TruSight Oncology Comprehensive

illumina®

(US) Package Insert

FOR IN VITRO DIAGNOSTIC USE.

Intended Use

TruSight™ Oncology Comprehensive is a qualitative *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, and deletions from DNA, and fusions in 24 genes and splice variants in one gene from RNA. The test also reports a Tumor Mutational Burden (TMB) score.

The test is intended to be used as a companion diagnostic to identify cancer patients who may benefit from treatment with the targeted therapies 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 in oncology for patients with solid malignant neoplasms. Genomic findings other than those listed in Table 1 of the intended use statement are not conclusive or prescriptive for labeled use of any specific therapeutic product.

Table 1 Companion Diagnostic Indications

Tumor Type	Biomarker(s) Detected	Therapy
Solid Tumors	*NTRK1/2/3* fusions	VITRAKVI® (larotrectinib)
Non-Small Cell Lung Cancer (NSCLC)	*RET* fusions	RETEVMO® (selpercatinib)

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. Analysis of a cancer'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 cancer causation or progression, and many cancers 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 fusions, and splice variants. Another consequence of cancer gene

mutations is the production of neoantigens that can elicit cancer-specific immune responses. The mutational state of a cancer can be represented by TMB, a genomic signature associated with genomic instability and cancer neoantigen presentation.

TruSight Oncology Comprehensive is a qualitative next-generation sequencing (NGS) comprehensive genomic profiling test indicated as a companion diagnostic to identify cancer patients for treatment with the targeted therapies listed in Table 1, in accordance with the approved therapeutic product labeling. In addition, the assay broadly assesses genomic variants in a large panel of cancer-related genes listed in Table 2 that may inform patient management in accordance with professional guidelines. The assay detects small DNA variants in 517 genes, RNA fusions in 24 genes, and RNA splice variants in EGFR, as indicated in Table 2, and assesses a TMB score. The assay provides coding sequence coverage for all genes except TERT, where only the promoter region is covered. These assay targets include content cited by professional organizations and other major US guidelines. Independent consortia publications and late-stage pharmaceutical research also influenced the design of the TSO Comprehensive assay. The output of the test includes three levels.

- **Level 1**—CDx claims noted in Table 1 of the Intended Use.
- **Level 2**—Cancer Mutations with Evidence of Clinical Significance.
- **Level 3**—Cancer Mutations with Potential Clinical Significance.

Genomic findings other than those listed in Table 1 of the intended use statement are not prescriptive or conclusive for labeled use of any specific therapeutic product.

For a list of regions that are excluded from variant calling, refer to the *TruSight Oncology Comprehensive Block List (document # 200009524)* available from the Illumina support website.

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

All fusions containing one or two genes that are indicated for fusion detection in Table 2 (ie, marked as F for variant type next to gene name) are eligible for reporting.

Table 2 TSO Comprehensive 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, F	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	185	668	FOXL2	S	360	84295	PHF6	S
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

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
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
51	675	BRCA2	S	226	126961	HIST2H3C	S	401	5879	RAC1	S
52	23476	BRD4	S	227	653604	HIST2H3D	S	402	5885	RAD21	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
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, F	248	3624	INHBA	S	423	54894	RNF43	S
74	1021	CDK6	S	249	3631	INPP4A	S	424	6098	ROS1	S
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
91	64109	CRLF2	S	266	3792	KEL	S	441	4068	SH2D1A	S

No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type	No.	Entrez ID	Gene	Variant Type
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[1]	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	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	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, F	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

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170	2251	FGF6	S	345	57144	PAK5	S	N/A	N/A	N/A	N/A
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

[1] Fusions containing the ALK gene are not eligible for reporting.
[2] Fusions containing the ROS1 gene are not eligible for reporting.

Principles of Procedure

The TSO Comprehensive assay is a distributed test that is performed manually 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 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 adapter sequences are incorporated into each library to enable the capture of library fragments onto the surface of the flow cell during sequencing. The adapters include i5 and i7 index sequences to identify each individual sample and, 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 (US) analysis module (TSO Comprehensive [US] Analysis Module) resides on the NextSeq 550Dx instrument as part of the Local Run Manager software to facilitate TSO Comprehensive 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 and calculate a TMB score from DNA, and to call fusions and a splice variant from RNA. Multiple outputs are generated by the analysis software module including sequencing metrics and Variant Call Format (VCF) files. VCF files contain information about variants found at specific positions in a reference genome. Sequencing metrics and individual output files are generated for each sample.
- **Tertiary Analysis**—Tertiary analysis, performed by the TSO Comprehensive (US) analysis module, consists of a TMB score calculation, companion diagnostic (CDx) calling, tumor profiling of variants into two levels of clinical significance using a Knowledge Base and the tissue type, and results report generation. Tumor profiling can also be referred to as comprehensive genomic profiling for cancer variants. The interpreted variant results and the TMB score are summarized in the TSO Comprehensive (US) results report. For details on secondary and tertiary analysis, refer to the *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*.

Limitations of the Procedure

For *in vitro* diagnostic use only.

- For prescription use only. The test must be ordered by a qualified medical professional in accordance with clinical laboratory regulations.
- Genomic findings listed in the TSO Comprehensive results report under Cancer Mutations with Evidence of Clinical Significance (Level 2) and Cancer Mutations with Potential Clinical Significance (Level 3) are not prescriptive or conclusive for labeled use of any specific therapeutic product, and 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. Quality can be impacted by:
  - 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).

  - High amounts of necrotic tissue (≥ 25%) can interfere with the ability of the TSO Comprehensive assay to detect RNA fusions.
  - Somatic driver mutations may not be detected reliably if tumor content (by area) is less than 20%.
- The performance of TSO Comprehensive in samples obtained from patients that have had organ or tissue transplantation has not been evaluated.
- Accuracy of small DNA tumor profiling variants below 5% variant allele frequency (VAF) has not been established.
- Accuracy for the EGFRvIII splice variant from RNA has only been established in brain tissue. Accuracy for EGFRvIII in other tissue types has not been established.
- The Limit of Detection (LoD) for insertions of 1-2 base pairs with dinucleotide repeats has been established with cell lines.
- Contamination detection can be impacted by:
  - In highly rearranged genomes with deletions and loss of heterozygosity, TSO Comprehensive software can erroneously classify a DNA sample as contaminated (CONTAMINATION_SCORE > 3106 and p-value > 0.049).
  - Contamination during the procedure can result from nucleic acid from previous sample processing steps. Good laboratory practice and following all precautions and guidelines in this Package Insert will help avoid cross-contamination between samples.
  - TSO Comprehensive does not have a metric to detect contamination for RNA libraries. If the same fusion is detected in multiple samples in the same run, repeat TSO Comprehensive testing is suggested.
- A negative result does not rule out the presence of a mutation below the limits of detection (LoD) of the assay. Alterations at allele frequencies below the established LoD may not be detected consistently.
- The overall negative percent agreement for NTRK fusions between drug trial enrollment assays and TSO Comprehensive was 96.3% (95%CI: 93.1%, 98.3%). False positive results for gene fusions such as NTRK may be due to non-specific detection.
- The sensitivity for detection of small DNA variants can be impacted by:
  - Low complexity genomic context.
  - Increasing variant length.
  - Phasing: MNVs (more than 3 base pairs), delins (one or more nucleotides replaced by one or more nucleotides and is not an SNV or MNV), and certain deletions require a phasing approach to be detected. A predefined set of MNVs, delins, and deletions are detected for the EGFR and RET genes using a phasing approach (refer to Appendix D, MNVs, delins, and Deletions in EGFR and RET Detectable by Phased Variant Caller in the *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*. The phasing approach for small variant calling is limited to only these variants. The detection rate of these variants (for example, RET E632_V637delinsAA) may not be as high as observed for tumor profiling variants that do not require phasing.

- The clinical significance of TMB measurement has not been established. TMB is reported as mutations per megabase (Muts/Mb). TMB is a function of characteristics of a patient's specimen and testing parameters; therefore, TMB may differ across specimens (eg, primary vs metastatic, tumor content) and targeted panels. The TMB calculation will increase or decrease dependent on the variables listed below:
  - The size and specific region to calculate TMB may result in different values.
  - The percentage of tumor in the sample.
  - Methods of germline filtering.
  - The read depth and other bioinformatic test specifications.
- 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.
- TSO Comprehensive performance has been demonstrated for insertions and deletions up to 24 bp.
- Fusion calling algorithms in the TSO Comprehensive assay software may not consider evidence from reads that extend outside of annotated gene boundaries.
- 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 41).
  - A single gene spanning both breakpoints.
  - 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.
  - Small median insert sizes. A minimum median insert size of 80 bp is required, but sensitivity decreases in the 80–100 bp range.
  - 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 low depth outcome in that region for small DNA variants.
- Annotation or Knowledge Base errors can cause a false positive or false negative result, including listing a variant in the wrong cancer mutation level (between Level 2 and 3), or the annotation information in the report could be incorrect. Annotation or Knowledge Base errors do not impact variants reported in the CDx Results. The possibility of error exists from the following three sources:
  - TSO Comprehensive 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 errors.
  - Knowledge Base error due to the curation or leveling process.
  - The report reflects the knowledge at the time when the Knowledge Base version was curated.

- TSO Comprehensive is designed to report somatic variants and is not intended to report germline variants. When reporting variants with evidence of clinical significance or variants with potential clinical significance, as a tumor-only test, germline (inherited) variants may be inadvertently reported since TSO Comprehensive uses a Knowledge Base to report somatic variants without explicitly annotating germline or somatic origin.
- The Knowledge Base 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 Knowledge Base.

Product Components

The TSO Comprehensive test consists of the following components:

- TruSight Oncology Comprehensive (Illumina catalog # 20032573)—The kit includes reagents with sufficient volume to generate 24 DNA and 24 RNA libraries. This volume includes patient samples and controls. Controls sold separately (refer to *Reagents Required, Not Provided* on page 17).
- Knowledge Base—Available for download on the Illumina Lighthouse Portal.
- Local Run Manager TruSight Oncology Comprehensive (US) analysis module (Illumina catalog # 20089253), which includes the following components and supports tumor profiling and CDx claims:
  - TSO Comprehensive Claims Packages
  - TSO Comprehensive Software Suite
  - TSO Comprehensive USB Kit

  An Illumina service representative installs the appropriate version of the Local Run Manager TruSight Oncology Comprehensive (US) analysis module on the NextSeq 550Dx instrument. For information about the analysis module, refer to *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*.

Reagents

Reagents Provided

The following reagents are provided with the TSO Comprehensive 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
Adapter Ligation Buffer 1 (ALB1)	20031437	2	1.73 ml	Buffered aqueous solution containing salts	-25°C to -15°C

Reagent	Part Number	Quantity	Volume	Active Ingredients	Storage Temperature
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.

⚠ CAUTION

Use Unique Index Primers (UPxx) for RNA or DNA samples. Do not combine CPxx and UPxx index primers together in the same library.

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

TruSight Oncology Comp CP Index Primers, PN 20031126

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

⚠ CAUTION

Use Combinatorial Index Primers (CPxx) for DNA samples only. Do not combine CPxx and UPxx index primers together in the same library.

Index Primer	Part Number	Quantity	Volume	i7 Index	i7 Sequence	i5 Index	i5 Sequence	Storage Temperature
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

Index Primer	Part Number	Quantity	Volume	i7 Index	i7 Sequence	i5 Index	i5 Sequence	Storage Temperature
CP06	20031466	1	20 µl	D709	CGGCTATG	D507	ACGTCCTG	-25°C to -15°C
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
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 Controls:
  - TruSight Oncology DNA Control (Illumina catalog # 20065041)
  - TruSight Oncology RNA Control (Illumina catalog # 20065042)
- Ethanol (EtOH) 100% (200 proof), molecular biology grade
- RNase/DNase-free water

Post-Amp Reagents

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

Reagent Storage and Handling

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

Box	Part Number	Lab Area
TruSight Oncology Comp 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.
- FSM, SSM, ERA1-B, and TCB1 can have product-related particulates. Follow the specific handling guidelines for each reagent. After performing FSM and SSM mixing steps, remaining white product-related particulates will not impact performance.
- Stability of the TSO Comprehensive assay has been evaluated and performance demonstrated for up to four uses of the kit. Reagents are stable when stored at the indicated temperatures until the specified expiration date listed on the box label.

Equipment and Materials

Equipment and Materials Required, Not Provided

Pre-Amp Equipment and Materials

Equipment	Supplier
Ultrasonicator with associated accessories Refer to *Ultrasonicator Configuration Settings for DNA Fragmentation* on page 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 • Refer to *Thermal Cycler Ramp Rate* on page 24	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 • 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

Equipment	Supplier
Precision pipettes capable of accurately delivering volumes between 2 µl to 1000 µl with the following specifications: • Single- or multichannel pipette with increment of 0.02 µl • Single- or multichannel pipette with increment of 0.1 µl, 0.2 µl, or 0.5 µl • Single- or multichannel pipette with increment of 1 µl or 2 µl Pipettes must be calibrated regularly and accurate within 5% of stated volume.	General lab supplier
Pipette-aid	General lab supplier
Ice or cold block	General lab supplier
10 ml serological pipettes	General lab supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable • Suitable for skirted or semi-skirted PCR plates • Strong adhesive that withstands multiple temperature changes of -20°C to 100°C • DNase/RNase-free	General lab supplier
Microcentrifuge tubes with capacity of 1.7 ml, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
Compatible aerosol-resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859 or equivalent
96-well PCR plates compatible with thermal cycler, 0.2 ml (polypropylene wells)	General lab supplier

Post-Amp Equipment and Materials

Equipment	Supplier
NextSeq 550Dx Instrument	Illumina, catalog # 20005715
Centrifuge (plate centrifuge) with the following capabilities: • Centrifugation of 96-well microplates • 280 × g	General lab supplier

Equipment	Supplier
Thermal cycler with the following specifications: • Heated lid (100°C) • Encompass a 4°C to 99°C temperature range • ±0.25°C temperature accuracy • Compatible with 96-well PCR plates, 0.2 ml • Refer to *Thermal Cycler Ramp Rate* on page 24	General lab supplier
Vortexer	General lab supplier
Microsample incubator with insert for 96-well MIDI plates	General lab supplier
Dry heat block with the following specifications: • 25°C to 99°C temperature range • ±5°C temperature accuracy • Make sure that the microcentrifuge tubes are compatible with the heat block	General lab supplier
Plate shaker with the following 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 capable of accurately delivering volumes between 2 µl to 1000 µl with the following specifications: • Single- or multichannel pipette with increment of 0.02 µl • Single- or multichannel pipette with increment of 0.1 µl, 0.2 µl, or 0.5 µl • Single- or multichannel pipette with increment of 1 µl or 2 µl Pipettes must be calibrated regularly and accurate within 5% of stated volume.	General lab supplier
Pipette-aid	General lab supplier
10 ml serological pipettes	General lab supplier

Equipment	Supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable • Suitable for skirted or semi-skirted PCR plates • Strong adhesive that withstands multiple temperature changes of -20°C to 100°C • DNase/RNase-free	General lab supplier
Microcentrifuge tubes with capacity of 2 ml, nuclease-free	General lab supplier
Microcentrifuge tubes with capacity of 1.7 ml, nuclease-free	General lab supplier
Nuclease-free reagent reservoirs (disposable trough, 50 ml) (or equivalent)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
Compatible aerosol-resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, part # AB-0859 or equivalent
96-well PCR plates compatible with thermal cycler, 0.2 ml (polypropylene wells)	General lab supplier
Ice or cold block	General lab supplier

Ultrasonicator Configuration Settings for DNA Fragmentation

DNA fragmentation, or shearing, influences assay performance by determining the distribution of fragment size, which in turn affects sequencing coverage. Several focused-ultrasonication configurations were evaluated and optimized for the TSO Comprehensive assay (Table 3).

- Shearing time was adjusted to maximize the MEDIAN_EXON_COVERAGE metric outlined in *Quality Control* on page 77. Shearing times (refer to Table 3) and MEDIAN_INSERT_SIZE results differed across configurations.
- Configurations 1–4 were tested with 8-strip glass tubes. Configuration 5 used a single glass tube. Tube volume capacities are shown in Table 3.
- Optimization of configurations 3, 4, and 5 (smaller water bath volumes) used pulsing and were sheared in smaller volume tubes. Tube volume capacities affect shearing parameters.
- Configuration 4 (line transducer, medium-size water bath volume, degassed water) required a long pulse delay time (40 seconds) to achieve similar MEDIAN_EXON_COVERAGE as configuration 1 and 2 at nominal 40 ng input.

- Optimal settings for configuration 3 resulted in a slightly larger fragment size distribution compared to the other configurations (MEDIAN_INSERT_SIZE was approximately 5–10 base pairs larger).
- Configurations 3 and 5 used non-degassed water and the smallest water bath sizes and needed increased DNA input (50 ng for configuration 3, 60 ng for configuration 5) to achieve similar MEDIAN_EXON_ COVERAGE relative to the other 3 configurations, which used the nominal 40 ng input.
- Configurations 3 and 5 have more damage and/or denaturation and therefore a reduced effective mass of usable dsDNA molecules for library preparation.

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

Table 3 Focused-Ultrasonicator Configurations Evaluated

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

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

Thermal Cycler Ramp Rate

Thermal cycling ramp rate affects the assay Median Insert Size (RNA) QC metric and 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°C/s to 3°C/s to obtain comparable results to other models with lower default ramp rates.

Specimen Collection, Transport, and Storage

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

Sample Requirements

FFPE Tissue

The TSO Comprehensive 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 must not be decalcified. Before performing the TSO Comprehensive assay, the tissue sample should be examined by a pathologist to make sure that it is appropriate for this test. A minimum of 20% tumor content (by area) is required to detect somatic driver mutations. Tumor content for RNA variants depends on the extent of expression. Refer to *Tumor Content* on page 119.

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. This volume is equivalent to a cumulative viable tissue area of ≥ 200 mm^2 using 5 µm thick sections, or ≥ 100 mm^2 using 10 µm thick sections. Cumulative tissue area is the sum of the viable tissue area in all sections submitted for extraction. For example, a cumulative tissue area of 200 mm^2 can 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 can decrease the amount of nucleic acid yield. To minimize the possibility of false negative results, the tissue can be macrodissected to eliminate necrotic tissue and achieve a desirable viable tumor content.

High amounts of necrotic tissue (≥ 25% by area) can interfere with the ability of the TSO Comprehensive assay to detect RNA variants. If sample sections contain more than 25% necrosis in total tissue area, the necrotic tissue must be macrodissected. Refer to *Interfering Substances* on page 120 for more information.

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

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 124).

- Do not increase Proteinase K or equivalent enzyme during extraction from the standard concentration provided in an extraction kit (refer to *Interfering Substances* on page 120).
- Store extracted stock nucleic acid following the instructions from the extraction kit manufacturer.
- Store extracted DNA for up to 28 days at -25°C to -15°C.
- Store extracted RNA for up to 28 days at -85°C to -65°C.
- To avoid changes in concentration over time, measure DNA and RNA within 28 days of 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 32 days, depending on the type of library (refer to Table 4).

Table 4 Library Storage Times

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

Warnings and Precautions

⚠ CAUTION

Federal law restricts this device to sale by or on the order of a physician or other practitioner licensed by the law of the State in which they practice, to use or order the use of the device.

Safety

⚠ WARNING

1. **This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.** For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.
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 fully 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 tube strip, trough, or reservoir.
   - Mix samples with a pipette and centrifuge the plate when indicated.
   - Use a microplate shaker. Do not vortex the plates.
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 36.
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 36, 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. FSM, SSM, ERA1-B, and TCB1 can have product-related particulates. Follow handling guidelines for each specific reagent. After performing FSM and SSM mixing steps, remaining white product-related particulates will not impact performance.
13. Prepare fresh master mixes and discard the remaining volume after use.
14. 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.
15. Transfer the specified volume of eluate. Transferring less than the specified volume of eluate during the elution steps may impact results.
16. 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.
17. Do not pipette less than 2 µl sample input.
18. Do not use a trough to dispense reagents for steps that require less than 10 µl material to be added to each sample well.
19. Use a fine-tipped pipette when transferring fragmented gDNA samples from the ultrasonicator tubes to the Library Prep (LP) plate.
20. Do not combine SUA1 and UMI adapters together.
21. Use SUA1 adapters with RNA samples.
22. Use UMI adapters with DNA samples.
23. Assign different index primers to each library sample to identify uniquely each library when it is pooled for sequencing on a single flow cell.
24. Do not combine CPxx and UPxx index primers in the same library.
25. 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 (US) analysis module before beginning library preparation. Record sample IDs, indexing, and plate well orientation for reference during library preparation.
26. For libraries derived from RNA samples, use only UPxx indexes.
27. For libraries derived from DNA samples, use UPxx indexes or CPxx indexes.

28. Sequence a maximum of 8 RNA libraries and 8 DNA libraries per flow cell. Sequence a minimum of three libraries. Follow guidelines in *Number of Libraries and Selecting Indexes* on page 32.
29. After the bind step in *Capture Targets One* on page 56 and *Capture Targets Two* on page 60, proceed immediately to the wash step to prevent bead pellets from drying.
30. During wash steps, remove all 80% ethanol 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 36.
32. During the *Normalize Libraries* on page 66 procedure, thoroughly resuspend the library bead pellet to achieve consistent cluster density on the flow cell.

