, and gene amplifications from DNA, and gene fusions and splice variants from RNA. The test also reports a Tumor Mutational Burden (TMB) score and Microsatellite Instability (MSI) status.

The test is intended as a companion diagnostic to identify cancer patients for treatment with the targeted therapy listed in Table 1, in accordance with the approved therapeutic product labeling. In addition, the test is intended to provide tumor profiling information for use by qualified health care professionals in accordance with professional guidelines and is not conclusive or prescriptive for labeled use of any specific therapeutic product.

Table 1 Companion Diagnostics Indication

Tumor Type	Biomarkers	Targeted Therapy
Solid Tumors	NTRK1, NTRK2, and NTRK3 Gene Fusions	VITRAKVI® (larotrectinib)

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Summary and Explanation of the Assay

Clinical Description

Cancer is a leading cause of death worldwide and has the potential to originate in any tissue.[1] Analysis of a tumor's genetic basis is important for identifying patients that can benefit from targeted therapies and for developing new methods of treatment. Numerous genes have been implicated in tumor causation or progression, and many tumors carry a variety of variants affecting these genes and their functions. These variants can include gene mutations such as single-nucleotide variants (SNVs), multi-nucleotide variants (MNVs), insertions or deletions, gene amplifications, gene fusions, and splice variants. Another consequence of tumor gene mutations is the presentation of neoantigens that elicit tumor-specific immune responses. The mutational state of a tumor can be represented by TMB and MSI, which are genomic signatures that are associated with tumor neoantigen presentation.

TruSight Oncology Comprehensive is a next-generation sequencing (NGS) comprehensive genomic profiling (CGP) test that broadly assesses genomic variants in a large panel of cancer-related genes listed in Table 2. The assay detects small variants in 517 genes, plus gene amplifications, fusions, and splice variants as indicated in Table 2. The assay provides coding sequence coverage for all genes except TERT, where only the promoter region is covered, and assesses TMB score and MSI status. These assay targets include content cited by professional organizations such as the European Society for Medical Oncology (ESMO) and other major US guidelines.[2] Independent consortia publications and late-stage pharmaceutical research also influenced the design of the TSO Comprehensive assay.

For a list of regions that are excluded from variant calling, refer to the *TruSight Oncology Comprehensive Block List (document # 200009524)* available at the Illumina support site. The block list is referred to as blacklist in some files.

In Table 2, four variant type categories are identified: Small DNA variant (S), gene amplification (A), fusion (F), and splice variant (Sp). Small DNA variants include SNVs, MNVs, and insertions and deletions.

Table 2 TSO Comprehensive (EU) Assay Gene Panel

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
1	25	ABL1	S	176	2261	FGFR3	S, F	351	7849	PAX8	S
2	27	ABL2	S	177	2264	FGFR4	S	352	55193	PBRM1	S
3	84142	ABRAXAS1	S	178	2271	FH	S	353	5133	PDCD1	S
4	90	ACVR1	S	179	201163	FLCN	S	354	80380	PDCD1LG2	S
5	91	ACVR1B	S	180	2313	FLI1	S	355	5156	PDGFRA	S
6	25960	ADGRA2	S	181	2321	FLT1	S	356	5159	PDGFRB	S
7	207	AKT1	S	182	2322	FLT3	S	357	5163	PDK1	S
8	208	AKT2	S	183	2324	FLT4	S	358	5170	PDPK1	S
9	10000	AKT3	S	184	3169	FOXA1	S	359	5241	PGR	S
10	238	ALK	S, F	185	668	FOXL2	S	360	84295	PHF6	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
11	242	ALOX12B	S	186	2308	FOXO1	S	361	8929	PHOX2B	S
12	139285	AMER1	S	187	27086	FOXP1	S	362	5287	PIK3C2B	S
13	29123	ANKRD11	S	188	10818	FRS2	S	363	5288	PIK3C2G	S
14	22852	ANKRD26	S	189	8880	FUBP1	S	364	5289	PIK3C3	S
15	324	APC	S	190	2534	FYN	S	365	5290	PIK3CA	S
16	367	AR	S	191	2559	GABRA6	S	366	5291	PIK3CB	S
17	369	ARAF	S	192	2623	GATA1	S	367	5293	PIK3CD	S
18	10139	ARFRP1	S	193	2624	GATA2	S	368	5294	PIK3CG	S
19	8289	ARID1A	S	194	2625	GATA3	S	369	5295	PIK3R1	S
20	57492	ARID1B	S	195	2626	GATA4	S	370	5296	PIK3R2	S
21	196528	ARID2	S	196	2627	GATA6	S	371	8503	PIK3R3	S
22	84159	ARID5B	S	197	348654	GEN1	S	372	5292	PIM1	S
23	171023	ASXL1	S	198	79018	GID4	S	373	5336	PLCG2	S
24	55252	ASXL2	S	199	2735	GLI1	S	374	10769	PLK2	S
25	472	ATM	S	200	2767	GNA11	S	375	5366	PMAIP1	S
26	545	ATR	S	201	10672	GNA13	S	376	5378	PMS1	S
27	546	ATRX	S	202	2776	GNAQ	S	377	5395	PMS2	S
28	6790	AURKA	S	203	2778	GNAS	S	378	10957	PNRC1	S
29	9212	AURKB	S	204	2874	GPS2	S	379	5424	POLD1	S
30	8312	AXIN1	S	205	26585	GREM1	S	380	5426	POLE	S
31	8313	AXIN2	S	206	2903	GRIN2A	S	381	5468	PPARG	S
32	558	AXL	S, F	207	2913	GRM3	S	382	8493	PPM1D	S
33	567	B2M	S	208	2932	GSK3B	S	383	5518	PPP2R1A	S
34	8314	BAP1	S	209	3020	H3F3A	S	384	5520	PPP2R2A	S
35	580	BARD1	S	210	3021	H3F3B	S	385	5537	PPP6C	S
36	27113	BBC3	S	211	440093	H3F3C	S	386	639	PRDM1	S
37	8915	BCL10	S	212	3082	HGF	S	387	80243	PREX2	S
38	596	BCL2	S, F	213	3006	HIST1H1C	S	388	5573	PRKAR1A	S
39	598	BCL2L1	S	214	3017	HIST1H2BD	S	389	5584	PRKCI	S
40	10018	BCL2L11	S	215	8350	HIST1H3A	S	390	5591	PRKDC	S
41	599	BCL2L2	S	216	8358	HIST1H3B	S	391	5071	PRKN	S
42	604	BCL6	S	217	8352	HIST1H3C	S	392	5652	PRSS8	S
43	54880	BCOR	S	218	8351	HIST1H3D	S	393	5727	PTCH1	S
44	63035	BCORL1	S	219	8353	HIST1H3E	S	394	5728	PTEN	S
45	613	BCR	S	220	8968	HIST1H3F	S	395	5781	PTPN11	S
46	330	BIRC3	S	221	8355	HIST1H3G	S	396	5789	PTPRD	S
47	641	BLM	S	222	8357	HIST1H3H	S	397	5802	PTPRS	S
48	657	BMPR1A	S	223	8354	HIST1H3I	S	398	11122	PTPRT	S
49	673	BRAF	S, F	224	8356	HIST1H3J	S	399	9444	QKI	S
50	672	BRCA1	S	225	333932	HIST2H3A	S	400	11021	RAB35	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
51	675	BRCA2	S	226	126961	HIST2H3C	S	401	5879	RAC1	S
52	23476	BRD4	S	227	653604	HIST2H3D	S	402	5885	RAD21	S
53	83990	BRIP1	S	228	8290	HIST3H3	S	403	10111	RAD50	S
54	694	BTG1	S	229	6927	HNF1A	S	404	5888	RAD51	S
55	695	BTK	S	230	3190	HNRNPK	S	405	5890	RAD51B	S
56	811	CALR	S	231	10481	HOXB13	S	406	5889	RAD51C	S
57	84433	CARD11	S	232	3265	HRAS	S	407	5892	RAD51D	S
58	841	CASP8	S	233	3283	HSD3B1	S	408	5893	RAD52	S
59	865	CBFB	S	234	3320	HSP90AA1	S	409	8438	RAD54L	S
60	867	CBL	S	235	23308	ICOSLG	S	410	5894	RAF1	S, F
61	595	CCND1	S	236	3399	ID3	S	411	5903	RANBP2	S
62	894	CCND2	S	237	3417	IDH1	S	412	5914	RARA	S
63	896	CCND3	S	238	3418	IDH2	S	413	5921	RASA1	S
64	898	CCNE1	S	239	3459	IFNGR1	S	414	5925	RB1	S
65	29126	CD274	S	240	3479	IGF1	S	415	8241	RBM10	S
66	80381	CD276	S	241	3480	IGF1R	S	416	9401	RECQL4	S
67	972	CD74	S	242	3481	IGF2	S	417	5966	REL	S
68	973	CD79A	S	243	9641	IKBKE	S	418	5979	RET	S, F
69	974	CD79B	S	244	10320	IKZF1	S	419	6009	RHEB	S
70	79577	CDC73	S	245	3586	IL10	S	420	387	RHOA	S
71	999	CDH1	S	246	3575	IL7R	S	421	253260	RICTOR	S
72	51755	CDK12	S	247	3623	INHA	S	422	6016	RIT1	S
73	1019	CDK4	S	248	3624	INHBA	S	423	54894	RNF43	S
74	1021	CDK6	S	249	3631	INPP4A	S	424	6098	ROS1	S, F
75	1024	CDK8	S	250	8821	INPP4B	S	425	8986	RPS6KA4	S
76	1026	CDKN1A	S	251	3643	INSR	S	426	6198	RPS6KB1	S
77	1027	CDKN1B	S	252	3660	IRF2	S	427	6199	RPS6KB2	S
78	1029	CDKN2A	S	253	3662	IRF4	S	428	57521	RPTOR	S
79	1030	CDKN2B	S	254	3667	IRS1	S	429	861	RUNX1	S
80	1031	CDKN2C	S	255	8660	IRS2	S	430	862	RUNX1T1	S
81	1050	CEBPA	S	256	3716	JAK1	S	431	23429	RYBP	S
82	1058	CENPA	S	257	3717	JAK2	S	432	6389	SDHA	S
83	1106	CHD2	S	258	3718	JAK3	S	433	54949	SDHAF2	S
84	1108	CHD4	S	259	3725	JUN	S	434	6390	SDHB	S
85	1111	CHEK1	S	260	7994	KAT6A	S	435	6391	SDHC	S
86	11200	CHEK2	S	261	5927	KDM5A	S	436	6392	SDHD	S
87	23152	CIC	S	262	8242	KDM5C	S	437	26040	SETBP1	S
88	64326	COP1	S	263	7403	KDM6A	S	438	29072	SETD2	S
89	1387	CREBBP	S	264	3791	KDR	S	439	23451	SF3B1	S
90	1399	CRKL	S	265	9817	KEAP1	S	440	10019	SH2B3	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
91	64109	CRLF2	S	266	3792	KEL	S	441	4068	SH2D1A	S
92	1436	CSF1R	S	267	3799	KIF5B	S, F	442	55164	SHQ1	S
93	1441	CSF3R	S	268	3815	KIT	S	443	9353	SLIT2	S
94	1452	CSNK1A1	S	269	9314	KLF4	S	444	84464	SLX4	S
95	10664	CTCF	S	270	89857	KLHL6	S	445	4087	SMAD2	S
96	1493	CTLA4	S	271	4297	KMT2A	S	446	4088	SMAD3	S
97	1495	CTNNA1	S	272	3845	KRAS	S	447	4089	SMAD4	S
98	1499	CTNNB1	S	273	3916	LAMP1	S	448	6597	SMARCA4	S
99	8452	CUL3	S	274	9113	LATS1	S	449	6598	SMARCB1	S
100	1523	CUX1	S	275	26524	LATS2	S	450	6602	SMARCD1	S
101	7852	CXCR4	S	276	4004	LMO1	S	451	8243	SMC1A	S
102	1540	CYLD	S	277	53353	LRP1B	S	452	9126	SMC3	S
103	1616	DAXX	S	278	4067	LYN	S	453	6608	SMO	S
104	54165	DCUN1D1	S	279	8216	LZTR1	S	454	9627	SNCAIP	S
105	4921	DDR2	S	280	9863	MAGI2	S	455	8651	SOCS1	S
106	51428	DDX41	S	281	10892	MALT1	S	456	6663	SOX10	S
107	1665	DHX15	S	282	5604	MAP2K1	S	457	64321	SOX17	S
108	23405	DICER1	S	283	5605	MAP2K2	S	458	6657	SOX2	S
109	22894	DIS3	S	284	6416	MAP2K4	S	459	6662	SOX9	S
110	3337	DNAJB1	S	285	4214	MAP3K1	S	460	23013	SPEN	S
111	1786	DNMT1	S	286	9175	MAP3K13	S	461	8405	SPOP	S
112	1788	DNMT3A	S	287	9020	MAP3K14	S	462	6708	SPTA1	S
113	1789	DNMT3B	S	288	4216	MAP3K4	S	463	6714	SRC	S
114	84444	DOT1L	S	289	5594	MAPK1	S	464	6427	SRSF2	S
115	1871	E2F3	S	290	5595	MAPK3	S	465	10274	STAG1	S
116	8726	EED	S	291	4149	MAX	S	466	10735	STAG2	S
117	51162	EGFL7	S	292	4170	MCL1	S	467	6774	STAT3	S
118	1956	EGFR	S, F, Sp	293	9656	MDC1	S	468	6775	STAT4	S
119	1964	EIF1AX	S	294	4193	MDM2	S	469	6776	STAT5A	S
120	1974	EIF4A2	S	295	4194	MDM4	S	470	6777	STAT5B	S
121	1977	EIF4E	S	296	9968	MED12	S	471	6794	STK11	S
122	6921	ELOC	S	297	100271849	MEF2B	S	472	83931	STK40	S
123	27436	EML4	S, F	298	4221	MEN1	S	473	51684	SUFU	S
124	56946	EMSY	S	299	4233	MET	S, A, Sp	474	23512	SUZ12	S
125	2033	EP300	S	300	23269	MGA	S	475	6850	SYK	S
126	4072	EPCAM	S	301	4286	MITF	S	476	6872	TAF1	S
127	2042	EPHA3	S	302	4292	MLH1	S	477	6926	TBX3	S
128	2044	EPHA5	S	303	4300	MLLT3	S	478	6929	TCF3	S
129	2045	EPHA7	S	304	4352	MPL	S	479	6934	TCF7L2	S
130	2047	EPHB1	S	305	4361	MRE11	S	480	7012	TERC	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
131	2064	ERBB2	S, A	306	4436	MSH2	S	481	7015	TERT	S
132	2065	ERBB3	S	307	4437	MSH3	S	482	80312	TET1	S
133	2066	ERBB4	S	308	2956	MSH6	S	483	54790	TET2	S
134	2067	ERCC1	S	309	4485	MST1	S	484	7030	TFE3	S
135	2068	ERCC2	S	310	4486	MST1R	S	485	7037	TFRC	S
136	2071	ERCC3	S	311	2475	MTOR	S	486	7046	TGFBR1	S
137	2072	ERCC4	S	312	4595	MUTYH	S	487	7048	TGFBR2	S
138	2073	ERCC5	S	313	4602	MYB	S	488	55654	TMEM127	S
139	2078	ERG	S, F	314	4609	MYC	S	489	7113	TMPRSS2	S, F
140	54206	ERRFI1	S	315	4610	MYCL	S	490	7128	TNFAIP3	S
141	2099	ESR1	S, F	316	4613	MYCN	S	491	8764	TNFRSF14	S
142	2113	ETS1	S	317	4615	MYD88	S	492	7150	TOP1	S
143	2115	ETV1	S, F	318	4654	MYOD1	S	493	7153	TOP2A	S
144	2118	ETV4	S, F	319	4665	NAB2	S	494	7157	TP53	S
145	2119	ETV5	S	320	4683	NBN	S	495	8626	TP63	S
146	2120	ETV6	S	321	8202	NCOA3	S	496	7186	TRAF2	S
147	2130	EWSR1	S	322	9611	NCOR1	S	497	84231	TRAF7	S
148	2146	EZH2	S	323	257194	NEGR1	S	498	7248	TSC1	S
149	54855	FAM46C	S	324	4763	NF1	S	499	7249	TSC2	S
150	2175	FANCA	S	325	4771	NF2	S	500	7253	TSHR	S
151	2176	FANCC	S	326	4780	NFE2L2	S	501	7307	U2AF1	S
152	2177	FANCD2	S	327	4792	NFKBIA	S	502	7422	VEGFA	S
153	2178	FANCE	S	328	7080	NKX2-1	S	503	7428	VHL	S
154	2188	FANCF	S	329	4824	NKX3-1	S	504	79679	VTCN1	S
155	2189	FANCG	S	330	4851	NOTCH1	S	505	8838	WISP3	S
156	55215	FANCI	S	331	4853	NOTCH2	S	506	7490	WT1	S
157	55120	FANCL	S	332	4854	NOTCH3	S	507	331	XIAP	S
158	355	FAS	S	333	4855	NOTCH4	S	508	7514	XPO1	S
159	2195	FAT1	S	334	4869	NPM1	S	509	7516	XRCC2	S
160	55294	FBXW7	S	335	4893	NRAS	S	510	10413	YAP1	S
161	2246	FGF1	S	336	3084	NRG1	S, F	511	7525	YES1	S
162	2255	FGF10	S	337	64324	NSD1	S	512	57621	ZBTB2	S
163	2259	FGF14	S	338	4914	NTRK1	S, F	513	51341	ZBTB7A	S
164	9965	FGF19	S	339	4915	NTRK2	S, F	514	463	ZFHX3	S
165	2247	FGF2	S	340	4916	NTRK3	S, F	515	7764	ZNF217	S
166	8074	FGF23	S	341	9688	NUP93	S	516	80139	ZNF703	S
167	2248	FGF3	S	342	256646	NUTM1	S	517	8233	ZRSR2	S
168	2249	FGF4	S	343	5058	PAK1	S	N/A	N/A	N/A	N/A
169	2250	FGF5	S	344	5063	PAK3	S	N/A	N/A	N/A	N/A
170	2251	FGF6	S	345	57144	PAK5	S	N/A	N/A	N/A	N/A

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
171	2252	FGF7	S	346	79728	PALB2	S	N/A	N/A	N/A	N/A
172	2253	FGF8	S	347	142	PARP1	S	N/A	N/A	N/A	N/A
173	2254	FGF9	S	348	5077	PAX3	S, F	N/A	N/A	N/A	N/A
174	2260	FGFR1	S, F	349	5079	PAX5	S	N/A	N/A	N/A	N/A
175	2263	FGFR2	S, F	350	5081	PAX7	S	N/A	N/A	N/A	N/A

Principles of Procedure

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

The TSO Comprehensive (EU) assay involves the following processes.

- **Library Preparation and Enrichment**—For RNA, 40 ng total is converted to double-stranded complementary DNA (cDNA). For genomic DNA (gDNA), 40 ng of gDNA is sheared into small fragments. Universal adapters for sequencing are ligated onto the cDNA and gDNA fragments. P5 and P7 index sequences are incorporated into each library to enable the capture of library fragments onto the surface of the flow cell during sequencing. The indexes include a unique sequence to identify each individual sample and, in the case of libraries from gDNA samples, 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 Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module resides on the NextSeq 550Dx instrument as part of the Local Run Manager (LRM) software to facilitate TSO Comprehensive (EU) run setup and to perform the secondary analysis of sequencing results. Secondary analysis 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. FASTQ intermediate files are generated which contain the sequencing reads for each sample and the quality scores, 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 for libraries generated from DNA and/or RNA samples to call small DNA variants, gene amplifications, TMB, and

MSI for DNA samples, and fusions and splice variants for RNA samples. 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. Refer to the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661) for details on secondary and tertiary analysis.

- **Tertiary Analysis**—Tertiary analysis, performed by the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module, consists of TMB and MSI calculations, companion diagnostic calling, tumor profiling of variants into two levels of clinical significance using a Knowledge Base (KB) and the tissue type, and results report generation. Tumor profiling can also be referred to as comprehensive genomic profiling. The interpreted variant results, as well as the TMB and MSI biomarker results, are summarized in the TSO Comprehensive (EU) results report.

Limitations of the Procedure

For *in vitro* diagnostic use only.

- For prescription use only. The test must be used in accordance with clinical laboratory regulations.
- Genomic findings listed in Table 2 of the intended use are not prescriptive or conclusive for labeled use of any specific therapeutic product.
- For variants listed in the TSO Comprehensive (EU) results report under Genomic Findings with Evidence of Clinical Significance and Genomic Findings with Potential Clinical Significance, clinical validation has not been performed.
- 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. 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 77). FFPE blocks that have been stored longer than five years have demonstrated lower validity.
- Performance of TSO Comprehensive (EU) in samples obtained from patients that have had organ or tissue transplantation has not been evaluated.
- In highly rearranged genomes with deletions and Loss of Heterozygosity, TSO Comprehensive (EU) software can erroneously classify a DNA sample as contaminated (CONTAMINATION_SCORE > 3106 and p-value > 0.049).
- A negative result does not rule out the presence of a mutation below the limits of detection (LoD) of the assay.
- The sensitivity for detection of small DNA variants can be impacted by:
  - Low complexity genomic context

  - Increasing variant length
- TMB scores can be inaccurate in the following contexts:
  - As tumor content reaches levels where germline and somatic variant allele frequencies (VAFs) converge.
  - In populations not well represented in public databases.
- The sensitivity for detection of fusions can be impacted:
  - By low library complexity resulting in decreased supporting reads due to deviations in the assay workflow (For example, follow the mixing steps in *Denature and Anneal RNA* on page 42).
  - When a single gene spans both breakpoints.
  - In cases where several fusions breakpoints are in close proximity to each other with one or multiple partners; the multiple breakpoints and partners might be reported as a single breakpoint and partner.
  - By small median insert sizes; a minimum median insert size of 80 bp is required, but sensitivity decreases in the 80 – 100 bp range.
  - By low sequence complexity or homologous genomic context around fusion breakpoints.
- Resolution of the genes involved in a fusion can be impacted when fusion breakpoints occur in genomic regions containing overlapping genes. The assay will report all genes, delimited by semicolons, if multiple genes are overlapping a breakpoint.
- Inconsistent coverage in the TERT Promoter region can result in a No Result due to low depth.
- Annotation or KB errors can cause a false positive or false negative result, including listing a variant in the wrong level (between Genomic Findings with Evidence of Clinical Significance and Genomic Findings with Potential Clinical Significance), or the annotation information in the report could be incorrect. The possibility of error exists from three sources:
  - TSO Comprehensive (EU) variant annotation. There is an error rate of approximately 0.0027%, based on an analysis of 2,448,350 variants from COSMIC v92, therefore there is a low possibility for error.
  - KB error due to the curation or tiering process.
  - The relevance of KB content changes over time. The report will reflect the knowledge at the time when the KB version was curated.
  - Variants reported in the CDx Results are not impacted by annotation or KB errors.
- TSO Comprehensive (EU) is designed to report somatic variants when reporting variants with evidence of clinical significance or variants with potential clinical significance. As a tumor-only test, germline (inherited) variant reporting is possible but unintentional. TSO Comprehensive (EU) uses a KB to report variants without explicitly annotating if they are of germline or somatic origin.
- The KB only includes therapeutic, diagnostic, and prognostic associations that are relevant for variants present within an established solid malignant neoplasm. Susceptibility or cancer risk associations are not included in the KB.

Product Components

The TruSight Oncology Comprehensive (EU) test consists of the following components:

- TruSight Oncology Comprehensive (EU) kit (Illumina catalog # 20063092): The kit includes reagents with sufficient volume to generate 24 DNA and 24 RNA libraries with controls, which include patient samples and controls. Controls sold separately (refer to *Reagents Required, Not Provided* on page 17).
- Knowledge Base: Updated regularly and available for download on the Illumina Lighthouse Portal.
- Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module (Illumina catalog # 20051843*), which includes the following components and supports tumor profiling and NTRK:
  - Claims Packages TSO Comprehensive (EU) v2.1.0 (PN 20079589)
  - TSO Comprehensive (EU) v2.3.6 Software Suite (PN 20079588)
  - TSO Comprehensive (EU) v2.3.6 USB Kit (PN 20079591)
- Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module (Illumina catalog # 20051843*), which includes the following components and supports tumor profiling and NTRK:
  - Claims Packages TSO Comprehensive (EU) v2.0.0 (PN 20051760)
  - TSO Comprehensive (EU) v2.3.5 Software Suite (PN 20075244)
  - TSO Comprehensive (EU) v2.3.5 USB Kit (PN 20075239)

  * Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module: An Illumina service representative installs the appropriate version of the TSO Comprehensive (EU) analysis module on the Local Run Manager NextSeq 550Dx instrument. Refer to Table 3 for the Workflow Guide and Analysis Module software version.

