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Evaluating Targeted Next-Generation Sequencing Assays and Reference Materials for *NTRK* Fusion Detection

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Neurotrophic tyrosine receptor kinase (*NTRK1/2/3*) fusions are oncogenic drivers in approximately 0.3% of solid tumors. High-quality testing to identify patients with *NTRK* fusion-positive tumors who could benefit from tropomyosin receptor kinase inhibitors is recommended, but the current *NTRK* testing landscape, including next-generation sequencing (NGS), is fragmented and availability of assays varies widely. The analytical and clinical performance of four commonly available RNA-based NGS assays, Archer's FusionPlex Lung panel (AFL), Illumina's TruSight Oncology 500 (TSO500), Thermo Fisher's Oncomine Precision Assay and Oncomine Focus Assay (OFA), were evaluated. Experiments were conducted using contrived samples [formalin-fixed, paraffin-embedded cell lines and SeraSeq formalin-fixed, paraffin-embedded reference material], *NTRK* fusion-negative clinical samples, and *NTRK* fusion-positive clinical samples, according to local assays. Estimated limit of detection varied across the four assays: 30 to 620 fusion copies for AFL (cell lines), versus approximately 30 to 290 copies for TSO500 and approximately 1 to 28 copies for OFA and Oncomine Precision Assay. All assays showed 100% specificity for *NTRK* fusions detection, but quality control pass rate was variable (AFL, 43%; TSO500, 77%; and OFA, 83%). The *NTRK* fusion detection rate in quality control–validated clinical

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samples was 100% for all assays. This comparison of the strengths and limitations of four RNA-based NGS assays will inform physicians and pathologists regarding optimal assay selection to identify patients with *NTRK* fusion-positive tumors. *(J Mol Diagn 2022, 24: 18—32; https://doi.org/10.1016/j.jmoldx.2021.09.008)*

Fusions of the neurotrophic tyrosine receptor kinase genes (*NTRK1/2/3*) are important oncogenic drivers across a large range of tumor types.[1,2] Although their prevalence is generally low (approximately 0.3%) across all solid tumors, they are found at high frequency (>90%) in rare tumor types, such as mammary analogue secretory carcinoma and secretory breast carcinoma.[2—4] *NTRK* fusions are promising molecular targets for new therapies, and several tropomyosine receptor kinase (TRK) inhibitors have recently been approved, which provide effective targeted treatment options for patients presenting with these genomic rearrangements.

Larotrectinib and entrectinib were the first two TRK inhibitors approved in the United States, Europe, and other countries for the treatment of *NTRK* fusion-positive advanced solid tumors[5]; a companion diagnostic to larotrectinib (FoundationOne CDx; Foundation Medicine, Cambridge, MA) was also subsequently approved (*https://www.fda.gov/drugs/fda-approves-companion-diagnostic-identify-ntrk-fusions-solid-tumors-vitrakvi*, last accessed November 15, 2021). Both entrectinib and larotrectinib demonstrated strong clinical efficacy in patients with *NTRK* fusion-positive tumors enrolled in basket trials (response rates of 64% and 78%, respectively), supporting the relevance of *NTRK* fusions as treatment targets and, therefore, the importance of identifying patients harboring these gene rearrangements.[6,7] Moreover, a third inhibitor, repotrectinib, has shown promising early efficacy[8]; and recently received a fast track designation in *NTRK* fusion—positive, tyrosine kinase inhibitor—pretreated advanced solid tumors from the US Food and Drug Administration (*https://www.targetedonc.com/view/fda-grants-fast-track-to-repotrectinib-in-ntrk-positive-advanced-solid-tumors*, last accessed November 15, 2021); other inhibitors are currently in development.[9]

Thus, it is essential to provide broad global access to high-quality testing to ensure patients with *NTRK* fusion—positive tumors who could benefit from these targeted agents are identified. However, the current testing landscape is fragmented and available assay options vary widely. Multiple technologies, such as immunohistochemistry, RT-PCR, fluorescence *in situ* hybridization, and next-generation sequencing (NGS), are capable of detecting *NTRK* fusions. Among these, NGS is preferred because of its high specificity, high-to-moderate sensitivity, and ability to simultaneously interrogate multiple actionable cancer gene mutations. Although DNA-based NGS can detect gene fusions by also targeting intronic regions, RNA-based NGS allows direct detection of exon-exon junctions indicative of a fusion. Previous studies[10—12] showed that targeted RNA sequencing of formalin-fixed, paraffin-embedded (FFPE) tumor samples was possible, by detecting a range of gene fusions, including *ETV6:NTRK3* and *TPM3:NTRK1*, in a range of solid tumor types. The European Society for Medical Oncology guidelines on *NTRK* fusion detection now recommend the preferential use of RNA-based sequencing for tumor types where *NTRK* fusions are uncommon (eg, colorectal cancer) and suggest that it can be used as a confirmatory technique in tumor types where these fusions are more frequent (eg, mammary analogue secretory carcinoma).[13]

To get a better understanding of the techniques currently available for *NTRK* testing, the analytical performance of four commonly available RNA-based NGS assays was assessed: Archer's FusionPlex Lung panel (AFL; Archer, Boulder, CO; anchored multiplex PCR technology), TruSight Oncology 500 (TSO500; Illumina, San Diego, CA; hybrid-capture technology), Oncomine Precision Assay (OPA; Thermo Fisher, Waltham, MA; amplicon-based enrichment), and Oncomine Focus Assay (OFA; Thermo Fisher; amplicon-based enrichment). As a solution to the rarity of clinical samples, which makes it difficult to develop or validate NGS performance for these alterations, the experiments were performed with contrived *NTRK* fusion-positive samples, FFPE cell lines, and SeraSeq FFPE *NTRK* Fusion RNA reference materials (SeraCare, Milford, MA). Although contrived samples are not a perfect substitution of clinical samples, and do not share all of the same properties (ie, they are subject to less sample-dependent variability), they are reliable tools for the characterization of analytical assay performance within and between assay methods.

Finally, the ability of these assays to detect *NTRK* fusions in clinical tumor samples deemed to be *NTRK* fusion positive by local testing was compared.

Materials and Methods

RNA Quantitation

For all assays, samples were quantitated using Thermo Fisher's Qubit RNA Broad Range (catalog number Q10211) or High Sensitivity Assay Kits (catalog number Q32855) and a Qubit 2.0—4.0 fluorometer (catalog number Q33238).