Procedural Notes

- The TSO Comprehensive 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 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.
- Reagents, samples, and libraries must be kept cold at certain steps in the Instructions for Use. This is defined as keeping on ice or equivalent.

Thermal Cycler Programs

- Program thermal cycler programs on 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 the risk of cross-contamination and evaporation.
- Place the plate on a flat surface before slowly removing the seal.
- Before unsealing, if any fluid or condensation is observed on the seal or side walls of the plate wells, centrifuge at 280 × g for 1 minute.
- Use adhesive plate seals that are effective at -20°C to 100°C, and suitable for skirted or semi-skirted PCR plates.

Equipment

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

Plate Type and Plate Transfers

- Proper plate type is required for optimal assay performance and storage.
- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the destination plate.
- Multichannel pipettes can be used when transferring samples between tube strips or plates.
- Use the following guidelines when shaking plates.
  - Use a plate shaker to shake plates. Do not vortex plates.
  - Shake PCR plates at 1200 rpm.
  - Shake MIDI plates at 1800 rpm.
  - Follow manufacturer instructions to make sure that the plate shaker holds the plate securely.

Centrifugation

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

Handling Reagents

- Tightly recap all reagent tubes immediately after use to limit evaporation and prevent contamination.
- Return reagents to the specified storage temperature when they are no longer needed for a procedure.
- Follow the reagent preparation that precedes each procedure section of the *Instructions for Use* on page 36.
- Make sure to prepare the required volume of master mix, elution mix, and 80% ethanol for the number of samples you process.
- Volumes provided in master mix and solution tables contain overage. Overage volume calculations are as follows.
  - Table 13
    - Volume of 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 20
    - 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 28
    - 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 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 35
    - 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 36
    - 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 assay includes SUA1 and UMI adapters.
- SUA1 adapters are for use with RNA samples. Not for use with DNA samples.
- UMI adapters are for use with DNA samples. Not for use with RNA samples.

Handling Beads

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

Number of Libraries and Selecting Indexes

Before run setup, plan the number of sample libraries and sample indexes for the sequencing run. The following sample number guidelines include positive controls but exclude negative/no-template controls (NTCs). NTCs must be added to the planned run as an additional sample.

For TSO Comprehensive, follow the guidelines in Table 5 and Table 6 to determine the number of RNA and/or DNA libraries to sequence on one flow cell. Refer to Table 5 when sequencing RNA *or* DNA libraries separately. Refer to Table 6 when sequencing RNA *and* DNA libraries on the same flow cell.

Table 5 Sequencing RNA *or* DNA Libraries

Library Type	Minimum*	Maximum*
RNA-only	3	16
DNA-only	3	8

* NTCs do not contribute to the plexity.

Table 6 Sequencing RNA *and* DNA Libraries on the Same Flow Cell

Library Type	Minimum*	Maximum*
RNA	3	8
DNA	3	8

* NTCs do not contribute to the plexity.

For optimal reagent usage when sequencing DNA ***and*** RNA libraries with TSO Comprehensive on the NextSeq 550Dx instrument, sequence 8 RNA libraries and 8 DNA libraries per flow cell.

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 (US) analysis module. During library preparation, add the index primer to each sample library. ***Use a different index primer mix for each sample library.***

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 (US) analysis module. ***Mismatches cause incorrect result reporting due to loss of positive sample identification.***

There are two types of indexes in the TSO Comprehensive 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 requirements apply:

- 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 DNA and RNA Controls

TSO Comprehensive requires 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.

The positive control input amount is 40 ng for DNA and RNA.

Include the appropriate 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 DNA and RNA 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 following tables 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 7 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 8 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

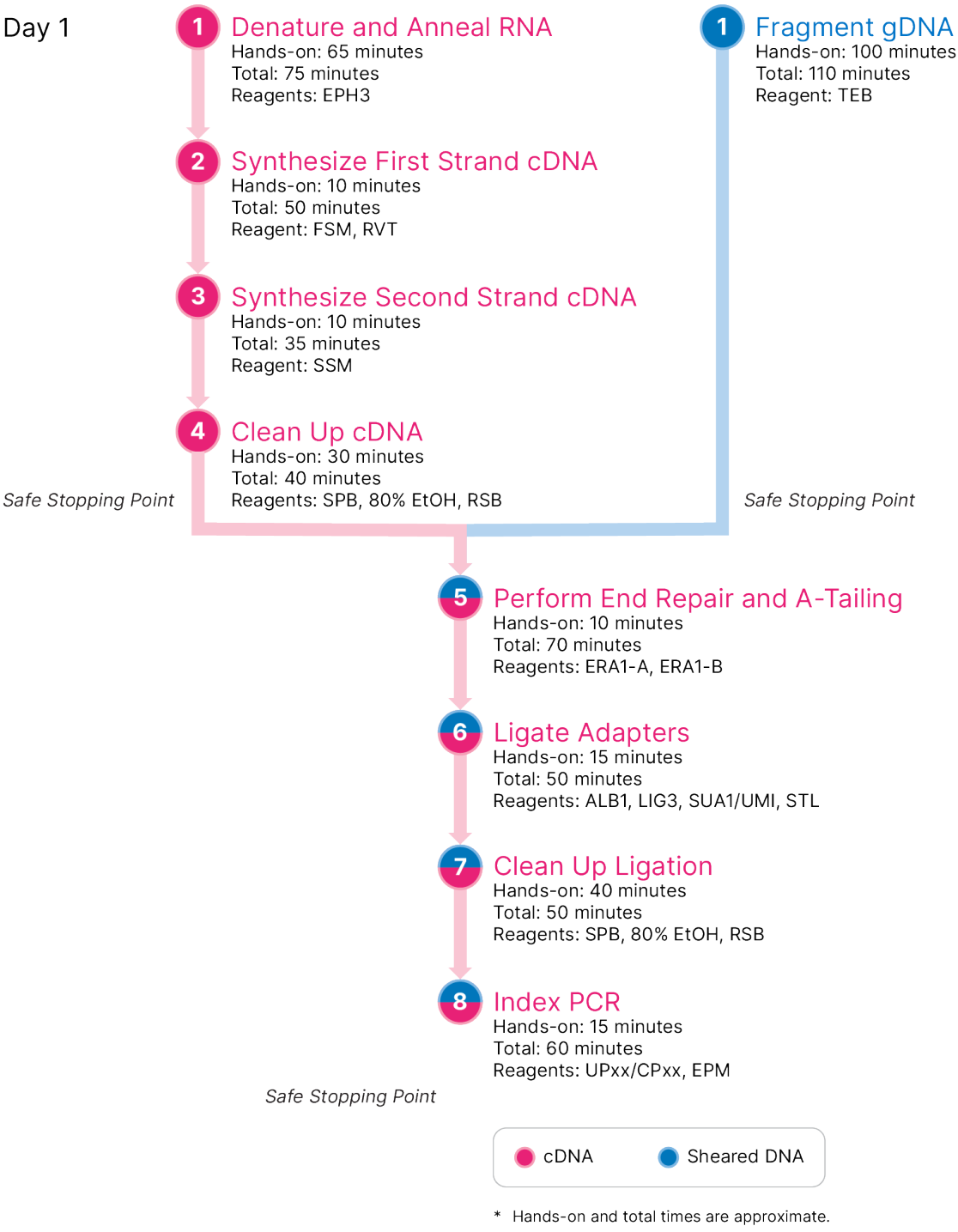
An overview of the TSO Comprehensive workflow is shown in Figure 1 and Figure 2.

Library Prep Workflow

Figure 1 illustrates the library prep workflow for TSO Comprehensive. Libraries from RNA and DNA samples can be prepared simultaneously in separate wells. Positive controls and NTCs are processed identically to samples. Safe stopping points are marked between steps.

Before starting the protocol, enter run and sample information into the TSO Comprehensive (US) analysis module. Refer to the *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*.

Figure 1 TSO Comprehensive Workflow (Part 1)



* Hands-on and total times are approximate.

Enrichment Workflow

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

Figure 2 TSO Comprehensive Workflow (Part 2)

Program Thermal Cyclers

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

Table 9 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 • Hold at 4°C
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 • Hold at 4°C
Synthesize Second Strand cDNA	2ndSS	30°C	50 µl	• 16°C for 25 minutes • 4°C for 1 minute • Hold at 4°C

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

Table 10 Post-amplification Thermal Cycler Programs

Procedural Step	Program Name	Lid Temperature	Reaction Volume	Thermal Cycler Parameters

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

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

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 • Hold at 57°C 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
				• Hold at 10°C

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. Make sure pre-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 39.
3. Follow manufacturer instructions to set up the ultrasonicator.
4. If processing DNA samples only, proceed directly to *Fragment gDNA* on page 45.
5. Remove RNA controls from storage.
6. Remove the reagent tubes from the box and follow thaw instructions.

Table 11 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 cold.	Synthesize First Strand cDNA
SSM	-25°C to -15°C	Thaw to room temperature.	Synthesize Second Strand cDNA

Table 12 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 on product performance.
   - RVT—Centrifuge briefly, and then pipette to mix. Keep cold.

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

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

   Table 13 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 10 times to mix.
4. Keep the FSM + RVT Master Mix cold until *Synthesize First Strand cDNA* on page 42.

Procedure

1. Keep extracted RNA samples and RNA controls cold while thawing.
   Process RNA controls as samples for the remainder of the protocol.
2. Keep RNA cold 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 (US) 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

    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 39.
14. When the samples reach 4°C, hold for 1 minute. 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. Make sure that 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 39.
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 SSM:

a. Invert 10 times to mix.
b. Centrifuge briefly. The reagent may contain white product-related particulates. No action is required. There is no impact on product performance.

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 39.
7. When the samples reach 4°C, hold for 1 minute and 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% EtOH. 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 as follows.

   Table 14 Prepare Fresh 80% EtOH

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

3. Vortex fresh 80% EtOH to mix.
4. 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 1.15 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. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each sample well.

Wash

1. Wash beads as follows.
   a. Keep the BIND1 MIDI plate on the magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Without disturbing the bead pellet, use a pipette set at 200 µl to remove and discard all supernatant from each sample well.
2. Wash beads a **second** time.
3. Use a pipette with fine tips to remove residual EtOH from each well.
4. Discard unused 80% EtOH.

Elute

1. Remove the 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 45, 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 cold.
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 48.

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 15 TruSight Oncology Comp Library Prep (Refrigerate) (PN 20031119)

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

Fragment gDNA

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

Preparation

1. Follow recommendations in *Nucleic Acid Extraction, Quantification, and Storage* on page 24 to quantify samples.
2. Prepare the following reagent.
   - TEB—Invert or vortex to mix.

Procedure

Prepare the Plate

Select one of the following 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. Keep cold and set aside for use in *Transfer Fragmented DNA* on page 47.
- **[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 keep cold until *Transfer Fragmented DNA* on page 47.
- **[Option 3]** Process gDNA-only samples.
  a. Label a new 96-well MIDI plate LP (Library Preparation).
  If you are stopping at the *SAFE STOPPING POINT* on page 47, use a PCR plate.
  b. Cover and set aside for use in *Transfer Fragmented DNA* on page 47.

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 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 of each gDNA sample into a separate well of the ultrasonicator tube.

   ⚠ CAUTION

   Load the gDNA into the tube slowly, making sure that there are no air gaps at the bottom of the tube. For more information, refer to *Assay* on page 27 and manufacturer instructions.

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

Transfer Fragmented DNA

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

SAFE STOPPING POINT

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

Prepare for Protocol Steps

Make sure that post-amplification thermal cycler programs are set. Refer to *Program Thermal Cyclers* on page 39.

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

Table 16 TruSight Oncology Comp Library Prep (Freeze) (PN 20031118)

Reagent	Storage	Thaw Instructions	Protocol Step
ERA1-A	-25°C to -15°C	Keep cold.	Perform End Repair and A-Tailing
ERA1-B	-25°C to -15°C	Thaw to room temperature.	Perform End Repair and A-Tailing
ALB1	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters
LIG3	-25°C to -15°C	Keep cold.	Ligate Adapters
SUA1 (blue cap)	-25°C to -15°C	Thaw to room temperature.	Ligate Adapters

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

Table 17 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 Ligation
RSB	2°C to 8°C	Bring to room temperature.	Clean Up Ligation

Table 18 TruSight Oncology Comp UP Index Primers (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 19 TruSight Oncology Comp CP Index Primers (PN 20031126)

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

Perform End Repair and A-Tailing

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

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

Preparation

1. Preheat 2 microsample incubators with MIDI heat block inserts as follows.
   a. Preheat a microsample incubator to 30°C.
   b. Preheat a microsample incubator to 72°C.
2. Prepare the following reagents.
   a. ERA1-A—Centrifuge briefly, and then pipette to mix. Keep cold.
   b. 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 20 ERA1 Master Mix*

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

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

4. Pipette slowly 10 times to ensure homogeneity, and then centrifuge briefly. Keep ERA1 master mix cold.
5. To prepare the plate, select one of the following options:
   - **[Option 1]** If samples are in a MIDI plate, prepare as follows.
     a. Relabel the MIDI plate LP2 (Library Preparation 2).
     b. If some samples are in separate MIDI plates, move all samples to separate wells of the same MIDI plate according to the plate layout.
   - **[Option 2]** If the plate is frozen, prepare as follows.
     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.
7. Incubate at 72°C for 20 minutes.
8. Keep the LP2 MIDI plate cold for 5 minutes.

Ligate Adapters

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

The TSO Comprehensive assay includes SUA1 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 cold.
   - 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 or equivalent.
2. Add 60 µl ALB1 to each sample well of the LP2 MIDI plate. ALB1 is a viscous solution. Pipette slowly to minimize bubble formation.
3. Add 5 µl LIG3 to each sample well.
4. Add adapters as follows.
   Do ***not*** combine different types of adapters together.
   - **[RNA sample wells]**—Add 10 µl SUA1 (blue cap) to each sample derived from RNA.
   - **[DNA sample wells]**—Add 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 21 Prepare Fresh 80% Ethanol

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
100% EtOH, 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 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SPB has been added to each sample well.
3. Apply adhesive plate seal to the LP2 MIDI plate.
   Seal edges and wells completely.
4. Shake at 1800 rpm for 2 minutes.
5. Incubate at room temperature for 5 minutes.
6. Place the LP2 MIDI plate on the magnetic stand for 10 minutes.
7. Without disturbing the bead pellet, use a pipette set at 200 µl to remove and discard all supernatant from each sample well.

Wash

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

Elute

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

Index PCR

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

Preparation

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

2. Make sure that indexes for each sample match the run planned on the TSO Comprehensive (US) analysis module during run setup. Make sure to follow instructions regarding index selection in *Number of Libraries and Selecting Indexes* on page 32.

   ⚠ 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 39.
   If continuing with *Set Up First Hybridization* on page 54, 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 39.
2. Remove the reagent tubes from the box and follow thaw instructions.

Table 22 TruSight Oncology Comp Enrichment (Refrigerate) (PN 20031123)

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

Table 23 TruSight Oncology Comp Enrichment (Freeze) (PN 20031121)

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

Table 24 TruSight Oncology Comp Content Set (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 52. 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. 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 to prevent evaporation.
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]**—Add 5 µl OPR1 (red cap) to each library derived from RNA.
   - **[DNA library wells]**—Add 5 µl OPD2 (white cap) to each library derived from DNA.
7. Apply adhesive plate seal to the HYB1 PCR plate.
   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 39.
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

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

Table 25 TruSight Oncology Comp Enrichment (Refrigerate) (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
TCB1	2°C to 8°C	Bring to room temperature.	Set Up Second Hybridization

Reagent	Storage	Thaw Instructions	Protocol Step
RSB	2°C to 8°C	Bring to room temperature.	Capture Targets Two Clean Up Amplified Enriched Library

Table 26 TruSight Oncology Comp Enrichment (Freeze) (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 27 TruSight Oncology Comp Content Set (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 28 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 58 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 to allow sufficient material per sample.
5. After adding SMB to each sample well, discard any remaining material.
6. Set pipette to 50 µl and transfer entire volume of each library from the HYB1 PCR plate to the corresponding well in the CAP1 MIDI plate.
7. Discard the empty HYB1 PCR plate.
8. Apply adhesive plate seal to the CAP1 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
9. Shake at 1800 rpm for 2 minutes.
10. Incubate in the preheated microsample incubator at 57°C for 25 minutes.
11. Place the CAP1 MIDI plate on a magnetic stand for 2 minutes.
12. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

⚠ CAUTION

Proceed immediately to the next step (*Wash* on page 58). Do not allow the bead pellet to sit for an extended amount of time without liquid present.

Wash

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

Elute

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

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

Set Up Second Hybridization

This step binds targeted regions of the enriched 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 the same probe used during the first hybridization to each well. Add only one probe set per well.
   Do *not* combine different types of probes together.
   - **[RNA library wells]**—Add 5 µl OPR1 (red cap) to each library derived from RNA.
   - **[DNA library wells]**—Add 5 µl OPD2 (white cap) to each library derived from DNA.
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 39.
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 29 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 61 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 to allow sufficient material per sample.
5. After adding SMB to each sample well, discard any remaining material.

6. Set pipette to 50 µl and transfer entire volume of each library from the ELU1 PCR plate to the corresponding well of the CAP2 MIDI plate.
7. Discard the empty ELU1 PCR plate.
8. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely to prevent evaporation.
9. Shake at 1800 rpm for 2 minutes.
10. Incubate in a microsample incubator at 57°C for 25 minutes.
    If continuing with *Amplify Enriched Library* on page 63, follow thaw instructions for reagents in the Prepare for Protocol Steps section.
11. Place on a magnetic stand for 2 minutes.
12. Keep the CAP2 MIDI plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

    ⚠ CAUTION

    Proceed immediately to the next step (*Wash* on page 61). Do not allow the bead pellet to sit for an extended amount of time without liquid present.

Wash

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

Elute

1. Remove the CAP2 MIDI plate from the magnetic stand.
2. Vortex fresh EE2 + HP3 Elution Mix, and then centrifuge briefly.
3. Add 22 µl EE2 + HP3 Elution Mix to each library well in the CAP2 MIDI plate.
4. Discard remaining EE2 + HP3 Elution Mix.
5. Apply adhesive plate seal to the CAP2 MIDI plate.
   Seal edges and wells completely.
6. Shake at 1800 rpm for 2 minutes.

7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate ELU2 (Elution 2).
9. Vortex ET2 to mix, and then centrifuge briefly.
10. Add 5 µl ET2 to each corresponding library well in the new ELU2 PCR plate.
11. Carefully transfer 20 µl eluate from each library well of the CAP2 MIDI plate to the corresponding well in the ELU2 PCR plate.
12. Discard empty CAP2 MIDI plate.
13. Apply adhesive plate seal to the ELU2 PCR plate.
    Seal edges and wells completely to prevent evaporation.
14. Shake at 1200 rpm for 2 minutes.
15. Return SMB, EE2, HP3, RSB, and ET2 to storage.

SAFE STOPPING POINT

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

Prepare for Protocol Steps

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

Table 30 TruSight Oncology Comp Enrichment (Freeze) (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 cold.	Amplify Enriched Library

Table 31 TruSight Oncology Comp Enrichment (Refrigerate) (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 39.
   If continuing with *Normalize Libraries* on page 66, 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 32 Prepare Fresh 80% Ethanol

Reagent	4 Libraries	8 Libraries	16 Libraries	24 Libraries	48 Libraries
100% EtOH, 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.
   If using a trough to dispense SPB, include a 1.15 overage factor when aliquoting sufficient material per sample. Discard any remaining material after SPB has been added to each sample well.
5. Transfer 50 µl each library from the ELU2 PCR plate to the corresponding well of the BIND2 MIDI plate.
6. Discard empty ELU2 PCR plate.
7. Apply adhesive plate seal to the BIND2 MIDI plate.
   Seal edges and wells completely.
8. Shake at 1800 rpm for 2 minutes.
9. Incubate at room temperature for 5 minutes.
10. Place the BIND2 MIDI plate on magnetic stand for 5 minutes.
11. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

Wash

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

Elute

1. Remove the BIND2 MIDI plate from the magnetic stand.
2. Invert or vortex to mix RSB.
3. Add 32 µl RSB to each library well.

4. Apply adhesive plate seal to the BIND2 MIDI plate.
   Seal edges and wells completely.
5. Shake at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 2 minutes.
7. Place on a magnetic stand for 2 minutes.
8. Label a new 96-well PCR plate PL (Purified Libraries).
9. Transfer 30 µl each eluate from the BIND2 MIDI plate to the corresponding well of the PL PCR plate.
10. Discard the empty BIND2 MIDI plate.
11. Apply adhesive plate seal to the PL PCR plate.
12. Return SPB and RSB to storage.