Table 3 Workflow Guide for TSO Comprehensive Analysis Module Software Version

Workflow Guide	Tissue	TSO Comprehensive Software Version
200008661	FFPE	v2.3.5 or v2.3.6

Reagents

Reagents Provided

The following reagents are provided with the TSO Comprehensive (EU) kit.

TruSight Oncology Comp RNA Library Prep, PN 20031127

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
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 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

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
Adapter 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
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

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.

Note Use Unique Index Primers (UPxx) for RNA or DNA samples.

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

Use Combinatorial Index Primers (CPxx) for DNA samples only (FFPE workflow).

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 1 (LNB1)	20031481	1	1.04 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C
Library Normalization Wash 1 (LNW1)	20031482	2	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	2°C to 8°C

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

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
PhiX Internal Control (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

Reagents Required, Not Provided

Pre-amp Reagents

- DNA and RNA Extraction and Purification Reagents—Refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 24 for reagent requirements.
- DNA and RNA Quantification Reagents—Refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 24 for reagent requirements.
- TruSight Oncology DNA Control (Illumina catalog # 20065041)
- TruSight Oncology RNA Control (Illumina catalog # 20065042)
- Ethanol 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)
- Ethanol 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 RNA Library Prep	20031127	Pre-amp
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

CAUTION

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

- 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

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.
- Stability of the TSO Comprehensive (EU) 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 *Optimization of Ultrasonicators to Fragment DNA* on page 22.	General lab supplier
Thermal cycler with the following specifications: • Heated lid capable of being set to 30°C and 100°C (or turned off if not capable of 30°C) • Encompass a 4°C to 99°C temperature range • ±0.25°C temperature accuracy • Compatible with 96-well PCR plates, 0.2 ml (polypropylene) • Refer to *Thermal Cycler Ramp Rate* on page 23	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
Centrifuge (plate centrifuge) with the following capabilities: • Centrifugation of 96-well microplates • Capable of 280 × g	General lab supplier
Plate shaker with the following capabilities: • 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 the bottom • For 96-well MIDI plates	General lab supplier
Precision pipettes • 20 µl single- or multichannel pipettes • 200 µl single- or multichannel pipettes • 1000 µl single- or multichannel pipette That meet the following requirements: • Calibrated regularly, within 5% accuracy.	General lab supplier

Equipment	Supplier
Pipette-aid	General lab supplier
10 ml serological pipettes	General lab supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable, optically clear polyester • Suitable for skirted or semiskirted PCR plates • Strong adhesive that withstands multiple temperature changes of -40°C to 110°C • DNase/RNase-free	General lab supplier
1.7 ml microcentrifuge tubes, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (PVC, disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
20 µl aerosol resistant pipette tips	General lab supplier
200 µl aerosol resistant pipette tips	General lab supplier
1000 µl 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, 0.2 ml (polypropylene)	General lab supplier

Post-amp Equipment and Materials

Equipment	Supplier
NextSeq 550Dx Instrument	Illumina, catalog # 20005715
Centrifuge (plate centrifuge) with the following capabilities: • Centrifugation of 96-well microplates • Capable of 280 × g	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 (polypropylene) • Refer to *Thermal Cycler Ramp Rate* on page 23	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 • Ensure microcentrifuge tubes are compatible with the heat block	General lab supplier
Plate shaker with the following capabilities: • 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 the bottom • For 96-well MIDI plates	General lab supplier
Precision pipettes • 20 µl single- or multichannel pipettes • 200 µl single- or multichannel pipettes • 1000 µl single- or multichannel pipette That meet the following requirements: • Calibrated regularly, within 5% accuracy.	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, optically clear polyester • Suitable for skirted or semiskirted PCR plates • Strong adhesive that withstands multiple temperature changes of -40°C to 110°C • DNase/RNase-free	General lab supplier
Microcentrifuge tubes, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (PVC, disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
20 µl aerosol resistant pipette tips	General lab supplier
200 µl aerosol resistant pipette tips	General lab supplier
1000 µl aerosol resistant pipette tips	General lab supplier

Equipment	Supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859 or equivalent
96-well PCR plates, 0.2 ml (polypropylene)	General lab supplier

Optimization of Ultrasonicators to Fragment DNA

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 (EU) assay (Table 4). Shearing time was adjusted to maximize the MEDIAN_EXON_COVERAGE metric outlined in *Quality Control* on page 77. Shearing times (bolded in Table 4) differed across configurations as did MEDIAN_INSERT_SIZE results. All three configurations were tested with 8-strip tubes; volumes used are shown in Table 4.

Optimization of configuration 3 (point transducer, non-degassed water, small water bath volume) used pulsing and had the shortest shearing time resulting in a slightly larger fragment size distribution compared to the other two configurations (MEDIAN_INSERT_SIZE was approximately 5 – 10 base pairs larger). Furthermore, configuration 3 needed an increased DNA input (50 ng) to achieve similar MEDIAN_EXON_COVERAGE relative to the other two configurations, which used the nominal 40 ng input. Configuration 3 has 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 ensure that the specified volume is retrieved as any loss of material can adversely affect performance.

Table 4 Focused-Ultrasonicator Configurations Evaluated

	Configuration		
Parameter	**1**	**2**	**3**
Transducer	Line	Point	Point
Water bath volume	5 L	5 L	85 ml
Water degassed	Yes	Yes	No
Water Chiller	Yes	Yes	Yes
Water Bath Temperature	7°C	7°C	12°C
Peak Incident Power (PIP)	450 W	175 W	50 W
% Duty Factor	30	10	30
Cycles per Burst	200	200	1000
Pulsing (10 sec bursts)	No	No	Yes
Shearing Time	**250 s**	**280 s**	**200 s** *
Sample Processing	1 – 8	1	1
Batch Size	1 – 96	1 – 96	1 – 8
Glass 8-Strip tube sample size	130 µl	130 µl	50 µl
DNA Input Equivalent (for median exon coverage)	40 ng	40 ng	50 ng

* The shearing time of 200 seconds consists of 10-second bursts with 20 repeats.

Thermal Cycler Ramp Rate

Thermal cycling ramp rate affects assay QC metrics—Usable MSI Sites, Median Bin Count CNV Target, Median Insert Size (RNA)—as well as supporting reads for splice variants and fusions. Optimization of thermal cycler ramp rate is recommended. For example, a tested model was adjusted from a default (and maximum) ramp rate of 5 degrees C/s to 3 degrees C/s to obtain comparable results to other models with lower default ramp rates.

Specimen Collection, Transport, and Storage

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

Sample Requirements

FFPE Tissue

The TSO Comprehensive (EU) assay requires 40 ng RNA and/or 40 ng DNA extracted from FFPE tissue. Using both RNA and DNA enables analysis of all claimed variant types. Tissue should be fixed using formalin fixative suitable for molecular analyses (for example, 10% neutral-buffered formalin). Tissue cannot be decalcified. Before performing the TSO Comprehensive (EU) assay, the tissue sample should be examined by a pathologist to ensure 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 30 % tumor content is required to detect MSI-high. Tumor content for gene amplifications and RNA variants depends on the extent of amplification or fusion expression (refer to *Tumor Content* on page 94).

For a high probability of extracting 40 ng RNA and 40 ng DNA from various solid tissue types, the recommended tissue volume is ≥ 1.0 mm^3, which 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 amount of nucleic acid yield. To minimize the possibility of false negative results, the tissue may be macrodissected to achieve a desirable viable tumor content.

High amount of necrotic tissue (≥ 25%) can interfere with the ability of the TSO Comprehensive (EU) assay to detect gene amplifications and RNA fusions.

Nucleic Acid Extraction, Quantification, and Storage

- Extract RNA and 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 86.
- Store extracted stock nucleic acid following the instructions from the extraction kit manufacturer.
- To avoid changes in concentration over time, measure DNA and RNA immediately before starting library preparation. Quantify RNA and 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 RNA sample prepared in RNase/DNase-free water (not provided), with a final volume of 8.5 µl (4.7 ng/µl).

- 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 30 days, depending on the type of library (Refer to Table 5).

Table 5 Library Storage Times

Library Type	Plate	Number of Days	Storage Temperature
cDNA	PCF PCR	≤ 7	-25°C to -15°C
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	≤ 30	-25°C to -15°C

Warnings and Precautions

Safety

1. **Some components of this assay contain potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. 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 Safety Data Sheets (SDS), visit support.illumina.com/sds.html.
2. Handle all specimens as if they are known to be infectious.
3. 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.

Laboratory

1. 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.
2. Perform Index PCR and Enrichment steps in a post-amplification area to prevent amplification product carryover.
3. 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.
4. 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.
5. Use nuclease-free microcentrifuge tubes, plates, pipette tips, and reservoirs.
6. Use calibrated equipment throughout the assay. Make sure to calibrate equipment to the speeds, temperatures, and volumes specified in this protocol.
7. Use precision pipettes to ensure accurate reagent and sample delivery. Calibrate regularly according to manufacturer specifications.
8. 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 dispensed from every tip.

9. Make sure to use equipment specified for the assay and to set programs as directed.
10. Stated temperatures for the thermal cycler and the microsample incubator indicate reaction temperature, not necessarily the set temperature of the equipment.

Assay

1. 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 strip tube, trough, or reservoir.
   - Mix samples with a pipette and centrifuge the plate when indicated.
   - Use a microplate shaker. Do not vortex the plates.
2. 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.
3. 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.
4. 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 37.
5. Failure to follow the procedures as outlined can result in erroneous results, or a significant reduction in library quality.
6. Unless a safe stopping point is specified in the *Instructions for Use* on page 37, proceed immediately to the next step.
7. Store the assay reagents or components at the specified temperature in designated pre-amplification and post-amplification areas.

8. Do not store reagents in a frost-free storage unit or in refrigerator door compartments.
9. Do not freeze reagents containing beads (LNB1, SPB, and SMB).
10. Do not use reagents that have been stored improperly.
11. 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.
12. Prepare fresh master mixes and discard the remaining volume after use.
13. 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.
14. Transfer the specified volume of eluate. Transferring less than the specified volume of eluate during the elution steps may impact results.
15. 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.
16. Do not pipette less than 2 µl sample input.
17. Do not use a trough to dispense reagents for steps that require less than 10 µl material to be added to each sample well.
18. Use a P20 pipette when transferring fragmented gDNA sample from the utrasonicator tubes to the Library Prep (LP) plate.
19. Do not combine UMI and SUA1 adapters together.
20. Use SUA1 adapters with RNA samples.
21. Use UMI adapters with DNA samples.
22. Assign different index primers to each library sample to uniquely identify each library when it is pooled for sequencing on a single flow cell.
23. Do not combine CPxx and UPxx index primers together in the same library.
24. Mismatches between the samples and indexing primers cause incorrect result reporting due to loss of positive sample identification. Enter sample IDs and assign indexes in the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module before beginning library preparation. Record sample IDs, indexing, and plate well orientation for reference during library preparation.
25. For libraries derived from RNA samples, use only UPxx indexes.
26. For libraries derived from DNA samples, use UPxx indexes or CPxx indexes.
27. Sequence 8 RNA libraries and 8 DNA libraries per flow cell. Refer to *Number of Libraries and Selecting Indexes* on page 34.

28. Sequence a minimum of three libraries. Follow guidelines in *Number of Libraries and Selecting Indexes* on page 34.
29. After the bind step in *Capture Targets One* on page 57 and *Capture Targets Two* on page 61, proceed immediately to the wash step to prevent bead pellets from drying.
30. During wash steps, ensure all 80% ethanol is removed from the bottom of the wells. Residual ethanol can impact results.
31. For optimal assay performance, follow the specified number of washes indicated in the *Instructions for Use* on page 37.
32. During the *Normalize Libraries* on page 67 procedure, thoroughly resuspend the library bead pellet to achieve consistent cluster density on the flow cell.

Procedural Notes

- The TSO Comprehensive (EU) workflow can be conducted according to the following schedule.
  - Day 1: cDNA Synthesis from RNA samples, 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 (EU) 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 RNA and DNA samples can be prepared simultaneously in separate wells.
- 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.

Thermal Cycler Programs

- Program thermal cycler programs on pre-amplification and 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 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 -40°C to 110°C, and suitable for skirted or semiskirted 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 37.
- 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 14
  - Volume of FSM = (7.2 µl) x (number of samples + controls) x (1.25).
  - Volume of RVT = (0.8 µl) x (number of samples + controls) x (1.25).
- Table 21
  - 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 29
  - 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 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 36
  - 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 37
  - 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

- The TSO Comprehensive (EU) assay includes UMI adapters and SUA1 adapters.
- SUA1 adapters are for use with RNA samples, not for DNA.
- UMI adapters are for use with DNA samples, not for RNA.

Handling Beads

- Three types of beads are included in the TSO Comprehensive (EU) 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.

Lab Tracking Form

- The *TruSight Oncology Comprehensive (EU) Lab Tracking Form (document # 200009022)* provides a checklist of the protocol steps.

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 negative/no-template controls (NTCs). NTCs must be added to the planned run as an additional sample.

For TSO Comprehensive (EU), follow the guidelines in Table 6 and Table 7 for determining the number of libraries to sequence on one flow cell.

Table 6 RNA or DNA Libraries for TSO Comprehensive (EU)

Library Type	Minimum*	Maximum
RNA	3	16
DNA	3	8

For optimal reagent usage when sequencing TSO Comprehensive (EU) on the NextSeq 550Dx instrument, sequence 8 RNA libraries + 8 DNA libraries per flow cell.

Table 7 RNA and DNA Libraries Combined for TSO Comprehensive (EU)

Number of DNA Libraries	Number of RNA Libraries
8	8

During library preparation, add the index primer to each sample library. ***Use a different index primer mix for each sample library***. Index primers uniquely identify each sample so that libraries can be pooled together for sequencing on one flow cell. (Compatible index combinations display on the Create Run screen during run setup on the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module.)

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

There are two types of indexes in the TSO Comprehensive (EU) assay.

- **UPxx indexes**—Use UPxx indexes for libraries derived from RNA or DNA samples.
- **CPxx indexes**—Use CPxx indexes for libraries derived from DNA samples. Do not use CPxx indexes for libraries derived from RNA or if sequencing three DNA libraries in total.

When sequencing only three libraries, the following is required.

- Libraries must be all DNA or all RNA.
- 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 Controls

TSO Comprehensive (EU) requires the use of the TruSight Oncology Controls, which consist of the TruSight Oncology DNA Control and the TruSight Oncology RNA Control as positive controls. Include the TruSight Oncology DNA Control for each DNA sequencing run and the TruSight Oncology RNA Control for each RNA sequencing run within a given library preparation event (include controls for combined DNA and RNA runs as well). A unique positive control is prepared for each planned sequenced run.

Include one NTC in each RNA and each DNA library preparation event. The NTC is sequenced repeatedly within one library preparation event. Follow these guidelines for the TruSight Oncology Controls:

- Prepare libraries from positive controls and no-template controls identically to samples.
- Use TEB for the DNA NTC.
- Use DNase/RNase-free water for the RNA 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 3 libraries.
- As the NTC is sequenced repeatedly, the indexes selected for this control cannot be repeated in the library preparation event.

The tables below show example plate layouts for library preparation. Each numbered column represents a single sequencing run. When sequencing DNA and RNA libraries together, each corresponding set of columns represents a single sequencing run (for example, column 1 and column 7). The NTC is sequenced for each column or set of columns.

Table 8 Library Preparation Event of a Single Run Including Six Patient Samples

	1	2	3	4	5	6	7
A	Pos DNA Control	empty	empty	empty	empty	empty	Pos RNA Control
B	DNA 1	empty	empty	empty	empty	empty	RNA 1
C	DNA 2	empty	empty	empty	empty	empty	RNA 2
D	DNA 3	empty	empty	empty	empty	empty	RNA 3
E	DNA 4	empty	empty	empty	empty	empty	RNA 4
F	DNA 5	empty	empty	empty	empty	empty	RNA 5
G	DNA 6	empty	empty	empty	empty	empty	RNA 6
H	DNA NTC	empty	empty	empty	empty	empty	RNA NTC

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

Instructions for Use

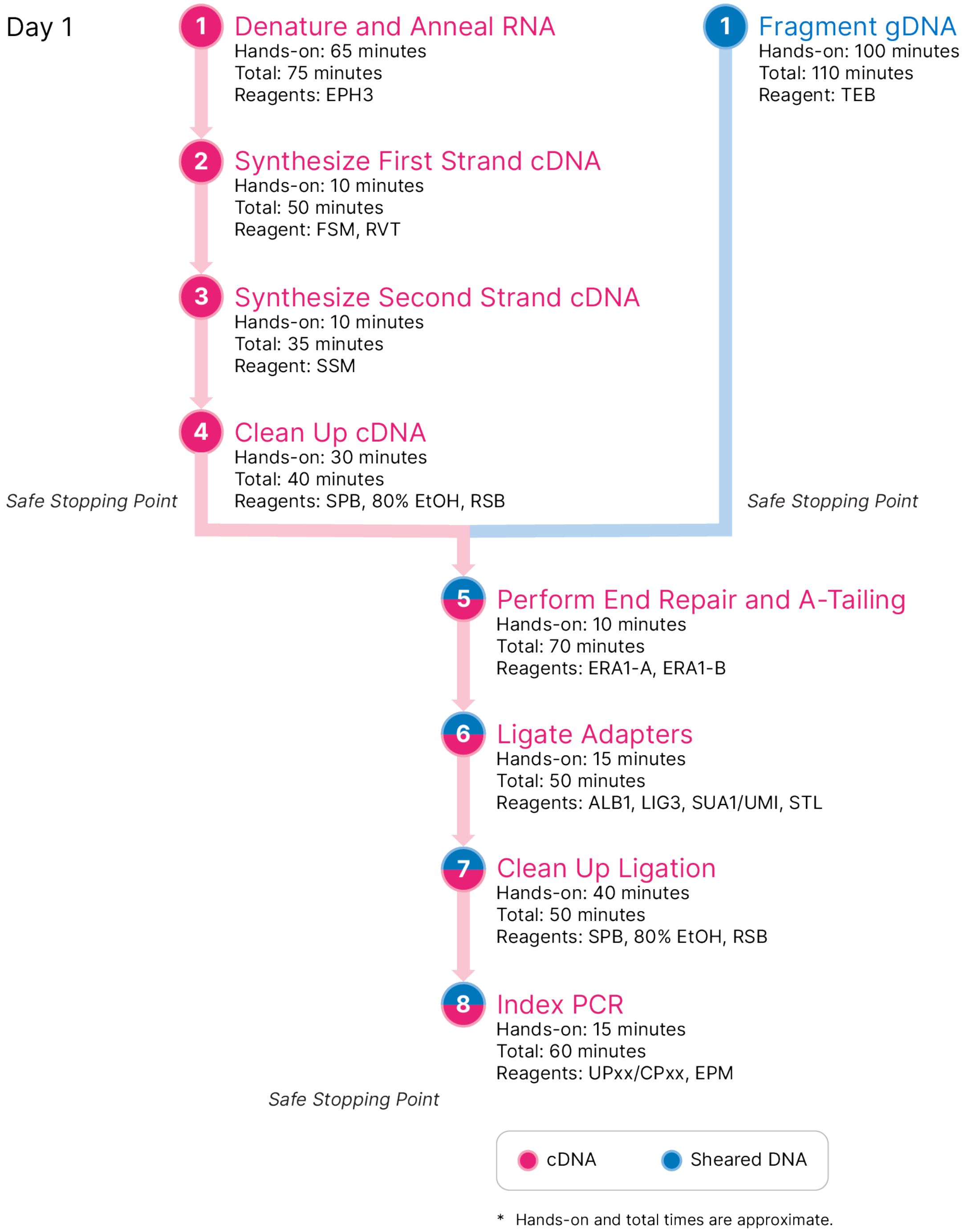
An overview of the TSO Comprehensive (EU) workflow is shown in Figure 1 and Figure 2.

Library Prep Workflow

Figure 1 illustrates the library prep workflow for TSO Comprehensive (EU). Libraries from RNA and DNA samples can be prepared simultaneously in separate wells. Positive controls and no-template controls are processed identically to samples. Safe stopping points are marked between steps.

Before starting the protocol, enter run and sample information into a v2 sample sheet to be used with the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module. Refer to the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661).

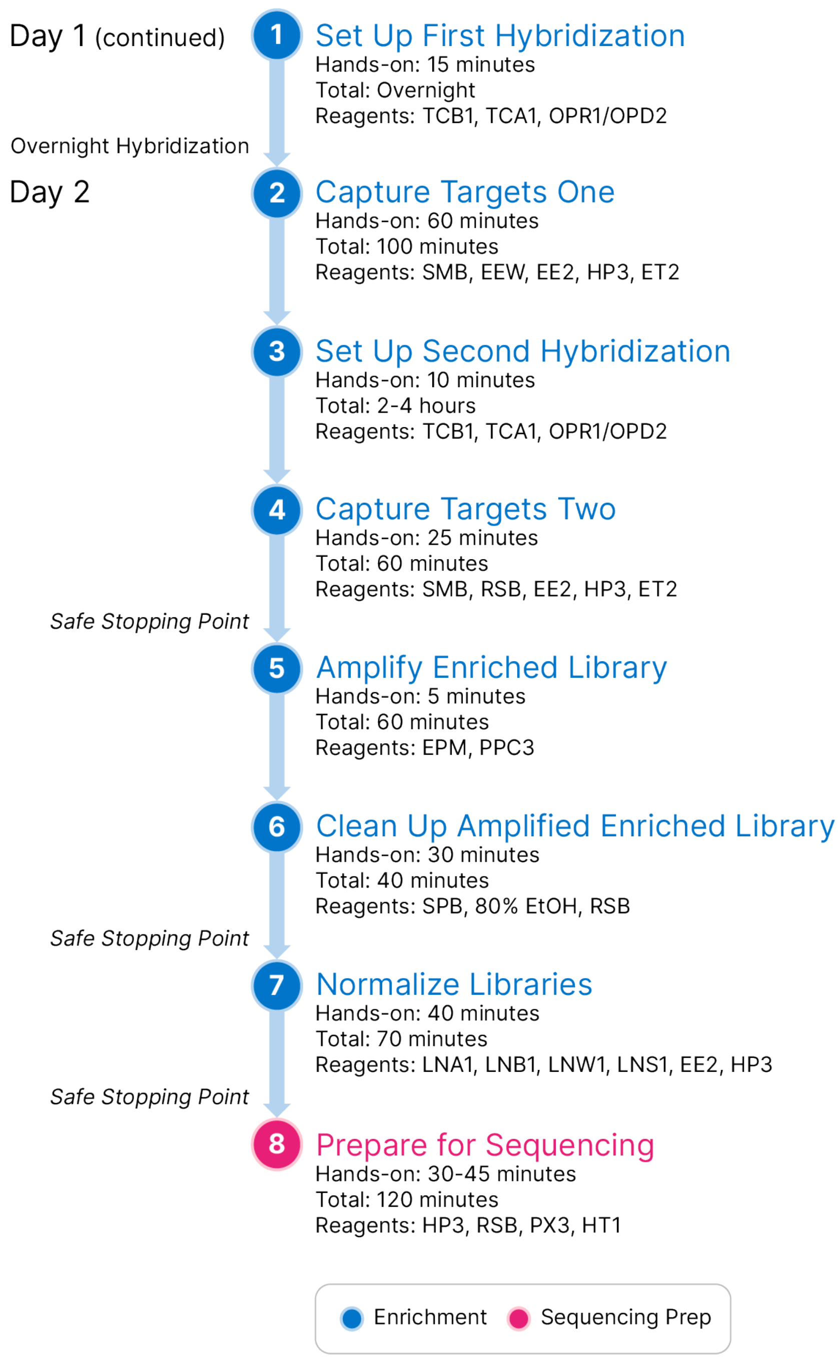
Figure 1 TSO Comprehensive (EU) Workflow (Part 1)



Enrichment Workflow

Figure 2 illustrates the enrichment workflow for TSO Comprehensive (EU). Safe stopping points are marked between steps.