Fusion Copy Number Determination (ddPCR)

Fusion copy numbers of the FFPE cell lines and reference neat samples were determined per ng of cDNA for *TPM3:NTRK1*, *AFAP1:NTRK2*, and *ETV6:NTRK3* (E4N15) using Thermo Fisher's TaqMan Fusion assays (catalog

number 4351372; assay identifiers Hs03024839_ft, Hs04421278_ft, and Hs04396683_ft, respectively; FAM dye) multiplexed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog number 4448489; assay identifier Hs99999905_m1). The positive control was Universal Human Reference RNA (Agilent, Santa Clara, CA; catalog number 750500) cDNA spiked with 100 copies of the gBlock Gene Fragments [Integrated DNA Technologies, Coralville, IA; custom sequences (Table 1)], and the negative controls were Universal Human Reference RNA cDNA and a no-template control.

Sample inputs ranged from 10 to 20 ng of cDNA (converted from RNA) in duplicates. The samples were run on a Bio-Rad (Hercules, CA) QX200 droplet digital PCR (ddPCR) system (catalog number 1864001). The expected fusion copy numbers of the reference samples were compared with the manufacturer's certificate of analysis (CofA) per lot number. Of note, the SeraCare CofA testing utilized the Agencourt FormaPure Extraction Kit (Beckman Coulter, Brea, CA), which differs from the extraction kits used in this study.

Assays

Archer FusionPlex Lung Panel

The AFL (catalog number AB0121) NGS assay is designed to detect key fusions in seven genes, including *NTRK1/2/3*, skipping events in *EGFR* vIII and *MET* exon 14, and select point mutations in 14 key lung cancer–associated genes. The AFL uses Archer's Anchor Multiplex PCR chemistry to target regions of interest. Because of the use of one gene-specific and one universal primer, both known and unknown gene fusion partners can be detected.

The sample input for our analyses (recommended input: 20 to 250 ng) was 200 ng of RNA isolated using the Promega ReliaPrep FFPE Total RNA Miniprep system (Promega, Madison, WI; catalog number Z1002) as per manufacturer's instructions. Reagent preparation and DNA synthesis, ligation, and amplification were performed according to the official assay protocol. Libraries were multiplexed for sequencing on an Illumina MiSeq (catalog number SY-410-1003) to target a minimum of 500,000 reads per sample. Data were analyzed with the Archer Analysis software version 5.1_RC_11. Samples had to pass the following quality control (QC) criteria: i) presequencing RNA Ct $\leq$28; ii) unique RNA start sites (control) $\geq$10; and iii) library quantity $>$2 nmol/L.

Illumina's TruSight Oncology 500

The TSO500 assay from Illumina (catalog number 20028216) can detect copy number variants, gene fusions, insertions/deletions, single-nucleotide variants, and transcript variants from the RNA of 55 genes and the DNA of 523 genes found across a large range of cancers, including fusions of the *NTRK1/2/3* genes. Only the RNA component of the assay was processed: sample input for this assay was 40 ng RNA, as per manufacturer's instructions, isolated using the AllPrep DNA/RNA FFPE Mini Kit from Qiagen (Hilden, Germany; catalog number 80234). Library was prepared and enriched as recommended by the assay protocol. Libraries were multiplexed for sequencing on an Illumina NextSeq (catalog number SY-415-1001) with up to 16 RNA libraries per flowcell. Data were analyzed using the TSO500 Local App software version 2.0.1.4 (Illumina). Samples had to pass the following QC criteria: i) median insert size $\geq$80; ii) median CV value of target region coverage across all genes with coverage above 500$\times$ $\leq$93; and iii) total on-target reads $\geq$9,000,000.

Thermo Fisher's Oncomine Focus Assay

The OFA (Thermo Fisher; catalog number A42008) is a targeted multibiomarker NGS assay that enables the simultaneous detection of multiple variants across 52 tumor-related genes from DNA or RNA samples. In particular, the OFA can detect fusions in 23 genes, including *NTRK1/2/3*, using amplicon-based enrichment from RNA (Thermo Fisher white paper: An Approach for Establishing Oncomine Focus Assay Performance. Waltham, MA). Because of the use of specific primers binding to both *NTRK* and its partner gene, only known gene fusions can be detected.

Only the RNA portion of the assay was sequenced for OFA in this study. As recommended by the protocol, the sample input for this assay was 10 ng RNA isolated with Thermo Fisher's RecoverAll Total Nucleic Acid (catalog number AM1975) Isolation Kit, according to the manufacturer's instructions. Library preparation, comprising reverse transcription, amplification, and ligation steps, was performed in line with the OFA protocol. Libraries were multiplexed for sequencing on an Ion S5 system (Thermo Fisher; catalog number A27212) with up to 22 sample pairs per 540 chip. Data were analyzed with the Oncomine Focus RNA Fusions panel version 1.4 from the Ion Reporter software version 5.10 (Thermo Fisher), using the Oncomine Focus w2.4 – DNA and Fusions – Single Sample workflow. Samples had to pass the following QC criteria: mapped fusion panel reads $\geq$20,000; and fusion sample overall call: negative/positive (ie, no fusion detected and fusion imbalance score negative).

Thermo Fisher's Oncomine Precision Assay Genexus

The OPA (catalog number A46291) is an NGS assay designed to analyze mutations, copy number variations, and fusion variants across 50 key genes; in particular, 18 fusion variants, including *NTRK1/2/3* fusions, can be detected from RNA samples. Contrary to the OFA, the OPA is fully automated, has a rapid turnaround, and incorporates a 5′/3′ expression imbalance caller for the detection of novel fusions, including *NTRK1/2/3*. The assay also differs from OFA in that it utilizes molecular barcodes to increase sequencing resolution and is designed for use on the Genexus automated sequencer (Thermo Fisher; catalog number A45727).