SAFE STOPPING POINT

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

Prepare for Protocol Steps

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

   Table 33 TruSight Oncology Comp Enrichment (Freeze) (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 34 TruSight Oncology Comp Enrichment (Refrigerate) (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 69, 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. Pulse vortex LNB1 for 1 minute to resuspend beads.
   Invert LNB1 tube to make sure that all beads are resuspended.
3. Repeat pulse vortexing for 1 minute if a pellet is observed.
4. Use a pipette set at 800 µl to pipette LNB1 up and down 10 times to ensure resuspension.
5. Immediately prepare fresh LNA1 + LNB1 Master Mix in a conical tube.

   ⚠ CAUTION

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

   Table 35 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.
6. Vortex LNA1 + LNB1 master mix. Set aside for the *Bind* on page 67 step.

7. Prepare fresh EE2 + HP3 Elution Mix in a microcentrifuge tube.

   Table 36 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.

8. Vortex fresh elution mix, and then centrifuge briefly. Set aside for the *Elute* on page 68 step.
9. If the PL PCR plate was stored, thaw to room temperature, centrifuge at 280 × g for 1 minute. Pipette to mix.
10. Label a new 96-well MIDI plate BBN (Bead-Based Normalization).
11. Set out the magnet.

Procedure

Bind

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

Wash

1. Wash beads as follows.
   a. Remove the BBN MIDI plate from the magnetic stand.
   b. Add 45 µl LNW1 to each library well.
   c. Apply adhesive plate seal to the BBN MIDI plate.
      Seal edges and wells completely.
   d. Shake at 1800 rpm for 5 minutes.
   e. Place the BBN MIDI plate on a magnetic stand for 2 minutes.

f. Keep the plate on the magnetic stand. Without disturbing the bead pellet, use a pipette set to 200 µl to remove and discard all supernatant from each well.

2. Wash beads a ***second*** time.
3. Use a pipette with fine tips to remove residual supernatant from each well.

Elute

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

   ⚠ CAUTION

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

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

SAFE STOPPING POINT

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

Prepare for Protocol 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 keep cold.
2. Follow preparation instructions in the *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)* for other consumables in the kit.
   - NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles)
   - NextSeq 550Dx Buffer Cartridge v2 (300 cycles)
   - NextSeq 550Dx High Output Flow Cell Cartridge v2.5 (300 cycles)
3. Remove the reagent tubes from the box and follow thaw instructions.

Table 37 TruSight Oncology Comp Enrichment (Freeze) (PN 20031121)

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

Table 38 TruSight Oncology Comp Enrichment (Refrigerate) (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

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

Preparation

1. Review the guidelines for *Number of Libraries and Selecting Indexes* on page 32.
2. Label a microcentrifuge tube dHP3 (diluted HP3).
3. Label a microcentrifuge tube dPhiX (diluted PhiX).
4. Preheat a heat block to 96°C for microcentrifuge tubes.
5. Prepare an ice bucket or equivalent.

Dilute and Denature PhiX Control

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

Pool, Denature, and Dilute Libraries for TSO Comprehensive

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.

NOTE After sequencing, if run-level or systemic library QC failures are observed and troubleshooting implicates bead-based normalized libraries from the NL PCR plate, use the *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74 to resequence with an adjusted second dilution.

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 71.
   - **[Option 2]** Sequence libraries derived from DNA samples only. Refer to *Option 2: DNA-Only Libraries* on page 72.
   - **[Option 3]** Sequence libraries derived from RNA samples only. Refer to *Option 3: RNA-Only Libraries* on page 73.

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 the PRL and PDL tubes to mix.
7. Centrifuge the PRL and PDL tubes briefly.
8. Incubate the PRL and PDL tubes in a heat block at 96°C for 2 minutes.
9. Keep the PRL and PDL tubes cold for 5 minutes.
10. Vortex the PRL and PDL tubes to mix, and then centrifuge briefly.
11. Keep the PRL and PDL tubes cold.

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 the DIL1 tube.
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.

7. **[Troubleshooting]** After sequencing, if run-level or systemic library QC failures associated with bead-based normalization are observed, proceed to *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74.

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 the NL PCR plate at 280 × g for 1 minute, and then store at -25°C to -15°C for up to 32 days.
11. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Option 2: DNA-Only Libraries

1. Label a screw top microcentrifuge tube PDL (Pooled DNA Libraries).
2. Transfer 10 µl of 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. Vortex the PDL tube to mix, and then centrifuge briefly.
5. Incubate the PDL tube in a heat block at 96°C for 2 minutes.
6. Keep the PDL tube cold for 5 minutes.
7. Vortex the PDL tube to mix, and then centrifuge briefly.
8. Keep the PDL tube cold.

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 the DIL1 to mix, and then centrifuge briefly.
6. **[Troubleshooting]** After sequencing, if run-level or systemic library QC failures associated with bead-based normalization are observed, proceed to *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74.

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 the NL PCR plate at 280 × g for 1 minute, and then store at -25°C to -15°C for up to 32 days.
11. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Option 3: RNA-Only Libraries

1. Label a microcentrifuge tube PRL (Pooled RNA Libraries).
2. Transfer 10 µl of 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 to prevent evaporation.
4. Vortex the PRL tube to mix.
5. Centrifuge the PRL tube briefly.
6. Incubate the PRL tube in a heat block at 96°C for 2 minutes.
7. Keep the PRL tube cold for 5 minutes.

8. Vortex the PRL tube to mix, and then centrifuge briefly.
9. Keep the PRL tube cold.

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 the DIL1 to mix, and then centrifuge briefly.
6. **[Troubleshooting]** After sequencing, if run-level or systemic library QC failures associated with bead-based normalization are observed, proceed to *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74.

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 to mix, 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 the NL PCR plate at 280 × g for 1 minute, and then store at -25°C to -15°C for up to 32 days.
11. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Troubleshooting Workflow: Prepare Adjusted Second Dilution

Use this troubleshooting workflow when, after sequencing, run-level or systemic library QC failures associated with bead-based normalization are observed (refer to *Troubleshooting* on page 81 for more information). Cluster densities outside the range of 170–220 K/mm² should not be adjusted unless associated with a QC failure. After completing the ***Prepare First Dilution*** step for the selected nucleic acid option, prepare a second dilution using adjusted DIL1 and HT1 volumes.

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

1. Use Table 39 to find the cluster density range (K/mm²) inclusive of the value observed in the sequencing run. Note the corresponding adjusted volume of DIL1 (µl) and HT1 (µl).
   The combined reagent volume is always 1700 µl for DNA and RNA or DNA-only libraries, and 1686 µl for RNA-only libraries, matching the nominal dilution volume.

   Table 39 Volume Adjustments Based on Cluster Density

Cluster Density Range (K/mm²)	Volume DIL1 (µl)	Volume HT1 (µl)	
		DNA and RNA or DNA-Only Libraries	RNA-Only Libraries
≤ 50	199	1501	1487
51–70	169	1531	1517
71–90	123	1577	1563
91–110	95	1605	1591
111–130	76	1624	1610
131–150	63	1637	1623
151–169	53	1647	1633
170–220	40	1660	1646
221–240	31	1669	1655
241–260	28	1672	1658
261–280	25	1675	1661
281–300	22	1678	1664
≥ 300	21	1679	1665

2. Label a 2.0 ml microcentrifuge tube TDIL2 (Troubleshooting Dilution 2).
3. Transfer the volume of DIL1 from Table 39 to the empty TDIL2 tube.
4. Discard the DIL1 tube.
5. Add the volume of prechilled HT1 from Table 39 to the TDIL2 tube.
6. Vortex prepared 20 pM dPhiX to mix, and then centrifuge briefly.

7. Add 20 pM dPhiX as follows.
   - **[DNA and RNA libraries]**—Add 2.5 µl prepared 20 pM dPhiX to the TDIL2 tube.
   - **[DNA-only libraries]**—Add 2.5 µl prepared 20 pM dPhiX to the TDIL2 tube.
   - **[RNA-only libraries]**—Add 16.7 µl prepared 20 pM dPhiX to the TDIL2 tube.
8. Vortex to mix, and then centrifuge briefly.
9. Load 1300 µl TDIL2 into the thawed NextSeq 550Dx High Output Reagent Cartridge v2 (300 cycles).
   For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.
10. Discard the TDIL2 tube.
11. Centrifuge the NL PCR plate at 280 × g for 1 minute, and then store at -25°C to -15°C for up to 32 days.
12. Proceed to sequencing.
    For more information, refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Interpretation of Results

The sequencing results from the TSO Comprehensive 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 *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*.

Companion Diagnostic Results

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

- **Positive**—A variant or biomarker 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 is 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 results are reported in the Companion Diagnostic Results section of the JSON report. Only positive results are listed in the Companion Diagnostic Results section of the PDF report.

Tumor Profiling Variants

TSO Comprehensive is designed to report somatic variants when reporting cancer mutations with evidence of clinical significance (Level 2) or cancer mutations with evidence of potential clinical significance (Level 3) in solid malignant neoplasms. The TSO Comprehensive assay software uses a Knowledge Base 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 Knowledge Base also considers if associations are established (or not) in the tested tumor type. Susceptibility or cancer risk associations are not included in the Knowledge Base. Common polymorphisms are removed.

For Tumor Profiling variants, positive results are classified into Level 2 or Level 3 according to the installed Knowledge Base 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 report.

Quality Control

Sequencing run, library, and control validity is determined automatically and reported by the TSO Comprehensive (US) analysis module. Refer to Table 40 and Table 41 for the QC metrics and specifications applied for sequencing run, library, and controls. Positive controls are evaluated for variant calling and biomarker calling. If positive controls do not meet the variant calling specifications, the software automatically invalidates 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, the software automatically invalidates patient samples based on control sample results. For detailed analysis information, refer to *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*. The TSO Comprehensive report, which is available in PDF and JSON formats, summarizes quality control results. The report files are in the analysis folder. Refer to *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)* for the location of the analysis folder (contains PDF and JSON reports) and the run folder.

Invalid sequencing runs and libraries should be repeated. For more information on repeating sequencing runs or tests of libraries, refer to *Troubleshooting* on page 81. Perform additional quality control measures in accordance with local, state, and/or federal regulations or accreditation requirements.

For nucleic acid quantification information and minimum input material requirements, refer to *Nucleic Acid Extraction, Quantification, and Storage* on page 24.

Table 40 TSO Comprehensive 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.	
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 is used to assess highly rearranged genomes. It is 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.	
RNA Libraries	MEDIAN_INSERT_SIZE (bp)	≥ 80	The median fragment length in the sample.	No fusions or splice variant results reported.

Output Type	Metric	Specification	Description	Impact of Specification Failure*
	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 Report Result Control Metrics

Output Type	Metric	Specification	Impact of Specification Failure*
Positive Control	DNA External Control	23 of 24 specified variants detected	The software automatically invalidates patient samples based on control sample results. DNA External Control: No small DNA variant or TMB results reported. RNA External Control: No fusions or splice variant results reported.
	RNA External Control	12 of 13 specified variants detected	
No-template control	DNA Median Exon Coverage	≤ 8	The software automatically invalidates patient samples based on control sample results. DNA Median Exon Coverage: No small DNA variant or TMB results reported. RNA Gene Above Median Cutoff: No fusions or splice variant results reported.
	RNA Gene Above Median Cutoff	≤ 1	

* Successful results show PASS.

Troubleshooting

Use Table 42 to troubleshoot issues in the workflow. If a sequencing run or library preparation for a sample fails two times, additional troubleshooting may be necessary. Contact Illumina Technical Support.

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

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

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

- Inspect the total PF reads (expanded metric) for each library from the `MetricsOutput.tsv`. Similar values indicate a systemic issue, whereas low total PF reads for one or a few samples, including the samples not passing library quality control, suggests a sample or well issue.
- Inspect the total PF reads for the DNA positive control in `<run folder>/<analysis folder>/Logs_Intermediates/DnaQCMetrics/<control ID>/<control ID-DNA.aligned.metrics.json>` and/or RNA positive control in `<run folder>/<analysis folder>/Logs_Intermediates/RnaQCMetrics/<control ID>/<control ID-RNA_QC.json` and compare the metric to historic values. A reduction in this metric for a positive control might be indicative of a systemic issue.

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

Table 42 TSO Comprehensive Troubleshooting

Observation	Possible Cause	Recommended Action
Sequencing run does not pass run quality control specifications.	• Pooling error. • Dilution error. • Issues with sequencing consumables preparation (for example, not thawed adequately, condensation/debris on flow cell).	Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 69.
	Bead-based normalization (high cluster density).	If none of the other possible causes for run QC failure are suspected, the concentration of beads from the LNB1 reagent tube may have caused high cluster density. This could be due to a subsequent use of LNB1 after insufficient bead mixing or a volume error resulting in a higher concentration of beads. Find and record the cluster density value from `RunCompletionStatus.xml` in the run folder. If the cluster density is above 220 K/mm² and a systemic bead-based normalization is suspected, resequence libraries from the Normalized Libraries (NL) PCR plate and follow the instructions for an adjusted second dilution. Refer to *Prepare for Sequencing* on page 69 and *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74.
	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 *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)* for help with report generation. Contact Illumina Technical Support for additional help.

Observation	Possible Cause	Recommended Action
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.
	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 69. • Repeat enrichment steps for libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 54. • Start library preparation from the beginning of the workflow. Refer to *Fragment gDNA* on page 45.
	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 45. Contamination may have occurred during sample extraction. It may be necessary to repeat extraction to make sure that the sample is free from contamination.

Observation	Possible Cause	Recommended Action
DNA library does not pass quality control specifications (continued).	Sample may be overly fragmented or have nucleic acid damage that impacts the ability to generate sufficient unique libraries.	Review *Ultrasonicator Configuration Settings for DNA Fragmentation* on page 22 and ultrasonicator manufacturer settings for use and operation (including water level and tube type). Ensure appropriate sample input into the assay. Refer to *Sample Requirements* on page 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 69. • Repeat enrichment steps for libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 54. • Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 41.
	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
DNA and/or RNA library does not pass quality control specifications.	• Pooling error. • Dilution error. • Incomplete heat denaturation of PRL/PDL.	Rule out DNA- or RNA-related causes listed above (eg, sample input, sample quality, equipment). Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 69.
	Bead-based normalization (low cluster density).	If none of the other possible causes for library QC failure are suspected, the concentration of beads from the LNB1 reagent tube may have caused low cluster density due to insufficient mixing. Find and record the cluster density value from `RunCompletionStatus.xml` in the run folder. If the cluster density is below 170 K/mm² and a systemic bead-based normalization is suspected (including evidence from total PF reads for the positive controls), resequence libraries from the Normalized Libraries (NL) PCR plate and follow the instructions for an adjusted second dilution. Refer to *Prepare for Sequencing* on page 69 and *Troubleshooting Workflow: Prepare Adjusted Second Dilution* on page 74.
	Error in library preparation workflow during or prior to index PCR step.	Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 41 or *Fragment gDNA* on page 45.
	• Incorrect use of enrichment probes (for example, OPR1 probes used for DNA samples, OPD2 probes used for RNA samples). • Error in library preparation workflow during or after first hybridization step.	A systemic probe pool error (eg, swapping OPR1 and OPD2 across DNA and RNA samples) might result in normal total passing filter reads for DNA and RNA, but median exon coverage (DNA) and total on-target reads (RNA) fail QC. Repeat enrichment steps for libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 54.

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.
	Use or equipment error in the assay workflow.	• Resequence libraries from the Normalized Libraries (NL) PCR plate. Refer to *Prepare for Sequencing* on page 69. • Repeat enrichment steps for libraries from the Amplified Libraries Samples (ALS) PCR plate. Refer to *Set Up First Hybridization* on page 54. • Start library preparation from the beginning of the workflow. Refer to *Denature and Anneal RNA* on page 41 or *Fragment gDNA* on page 45.
NTC Failure (DNA/RNA).	Cross-contamination occurred or contamination of work area.	Review Warnings and Precautions section for information on decontaminating work areas and avoiding cross-contamination. Review plate layout and library indexing to make sure that 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.	
Positive and/or negative controls in the sequencing run were analyzed as non-control samples.	Incorrect assignment of Cancer Type during sequencing run setup.	Requeue analysis with controls correctly identified as instructed in the *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)*.

Performance Characteristics

TSO Comprehensive is a targeted NGS panel detecting alterations in 517 genes. Small DNA variants (single nucleotide variants [SNVs], multi-nucleotide variants [MNVs], insertions, and deletions) are eligible for reporting from all 517 genes. Tumor Mutational Burden is reported as a score based on a count of non-driver somatic variants per megabase (refer to *Local Run Manager TruSight Oncology Comprehensive (US) Analysis Module Workflow Guide (document # 200061834)* for details). Fusions are eligible for reporting from the 24 genes indicated in *Summary and Explanation of the Assay* on page 1. Fusions containing the ALK or ROS1 gene are not eligible for reporting. All other fusions containing one or two genes that are indicated for fusion detection in Table 2 are eligible for reporting. Splice variants in the EGFR gene are eligible for reporting. To be reported in Level 2 or 3, variants must be detected and have evidence in the TSO Comprehensive assay Knowledge Base based on the tested tissue type. To be reported in Level 1, NTRK and RET fusions require the fusion partner to be 5' and the NTRK or RET kinase domain to be intact.

For CDx claims, FFPE samples with specific CDx positive variants were included in studies. For tumor profiling, a representative approach to validation of variants was conducted. Small DNA variant testing included SNVs, MNVs, insertions, and deletions. For RNA fusions, testing was done at the gene level. TMB does not have a qualitative cutoff, so only quantitative analysis was performed in each study.

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

Table 43 provides definitions of metrics calculated in various studies.

Table 43 Metrics Definitions

Term	Definition
Positive Percent Agreement (PPA)	The percentage of comparator method positive results in which TSO Comprehensive is positive.
Negative Percent Agreement (NPA)	The percentage of comparator method negative results in which TSO Comprehensive is negative.
Percent Positive Call (PPC)	Percentage of observations that are positive for a target among observations expected to be positive for the target.
Percent Negative Call (PNC)	Percentage of observations that are negative for a target among observations expected to be negative for the target.

Accuracy Study for NTRK1, NTRK2, NTRK3 Fusion Detection

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

A retrospective, noninterventional study was conducted. Larotrectinib clinical trial samples and supplemental samples were tested with the TSO Comprehensive assay at one external site and with a comparator method at a central laboratory. Accuracy of the TSO Comprehensive assay NTRK fusion calls was estimated relative to the comparator 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 assay and/or the comparator 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 comparator 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.

Of the 499 samples, 85 samples had invalid TSO Comprehensive assay results (invalid rate of 17%). Of the 85 samples, 53 also had invalid comparator method results. The comparator method had seven additional invalid results. Thus, 407 of the 499 samples had valid results by both methods. The agreement analysis is shown in Table 44. Based on valid test 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 44 Concordance Between the TSO Comprehensive Assay and Comparator Method for Detection of NTRK Fusions

		Comparator Method Result		
		NTRK Fusion Positive	**NTRK Fusion Negative**	**Total**
TSO Comprehensive Assay Result	NTRK Fusion Positive	114	16[1]	130
	NTRK Fusion Negative	4	273	277
	Total	118	289	407
Agreement Statistics	PPA% (n/N; 95% CI[2])	96.6% (114/118; 91.5%, 99.1%)		
	NPA% (n/N; 95% CI[2])	94.5% (273/289; 91.2%, 96.8%)		

[1] Six of the 16 samples were TSO Comprehensive positive due to an isolated contamination event during study testing.
[2] CI based on Clopper-Pearson (exact) method.

In Table 44, 16 samples were positive for NTRK fusions by TSO Comprehensive but negative by the comparator method leading to an NPA of 94.5%. Of these 16 samples, six were positive due to a putative isolated contamination event that occurred during the study. These six samples all contained the same fusion, were in the same library preparation plate as a sample with an identical fusion with high supporting reads. This putative contamination was investigated during an additional cross-contamination study (*Cross Contamination* on page 123) and a study to understand discordant results described in the following paragraph. Of the remaining 10 samples positive by TSO Comprehensive and negative by the comparator method, seven samples were local test (LT) positive and three samples were LT negative. Six of these seven LT positive subjects showed clinical benefit with larotrectinib suggesting that the samples did have NTRK fusions.

Testing of discordant results was performed for samples in both the NTRK Accuracy and Bridging studies due to low NPA values in both studies, possibly due to a suspected contamination event. A total of 142 RNA samples, comprised of 107 concordant samples (53 concordant negative, 54 concordant positive), 26 discordant samples, and nine samples with unknown NTRK status from any of the methods used (TSO Comprehensive, comparator method, and LT) were re-tested with the TSO Comprehensive assay. Of the 142 samples, 133 produced valid results. Of the six samples that were suspected to be contaminated, presented as positive in the original Accuracy study in Table 44, only four were available for retesting. All four of those samples were NTRK fusion-negative upon re-testing. Additionally, six out of the remaining 126 samples with valid retest results changed NTRK fusion status upon retesting. Of those samples, three were concordant NTRK fusion-positives by original testing, changing to negative upon retest. None of the concordant NTRK fusion-negatives from the original testing changed to positives. Further, when a sensitivity analysis based on the results from this testing of discordants was performed, the PPA in the imputed data set was estimated to be 93.3% (95% CI: 87.9%, 98.7%) and NPA was estimated at 97.3% (95% CI: 95.4%, 99.3%). Note: 95% CI was calculated according to method described by Gelman, Andrew[1]. The PPA and NPA from the imputed data set are within the confidence limits of the original estimates (Table 44).