Figure 2 TSO Comprehensive (EU) Workflow (Part 2)



Program Thermal Cyclers

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

Table 10 Pre-amplification Thermal Cycler Programs

Procedural Step	Program Name	Lid Temperature	Reaction Volume	Thermal Cycler Parameters
Denature and Anneal RNA	LQ-RNA	100°C	17 µl	• 65°C for 5 minutes • 4°C for 1 minute • 4°C Hold
Synthesize First Strand cDNA	1stSS	100°C	25 µl	• 25°C for 10 minutes • 42°C for 15 minutes • 70°C for 15 minutes • 4°C for 1 minute • 4°C Hold
Synthesize Second Strand cDNA	2ndSS	30°C	50 µl	• 16°C for 25 minutes • 4°C for 1 minute • 4°C Hold

NOTE If the lid temperature for 2ndSS cannot be set to 30°C, turn off the preheated lid heat option.

Table 11 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
				• 10°C Hold

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 • 57°C Hold for 8 to 24 hours

Procedural Step	Program Name	Lid Temperature	Reaction Volume	Thermal Cycler Parameters
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 • 57°C Hold for 1.5 to 4 hours

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

Prepare for Protocol Steps

1. Thoroughly decontaminate work areas with an RNase/DNase-inhibiting cleaner.

   ⚠ CAUTION

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

2. Set pre-amplification thermal cycler programs. Refer to *Program Thermal Cyclers* on page 40.
3. Follow manufacturer instructions to set up the ultrasonicator.
4. If processing DNA samples only, proceed directly to *Fragment gDNA* on page 46.
5. Remove RNA controls from storage.
6. Remove the reagent tubes from the box and follow thaw instructions.

Table 12 TruSight Oncology Comp RNA Library Prep (PN 20031127)

Reagent	Storage	Thaw Instructions	Protocol Step
EPH3	-25°C to -15°C	Thaw to room temperature	Denature and Anneal RNA
FSM	-25°C to -15°C	Thaw to room temperature	Synthesize First Strand cDNA
RVT	-25°C to -15°C	Keep on ice	Synthesize First Strand cDNA
SSM	-25°C to -15°C	Thaw to room temperature	Synthesize Second Strand cDNA

Table 13 TruSight Oncology Comp Library Prep (Refrigerate) (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 cDNA
RSB	2°C to 8°C	Bring to room temperature.	Clean Up cDNA

Denature and Anneal RNA

This process denatures purified RNA and primes with random hexamers in preparation for cDNA synthesis.

Preparation

1. Prepare the following reagents.
   - EPH3—Set aside.
   - FSM—Vortex to mix. Centrifuge briefly, and then pipette to mix.
     The reagent may contain white product-related particulates. No user action is required. There is no impact to product performance.
   - RVT—Centrifuge briefly, and then pipette to mix. Keep on ice.

NOTE RVT is a viscous solution. Minimize bubble formation while pipetting.

2. In a microcentrifuge tube, combine the following volumes to prepare a FSM + RVT Master Mix.

   Table 14 FSM + RVT Master Mix

Master Mix Component	4 Libraries (µl)	8 Libraries(µl)	16 Libraries (µl)	24 Libraries (µl)
FSM	36	72	144	216
RVT	4	8	16	24

   This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.
3. Pipette ten times to mix.
4. Place the FSM + RVT Master Mix on ice until *Synthesize First Strand cDNA* on page 43.

Procedure

1. Thaw extracted RNA samples and RNA controls on ice.
   Process RNA controls as samples for the remainder of the protocol.
2. Store RNA on ice when not in use. Refer to *Sample Requirements* on page 24 to quantify samples.
3. Pipette each RNA sample 10 times to mix.
4. Use RNase/DNase-free water to prepare 40 ng of each RNA sample in a final volume of 8.5 µl (4.7 ng/µl).

For RNA controls, use the concentration provided on the tube label.

5. Label a new 96-well PCR plate CF (cDNA Fragments).
6. Add 8.5 µl each RNA sample to a unique well of the CF PCR plate.
7. Make sure that sample plate layout and indexes for each sample match the run planned in the TSO Comprehensive (EU) analysis module during run setup.
8. Vortex EPH3 to mix, and then centrifuge briefly.
9. Add 8.5 µl EPH3 to each sample well.
10. Apply adhesive plate seal to the CF PCR plate.

CAUTION

Make sure to seal edges and wells completely to prevent evaporation.

11. Shake at 1200 rpm for 1 minute.
12. Centrifuge at 280 × g for 1 minute.
13. Place on the thermal cycler and run the LQ-RNA program.
    Refer to *Program Thermal Cyclers* on page 40.
14. When the samples reach 4°C, hold for one minute and then proceed immediately to the next step.

Synthesize First Strand cDNA

This process reverse transcribes the RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase.

Procedure

1. Remove the CF PCR plate from the thermal cycler.
2. Pipette 10 times to mix FSM + RVT master mix. Ensure FSM + RVT mix is completely homogenous.
3. Add 8 µl FSM + RVT master mix to each sample well.
4. Pipette 10 times to mix.
5. Discard remaining FSM + RVT master mix.
6. Apply adhesive plate seal to the CF PCR plate.
   Seal edges and wells completely to prevent evaporation.
7. Shake at 1200 rpm for 1 minute.
8. Centrifuge at 280 × g for 1 minute.
9. Place on a thermal cycler and run the 1stSS program.
   Refer to *Program Thermal Cyclers* on page 40.
10. When the samples reach 4°C, proceed immediately to the next step.
    First strand samples can be held at 4°C for up to 5 minutes.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes double-stranded cDNA.

Preparation

1. Prepare the following reagent.
   - SSM—Invert 10 times to mix. Centrifuge briefly.

Procedure

1. Remove the CF PCR plate from the thermal cycler.
2. Add 25 µl SSM to each sample well.
3. Apply adhesive plate seal to the CF PCR plate.
   Seal edges and wells completely to prevent evaporation.
4. Shake at 1200 rpm for 1 minute.
5. Centrifuge at 280 × g for 1 minute.
6. Place on a thermal cycler and run the 2ndSS program.
   Refer to *Program Thermal Cyclers* on page 40.
7. When the samples reach 4°C, hold for one minute and then proceed immediately to the next step.

Clean Up cDNA

This process uses SPB to purify the cDNA from unwanted reaction components. The beads are washed twice with fresh 80% ethanol. The cDNA is 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 15 Prepare Fresh 80% EtOH

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries
100% Ethanol alcohol, 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. Label a new 96-well MIDI plate BIND1 (cDNA Binding).
5. Cover and set aside.
6. Set out the magnet.

Procedure

Bind

1. Remove the CF PCR plate from the thermal cycler.
2. Vortex SPB for 1 minute to resuspend beads.
3. Immediately add 90 µl SPB to each sample well of the BIND1 MIDI plate.
   If using a trough to dispense SPB, include a 1.05 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SPB has been added to each sample well.
4. Transfer the entire volume (50 µl) of each sample from the CF PCR plate to the corresponding well of the BIND1 MIDI plate.
5. Discard empty CF PCR plate.
6. Apply adhesive plate seal to the BIND1 MIDI plate.
   Seal edges and wells completely.
7. Shake at 1800 rpm for 2 minutes.
8. Incubate at room temperature for 5 minutes.
9. Place the BIND1 MIDI plate on a magnetic stand for 5 minutes.
10. Use a P200 pipette set to 200 µl to remove and discard all supernatant from each sample well without disturbing the bead pellet.

Wash

1. Wash beads as follows.
   a. Keep on the magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well.
2. Wash beads a ***second*** time.
3. Remove residual EtOH from each well.
   Use a P20 pipette with fine tips.
4. Discard unused 80% EtOH.

Elute

1. Remove the BIND1 MIDI plate from the magnetic stand.
2. Invert or vortex RSB to mix.

3. Add 22 µl RSB to each sample well.
4. Apply adhesive plate seal to the BIND1 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 MIDI plate PCF (Purified cDNA Fragments).
   If you are stopping at the *SAFE STOPPING POINT* on page 46, use a PCR plate.
9. Transfer 20 µl eluate from each sample well of the BIND1 MIDI plate to the corresponding well of the PCF plate.
10. Discard empty BIND1 MIDI plate.
11. Add 30 µl RSB to each sample well of the PCF plate.
12. Pipette to mix 10 times.
13. Apply adhesive plate seal to the PCF plate and keep it on ice.
14. Return EPH3, FSM, RVT, and SSM to storage.
15. If you are processing samples derived from RNA (cDNA) only, and not stopping at the safe stopping point, proceed to *Perform End Repair and A-Tailing* on page 49.

SAFE STOPPING POINT

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

Prepare for Protocol Steps

1. Remove DNA controls from storage.
2. Remove the reagent tube from the box and follow thaw instructions.

Table 16 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. Make sure to follow recommendations in *Nucleic Acid Extraction, Quantification, and Storage* on page 24 to quantify samples.

2. Prepare the following reagent.
   - TEB—Invert or vortex to mix.

Procedure

Prepare the Plate

1. Select one of the following three options to prepare the plate.
   - **Option #1:** Process gDNA samples simultaneously with cDNA samples in the PCF MIDI plate.
     a. Label the PCF MIDI plate LP (Library Preparation).
     b. Place on ice and set aside for use in *Transfer Fragmented DNA* on page 48.
   - **Option #2:** Process gDNA samples simultaneously with cDNA samples and the PCF PCR plate is frozen.
     a. Thaw the PCF PCR plate to room temperature.
     b. Centrifuge at 280 × g for 1 minute.
     c. Pipette 10 times to mix.
     d. Label a new 96-well MIDI plate LP (Library Preparation).
     e. Transfer the entire 50 µl each sample from the PCF PCR plate to the corresponding well of the LP MIDI plate.
     f. Discard PCF PCR plate.
     g. Apply adhesive plate seal and place on ice until *Transfer Fragmented DNA* on page 48.
   - **Option #3:** Process gDNA only samples.
     a. Label a new 96-well MIDI plate LP (Library Preparation).
     b. If you are stopping at the *SAFE STOPPING POINT* on page 48, use a PCR plate.
     c. Cover and set aside for use in *Transfer Fragmented DNA* on page 48.

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 controls, use the concentration provided on the tube label. To prevent sample loss, do not pipette less than 2 µl of sample into this dilution.

Sample Type	Input Amount (ng)	Minimum Concentration (ng/µl)
FFPE	40	3.33
Control	40	Refer to tube label

Fragment

1. Add 52 µl 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 27 and manufacturer instructions.

2. Record the orientation of the strip.
3. Fragment gDNA into fragments with an 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) analysis module.
2. Follow ultrasonicator manufacturer instructions to recover the sample.
   For some ultrasonicator tube types, centrifugation can be necessary to consolidate the sample in the tube.
3. For each fragmented gDNA sample, use a p20 pipette with fine tips to perform three transfers of 16.7 µl into an empty well of the LP MIDI plate.
4. Apply adhesive plate seal to the LP MIDI plate.

SAFE STOPPING POINT

If you are stopping, apply 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 Steps

Make sure that post-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 40.

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

Table 17 TruSight Oncology Comp Library Prep (Freeze) Box (PN 20031118)

Reagent	Storage	Thaw Instructions	Protocol Step
ERA1-A	-25°C to -15°C	Keep on ice.	Perform End Repair and A-Tailing

Reagent	Storage	Thaw Instructions	Protocol Step
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 on ice.	Ligate Adapters
SUA1 (blue cap)	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters
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 on ice.	Index PCR

Table 18 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 19 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 20 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-tail 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 2 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 on ice.
   - 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 21 ERA1 Master Mix[1]

Master Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
ERA1-B	35 µl	69 µl	138 µl	207 µl	415 µl
ERA1-A	13.5 µl	27 µl	54 µl	81 µl	161 µl

[1] This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.

4. Pipette slowly 10 times to ensure homogeneity, centrifuge briefly, and then place ERA1 master mix on ice.
5. Select the appropriate option of the two following options to prepare the plate.
   - **Option #1:** If samples are in a MIDI plate:
     - Relabel the MIDI plate LP2 (Library Preparation 2).

   If some samples are in separate MIDI plates, move all samples to separate wells of the same MIDI plate according to the plate layout.
   - **Option #2:** If the plate is frozen:
     a. Thaw the PCF PCR plate or 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 PCF PCR plate or the LP PCR plate to the corresponding well of the LP2 MIDI plate.
     f. Discard PCF PCR or 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, and incubate at 72°C for 20 minutes.
7. Place the LP2 MIDI plate on ice for 5 minutes.

Ligate Adapters

This process ligates adapters to the ends of the cDNA and/or gDNA fragments.

The TSO Comprehensive (EU) assay includes SUA1 adapters and UMI adapters.

- Use SUA1 adapters with RNA samples.
- Use UMI adapters with DNA samples.

Preparation

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 on ice.
   - SUA1—Vortex to mix for a minimum of 10 seconds, and then centrifuge briefly.
   - 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.
2. Add 60 µl ALB1 to each sample well of the LP2 MIDI plate. ALB1 is a viscous solution; minimize bubble formation while pipetting.
3. Add 5 µl LIG3 to each sample well.
4. Add adapters.
   Do *not* combine different types of adapters together.
   - **RNA sample wells**—10 µl SUA1 (blue cap) to each sample derived from RNA.
   - **DNA sample wells**—10 µl UMI (white cap) to each sample derived from DNA.
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 cDNA or 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 22 Prepare Fresh 80% Ethanol

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
100% Ethanol alcohol, pure	2 ml	4 ml	8 ml	12 ml	24 ml
RNase/DNase-free water	500 µl	1 ml	2 ml	3 ml	6 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 1.05 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. Use a P200 pipette set at 200 µl to remove and discard all supernatant from each sample well without disturbing the bead pellet.

Wash

1. Wash beads as follows.
   a. Keep on the magnetic stand and add 200 µl fresh 80% EtOH to each sample well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well without disturbing the bead pellet.
2. Wash beads a ***second*** time.
3. Remove residual EtOH from each well.
   Use a P20 pipette with fine tips.
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 on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate LS (Library Samples).
9. Transfer 25 µl of 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 cDNA and/or DNA fragments flanked by adapters required for cluster generation.

Preparation

1. Prepare the following reagents.
   - EPM—Keep on ice.
   - UPxx—Vortex to mix and centrifuge briefly. UPxx is the index primer selected on the Create Run screen in the Local Run Manager software during run setup.
   - CPxx—Vortex to mix and centrifuge briefly. CPxx is the index primer selected on the Create Run screen in the Local Run Manager software during run setup.

2. Make sure that indexes for each sample match the run planned on the TSO Comprehensive (EU) analysis module during run setup. Make sure to follow instructions regarding index selection in *Number of Libraries and Selecting Indexes* on page 34.

   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 40.

NOTE If continuing with *Set Up First Hybridization* on page 55, 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 Steps

1. Make sure that post-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 40.
2. Remove the reagent tube from the box and follow thaw instructions.

Table 23 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123)

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

Table 24 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

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

Table 25 TruSight Oncology Comp Content Set Box (PN 20031122)

Reagent	Storage	Thaw Instructions	Protocol Step
OPR1 (red cap)	-25°C to -15°C	Thaw to room temperature.	Set Up First Hybridization
OPD2 (white cap)	-25°C to -15°C	Thaw to room temperature.	Set Up First Hybridization

Set Up First Hybridization

During this process, a pool of oligos hybridizes to cDNA libraries, and a pool of oligos hybridizes to gDNA libraries prepared in *Index PCR* on page 53. Enrichment of targeted regions requires two hybridization steps. In the first hybridization, oligos hybridize to cDNA and/or 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.
   - OPR1—Vortex to mix, and then centrifuge briefly.
   - OPD2—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. Then pipette to mix.
3. Label a new 96-well PCR plate HYB1 (Hybridization 1).

Procedure

1. Transfer 20 µl each cDNA and/or gDNA library from the ALS PCR plate to the corresponding well in the HYB1 PCR plate.
2. Apply adhesive plate seal to the ALS PCR plate and set aside.
   Seal edges and wells completely.
3. Inspect TCB1 for precipitates. If present, warm the tube again and vortex the tube until the crystals dissolve.
4. Add 15 µl TCB1 to each library well in the HYB1 PCR plate.
5. Add 10 µl TCA1 to each library well in the HYB1 PCR plate.
6. Add probes.
   Do ***not*** combine different types of probes together. Add only one probe set per well.
   - RNA library wells— 5 µl OPR1 (red cap) to each library derived from RNA.
   - DNA TSO Comprehensive (EU) library wells— 5 µl OPD2 (white cap) to each library derived from DNA for TSO Comprehensive (EU) enrichment.
7. Apply adhesive plate seal to the HYB1 PCR plate.

   CAUTION

   Make sure to seal edges and wells completely to prevent evaporation.
8. Shake at 1200 rpm for 2 minutes.
9. Place on the thermal cycler and run the HYB1 program.
   Refer to *Program Thermal Cyclers* on page 40.
10. Hybridize at 57°C for a minimum of 8 hours to a maximum of 24 hours.
11. Return hybridization reagents to storage.
12. Store the ALS PCR plate at -25°C to -15°C for up to 30 days.

Prepare for Protocol Steps

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

Table 26 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123)

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

Reagent	Storage	Thaw Instructions	Protocol Step
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 27 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

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 28 Assay Content Set Box (PN 20031122)

Reagent	Storage	Thaw Instructions	Protocol Step
OPR1 (red cap)	-25°C to -15°C	Thaw to room temperature.	Set Up Second Hybridization
OPD2 (white 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 29 EE2 + HP3 Elution Mix for Capture Targets One

Elution Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	114 µl	228 µl	456 µl	684 µl	1368 µl
HP3	6 µl	12 µl	24 µl	36 µl	72 µl

This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.

4. Vortex EE2 + HP3 elution mix, and then centrifuge briefly. Set aside for the *Elute* on page 59 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.
4. Immediately add 150 µl SMB to each library well of the CAP1 MIDI plate.
   If using a trough to dispense SMB, include a 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SMB has been added to each sample well.
5. 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.
6. Discard the empty HYB1 PCR plate.
7. Apply adhesive plate seal to the CAP1 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
8. Shake at 1800 rpm for 2 minutes.
9. Incubate in the preheated microsample incubator at 57°C for 25 minutes.
10. Place on a magnetic stand for 2 minutes.
11. While keeping the CAP1 MIDI plate on the magnetic stand, use a P200 µl pipette set to 200 µl to remove and discard all supernatant without disturbing the bead pellet.

⚠ CAUTION

Proceed immediately to the next step (*Wash* on page 59). 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. Set pipette volume to 150 µl, and pipette to mix a minimum of 10 times. Make sure that all beads are resuspended.

   > CAUTION
   >
   > Make sure that no bead pellets are present by carefully aspirating total bead solution of well into the tip. Then look at bottom of each well for a pellet. Angle pipette tip towards bead pellet during wash steps to dislodge pellet. Make sure that the bead pellet is fully in solution. The solution should look dark brown and have a homogenous consistency.

   d. Apply adhesive plate seal to the CAP1 MIDI plate.
   e. Seal edges and wells completely to prevent evaporation.
   f. Shake at 1800 rpm for 4 minutes.
   g. Incubate in a microsample incubator at 57°C for 5 minutes.
   h. Place on a magnetic stand for 2 minutes.
   i. Keep on the magnetic stand and remove and discard all supernatant from each well without disturbing the bead pellet.
2. Wash beads a ***second*** time.
3. Wash beads a ***third*** time.
4. Remove residual supernatant from each well.
   Use a P20 pipette with fine tips.

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.
14. Seal edges and wells completely to prevent evaporation.
15. Shake at 1200 rpm for 2 minutes.
16. Return EEW to storage.

Set Up Second Hybridization

This step binds targeted regions of the enriched cDNA and/or 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.
   - OPR1—Vortex to mix, and then centrifuge briefly.
   - OPD2—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 probes.
   Do ***not*** combine different types of probes together.
   - RNA library wells— 5 µl OPR1 (red cap) to each library derived from RNA.
   - DNA TSO Comprehensive (EU) library wells— 5 µl OPD2 (white cap) to each library derived from DNA for TSO Comprehensive (EU) 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 40.
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 30 EE2 + HP3 Elution Mix for Capture Targets Two

Elution Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	114 µl	228 µl	456 µl	684 µl	1368 µl
HP3	6 µl	12 µl	24 µl	36 µl	72 µl

This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.

4. Vortex to mix, and then centrifuge briefly. Set aside for the *Elute* on page 62 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 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SMB has been added to each sample well.

5. 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.
6. Discard the empty ELU1 PCR plate.
7. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
8. Shake at 1800 rpm for 2 minutes.
9. Incubate in a microsample incubator at 57°C for 25 minutes.

NOTE If continuing with *Amplify Enriched Library* on page 63, follow thaw instructions for reagents in the Prepare for Protocol Steps section.

10. Place on a magnetic stand for 2 minutes.
11. Keep the CAP2 MIDI plate on the magnetic stand and use a P200 pipette set to 200 µl to remove and discard all supernatant from each library well without disturbing the bead pellet.

CAUTION

Proceed immediately to the next step (*Wash* on page 62). 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 on the magnetic stand for 2 minutes.
7. Keep the CAP2 MIDI plate on the magnetic stand and remove and discard all supernatant without disturbing the bead pellet.
8. Remove residual supernatant from each well.
   Use a P20 pipette with fine tips.

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, 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. Return RSB to storage.

Prepare for Protocol Steps

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

Table 31 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

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 on ice.	Amplify Enriched Library

Table 32 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123

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 40.

Note If continuing with *Normalize Libraries* on page 67, follow the thaw instructions in the Prepare for Protocol Steps 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 33 Prepare Fresh 80% Ethanol

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
100% Ethanol alcohol, pure	2 ml	4 ml	8 ml	12 ml	24 ml
RNase/DNase-free water	500 µl	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.
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 plate on magnetic stand for 5 minutes.
11. Use a P200 pipette set at 200 µl to remove and discard ***all*** supernatant from each library well without disturbing the bead pellet.

Wash

1. Wash beads as follows.
   a. Keep on magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each sample well without disturbing the bead pellet.
2. Wash beads a ***second*** time.
3. Remove residual EtOH from each well.
   Use a P20 pipette with fine tips.
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 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. Return RSB to storage.

Prepare for Protocol Steps

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

Table 34 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

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 35 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123

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 71, follow the thaw instructions in the Prepare for Protocol Steps 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. Vortex LNB1 for 1 minute to resuspend beads.
   Invert LNB1 tube to make sure that all beads are resuspended.
3. Using a P1000 set at 800 µl, pipette LNB1 up and down 10 times to ensure resuspension.
4. 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 36 LNA1 + LNB1 Master Mix

Master Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
LNA1	305 µl	610 µl	1219 µl	1829 µl	3658 µl
LNB1	55 µl	110 µl	221 µl	331 µl	662 µl

   This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.
5. Vortex LNA1 + LNB1 master mix. Set aside for *Bind* on page 68 step.
6. Prepare fresh EE2 + HP3 Elution Mix in a microcentrifuge tube.

Table 37 EE2 + HP3 Elution Mix for Normalize Libraries

Elution Mix Component	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
EE2	152 µl	304 µl	608 µl	912 µl	1824 µl
HP3	8 µl	16 µl	32 µl	48 µl	96 µl

This table includes volume overage. Refer to *Handling Reagents* on page 31 for calculations.

7. Vortex fresh elution mix, and then centrifuge briefly. Set aside for the *Elute* on page 69 step.
8. If the PL PCR plate was stored, thaw to room temperature, centrifuge at 280 × g for 1 minute, and then pipette to mix.
9. Label a new 96-well MIDI plate BBN (Bead-Based Normalization).
10. 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 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 plate on a magnetic stand for 2 minutes.
9. Keep on a magnetic stand and use a P200 pipette to remove and discard all supernatant from each well without disturbing the bead pellet.

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.
   d. Seal edges and wells completely.
   e. Shake at 1800 rpm for 5 minutes.
   f. Place on a magnetic stand for 2 minutes.
   g. Remove and discard all supernatant from each well without disturbing the bead pellet.
2. Wash beads a *second* time.
3. Remove residual supernatant from each well.
   Use a P20 pipette with fine tips.

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 30 days.

Prepare for Protocol Steps

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, and then place on ice.
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 tube from the box and follow thaw instructions.