Table 1 gBlock Gene Fragments

TPM3:NTRK1
5′-AAGATAAATATGAGGAAGAAATCAAGATTCTTACTGATAAACTCAAGGAGGCAGAGACCCGTGCTGAGTTTGCTGAGAGATCGGTAG
CCAAGCTGGAAAAGACAATTGATGACCTGGAAGACACTAACAGCACATCTGGAGACCCGGTGGAGAAGAAGGACGAAACACCTT
TTGGGGTCTCGGTGGCTGTGGGCCTGGCCGTCTTTGCCTGCCTCTTCCTTTCTACGCTGCTCCTTGTGCTC-3′
AFAP1:NTRK2
5′-CGCAGCTCAAGGGTAAAAAGCCCCCCGTGGCGTCTAATGGGGTCACAGGAAAAGGGAAGACTCTGAGCAGTCAGCCAAAGAAA
GCGGATCCCGCGGCTGTTGTGAAAAGGACGGGTTCGAGTGCAAACCCAAATTATCCTGATGTAATTTATGAAGATTATGGAACTGCA
GCGAATGACATCGGGGACACCACGAACAGAAGTAATGAAATCCCTTCCACAGACGTCACTGATAAAACCGG-3′
ETV6:NTRK3 (E4N15)
5′-TCTGAAGCAGAGGAAACCTCGGATTCTTTTTTCACCATTCTTCCACCCTGGAAACTCTATACACACACAGCCGGAGGTCATACTGCA
TCAGAACCATGAAGAAGATGTGCAGCACATTAAGAGGAGAGACATCGTGCTGA-3′

Nucleic acid was extracted using either the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher; catalog number AM1975) for cell lines or SeraSeq reference material or with the MagMAX FFPE DNA/RNA Ultra Kit (Thermo Fisher; catalog number A31881) for clinical samples, as per manufacturer's instructions, and run on the KingFisher Duo primer automated extraction system (Thermo Fisher Scientific; catalog number 5400110). The OPA GX (Thermo Fisher Scientific; catalog number A46291) was used for library preparation, as per the official protocol, using a total RNA input volume of 20 μL per sample (minimum, 0.67 ng/μL sample concentration). Of note, an RNA input of 5 ng was utilized for the SeraSeq reference sample during the sensitivity experiment, which is below the manufacturer's recommendation of 10 ng; nonetheless, the assay has been validated for use in this range. The analysis was performed using the Genexus software version 6.2 (Thermo Fisher). The Torrent Browser from the Torrent Suite software version 6.2.1 (Thermo Fisher Scientific) was used to perform initial QC, including chip loading density, median read length, and number of mapped reads. Samples had to pass the following QC criteria: detection of five inset RNA controls, including *ANKRD17*, *E1F2B1*, *ALAS1*, *G6PD*, *TBP*, *HMBS*, and *TRIM27*. The threshold for positive detection was defined at ≥25 RNA reads, originating from at least two unique PCR products, as determined by molecular barcoding.

Analytical Performance

Cell Lines and Reference Materials (Fusion Copy Number Determination, Pilot Study, and Sensitivity/Precision Analysis)

FFPE human cell lines KM-12 (originating from colorectal cancer and obtained under material transfer agreement from National Cancer Institute Division of Cancer Treatment and Diagnosis' repository; *https://dctd.cancer.gov*, last accessed November 15, 2021) and IMS-M2 [originating from acute myeloid leukemia and initially provided by Dr. Brunangelo Falini (University of Perugia, Perugia, Italy)] were used for the detection of *NTRK1* and *NTRK3* gene fusions, respectively (Table 2). Because of the rarity of *NTRK2* fusions, an FFPE mouse cell line (Ba/F3-AFAP1-NTRK2, an IL-3–dependent murine pro-B cell line) engineered by Biosettia, Inc. (San Diego, CA) was used for this marker. Cells were formalin fixed and paraffin embedded before processing with the NGS assays to mimic clinical sample quality and sample processing workflows; cell line pellets were formalin fixed and paraffin embedded separately. FFPE sections from each cell line were then processed through the different extraction kits. The RNA obtained from each extraction was quantitated via the Qubit system, and the fusion copy number was determined for all *NTRK* fusion–positive cell line samples. In cases where RNA from multiple extractions of the same cell line was combined to obtain enough material for serial dilutions, the quantitation and ddPCR were performed on the pooled RNA sample. To dilute fusion copies to the desired quantity, RNA from *NTRK* fusion-positive cell lines was subsequently mixed with RNA from a cell line that does not express any *NTRK* gene fusion (ML-2, originating from acute myeloid leukemia and available from the Leibniz Institute DSMZ GmbH, Germany).

For the reference materials, three lots of SeraCare's (Milford, MA) SeraSeq FFPE *NTRK* Fusion RNA Reference Material (SeraSeq *NTRK*; material number 0710-1031; lot 1: 10373554, lot 2: 10468254, and lot 3: 10485318) were used. The SeraCare *NTRK* standard includes a range of *NTRK1/2/3* fusions, as described in Table 2. SeraCare's Seraseq FFPE WT (DNA/RNA) Reference Material (material number 0710-0137), which does not include any *NTRK* fusion, was used to prepare serial dilutions of gene fusions for sensitivity and precision studies. Of note, the Horizon Discovery (Cambridge, UK) 5-Fusion Multiplex positive control (catalog number HD796), another reference material for *NTRK* fusions, has been unavailable since mid-2019, so could not be used for testing in this study.

ddPCR and Pilot Study

The four FFPE cell lines (three *NTRK* fusion positive, one *NTRK* fusion negative) and reference samples, including the *NTRK* fusion-negative control, were first processed neat in duplicates to determine the ability of each of the four assays to detect the different *NTRK* fusions. Each assay used RNA

Table 2 Contrived Samples Used for the Pilot, Specificity, Sensitivity, and Precision Analysis

Sample type/name	Fusion	Provenance	Experiment
Cell lines			
KM-12	*TPM3:NTRK1*	CRC tissue (NCI-DCTD repository)	ddPCR Pilot Sensitivity/precision Sensitivity/precision per fusion copy number
IMS-M2	*ETV6:NTRK3*	AML tissue (University of Perugia)	ddPCR Pilot Sensitivity/precision Sensitivity/precision per fusion copy number
BaF3 AFAP1-NTRK2	*AFAP1:NTRK2*	Glioma tissue (Biosettia, Inc.)	ddPCR Pilot Sensitivity/precision Sensitivity/precision per fusion copy number
ML-2	*NTRK* fusion negative	AML tissue (Leibniz DSMZ GmbH)	Pilot (negative control) Sensitivity/precision (dilution of *NTRK* fusion-positive cell lines)
SeraSeq FFPE RNA reference material			
SeraSeq FFPE *NTRK* fusion-positive reference material	*IRF2BP2:NTRK1* *LMNA:NTRK1* *SQSTM1:NTRK1* *TFG:NTRK1* *TPM3:NTRK1* *AFAP1:NTRK2* *NACC2:NTRK2* *PAN3:NTRK2* *QKI:NTRK2* *TRIM24:NTRK2* *BTBD1:NTRK3* *ETV6:NTRK3* (E4N14) *ETV6:NTRK3* (E4N15) *ETV6:NTRK3* (E5N14) *ETV6:NTRK3* (E5N15)	Commercial product	Pilot (all) Sensitivity/precision (all) ddPCR and sensitivity/precision per fusion copy number [*TPM3:NTRK1*; *AFAP1:NTRK2*; *ETV6:NTRK3* (E4N15)]
SeraSeq FFPE WT (DNA/RNA) reference material	*NTRK* fusion negative	Commercial product	Pilot (negative control) Sensitivity/precision (dilution of SeraSeq *NTRK* reference materials)

Exon numbers correspond to ENST00000394480.6 for *NTRK3*.