Accuracy Study for RET Gene Fusions Detection in NSCLC

The accuracy study was conducted using samples obtained from the RETEVMO (selpercatinib) clinical trial (LIBRETTO-001) and supplemental samples obtained from commercial sources. Across both sets of samples, 219 samples were tested using both the TSO Comprehensive assay and the comparator method. Of the 219 samples, 14 samples had invalid TSO Comprehensive assay results (invalid rate of 6.4%). Of these 14, eight also had invalid comparator method results. The comparator method had 15 additional invalid results and 22 samples with no calls. Thus, 168 of the 219 samples had valid results by both methods. The agreement analysis is shown in Table 45. Based on valid test results, the Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) between the TSO Comprehensive assay and comparator method were 100.0% (69/69; 95% CI: 94.8%, 100.0%), and 97.0% (96/99; 95% CI: 91.4%, 99.4%), respectively.

Table 45 RET Accuracy Study: Concordance Between the TSO Comprehensive Assay and Comparator Method for Detection of RET Fusions in NSCLC

		Comparator Method Result		
		RET Fusion Positive	**RET Fusion Negative**	**Total**
TSO Comprehensive Assay Result	RET Fusion Positive	69	3	72
	RET Fusion Negative	0	96	96
	Total	69	99	168
Agreement Statistics	PPA% (n/N; 95% CI*)	100.0% (69/69; 94.8%, 100.0%)		
	NPA% (n/N; 95% CI*)	97.0% (96/99; 91.4%, 99.4%)		

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

Accuracy for Tumor Profiling

The detection of variants by TSO Comprehensive assay was compared to the results of comparator methods. Small DNA variants were compared to an externally validated targeted NGS method. TMB score was compared to a whole exome sequencing (WES) method. RNA splice variants were compared against a quantitative PCR (qPCR) method. Fusions were compared against a composite method consisting of an RNA whole exome NGS panel, a targeted NGS fusion panel, and droplet digital PCR (ddPCR).

Small DNA Variant Detection

The detection of small DNA variants was compared to another externally validated NGS (evNGS) panel assay (comparator method). The comparison between small DNA variants, consisting of single nucleotide variants (SNVs), multi-nucleotide variants (MNVs), insertions, and deletions, was based on 414 FFPE samples from 16 different tissue types that were valid for both TSO Comprehensive and the comparator method (Table 46).

Table 46 Number of Evaluable Samples by Cancer/Tissue Type

Tissue Type / Cancer Type	Number of Samples
Bladder	19
Brain	21
Breast	30
CRC	52
Gastric	24
Head and Neck	1
Liver	21
Medullary Thyroid	38
NSCLC	57
Ovary	16
Pancreas	24
Papillary Thyroid	26
Prostate	36
Skin	25
Soft Tissue	2
Uterus	22
Total	*414*

The evNGS method uses a 5% VAF filter during reporting to prevent artifacts from being output to end users. The evNGS method is therefore not fully validated for variants below 5% VAF; however, these variant calls (below 5% VAF) are available in the evNGS variant calling output. Therefore, accuracy is assessed across the full VAF range. When variants below 5% VAF are a significant source of discordance this will be mentioned in the assessment.

A summary of accuracy (PPA and NPA) for all Cancer Mutations with Evidence of Clinical Significance (Level 2) and Cancer Mutations with Potential Clinical Significance (Level 3) small variants was computed and is shown in Table 47. Level 2 deletions had a PPA of 66.7% (2/3). This low PPA was due to TSO Comprehensive detecting an EGFR clinically significant deletion with one nucleotide difference in the alternate sequence relative to the clinically significant deletion found by the comparator method.

The PPA values for Level 3 variants in Table 47 were driven by many Level 3 small DNA variants detected only by the evNGS method, which would not be reported as their VAFs were less than 5%. If only variants ≥ 5% VAF were considered, the PPA for Level 3 SNVs increased to 98% (348/355; 95% CI: 96.0%, 99.0%) and the PPA for Level 3 insertions and deletions were 96.1% (49/51; 95% CI: 86.8%, 98.9%) and 97.1% (133/137; 95% CI: 92.7%, 98.9%), respectively.

Table 47 Concordance Summary for Level 2 and Level 3 Small Variant Calls Evaluated by Variant Type

Variant Type	Clinical Significance	PPA (%) (n/N) (95% CI)	NPA (%) (n/N) (95% CI)
SNVs	Level 2	96.1 (99/103) (90.4, 98.5)	> 99.9 (9832/9833) (> 99.9, > 99.9)
	Level 3	76.9 (373/485) (73.0, 80.4)	> 99.9 (212,277/212,311) (> 99.9, > 99.9)
	All	80.3 (472/588) (76.9, 83.3)	> 99.9 (219,211/219,246) (> 99.9, > 99.9)
MNVs	Level 2	100.0 (5/5) (56.6, 100.0)	100.0 (9931/9931) (> 99.9, > 99.9)
	Level 3	90.0 (9/10) (59.6, 98.2)	> 99.9 (212,785/212,786) (> 99.9, > 99.9)
	All	93.3 (14/15) (70.2, 98.8)	> 99.9 (219,818/219,819) (> 99.9, > 99.9)
Insertions	Level 2	100.0 (1/1) (20.7, 100.0)	100.0 (9935/9935) (> 99.9, 100.0)
	Level 3	86.0 (49/57) (74.7, 92.7)	> 99.9 (212,738/212,739) (> 99.9, > 99.9)
	All	86.2 (50/58) (75.1, 92.8)	> 99.9 (219,775/219,776) (> 99.9, > 99.9)

Variant Type	Clinical Significance	PPA (%) (n/N) (95% CI)	NPA (%) (n/N) (95% CI)
Deletions	Level 2	66.7 (2/3) (20.8, 93.9)	> 99.9 (9932/9933) (> 99.9, > 99.9)
	Level 3	90.3 (139/154) 84.6, 94.0)	> 99.9 (212,624/212,642) (> 99.9, > 99.9)
	All	89.8 (141/157) (84.1, 93.6)	> 99.9 (219,658/219,677) (> 99.9, > 99.9)
All Variants	Level 2	95.5 (107/112) (90.0, 98.1)	> 99.9 (9822/9824) (> 99.9, > 99.9)
	Level 3	80.7 (570/706) (77.7, 83.5)	> 99.9 (212,036/212,090) (> 99.9, > 99.9)
	All	82.8 (677/818) (80.0, 85.2)	> 99.9 (218,960/219,016) (> 99.9, > 99.9)

A summary of accuracy (PPA and NPA) of Level 2 and Level 3 insertions and deletions binned by size is presented in Table 48.

Table 48 Concordance Summary for Level 2 and Level 3 Insertions and Deletions Binned by Size

Variant Type	Bin Size	Total Unique Variants	Concordant Positive Calls	Discordant Negative[1]	Discordant Positive[2]	Concordant Negative Calls	PPA (%) (n/N) (95% CI)	NPA (%) (n/N) (95% CI)
Insertions	1–5	46	47	7	1	219779	87.0 (47/54) (75.6, 93.6)	> 99.9 (219,779/219,780) (> 99.9, > 99.9)
	6–10	2	1	1	0	219832	50.0 (1/2) (9.5, 90.5)	100.0 (219,832/219,832) (> 99.9, 100.0)
	11–15	2	2	0	0	219832	100.0 (2/2) (34.2, 100.0)	100.0 (219,832/219,832) (> 99.9, 100.0)
	All	50	50	8	1	219775	86.2 (50/58) (75.1, 92.8)	> 99.9 (219,775/219,776) (> 99.9, > 99.9)
Deletions	1–5	129	132	15	18	219669	89.8 (132/147) (83.8, 93.7)	> 99.9 (219,669/219,687) (> 99.9, > 99.9)
	6–10	5	5	0	0	219829	100.0 (5/5) (56.6, 100.0)	100.0 (219,829/219,829) (> 99.9, 100.0)
	11–15	2	0	1	1	219832	0.0 (0/1) (0.0, 79.3)	> 99.9 (219,832/219,833) (> 99.9, > 99.9)
	16–20	3	4	0	0	219830	100.0 (4/4) (51.0, 100.0)	100.0 (219,830/219,830) (> 99.9, > 99.9)
	All	139	141	16	19	219658	89.8 (141/157) (84.1, 93.6)	> 99.9 (219,658/219,677) (> 99.9, > 99.9)

[1] Called only in evNGS.
[2] Called only in TSO Comprehensive.

Accuracy of all insertions and deletions (Table 48 and variants not reported as Level 2 or Level 3) up to and including 24 base pairs in length has also been demonstrated. The PPA for all insertions and deletions (Table 48 and variants not reported as Level 2 or Level 3, but with VAF ≥ 5%) was 82.7% (153/185; 95% CI: 76.6%, 87.5%) and 93.7% (402/429; 95% CI: 91.0%, 95.6%) respectively. Corresponding NPA values were > 99.9% (433,101,883/433,101,896; 95% CI: > 99.9%, > 99.9%) and > 99.9% (433,101,611/433,101,652; 95% CI: > 99.9%, > 99.9%).

A summary of accuracy (PPA and NPA) of Level 2 variants by gene is presented in Table 49.

Table 49 Concordance Summary of Level 2 Variants by Gene

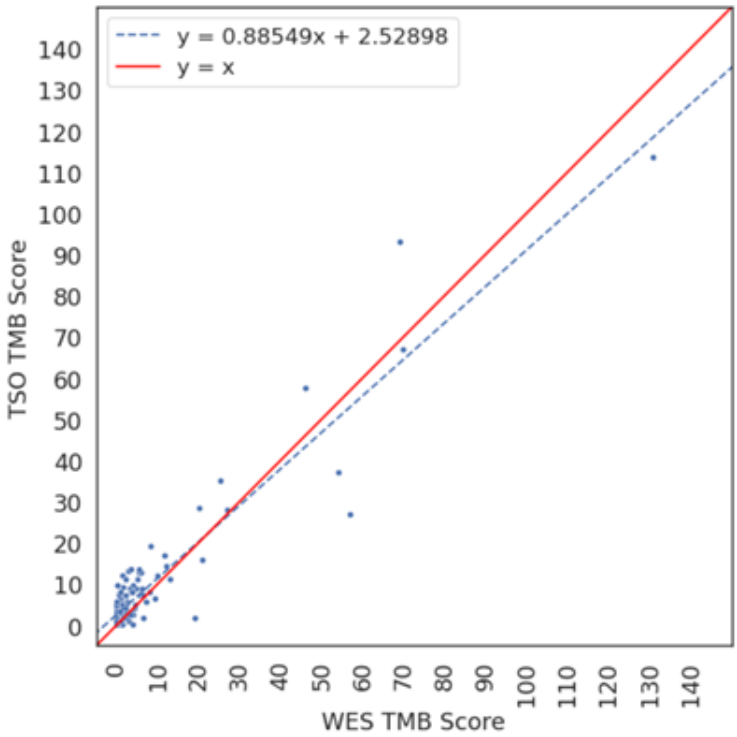
Gene	Variant	Concordant Positive Calls	Discordant Negative[1]	Discordant Positive[2]	Concordant Negative Calls	PPA (%) (95% CI)	NPA (%) (95% CI)
BARD1	W91*	0	1	0	413	0.0 (0.0, 79.3)	100.0 (99.1, 100.0)
BRAF	V600E	41	1	0	372	97.6 (87.7, 95.6)	100.0 (99.0, 100.0)
BRIP1	R162*	0	1	0	413	0.0 (0.0, 79.3)	100.0 (99.1, 100.0)
CDK12	L453Ifs*9	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
CHEK2	T367Mfs*15	1	0	0	413	100.0 (20.7,100.0)	100.0 (99.1, 100.0)
EGFR	L747_A750del	0	0	1	413	N/A	99.8 (98.6, > 99.9)
	L747_ T751delinsS	0	1	0	413	0.0 (0.0, 79.3)	100.0 (99.1, 100.0)
	L858R	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
KRAS	A146T	2	0	0	412	100.0 (34.2, 100.0)	100.0 (99.1, 100.0)
	Q61L	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	G12A	12	0	0	402	100.0 (75.8, 100.0)	100.0 (99.1, 100.0)
	G12C	18	0	1	395	100.0 (82.4, 100.0)	99.7 (98.6, > 99.9)
	G12F	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	G12S	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	G12V	7	0	0	407	100.0 (64.6, 100.0)	100.0 (99.1, 100.0)
	G13A	4	0	0	410	100.0 (51.0, 100.0)	100.0 (99.1, 100.0)
NRAS	G12A	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
PALB2	Q472*	0	1	0	413	0.0 (0.0, 79.3)	100.0 (99.1, 100.0)
PTEN	L320*	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
RET	A883F	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	C609Y	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	C630R	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	C634R	2	0	0	412	100.0 (34.2,100.0)	100.0 (99.1, 100.0)
	C634F	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)
	C634S	3	0	0	411	100.0 (43.9,100.0)	100.0 (99.1, 100.0)
	C634Y	2	0	0	412	100.0 (34.2, 100.0)	100.0 (99.1, 100.0)
	M918T	3	0	0	411	100.0 (43.9, 100.0)	100.0 (99.1, 100.0)
	V804M	1	0	0	413	100.0 (20.7, 100.0)	100.0 (99.1, 100.0)

[1] Called only in evNGS.
[2] Called only in TSO Comprehensive.

Tumor Mutational Burden Detection

Concordance of TMB score was determined by comparing the TMB scores (somatic mutations/megabase) between a validated Whole Exome Sequencing (WES) comparator method and TSO Comprehensive assay. 124 FFPE samples from 14 different tissue types across a range of TMB scores (0.8–115.7 Mut/Mb) with valid results by both TSO Comprehensive and WES comparator method were analyzed. Linear regression analysis with WES as the predictor and TSO Comprehensive as the outcome 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



RNA Splice Variant Detection

Accuracy for splice variant detection was calculated by comparing TSO Comprehensive assay results to a validated qPCR comparator method for EGFRvIII. Concordance analysis was performed on a total of 16 unique FFPE RNA samples from brain tissue. Table 50 summarizes the concordance study results for EGFRvIII. In summary, EGFR had a PPA and NPA of 100%.

Table 50 Summary of Concordance Analysis between TSO Comprehensive and qPCR Assay for EGFRvIII

TSO Comprehensive Results	qPCR Positive	qPCR Negative
Positive	3	0
Negative	0	13
PPA % (n/N; 95% CI)	100% (3/3; 44%, 100%)	
NPA % (n/N; 95% CI)	100% (13/13; 77%, 100%)	

RNA Fusion Detection

TSO Comprehensive assay fusions were compared to a validated composite method consisting of an RNA whole exome NGS panel, a targeted NGS fusion panel (tNGS), and droplet digital PCR (ddPCR). A breakdown of the composite methodology is shown in Table 51. In short, the NGS whole exome panel overlapped with all the genes for which TSO Comprehensive assay can detect fusions. However, because the limit of detection of the weNGS assay was 4–8x that of TSO Comprehensive assay (based on the number of supporting reads observed in the overlapping fusion calls), a composite method using two additional methods with greater sensitivity, but less breadth, for fusions (NGS Targeted Panel and ddPCR).

Table 51 Composite Reference Method Testing for RNA Fusions

		weNGS	
		Detected	**Not Detected**
TSO Comprehensive	**Detected**	Tested with tNGS if covered by assay; if not, tested with ddPCR	Tested with tNGS if covered by assay; if not, tested with ddPCR
	Not Detected	Tested with tNGS if covered by assay; if not, tested with ddPCR	Randomly selected samples tested with tNGS or ddPCR if fusions are not covered by tNGS

A total of 255 unique RNA samples representing 14 tissue types were tested. All samples passed TSO Comprehensive metrics. Of the 255 samples, 220 samples were not selected based on prior screening (hereafter known as uncharacterized samples), while 35 samples were selected for the study because they were fusion-positive with TSO Comprehensive assay or a predecessor assay (hereafter known as characterized samples). Performance for these 35 characterized samples was adjusted using a mean fusion prevalence. Therefore, the combined performance across characterized and uncharacterized samples was calculated using an inverse-variance weighted average to account for the adjustment for prevalence in the characterized samples. These concordance results are shown in Table 52. In summary, the PPA of the combined fusions was 87.29%.

Table 52 Summary of Performance for RNA Fusions (Combined for Characterized and Uncharacterized)

PPA (%) (95% CI*)	NPA (%) (95% CI*)
87.29 (67.83, 96.35)	99.99 (99.98, > 99.99)

* Confidence interval calculated by bootstrap.

A summary of the concordance data by characterization and by gene is also presented in Table 53 below, which shows PPA/NPA for all 24 fusions genes in the TSO Comprehensive panel. For the PPA/NPA values, characterized samples were adjusted by prevalence, while uncharacterized samples were not.

Table 53 Concordance for RNA Fusions by Gene and Sample Characterization

Gene	# TP	# FP	# FN	# TN	Characterized	PPA (%)(n/N) (95% CI)	NPA (%)(n/N) (95% CI)	PPV (%)(n/N) (95% CI)	NPV (%)(n/N) (95% CI)	Adjusted PPA (%)	Adjusted NPA (%)
AXL	2	0	0	33	YES	100.0 (2/2) (34.2, 100.0)	100.0 (33/33 (89.6, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0	100.0
AXL	1	0	0	219	NO	100.0 (1/1) (20.7, 100.0)	100.0 (219/219) (98.3, 100.0)	100.0 (1/1) (20.7, 100.0)	100.0 (219/219) (98.3, 100.0)	N/A	N/A
BCL2	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
BCL2	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
BRAF	2	0	0	33	YES	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0	100.0
BRAF	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
CDK4	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
CDK4	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
EGFR	1	3	0	31	YES	100.0 (1/1) (20.7, 100.0)	91.2 (31/34) (77.0, 97.0)	25.0 (1/4) (4.6, 69.9)	100.0 (31/31) (89.0, 100.0)	100.0	100.0
EGFR	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
EML4	1	0	1	33	YES	50.0 (1/2) (9.5, 90.5)	100.0 (33/33) (89.6, 100.0)	100.0 (1/1) (20.7, 100.0)	97.1 (33/34) (85.1, 99.5)	1.3	100.0
EML4	1	0	0	219	NO	100.0 (1/1) (20.7, 100.0)	100.0 (219/219) (98.3, 100.0)	100.0 (1/1) (20.7, 100.0)	100.0 (219/219) (98.3, 100.0)	N/A	N/A
ERG	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
ERG	6	0	1	213	NO	85.7 (6/7) (48.7, 97.4)	100.0 (213/213) (98.2, > 99.9)	100.0 (6/6) (61.0, 100.0)	99.5 (213/214) (97.4, 99.9)	N/A	N/A
ESR1	2	0	0	33	YES	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0	100.0

Gene	# TP	# FP	# FN	# TN	Characterized	PPA (%)(n/N) (95% CI)	NPA (%)(n/N) (95% CI)	PPV (%)(n/N) (95% CI)	NPV (%)(n/N) (95% CI)	Adjusted PPA (%)	Adjusted NPA (%)
ESR1	2	0	0	218	NO	100.0 (2/2) (34.2, 100.0)	100.0 (218/218) (98.3, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (218/218) (98.3, 100.0)	N/A	N/A
ETV1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
ETV1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
ETV4	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
ETV4	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
EWSR1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
EWSR1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
FGFR1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
FGFR1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
FGFR2	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
FGFR2	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
FGFR3	1	1	1	32	YES	50.0 (1/2) (9.5, 90.5)	97.0 (32/33) (84.7, 99.5)	50.0 (1/2) (9.5, 90.5)	97.0 (32/33) (84.7, 99.5)	0.2	100.0
FGFR3	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
FLI1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
FLI1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
KIF5B	2	0	0	33	YES	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0	100.0
KIF5B	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
NRG1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, 99.9)	100.0 (1/1) (20.7, 100.0)	100.0(34/34) (89.8, > 99.9)	100.0	100.0
NRG1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A

Gene	# TP	# FP	# FN	# TN	Characterized	PPA (%)(n/N) (95% CI)	NPA (%)(n/N) (95% CI)	PPV (%)(n/N) (95% CI)	NPV (%)(n/N) (95% CI)	Adjusted PPA (%)	Adjusted NPA (%)
NTRK1	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0(1/1) (20.7, 100.0)	100.0(34/34) (89.8, > 99.9)	100.0	100.0
NTRK1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
NTRK2	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
NTRK2	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
NTRK3	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0(34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
NTRK3	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0(220/220) (98.3, > 99.9)	N/A	N/A
PAX3	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
PAX3	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0(220/220) (98.3, > 99.9)	N/A	N/A
RAF1	1	0	0	34	YES	100.0(1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
RAF1	0	0	0	220	NO	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	100.0 (220/220) (98.3, > 99.9)	N/A	N/A
RET	2	0	0	33	YES	100.0 (2/2) (34.2, 100.0)	100.0 (33/33) (89.6, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0(33/33) (89.6, 100.0)	100.0	100.0
RET	2	0	0	218	NO	100.0 (2/2) (34.2, 100.0)	100.0 (218/218) (98.3, 100.0)	100.0 (2/2) (34.2, 100.0)	100.0 (218/218) (98.3, 100.0)	N/A	N/A
TMPRSS2	1	0	0	34	YES	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0 (1/1) (20.7, 100.0)	100.0 (34/34) (89.8, > 99.9)	100.0	100.0
TMPRSS2	6	0	1	213	NO	85.7 (6/7) (48.7, 97.4)	100.0 (213/213) (98.2, > 99.9)	100.0 (6/6) (61.0, 100.0)	99.5 (213/214) (97.4, 99.9)	N/A	N/A

Within-Laboratory Precision

Two studies were conducted to evaluate within-laboratory precision for TSO Comprehensive. Study 1 evaluated NTRK and RET fusions. Study 2 evaluated TMB.