Table 38 TruSight Oncology Comp Enrichment (Freeze) Box (PN 20031121)

Reagent	Storage	Thaw Instructions	Protocol Step
PhiX Internal Control (PX3 or PhiX)	-25°C to -15°C	Thaw to room temperature. Keep on ice.	Prepare for Sequencing

Table 39 TruSight Oncology Comp Enrichment (Refrigerate) Box (PN 20031123)

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

Preparation

1. Review the guidelines for *Number of Libraries and Selecting Indexes* on page 34.
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.

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 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. Place dPhiX on ice 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 for TSO Comprehensive (EU)

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 five times.

Use fresh tips for each library.

CAUTION

Make sure to mix libraries well for optimal performance.

3. Select one of the following options to pool, denature, and dilute the libraries.
   - **Option #1:** Sequence libraries derived from RNA samples and DNA samples simultaneously. Refer to *Option #1: DNA and RNA Libraries Together* on page 72.
   - **Option #2:** Sequence libraries derived from DNA samples only. Refer to *Option #2: DNA Only Libraries* on page 73.
   - **Option #3:** Sequence libraries derived from RNA samples only. Refer to *Option #3: RNA Only Libraries* on page 74.

Option #1: DNA and RNA Libraries Together

1. Label a microcentrifuge tube PRL (Pooled RNA Libraries).
2. Label a microcentrifuge tube PDL (Pooled DNA Libraries).
3. Transfer 10 µl each normalized RNA (cDNA) library from the NL plate to the PRL tube.
   Do not pool two libraries with the same index primer.
4. Transfer 10 µl each normalized DNA library from the NL plate to the PDL tube.
   Do not pool two libraries with the same index primer.
5. Apply adhesive plate seal to the NL PCR plate.
   Seal edges and wells completely.
6. Vortex PRL and PDL tubes to mix.
7. Centrifuge PRL and PDL tubes briefly.
8. Incubate PRL and PDL tubes in a heat block at 96°C for 2 minutes.
9. Place PRL and PDL tubes on ice for 5 minutes.
10. Vortex PRL and PDL tubes to mix, and then centrifuge briefly.
11. Return PRL and PDL 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. Add 5 µl PRL to DIL1.
4. Discard the PDL and PRL tubes.
5. Add 475 µl prechilled HT1 to the DIL1 tube (1:20 dilution).
6. Vortex DIL1 tube to mix, and then centrifuge briefly.

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

Option #2: DNA Only Libraries

1. Label a screw top microcentrifuge tube PDL (Pooled DNA Libraries).
2. Transfer 10 µl each normalized DNA library from the NL plate to the PDL tube.
   Do not pool two libraries with the same index primer.
3. Apply adhesive plate seal to the NL PCR plate.
   Seal edges and wells completely.
4. Apply Microseal 'B' to the NL PCR plate.
   Seal edges and wells completely.
5. Vortex PDL tube to mix.
6. Centrifuge PDL tube briefly.
7. Incubate PDL tube in a heat block at 96°C for 2 minutes.
8. Place PDL tube on ice for 5 minutes.
9. Vortex PDL tube to mix, and then centrifuge briefly.
10. Return PDL tube to ice.

Prepare First Dilution

1. Label a microcentrifuge tube DIL1 (Dilution 1).
2. Transfer 10 µl PDL to the empty DIL1 tube.
3. Discard the PDL tube.
4. Add 190 µl prechilled HT1 to the DIL1 tube (1:20 dilution).

5. Vortex DIL1 to mix, and then centrifuge briefly.

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, 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 then store at -25°C to -15°C for up to 30 days.
11. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Option #3: RNA Only Libraries

1. Label a microcentrifuge tube PRL (Pooled RNA Libraries).
2. Transfer 10 µl each normalized RNA (cDNA) library from the NL plate to the PRL tube.
   Do not pool two libraries with the same index primer.
3. Apply adhesive plate seal to the NL PCR plate.
   Seal edges and wells completely.
4. Vortex PRL tube to mix.
5. Centrifuge PRL tube briefly.
6. Incubate PRL tube in a heat block at 96°C for 2 minutes.
7. Place PRL tube on ice for 5 minutes.
8. Vortex PRL tube to mix, and then centrifuge briefly.
9. Return PRL tube to ice.

Prepare First Dilution

1. Label a microcentrifuge tube DIL1 (Dilution 1).
2. Transfer 10 µl PRL to the empty DIL1 tube.
3. Discard the PRL tube.
4. Add 190 µl prechilled HT1 to the DIL1 tube (1:20 dilution).

5. Vortex DIL1 to mix, and then centrifuge briefly.

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 1646 µl prechilled HT1 to the DIL2 tube (1:843 dilution).
5. Vortex prepared 20 pM dPhiX, and then centrifuge briefly.
6. Add 16.7 µl prepared 20 pM dPhiX to the DIL2 tube.
7. Vortex to mix, and then centrifuge briefly.
8. Load 1300 µl DIL2 into 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 30 days.
11. 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 (EU) 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 Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661).

Companion Diagnostic Results

For each companion diagnostic (CDx) intended use, there are three possible results:

- **Positive**—A variant is detected and classified as level 1 (CDx).
- **Not detected**—No variants or biomarkers associated with the CDx intended use are detected in the sample. The tumor type selected for the sample is appropriate for the CDx.
- **No result**—A determination of a variant status is not possible for one or more of the following reasons:
  - The CDx intended use was not applicable to the tested sample because the tumor type selected for the sample is not appropriate for tumor type of the CDx.
  - The sequencing run failed quality control specifications.
  - The library failed required quality control specifications.
  - The appropriate nucleic acid was not run.

All CDx intended use results are reported in the Companion Diagnostic Results section of the JSON report. Only the intended uses with a positive result are listed in the Companion Diagnostic Results section of the PDF report.

Tumor Profiling Variants

TSO Comprehensive (EU) is designed to report somatic variants when reporting variants with evidence of clinical significance or variants with potential clinical significance. The TSO Comprehensive (EU) assay software uses a KB that determines if each detected and eligible variant (Table 2) is clinically significant or potentially clinically significant based on evidence of therapeutic, diagnostic, or prognostic associations. The KB also takes into consideration if associations are established (or not) in the tested tumor type. Susceptibility or cancer risk associations are not included in the KB. Common polymorphisms are removed.

For Tumor Profiling variants, positive results are classified into Genomic Findings with Evidence of Clinical Significance or Genomic Findings with Potential Clinical Significance according to the installed KB and the identified tumor type.

Quality control failures lead to no results for the variant types that are relevant to the failed quality control metric. Refer to Table 40 and Table 41 for more information. Tumor Profiling positions with insufficient depth are listed in the Low Depth Report and not in the TSO Comprehensive (EU) report.

Quality Control

- For nucleic acid quantification information and minimum input material requirements, refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 24.
- Sequencing run and sample validity are determined automatically and reported by the TSO Comprehensive (EU) analysis module. For detailed analysis information, refer to the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661).

Table 40 TSO Comprehensive (EU) 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	≤ 3106 OR > 3106 and P_VALUE ≤ 0.049	A metric assessing the likelihood of contamination using the VAF of common variants. The contamination score is based on VAF distribution of SNPs. The contamination P value used to assess highly rearranged genomes, only applicable when contamination score is above Upper Spec Limit.	No DNA results reported.
	MEDIAN_INSERT_SIZE (bp)	≥ 70	The median fragment length in the sample.	No TMB or 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.	
	USABLE_MSI_SITES (count)	≥ 40	The number of MSI sites usable for MSI calling (Number of microsatellite sites with sufficient spanning reads to identify microsatellite instability).	No MSI results reported.
	COVERAGE_MAD (count)	≤ 0.210	The median of absolute deviations from the median of the normalized count of each CNV target region.	No gene amplification results reported.
	MEDIAN_BIN_COUNT_CNV_TARGET (count)	≥ 1.0	The median raw bin count per CNV target.	

Output Type	Metric	Specification	Description	Impact of Specification Failure*
RNA Libraries	MEDIAN_INSERT_SIZE (bp)	≥ 80.0	The median fragment length in the sample.	No fusions or splice variant results reported.
	MEDIAN_CV_GENE_500X (coefficient)	≤ 0.93	MEDIAN_CV_GENE_500X is a measure of coverage uniformity. For each gene with at least 500x coverage, the coefficient of variation in coverage across the gene body is computed. This metric is the median of these values. A high value indicates a high level of variation and indicates a problem in library preparation such as low sample input and/or probe pulldown issues. This metric is computed using all reads (including reads marked as duplicates).	
	TOTAL_ON_TARGET_READS (count)	≥ 9,000,000	The total number of reads that map to the target regions. This metric is computed using all reads (including reads marked as duplicates).	

* Successful results show PASS.

Table 41 TSO Comprehensive (EU) Report Result Control Metrics

Output Type	Metric	Specification	Impact of Specification Failure*
Positive Control	DNA External Control	23 of 24 specified variants detected	Manually invalidate patient samples based on control sample results. The analysis module software does not automatically invalidate patient samples based on control sample results.
	RNA External Control	12 of 13 specified variants detected	

Output Type	Metric	Specification	Impact of Specification Failure*
No-template control	DNA Median Exon Coverage for TSO Comprehensive (EU)	≤ 8	Manually invalidate patient samples based on control sample results. The analysis module software does not automatically invalidate patient samples based on control sample results.
	RNA Gene Above Median Cutoff	≤ 1	

* Successful results show PASS.

- The TSO Comprehensive (EU) report, which is available in PDF and JSON formats, summarizes quality control results. The reports are in the analysis folder. Refer to the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661) for the location of the analysis folder (contains PDF and JSON reports) and the run folder.
- Repeat sequencing runs that are invalid.
- Repeat tests of libraries with the following results:
  - Contaminated DNA libraries
  - Invalid RNA libraries
  - Tests can be repeated to obtain more variant or biomarker results for DNA libraries that were invalidated for one but not all variant types.
- Positive controls are evaluated for variant calling. If positive controls do not meet the variant calling specifications, manually invalidate the sequencing run. The analysis module software does not automatically invalidate patient samples based on control sample results.
- NTCs are evaluated against the median exon coverage for DNA and genes above median cutoff for RNA. If negative controls do not meet specifications, manually invalidate the library preparation event and all associated sequencing runs. The analysis module software does not automatically invalidate patient samples based on control sample results.
- Perform additional quality control measures in accordance with local, state, and/or federal regulations or accreditation requirements.

For more information on repeating sequencing runs or tests of libraries, refer to *Troubleshooting* on page 81.

Troubleshooting

Use the following table 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.

Observation	Possible Cause	Recommended Action
Sequencing run does not pass run Quality Control Specifications	Use or lab 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 71. • Reenrich libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 55. • Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 42 or *Fragment gDNA* on page 46.
	Instrument issue	Contact Illumina Technical Support.
Error with report generation or general instrument error (network error, errors loading/unloading reagents, etc.)	Software or instrument issue	Refer to the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661) 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 24 and *Nucleic Acid Extraction, Quantification, and Storage* on page 24.

Observation	Possible Cause	Recommended Action
DNA library does not pass quality control specifications (continued)	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 71. • Reenrich libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 55. • Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 46.
	CONTAMINATION_SCORE, CONTAMINATION_P_VALUE criteria are not met	Review Warnings and Precautions for information on avoiding cross-contamination. Review plate layout and library indexing to make sure that libraries of the same index were not sequenced together. For impacted libraries, start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 46. Contamination may have occurred during sample extraction. It may be necessary to repeat extraction to make sure that the sample is free from contamination.
	Usable MSI failed	Review 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 24 and *Nucleic Acid Extraction, Quantification, and Storage* on page 24. A new sample extraction and/or repeating the Fragment gDNA step may be necessary if the sample is overly fragmented or damaged.

Observation	Possible Cause	Recommended Action
	Sample may be overly fragmented or have nucleic acid damage that impacts the ability to generate sufficient unique libraries	Review 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 24 and *Nucleic Acid Extraction, Quantification, and Storage* on page 24. A new sample extraction and/or repeating the Fragment gDNA step may be necessary if the sample is overly fragmented or damaged.
RNA library does not pass quality control specifications	Requirements for sample input were not met	Ensure appropriate sample input and repeat library preparation from the Denature and Anneal RNA step. Refer to *Sample Requirements* on page 24 and *Nucleic Acid Extraction, Quantification, and Storage* on page 24.
	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 71. • Reenrich libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 55. • Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 42.
	Sample may be overly fragmented or have nucleic acid damage that impacts the ability to generate sufficient unique libraries	Ensure appropriate sample input. Refer to *Sample Requirements* on page 24 and *Nucleic Acid Extraction, Quantification, and Storage* on page 24. A new sample extraction may be necessary if the sample is overly fragmented or damaged.

Observation	Possible Cause	Recommended Action
Positive Control Failure (DNA/RNA)	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 71. • Reenrich libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 55. • Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 42 or *Fragment gDNA* on page 46.
	Use or equipment error in the assay workflow	
NTC Failure (DNA/RNA)	Cross contamination occurred or contamination of work area	Review Warnings and Precautions section for information on decontaminating work areas. Review Warnings and Precautions for information on avoiding cross-contamination. Review plate layout and library indexing to make sure that libraries 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	
Software indicates positive and/or negative controls were not included in sequencing run	Incorrect assignment of Tumor Type in Local Run Manager run planning	Requeue analysis with controls correctly identified as instructed in the Local Run Manager TruSight Oncology Comprehensive (EU) Analysis Module Workflow Guide (document # 200008661).

Performance Characteristics

TSO Comprehensive (EU) is a targeted NGS panel with 517 genes. Small DNA variants—single nucleotide variants (SNVs), multiple nucleotide variants (MNVs), insertions, and deletions—are eligible for reporting from all 517 genes. Gene amplifications are eligible for reporting from the MET and ERBB2 genes. Fusions are eligible for reporting from the 23 genes indicated in *TSO Comprehensive (EU) Assay Gene Panel* on page 2. Splice variants are eligible for reporting from the MET and EGFR genes. To be reported, variants must be detected and have evidence in the TSO Comprehensive (EU) assay KB and be eligible based on the tested tissue type. To be reported, NTRK fusions require the fusion partner to be 5' and the NTRK or RET kinase domain to be intact.

For small DNA variants, a representative approach to validation of the targeted genes in the panel was conducted with data representing SNVs, MNVs, insertions, and deletions. For gene amplifications, fusions, and splice variants, testing was done at the gene level. TMB and MSI were evaluated where indicated. For the NTRK fusions CDx claims, fusions in FFPE samples were tested in studies focused on performance specific to the claim (such as Limit of Detection, Within-Laboratory Precision, Reproducibility, Accuracy, and Clinical Performance).

Table 42 provides definitions of metrics calculated in various studies.

Table 42 Metrics Definitions

Term	Definition
Positive Percent Agreement (PPA)	The percentage of positives correctly identified from the total positives relative to an orthogonal method.
Negative Percent Agreement (NPA)	The percentage of negatives correctly identified from the total negatives relative to an orthogonal method.
Overall Percentage Agreement (OPA)	The percentage of positives and negatives correctly identified from the total observation relative to an orthogonal method.
Positive Percent Concordance (PPC)	The percentage of positive calls correctly identified from the total positives relative to a control condition in a direct pairwise comparison.
Negative Percent Concordance (NPC)	The percentage of negative calls correctly identified from the total negatives relative to a control condition in a direct pairwise comparison.
Positive Percent Call (PPC)	Percentage of observations that are positive for a target among observations expected to be positive for the target.
Negative Percent Call (NPC)	Percentage of observations that are negative for a target among observations expected to be negative for the target.

Cross Contamination

The cross-contamination study was conducted to evaluate if false positive results due to well-to-well contamination during sample library preparation, and run-to-run contamination between consecutive sequencing runs, occur for the TSO Comprehensive (EU) assay. Two DNA and two RNA samples with unique and non-overlapping variants were used to evaluate cross-contamination. Thirty-two DNA libraries and 32 RNA libraries were each prepared three times by two operators 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. To evaluate cross-contamination, small DNA variants (which also impact TMB) and RNA variants were evaluated (MSI and gene amplifications were not evaluated). The cross-contamination study showed zero contamination events observed by examining the detected variants in each sample, with no false positives detected.

Nucleic Acid Extraction Kit Evaluation

Three commercially available DNA and RNA extraction kits were evaluated with TSO Comprehensive (EU). The three extraction kits isolated both DNA and RNA from the same FFPE tissue sections. The kits differed in their deparaffinization agent and nucleic acid binding steps (Table 43). Kit 1 was the predominant extraction kit used to determine TSO Comprehensive (EU) performance.

Table 43 Kit Characteristics

Kit	Deparaffinization Agent	Nucleic Acid Binding
1	Proprietary	Column
2	Xylene	Column
3	Mineral Oil	Magnetic beads

Seven samples (5 FFPE tissue and 2 FFPE cell lines) were extracted in duplicate by 2 operators repeated on 3 days for each of the 3 extraction kits (7 samples x 3 extraction kits x 2 extraction operators x 3 extraction days x 2 extraction replicates).

Table 44 summarizes the effects of extraction kits on library validity and variant calling. For library validity, the largest rate difference between extraction kits was reported and significance was determined by a quantitative analysis of the library metrics. For variant calling, if the extraction kit means were significantly different, the difference was reported.

Extraction kits were observed to affect the library validity metrics for small DNA variants/TMB and MSI. Library validity metrics for gene amplifications and RNA were not significantly different between extraction kits. Extraction kits did not affect variant calling for small DNA variants and TMB score. For MSI score and gene amplifications, no false positives were detected, and a quantitative analysis found no significant differences in the negative samples. Extraction kits were observed to have different supporting read values so that fusions and splice variants near the LoD may be missed due to extraction kit selection.

The extraction kit selected should be used in the laboratory's verification of TSO Comprehensive (EU) performance characteristics and to yield adequate library validity.

Table 44 Extraction Kit Impacts on Library Validity and Variant Calling

Variant Type	Library Validity Rate (Largest Difference)	Variant Calling (Largest Mean Difference in underlying variable)
Small DNA Variants	Kit 2 significantly lower to Kit 3 (10%)	Not significant
TMB		Not significant
MSI	Kit 1 significantly lower to Kit 3 (14%)	No false positives detected False negatives not evaluated
Gene Amplification	Not significant (5%)	No false positives detected False negatives not evaluated
Fusions	Not significant (3%)	Kit 1 significantly lower to Kit3 (11%)
Splice Variants		Kit 1 significantly lower to Kit3 (11%)

Interfering Substances

The impact of potential endogenous and exogenous substances on the performance of the TSO Comprehensive (EU) assay was evaluated on 16 unique FFPE samples from brain, thyroid, colon, breast, lung, prostate, skin, and soft tissue types. Endogenous substances, melanin, and hemoglobin, were spiked into the samples during the nucleic acid extraction process. Exogenous substances (ethanol, xylene, and Proteinase K) were present during the nucleic acid extraction process, and they were also spiked into the purified nucleic acid before library preparation. Addition of extra Proteinase K during the extraction process was also evaluated where interference was observed with spiked Proteinase K. There was a no-spiked endogenous control, and buffer or water-spiked exogenous control for each of the 16 unique samples. The effect of necrosis was assessed on a different set of eight FFPE samples from lung, brain, and colon tissues. There was a macrodissected no necrosis control for each necrosis sample. For all interferents, four replicates per sample per substance were tested with the TSO Comprehensive (EU) assay and compared to their respective control for detection of small DNA variants, gene amplifications, RNA fusions, and RNA splice variants, as well as for MSI status and TMB score.

DNA Variant Detection

Melanin (0.2 µg/ml), hemoglobin (2 mg/ml), ethanol (5%), Proteinase K (0.04 mg/ml), and xylene (0.0001%) do not interfere with TMB score, MSI status, small DNA variants, and gene amplifications.

RNA Variant Detection

The data support no interference of hemoglobin (2 mg/ml), melanin (0.2 µg/ml), ethanol (5%), and xylene (0.0001%) on RNA fusions or splice variants. Similarly, there was no interference on RNA variant detection when 0.02 mg/ml of Proteinase K were spiked into RNA before the library preparation event and when up to 2.6 mg/ml of Proteinase K are added to the sample during the RNA purification process.

Some false positives relative to the no interferent controls were observed between replicate libraries for RNA fusions with hemoglobin, melanin, ethanol and xylene, and for RNA splice variants with melanin and xylene. Similarly, some false negatives were observed on some replicate libraries for RNA splice variants with hemoglobin, melanin, xylene, and 0.02 mg/ml of Proteinase K. However, in all cases the false positives and false negatives were deemed to be sample issues because the observations for detected events showed supporting reads near the LoD. Therefore, false positives and false negatives across replicates were considered unrelated to interference and were attributed to random variation of the number of supporting reads for fusions and/or splice variants at or below the LoD.

Necrosis

The presence of necrotic tissue up to 70% does not interfere with TMB score, MSI status, small DNA variants, or RNA splice variants detection. RNA fusions and gene amplification detection are impacted in samples with ≥ 25% necrosis content in the tissue area. If the sample section contains more than 25% necrosis in total tissue area, then the necrotic tissue must be macrodissected.

Stability

Real-time Stability

Real-time stability was used to establish the shelf life of the TSO Comprehensive (EU) assay kit when stored per label conditions. The study design was based on the testing of 3 lots of reagents and used the classical stability study design described in CLSI EP25-A. The kits were stored in final kitted configuration for the duration of the study, at storage conditions defined on the product label. Frozen kit components were stored at -15°C to -25°C. Refrigerated kit components were stored at 2°C to 8°C. Room-temperature components were stored at 15°C to 30°C.

Kits were tested for appearance and functional kit release criteria at specified time points. Also, variant calling and sample QC metric trends were analyzed for the QC control material. Shelf life was determined for each reagent. Expiration dates are assigned based on date of manufacture and shelf life. Kit expiration is assigned based on the earliest expiring reagent.

Kit In-use Stability

In-use stability of the TSO Comprehensive (EU) assay kit was evaluated under standard use conditions over the course of the shelf life to support multiple kit uses. The reagent kit was subjected to multiple freeze/thaws and tested to support up to 4 uses of the kit. In addition, 8 RNA and 8 DNA libraries were prepared a total of 3 times to test the maximum number of libraries supported (24 DNA and 24 RNA libraries per kit). All functional kit release criteria were met for all freeze/thaw cycles and timepoints tested. Testing of FFPE samples with reagents aged ≥ 25 months was performed to assess the impact of in-use testing on variant calling. A qualitative analysis of targeted variants demonstrate that the in-use events did not affect variant calling.

Library Stability

The stability of libraries prepared with the TSO Comprehensive (EU) assay was evaluated using 8 FFPE DNA and 8 FFPE RNA samples from 9 different tissue types tested in triplicate through the assay. Libraries from the Normalized Library (NL) PCR plate were pooled and sequenced on day 0. The remaining volume of the libraries in the NL PCR plate were stored frozen (-25°C to -15°C), then repooled and sequenced on day 30. Any statistically significant results for small DNA variants between day 0 and day 30 were technically negligible. There were no statistical differences between day 0 and day 30 results for MSI status, TMB score, gene amplifications, RNA fusions, and RNA splice variants. The data indicate that libraries generated from the TSO Comprehensive (EU) assay are stable for up to 30 days at -25°C to -15°C.

Slide-mounted FFPE Tissue Stability

The stability of slide-mounted FFPE tissues for use with the TSO Comprehensive (EU) assay was evaluated by sectioning FFPE blocks (5 µm sections) from 16 unique samples representing 9 tissue types, mounting on slides, followed by storage at room temperature for 3 timepoints: 1 day (control), 4 weeks, and 8 weeks. Nucleic acids (both DNA and RNA) were extracted at the indicated timepoint then stored frozen until extractions for all timepoints were complete. Extracted RNA was stored at -65°C to -85°C and extracted DNA was stored at -25°C to -15°C. For each timepoint, three replicates per sample were tested with the TSO Comprehensive (EU) assay, and compared to the control for small DNA variants, MSI status, TMB score, gene amplifications, RNA fusions, and RNA splice variants. The data indicate that slide mounted FFPE tissues for use with the TSO Comprehensive (EU) assay are stable for up to 4 weeks.