AML, acute myeloid leukemia; CRC, colorectal cancer; ddPCR, droplet digital PCR; FFPE, formalin fixed, paraffin embedded; NCI-DCTD, National Cancer Institute - Division of Cancer Treatment and Diagnosis; *NTRK*, neurotrophic tyrosine receptor kinase; WT, wild type.

extracted with its respective, recommended extraction kit, with the exception of the OPA that used RNA extracted with Thermo Fisher's RecoverAll Total Nucleic Acid Isolation Kit.

In addition, fusion copy number was determined for all *NTRK* fusion-positive samples (cell lines and reference sample) by ddPCR. For the SeraSeq *NTRK* reference materials, *TPM3:NTRK1*, *AFAP1:NTRK2*, and *ETV6:NTRK3* (E4N15) fusions were selected to align with those expressed by the three *NTRK* fusion-positive cell lines. For AFL and OPA, two independent extractions with subsequent ddPCR were performed. Fusion copy numbers of these neat samples were subsequently determined per ng of RNA for select fusion genes.

Sensitivity/Precision

To determine sensitivity, five serial dilutions of *NTRK* fusion-positive samples in corresponding *NTRK* fusion-negative samples were performed. Cell line samples were diluted 1:4 after first diluting to $\leq$25 copies/ng RNA based on ddPCR quantitation for the cell lines harboring an *NTRK2* or *NTRK3* fusion; SeraSeq *NTRK* standards were diluted 1:5 from neat. Each serial dilution level was run in triplicates, resulting in a total of 15 runs. After obtaining the estimated limit of detection for each sample (defined as the lowest sensitivity level/dilution at which all three replicates were positive), two additional replicates from this dilution were run on different days (and by different operators for AFL, OFA, and TSO500) to determine the precision of the four assays. Sensitivity experiments with additional replicates for OPA were only performed on the SeraSeq reference materials (Fusion Caller) as there were not enough left-over cell line samples for this assay.

Table 3 Fusion Copy Number Determined by the AFL, TSO500, OPA, and OFA Assays (ddPCR) in *NTRK* Fusion–Positive Cell Lines and Corresponding Reference Materials

Sample ID	Fusion	Expected fusion copies/ng (SeraCare CofA*)	Actual fusion copies/ng RNA (ddPCR)					
			ReliaPrep 1 (AFL)	ReliaPrep 2 (AFL)	AllPrep (TSO500)	MagMAX 1 (OPA)	MagMAX 2 (OPA)	RecoverAll (OFA/OPA)
KM-12	*TPM3:NTRK1*	N/A	16	—	7	2	—	15
BaF3-AFAP1-NTRK2	*AFAP1:NTRK2*	N/A	5160	—	1276	1380	—	3785
IMS-M2	*ETV6:NTRK3* (E4N15)	N/A	1264	—	247	447	—	897
SeraSeq *NTRK*	*TPM3:NTRK1*	473	—	—	19	—	—	99
lot 1	*AFAP1:NTRK2*	623	—	—	32	—	—	155
	ETV6:NTRK3 (E4N15)	476	—	—	7	—	—	36
SeraSeq *NTRK*	*TPM3:NTRK1*	151	7	—	—	—	—	—
lot 2	*AFAP1:NTRK2*	263	4	—	—	—	—	—
	ETV6:NTRK3 (E4N15)	222	1.2	—	—	—	—	—
SeraSeq *NTRK*	*TPM3:NTRK1*	502	83	25	—	31	34	71
lot 3	*AFAP1:NTRK2*	571	112	57	—	44	48	131
	ETV6:NTRK3 (E4N15)	475	28	16	—	15	16	38

*SeraCare uses Agencourt Formapure Extraction kit for quality control, as reported on the reference material lot CofA.

—, No data are available for this condition; AFL, Archer's FusionPlex Lung panel; CofA, certificate of analysis; ddPCR, droplet digital PCR; ID, identifier; N/A, not applicable; *NTRK*, neurotrophic tyrosine receptor kinase; OFA, Oncomine Focus Assay; OPA, Oncomine Precision Assay; TSO500, TruSight Oncology 500.

Specificity

The 30 FFPE *NTRK* fusion-negative tumor samples (confirmed as *NTRK* fusion negative by an NGS laboratory-developed test) tested in the specificity analyses for AFL, TSO500, and OFA were purchased from Indivumed GmbH (Hamburg, Germany) and Discovery Life Sciences, Inc. (formerly Conversant Bio, Los Osos, CA) and originally derived from a large range of solid tumors: brain ($n = 2$), breast ($n = 3$), colon ($n = 5$), endometrium ($n = 1$), head ($n = 1$), liver ($n = 1$), lung ($n = 5$), ovary ($n = 1$), prostate ($n = 1$), salivary gland ($n = 3$), soft tissue ($n = 5$), and thyroid ($n = 2$). The specificities of AFL, TSO500, and OFA (not OPA) were evaluated by processing each of the 30 *NTRK* fusion-negative samples once on each of the assays and evaluating the rate of false-positive results. Each assay had

Table 4 Results of the Pilot Study Evaluating the Ability of the AFL, TSO500, OPA, and OFA Assays to Detect *NTRK* Fusions in Cell Line Samples and Reference Materials

Sample ID	Sample type	Expected fusion	AFL	TSO500	OPA	OFA
KM-12	Cell line	*TPM3:NTRK1*	Detected	Detected	Detected	Detected
BaF3-AFAP1-NTRK2	Cell line	*AFAP1:NTRK2*	Detected	Detected	Detected	Detected
IMS-M2	Cell line	*ETV6:NTRK3*	Detected	Detected	Detected	Detected
ML-2	Cell line	None	*No NTRK fusion detected*			
SeraSeq *NTRK*	Reference material	*IRF2BP2:NTRK1*	Detected	Detected	Detected	Detected
		LMNA:NTRK1	Detected	Detected	Detected	*Not detected (missing from panel)*
		SQSTM1:NTRK1	Detected	Detected	Detected	Detected
		TFG:NTRK1	Detected	Detected	Detected	*Not detected (missing from panel)*
		TPM3:NTRK1	Detected	Detected	Detected	Detected
		AFAP1:NTRK2	Detected	Detected	Detected	Detected
		NACC2:NTRK2	Detected	Detected	Detected	Detected
		PAN3:NTRK2	Detected	Detected	Detected	*Not detected (missing from panel)*
		QKI:NTRK2	Detected	Detected	Detected	Detected
		TRIM24:NTRK2	Detected	Detected	Detected	Detected
		BTBD1:NTRK3	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E4N14)	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E4N15)	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E5N14)	Detected	*Not reported*	Detected	Detected
		ETV6:NTRK3 (E5N15)	Detected	*Not reported*	Detected	Detected
SeraSeq WT	Reference material	None	*No NTRK fusion detected*			

Cell line and reference samples were processed neat in duplicates. The *LMNA:NTRK1*, *TFG:NTRK1*, and *PAN3:NTRK2* fusions were confirmed as missing from the OFA panel. All *ETV6:NTRK3* breakpoints not reported by TSO500 were present, but the software was unable to distinguish all of them.