Study 1 - CDx Within-Laboratory Precision

Within-laboratory precision was evaluated for NTRK1, NTRK2, and NTRK3 fusions in six FFPE samples from five tumor types (lower grade glioma, glioblastoma multiforme, colorectal cancer, myofibroblastic sarcoma, secretory breast cancer) and RET fusions in four samples from three tumor types (non-small cell lung cancer, thyroid cancer, and atypical Spitz tumor from skin tissue specimen). Each sample was tested at two variant levels: ~1x LoD (low variant level) and ~2–3x LoD (high variant level) with the exception of 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 duplicate 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 sample per level. 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).

For NTRK fusions, at the higher variant level (~2 – 3x LoD), the TSO Comprehensive assay demonstrated 100% PPC for all fusions except one (LMNA-NTRK1 had 99.0% PPC). It should be noted that LMNA-NTRK1 at the high level had more than 54 observations; this was due to the preparation of two dilution mixtures targeting to the same variant level (Table 54). Therefore, the observations were combined. At the low variant level (~1x LoD), the PPC for RNA fusions ranged from 94.4% to 100%. For variants with PPC < 95% (BCAN-NTRK1), the supporting reads were below the respective Limits of Detection (53.2 supporting reads for BCAN-NTRK1). 100% PNC was achieved for all variants at both levels (Table 54).

Table 54 Qualitative Results for Targeted NTRK RNA Fusions

Variant Level	Fusion	Mean Supporting Reads	PPC (%) (n/N) (95% CI)	PNC (%) (n/N) (95% CI)
~1x LoD	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)
	LMNA-NTRK1	12.2	98.1 (51/52) (89.9, 99.7)	100.0 (539/539) (99.3, 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)	
	KANK1-NTRK3	13.5	100.0 (54/54) (93.4, 100.0)	100.0 (591/591) (99.4,100.0)

Variant Level	Fusion	Mean Supporting Reads	PPC (%) (n/N) (95% CI)	PNC (%) (n/N) (95% CI)
~2-3x LoD	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)
	LMNA-NTRK1	35.1	99.0 (103/104) (94.8, 99.8)	100.0 (431/431) (99.1, 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)	
	KANK1-NTRK3	39.2	100.0 (54/54) (93.4, 100.0)	100.0 (535/535) (99.3, 100.0)

For RET fusions, at the higher variant level (~2 – 3x LoD), the TSO Comprehensive assay demonstrated 100% PPC for all fusions (Table 55). It should be noted that KIF5B-RET at the low level had more than 54 observations; this was due to the preparation of two dilution mixtures targeting to the same variant level. Therefore, the observations were combined. At the low variant level (~1x LoD), the PPC for RET fusions ranged from 90.7% to 98.1%. For variants with PPC < 95% (NCOA4-RET), the supporting reads were below the respective Limits of Detection (15.8 supporting reads for NCOA4-RET). 100% PNC was achieved for all variants at both levels (Table 55).

Table 55 Qualitative Results for Targeted RET RNA Fusions

Variant Level	Targeted Fusions	Mean Supporting Reads	PPC (%) (n/N) (95% CI)	PNC (%) (n/N) (95% CI)
~1x LoD	NCOA4-RET[1]	13.3	90.7 (49/54) (80.1, 96.0)	100.0 (537/537) (99.3, 100.0)
	CCDC6-RET[2]	18.7	98.1 (53/54) (90.2, 99.7)	100.0 (591/591) (99.4, 100.0)
	KIF5B-RET (sample 1)[3]	17.3	95.4 (103/108) (89.6, 98.0)	100.0 (430/430) (99.1, 100.0)
	KIF5B-RET (sample 2)[3]	17.3	96.2 (51/53) (87.2, 99.0)	

Variant Level	Targeted Fusions	Mean Supporting Reads	PPC (%) (n/N) (95% CI)	PNC (%) (n/N) (95% CI)
~3x LoD	NCOA4-RET[1]	24.8	100.0 (54/54) (93.4, 100.0)	100.0 (481/481) (99.2, 100.0)
	CCDC6-RET[2]	N/A	Not tested[4]	100.0 (589/589) (99.4, 100.0)
	KIF5B-RET (sample 1)[3]	43.8	100.0 (54/54) (93.4, 100.0)	100.0 (428/428) (99.1, 100.0)
	KIF5B-RET (sample 2)[3]	44.6	100.0 (53/53) (93.2, 100.0)	

[1] Thyroid tissue
[2] Atypical Spitz tumor
[3] Lung Tissue from NSCLC
[4] CCDC6-RET was tested at one level only (ie, ~1x C95) due to insufficient materials.

Restricted maximum likelihood (REML) variance components analysis was performed to evaluate total variation of the underlying continuous variable (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 Table 56 for NTRK RNA fusions and Table 57 for RET RNA fusions.

The residual component was the largest contributor to total variance for RNA fusions at both levels supporting the conclusion that detection of these variants by TSO Comprehensive is robust to operators, lots, instruments, and days.

Table 56 Quantitative SD and CV Results for Targeted NTRK 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)
~1x LoD	TPM3-NTRK1	54	20.2	2.33 (12)	0.94 (5)	3.31 (16)	0.83 (4)	5.70 (28)	7.10 (35)
	BCAN-NTRK1	54	22.1	3.38 (15)	1.41 (6)	1.78 (8)	0.00 (0)	6.03 (27)	7.28 (33)
	LMNA-NTRK1	52	12.2	1.36 (11)	1.25 (10)	1.59 (13)	0.00 (0)	4.74 (39)	5.33 (44)
	ETV6-NTRK2	54	20.3	0.00 (0)	3.18 (16)	4.36 (21)	0.00 (0)	8.30 (41)	9.90 (49)
	ETV6-NTRK3	54	16.2	2.28 (14)	2.36 (15)	2.17 (13)	0.00 (0)	4.65 (29)	6.10 (38)
	ETV6-NTRK3 (cell line)	54	23.1	4.55 (20)	1.18 (5)	0.00 (0)	0.00 (0)	6.73 (29)	8.21 (36)
	KANK1-NTRK3	54	13.5	0.74 (5)	0.11 (1)	1.09 (8)	0.00 (0)	4.22 (31)	4.42 (33)
2~3x LoD	TPM3-NTRK1	54	57.1	11.21 (20)	1.18 (2)	5.68 (10)	2.03 (4)	11.86 (21)	17.44 (31)
	BCAN-NTRK1	54	53.2	8.22 (15)	0.76 (1)	5.59 (11)	2.89 (5)	11.34 (21)	15.37 (29)
	LMNA-NTRK1	104	35.1	1.47 (4)	5.92 (17)	8.11 (23)	2.92 (8)	10.69 (30)	15.03 (43)
	ETV6-NTRK2	54	52	0.00 (0)	4.07 (8)	7.07 (14)	5.72 (11)	12.91 (25)	16.31 (31)
	ETV6-NTRK3	54	41.7	7.16 (17)	0.40 (1)	6.40 (15)	0.00 (0)	10.74 (26)	14.41 (35)
	ETV6-NTRK3 (cell line)	54	28.3	7.93 (28)	1.02 (4)	0.00 (0)	0.00 (0)	9.05 (32)	12.08 (43)
	KANK1-NTRK3	54	39.2	5.10 (13)	0.00 (0)	4.78 (12)	0.00 (0)	9.44 (24)	11.74 (30)

Table 57 Quantitative SD and CV Results for Targeted RET 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)
~1x LoD	NCOA4-RET	54	13.3	1.67 (13)	0.00 (0)	0.00 (0)	1.67 (13)	5.09 (38)	5.61 (42)
	CCDC6-RET	54	18.7	0.00 (0)	1.14 (6)	5.44 (29)	0.00 (0)	6.17 (33)	8.30 (44)
	KIF5B-RET (Sample 1)	108	17.3	2.11 (12)	2.50 (14)	2.89 (17)	3.52 (20)	7.09 (41)	9.04 (52)
	KIF5B-RET (Sample 2)	53	17.3	2.05 (12)	3.72 (22)	3.65 (21)	2.41 (14)	5.95 (34)	8.52 (49)
2~3x LoD	NCOA4-RET	54	24.8	3.05 (12)	0.00 (0)	5.92 (24)	0.00 (0)	6.78 (27)	9.50 (38)
	KIF5B-RET (Sample 1)	54	43.8	4.15 (9)	0.96 (2)	12.57 (29)	6.52 (15)	15.23 (35)	21.23 (48)
	KIF5B-RET (Sample 2)	53	44.6	5.37 (12)	4.97 (11)	13.73 (31)	0.00 (0)	12.41 (28)	19.90 (45)

Study 2 - TMB Within-Laboratory Precision

Five NSCLC FFPE DNA samples for TMB were used to evaluate precision at different levels across a range of scores. Each of the samples was run in duplicate across three operators, three days, with three library preparations for three reagent lots using three NextSeq 550Dx instruments generating 54 observations per level. Some levels had fewer than 54 observations due to invalid libraries that did not meet the necessary QC metrics.

The total variation of TMB 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 58 for TMB by level. In summary, the residual component remained the largest contributor to total variance for TMB scores.

The variation in TMB scores tends to increase with the mean as expected from theoretical distributions of count data. The residual component remained the largest contributor to total variance for TMB scores supporting the conclusion that the scores are robust to operators, lots, instruments, and days.

However, TMB is a complex biomarker and analytical performance may vary from 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, insertion, or deletion) and VAF level (closeness to inclusion cutoff).

Table 58 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)

Reproducibility

Multiple studies were conducted to evaluate reproducibility for the TSO Comprehensive assay.

Study 1 - CDx Reproducibility

Reproducibility of the TSO Comprehensive assay was assessed across three testing sites (one internal, two external) with two operators per site, two within-run replicates, and three non-consecutive testing days. Testing was conducted with a reproducibility panel with RNA samples containing specific known NTRK1–3 and RET fusion variants from formalin-fixed, paraffin embedded (FFPE) tissue specimens and cell lines. Specifically, the panel included seven members from FFPE samples in three different tissue types (brain, soft tissue, and breast) and five members from three cell lines containing NTRK1–3 RNA fusion variants, and four members from FFPE samples in three different tissue types (papillary thyroid, unclassified thyroid, and lung) containing RET RNA fusion variants. High-level panel members were targeted at approximately two to three 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 over the course of three non-consecutive testing days, generating six observations per target per panel member. From all three sites, 36 observations were generated per panel member (three sites/instruments × two operators × three start days x two within-run replicates).

PPC and PNC for targeted RNA fusion variants, at both high and low levels were determined. Analyses were performed to estimate PPC and PNC (with associated 95% CIs) in the targeted high-level and low-level panel members by combining TSO Comprehensive assay observations for a given target in a group of panel members representing the applicable RNA fusions across sites/instruments, operators, and runs. For each targeted variant, TSO Comprehensive 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 calculate PNC.

NTRK 1/2/3 Fusions

For the NTRK fusion panel members at 2-3x LoD, PPC values ranged from 94.4% to 100.0%, with the lowest PPC value being the BCAN-NTRK1 panel member (PPC = 94.4% [34/36; 95% CI: 81.9%, 98.5%]), which was below the respective LoD (53.1 supporting reads), causing several discordant samples at both the low and high levels (Table 59). PNC values for the high-level NTRK fusion panel members were all 100.0% (Table 60). For the low-level NTRK fusion panel members, PPC values ranged from 80.6% (BCAN-NTRK1) to 100.0%, and all PNC values were 100.0%. Further, none of the individual sites showed any additional discordance.

Table 59 PPC of TSO Comprehensive Assay for Detection of NTRK Fusions in High- and Low-Level Targeted Panel Members

Variant Level	Targeted Fusions	N	Mean Supporting Reads	PPC (%) (n/N)	95% CI
~2-3x LoD	LMNA-NTRK1	36	37.9	100.0 (36/36)	(90.4, 100.0)
	BCAN-NTRK1	36	33.6	94.4 (34/36)	(81.9, 98.5)
	ETV6-NTRK2	36	24.6	100.0 (36/36)	(90.4, 100.0)
	TRIM24-NTRK2*	36	36.6	100.0 (36/36)	(90.4, 100.0)
	ETV6-NTRK3	36	56.4	100.0 (36/36)	(90.4, 100.0)
	BTBD1-NTRK3	35	32.9	100.0 (35/35)	(90.1, 100.0)
~1x LoD	LMNA-NTRK1	36	13.8	94.4 (34/36)	(81.9, 98.5)
	BCAN-NTRK1	36	16.9	80.6 (29/36)	(65.0, 90.2)
	ETV6-NTRK2	35	15.2	94.3 (33/35)	(81.4, 98.4)
	STRN-NTRK2*	36	13.6	100.0 (36/36)	(90.4, 100.0)
	ETV6-NTRK3	36	24.8	100.0 (36/36)	(90.4, 100.0)
	BTBD1-NTRK3	36	18.1	100.0 (36/36)	(90.4, 100.0)

* Two different fusions (STRN-NTRK2 and TRIM24-NTRK2) were used for the low- and high-level panel members representing NTRK2 fusions due to limited NTRK2 fusion-positive FFPE tissue.

Table 60 PNC of TSO Comprehensive Assay for Detection of NTRK Fusions in High- and Low-Level Targeted Panel Members

Variant Level	Targeted Fusions	N*	PNC (%) (n/N)	95% CI
~2-3 LoD	LMNA-NTRK1	180	100.0 (180/180)	(97.9, 100.0)
	BCAN-NTRK1	251	100.0 (251/251)	(98.5, 100.0)
	ETV6-NTRK2	251	100.0 (251/251)	(98.5, 100.0)
	TRIM24-NTRK2	216	100.0 (216/216)	(98.2, 100.0)
	ETV6-NTRK3	144	100.0 (144/144)	(97.4, 100.0)
	BTBD1-NTRK3	216	100.0 (216/216)	(98.2, 100.0)
~1x LoD	LMNA-NTRK1	213	100.0 (213/213)	(98.2, 100.0)
	BCAN-NTRK1	249	100.0 (249/249)	(98.5, 100.0)
	ETV6-NTRK2	250	100.0 (250/250)	(98.5, 100.0)
	STRN-NTRK2	249	100.0 (249/249)	(98.5, 100.0)
	ETV6-NTRK3	177	100.0 (177/177)	(97.9, 100.0)
	BTBD1-NTRK3	249	100.0 (249/249)	(98.5, 100.0)

* 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.

Table 61 shows the variance components analysis of supporting reads of NTRK fusions 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 61 Variance Components Analysis of Supporting Reads NTRK 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)
~2-3x LoD	LMNA-NTRK1	36	37.9	3.52 (9)	3.37 (9)	6.93 (18)	9.04 (24)	12.39 (33)
	BCAN-NTRK1	36	33.6	13.75 (41)	7.87 (23)	5.40 (16)	8.95 (27)	18.98 (57)
	ETV6-NTRK2	36	24.6	8.03 (33)	3.50 (14)	4.20 (17)	4.86 (20)	10.86 (44)
	TRIM24-NTRK2	36	36.6	11.44 (31)	4.24 (12)	6.82 (19)	6.87 (19)	15.57 (43)
	ETV6-NTRK3	36	56.4	11.49 (20)	10.20 (18)	9.25 (16)	8.69 (15)	19.93 (35)
	BTBD1-NTRK3	35	32.9	1.49 (5)	2.65 (8)	2.16 (7)	10.47 (32)	11.11 (34)

Variant Level	Fusion	N	Mean Supporting Reads	Site SD (%CV)	Operator SD (%CV)	Day SD (%CV)	Replicate SD (%CV)	Total SD (%CV)
~1x LoD	LMNA-NTRK1	36	13.8	1.79 (13)	0.00 (0)	2.74 (20)	4.37 (32)	5.47 (40)
	BCAN-NTRK1	36	16.9	2.92 (17)	2.98 (18)	4.61 (27)	5.82 (34)	8.52 (50)
	ETV6-NTRK2	35	15.2	0.00 (0)	3.41 (22)	3.83 (25)	4.39 (29)	6.75 (45)
	STRN-NTRK2	36	13.6	1.77 (13)	0.61 (5)	2.33 (17)	2.57 (19)	3.95 (29)
	ETV6-NTRK3	36	24.8	6.03 (24)	3.46 (14)	0.00 (0)	6.39 (26)	9.44 (38)
	BTBD1-NTRK3	36	18.1	0.93 (5)	0.00 (0)	0.00 (0)	6.64 (37)	6.71 (37)

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

RET Fusions

For the RET fusion panel members at 2-3x LoD, PPC and PNC values were 100.0% (Table 62 and Table 63). For the low-level RET fusion panel members, PPC values were 97.2% and 97.1%, and PNC values were both 100.0%. Further, none of the individual sites showed any additional discordance.

Table 62 PPC of TSO Comprehensive Assay for Detection of RET Fusions in High- and Low- Level Targeted Panel Members

Variant Level	Targeted Fusion	N	Mean Supporting Reads	PPC (%) (n/N)	95% CI
~1x LoD	NCOA4-RET[2]	36	15.8	97.2 (35/36)	(85.8, 99.5)
	KIF5B-RET[1,3]	34	16.6	97.1 (33/34)	(85.1, 99.5)
~2-3x LoD	NCOA4-RET[2]	36	36.7	100.0 (36/36)	(90.4, 100.0)
	CCDC6-RET[1,2]	36	33.4	100.0 (36/36)	(90.4, 100.0)

[1] Two different fusions (KIF5B-RET and CCDC6-RET) were used for low- and high-level panel members due to sample insufficiency.
[2] Thyroid tissue.
[3] Lung tissue from NSCLC.

Table 63 PNC of TSO Comprehensive Assay for Detection of RET Fusions in High- and Low- Level Targeted Panel Members

Variant Level	Targeted Fusions	N*	PNC (%) (n/N)	95% CI
~1x LoD	NCOA4-RET	213	100.0 (213/213)	(98.2, 100.0)
	KIF5B-RET	251	100.0 (251/251)	(98.5, 100.0)
~2-3x LoD	NCOA4-RET	215	100.0 (215/215)	(98.2, 100.0)
	CCDC6-RET	251	100.0 (251/251)	(98.5, 100.0)

* 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.

Table 64 shows the variance components analysis of supporting reads of RET fusions 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 64 Variance Components Analysis of Supporting Reads in Targeted RET 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)
~1x LoD	NCOA4-RET	36	15.8	2.08 (13)	1.03 (7)	0.00 (0)	5.11 (32)	5.61 (36)
	KIF5B-RET	34	16.6	2.07 (12)	0.00 (0)	1.58 (10)	5.83 (35)	6.39 (39)
~2-3x LoD	NCOA4-RET	36	36.7	4.64 (13)	4.09 (11)	6.17 (17)	5.20 (14)	10.17 (28)
	CCDC6-RET	36	33.4	7.25 (22)	2.56 (8)	6.53 (20)	5.51 (16)	11.49 (34)

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

Study 2 – Tumor Profiling Reproducibility

A second study was performed to assess the reproducibility of the TSO Comprehensive assay across three testing sites (two external and one internal) to assess panel wide reproducibility for tumor profiling. At each site, each operator (2) using a single dedicated instrument tested panel members in duplicate using four unique reagent lots over the course of four non-consecutive testing days, generating eight observations per target per panel member. In total, 48 observations were generated per panel member (three sites x two operators/instruments x four lots x two sequencing runs).

Testing was conducted using extracted DNA and RNA samples from FFPE tissue specimens and one FFPE cell line (with one FFPE tissue specimen and the FFPE cell line used to create two panel members each). Specimens by tumor type are shown in Table 65.

Table 65 Number of Specimens per Tumor Type

Tumor Type	Number of Specimens
Bladder	1
Bone	1
Brain	1
Breast	3
Colon	6
FFPE Cell Line	1
Jejunum	1
Kidney	1
Liver	1
Lung-NOS	2
Lung-NSCLC	4
Ovary	2
Prostate	4
Skin	1
Soft Tissue	3
Stomach	2
Thyroid	1
Unknown	4
Uterus	1

A total of 42 panel members were tested, comprised of DNA panel members with small DNA variants (SNVs, MNVs, insertions, and deletions) and different TMB scores, and RNA panel members that cover the 24 fusion genes and EGFR splice variants in the tumor profiling claims. Variants for which a sample was selected were targeted to a specified level by mixing the nucleic acid from the sample with nucleic acid from normal tissue. Both targeted and non-targeted variants were included in the analysis. Mean observed variant levels were categorized as <2×LoD, ~2–3×LoD, or >3×LoD.