Nucleic Acid Input Titration Guardbanding

Nucleic acid input for the TSO Comprehensive (EU) assay was evaluated by testing DNA from 33 FFPE samples encompassing 17 tissue types, at input levels ranging from 10 ng to 500 ng, and testing RNA from 5 FFPE samples from 5 tissue types at input levels ranging from 10 ng to 85 ng. Library QC metrics were evaluated and were sample-dependent. The DNA results demonstrated that some but not all DNA sample QC metrics respond to increased input above the nominal 40 ng input:

- MEDIAN_INSERT_SIZE did not respond to input above 30 ng.
- MEDIAN_EXON_COVERAGE showed a positive correlation with increasing input.
- PCT_EXON_50X increased with increasing input up to 80 ng.
- USABLE_MSI_SITES increased with increasing input. Some samples with fewer than 40 USABLE_MSI_SITES at 40 ng met the specification at higher inputs, which would allow an MSI score to be calculated.
- MEDIAN_BIN_COUNT_CNV_TARGET increased with increasing input.
- Increasing input to increases COVERAGE_MAD toward the upper specification limit.

RNA sample QC metrics increased (MEDIAN_INSERT_SIZE and TOTAL_ON_TARGET_READS) or decreased (MEDIAN_CV_GENE_500X) from 10 ng to 40 ng but in general did not change between 40 ng and 85 ng input.

Limit of Blank

The percentage of false positives (out of the total expected negatives) were assessed by replicate testing of FFPE normal or benign, adjacent tissue that should not contain somatic variants for small DNA variants, gene amplifications, MSI, RNA fusions, and RNA splice variants. False positives were not analyzed for TMB as there is no clinical cutoff. Six DNA and 6 RNA FFPE samples were run in duplicate with 2 operators across 3 days for each of the 2 reagent lots. A subset of samples was re-pooled and re-sequenced in a 3x DNA only and a 3x RNA only format to evaluate false positives with several multiplex configurations supported by this device. In addition, there were 30 additional RNA samples run in duplicate that were processed with 1 reagent lot, divided between 2 operators. In total, there were 168 possible observations for DNA and 228 observations for RNA reduced by invalid libraries for each variant type. The percentage of false positives was calculated at the gene level for amplifications and at the position level (approximately 1.9 million positions) for small DNA variants. The percentage of false positives for DNA variant types is shown in Table 45. The percentage of false positives for RNA fusions and splice variants was 0% as shown in Table 46.

Table 45 False Positives by DNA Variant Type

Variant Type	False Positives
Gene Amplifications	0% (0/9912)
Small DNA Variants	0.0001% (271/295,801,567)
MSI	0% (0/156)
TMB	N/A*

* False positives are not applicable because TMB is reported as a score and does not have a qualitative outcome.

Table 46 False Positives by RNA Variant Type

Variant Type	False Positives
Fusion	0% (0/226)
Splice Variant	0% (0/226)

Limit of Detection

Two studies were conducted to assess the Limits of Detection for TSO Comprehensive (EU). Study 1 evaluated RET small DNA variants, RET fusions, and NTRK1 – 3 Fusions. Study 2 evaluated other tumor profiling variants.

Study 1

The Limits of Detection (LoDs) of NTRK1, NTRK3, and RET small DNA variants and NTRK1 – 3 and RET fusions were determined. The LoD is the lowest analyte value (for example, variant allele frequency or supporting reads) that can be detected consistently (95% detection limit or a type II error of 5%). FFPE tissues with RET small DNA variants (medullary thyroid cancer), RET fusions (Papillary Thyroid Cancer, Atypical Spitz Tumor), and NTRK1 – 3 fusions (Low-Grade Glioma, Glioblastoma Multiforme, Myofibroblastic Sarcoma, Sarcoma, Secretory Breast Cancer, Colon Cancer), as well as an FFPE-treated cell line with NTRK1 and NTRK3 small DNA variants were used in the study. Each sample was diluted to at least 5 test levels (ranging from approximately 0.01 – 0.10 VAF for small DNA variants and 2 – 25 supporting reads for fusions). There were 18 observations for each test level per lot per variant generated by 3 operators and 3 sequencing instruments initiating library preparation on 3 non-consecutive days with 2 replicates of each sample test level. Two reagent lots were tested.

For DNA variants, the 2 lots were analyzed independently using probit regression or the hit rate approach (lowest test level with a hit rate (point estimate) ≥ 95%) to determine the LoD for each variant by lot. The larger LoD across the two reagent lots was taken as the limit of detection for the variant (Table 47).

For RNA fusions, FFPE cell lines were used to estimate the LoD values for each fusion gene. The LoDs were then verified with FFPE tissues using duplicate library preparations across 3 operators, 3 instruments, and 3 reagent lots to generate 54 observations per variant near the LoD established with FFPE cell lines. The claimed limits of detection for each fusion (Table 48) are the lowest mean supporting reads that reached a hit rate (point estimate) ≥ 95%.

Table 47 Limit of Detection for NTRK1, NTRK3, and RET Small DNA Variants

Marker	Chr	Position	Reference	Alternative	Limit of Detection (Variant Allele Frequency)
NTRK1 G595R (SNV)*	Chr1	156846342	G	A	0.038
NTRK3 F617L (SNV)*	Chr15	88476283	A	G	0.032
NTRK3 G623R (SNV)*	Chr15	88476265	C	T	0.036
NTRK3 G696A (SNV)*	Chr15	88472468	C	G	0.027
RET C618R (SNV)	Chr10	43609096	T	C	0.053
RET M918T (SNV)	Chr10	43617416	T	C	0.045

Marker	Chr	Position	Reference	Alternative	Limit of Detection (Variant Allele Frequency)
RET C634Y (MNV)	Chr10	43609949	GC	AT	0.045
RET D898_E901del (deletion)*	Chr10	43615611	GAGATGTTTATGA	G	0.055

Chr = Chromosome
* These DNA variants were analyzed by probit regression; the other DNA variants were analyzed by the hit rate approach.

Table 48 Limit of Detection for NTRK and RET Fusions

Gene	Fusion	Limit of Detection (Supporting Reads)
NTRK1	TPM3-NTRK1	20.2
	BCAN-NTRK1	53.2
NTRK2	STRN-NTRK2	13.6
	ETV6-NTRK2	20.3
NTRK3	KANK1-NTRK3	13.5
	ETV6-NTRK3	16.2
RET	NCOA4-RET	15.8
	KIF5B-RET	16.6

Study 2

The Limits of Detection (LoDs) of tumor profiling variants reported by TSO Comprehensive (EU) were evaluated. The LoD is the lowest analyte value (variant allele frequency, fold change, or supporting reads) that can be detected consistently (95% hit rate or a type II error of 5%). FFPE samples from 17 tissue types containing variants were diluted to multiple test levels. Six observations were generated per level by two operators each using a different reagent lot and instrument.

DNA Variants

The LoDs of 10 small DNA variants classes (25 variants in total) and 2 DNA gene amplifications (ERBB2 and MET) were determined and summarized as ranges (Table 49). RET variants from the Study 1 LoD are also included. Two of 3 insertions greater than 5 bp had LoDs of 0.034 and 0.036 VAF with the third having an LoD of 0.215 VAF. The latter was an insertion in a low complexity region where the insertion adds additional repeats, impacts alignment, and requires more reads for consistent detection. Therefore, some low complexity genomic contexts may impact detection of insertions > 5bp.

Table 49 Limit of Detection for Small DNA Variants and Gene Amplifications

Type (Unit of Measure for LoD)	Variant Class / Genomic Context	Number of Variants	Range
Small DNA Variants (variant allele frequency)	SNVs	5	0.016–0.064
	MNVs	3	0.022–0.048
	Insertion (1-2bp) near homopolymer repeats	2	0.086–0.104
	Insertion (1-2bp) near dinucleotide repeats	2	0.038–0.051
	Insertion (3-5bp)	2	0.030–0.056
	Insertion (> 5bp and up to 25bp)	3	0.034–0.215
	Deletion (1-2bp) near homopolymer repeats	2	0.094–0.100
	Deletion (1-2bp) near dinucleotide repeats	2	0.033–0.070
	Deletion (3-5bp)	2	0.028–0.064
	Deletion (> 5 and up to 25bp)	2	0.047–0.055
Gene Amplifications (fold change)	By gene (ERBB2, MET)	2	2.034–2.195

Fusions

LoDs were determined for 18 fusions, accounting for 20 genes in the TSO Comprehensive (EU) panel, which ranged from 10 to 54.7 supporting reads (Table 50). An additional 3 genes (NTRK1 – 3) were tested in the other study. The RET gene was tested here and in the other LoD study. Sixteen fusions with LoDs determined had data consistent with a common LoD of 16 supporting reads using a two-sided, 95% upper confident limit (UCL). Two fusions had LoDs of 24.7 and 44.2 support reads that were not consistent with the common LoD.

The fusion FGFR2-SRPK2 with a LoD value of 24.7 supporting reads had repeat overlap regions in the breakpoint as annotated by the TSO Comprehensive (EU) assay software. Repeat regions within a breakpoint typically have lower levels of evidence as reads may map elsewhere in the genome or may remain unaligned. In addition, repeat regions make the process of assembly (used to identify fusion sequences) more challenging and require additional evidence to construct the correct sequence. SEPT14-EGFR is another example of a fusion with homologous sequence in the breakpoint.

The fusion BCL2-IGHJ5 with an LoD value of 44.2 supporting reads had a very short gene (IGHJ5) with the breakpoint near the start of an exon requiring gapped short alignments. Consequently, more reads were required for consistent detection.

Table 50 Limit of Detection for Fusions

Fusion	Gene A Breakpoint	Gene B Breakpoint	LoD	Common LoD
NCOA4-RET	51582937	43612030	10.0	yes
TMPRSS2-ERG	39817543	42880007	13.2	yes
KIF5B-RET	32311775	43612032	14.5	yes
ACPP-ETV1	132036419	14028762	17.2	yes
FGFR3-TACC3	1801536	1736997	17.5	yes
EML4-ALK	29446394	42553391	20.2	yes
FGFR1-GSR	38274821	30569602	23.7	yes
EGFR-GALNT13	55087056	155295102	24	yes
ESR1-CCDC170	151857451	152023138	24.3	yes
FGFR2-SRPK2	123353223	104926165	24.7	no
HNRNPUL1-AXL	41743847	41782201	26.3	yes
CD74-ROS1;GOPC	149784243	117645578	28.2	yes
SPIDR-NRG1	32453345	48353103	28.2	yes
RAF1-VGLL4	12641189	11606492	28.5	yes
DHX8;ETV4-STAT3	41613847	40474300	30.5	yes
MKRN1-BRAF	140487383	140158806	31.2	yes
BCL2-IGHJ5	60793496	106330066	44.2	no
PAX3-FOXO1	41134997	223084859	54.7	yes

Splice Variants

The 2 RNA Splice variants, MET and EGFR, had LoDs of 18.7 and 24.8 supporting reads, respectively.

Tumor Content

The results in the study inform recommendations for tumor content for clinical specimens. In general, the greater the tumor content, the higher the “signal” (VAF, fold-change, or supporting reads) for variants in the tumor. Minimum tumor content recommendations are based on the following observations. LoD values for small DNA variants are no greater than 0.104 VAF (with the exception of the TP53 insertion). To detect driver mutations in the tumor (0.50 variant allele frequency), 20% tumor content is recommended, so that these mutations would have 0.10 VAF and be at or above LoD. At 20% tumor content, genes amplified to 5.5 fold change (11 copies), would be consistently detected based on a Limit of Detection of 1.8 fold change. At 20% tumor content, fusions with 80 supporting reads would be consistently detected based on a Limit of Detection of 16 supporting reads.

Reproducibility

Two studies were conducted to evaluate Reproducibility for the TSO Comprehensive (EU) assay. Study 1 evaluated RET small DNA variants in addition to NTRK and RET fusion variants. Study 2 evaluated additional tumor profiling variants.

Study 1

This study was performed to assess the reproducibility of the TSO Comprehensive (EU) assay across 3 testing sites (1 internal, 2 external) with 2 operators per site, 2 within-run replicates, and 3 non-consecutive testing days. Testing was conducted with a reproducibility panel including DNA samples containing specific known RET small DNA variants and RNA samples containing specific known NTRK1 – 3 and RET fusion variants from formalin-fixed, paraffin embedded (FFPE) tissue specimens and cell lines. The panel contained DNA and RNA panel members with low variant levels and high variant levels with the same number of low and high-level panel members for each variant class. High-level panel members were targeted at approximately 2 to 3 times the LoD and low-level panel members were targeted at approximately the LoD. At each site, each operator tested the panel members in duplicate 3 times, generating 6 observations per target per panel member. From all 3 sites, 36 observations were generated per panel member (3 sites/instruments × 2 operators × 2 within-run replicates × 3 start days).

Percent positive calls (PPCs) and percent negative calls (PNCs) for targeted small DNA variants and targeted RNA fusion variants at the high level were determined as the primary endpoints. PPCs and PNCs for targeted small DNA variants and targeted RNA fusion variants at the low level were calculated as secondary endpoints. Two-sided 95% confidence intervals (CIs) associated with all endpoints were calculated using the Wilson score method. Primary analyses were performed to estimate PPC and PNC (with associated 95% CIs) in the targeted high-level panel members by combining TSO Comprehensive (EU) assay observations for a given target in a group of panel members representing the applicable variant class (for example, small DNA variants and RNA fusions) across sites/instruments, operators, and runs. For each targeted variant, TSO Comprehensive (EU) assay observations in other panel members at the high level targeted for the same variant type but not containing the same variant as determined by the majority rule were combined to calculated PNC. The overall PPC and PNC for the low-level targeted panel members were determined in a similar manner.

RET Small DNA Variants

For the high-level small DNA variant panel members, the overall PPC was 100.0% (207/207; 95% CI: 98.2% to 100.0%) (Table 51). The overall PNC for the high-level small DNA variant panel members was 100.0% (1035/1035; 95% CI: 99.6% to 100.0%) (Table 52). For low-level targeted small DNA variant panel members, the overall PPC for the low-level targeted small DNA variant panel members was 99.1% (210/212; 95% CI: 96.6% to 99.7%), and the overall PNC was 100.0% (1026/1026; 95% CI: 99.6% to 100.0%).

Table 51 PPC of TSO Comprehensive (EU) Assay for Detection of RET Small DNA Variants in High- and Low-Level Targeted Panel Members

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n	Mean VAF	Percent Positive Calls (%)	95% CI*
High	SNV	chr10_43617416_T_C	RET M918T	34	0.156	100.0% (34/34)	(89.8%, 100.0%)
High	SNV	chr10_43609949_G_C	RET C634S	36	0.140	100.0% (36/36)	(90.4%, 100.0%)
High	SNV	chr10_43614996_G_A	RET V804M	33	0.116	100.0% (33/33)	(89.6%, 100.0%)
High	MNV	chr10_43609949_GC_AT	RET C634Y	35	0.195	100.0% (35/35)	(90.1%, 100.0%)
High	Deletion	chr10_43615611_ GAGATGTTTATGA_G	RET D898_ E901del	33	0.199	100.0% (33/33)	(89.6%, 100.0%)
High	Insertion	chr10_43609946_T_ TGTGCCGCAC	RET C634_ T636dup	36	0.095	100.0% (36/36)	(90.4%, 100.0%)
High	All small DNA variants high	All small DNA variants high	All small DNA variants high	207	N/A	100.0% (207/207)	(98.2%, 100.0%)
Low	SNV	chr10_43617416_T_C	RET M918T	35	0.042	100.0% (35/35)	(90.1%, 100.0%)

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n	Mean VAF	Percent Positive Calls (%)	95% CI*
Low	SNV	chr10_43601830_G_A	RET V292M	35	0.033	94.3% (33/35)	(81.4%, 98.4%)
Low	SNV	chr10_43613840_G_C	RET E768D	36	0.044	100.0% (36/36)	(90.4%, 100.0%)
Low	MNV	chr10_43609949_GC_AT	RET C634Y	36	0.071	100.0% (36/36)	(90.4%, 100.0%)
Low	Deletion	chr10_43615611_ GAGATGTTTATGA_G	RET D898_ E901del	34	0.065	100.0% (34/34)	(89.8%, 100.0%)
Low	Insertion	chr10_43609946_T_ TGTGCCGCAC	RET C634_ T636dup	36	0.037	100.0% (36/36)	(90.4%, 100.0%)
Low	All small DNA variants low	All small DNA variants low	All small DNA variants low	212	N/A	99.1% (210/212)	(96.6%, 99.7%)

Abbreviations: N/A, not applicable; VAF, variant allele frequency.
* 95% 2-sided confidence interval calculated via the Wilson score method.

Table 52 PNC of TSO Comprehensive (EU) Assay for Detection of RET Small DNA Variants in High- and Low-Level Targeted Panel Members

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n[1]	Percent Negative Calls (%)	95% CI[2]
High	SNV	chr10_43617416_T_C	RET M918T	173	100.0% (173/173)	(97.8%, 100.0%)
High	SNV	chr10_43609949_G_C	RET C634S	171	100.0% (171/171)	(97.8%, 100.0%)
High	SNV	chr10_43614996_G_A	RET V804M	174	100.0% (174/174)	(97.8%, 100.0%)
High	MNV	chr10_43609949_GC_AT	RET C634Y	172	100.0% (172/172)	(97.8%, 100.0%)
High	Deletion	chr10_43615611_ GAGATGTTTATGAG	RET D898_ E901del	174	100.0% (174/174)	(97.8%, 100.0%)
High	Insertion	chr10_43609946_T_ TGTGCCGCAC	RET C634_ T636dup	171	100.0% (171/171)	(97.8%, 100.0%)
High	All small DNA variants high	All small DNA variants high	All small DNA variants high	1035	100.0% (1035/1035)	(99.6%, 100.0%)
Low	SNV	chr10_43617416_T_C	RET M918T	177	100.0% (177/177)	(97.9%, 100.0%)
Low	SNV	chr10_43601830_G_A	RET V292M	143	100.0% (143/143)	(97.4%, 100.0%)
Low	SNV	chr10_43613840_G_C	RET E768D	176	100.0% (176/176)	(97.9%, 100.0%)
Low	MNV	chr10_43609949_GC_AT	RET C634Y	176	100.0% (176/176)	(97.9%, 100.0%)

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n[1]	Percent Negative Calls (%)	95% CI[2]
Low	Deletion	chr10_43615611_ GAGATGTTTATGA_G	RET D898_ E901del	178	100.0% (178/178)	(97.9%, 100.0%)
Low	Insertion	chr10_43609946_T_ TGTGCCGCAC	RET C634_ T636dup	176	100.0% (176/176)	(97.9%, 100.0%)
Low	All small DNA variants low	All small DNA variants low	All small DNA variants low	1026	100.0% (1026/1026)	(99.6%, 100.0%)

[1] All observations pooled from panel member-variant combinations for which the majority call is negative, ie, targeted variants harboring fusions with less than 50% calls positive.
[2] 95% 2-sided confidence interval calculated via the Wilson score method.

Table 53 shows the variance components analysis of variant allele frequencies (VAFs) across the approximately 36 observations for each panel member. The standard deviation (SD) and percent coefficient of variation (%CV; total and for each source) were calculated and presented for each targeted RET small DNA variant.

Table 53 TSO Comprehensive (EU) Assay Variance Components Analysis of VAF in Targeted Small DNA Variants Panel Members

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n	Mean VAF	Site SD %CV)	Operator SD (%CV)	Day SD (%CV)	Replicate SD (%CV)	Total SD (%CV)
High	SNV	chr10_43617416_T_C	RET M918T	34	0.156	0.011 (7.2%)	0.000 (0.0%)	0.000 (0.0%)	0.017 (10.8%)	0.020 (13.0%)
High	SNV	chr10_43609949_G_C	RET C634S	36	0.140	0.006 (4.6%)	0.000 (0.0%)	0.005 (3.7%)	0.014 (10.2%)	0.017 (11.8%)
High	SNV	chr10_43614996_G_A	RET V804M	33	0.116	0.005 (4.1%)	0.000 (0.0%)	0.002 (1.7%)	0.012 (10.7%)	0.013 (11.6%)
High	MNV	chr10_43609949_GC_AT	RET C634Y	35	0.195	0.000 (0.0%)	0.000 (0.0%)	0.009 (4.4%)	0.012 (6.0%)	0.015 (7.5%)
High	Deletion	chr10_43615611_GAGATGTTTATGA_G	RET D898_E901del	33	0.199	0.000 (0.0%)	0.000 (0.0%)	0.011 (5.5%)	0.017 (8.6%)	0.020 (10.2%)
High	Insertion	chr10_43609946_T_TGTGCCGCAC	RET C634_T636dup	36	0.095	0.003 (3.0%)	0.000 (0.0%)	0.000 (0.0%)	0.009 (9.6%)	0.010 (10.1%)
Low	SNV	chr10_43617416_T_C	RET M918T	35	0.042	0.000 (0.0%)	0.000 (0.0%)	0.000 (0.0%)	0.009 (22.2%)	0.009 (22.2%)
Low	SNV	chr10_43601830_G_A	RET V292M	35	0.033	0.000 (0.0%)	0.003 (9.8%)	0.002 (6.2%)	0.007 (21.7%)	0.008 (24.6%)

Variant Level	Variant Type	Targeted Variant (Nucleotide)	Targeted Variant (Amino Acid)	n	Mean VAF	Site SD %CV)	Operator SD (%CV)	Day SD (%CV)	Replicate SD (%CV)	Total SD (%CV)
Low	SNV	chr10_43613840_G_C	RET E768D	36	0.044	0.003 (6.0%)	0.000 (0.0%)	0.000 (0.0%)	0.008 (17.5%)	0.008 (18.5%)
Low	MNV	chr10_43609949_GC_AT	RET C634Y	36	0.071	0.000 (0.0%)	0.008 (10.7%)	0.000 (0.0%)	0.011 (14.9%)	0.013 (18.4%)
Low	Deletion	chr10_43615611_GAGATGTTTATGA_G	RET D898_E901del	34	0.065	0.002 (2.5%)	0.006 (9.9%)	0.004 (6.4%)	0.010 (16.2%)	0.013 (20.2%)
Low	Insertion	chr10_43609946_T_TGTGCCGCAC	RET C634_T636dup	36	0.037	0.005 (13.8%)	0.000 (0.0%)	0.003 (9.1%)	0.006 (15.9%)	0.008 (22.9%)

NTRK 1 – 3 and RET Fusions

For the high-level RNA fusion panel members, the overall PPC was 99.3% (285/287; 95% CI: 97.5% to 99.8%) (Table 54). The PPC was 100% for each high-level panel member except for the BCAN-NTRK1 panel member (PPC = 94.4% [34/36; 95% CI: 81.9% to 98.5%]). The overall PNC for the high-level RNA fusion panel members was 100.0% (1724/1724; 95% CI: 99.8% to 100.0%) (Table 55). For the low-level targeted RNA fusion panel members, the overall PPC was 95.4% (272/285; 95% CI: 92.3%, 97.3%), and the overall PNC was 100.0% (1851/1851; 95% CI: 99.8% to 100.0%).

Table 54 PPC of TSO Comprehensive (EU) Assay for Detection of NTRK and RET Fusions in High- and Low-Level Targeted Panel Members

Variant Level	Targeted Fusion	n	Mean Supporting Reads	Percent Positive Calls (%)	95% CI*
High	LMNA-NTRK1	36	37.9	100.0% (36/36)	(90.4%, 100.0%)
High	BCAN-NTRK1	36	33.6	94.4% (34/36)	(81.9%, 98.5%)
High	ETV6-NTRK2	36	24.6	100.0% (36/36)	(90.4%, 100.0%)
High	TRIM24-NTRK2	36	36.6	100.0% (36/36)	(90.4%, 100.0%)
High	ETV6-NTRK3	36	56.4	100.0% (36/36)	(90.4%, 100.0%)
High	BTBD1-NTRK3	35	32.9	100.0% (35/35)	(90.1%, 100.0%)
High	NCOA4-RET	36	36.7	100.0% (36/36)	(90.4%, 100.0%)
High	CCDC6-RET	36	33.4	100.0% (36/36)	(90.4%, 100.0%)
High	All Fusions High	287	36.5	99.3% (285/287)	(97.5%, 99.8%)
Low	LMNA-NTRK1	36	13.8	94.4% (34/36)	(81.9%, 98.5%)
Low	BCAN-NTRK1	36	16.9	80.6% (29/36)	(65.0%, 90.2%)

Variant Level	Targeted Fusion	n	Mean Supporting Reads	Percent Positive Calls (%)	95% CI*
Low	ETV6-NTRK2	35	15.2	94.3% (33/35)	(81.4%, 98.4%)
Low	STRN-NTRK2	36	13.6	100.0% (36/36)	(90.4%, 100.0%)
Low	ETV6-NTRK3	36	24.8	100.0% (36/36)	(90.4%, 100.0%)
Low	BTBD1-NTRK3	36	18.1	100.0% (36/36)	(90.4%, 100.0%)
Low	NCOA4-RET	36	15.8	97.2% (35/36)	(85.8%, 99.5%)
Low	KIF5B-RET	34	16.6	97.1% (33/34)	(85.1%, 99.5%)
Low	All Fusions Low	285	16.8	95.4% (272/285)	(92.3%, 97.3%)

* 95% 2-sided confidence interval (CI) calculated via the Wilson Score method.