AFL, Archer's FusionPlex Lung panel; ID, identifier; *NTRK*, neurotrophic tyrosine receptor kinase; OFA, Oncomine Focus Assay; OPA, Oncomine Precision Assay; TSO500, TruSight Oncology 500; WT, wild type.

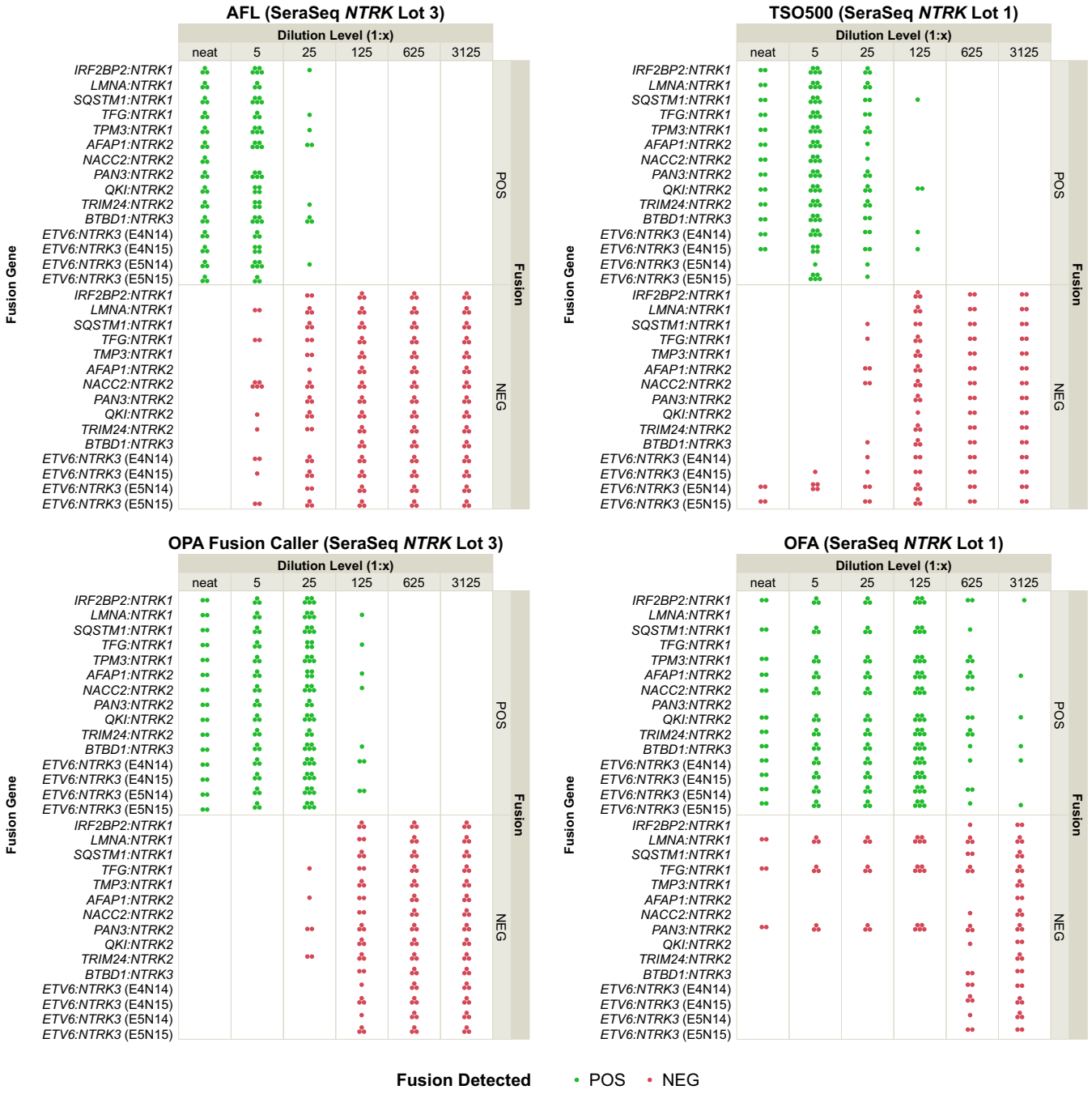

Figure 1 Sensitivity/precision of Archer's FusionPlex Lung panel (AFL), TruSight Oncology 500 (TSO500), Oncomine Precision Assay (OPA), and Oncomine Focus Assay (OFA) for the SeraSeq neurotrophic tyrosine receptor kinase (*NTRK*) reference materials at a range of dilutions. For each assay, and across the six different dilution levels, dots representing individual replicates are displayed on the basis of whether the assay was able (green; **top of panels**) or unable (red; **bottom of panels**) to detect the SeraSeq *NTRK* reference material (two lots). For precision experiments, two additional replicates were processed for the lowest sensitivity level, where most expected fusions in all three replicates were positive (POS; processed on minimum three different runs). For the OPA, 5 ng RNA input instead of 10 ng was used for two of three replicates. Note: The OPA expression imbalance caller failed to detect any of the *NTRK* fusions in this sample type. NEG, negative.

different criteria for failing the quality check, as described above.

Clinical Sample Performance

Fourteen additional clinical samples (provided by Samsung Medical Center, Seoul, Republic of Korea; Les Hospices Civils de Lyon, Lyon, France; Nova Scotia Health Authority, Halifax, NS, Canada; and William Osler Health System, Brampton, ON, Canada), identified as *NTRK* fusion positive by local assays, were analyzed during the clinical sample comparison study. These samples originated from several different tumor types [parotid gland tumor ($n = 3$), mammary analogue secretory carcinoma ($n = 2$), and

lipofibromatosis-like neural tumor, anaplastic glioma, parapharyngeal region tumor, papillary thyroid carcinoma and colon, cecal, rectal, sigmoid colon, and splenic flexure cancers (all $n = 1$)]. Samples were obtained according to best practice at each of the contributing institutions and subject to material transfer agreement with the central site.