PPC for small DNA variants and RNA variants were calculated by combining observations across sequencing runs and sites. PNCs were similarly calculated for small DNA variants and RNA variants. For each targeted variant, TSO Comprehensive 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 (< 50% of calls were positive) were combined across sites, operators/instruments, days, reagent lots, and sequencing runs to calculate PNC. It should be noted that some levels had fewer than 48 observations; unless otherwise stated, these were due to invalid libraries that did not meet the necessary QC metrics.

Small DNA Variants

Table 66 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. PNCs were 100% across small DNA variants.

Table 66 PPC of TSO Comprehensive 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]	PPC (%) (n/N)	95% CI
< 2x LoD	INSERTION	chr1_27024001_C_CG	ARID1A Q372fs*28	8.4	100.0 (4/4)[3]	(51.0, 100.0)
	INSERTION	chr17_7578470_C_CGGGCGG	TP53 P152_P153dup	15.7	100.0 (2/2)[4]	(34.2, 100.0)
~2-3x LoD	DELETION	chr5_112175751_CT_C	APC L1488fsTer19	18.1	100.0 (28/28)	(87.9, 100.0)
	DELETION	chr5_112175675_AAG_A	APC S1465WfsTer3	16.6	100.0 (40/40)	(91.2, 100.0)
	INSERTION	chr5_112175951_G_GA	APC T1556NfsTer3	22.7	100.0 (32/32)	(89.3,100.0)
	INSERTION	chr5_112175675_A_AAG	APC S1465fs*9	10.0	100.0 (48/48)	(92.6, 100.0)
	SNV	chr7_140453136_A_T	BRAF V600E	4.5	91.3 (42/46)[5]	(79.7, 96.6)
	DELETION	chr7_55242465_GGAATTAAGAGAAGCA_G	EGFR E746_A750del	11.2	100.0 (46/46)	(92.3, 100.0)
	SNV	chr7_55259515_T_G	EGFR L858R	4.5	100.0 (38/38)	(90.8, 100.0)
	DELETION	chr22_41574678_GC_G	EP300 H2324fs*29	24.5	100.0 (44/44)	(92.0, 100.0)
	INSERTION	chr17_37880981_A_AGCATACGTGATG	ERBB2 Y772_A775dup	7.5	100.0 (36/36)	(90.4, 100.0)
	SNV	chr2_209113112_C_T	IDH1 R132H	15.5	100.0 (36/36)	(90.4, 100.0)
	MNV	chr12_25398284_CC_AT	KRAS G12I	11.1	100.0 (38/38)	(90.8, 100.0)

Observed Variant Level[1]	Variant Type	Targeted Variant (nucleotide)	Targeted Variant (amino acid)	Mean VAF (%)[2]	PPC (%) (n/N)	95% CI
	INSERTION	chr9_139399350_C_CG	NOTCH1 R1598fs*12	14.6	100.0 (48/48)	(92.6, 100.0)
	DELETION	chr10_89720798_ GTACT_G	PTEN T319fs*1	15.7	100.0 (44/44)	(92.0, 100.0)
	INSERTION	chr17_7574029_C_ CGGAT	TP53 R333HfsTer5	15.4	100.0 (48/48)	(92.6, 100.0)
> 3x LoD	MNV	chr7_140453136_AC_TT	BRAF V600K	13.0	100.0 (46/46)	(92.3, 100.0)
	INSERTION	chr4_153332910_C_ CAGG	FBXW7 T15_ G16insP	13.0	100.0 (44/44)	(92.0, 100.0)
	DELETION	chr5_112175751_CT_C	APC L1488fs*18	15.0	100.0 (46/46)	(92.3, 100.0)
	DELETION	chr17_7574002_CG_C	TP53 R342fs*3	15.8	100.0 (44/44)	(92.0, 100.0)

[1] Variant level calculated from mean observed variant allele frequency.
[2] Mean variant allele frequency calculated from observed assay results.
[3] The sample with this variant (TPSAD6) had only four valid observations at one site due to numerous failed libraries (n=60) due to sample quality; as such, they failed several DNA QC metrics
[4] The sample with this variant (TPSBD1) had only two valid observations at one site due to numerous failed libraries (n=60) due to sample quality; as such, they failed several DNA QC metrics
[5] BRAF V600E had four discordant observations due to a low VAF in those replicates (> 0.02)

Table 67 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. 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 (N=4 and N=2, respectively).

Table 67 Variance Components Analysis of VAF for Targeted Small DNA Variants

Targeted Variant (nucleotide)	Targeted Variant (amino acid)	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	IDH1 R132H	36	15.5	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	FBXW7 T15_G16insP	44	13.0	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	APC S1465fs*9	48	10.0	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	APC S1465WfsTer3	40	16.6	0.000 (0.0)	0.000 (0.0)	0.024 (14.2)	0.000 (0.0)	0.011 (6.7)	0.026 (15.7)
chr5_112175751_CT_C	APC L1488fsTer19	28	18.1	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	APC L1488fs*SESe18	46	15.5	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	APC T1556NfsTer3	32	22.7	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	EGFR E746_A750del	46	11.2	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	EGFR L858R	38	4.5	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	BRAF V600E	46	4.5	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	BRAF V600K	46	13.0	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	NOTCH1 R1598fs*12	48	14.6	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	PTEN T319fs*1	44	15.7	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	KRAS G12I	38	11.1	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	TP53 R342fs*3	44	15.8	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	TP53 R333HfsTer5	48	15.4	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	ERBB2 Y772_A775dup	36	7.5	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	EP300 H2324fs*29	44	24.5	0.006 (2.4)	0.002 (0.6)	0.019 (7.9)	0.000 (0.0)	0.005 (2.1)	0.021 (8.6)

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

TMB

To evaluate the reproducibility of TMB scores, a quantitative analysis of the score was conducted in 18 targeted TMB panel members, which represented a range of expected TMB scores (0.8–123.1 Mut/Mb). Table 68 shows the variance component analysis of TMB score results for each source of variation and total variation in the TMB panel members. Total SD of TMB score ranged from 0.2 (%CV =29) for panel member TPSAD10 to 1.9 (%CV = 24) for panel member TPSAD3. There were two TMB panel members (TPSAD6 and TPSBD1 – not included in Table 68) for which the number of observations was too small (N = 4 and N=2, respectively) for a variance components model to be fitted. For these panel members, overall SD for TPSAD6 was 0.4 and TPSBD1 was 1.7.

Table 68 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)
TPSAD1	38	2.7	0.0 (0)	0.0 (0)	0.6 (22)	0.1 (4)	0.5 (19)	0.8 (29)
TPSAD2	46	3.5	0.0 (0)	0.9 (25)	1.0 (28)	0.0 (0)	0.8 (24)	1.5 (44)
TPSAD3	36	8.2	1.1 (14)	0.0 (0)	1.1 (13)	0.7 (9)	0.9 (11)	1.9 (24)
TPSAD4	46	1.6	0.0 (0)	0.0 (0)	0.5 (33)	0.0 (0)	0.5 (33)	0.7 (46)
TPSAD5	38	4.8	0.2 (5)	0.0 (0)	0.8 (17)	0.3 (6)	0.7 (14)	1.1 (23)
TPSAD7	32	6.0	0.1 (2)	0.1 (1)	0.4 (6)	0.0 (0)	0.4 (6)	0.6 (9)
TPSAD10	44	0.8	0.0 (0)	0.0 (0)	0.0 (0)	0.1 (6)	0.2 (28)	0.2 (29)
TPSAD11	48	1.5	0.0 (0)	0.3 (18)	0.4 (24)	0.0 (0)	0.6 (38)	0.7 (48)
TPSBD2	46	4.0	0.0 (0)	0.0 (0)	0.6 (14)	0.0 (0)	0.5 (12)	0.7 (18)
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)
TPSBD5	44	5.4	0.0 (0)	0.3 (5)	0.7 (13)	0.0 (0)	0.6 (11)	1.0 (18)
TPSBD6	40	123.1	0.3 (0)	0.0 (0)	1.0 (1)	0.0 (0)	0.9 (1)	1.4 (1)
TPSBD7	46	12.6	0.0 (0)	0.0 (0)	0.7 (5)	0.0 (0)	1.1 (8)	1.3 (10)
TPSBD8	44	5.6	0.0 (0)	0.0 (0)	0.2 (4)	0.0 (0)	0.1 (2)	0.3 (4)
TPSBD12	47	6.3	0.4 (6)	0.0 (0)	1.1 (17)	0.0 (0)	0.6 (10)	1.3 (20)

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

RNA Variants

Table 69 shows PPC for targeted RNA variants. PPC ranged from 91.7% (for KIF5B-RET) to 100% for most RNA variants. PNC was 100% for each targeted RNA variant, except for the FGFR2-SRPK2 fusion (PNC = 99.60% [984/988; 95% CI: 98.96%, 99.84%]).

Table 69 PPC of TSO Comprehensive Assay for Detection of RNA Variants in Targeted Panel Members

Observed Variant Level[1]	Variant Type	Targeted Variant	Mean Supporting Reads[2]	PPC (%) (n/N)	95% CI
< 2x LoD	Fusion	KIF5B-RET	11.6	91.7 (44/48)	(80.4, 96.7)
	Fusion	RAF1-VGLL4	15.9	100.0 (46/46)	(92.3, 100.0)
~2–3x LoD	Fusion	DHX8;ETV4-STAT3	48.9	100.0 (46/46)	(92.3, 100.0)
	Fusion	ESR1-CCDC170	45.1	100.0 (46/46)	(92.3, 100.0)
	Fusion	FGFR1-GSR	61.1	100.0 (46/46)	(92.3, 100.0)
	Fusion	FGFR2-SRPK2	53.4	100.0 (48/48)	(92.6, 100.0)
	Fusion	FGFR3-TACC3	53.5	100.0 (48/48)	(92.6, 100.0)
	Fusion	HNRNPUL1-AXL	58.0	100.0 (48/48)	(92.6, 100.0)
	Fusion	MKRN1-BRAF	33.4	100.0 (48/48)	(92.6, 100.0)
	Fusion	TMPRSS2-ERG	43.5	97.9 (47/48)	(89.1, 99.6)
> 3x LoD	Fusion	ACPP-ETV1	44.7	100.0 (46/46)	(92.3, 100.0)
	Fusion	BCL2-IGHJ5	124.9	100.0 (46/46)	(92.3, 100.0)
	Fusion	EGFR-GALNT13	49.8	100.0 (46/46)	(92.3, 100.0)
	Fusion	EML4-ALK	49.3	100.0 (48/48)	(92.6, 100.0)
	Fusion	PAX3-FOXO1	70.1	100.0 (48/48)	(92.6, 100.0)
	Fusion	SPIDR-NRG1	51.5	100.0 (48/48)	(92.6, 100.0)
	Splice Variant	EGFRvIII	64.0	100.0 (46/46)	(92.3, 100.0)
	Fusion	CDK4-DPY19L2	55.7	97.8 (45/46)	(88.7, 99.6)
	Fusion	EWSR1-FLI1	31.6	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.

Table 70 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 70 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)
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)
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)
EGFRvIII splice variant	46	64.0	12.70 (20)	0.42 (1)	17.69 (28)	0.00 (0)	2.34 (4)	21.90 (34)
NRG1-EGFR	46	9.4	< 0.01 (< 0.1)	2.27 (24)	3.64 (39)	1.92 (20)	2.16 (23)	5.17 (55)
CDK4-DPY19L2	45	55.7	3.14 (6)	0.00 (0)	9.67 (17)	0.00 (0)	11.80 (21)	15.58 (28)
EWSR1-FLI1	48	31.6	7.51 (24)	3.03 (10)	7.40 (23)	0.00 (0)	1.55 (5)	11.08 (35)

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

Study 3 - Controls Reproducibility

Reproducibility of the TruSight Oncology DNA Control and TruSight Oncology RNA Control was evaluated in two separate studies.

For the TSO DNA Control, reproducibility was evaluated at three testing sites, with two operators/instruments using three reagent lots over three non-consecutive days, testing three lots of the DNA controls in duplicate, for a total of 108 observations across all sites. A total of 112 valid observations were assessed due to repeat testing at one of the sites. Controls were evaluated for library validity based on the QC metric used by the TSO Comprehensive assay (23 out of 24 variant calls required to pass). In summary, 98.2% of the DNA control libraries passed (110/112; 95% CI: 93.7%, 99.5%) with only two failing observations at one of the sites.

For the TSO RNA Control, reproducibility was evaluated at three testing sites, with two operators/instruments using four reagent lots over four non-consecutive days, testing three lots of RNA controls in duplicate across two sequencing runs for a total of 96 observations across all sites. Controls were evaluated for library validity based on the QC metric used by the TSO Comprehensive assay (12 out of 13 variant calls required to pass). In summary, 100.0% of the RNA control libraries passed (96/96; 95% CI: 96.2%, 100%), with no failing observations at any of the sites.

Limit of Blank

The percentage of false positives (out of the total expected negatives) were assessed by replicate testing of FFPE normal or benign tumor-adjacent tissue that should not contain somatic variants for small DNA variants, RNA fusions, and RNA splice variants. Six (6) DNA and six (6) RNA FFPE samples were run in duplicate with two operators across three days for each of the two reagent lots. There were 30 additional RNA samples run in duplicate that were processed with one reagent lot, divided between two 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 (shown in Table 71) was calculated at the position level (approximately 1.9 million positions per sample) for small DNA variants and was calculated at the sample level for RNA variants. False positive rate for small DNA variants was 0.0001% (271/295,801,567), and 0% for both RNA fusions and splice variants. False positives were not analyzed for TMB as there is no quantitative cutoff.

Further analysis of the 271 small DNA variant false positives showed that, when they were leveled by the TruSight Oncology Comprehensive assay Knowledge Base it was shown that none of the false positives were clinically significant (Level 2). In addition, there were four false positives in Level 3 arising from two variants across four (2.4%) observations out of the 168 observations. The percentage of false positives for RNA fusions and splice variants was 0% as shown in Table 72.

Table 71 False Positives by DNA Variant Type

Variant Type	False Positives
Small DNA Variants	0.0001% (271/295,801,567)
TMB	N/A*

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

Table 72 False Positives by RNA Variant Type

Variant Type	False Positives
Fusion	0% (0/227)
Splice Variant	0% (0/227)

Limit of Detection

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

Study 1 - CDx Limit of Detection

The Limit of Detection (LoD) of NTRK1–3 and RET fusions were determined. Seven FFPE samples in four different tissue types (brain, soft tissue, breast, and colon) and two FFPE-treated cell lines were tested for NTRK1–3 fusions. Four FFPE samples in three different tissue types (papillary thyroid, skin, and lung) and one FFPE-treated cell line were tested for RET fusions. FFPE cell lines were first used to estimate the LoD values for each fusion gene, then verified with clinical FFPE samples. For verification, there were 18 observations for each test level per lot per variant generated by three operators and three sequencing instruments initiating library preparation on three non-consecutive days with two replicates of each sample per test level, and three reagent lots (54 total observations per variant). The claimed limits of detection for each fusion (Table 73) are the lowest mean supporting reads that reached a hit rate (point estimate) ≥ 95%.

Table 73 Limit of Detection for NTRK and RET Fusions

Gene	Fusion	Tumor Type	Limit of Detection (Supporting Reads)
NTRK1	LMNA-NTRK1	Colon cancer	12.2
	TPM3-NTRK1	Glioblastoma multiforme	20.2
	BCAN-NTRK1	Low-grade glioma	53.2
NTRK2	STRN-NTRK2	Sarcoma	13.6
	ETV6-NTRK2	Myofibroblastic sarcoma	20.3
NTRK3	KANK1-NTRK3	Colon cancer	13.5
	ETV6-NTRK3	Secretory breast cancer	16.2
RET	NCOA4-RET	Papillary thyroid	15.8
	KIF5B-RET	Non-small cell lung cancer	16.6
	CCDC6-RET	Atypical Spitz tumor	18.7

Study 2 - Tumor Profiling Limit of Detection

The Limit of Detection (LoD) of the claimed tumor profiling variants (SNVs, MNVs, insertions, deletions in DNA; fusions and a splice variant in RNA) were evaluated. Twenty-three FFPE samples from 17 tissue types, as well as two cell lines, were used to evaluate small DNA variants from different genomic contexts. Two FFPE samples in two different tissues (lung and unknown) were used to assess RNA splice variants, and 21 samples in 21 different tissue types were used to assess RNA fusions variants. Samples were diluted to multiple test levels, with six observations generated per level by two operators, each using a different reagent lot and instrument.

DNA Variants

The LoDs of 10 small DNA variants types with different genomic context (25 variants in total) were determined and summarized as ranges (Table 74). One of three insertions greater than 5 bp had an LoD of 21.5% VAF, which was different than the LoDs for the other two insertions in the same category (3.4% and 3.6% VAF). This was due to that insertion being in a low complexity region where the insertion adds additional repeats, impacts alignment, and requires more reads for consistent detection.

Table 74 Limit of Detection for Small DNA Variants

Variant Types / Genomic Context	Number of Variants	Range (VAF)
SNVs	5	1.6%–6.4%
MNVs	3	2.2%–4.8%
Insertion (1–2 bp) near homopolymer repeats	2	8.6%–10.4%
Insertion (1–2 bp) near dinucleotide repeats*	2	3.8%–5.1%
Insertion (3–5 bp)	2	3.0%–5.6%
Insertion (> 5 bp and up to 25 bp)	3	3.4%–21.5%
Deletion (1–2 bp) near homopolymer repeats	2	9.4%–10.0%
Deletion (1–2 bp) near dinucleotide repeats	2	3.3%–7.0%
Deletion (3–5 bp)	2	2.8%–6.4%
Deletion (> 5 and up to 25 bp)	2	4.7%–5.5%

* LoD for this specific variant type was determined using cell lines.

Analysis of non-targeted variants was performed from Study 1 samples that had at least five testing levels. Each non-targeted variant was analyzed individually and an LoD estimated only for variants with at least one level > 0% and ≤ 95% hit rate, and at least one level ≥ 95% hit rate. Table 75 shows percentiles along with the minimum and maximum LoDs observed by type for the non-targeted variants. The non-targeted variants provide more variants by type than were tested in Study 2 and are consistent with the LoD ranges from Table 75.

Table 75 Summary Statistics for Limits of Detection by Class of Non-targeted Variants (from Study 1)

Class	N	Min	25%	50%	75%	90%	Max
SNV	862	2.0%	4.7%	5.9%	7.9%	9.7%	59.2%
MNV	5	3.8%	4.0%	5.0%	8.6%	9.5%	9.5%
Insertion	24	3.9%	6.0%	8.4%	9.7%	16.6%	26.1%
Deletion	24	3.4%	6.3%	8.1%	8.9%	12.4%	16.7%

Fusions

LoDs were determined for 18 fusions, accounting for 20 non-CDx genes in the TSO Comprehensive panel, which ranged from 8.3 to 31.3 supporting reads (Table 76). An additional three genes (NTRK1–3) were tested in Study 1. The RET gene was tested both here and in Study 1, and the LoD values shown in Table 76 represent the conservative values obtained from Study 1.