Table 55 PNC of TSO Comprehensive (EU) Assay for Detection of NTRK and RET Fusions in High- and Low-Level Non-Targeted Panel Members

Variant Level	Targeted Fusions	n[1]	Percent Negative Calls (%)	95% CI[2]
High	LMNA-NTRK1	180	100.0% (180/180)	(97.9%, 100.0%)
High	BCAN-NTRK1	251	100.0% (251/251)	(98.5%, 100.0%)
High	ETV6-NTRK2	251	100.0% (251/251)	(98.5%, 100.0%)
High	TRIM24-NTRK2	216	100.0% (216/216)	(98.2%, 100.0%)
High	ETV6-NTRK3	144	100.0% (144/144)	(97.4%, 100.0%)
High	BTBD1-NTRK3	216	100.0% (216/216)	(98.2%, 100.0%)
High	NCOA4-RET	215	100.0% (215/215)	(98.2%, 100.0%)
High	CCDC6-RET	251	100.0% (251/251)	(98.5%, 100.0%)
High	All Fusions - High	1724	100.0% (1724/1724)	(99.8%, 100.0%)
Low	LMNA-NTRK1	213	100.0% (213/213)	(98.2%, 100.0%)
Low	BCAN-NTRK1	249	100.0% (249/249)	(98.5%, 100.0%)
Low	ETV6-NTRK2	250	100.0% (250/250)	(98.5%, 100.0%)
Low	STRN-NTRK2	249	100.0% (249/249)	(98.5%, 100.0%)

Variant Level	Targeted Fusions	n[1]	Percent Negative Calls (%)	95% CI[2]
Low	ETV6-NTRK3	177	100.0% (177/177)	(97.9%, 100.0%)
Low	BTBD1-NTRK3	249	100.0% (249/249)	(98.5%, 100.0%)
Low	NCOA4-RET	213	100.0% (213/213)	(98.2%, 100.0%)
Low	KIF5B-RET	251	100.0% (251/251)	(98.5%, 100.0%)
Low	All Fusions - Low	1851	100.0% (1851/1851)	(99.8%, 100.0%)

[1] All observations pooled from panel member-variant combinations for which the majority call is negative, ie. targeted variants harboring fusions with less than 50% calls positive.
[2] 95% 2-sided confidence interval (CI) calculated via the Wilson Score method.

Table 56 shows the variance components analysis of supporting reads across the approximately 36 observations within each targeted fusion. The SD and %CV (total and for each source) were calculated and presented for each targeted fusion.

Table 56 TSO Comprehensive (EU) Assay Variance Components Analysis of Supporting Reads in Targeted RNA Fusion Panel Members

Variant Level	Fusion	n	Mean Supporting Reads	Site SD (%CV)	Operator SD (%CV)	Day SD (%CV)	Replicate SD (%CV)	Total SD (%CV)
High	LMNA-NTRK1	36	37.9	3.52 (9%)	3.37 (9%)	6.93 (18%)	9.04 (24%)	12.39 (33%)
High	BCAN-NTRK1	36	33.6	13.75 (41%)	7.87 (23%)	5.40 (16%)	8.95 (27%)	18.98 (57%)
High	ETV6-NTRK2	36	24.6	8.03 (33%)	3.50 (14%)	4.20 (17%)	4.86 (20%)	10.86 (44%)
High	TRIM24-NTRK2	36	36.6	11.44 (31%)	4.24 (12%)	6.82 (19%)	6.87 (19%)	15.57 (43%)
High	ETV6-NTRK3	36	56.4	11.49 (20%)	10.20 (18%)	9.25 (16%)	8.69 (15%)	19.93 (35%)
High	BTBD1-NTRK3	35	32.9	1.49 (5%)	2.65 (8%)	2.16 (7%)	10.47 (32%)	11.11 (34%)
High	NCOA4-RET	36	36.7	4.64 (13%)	4.09 (11%)	6.17 (17%)	5.20 (14%)	10.17 (28%)
High	CCDC6-RET	36	33.4	7.25 (22%)	2.56 (8%)	6.53 (20%)	5.51 (16%)	11.49 (34%)
Low	LMNA-NTRK1	36	13.8	1.79 (13%)	0.00 (0%)	2.74 (20%)	4.37 (32%)	5.47 (40%)

Variant Level	Fusion	n	Mean Supporting Reads	Site SD (%CV)	Operator SD (%CV)	Day SD (%CV)	Replicate SD (%CV)	Total SD (%CV)
Low	BCAN-NTRK1	36	16.9	2.92 (17%)	2.98 (18%)	4.61 (27%)	5.82 (34%)	8.52 (50%)
Low	ETV6-NTRK2	35	15.2	0.00 (0%)	3.41 (22%)	3.83 (25%)	4.39 (29%)	6.75 (45%)
Low	STRN-NTRK2	36	13.6	1.77 (13%)	0.61 (5%)	2.33 (17%)	2.57 (19%)	3.95 (29%)
Low	ETV6-NTRK3	36	24.8	6.03 (24%)	3.46 (14%)	0.00 (0%)	6.39 (26%)	9.44 (38%)
Low	BTBD1-NTRK3	36	18.1	0.93 (5%)	0.00 (0%)	0.00 (0%)	6.64 (37%)	6.71 (37%)
Low	NCOA4-RET	36	15.8	2.08 (13%)	1.03 (7%)	0.00 (0%)	5.11 (32%)	5.61 (36%)
Low	KIF5B-RET	34	16.6	2.07 (12%)	0.00 (0%)	1.58 (10%)	5.83 (35%)	6.39 (39%)

%CV: Percent coefficient of variation.
SD: Standard deviation.

Study 2

A second study was performed to assess the reproducibility of the TSO Comprehensive (EU) assay across 3 testing sites (2 external and 1 internal), 2 operators/instruments per site, 3 unique reagent lots, 4 testing days (non-consecutive), and 2 sequencing runs per sample library.

Testing was conducted using extracted DNA and RNA samples from 41 FFPE tissue specimens and 1 FFPE cell line (with 1 FFPE tissue specimen and the FFPE cell line used to create 2 panel members each). Tissue specimens consisted of the following types: bladder, bone, brain, breast, colon, jejunum, kidney, liver, lung, ovary, prostate, skin, soft tissue, stomach, thyroid, and uterus. A total of 44 panel members were tested including DNA panel members with small DNA variants (SNVs, MNVs, insertions, and deletions), gene amplifications, different TMB scores, high MSI scores, and RNA panel members with gene fusions and splice variants. Most panel members had known target variants at levels of approximately 2 to 3 times the variant-specific limit of detection (~2–3×LoD).

The LOD is the analyte concentration where observed assay results are positive (variant detected relative to the TSO Comprehensive (EU) assay cutoff) ≥ 95% of the time. Mean observed variant levels were categorized as approximately <2×LOD (observed variant levels at < 1.5×LOD), ~2–3×LOD (observed variant levels at 1.5×LOD to 3.4×LOD), and approximately >3×LOD (observed variant levels at > 3.4×LOD).

Percent positive calls (PPCs) for small DNA variants, gene amplifications, MSI-high (MSI-H), and RNA variants were calculated by combining observations across sequencing runs and sites. Percent negative calls (PNCs) were similarly calculated for small DNA variants, gene amplifications, and RNA variants. For each known target variant, TSO Comprehensive (EU) assay observations in panel members of the same variant type but containing other variants, not derived from the same source specimen, nor meeting the majority rule for that variant (ie, < 50% of calls were positive) were combined across sites, operators/instruments, days, reagent lots, and sequencing runs to calculate PNC. Two-sided 95% confidence intervals (CIs) were calculated using the Wilson score method.

Small DNA Variants

Table 57 shows PPCs for targeted small DNA variants. PPCs ranged from 91.3% for a BRAF SNV to 100% for the majority of small DNA variants.

Table 57 PPC of TSO Comprehensive (EU) Assay for Detection of Small DNA Variants in Combined Targeted Panel Members

Observed Variant Level[1]	Variant Type	Targeted Variant (nucleotide)	Targeted Variant (amino acid)	Mean VAF[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	DELETION	chr5_112175751_CT_C	APC L1488fsTer19	0.181	100.0% (28/28)	(87.9%, 100.0%)
~2-3xLOD	DELETION	chr5_112175675_AAG_A	APC S1465WfsTer3	0.166	100.0% (40/40)	(91.2%, 100.0%)
~2-3xLOD	INSERTION	chr5_112175951_G_GA	APC T1556NfsTer3	0.227	100.0% (32/32)	(89.3%, 100.0%)
~2-3xLOD	INSERTION	chr5_112175675_A_AAG	APC S1465fs*9	0.100	100.0% (48/48)	(92.6%, 100.0%)
<2xLOD	INSERTION	chr1_27024001_C_CG	ARID1A Q372fs*28	0.084	100.0% (4/4)	(51.0%, 100.0%)
~2-3xLOD	SNV	chr7_140453136_A_T	BRAF V600E	0.045	91.3% (42/46)	(79.7%, 96.6%)
~2-3xLOD	DELETION	chr7_55242465_GGAATTAAGAGAAGCA_G	EGFR E746_A750del	0.112	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	SNV	chr7_55259515_T_G	EGFR L858R	0.045	100.0% (38/38)	(90.8%, 100.0%)
~2-3xLOD	DELETION	chr22_41574678_GC_G	EP300 H2324fs*29	0.245	100.0% (44/44)	(92.0%, 100.0%)

Observed Variant Level[1]	Variant Type	Targeted Variant (nucleotide)	Targeted Variant (amino acid)	Mean VAF[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	INSERTION	chr17_37880981_A_ AGCATACGTGATG	ERBB2 Y772_ A775dup	0.075	100.0% (36/36)	(90.4%, 100.0%)
~2-3xLOD	SNV	chr2_209113112_C_T	IDH1 R132H	0.155	100.0% (36/36)	(90.4%, 100.0%)
~2-3xLOD	MNV	chr12_25398284_CC_AT	KRAS G12I	0.111	100.0% (38/38)	(90.8%, 100.0%)
~2-3xLOD	INSERTION	chr9_139399350_C_CG	NOTCH1 R1598fs*12	0.146	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	DELETION	chr10_89720798_ GTACT_G	PTEN T319fs*1	0.157	100.0% (44/44)	(92.0%, 100.0%)
<2xLOD	INSERTION	chr17_7578470_C_ CGGGCGG	TP53 P152_ P153dup	0.157	100.0% (2/2)	(34.2%, 100.0%)
~2-3xLOD	INSERTION	chr17_7574029_C_ CGGAT	TP53 R333HfsTer5	0.154	100.0% (48/48)	(92.6%, 100.0%)

[1] Variant level calculated from mean observed variant allele frequency.
[2] Mean variant allele frequency calculated from observed assay results.
[3] 95% two-sided confidence interval calculated via the Wilson Score Method.

PNCs were 100% across small DNA variants.

Table 58 shows the variance component analysis of VAF results for each source of variation and total variation in all panel members with targeted small DNA variants.

Table 58 Variance Components Analysis of VAF for Targeted Small DNA Variants

Targeted Variant	N	Mean VAF	Site SD (%CV)	Operator (Site) SD (%CV)	Day(Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
chr2_209113112_C_T	36	0.155	0.008 (4.9)	0.006 (4.1)	0.034 (22.1)	0.000 (0.0)	0.016 (10.2)	0.039 (25.2)
chr4_153332910_C_ CAGG	44	0.130	0.000 (0.0)	0.000 (0.0)	0.013 (10.3)	0.014 (11.1)	0.008 (6.1)	0.021 (16.3)
chr5_112175675_A_AAG	48	0.100	0.000 (0.0)	0.000 (0.0)	0.010 (10.4)	0.003 (2.9)	0.003 (3.3)	0.011 (11.3)
chr5_112175675_AAG_A	40	0.166	0.000 (0.0)	0.000 (0.0)	0.024 (14.2)	0.000 (0.0)	0.011 (6.7)	0.026 (15.7)

Targeted Variant	N	Mean VAF	Site SD (%CV)	Operator (Site) SD (%CV)	Day(Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
chr5_112175751_CT_C	28	0.181	0.000 (0.0)	0.000 (0.0)	0.029 (15.8)	0.019 (10.8)	0.008 (4.7)	0.036 (19.7)
chr5_112175751_CTTTA_C	46	0.155	0.000 (0.0)	0.009 (5.6)	0.023 (14.9)	0.015 (9.7)	0.008 (5.5)	0.030 (19.4)
chr5_112175951_G_GA	32	0.227	0.000 (0.0)	0.006 (2.5)	0.034 (15.1)	0.000 (0.0)	0.011 (4.9)	0.036 (16.1)
chr7_55242465_GGAATTAAGAGAAGCA_G	46	0.112	0.000 (0.0)	0.004 (3.8)	0.015 (13.7)	0.005 (4.1)	0.008 (6.9)	0.018 (16.3)
chr7_55259515_T_G	38	0.045	0.003 (6.0)	0.000 (0.0)	0.012 (27.3)	0.000 (0.0)	0.003 (6.8)	0.013 (28.8)
chr7_140453136_A_T	46	0.045	0.000 (0.0)	0.000 (0.0)	0.016 (34.9)	0.000 (0.0)	0.006 (12.2)	0.017 (36.9)
chr7_140453136_AC_TT	46	0.130	0.000 (0.0)	0.004 (2.9)	0.017 (13.4)	0.003 (2.6)	0.006 (4.9)	0.019 (14.8)
chr9_139399350_C_CG	48	0.146	0.015 (10.2)	0.000 (0.0)	0.012 (8.2)	0.000 (0.0)	0.004 (2.8)	0.020 (13.4)
chr10_89720798_GTACT_G	44	0.157	0.000 (0.0)	0.003 (2.0)	0.021 (13.6)	0.002 (1.6)	0.010 (6.4)	0.024 (15.3)
chr12_25398284_CC_AT	38	0.111	0.000 (0.0)	0.000 (0.0)	0.019 (16.8)	0.003 (2.5)	0.008 (7.3)	0.020 (18.5)
chr17_7574002_CG_C	44	0.158	0.007 (4.2)	0.000 (0.0)	0.021 (13.5)	0.013 (8.6)	0.013 (8.2)	0.029 (18.4)
chr17_7574029_C_CGGAT	48	0.154	0.000 (0.0)	0.000 (0.0)	0.017 (11.0)	0.006 (3.8)	0.010 (6.6)	0.021 (13.4)
chr17_37880981_A_AGCATACGTGATG	36	0.075	0.013 (16.9)	0.006 (8.1)	0.013 (16.7)	0.000 (0.0)	0.004 (4.7)	0.019 (25.5)
chr22_41574678_GC_G	44	0.245	0.006 (2.4)	0.002 (0.6)	0.019 (7.9)	0.000 (0.0)	0.005 (2.1)	0.021 (8.6)

There were two small DNA targeted variants for which the number of observations was too small for a variance components model to be fitted. For these two targeted variants, overall SDs were 0.027 for variant chr1_27024001_C_CG and 0.001 for variant chr17_7578470_C_CGGGCGG.

Gene Amplifications

Table 59 shows PPCs for targeted gene amplifications. PPCs were 100.0% for MET and 100.0% for ERBB2.

Table 59 PPC of TSO Comprehensive (EU) Assay for Detection of Gene Amplifications in Combined Targeted Panel Members

Observed Variant Level[1]	Targeted Variant	Mean Observed Fold-change[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	MET	5.14	100.0% (48/48)	(92.6%, 100.0%)
<2xLOD	ERBB2	2.33	100.0% (47/47)	(92.4%, 100.0%)

[1] Variant level calculated from mean observed fold-change.
[2] Mean fold-change calculated from observed assay results.
[3] 95% two-sided confidence interval calculated via the Wilson Score Method.

PNCs were 100% across gene amplifications.

Table 60 shows the variance component analysis of fold-change results for each source of variation and total variation in all panel members with targeted gene amplifications.

Table 60 Variance Components Analysis of Fold-Change for Targeted Gene Amplifications

Targeted Variant	N	Mean Fold-change	Site SD (%CV)	Operator (Site) SD (%CV)	Day (Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
ERBB2	47	2.33	0.02 (0.6)	0.01 (0.4)	0.02 (0.9)	0.01 (0.4)	0.01 (0.5)	0.03 (1.3)
MET	48	5.14	0.05 (1.0)	0.12 (2.4)	0.14 (2.6)	0.00 (0.0)	0.03 (0.6)	0.19 (3.7)

MSI

Table 61 shows PPCs for targeted MSI-H panel members. PPCs were 100% for both MSI-H panel members.

Table 61 PPC of TSO Comprehensive (EU) Assay for Detection of MSI-H status in Combined Targeted Panel Members

Panel Member	Mean MSI Score[1]	N	Percent Positive Call (%)	95% CI[2]
TPSBD4	60.5	36	100.0% (36/36)	(90.4%, 100.0%)
TPSBD6	55.7	32	100.0% (32/32)	(89.3%, 100.0%)
All Members		68	100.0% (68/68)	(94.7%, 100.0%)

[1] Mean observed MSI Score calculated from observed assay results.

[2] 95% two-sided confidence interval calculated via the Wilson Score Method.

Table 62 shows the variance component analysis of MSI score results for each source of variation and total variation in all panel members targeted for MSI-H status.

Table 62 Variance Components Analysis of MSI Score for Targeted MSI-H Panel Members

Panel Member	N	Mean MSI Score	Site SD (%CV)	Operator (Site) SD (%CV)	Day (Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
TPSBD4	36	60.5	0.0 (0)	0.0 (0)	2.1 (3)	0.0 (0)	2.1 (3)	3.0 (5)
TPSBD6	32	55.7	0.0 (0)	1.3 (2)	1.0 (2)	0.8 (1)	2.9 (5)	3.4 (6)

TMB

To evaluate the reproducibility of TMB scores, a quantitative analysis of the score was conducted in targeted TMB panel members, which represented a range of expected TMB scores. Table 63 shows the variance component analysis of TMB score results for each source of variation and total variation in the TMB panel members. Total SDs of TMB score were 1.0 (%CV = 13) for one panel member (mean TMB score = 7.6) and 1.1 (%CV = 2) for another panel member (mean TMB score = 63.2).

Table 63 Variance Components Analysis of TMB Score for Targeted TMB Panel Members

Panel Member	N	Mean TMB Score	Site SD (%CV)	Operator (Site) SD (%CV)	Day (Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
TPSBD3	28	7.6	0.2 (2)	0.0 (0)	0.8 (10)	0.0 (0)	0.5 (7)	1.0 (13)
TPSBD4	44	63.2	0.3 (1)	0.6 (1)	0.4 (1)	0.0 (0)	0.7 (1)	1.1 (2)

There was 1 TMB panel member for which the number of observations was too small (N = 2) for a variance components model to be fitted. For this panel member, overall SD was 1.7.

RNA Variants

Table 64 shows PPCs for targeted RNA variants. PPCs ranged from 91.7% for KIF5B-RET to 100% for most RNA variants.

Table 64 PPC of TSO Comprehensive (EU) Assay for Detection of RNA Variants in Combined Targeted Panel Members

Observed Variant Level[1]	Variant Type	Targeted Variant	Mean Supporting Reads[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	Fusion	ACPP-ETV1	44.7	100.0% (46/46)	(92.3%, 100.0%)

Observed Variant Level[1]	Variant Type	Targeted Variant	Mean Supporting Reads[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	Fusion	BCL2-IGHJ5	124.9	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	CD74-ROS1;GOPC	56.6	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	Fusion	DHX8;ETV4-STAT3	48.9	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	EGFR-GALNT13	49.8	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	EML4-ALK	49.3	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	Fusion	ESR1-CCDC170	45.1	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	FGFR1-GSR	61.1	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	FGFR2-SRPK2	53.4	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	Fusion	FGFR3-TACC3	53.5	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	Fusion	HNRNPUL1-AXL	58.0	100.0% (48/48)	(92.6%, 100.0%)
<2xLOD	Fusion	KIF5B-RET	11.6	91.7% (44/48)	(80.4%, 96.7%)
<2xLOD	Fusion	MKRN1-BRAF	33.4	100.0% (48/48)	(92.6%, 100.0%)
<2xLOD	Fusion	PAX3-FOXO1	70.1	100.0% (48/48)	(92.6%, 100.0%)
<2xLOD	Fusion	RAF1-VGLL4	15.9	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Fusion	SPIDR-NRG1	51.5	100.0% (48/48)	(92.6%, 100.0%)
~2-3xLOD	Fusion	TMPRSS2-ERG	43.5	97.9% (47/48)	(89.1%, 99.6%)

Observed Variant Level[1]	Variant Type	Targeted Variant	Mean Supporting Reads[2]	Percent Positive Call (%)	95% CI[3]
~2-3xLOD	Splice Variant	EGFR vIII	64.0	100.0% (46/46)	(92.3%, 100.0%)
~2-3xLOD	Splice Variant	MET exon 14 skipping	61.2	100.0% (48/48)	(92.6%, 100.0%)

[1] Variant level calculated from mean observed supporting reads.
[2] Mean supporting reads calculated from observed assay results.
[3] 95% two-sided confidence interval calculated via the Wilson Score Method.

PNC was 100% for each targeted RNA variant, except for the FGFR2-SRPK2 fusion (PNC = 99.60% (984/988; 95% CI: 98.96% to 99.84%).

Table 65 shows the variance component analysis of supporting read results for each source of variation and total variation in all panel members with targeted RNA variants.

Table 65 Variance Components Analysis of Supporting Reads for Targeted RNA Variants

Targeted Variant	N	Mean Supporting Reads	Site SD (%CV)	Operator (Site) SD (%CV)	Day (Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
ACPP-ETV1	46	44.7	10.38 (23)	0.00 (0)	13.01 (29)	5.90 (13)	2.28 (5)	17.80 (40)
BCL2-IGHJ5	46	124.9	38.22 (31)	13.24 (11)	29.08 (23)	9.51 (8)	8.30 (7)	51.39 (41)
CD74-ROS1;GOPC	48	56.6	0.00 (0)	3.98 (7)	17.18 (30)	0.00 (0)	3.00 (5)	17.89 (32)
DHX8;ETV4-STAT3	46	48.9	18.27 (37)	13.42 (27)	17.01 (35)	0.00 (0)	1.50 (3)	28.38 (58)
EGFR-GALNT13	46	49.8	0.00 (0)	6.90 (14)	14.86 (30)	2.08 (4)	2.82 (6)	16.75 (34)
EML4-ALK	48	49.3	0.00 (0)	12.18 (25)	19.10 (39)	8.83 (18)	1.94 (4)	24.39 (49)
ESR1-CCDC170	46	45.1	2.30 (5)	0.00 (0)	12.37 (27)	0.00 (0)	8.08 (18)	14.95 (33)
FGFR1-GSR	46	61.1	8.57 (14)	1.31 (2)	11.15 (18)	9.23 (15)	5.18 (8)	17.65 (29)
FGFR2-SRPK2	48	53.4	3.18 (6)	10.90 (20)	15.85 (30)	15.29 (29)	3.10 (6)	24.97 (47)

Targeted Variant	N	Mean Supporting Reads	Site SD (%CV)	Operator (Site) SD (%CV)	Day (Site, Operator) SD (%CV)	Lot SD (%CV)	Run SD (%CV)	Total SD (%CV)
FGFR3-TACC3	48	53.5	17.43 (33)	0.00 (0)	12.38 (23)	5.81 (11)	3.46 (6)	22.42 (42)
HNRNPUL1-AXL	48	58.0	0.00 (0)	12.15 (21)	18.22 (31)	0.00 (0)	3.96 (7)	22.26 (38)
KIF5B-RET	48	11.6	0.89 (8)	0.00 (0)	3.97 (34)	1.44 (12)	1.09 (9)	4.45 (38)
MKRN1-BRAF	48	33.4	6.98 (21)	8.19 (25)	13.02 (39)	6.63 (20)	4.00 (12)	18.58 (56)
PAX3-FOXO1	48	70.1	12.45 (18)	10.79 (15)	17.91 (26)	3.02 (4)	2.42 (3)	24.65 (35)
RAF1-VGLL4	46	15.9	1.46 (9)	1.52 (10)	3.80 (24)	4.42 (28)	1.23 (8)	6.32 (40)
SPIDR-NRG1	48	51.5	4.78 (9)	0.00 (0)	10.69 (21)	5.94 (12)	3.29 (6)	13.54 (26)
TMPRSS2-ERG	48	43.5	5.63 (13)	8.81 (20)	9.98 (23)	0.00 (0)	6.21 (14)	15.73 (36)
EGFR vIII splice variant	46	64.0	12.70 (20)	0.42 (1)	17.69 (28)	0.00 (0)	2.34 (4)	21.90 (34)
MET exon 14 skipping splice variant	48	61.2	11.42 (19)	3.43 (6)	19.84 (32)	7.55 (12)	2.10 (3)	24.43 (40)

Within-laboratory Precision

Two studies were conducted to evaluate within-laboratory precision for TSO Comprehensive (EU). Study 1 evaluated NTRK and RET fusions, and RET small DNA variants. Study 2 evaluated TMB and MSI.