The 14 clinical *NTRK* fusion–positive samples were processed once on each of the four assays using their respective recommended extraction kit, with the exception of one sample failing extraction for OPA because of human error, where remaining RNA extracted with Thermo Fisher's RecoverAll Total Nucleic Acid Isolation Kit was used instead. Quality checks of the results, specific to each assay, were performed, as described in *Materials and Methods*. The *NTRK* fusions detected by the four NGS assays were then compared with the fusions previously identified by local testing.

Results

Fusion Copy Number Determination (ddPCR)

Besides giving different RNA yields (data not shown), the different extraction methods used in this study also yielded variable fusion copies/ng RNA (Table 3). The ReliaPrep (AFL) and RecoverAll (OFA/OPA) extraction kits yielded similar results, whereas the AllPrep (TSO500) and MagMAX (OPA) kits yielded about one-third of the fusion copies/ng RNA achieved with the other methods. Results for ReliaPrep (AFL) showed variability between the two different extraction events or between SeraSeq curls within the same lot, whereas MagMax-extracted RNA (OPA) showed consistent fusion copy number recovery.

In addition, the SeraSeq *NTRK* reference samples yielded variable results across lots. Lot 2 had a low number of fusion copies/ng RNA versus lot 1 and not all expected fusions were detected by AFL; after providing this feedback to SeraCare, a new lot 3 was released shortly after, which performed similarly to lot 1. All extraction methods and assays yielded substantially fewer fusion copies/ng RNA than expected based on the SeraSeq CofA's control assay.

Pilot Study

The assays were able to detect the majority of expected *NTRK* gene fusions (Table 4). Although all *NTRK* fusions from cell line samples were detected by the four assays, only the AFL assay and OPA detected 100% of *NTRK* fusions in the reference materials: the TSO500 assay and OFA missed two and three SeraSeq *NTRK* fusions in these samples, respectively. The two gene fusions not reported by the TSO500 assay were *ETV6:NTRK3* fusions (E5N14 and E5N15). After further analysis of the supporting reads generated through the assay, all four *ETV6:NTRK3* breakpoints were present in the underlying raw data, but the TSO500 software was unable to distinguish all of them from the heterogeneous mix, thus not reporting all different breakpoints even though all had supporting reads. The OFA failed to detect the *LMNA:NTRK1*, *TFG:NTRK1*, and *PAN3:NTRK2* fusions; these three fusions are not included in the assay panel.

Sensitivity/Precision

The sensitivity and precision of the four assays were evaluated at a range of dilutions (Figure 1 and Supplemental Table S1) and according to fusion copy number (Figure 2 and Supplemental Table S2). The AFL and TSO500 assays detected most *NTRK* fusions up to a dilution level of 1:5 of the SeraSeq reference material, whereas OPA and OFA could still detect most *NTRK* fusions at dilution levels of 1:25 and 1:125, respectively. When normalized by fusion copy number, the AFL assay had an estimated limit of detection of 30 to 620 total fusion copies for cell lines and 710 to 5200 total fusion copies for the SeraSeq *NTRK* standards (200 ng RNA), versus 30 to 290 total fusion copies for TSO500 (40 ng RNA). OFA and OPA fusion caller showed a low limit of detection of approximately 1 to 28 total fusion copies and used little sample input (10 ng RNA).

After running two additional replicates at the estimated limit of detection, the precision for the TSO500, OPA, and OFA assays was deemed reproducible, with four/five or five/five detected/expected fusions across sample types. AFL precision was slightly below the limit of detection for SeraSeq *NTRK* samples; accordingly, reproducibility was lower, at three/five to five/five detected/expected fusions.

Specificity

All of the assays showed high specificity, with no *NTRK* fusions detected in any of the *NTRK* fusion-negative clinical samples. However, sample pass metrics for the three assays tested for specificity were variable: TSO500 and OFA had a high success rate (77% and 83%, respectively), whereas only 43% of samples on AFL passed all assay quality check metrics (Table 5). The original processing of samples on AFL using the recommended ReliaPrep extraction kit resulted in only eight samples (27%) passing QC criteria. After reprocessing 15 samples on AFL using RNA extracted with the AllPrep extraction kit, five additional samples passed QC metrics. The main reasons behind sample fails were presequencing Ct >28 (AFL), fusion reads $<20{,}000$ (OFA), and median insert size <80 bp (TSO500). It is unclear if the extraction kit lot used for the processing of AFL clinical samples was the reason for higher sample failure.

Although specificity assays were not run with OPA, internal validation experiments showed no false-positive *NTRK* fusions in a set of >30 pan-tumor samples (data not shown).