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 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 31.3 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 76 Limit of Detection for Fusions

Fusion	Gene A Breakpoint	Gene B Breakpoint	LoD
EWSR1-FLI1	29683123	128675261	8.3
TMPRSS2-PMFBP1	42866283	72153988	9.0
ACPP-ETV1	132036419	14028762	9.5
CDK4-DPY19L2	58145954	64041141	10.7
MKRN1-BRAF	140158806	140487383	11.0
RAF1-VGLL4	12641189	11606492	11.2
EGFR-GALNT13	55087056	155295102	12.3
EML4-ALK	42553391	29446394	12.8
SPIDR-NRG1	48353103	32453345	12.8
TMPRSS2-ERG	42880007	39817543	13.2
ESR1-CCDC170	152023138	151914240	13.5

Fusion	Gene A Breakpoint	Gene B Breakpoint	LoD
NCOA4-RET	51582937	43612030	15.8
DHX8;ETV4-STAT3	41613847	40474300	16.2
KIF5B-RET	32311775	43612032	16.6
FGFR3-TACC3	1801536	1736997	17.5
PAX3-FOXO1	223084859	41134997	19.0
FGFR1-GSR	38274821	30569602	23.7
FGFR2-SRPK2	123353223	104926165	24.7
HNRNPUL1-AXL	41782201	41743847	26.3
BCL2-IGHJ5	60793496	106330066	31.3

Splice Variants

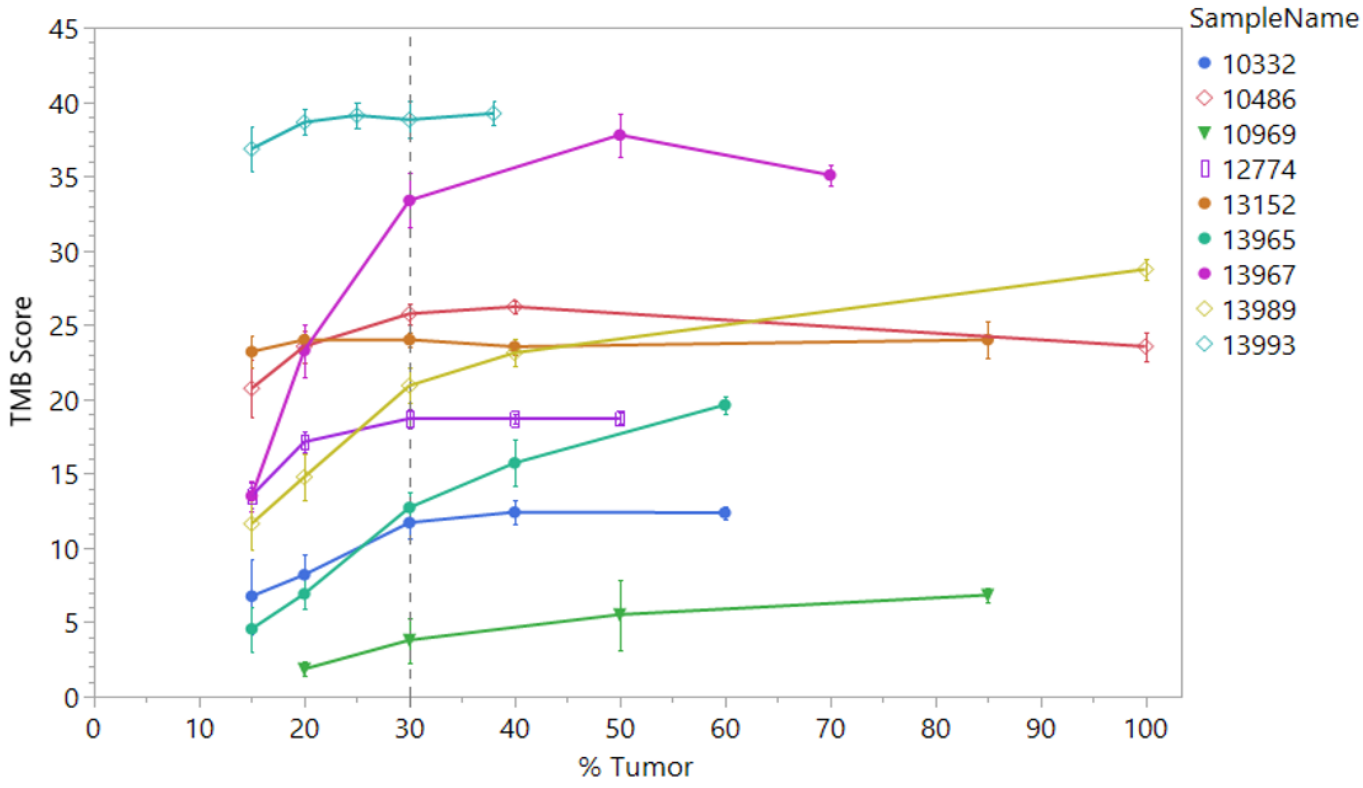
An EGFR splice variant (vIII) had a LoD of 16.7 supporting reads.

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 or supporting reads) for variants in the tumor. The minimum tumor content recommendations are due to the fact that LoD values for small DNA variants are no greater than 10.4% VAF (with the exception of the TP53 insertion). To detect driver mutations in the tumor (50% VAF), 20% tumor content is recommended, so that these mutations would have a 10.0% VAF and be at or above LoD. At 20% tumor content, fusions with 74 supporting reads would be consistently detected based on a Limit of Detection of 14.7 supporting reads.

Nine samples with a range of TMB scores were evaluated for the effect of tumor content on TMB score. These samples included four from bladder tissue, three from endometrial tissue, one from breast tissue, and one from skin. Each sample was tested at five dilution levels, with five replicates, and were each diluted with normal tissue from the same subject to mimic a range of tumor content for each sample. Figure 4 shows the observed correlation between TMB score versus tumor content, with each data point being the mean across replicates (the gray dashed line represents 30% tumor content for reference). In summary, TMB scores show minimal change above 30% tumor content, with all but the sample with the lowest TMB score showing a reduction in TMB score after 30%. However, as TSO Comprehensive does not have a qualitative cutoff for TMB (ie, Low vs. High), the tumor content required to maintain a TMB-high classification (above cutoff) status cannot be determined.

Figure 4 TMB Score vs. Percent (%) Tumor Content



Interfering Substances

The impact of potential endogenous and exogenous substances on the performance of the TSO Comprehensive assay was evaluated with endogenous substances (melanin and hemoglobin) that were spiked into the samples during the nucleic acid extraction process, as well as exogenous substances (ethanol, xylene, Proteinase K, and excess index primers) that were also spiked into the purified nucleic acid before library preparation. For all substances except index primers, four replicates of the sample libraries were generated by one or more operators. For index primers, 12 replicates of the sample libraries were generated by one operator. Replicates of each spiked substance, endogenous control, or exogenous control of the same sample were processed at the same time. For all substances except for index primers, eight FFPE samples from four different tissue types (medullary thyroid, breast, lung, colon) were used for DNA analysis and 13 FFPE samples from nine different tissue types were used for RNA analysis. For index primers, six FFPE samples from three different tissue types (thyroid, bladder, colon) were used for DNA analysis and five FFPE samples from four different tissue types (lung, thyroid, colon, breast) were used for RNA analysis. The effect of necrosis was also assessed on a different set of eight FFPE samples from three different tissue types (brain, colon, and lung) with a wide range of necrotic tissue (3%-70%). There was a macrodissected no necrosis control for each necrosis sample. Four replicates of DNA and RNA libraries and their controls were processed together. For all interferents, sample replicates per substance were compared to their respective control replicates.

Endogenous and Exogenous Substances with DNA

For small DNA variant detection, none of the substances tested [Melanin (0.2 µg/ml), hemoglobin (2 mg/ml), ethanol (5%), Proteinase K (0.04 mg/ml), xylene (0.0001%), and index primers (30% excess)] interfered with the detection of small DNA variants or with TMB score, and VAF levels were unaffected by the presence of any of the tested interferents.

Endogenous and Exogenous Substances with RNA

For RNA variant detection, none of the endogenous substances tested interfered with the detection of RNA fusion variants or splice variants, either quantitatively or qualitatively.

Splice variants were inhibited by excess index primers (30%), which should not be increased from the nominal amount stated in the Instructions for Use.

Proteinase K (0.04 mg/ml in nucleic acid) interfered with RNA fusions. Proteinase K was tested at 2.6 mg/ml and 5.2 mg/ml during the extraction process, which is 2x and 4x the standard concentration (1.3 mg/ml) per reaction used in many extraction kits. Fusions were inhibited at 4x, but not 2x Proteinase K. RNA splice variants were inhibited at 0.04 mg/ml, but not at 2.6 mg/ml or 5.2 mg/ml; however, quantitative analysis showed a decrease in supporting reads for splice variants at 2.6 mg/ml.

Necrosis with DNA and RNA

The presence of necrotic tissue up to 70% did not interfere with TMB and small DNA variants. RNA variants (supporting reads) were reduced in samples with ≥ 25% (by area) necrotic content in the tissue area.

Nucleic Acid Input Titration Guardbanding

TruSight Oncology Comprehensive assay (TSO Comprehensive assay) requires a fixed input of 40 ng of DNA or RNA sample. The performance of the assay was evaluated using six FFPE samples for DNA extracted from five different tissue types, and six FFPE samples for RNA extracted from four different tissue types to establish tolerances for nucleic acid input variation that could lead to assay failure or negatively impact output of the assay. Each FFPE sample was tested at five (5) input levels (20 ng, 30 ng, 40 ng (nominal), 80 ng, and 200 ng) in triplicate to evaluate assay performance. Results demonstrated that small DNA variant detection and TMB score were not affected by difference in input amount.

RNA fusions (35% decrease in supporting reads) and RNA splice variants (43% decrease in supporting reads) were affected by the 20 ng input condition as compared to the nominal 40 ng input condition, which could lead to inconsistent RNA variant detection at 20 ng input. In addition, as input increased from the nominal condition, additional RNA variants were observed. Two major splice variant species, detected in the nominal condition, were consistently detected in the 30 ng, 80 ng, and 200 ng inputs. Additionally, minor species (same gene, different breakpoints, fewer supporting reads) were detected at 80 ng and 200 ng inputs and not in the nominal condition. These minor species were a result of increasing supporting reads with increasing input. The minor species do not change the conclusion for each major splice variant that was detected, which would be reported if there were evidence for clinical significance.

The effect of increasing input amount on the QC metrics used to validate DNA and RNA libraries was evaluated in the same sample set. DNA results demonstrated that median exon coverage (MEDIAN_EXON_COVERAGE) increased with increasing input amount above the nominal input (40 ng) for all samples tested, median insert size (MEDIAN_INSERT_SIZE) increased with increasing input up to 40 ng for some of the samples tested, and percent exon coverage at 50X (PCT_EXON_50X) was not affected by input amounts above 30 ng for all samples tested. RNA results demonstrated that total on target reads (TOTAL_ON_TARGET_READS) showed a positive correlation with increasing input above the nominal amount (40 ng), but MEDIAN_CV_GENE_500X and MEDIAN_INSERT_SIZE were not impacted by increasing input.

Workflow Guardbanding

Guardbanding around nominal conditions was performed for steps throughout the assay workflow (Table 77). Six FFPE samples in five different tissue types for DNA (three samples per workflow step), and six FFPE samples in four different tissue types for RNA were tested to allow for analysis of small DNA variants, TMB, RNA fusions, and RNA splice variants with CDx and Tumor Profiling variants. For DNA small variants, TMB, and RNA fusions, libraries were generated in duplicate per library preparation event by two operators for each workflow testing condition and a nominal condition for a total of four observations per sample, per condition. For splice variants, libraries were generated with two replicates per library preparation event by two operators for each of two workflow testing conditions and a nominal condition per test step (eight observations per sample). Variant calling was evaluated both qualitatively (except for TMB) and quantitatively. Invalid rates were 11.4% (37 out of 324 DNA libraries failed) and 3.0% (28 out of 924 RNA libraries failed).

Table 77 Tested Workflow Steps

Workflow Step	Steps Tested
Synthesize First Strand cDNA (A)*	Pipette mixing step
Clean Up cDNA (B)*	Residual ethanol volumes
Perform End Repair and A-Tailing (C)	Incubation times
Ligate Adapters (D)	Incubation times for operators to add STL then wait to clean up.
Clean Up Ligation (E)	Residual ethanol volumes
Capture Targets (One and Two) (F-H)	• SMB bead mixing time for 1st and 2nd capture (F) • Time to transfer SMB to plate after mixing (G) • EEW pipette mixing (H)
Clean Up Amplified Enriched Library (I-J)	• Residual ethanol volumes (I) • Elution volume transfer to normalization (J)
Normalize Libraries (K)	Mixing time for LNB1

* Steps A and B apply to the RNA Workflow only.

Results show that small DNA variant calling was not affected by deviations relative to the nominal condition in the DNA workflow steps, as all PPC values for test conditions relative to the nominal condition were > 98%. There was also no significant effect on VAF scores relative to the nominal condition for small DNA variants. TMB score was affected by undermixing of reagent EEW relative to the nominal condition (Step H) but unaffected at any of the other DNA workflow steps relative to the nominal condition.

RNA fusion calling was unaffected by deviations in any of the tested steps, as all PPC values for test conditions relative to the nominal condition were > 90% with one exception. Reduced PPC at the Clean Up Ligation (Step E) was due to a variant under the respective LoD. Quantitative analysis showed underlying supporting reads were reduced by residual amounts of ethanol at after the ligation clean up stage (Step H). Underlying supporting reads for RNA fusions were unaffected at any of the other workflow steps relative to the nominal condition.

For RNA splice variants, PPC was low in steps A, C, F, and H – K, which was the result of the FFPE sample used, which contained a splice variant below the respective LoD. Quantitative analysis showed underlying supporting reads for splice variants were reduced by 35% in the presence of 4 µl of residual EtOH after Clean Up Ligation (Step E).

Cross Contamination

The cross-contamination study was conducted to evaluate 1) if false positive results were due to well-to-well contamination during sample library preparation or run-to-run contamination between consecutive sequencing runs, and 2) the sensitivity of the two QC metrics designed to detect contamination in DNA samples (CONTAMINATION_SCORE and P_VALUE). Analysis for 1) was performed both for small DNA variants (including TMB), and RNA variants, and analysis for 2) was performed for just DNA variants.

For 1), libraries were prepared from characterized FFPE samples in a checkerboard layout with alternating samples to evaluate well-to-well contamination, and with alternating indexes to evaluate sequencing run-to-run contamination when sequenced consecutively on the same NextSeq 550Dx instrument. The cross-contamination study showed zero contamination events observed by examining the detected variants in each sample, with no false positives detected. An additional study was also conducted to test cross-contamination with a single donor sample with an NTRK fusion at a high number of supporting reads (> 3000) relative to the distribution observed in the clinical study for NTRK in a donor-receiver layout, with the donor surrounded by RNA positive controls acting as the receiver, as they do not contain the specific NTRK fusion used by the donor. No cross-contamination was observed.

For 2), FFPE tumor DNA samples were mixed with varying amounts of FFPE normal DNA samples to create purposely contaminated samples. Contamination detection using the above-described metrics was evaluated. In total, 1112 contamination observations were generated, and contamination was detected in 95% (1054) of the observations. The detection rate was increased to 96% (939/976) when the percentage of contamination was between 10% to 90% (mass/mass). Of the 37 observations between 10% to 90% contamination where contamination was not detected, 12 did not meet the coverage specification to call small DNA variants and TMB.

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

Nucleic Acid Extraction Kit Evaluation

Three extraction kits for DNA and RNA were evaluated with the TSO Comprehensive assay. The three extraction kits isolated both DNA and RNA from the same FFPE tissue sections but differed in their deparaffinization agent and nucleic acid binding steps (Table 78). Four FFPE samples for DNA from two different tissue types and five FFPE samples for RNA from five different tissue types were used. Samples were subjected to nucleic acid extraction (three kits x two operators x three days x two replicates) to obtain DNA and RNA in the same event (36 total observations). For some FFPE samples with fusion genes, an alternative design was used (samples extracted in triplicate using the three extraction kits and tested in four library prep replicates for a total of 36 total observations). Mean differences between extraction kits were calculated with Kit 1 as the control because Kit 1 was used to extract most of the nucleic acids used for TSO Comprehensive assay analytical studies. The mean difference relative to Kit 1 was reported to illustrate how different extraction kits would affect the other TSO Comprehensive assay analytical studies.

Table 78 Kit Characteristics

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

Table 79 shows the summary of the effects of the extraction kits on library validity: that Kits 2 and 3 had no significant variation in library QC metrics in relation to Kit 1.

Table 79 Extraction Kit Impacts on Library Validity

Variant Type	Library QC Metric	Mean Difference Relative to Kit 1
DNA Small Variants/TMB	Median Exon Coverage (count) PCT Exon 50X (%) Median Insert Size (bp)	Kit 2 lower by 56 reads Kit 3 higher by 0.298% Kit 2 and Kit 3 lower by 3 bp
RNA (Fusions/Splice Variants)	Median Insert Size (bp) Log (Median CV Gene500X) Total on Target Reads	Kit 3 higher by 2 bp Kit 2 higher by 0.029 No significant difference

Qualitative analysis showed that there was no discernable difference in variant calling for any of the variant types, as all variant observations had PPCs > 95%. Table 80 shows the summary of the effects of the extraction kits on variant calling (quantitative analysis). For small DNA variants, the variance between kits was small compared to the residual, and there was no significant difference associated with kits for several levels of VAF analyzed. For TMB, between kit variance was small compared to the residual. For fusions and splice variants, there was a large increase in supporting reads (~51% increase in Kit 2 and 23% increase in Kit 3 for fusions, 48% increase in Kits 2 and 3 for splice variants).

Combined, these data demonstrate that different extraction methods may be used to extract nucleic acids for use with the TSO Comprehensive assay.

Table 80 Extraction Kit Impacts on Variant Calling

Variant Type (units)	Variant Calling (Mean Difference Relative to Kit 1)
Small DNA Variants (VAF)	Not technically significant Targeted variants: between kit variance was small relative to residual Non-targeted variants: No significant differences for the first two VAF bins. No meaningful differences when statistical significance observed.
TMB (mutation per megabase)	Not technically significant, between-kit variance was small relative to residual
Fusions (supporting reads)	Kit 2 had 51% and Kit 3 had 23% increase in supporting reads
Splice Variants (supporting reads)	Kit 2 and Kit 3 had 48% increase in supporting reads

Reagent Stability

Real-Time Stability

Real-time stability of the TSO Comprehensive assay reagents was evaluated using five FFPE samples for DNA from five different tissue types, and four FFPE samples for RNA from three different tissue types. Three (3) reagent lots were tested across six time points (three months, six months, nine months, 13 months, 19 months, and 25 months) with three operators performing library preparation events for each lot, including one replicate of each sample, for a total of three replicates per lot. For targeted small DNA variants, RNA fusions, and RNA splice variants, aggregate PPC was > 95%. In addition, there were no significant differences associated with time for TMB score. However, for RNA fusions, RNA splice variants, and small DNA variants, there was a significant difference associated with time in one of the lots tested; however, the significance did not cause the respective values (VAF and supporting reads) to fall below the allowable drift for each of the respective variants. These data support the stability claim of 24 months for the TSO Comprehensive assay reagents at their appropriate storage temperatures.

Kit In-Use Stability

Kit in-use stability of the TSO Comprehensive assay kit was evaluated under standard use conditions over the course of the shelf life (24 months). The frozen components of the reagent kit were subjected to multiple freeze/thaws and refrigerated reagents were brought up to room temperature to support up to four uses of the kit. Five FFPE samples for DNA from four different tissue types, and six FFPE samples for RNA from five different tissue types were used to evaluate in-use stability. For each of the time points, a kit was used to prepare a total of 24 DNA and 24 RNA libraries with a total of three library preparation events for each sample. For small DNA variants, RNA fusions, and RNA splice variants, PPC was 100%. In addition, there were no significant differences in TMB score, RNA splice variants, or RNA fusions associated with time. However, for

small DNA variants, several VAF bins were determined to have significant differences associated with time; however, these differences were negligible and pose a minimal risk to the assay. These data support up to 4 freeze/thaws of the kit with sufficient reagent volume for 24 DNA and 24 RNA libraries.

Transport Stability

Kit transport stability of the TruSight Oncology Comprehensive assay was evaluated using eight FFPE samples for DNA from five different tissue types, and 10 FFPE samples for RNA from five different tissue types to show functionality of the reagent kits after exposure to a real-world international shipping and distribution condition. Functional testing was performed post-shipping using the above-named samples and consisted of four library preps performed by two operators generating six replicates of each sample. The physical attributes (packaging) of the kits were also observed. Functional testing concluded that shipping did not negatively impact variant calling for small DNA variants (PPC = 100%) and RNA fusions (PPC = 97%), relative to the control condition. RNA splice variants did show a negative impact due to shipping (PPC = 75% in the test condition). In addition, both RNA fusions and RNA splice variants showed a significant difference in supporting reads associated with shipping (decrease of between 20%-24%). Further investigation of RNA fusions and splice variants using a new set of samples showed no significant difference in supporting reads associated with shipping after the retest, and RNA splice variant detection was also not affected (PPC = 100%). TMB also did not show a significant difference in score associated with shipping.

Sample Stability

Nucleic Acid Stability

The stability of nucleic acids (DNA and RNA) was evaluated using eight FFPE samples for DNA from four different tissue types, and nine FFPE samples for RNA from eight different tissue types. Extracted nucleic acid was aliquoted into two sets of single-use tubes to be frozen for two time points: T0 (control) and T1 (to support 28 days). All extracted RNA was stored at -85°C to -65°C, and all extracted DNA was stored at -25°C to -15°C until being tested. Libraries were generated by operators for each of the samples (four independent library preparation events) with two replicates (eight observations per sample per time point). Additional testing for CDx variants (RET and NTRK fusions) was conducted by two operators (two library preparation events) with two replicates per library preparation each for a total of eight observations per time point. Baseline (T0) was compared to T1 to support a stability claim of 28 days. Qualitative analysis showed that both targeted DNA and RNA variants had > 90% PPC, and quantitative analysis showed no significant differences across time points for either DNA or RNA variants. These data indicate that nucleic acids are stable for up to 28 days when stored at the recommended temperatures (RNA at -85°C to -65°C and DNA at -25°C to -15°C).

Library Stability

The stability of libraries using each of the six safe stopping points for the assay (refer to Table 4) was evaluated using eight FFPE samples for DNA from three different tissue types, and six FFPE samples for RNA in four different tissue types (PCF, LP, ALS, ELU2, and PL PCR plates), and five FFPE samples for DNA from four different tissue types, and four FFPE samples for RNA from three different tissue types (NL PCR plate). Libraries

were generated in duplicate by three operators for each of the FFPE samples for each stopping point condition (PCF, LP, ALS, ELU2, and PL PCR plates). Normalized libraries were generated in singlets by three operators. Operators stored the respective plates at -25°C to -15°C at each stopping point to support the claim for the storage time tested (T1). Control libraries (T0, no stopping points) were sequenced immediately at the end of the workflow. T1 was compared to T0 for small DNA variants, TMB, RNA fusions, and RNA splice variants with both CDx and Tumor Profiling variants. Qualitative analysis showed that both targeted DNA and RNA variants had > 90% PPC, and quantitative analysis showed no significant differences across time points for either DNA or RNA variants. These results support stability of 32 days for normalized libraries (NL), 30 days for pre-enrichment (ALS) and post-enrichment (PL PCR) plates, and 7 days for cDNA (PCF), fragmented gDNA (LP), and post-enrichment (ELU2 PCR) plates, all stored at -25°C to -15°C. These results indicate that libraries generated from the TSO Comprehensive assay are stable in accordance with the Instructions for Use.