Study 1

Within-laboratory precision was evaluated for NTRK1 – 3 fusions (Lower-grade glioma, Glioblastoma Multiforme, Myofibroblastic sarcoma, Secretory breast cancer), RET fusions (Thyroid cancer and skin tissue from an unknown cancer), and RET small DNA variants (medullary thyroid cancer) with FFPE tissues from the indicated cancers. Each sample was tested at two variant levels: ~1x LoD (low variant level) and ~2 – 3x LoD (high variant level) except for the sample harboring CCDC6-RET, which was only tested at the low variant level. Each of the samples at each test level was run in duplicates in each library preparation event across three (3) operators. Each operator started library preparation on three (3) non-consecutive start days and sequenced on three (3) designated NextSeq 550Dx instruments. Three (3) reagents lots were tested generating 54 observations per level. Some levels had fewer than 54 observations due to invalid libraries.

Qualitative Analysis

The qualitative concordance of variant calling was evaluated separately for the two variant levels for a given variant from pooled observations across all variables (operators, reagent lots, instruments, days, and replicates). The percent positive calls (PPC) and percent negative calls (PNC) and associated two-sided 95% confidence interval (Wilson score) are summarized in Table 66 (small DNA variants) and Table 67 (RNA fusions).

At the high variant level (~2 – 3x LoD), the TSO Comprehensive (EU) assay demonstrated 100% for PPC and PNC for all variants tested.

At the low variant level (~1x LoD), PPC for small DNA variants ranged from 83.3% to 98.1%, and the PPC for RNA fusions ranged from 90.7% to 100%. For variants with PPC < 95%, the mean VAFs (RET C634Y and RET D898_E901del) or supporting reads (NCOA4-RET and BCAN-NTRK1) were below the respective Limits of Detection. At the low variant level, 100% PNC was achieved for all variants.

Table 66 Qualitative Results for Targeted DNA Variant

Variant Level	Variant	Variant Type	Mean VAF	PPC (95% CI)	PNC (95% CI)
Low (~1x LoD)	RET C634Y	MNV	0.028	83.3% (45/54) (71.3% – 91.0%)	100.0% (215/215) (98.2% – 100.0%)
	RET D898_E901del	DELETION	0.048	87.0% (47/54) (75.6% – 93.6%)	100.0% (215/215) (98.2% – 100.0%)
	RET C618R	SNV	0.045	94.4% (51/54) (84.9% – 98.1%)	100.0% (215/215) (98.2% – 100.0%)
	RET M918T	SNV	0.042	96.2% (51/53) (87.2% – 99.0%)	100.0% (216/216) (98.3 – 100.0%)
	RET D631_ L633delinsE*	DELETION	0.056	98.1% (53/54) (90.2% – 99.7%)	100.0% (215/215) (98.2% – 100.0%)

Variant Level	Variant	Variant Type	Mean VAF	PPC (95% CI)	PNC (95% CI)
High (~3x LoD)	RET C634Y	MNV	0.095	100.0% (54/54) (93.4% – 100.0%)	100.0% (192/192) (98.0% – 100.0%)
	RET D898_E901del	DELETION	0.088	100.0% (54/54) (93.4% – 100.0%)	100.0% (192/192) (98.0% – 100.0%)
	RET C618R	SNV	0.146	100.0% (54/54) (93.4% – 100.0%)	100.0% (192/192) (98.0% – 100.0%)
	RET M918	SNV	0.078	100.0% (52/52) (93.1% – 100.0%)	100.0% (194/194) (98.1% – 100.0%)
	RET D631_ L633delinsE*	DELETION	0.161	100.0% (32/32) (89.3% – 100.0%)	100.0% (214/214) (98.2% – 100.0%)

* Nucleotide changes are listed for each variant in the Limit of Detection section except for RET D631_L633delinsE, which is Chromosome 10, Position 43609940, Reference ACGAGCT, Alternative A.

Table 67 Qualitative Results for Targeted RNA Fusions

Variant Level	Fusion	Mean Supporting Reads	PPC (95% CI)	PNC (95% CI)
Low	TPM3-NTRK1	20.2	100.0% (54/54) (93.4%, 100.0%)	100.0% (537/537) (99.3%, 100.0%)
	BCAN-NTRK1	22.1	94.4% (51/54) (84.9%, 98.1%)	100.0% (591/591) (99.4%, 100.0%)
	ETV6-NTRK2	20.3	100.0% (54/54) (93.4%, 100.0%)	100.0% (591/591) (99.4%, 100.0%)
	ETV6-NTRK3	16.2	100.0% (54/54) (93.4%, 100.0%)	100.0% (537/537) (99.3%, 100.0%)
	ETV6-NTRK3 (FFPE cell line)	23.1	98.1% (53/54) (90.2%, 99.7%)	
	NCOA4-RET	13.3	90.7% (49/54) (80.1%, 96.0%)	100.0% (537/537) (99.3%, 100.0%)
	CCDC6-RET	18.7	98.1% (53/54) (90.2%, 99.7%)	100.0% (591/591) (99.4%, 100.0%)
High	TPM3-NTRK1	57.1	100.0% (54/54) (93.4%, 100.0%)	100.0% (481/481) (99.2%, 100.0%)
	BCAN-NTRK1	53.2	100.0% (54/54) (93.4%, 100.0%)	100.0% (535/535) (99.3%, 100.0%)
	ETV6-NTRK2	52.0	100.0% (54/54) (93.4%, 100.0%)	100.0% (535/535) (99.3%, 100.0%)
	ETV6-NTRK3	41.7	100.0% (54/54) (93.4%, 100.0%)	100.0% (481/481) (99.2%, 100.0%)
	ETV6-NTRK3 (FFPE cell line)	28.3	100.0% (54/54) (93.4%, 100.0%)	
	NCOA4-RET	24.8	100.0% (54/54) (93.4%, 100.0%)	100.0% (481/481) (99.2%, 100.0%)
	CCDC6-RET	N/A	Not tested	100.0% (589/589) (99.4%, 100.0%)

Quantitative Analysis

Restricted maximum likelihood (REML) variance components analysis was performed to evaluate total variation of the underlying continuous variable (VAF for small DNA variants and supporting reads for RNA fusions) and estimate the components of precision [standard deviation (SD), coefficient of variation (CV)] for each source of variation [operators, instruments, days, reagent lots, residual and total]. The results are presented in Table 68 for small DNA variants and Table 69 for RNA fusions.

The variation in VAF increased with the mean as expected for a binomial proportion. The variation in supporting reads increased with the mean as expected with count data. The residual component was the largest contributor to total variance for both small DNA variants and RNA fusions at both levels supporting the conclusion that detection of these variants by TSO Comprehensive (EU) is robust to operators, lots, instruments, and days.

Table 68 Quantitative SD and CV Results for Targeted Small DNA Variants

VAF Level	Variant	Variant Type	N Valid Attempts	Mean VAF	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
Low (~1x LoD)	RET D898_ E901del	DELETION	54	0.048	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.004 (8.7)	0.014 (30.0)	0.015 (31.2)
	RET C618R	SNV	54	0.046	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.000 (0.0)	0.014 (31.3)	0.014 (31.3)
	RET M918T	SNV	53	0.042	0.000 (0.0)	0.001 (3.0)	0.000 (0.0)	0.000 (0.0)	0.011 (25.6)	0.011 (25.7)
	RET C634Y	MNV	54	0.028	0.000 (0.0)	0.000 (0.0)	0.001 (3.3)	0.000 (0.0)	0.009 (30.7)	0.009 (30.9)
	RET D631_ L633delinsE	DELETION	54	0.056	0.000 (0.0)	0.002 (3.0)	0.006 (11.6)	0.000 (0.0)	0.010 (18.5)	0.012 (22.0)

VAF Level	Variant	Variant Type	N Valid Attempts	Mean VAF	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
High (~3x LoD)	RET D898_ E901del	DELETION	54	0.088	0.000 (0.0)	0.000 (0.0)	0.001 (1.4)	0.006 (7.0)	0.017 (19.2)	0.018 (20.5)
	RET C618R	SNV	54	0.146	0.003 (1.7)	0.000 (0.0)	0.020 (13.7)	0.002 (1.1)	0.018 (12.6)	0.027 (18.7)
	RET M918T	SNV	52	0.078	0.002 (3.1)	0.000 (0.0)	0.000 (0.0)	0.007 (9.1)	0.018 (23.1)	0.020 (25.0)
	RET C634Y	MNV	54	0.095	0.000 (0.0)	0.002 (2.5)	0.002 (2.1)	0.000 (0.0)	0.014 (15.0)	0.015 (15.3)
	RET D631_ L633delinsE	DELETION	52*	0.164	0.000 (0.0)	0.000 (0.0)	0.005 (3.0)	0.000 (0.0)	0.020 (12.1)	0.020 (12.4)

Table 69 Quantitative SD and CV Results for Targeted RNA Fusions

Supporting Reads Level	Fusion	N Valid Attempts	Mean Supporting Reads	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
Low	TPM3-NTRK1	54	20.2	2.3 (11.5)	0.9 (4.7)	3.3 (16.4)	0.8 (4.1)	5.7 (28.2)	7.1 (35.2)
	BCAN-NTRK1	54	22.1	3.4 (15.3)	1.4 (6.4)	1.8 (8.0)	0.0 (0.0)	6.0 (27.2)	7.3 (32.9)
	ETV6-NTRK2	54	20.3	0.0 (0.0)	3.2 (15.7)	4.4 (21.5)	0.0 (0.0)	8.3 (40.8)	9.9 (48.7)
	ETV6-NTRK3	54	16.2	2.3 (14.0)	2.4 (14.6)	2.2 (13.4)	0.0 (0.0)	4.7 (28.7)	6.1 (37.5)
	ETV6-NTRK3 (cell line)	54	23.1	4.6 (19.7)	1.2 (5.1)	0.0 (0.0)	0.0 (0.0)	6.7 (29.1)	8.2 (35.5)
	NCOA4-RET	54	13.3	1.7 (12.6)	0.0 (0.0)	0.0 (0.0)	1.7 (12.6)	5.1 (38.3)	5.6 (42.2)
	CCDC6-RET	54	18.7	0.0 (0.0)	1.1 (6.1)	5.4 (29.1)	0.0 (0.0)	6.2 (33.0)	8.3 (44.4)

Supporting Reads Level	Fusion	N Valid Attempts	Mean Supporting Reads	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
High	TPM3-NTRK1	54	57.1	11.2 (19.6)	1.2 (2.1)	5.7 (9.9)	2.0 (3.5)	11.9 (20.8)	17.4 (30.5)
	BCAN-NTRK1	54	53.2	8.2 (15.5)	0.8 (1.4)	5.6 (10.5)	2.9 (5.4)	11.3 (21.3)	15.4 (28.9)
	ETV6-NTRK2	54	52	0.0 (0.0)	4.1 (7.8)	7.1 (13.6)	5.7 (11.0)	12.9 (24.9)	16.3 (31.4)
	ETV6-NTRK3	54	41.7	7.2 (17.2)	0.4 (1.0)	6.4 (15.4)	0.0 (0.0)	10.7 (25.8)	14.4 (34.6)
	ETV6-NTRK3 (cell line)	54	28.3	7.9 (28.0)	1.0 (3.6)	0.0 (0.0)	0.0 (0.0)	9.1 (32.0)	12.1 (42.6)
	NCOA4-RET	54	24.8	3.1 (12.3)	0.0 (0.0)	5.9 (23.9)	0.0 (0.0)	6.8 (27.3)	9.5 (38.3)

Study 2

Within-laboratory precision was evaluated for TMB and MSI. Five NSCLC FFPE DNA samples for TMB and seven CRC FFPE samples for MSI, including both microsatellite stable (MSS) and MSI-high, were used to evaluate precision at different levels across the range of scores. Each of the samples were run in duplicate across three (3) operators, three (3) days, with three (3) library preparations for three (3) reagent lots using three NextSeq 550Dx instruments generating 54 observations per level.

Qualitative concordance was evaluated for MSI-status. The TSO Comprehensive (EU) assay demonstrated 100% concordance for percent positive calls and percent negative calls for MSI status. For TMB, the TSO Comprehensive (EU) assay reports a TMB score; qualitative concordance is not applicable.

The total variation of TMB and MSI score, along with the contribution by source (instruments, operators, lots, days, and residual), was quantified using a variance components model across a range of scores. The standard deviation (SD) and coefficient of variation (CV) are presented in Table 70 for TMB and Table 71 for MSI by level. Some levels had fewer than 54 observations due to invalid libraries.

Table 70 Quantitative TMB Score SD and CV Results

Level	Mean TMB Score	N Valid Attempts	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
L1	0.3	52	0.00 (0%)	0.06 (23%)	0.00 (0%)	0.08 (30%)	0.40 (146%)	0.41 (151%)
L2	8.4	53	0.00 (0%)	0.14 (2%)	0.00 (0%)	0.00 (0%)	0.71 (8%)	0.73 (9%)
L3	15.1	54	0.00 (0%)	0.00 (0%)	0.20 (1%)	0.00 (0%)	1.16 (8%)	1.18 (8%)
L4	20.3	53	0.00 (0%)	0.00 (0%)	0.06 (0%)	0.00 (0%)	0.56 (3%)	0.57 (3%)
L5	42.3	54	0.00 (0%)	0.00 (0%)	0.15 (0%)	0.00 (0%)	1.37 (3%)	1.38 (3%)

Table 71 Quantitative MSI Score SD and CV Results

MSI Status	Level	Mean MSI Score (%)	N Valid Attempts	Operator SD (%CV)	Instrument SD (%CV)	Lot SD (%CV)	Day SD (%CV)	Residual SD (%CV)	Total SD (%CV)
MS-Stable	L1	0.80	53	0.35 (43%)	0.00 (0%)	0.15 (18%)	0.00 (0%)	0.52 (66%)	0.64 (81%)
	L2	5.90	53	0.47 (8%)	0.00 (0%)	0.84 (14%)	0.00 (0%)	1.26 (21%)	1.58 (27%)
MSI-High	L3	48.68	53	0.19 (0%)	0.00 (0%)	0.00 (0%)	1.19 (2%)	2.48 (5%)	2.76 (6%)
	L4	56.85	54	1.66 (3%)	0.00 (0%)	1.92 (3%)	0.00 (0%)	3.07 (5%)	3.98 (7%)
	L5	72.62	54	0.00 (0%)	0.47 (1%)	0.34 (0%)	0.62 (1%)	1.28 (2%)	1.54 (2%)
	L6	75.29	54	0.00 (0%)	0.42 (1%)	0.09 (0%)	0.00 (0%)	1.46 (2%)	1.52 (2%)
	L7	78.38	54	0.00 (0%)	0.00 (0%)	0.00 (0%)	0.45 (1%)	0.95 (1%)	1.06 (1%)

The variation in TMB scores tend to increase with the mean as expected from theoretical distributions of count data. The variation in MSI scores for Levels near MSI Score = 50 are greater than the variation of MSI scores closer to 0, or 100 consistent with variability from theoretical distributions of proportion data. The residual component remained the largest contributor to total variance for both MSI and TMB scores supporting the conclusion that the scores are robust to operators, lots, instruments, and days.

C5 and C95 values around the cutoff of 20.00% were determined for MSI using a precision profile (Table 72).

Table 72 C5-C95 Intervals for MSI

Score	C5	C95
MSI	17.17%	23.32%

However, because both MSI and TMB are complex biomarkers, analytical performance may vary sample to sample. That is, TMB variation depends not only on the TMB value but also on the composition of variants in the sample, such as variant type (SNV, Indel), and VAF level (closeness to inclusion cutoff). Likewise, MSI variation depends not only on the MSI value but also on the composition of the sites in the sample, such as the number of sites that are unstable and the amount of instability per site.

The impact of tumor content on TMB and MSI scores was evaluated. For most samples, tumor content ≥ 30% had negligible impact on TMB scores above approximately 10 mutations per megabase. TMB scores remained relatively unchanged with increasing tumor content. For MSI-high samples, tumor content exhibited a positive, linear correlation with MSI score. MSI-high samples remained MSI-H on average when tumor content was ≥ 30%. Endometrial samples behaved distinctly different than the other tissue types and were found to need a greater amount of tumor content to be called MSI-H.

Accuracy for Tumor Profiling

The detection of variants by TSO Comprehensive (EU) assay was compared to the results of reference methods. DNA small variants and TMB were compared to an external validated whole exome NGS method. Gene amplifications were compared against the same whole exome NGS method or validated Dual In-Situ Hybridization (DISH) method for HER2 amplifications. MSI was evaluated against a validated MSI-PCR test. RNA splice variants were compared against a validated quantitative PCR (qPCR) method. ROS1 and ALK fusions were compared against validated FISH assays. All other fusions were compared against a composite method consisting of a validated RNA whole exome NGS assay (RNGS1), a targeted NGS panel (RNGS2), and droplet digital PCR (ddPCR).

Small DNA Variant Detection

The detection of small DNA variants by the TSO Comprehensive (EU) assay was compared to the results of whole exome sequencing (WES) that uses WES with matched tumor normal sample pairs for germline and somatic small variant calling. The comparison between small variants, consisting of single nucleotide variants (SNVs), insertions, and deletions, was based on 124 samples from 14 different tissue types that were valid for both TSO Comprehensive (EU) and WES. TSO Comprehensive (EU) but not the WES assay can detect multi-nucleotide variants (MNVs, 2 – 3bp) which requires phasing. TSO Comprehensive (EU) MNVs were evaluated as individual SNVs against WES. A summary of concordance at the variant level including Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) for all variant calls is shown in Table 73.

Table 73 Concordance Summary for Small Variant Calls Evaluated by Germline or Somatic Status

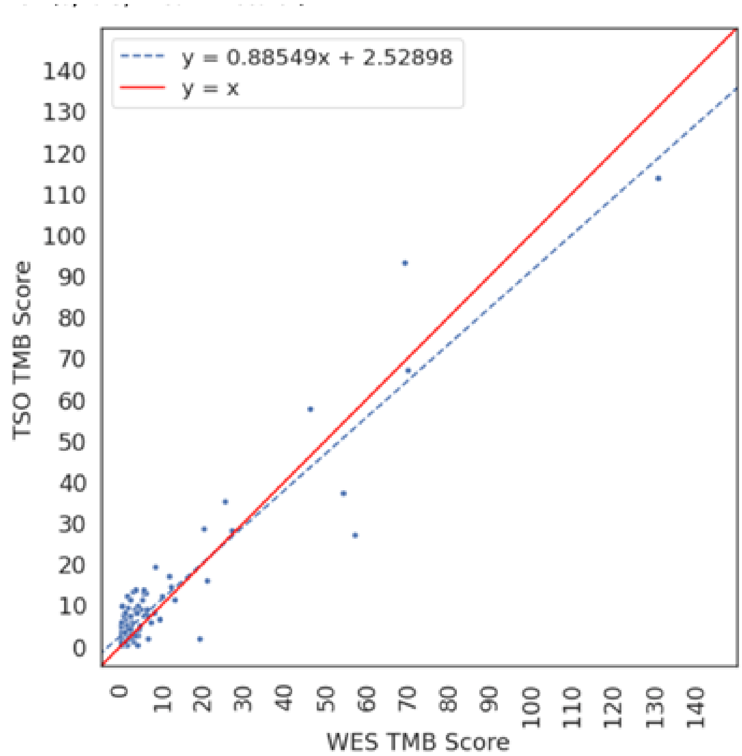
	WES Somatic Called	WES Germline Called	WES Not Called
TSO Comprehensive (EU) Called	382	33,163	426
TSO Comprehensive (EU) Not Called	69	61	70,000,481
Total	451	33,224	70,000,907
Percent Agreement	PPA: 85% (382/451), 95% CI: [81% – 87%]	PPA: > 99% (33,163/33,224) 95% CI: [99.8% – 99.9%]	NPA: > 99% (70,000,481/70,000,907) 95% CI: [99.999% – 99.999%]

In total, TSO Comprehensive (EU) called 426 variants that were not detected in the WES method. Two hundred and four (48%) of these variants had variant allele frequencies below the threshold for calling in the WES method. Of the remaining potential false positive variants, there was evidence of the variant call in the WES method with low support. Also, many of the variants had very low-level WES evidence in the matched normal samples. This result suggests that these variants were missed in the tumor by WES because of tumor in normal contamination.

Tumor Mutational Burden Detection

TMB concordance was determined by comparing the TMB scores (somatic mutations/megabase) between the WES method and TSO Comprehensive (EU) for 124 samples with available data by both TSO Comprehensive (EU) and WES. Linear regression analysis with WES as the predictor had a y-intercept of 2.53, a slope of 0.89, and Pearson's correlation coefficient of 0.94 (Figure 3).

Figure 3 TMB Score correlation between WES and TSO Comprehensive (EU)



Gene Amplification Detection

The detection of gene amplifications by the TSO Comprehensive (EU) assay was compared to the results of the same WES assay using either tumor normal matched samples or tumor only samples. In total, there were 420 samples of which 183 used the orthogonal tumor/normal method and 237 used the tumor only method. The samples were from 14 tissue types and contained amplifications from 55 genes. TSO Comprehensive (EU) reports gene amplifications from the MET and ERBB2 genes. However, accuracy was assessed for all 55 genes. A summary of the gene amplification calls is shown in Table 74.

Table 74 Gene Amplification Calls

	WES Positive	WES Negative
TSO Comprehensive (EU) Positive	337	415
TSO Comprehensive (EU) Negative	28	24,000
Total	365	24,415
Percent Agreement	PPA: 92% (337/365) 95% CI: [89%, 95%]	NPA: 98.3% (24,000/24,415) 95% CI: [98.1%, 98.5%]

ERBB2 (HER2) amplifications in gastric and breast tissues were analyzed separately from other gene amplifications using a Dual In-Situ Hybridization Method (DISH). In total, 116 breast and gastric samples, of which 64 had been previously characterized as HER2 positive by IHC or FISH were tested. One sample failed in extraction, 3 samples failed validity for TSO Comprehensive (EU), and 3 samples failed validity for DISH assay. Of the 108 samples, 20 (18.5%) had borderline scores (between 1.5 and 2.5) near the DISH cutoff of 2.0. Concordance results including PPA, NPA for all samples and excluding borderline HER2 DISH cases are shown in Table 75.

Table 75 Summary of Concordance Between TSO Comprehensive (EU) and HER2 DISH Including for HER2 Gene Amplification

HER2 Gene Amplification (Breast and Gastric tissues)	HER2 DISH Amplified	HER2 DISH not Amplified
TSO Comprehensive (EU) Positive	17 (including 1 borderline)	13 (including 1 borderline)
TSO Comprehensive (EU) Negative	10 (including 6 borderline)	68 (including 12 borderline)
Percent Agreement Including Borderline cases	PPA: 63% (17/27) 95% CI: [44%, 78%]	NPA: 84% (68/81) 95% CI: [74%, 90%]
Percent Agreement Excluding Borderline cases	PPA: 80% (16/20) 95% CI: [58%, 92%]	NPA: 82% (56/68) 95% CI: [72%, 90%]

Microsatellite Instability Detection

The detection of microsatellite instability by the TSO Comprehensive (EU) assay was compared to the results of a validated MSI-PCR test that uses tumor normal matched samples for testing. A total of 195 samples, meeting the tumor content requirement of ≥ 30% and representing 14 tissue types, were compared. MSI-PCR evaluates 5 sites and has 3 outcomes—MSS (no unstable sites), MSI-Low (one unstable site), and MSI-High (two or more unstable sites). TSO Comprehensive (EU) evaluates up to 130 microsatellite sites and only classifies samples as MSS or MSI-High (≥ 20% unstable sites). MSI-Low were grouped with MSS outcomes for MSI-PCR. Concordance analysis is shown in Table 76.