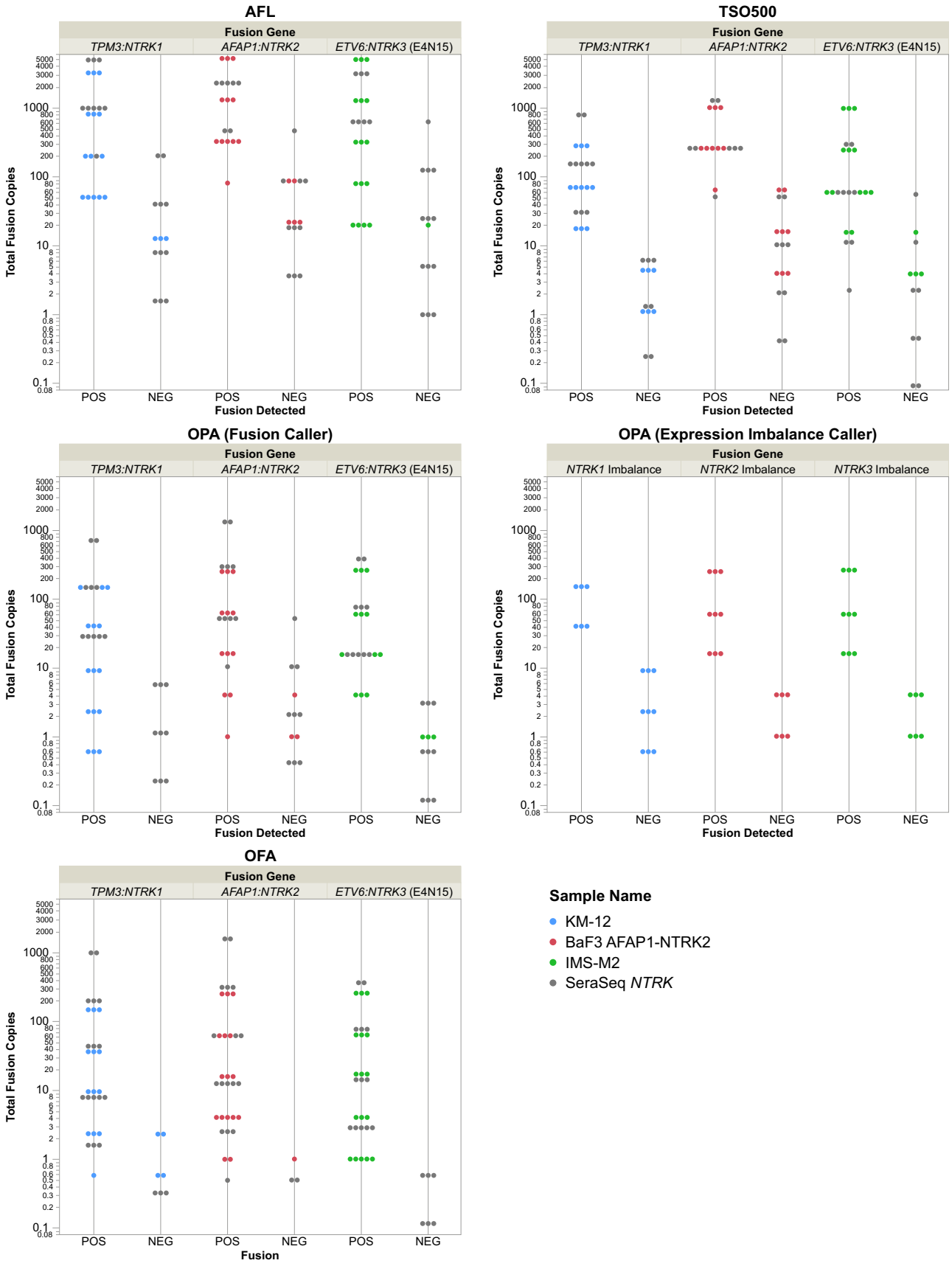
AFL
TSO500
OPA (Fusion Caller)
OPA (Expression Imbalance Caller)
OFA
Fusion Gene
TPM3:NTRK1
AFAP1:NTRK2
ETV6:NTRK3 (E4N15)
NTRK1 Imbalance
NTRK2 Imbalance
NTRK3 Imbalance
Total Fusion Copies
Fusion Detected
Fusion
POS
NEG
Sample Name
KM-12
BaF3 AFAP1-NTRK2
IMS-M2
SeraSeq NTRK

Table 5 Specificity of the AFL, TSO500, OPA, and OFA Assays in 30 *NTRK* Fusion-Negative Clinical Samples

Result	AFL	TSO500	OPA	OFA
Specificity, %	100	100	Specificity samples not processed on OPA.	100
Passed samples evaluated for specificity, *n* (%)	13 (43)*	23 (77)†	Internal validation data showed no false-positive *NTRK* fusion in >30 pan-tumor samples (data not shown)	25 (83)‡

*Five samples were reprocessed with AFL using RNA extracted with Qiagen AllPrep (TSO500-recommended extraction kit). AFL sample quality control failures were due to: presequencing quality control: Ct >28 (76%); unique RNA start sites (control) <10 (18%); and library quantity <2 nmol/L (6%).

†TSO500 sample quality control failures were due to: median insert size <80 bp (44%); median CV gene 500× >93 (33%); and unspecified cause (22%).

‡Nineteen samples had an RNA mean read length below the recommended 90 bp (41 to 89 bp; average, 69 bp). OPA sample quality control failures were due to: mapped fusion panel reads <20,000 (80%); and other not specified fusion sample quality control (20%).

AFL, Archer's FusionPlex Lung panel; *NTRK*, neurotrophic tyrosine receptor kinase; OFA, Oncomine Focus Assay; OPA, Oncomine Precision Assay; TSO500, TruSight Oncology 500.

Clinical Sample Performance

Table 6 presents the *NTRK* gene fusions detected by the four assays in 12 clinical samples that had *NTRK* fusions previously identified by local assays (two samples, for which none of the NGS assays were able to detect these fusions, were excluded from the analysis). Most of the results generated by the four assays successfully passed QC (AFL, 83%; TSO500, 75%; OPA, 100%; and OFA, 80%), and expected fusions were covered by the respective assay panels, except OFA, which panel lacked two of the expected fusions. Of these validated samples, *NTRK* fusion detection rates for samples that passed QC were 100% for all assays on the targets that were in the panels (the OPA imbalance caller showed an *NTRK* fusion detection rate of 67%). In addition, several results that did not meet QC pass criteria also showed that the relevant *NTRK* fusion had been detected.

Discussion

The development and approval of TRK inhibitors, such as entrectinib and larotrectinib, as well as other targeted therapies for rare biomarkers [eg, Ret proto-oncogene (*RET*) and ROS1 proto-oncogene 1 (*ROS1*)] means that testing for oncogenic gene fusions is now recommended in the diagnostic process.[14] Currently, as only one companion diagnostic assay for *NTRK* fusion detection (FoundationOne CDx) is approved by the US Food and Drug Administration, clinical laboratories use a range of laboratory-developed NGS assays to detect *NTRK* fusions. To get a better understanding of the options that are available for *NTRK* testing in clinical practice, the analytical performance of the four most commonly used RNA-based NGS assays was compared for the detection of *NTRK* fusions. Because obtaining clinical samples for validation of a new *NTRK* fusion detection assay is extremely difficult, preclinical, contrived sample options (FFPE cell lines and SeraSeq reference materials) were evaluated in addition to clinical samples, although they are not a perfect substitution. Laboratories wanting to perform *NTRK* fusion testing by NGS should aim to process clinical samples in addition to contrived samples, to further characterize variability parameters specific to clinical samples (eg, tumor percentage, tumor and tissue type, and differences between patients). Nevertheless, the results from this comparative analysis demonstrate performance correlations between the different sample types.