Slide-Mounted FFPE Tissue Stability

Stability of FFPE slides was evaluated using eight FFPE samples for DNA from three different tissue types and nine FFPE samples for RNA from eight different tissue types. FFPE blocks with targeted variants were sectioned (eight sections / time points, 5 µm each), mounted on slides, randomized into two time points (T0 and T1), and stored in a calibrated incubator set at 22°C. Nucleic acid was extracted from the samples at T0 (one day) and T1 (to support a claim of 28 days) after sections were cut to support a stability claim of 28 days. Libraries were generated by four operators for each of the FFPE samples (four independent library preparation events) with two replicates per sample for a total of eight observations per time point. Additional testing for CDx variants (RET and NTRK fusions) was conducted by two operators (two library preparation events) with two replicates per library preparation each for a total of eight observations per time point. Baseline was compared to T1 for small DNA variants, TMB, RNA fusions, and RNA splice variants with CDx and Tumor Profiling variants for both qualitative and quantitative analysis. Qualitative analysis showed that targeted DNA variants had a 100% PPC in the test condition, with an aggregate PPC of > 99% for all variants, and targeted RNA variants had a PPC of 95% in the test condition. RNA fusions did have a higher-than-normal false positive rate in both the baseline and test condition due to one sample (NTRK1 fusion) having a level of supporting reads below the LoD. Quantitative analysis also showed a statistically significant difference associated with time among small DNA variants at high VAF levels (> 0.1); however, these were deemed negligible due to the sample size analyzed. There was also a significant difference associated with time in RNA fusions and splice variants, which saw a decrease in supporting reads (between 25%-29% reduction) but did not affect variant calling. These data indicate that slide mounted FFPE tissue is stable for up to 28 days when stored at room temperature.

Summary of Analytical Validation for Tumor Profiling Claims

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

- Small DNA variants—SNVs, MNVs, insertions, and deletions
- TMB
- 24 genes for which fusions can be detected (refer to Table 2)
- EGFR splice variants

NTRK Clinical Performance Bridging Study

To validate the TSO Comprehensive assay as a companion diagnostic (CDx) for the selection of subjects for treatment with VITRAKVI (larotrectinib), samples from subjects enrolled in the larotrectinib clinical trials (NCT02122913, NCT02576431, 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 assay Clinical Bridging Study.

NCT02122913 was a multicenter, open-label, Phase 1, dose escalation study in adult subjects 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 subjects with documented NTRK fusion-positive cancer and for subjects 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 subjects 12 years of age 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 subjects aged from birth to 21 years with advanced solid or primary central nervous system (CNS) tumors.

Of the NTRK fusion-positive subjects included in the TSO Comprehensive assay study, the larotrectinib primary analysis set (PAS) comprised of 55 subjects, and the larotrectinib extended primary efficacy set (ePAS4) comprised of 164 subjects.

The Clinical Bridging Study was conducted to assess the clinical effectiveness of the TSO Comprehensive assay in identifying NTRK1, NTRK2, or NTRK3 fusion-positive subjects for treatment with larotrectinib, and to assess the concordance between the TSO Comprehensive assay and the local tests (LTs) (used to determine NTRK fusion status for the larotrectinib clinical trials).

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

As of the data cutoff date 15 JUL 2019, 279 subjects were enrolled. Of the 279 subjects, 208 were NTRK fusion-positive. Of the 208 positive subjects, 55 subjects comprised the larotrectinib primary analysis set (PAS), and 164 subjects comprised the larotrectinib extended primary analysis set (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 three clinical studies. ORR was assessed based on the proportion of subjects with best overall response of confirmed complete response or confirmed partial response based on RECIST, version 1.1 criteria. Based on clinical trial results, ORR was 74.5% (95% CI: 61.0%, 85.3%) in the larotrectinib PAS and 72.6% (95% CI: 65.1%, 79.2%) in the larotrectinib ePAS4 cohort. The results included subjects with 16 different tumor types.

Concordance Results

There were 437 samples tested by TSO Comprehensive. Of those, 55 had invalid TSO Comprehensive results (invalid rate of 12.6%). Agreement of TSO Comprehensive results relative to the LT results among samples with valid results are shown in Table 81.

Table 81 Concordance Between the TSO Comprehensive Assay and LT for Detection of NTRK Fusions

		LT Result		
		NTRK Fusion Positive	**NTRK Fusion Negative**	**Total**
TSO Comprehensive Assay Result	NTRK Fusion Positive	123	9[1]	132
	NTRK Fusion Negative	15	235	250
	Total	138	244	382
Agreement Statistics	PPA% (n/N; 95% CI[2])	89.1% (123/138; 82.7%, 93.8%)		
	NPA% (n/N; 95% CI[2])	96.3% (235/244; 93.1%, 98.3%)		

[1] Five of the nine samples were TSO Comprehensive positive due to an isolated contamination event during study testing.
[2] CI based on Clopper-Pearson (exact) method.

Agreements between the TSO Comprehensive assay and LTs by type (for example, RNA NGS, FISH) are shown in Table 82.

Table 82 Concordance Between the TSO Comprehensive Assay and LT for Detection of NTRK Fusions by LT Type

LT 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)
RNA NGS[2]	PPA	91.5 (75/82)	(83.2, 96.5)
	NPA	96.9 (218/225)[3]	(93.7, 98.7)
FISH	PPA	80.0 (8/10)	(44.4, 97.5)
	NPA	Not calculated (1/1)	Not calculated
PCR	PPA	100.0 (8/8)	(63.1, 100.0)
	NPA	Not calculated (0/0)	Not calculated

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.
[3] Five of the TSO Comprehensive positive results were due to an isolated contamination event during the study.

Of the 382 samples with valid TSO Comprehensive assay and LT results, 24 had discordant results with the LTs: 15 were positive by the LTs and negative by the TSO Comprehensive assay and nine were negative by the LTs and positive by the TSO Comprehensive assay. Of the 24 samples with discordant results, eight were tested with a DNA NGS LT, 14 with an RNA NGS LT, and two with FISH.

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

Testing of discordant results was performed for samples in both the NTRK Accuracy and Bridging studies due to low NPA values in both studies, possibly due to a suspected contamination event. A total of 142 RNA samples from both studies, comprised of 107 concordant samples (53 concordant negative, 54 concordant positive), 26 discordant samples, and nine samples with unknown NTRK status from any of the methods used (TSO Comprehensive, comparator method, and LT), were retested with the TSO Comprehensive assay. Of the 142 samples, 133 produced valid results. Of the five suspected contaminated samples that tested NTRK fusion-positive by TSO Comprehensive assay, but negative by the LTs, in the original Bridging study (Table 81), three of them were available for retesting. All three of those samples were NTRK fusion-negative when re-tested by the TSO Comprehensive assay. Additionally, six out of the remaining 127 samples with valid retest results changed TSO Comprehensive assay status upon retesting. Of those samples, three were concordant NTRK fusion-positives by original testing, changing to negative upon re-test. None of the concordant NTRK fusion-negatives from the original testing changed to positives. In a sensitivity analysis based on the testing of discordant samples, the PPA in the imputed data set was estimated to be 86.5% (95% CI: 80.2%, 92.8%) and

NPA was estimated at 98.8% (95% CI: 97.5%, 100%). Note: 95% CI was calculated according to method described by Gelman, Andrew[1]. The PPA and NPA from the imputed data set are within the confidence limits of the original estimates (Table 81).

Clinical Efficacy Results

Within the efficacy primary analysis set (PAS), the estimated efficacy of larotrectinib in the TSO Comprehensive positive, LT positive population (34 subjects, ORR=85.3%; 95% CI: 68.9%, 95.0%) is higher than in the NDA efficacy population (55 subjects, ORR=74.5%; 95% CI: 61.0%, 85.3%) (Table 83). Of the 34 TSO Comprehensive positive subjects in the PAS, 10 (29.4%) subjects achieved a complete response / surgical complete response, and 19 (55.9%) subjects achieved a partial response.

Of the five TSO Comprehensive negative, LT positive population, one showed a complete response and one showed a partial response with larotrectinib therapy.

The estimated efficacy for TSO Comprehensive positive population (TSO Comprehensive+) is shown in Table 84. At any value of NPA within the observed 95% CI (93%, 98%) and c ≥ 0.5 when ORR (TSO Comprehensive+, LT-) is approximately 0.5 or greater than ORR (TSO Comprehensive+, LT+), the lower limit of the two-sided CI for ORR is exceeding 30%, which was considered clinically significant for the larotrectinib clinical study PAS population.

Table 83 Overall Response Rate (ORR) for LT Positive Subjects by LT and TSO Comprehensive Results in Efficacy Primary Analysis Set (PAS)

		LT Fusion Positive N=55	TSO Comprehensive Positive and LT Positive N=34	TSO Comprehensive Negative and LT Positive N=5
Best overall response, n (%)	Complete response	11 (20.0%)	8 (23.5%)	1 (20.0%)
	Surgical complete response	3 (5.5%)	2 (5.9%)	0
	Partial response	27 (49.1%)	19 (55.9%)	1 (20.0%)
	Stable disease	7 (12.7%)	4 (11.8%)	1 (20.0%)
	Progressive disease	5 (9.1%)	1 (2.9%)	2 (40.0%)
	Not evaluable	2 (3.6%)	0	0
Overall response rate	Number of patients, n	55	34	5
	Number of patients with CR + sCR + PR, n	41	29	2
	ORR% (95% CI*)	74.5% (61.0%, 85.3%)	85.3% (68.9%, 95.0%)	40.0% (5.3%, 85.3%)

* The two-sided 95% confidence interval was calculated using the Clopper-Pearson exact method.

Table 84 Estimated Efficacy in TSO Comprehensive Positive Population.

	Estimated overall response rate and 95% CI[2] in the TSO Comprehensive Positive Population				
NPA (%)	**c[1] = 0**	**c = 0.25**	**c = 0.5**	**c = 0.75**	**c = 1**
99.0%	19% (0%, 38%)	36% (19%, 53%)	52% (39%, 66%)	69% (58%, 80%)	85% (75%, 95%)
98.0%	11% (2%, 19%)	29% (17%, 42%)	48% (37%, 59%)	67% (56%, 77%)	85% (75%, 96%)
97.0%	7% (2%, 12%)	27% (15%, 38%)	46% (35%, 58%)	66% (55%, 77%)	85% (74%, 96%)
96.0%	6% (2%, 9%)	26% (14%, 37%)	45% (34%, 57%)	65% (54%, 77%)	85% (74%, 96%)
95.0%	5% (2%, 7%)	25% (13%, 36%)	45% (34%, 56%)	65% (54%, 77%)	85% (74%, 97%)
94.0%	4% (2%, 6%)	24% (13%, 36%)	45% (33%, 56%)	65% (54%, 77%)	85% (74%, 97%)
93.0%	3% (2%, 5%)	24% (12%, 35%)	44% (33%, 56%)	65% (53%, 76%)	85% (74%, 97%)

ORR in the TSO Comprehensive positive population (CDx+) estimated by sensitivity analysis was based on:
The ORR = 85% in the CDx positive, LT positive population from the primary efficacy analysis.
The ORR in the CDx positive but LT negative population was assumed to be c-times the ORR in the CDx positive, LT positive population, where c varies in the set (0, 0.25, 0.5, 0.75, 1).
The NPA was varied to be inclusive of the confidence interval calculated from the primary concordance analysis.
The PPA = 89% based on the complete case concordance analysis excluding the invalid CDx results.
NTRK fusion frequency = 0.32% based on literature.
[1] c is the ratio of efficacy in TSO Comprehensive positive, LT negative over TSO Comprehensive positive, LT positive patient populations.
[2] The two-sided 95% confidence interval was calculated using the delta method.

Within the ePAS4 cohort, the efficacy of larotrectinib in the TSO Comprehensive positive, LT positive population (97 subjects, ORR=78.4%; 95% CI: 68.8%, 86.1%) was similar to the efficacy of larotrectinib in the total ePAS4 population (164 subjects, ORR=72.6%; 95% CI: 65.1%, 79.2%) (Table 85). Of the 97 TSO Comprehensive positive subjects in ePAS4, 28 (28.9%) subjects achieved a complete response/surgical complete response and 48 (49.5%) subjects achieved a partial response.

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

Table 85 NTRK Clinical Bridging Study: ORR for LT Positive Subjects by LT and TSO Comprehensive Results in ePAS4

		LT Positive N=164	TSO Comprehensive Positive and LT Positive N=97	TSO Comprehensive 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.
54 patients have missing TSO Comprehensive assay results.
* The two-sided 95% confidence interval was calculated using the (exact) Clopper-Pearson method.

RET Clinical Performance Bridging Study

To validate the TSO Comprehensive assay as a companion diagnostic (CDx) for the selection of subjects for treatment with RETEVMO (selpercatinib), RET fusion-positive samples from subjects enrolled in the LIBRETTO-001 clinical trial (protocol no. LOXO-RET-17001, NCT03157128) using a data cutoff of Dec 2019, and supplemental samples from commercial sources were used in a Clinical Bridging Study.

The study was conducted to assess the concordance between the TSO Comprehensive assay and local tests (LTs) (used to determine RET gene fusion status for enrollment in the LIBRETTO-001 clinical trial, protocol no. LOXO-RET-17001; NCT03157128), and to assess the clinical efficacy of the selpercatinib in the RET fusion-positive subjects identified by the TSO Comprehensive assay. RET fusion status of the subjects was determined by testing tissue or blood using various local tests (including NGS, PCR [polymerase chain reaction], or FISH). The primary analysis was performed using 57 samples from the LIBRETTO-001 clinical trial from NSCLC subjects who had received prior therapy and were LT positive and enrolled in the study. A smaller secondary analysis set (n=29, 21 of 29 subjects with clinical outcome data) of LT positive samples from NSCLC subjects who were treatment-naïve and enrolled in the clinical trial were also evaluated. One hundred and twenty-nine LT negative samples were supplemented in the bridging study as there were no screen failed

samples collected in the LIBRETTO-001 clinical trial; of these supplemental samples, 113 samples had valid TSO Comprehensive assay results and were included in the analysis. These supplemental samples were tested with a representative LT assay.

Concordance Results

For the primary analysis group, including the previously treated NSCLC subjects and supplemental negative samples, 186 samples with valid LT results were tested with the TSO Comprehensive assay. Of those, 16 had invalid TSO Comprehensive results (invalid rate of 8.6%). Agreement of TSO Comprehensive results relative to LT results among samples with valid results are shown in Table 86. Of the 57 RET fusion-positive by the LT, 53 were RET fusion-positive by the TSO Comprehensive assay. The PPA was 93.0% (53/57; 95% CI: 83.0%, 98.1%). The remaining 113 of 170 samples were RET fusion-negatives by both the representative LT and the TSO Comprehensive assay. The NPA was 100.0% (113/113; 95% CI: 96.8%, 100.0%).

Table 86 RET Clinical Bridging Study: Concordance Between the TSO Comprehensive Assay and LT for Detection of RET Fusions in Previously Treated NSCLC Patients and Supplemental Negative Samples

		LT Result		
		RET Fusion Positive	**RET Fusion Negative**	**Total**
TSO Comprehensive Assay Result	RET Fusion Positive	53	0	53
	RET Fusion Negative	4	113	117
	Total	57	113	170
Agreement Statistics	PPA% (n/N; 95% CI*)	93.0% (53/57; 83.0%, 98.1%)		
	NPA% (n/N; 95% CI*)	100.0% (113/113; 96.8%, 100.0%)		

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

For the secondary analysis group with the treatment-naïve subjects and supplemental negative samples, 156 samples with valid LT results were tested with TSO Comprehensive assay. Of those, 14 had invalid TSO Comprehensive results (invalid rate of 9.0%). Agreement of TSO Comprehensive results relative to LT results among samples with valid results are shown in Table 87. Of the 29 RET fusion-positive by the LT, 28 were RET fusion-positive by the TSO Comprehensive assay. The PPA was 96.6% (28/29; 95% CI: 82.2%, 99.9%). The remaining 113 of 142 samples were RET fusion-negatives by both the representative LT and the TSO Comprehensive assay. The NPA was 100.0% (113/113; 95% CI: 96.8%, 100.0%).

Table 87 RET Clinical Bridging Study: Concordance Between the TSO Comprehensive Assay and LT for Detection of RET Fusions in the Treatment-Naïve NSCLC Patients and Supplemental Negative Sample

		LT Result		
		RET Fusion Positive	**RET Fusion Negative**	**Total**
TSO Comprehensive Assay Result	RET Fusion Positive	28	0	28
	RET Fusion Negative	1	113	114
	Total	29	113	142
Agreement Statistics	PPA% (n/N; 95% CI*)	96.6% (28/29; 82.2%, 99.9%)		
	NPA% (n/N; 95% CI*)	100.0% (113/113; 96.8%, 100.0%)		

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

For the combined group, which included both previously treated and treatment-naïve subjects' samples as well as supplemental LT negative samples, there were 215 samples with valid LT results tested with TSO Comprehensive assay. Of those, 16 had invalid TSO Comprehensive results (invalid rate of 7.4%). Agreement of the TSO Comprehensive assay relative to the LT among samples with valid results are shown in Table 88. Of the 86 RET fusion-positive by the LT, 81 were RET fusion-positive by the TSO Comprehensive assay. The PPA was 94.2% (81/86; 95% CI: 87.0%, 98.1%). The remaining 113 of 199 samples were RET fusion-negatives by both the representative LT and the TSO Comprehensive assay. The NPA was 100.0% (113/113; 95% CI: 96.8%, 100.0%).

Table 88 RET Clinical Bridging Study: Concordance Between the TSO Comprehensive Assay and LT for Detection of RET Fusions in the Previously Treated, Treatment-Naïve NSCLC Patients and Supplemental Negative Samples

		LT Result		
		RET Fusion Positive	**RET Fusion Negative**	**Total**
TSO Comprehensive Assay Result	RET Fusion Positive	81	0	81
	RET Fusion Negative	5	113	118
	Total	86	113	199
Agreement Statistics	PPA% (n/N; 95% CI*)	94.2% (81/86; 87.0%, 98.1%)		
	NPA% (n/N; 95% CI*)	100.0% (113/113; 96.8%, 100.0%)		

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

Clinical Efficacy Results

Table 89 shows the objective response rate (ORR) for selpercatinib in the RET fusion-positive subject population as detected by the TSO Comprehensive assay. The percentage of subjects with a response in the primary analysis set was 71.7% (38/53; 95% CI: 57.7%, 83.2%). The primary analysis set included subjects who

had previously received at least platinum-based chemotherapy. The ORR in the secondary analysis set, which included the treatment-naïve subjects, was 81.0% (17/21; 95% CI: 58.1%, 94.6%). The ORR in the combined analysis set was 74.3% (55/74; 95% CI: 62.8%, 83.8%), and the combined analysis set included both the previously treated with platinum-based chemotherapy and treatment-naïve subject subpopulations.

Table 89 RET Clinical Bridging Study: Clinical Efficacy in the TSO Comprehensive Assay Positive Subjects

	ORR (%) (n/N)	95% CI[1]
Primary Analysis Set[2]	71.7 (38/53)	(57.7, 83.2)
Secondary Analysis Set[3]	81.0 (17/21)	(58.1, 94.6)
Combined Analysis Set[4]	74.3 (55/74)	(62.8, 83.8)

[1] CI based on Clopper-Pearson (exact) method.
[2] Subjects previously treated with platinum-based chemotherapy.
[3] Treatment-naïve subjects.
[4] Subjects previously treated with platinum-based chemotherapy plus treatment-naïve subjects.

References

1. Gelman, Andrew. *Bayesian Data Analysis, Third Edition*. United Kingdom, Taylor & Francis, 2014.

Revision History

Document	Date	Description of Change
Document # 200061832 v03	May 2026	Added • Adjusted second dilution troubleshooting workflow. • Instruction for SPB overage calculation to the Clean Up Amplified Enrichment Library step. • Additional instruction to the preparation of LNB1. Updated • Troubleshooting instructions. • SPB overage calculation from 1.05x to 1.15x. • Language and format for editorial consistency. Corrected typographical errors: • PhiX to dPhiX in dilute and denature PhiX control. • Confidence interval for ORR within the NTRK clinical bridging study table.
Document # 200061832 v02.1	May 2025	Corrected typographical errors.
Document # 200061832 v02	March 2025	Corrected • Equipment and materials list. • Typographical errors.
Document # 200061832 v01	December 2024	• Updated the time at which NL PCR Plate may be stored from 30 to 32 days. • Added RSB prep in support of Clean Up Ligation. • Clarified samples / tissue types tested in Kit In-Use Stability section.
Document # 200061832 v00	October 2024	Initial release

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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

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