Table 76 Summary Of Concordance Analysis between TSO Comprehensive (EU) and MSI-PCR for DNA Microsatellite Instability

MSI Instability	PCR MSI-High	PCR MSI-Low	PCR MSS
TSO Comprehensive (EU) Unstable (MSI-High)	40	2	0
TSO Comprehensive (EU) Stable (MSS)	3	0	150
Total	43	2	150
Percent Agreement	PPA: 93% (40/43) 95% CI: [81%, 98%]	NPA: 99% (150/152) 95% CI: [95%, > 99%]	

RNA Splice Variant Detection

Accuracy for splice variant detection was calculated by comparing TSO Comprehensive (EU) results to qPCR assays for EGFRvIII and Met Exon 14del including one known positive RNA for each of the splice variants. Concordance analysis was performed on a total of 230 unique FFPE RNA samples from 14 tissue types with available data by both TSO Comprehensive (EU) and the reference method. All samples were tested for MET Exon 14del, while EGFRvIII were tested only in brain tissue respectively. Three samples called positive for MET Exon 14del by qPCR but not by TSO Comprehensive (EU) had average Ct > 37 and were below TSO Comprehensive (EU) LoD level. Table 77 summarizes the concordance study results.

Table 77 Summary of Concordance Analysis Between TSO Comprehensive (EU) and qPCR Assay for RNA Splice Variants

RNA Splice Variants	qPCR Positive	qPCR Negative
TSO Comprehensive (EU) Positive (EGFRvIII)	3	0
TSO Comprehensive (EU) Negative (EGFRvIII)	0	13
TSO Comprehensive (EU) Positive (Met Exon 14Del)	1	0
TSO Comprehensive (EU) Negative (Met Exon 14Del)	3	217
Total	7	230
Percent Agreement	PPA: 57% (4/7) 95% CI: [25%, 84%]	NPA: 100% (230/230) 95% CI: [98%, 100%)

RNA Fusion Detection

Comparison to a Composite Method

TSO Comprehensive (EU) fusions were compared to a composite method consisting of RNA whole exome sequencing using an NGS panel (RNGS1), a targeted NGS fusion panel (RNGS2), and droplet digital PCR (ddPCR).

The RNGS1 method overlaps with all the genes for which TSO Comprehensive (EU) can detect fusions. However, the limit of detection of the RNGS1 method was 4X – 8X that of TSO Comprehensive (EU) based on the number of supporting reads observed in the overlapping fusion calls. Hence, a composite method using two additional methods with greater sensitivity but less breadth for fusions were used with the WES (RNGS1) method.

A total of 255 unique RNA samples representing 14 tissue types and passing TSO Comprehensive (EU) metrics were tested with RNGS1. Two samples were invalid for RNGS1 sample QC and were excluded from additional analysis. Of the 82 fusions called by TSO Comprehensive (EU), 4 were excluded from evaluation due to RNGS1 sample QC failures, and 7 additional fusions were not callable due to absence of the targets in the RNGS1 panel. Of the remaining 71 fusions called by TSO Comprehensive (EU), 9 fusions were confirmed by RNGS1. RNGS1 called 4 fusions not called by TSO Comprehensive (EU).

From the 62 fusions that were TSO Comprehensive (EU) positive and not detected by RNGS1, 13 overlapped with and were confirmed by RNGS2. One fusion was called by RNGS2 but not called by TSO Comprehensive (EU).

Droplet digital PCR was then used for fusions called by TSO Comprehensive (EU), not called or not callable by RNGS1, and not evaluable by RNGS2 (49). In addition, ddPCR was used for reevaluation of 2 of the 4 false negative fusions for TSO Comprehensive (EU) with RNGS1 and 2 of 9 concordant fusions for TSO Comprehensive (EU) and RNGS1. Five fusions negative samples were included with testing of each positive fusion sample to ensure specificity. Eighteen fusions were not tested with ddPCR due to inability to design primers/probes, multiple gene partners for the fusion, or insufficient remaining FFPE material. For ddPCR, primers and probes were designed against the observed breakpoints in the TSO Comprehensive (EU) assay.

In total 52 fusions were detected by ddPCR, 41 of those fusions were called by TSO Comprehensive (EU) but not called or not callable by RNGS1. Nine fusions were called by ddPCR but negative in TSO Comprehensive (EU) or RNGS1. Two ddPCR positive fusions confirmed the 2 concordant fusions for TSO Comprehensive (EU) and RNGS1. No fusion was detected by ddPCR for the 2 reevaluated TSO Comprehensive (EU) false negatives with RNGS1; however, these were counted as false negatives based on the RNGS1 comparison.

The composite concordance results methods RNGS1, RNGS2, and ddPCR for fusions are shown in Table 78.

The 63 fusions concordant with the composite method represented 43 genes in the TSO Comprehensive (EU) panel. However, fusions are eligible for reporting only from the 23 genes indicated in *TSO Comprehensive (EU) Assay Gene Panel* on page 2.

Table 78 Cross Tabulation of TSO Comprehensive (EU) Versus Composite Method Outcomes for RNA Fusions (253 samples)

Fusions	Composite Method Positive	Composite Method Negative
TSO Comprehensive (EU) Positive	63[1]	18
TSO Comprehensive (EU) Negative	14[2]	13,821
Total	77	13,839
Percent Agreement	PPA: 82% (63/77) 95% CI: [72%, 89%]	NPA: 99.9% (13821/13839) 95% CI: [99.8%, 99.9%]

[1] 63 TSO Comprehensive (EU) true positives = 9 positives concordant with RNGS1 + 13 positive concordant with RNGS2 + 41 positive concordant with ddPCR.
[2] 14 TSO Comprehensive (EU) false negatives = 4 negatives not concordant with RNGS1 + 1 negative not concordant with RNGS2 + 9 negative not concordant with ddPCR.

Comparison to FISH Method for ROS1 and ALK Fusions

Twenty-five NSCLC samples were tested by FISH for both ROS1 and ALK fusions and 5 additional NSCLC samples were tested for ROS1 fusion, respectively. Eight samples failed FISH for ROS1 due to inadequate tissue. Two ROS1, and one ALK fusions were detected by both TSO Comprehensive (EU) and FISH. No discordant results were observed. Table 79 summarizes the concordance results of TSO Comprehensive (EU) and FISH method for ROS1 and ALK Fusions.

Table 79 Summary of the Concordance Results of TSO Comprehensive (EU) and FISH method for ROS1 and ALK Fusions

ALK+ROS1	FISH Positive	FISH Negative
TSO Comprehensive (EU) Positive	3	0
TSO Comprehensive (EU) Negative	0	44
Total	3	44
Percent Agreement	PPA: 100% (3/3) 95% CI: [44%, 100%]	NPA: 100% (44/44) 95% CI: [92%, 100%]

Sample Validity

Sample validity (first attempt) was measured for 181 unique RNA and 272 unique DNA samples from FFPE blocks ≤ 5 years of age. These samples were selected based on tissue type and available material; assay validity was unknown. The library QC metrics must pass for the variant type to be considered valid. Sample validity were evaluated separately for each of the variant types (small DNA variants/TMB, MSI, gene amplifications, fusions/splice variants) and are shown in Table 80.

Table 80 Sample Validity

Variant Type	Sample Validity
Fusions/Splice Variants (RNA)	76%
Small DNA Variants/TMB	75%
MSI	72%
Gene Amplification	94%

Summary of Analytical Validation for Tumor Profiling Claims

Based on Limit of Detection, Precision, Reproducibility, and Accuracy data, TSO Comprehensive (EU) is analytically validated for the following:

- Small DNA variants—SNVs, MNVs, insertions, and deletions
- TMB
- MSI
- MET and ERBB2 (HER2) gene amplifications (Refer to *TSO Comprehensive (EU) Assay Gene Panel* on page 2).
- 23 genes for which fusions can be detected (Refer to *TSO Comprehensive (EU) Assay Gene Panel* on page 2).
- EGFR and MET splice variants (Refer to *TSO Comprehensive (EU) Assay Gene Panel* on page 2).

NTRK Clinical Performance

To validate the TSO Comprehensive (EU) assay as a companion diagnostic (CDx) for the selection of patients for treatment with VITRAKVI® (larotrectinib), samples from patients enrolled in the larotrectinib clinical trials (NCT02122913, NAVIGATE NCT02576431, SCOUT NCT02637687; referred to collectively as the larotrectinib trial samples) using a data cutoff of 15 JUL 2019, supplemented with commercially sourced FFPE tissue specimens, were tested to support a TSO Comprehensive (EU) assay Accuracy Study and Clinical Bridging Study.

NCT02122913 was a multicenter, open-label, Phase 1, dose escalation study in adult patients with advanced solid tumors (all-comers) unselected for NTRK fusion positive cancer. Following the dose escalation portion of the study, a dose expansion was initiated for patients with documented NTRK fusion positive cancer and for patients whom the investigator believed might benefit from a highly selective TRK inhibitor. NAVIGATE NCT02576431 is an ongoing, multicenter, open-label, Phase 2, basket study in patients aged 12 and older with recurrent advanced solid tumors with a documented NTRK fusion as assessed by an outside laboratory. SCOUT NCT02637687 is an ongoing, multicenter, open-label, Phase 1/2 study in pediatric patients aged from birth to 21 years with advanced solid or primary central nervous system (CNS) tumors.

Of the NTRK fusion positive patients included in the TSO Comprehensive (EU) assay study, 164 formed the larotrectinib extended primary efficacy set (ePAS4).

Accuracy Study for NTRK1, NTRK2, NTRK3 Fusion Detection

The accuracy of the TSO Comprehensive (EU) assay for detecting NTRK fusions (NTRK1, NTRK2, or NTRK3) in patients with solid tumors was demonstrated by assessing the concordance of NTRK fusion results between the TSO Comprehensive (EU) assay and a validated orthogonal method based on NGS.

A retrospective, noninterventional study was conducted. Larotrectinib trial samples and supplemental samples were tested with the TSO Comprehensive (EU) assay at one external site and with an orthogonal method at a central laboratory. Accuracy of the TSO Comprehensive (EU) assay NTRK fusion calls was estimated relative to the orthogonal method; positive percent agreement (PPA), negative percent agreement (NPA), and the associated two-sided 95% confidence intervals (CIs) were calculated.

There were 516 samples tested with the TSO Comprehensive (EU) assay and/or the orthogonal method. Of these samples, 499 were tested by both methods. Seventeen of the 516 samples were not tested with one of the assays due to failed extraction, unknown reason (for the orthogonal method), or protocol deviation. Of the 499 samples tested by both methods, 170 (34.1%) were larotrectinib trial samples and 329 (65.9%) were supplemental samples.

A cross-tabulation of results for the 499 samples is shown in Table 81. Of the 499 samples, 85 samples had invalid TSO Comprehensive (EU) assay results; of these 85, 53 also had invalid orthogonal method results. An additional 7 samples had invalid orthogonal method results. Thus, 407 of the 499 samples had valid results by both methods.

Table 81 NTRK Accuracy Study: Cross Tabulation of TSO Comprehensive (EU) Result Versus Orthogonal Method Result for NTRK Fusion Detection

	Orthogonal Method Result			
TSO Comprehensive (EU) Assay Result	**NTRK Fusion Positive**	**NTRK Fusion Negative**	**Invalid**	**Total**
NTRK Fusion Positive	114	16	1	131
NTRK Fusion Negative	4	273	6	283
Invalids*	4	28	53	85
Total	122	317	60	499

* TSO Comprehensive (EU) assay invalid results come from sample and run level.

The agreement analyses, excluding and including invalid TSO Comprehensive (EU) assay results, are shown in Table 82. Excluding invalid TSO Comprehensive (EU) assay results, PPA was 96.6% (114/118; 95% CI: 91.5% – 99.1%) and NPA was 94.5% (273/289; 95% CI: 91.2% – 96.8%).

Table 82 NTRK Accuracy Study: PPA and NPA of the TSO Comprehensive (EU) Assay Compared to Orthogonal Method Result for Detecting NTRK Fusions

	Excluding Invalid TSO Comprehensive (EU) Assay Results		Including Invalid TSO Comprehensive (EU) Assay Results	
Agreement Measure	Agreement, % (n/N)	95% CI*	Agreement, % (n/N)	95% CI*
PPA	96.6% (114/118)	91.5% – 99.1%	93.4% (114/122)	87.5% – 97.1%
NPA	94.5% (273/289)	91.2% – 96.8%	86.1% (273/317)	81.8% – 89.7%

* 95% CI based on (exact) Clopper-Pearson method.

Clinical Bridging Study for NTRK1, NTRK2, NTRK3 Fusion Detection

The clinical validity of the TSO Comprehensive (EU) assay for detecting NTRK1, NTRK2, or NTRK3 fusions in patients with solid tumors who may benefit from treatment with larotrectinib was demonstrated in a clinical bridging study. The study was conducted to assess the clinical effectiveness of the TSO Comprehensive (EU) assay to identify NTRK1, NTRK2, or NTRK3 fusion positive patients for treatment with larotrectinib, and to assess the concordance between the TSO Comprehensive (EU) assay and local test (LT) methods (used to determine NTRK fusion status for the larotrectinib clinical trials).

LT methods included NGS, fluorescent in situ hybridization (FISH), polymerase chain reaction (PCR), and NanoString assays. NTRK fusions (ETV6 NTRK3) were inferred for patients with infantile fibrosarcoma who had a documented ETV6 translocation identified by FISH. Most the 235 larotrectinib trial patients with known NTRK fusion status had been tested by NGS methods.

The NAVIGATE NCT02576431 and SCOUT NCT02637687 studies are continuing to enroll. As of the data cutoff date 15 JUL 2019, 279 patients were enrolled. Of the 279 patients, 208 were NTRK fusion positive. Of the 208 positive patients, 164 formed the larotrectinib ePAS4.

The primary endpoint for the larotrectinib analysis of efficacy was overall response rate (ORR) according to independent review committee (IRC) assessment in a pooled data set from the 3 clinical studies. ORR was assessed based on the proportion of patients with best overall response of confirmed complete response or confirmed partial response based on RECIST, version 1.1 criteria. ORR in the larotrectinib ePAS4 was 72.6% (95% CI [65.1%, 79.2%]) and included patients with 16 different tumor types.

Sample Accounting

The sample set included representation of a wide range of tumor types and pediatric and adult patient samples.

There were 279 patients enrolled in the larotrectinib studies as of 15 JUL 2019. Of these, 235 patients had known NTRK fusion status as determined by an LT method: 208 were positive and 27 were negative. For 44 patients, the NTRK fusion status was unknown, as testing was not required for patient eligibility in the dose escalation phases of the NCT02122913 and SCOUT NCT02637687 studies. For the TSO Comprehensive (EU) assay clinical bridging study, samples from larotrectinib trial patients enrolled as of 15 JUL 2019 with known NTRK fusion status (208 positive patients and 27 negative patients) and the supplemental samples determined to be NTRK fusion negative by representative LT methods were eligible for this study.

Of the 208 positive larotrectinib trial samples, 154 had a sample available for TSO Comprehensive (EU) assay testing. Of these, 138 had valid results. Fifteen samples were invalid due to failing sample sequencing quality metrics and 1 sample was not tested due to a protocol deviation. Of the 27 negative larotrectinib trial samples, 24 had a sample available for testing. Of these, 22 had valid TSO Comprehensive (EU) assay results. Two samples were invalid due to failing sample sequencing quality metrics.

Supplemental samples were screened using one of two representative LT methods. More than 350 samples were procured and examined for tumor content. Of the supplemental samples meeting sample requirements, 266 were successfully extracted and confirmed to be NTRK fusion negative by a representative LT method. Of these samples, 260 were available for TSO Comprehensive (EU) assay testing of which 222 had valid results. There were 38 samples that were invalid due to failing the sample sequencing metrics (n = 25) or failing run sequencing (n = 13). The total NTRK fusion negative set was comprised of 222 supplemental samples and 22 larotrectinib trial samples.

Concordance Results

Agreement of TSO Comprehensive (EU) results relative to LT methods results, with and without invalid TSO Comprehensive (EU) results, are shown in Table 83.

Table 83 NTRK Clinical Bridging Study: Concordance Between the TSO Comprehensive (EU) Assay and LT Methods for Detection of NTRK Fusions

	Excluding TSO Comprehensive (EU) Assay Invalid Results		Including TSO Comprehensive (EU) Assay Invalid Results	
Agreement Measure	**% Agreement (n/N)**	**95% CI***	**% Agreement (n/N)**	**95% CI***
PPA	89.1% (123/138)	82.7% – 93.8%	80.4% (123/153)	73.2% – 86.4%
NPA	96.3% (235/244)	93.1% – 98.3%	82.7% (235/284)	77.8% – 87.0%
OPA	93.7% (358/382)	90.8% – 95.9%	81.9% (358/437)	78.0% – 85.4%

* The two-sided 95% CIs were calculated using the (exact) Clopper-Pearson method.

Sensitivity analysis against the missing TSO Comprehensive (EU) assay results demonstrated the robustness of the agreement analysis. Missing TSO Comprehensive (EU) assay results for the LT NTRK fusion positive patients (n = 70) were imputed using a logistic regression model. Agreement estimates, including the imputed values, are shown in Table 84.

Table 84 NTRK Clinical Bridging Study: Concordance Between the TSO Comprehensive (EU) Assay and LT Methods for Detection of NTRK Fusions Including Imputed Values for LT Positive Patients With Missing TSO Comprehensive (EU) Assay Results

Agreement Measure	% Agreement	95% CI*
PPA	85.2%	78.6% – 91.7%
NPA	96.3%	93.9% – 98.7%
OPA	91.2%	87.9% – 94.5%

Missing TSO Comprehensive (EU) assay results for LT fusion negative patients were not imputed.
* The two-sided 95% CIs were calculated based on the multiple imputation Boot method. The multiple imputation Boot method is a bootstrap step nested in the multiple imputation (Schomaker and Heumann 2018).

Agreements between the TSO Comprehensive (EU) assay and LTs by method type (for example, RNA NGS, FISH) are shown in Table 85.

Table 85 NTRK Clinical Bridging Study: Concordance Between the TSO Comprehensive (EU) Assay and LT Methods for Detection of NTRK Fusions by LT Method Type

LT Method Type	Measure of Agreement	% Agreement (n/N)	95% CI[1]
DNA NGS	PPA	84.2% (32/38)	68.7% – 94.0%
	NPA	88.9% (16/18)	65.3% – 98.6%
	OPA	85.7% (48/56)	73.8% – 93.6%
RNA NGS[2]	PPA	91.5% (75/82)	83.2% – 96.5%
	NPA	96.9% (218/225)	93.7% – 98.7%
	OPA	95.4% (293/307)	92.5% – 97.5%
FISH	PPA	80.0% (8/10)	44.4% – 97.5%
	NPA	Not calculated (1/1)	Not calculated
	OPA	81.8% (9/11)	48.2% – 97.7%
PCR	PPA	100.0% (8/8)	63.1% – 100.0%
	NPA	Not calculated (0/0)	Not calculated
	OPA	100.0% (8/8)	63.1% – 100.0%

Not calculated: for subgroups with sample count < 5, agreement statistics were not calculated.
[1] The two-sided 95% CIs were calculated using the (exact) Clopper-Pearson method.
[2] Includes NGS methods that use RNA only and both DNA and RNA.

Of the 437 samples tested with the TSO Comprehensive (EU) assay, 24 had discordant results with the LTs: 15 were positive by the LTs and negative by the TSO Comprehensive (EU) assay and 9 were negative by the LTs and positive by the TSO Comprehensive (EU) assay. Of the 24 samples with discordant results, 8 were tested with a DNA NGS LT method, 14 with an RNA NGS LT method, and 2 with FISH.

A validated independent NGS method confirmed TSO Comprehensive (EU) assay results in 14 of the 24 samples with discordant results. For the remaining 10 samples, TSO Comprehensive (EU) assay results were discordant with both the LT and the independent NGS method.

Clinical Efficacy Results

Within the ePAS4 cohort, the efficacy of larotrectinib in the TSO Comprehensive (EU) positive, LT positive population (97 patients, ORR=78.4%, 95% CI [68.8%, 86.1%]) was similar to the efficacy of larotrectinib in the total ePAS4 population (164 patients, ORR=72.6%, 95% CI [65.1%, 79.2%]) (Table 86). Of the 97 TSO Comprehensive (EU) positive patients in ePAS4, 28 (28.9%) patients achieved a complete response/surgical complete response and 48 (49.5%) patients achieved a partial response.

Of the 13 TSO Comprehensive (EU) negative, LT positive population, 1 (7.7%) showed a complete response and 2 (15.4%) showed a partial response with larotrectinib therapy.

Table 86 NTRK Clinical Bridging Study: ORR for LT Positive Patients by LT and TSO Comprehensive (EU) Results in ePAS4

		LT Fusion Positive N=164	**TSO Comprehensive (EU) Positive and LT Positive N=97**	**TSO Comprehensive (EU) Negative and LT Positive N=13**
Best overall response, n (%)	Complete response	31 (18.9%)	22 (22.7%)	1 (7.7%)
	Surgical complete response	8 (4.9%)	6 (6.2%)	0
	Partial response	80 (48.8%)	48 (49.5%)	2 (15.4%)
	Stable disease	25 (15.2%)	13 (13.4%)	4 (30.8%)
	Progressive disease	13 (7.9%)	6 (6.2%)	5 (38.5%)
	Not evaluable	7 (4.3%)	2 (2.1%)	1 (7.7%)
Overall response rate	Number of patients, n	164	97	13
	Number of patients with CR + sCR + PR, n	119	76	3
	ORR% (95% CI*)	72.6% (65.1%, 79.2%)	78.4% (68.8%, 86.1%)	23.1% (5.0%, 53.8%)

Abbreviations: CR = Complete Response, PR = Partial Response, sCR = Surgical Complete Response.
* The two-sided 95% confidence interval was calculated using the (exact) Clopper-Pearson method.
54 patients have missing TSO Comprehensive (EU) assay results.

The data from this study supports the safety and effectiveness of the TSO Comprehensive (EU) assay when used to identify patients with solid tumors with NTRK fusions who may be eligible for treatment with larotrectinib.

References

1. American Society of Clinical Oncology. www.asco.org. Accessed October 3 2016.
2. European Society for Medical Oncology. www.esmo.org. Accessed October 3 2016.

Revision History

Revision	Date	Description of Change
v06	February 2023	• Additional statements in Limitations section • Language updates for convention, grammar, and clarity • Correction of Tables 21, 28, 29, 32, 35, 36, 72 • Statement on presence of precipitates in FSM reagent • Updated thermal cycler and trough specs in the Equipment and Materials list
v05	September 2022	• Added TSO Comprehensive analysis module v2.3.5 PNs • Removed TSO Comprehensive analysis module v2.3.3 PNs • Updated terminology in Limit of Blank section
v04	June 2022	• Added TSO Comprehensive analysis module v2.3.5 PNs • Removed TSO Comprehensive analysis module v2.3.3 PNs • Updated terminology in Limit of Blank section
v03	April 2022	• Added performance characteristic information related to NTRK fusions • Added FOR EXPORT ONLY marking • Updated intended use statement to add the NTRK1-3 CDx claim • Expanded product components information to include software component PNs
v02	February 2022	• Corrected table reference error • Added limitation related to germline and somatic variants • Clarified language around gene amplification detection
v01	December 2021	• Updated limitations of the procedure • Clarified the magnetic stand and thermal cycler specifications in the equipment and materials lists
v00	November 2021	Initial release

Patents and Trademarks

Contact Information

Illumina, Inc.
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

CE

IVD

EC REP

Illumina Netherlands B.V.
Steenoven 19
5626 DK Eindhoven
The Netherlands

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