The variations in RNA sample input (AFL, 200 ng; TSO500, 40 ng; OPA, 5 or 10 ng; and OFA, 10 ng) and sensitivity between the four assays are likely explained by their inherent technology. Indeed, the OFA and OPA use an amplicon-based capture technology, which is highly sensitive and therefore requires little sample input.[15,16] However, OFA and OPA can only detect known fusions with corresponding primers included in the assay and therefore can miss samples with novel fusion genes. This is reflected by the results of this study, where the older OFA missed several of the *NTRK* fusions in its panel, whereas the updated OPA panel was able to detect most *NTRK* fusions. Although the OPA can also theoretically detect unknown fusions by expression imbalance of the gene of interest, this particular chemistry demonstrated a low overall sensitivity as it only detected 67% of *NTRK* fusions in the clinical samples (specificity not evaluated). Interestingly, the OPA expression imbalance caller was not able to detect any *NTRK* fusion in the *NTRK* fusion-positive reference sample. This technology compares amplicons tiled across the entire wild-type *NTRK* gene versus a direct amplification of the *NTRK* gene fusion; it is thus more susceptible to background

Figure 2 Sensitivity/precision of Archer's FusionPlex Lung panel (AFL), TruSight Oncology 500 (TSO500), Oncomine Precision Assay (OPA), and Oncomine Focus Assay (OFA) for cell lines and select SeraSeq neurotrophic tyrosine receptor kinase (*NTRK*) reference materials, according to total fusion copy number (determined by droplet digital PCR). The ability of each assay to detect the *TPM3:NTRK1*, *AFAP1:NTRK2*, and *ETV6:NTRK3* fusions was evaluated in three cell lines (KM-12, blue; BaF3 AFAP1-NTRK2, red; IMS-M2, green) and the Sera Seq *NTRK* reference materials (gray). For each tested fusion, dots representing individual replicates are displayed on the left-hand side bars if the fusion was detected, or on the right-hand side bar if the assay failed to detect the fusion. To evaluate the precision of each assay across samples, two additional replicates were processed for the lowest sensitivity level at which all three initial replicates were positive (POS; processed on minimum three different values). No expression imbalance was detected in SeraSeq *NTRK*. NEG, negative.

Table 6 Detection of *NTRK* Fusions by the AFL, TSO500, OPA, and OFA Assays on *NTRK* Fusion-Positive Clinical Samples

Local test used	Tumor type	Tumor, %*	Age of FFPE block	Local test *NTRK* fusion detected
Nanostring	Lipofibromatosis-like neural tumor	40	2018	*TPM3:NTRK1*
RNA sequencing with ArcherDx	Anaplastic glioma	70	2019	*TLE4:NTRK2*
Oncomine Comprehensive version 3 panel	Parapharyngeal region tumor	70	2012	*ETV6:NTRK3*
Oncomine Comprehensive version 3 panel	Parotid gland tumor	60	2009	*ETV6:NTRK3*
Oncomine Comprehensive version 3 panel	Parotid gland tumor	75	2020	*ETV6:NTRK3*
OFA and Illumina's Comprehensive RNA fusion panel	Parotid gland tumor	60	2017	*ETV6:NTRK3*
Archer FusionPlex	Colon cancer	60	2013	*TPM3:NTRK1*
Archer FusionPlex	Cecal cancer	20	2013	*LMNA:NTRK1*
Nanostring	MASC	15	2017	*ETV6:NTRK3*
RNA sequencing with Illumina	MASC	25	2017	*ETV6:NTRK3*
Oncomine Comprehensive assay	Papillary thyroid carcinoma	70	2018	*TPR:NTRK1*
Oncomine Comprehensive assay	Splenic flexure cancer	80	2019	*ETV6:NTRK3*
		QC passing success rate/*NTRK* fusions on panel[§]		
		NTRK fusion detection (sensitivity based on QC passed samples)		

(*table continues*)

NTRK fusions were initially characterized via local assays.

*Samples below 70% tumor content were macrodissected before processing.

[†]Cross-sample contamination was likely due to human error.

[‡]OFA panel only has the L2:N11 isoform for *LMNA:NTRK1* gene fusion.

[§]Two samples were excluded from the analysis; because none of the next-generation sequencing assays were able to detect these fusions, it was surmised that local tests may have given false-positive results (local test used: Oncomine Comprehensive; sample 1: rectal cancer, 70% tumor, 2014; sample 2: sigmoid colon cancer, 5% tumor, 2014).

AFL, Archer's FusionPlex Lung panel; FFPE, formalin fixed, paraffin embedded; MASC, mammary analogue secretory carcinoma; *NTRK*, neurotrophic tyrosine receptor kinase; OFA, Oncomine Focus Assay; OPA, Oncomine Precision Assay; QC, quality control; TSO500, TruSight Oncology 500.

NTRK expression not originating from the tumor tissue, similar to what has been previously reported for *ROS1*.[17] It is unclear if this can entirely explain the low sensitivity of the expression imbalance assay or whether other technical differences of this synthetic material also had an impact. Therefore, additional studies are needed to determine whether the expression imbalance caller of the OPA is a reliable method for fusion detection in clinical practice.

On the other hand, the TSO500 software, which uses hybrid-capture technology, was unable to distinguish the four *ETV6:NTRK3* breakpoints despite detecting supporting reads for all. Despite this limitation in contrived samples, the identification of *NTRK* fusion-positive tumors by this assay would not be affected, because at least one breakpoint would always be reported if multiple breakpoints occurred between the same gene partners. Finally, the AFL assay, using the anchored multiplex PCR technology, requires more RNA input than the other methods and had a lower sensitivity. Of note, this assay would likely have been less sensitive with RNA sample inputs of <200 ng (allowable range, 20 to 250 ng).[15,16] The possibility to function with a minimal amount of RNA can be critical in some practices, particularly when working with small biopsies. Consequently, the ability to maintain sensitivity at low RNA inputs and to minimize sample failures may lead to higher real-world or clinical sensitivity, and should be considered when selecting the most appropriate assay for practice.

All tested NGS assays showed high specificity, with no false-positive results reported, but the AFL assay was associated with a high rate of QC failure (57%). It is unclear if these sample failures were caused by aging FFPE samples and resulting lower quality of the RNA extracted from these samples, the associated extraction kit, or the AFL assay itself; although, the susceptibility of AFL to RNA and cDNA of suboptimal quality has been previously described.[18–22] Rigorous QC metrics for reagents and equipment in a clinical laboratory would reduce the potential risk of wasting valuable clinical samples because of poor result quality. Nevertheless, *NTRK* gene fusions could be detected reliably even when samples failed manufacturer's sequencing QC criteria. This stresses the importance of developing and

Table 6 (*continued*)

AFL	TSO500	OPA	OPA	OFA
NTRK fusion	*NTRK* fusion	*NTRK* (fusion caller)	*NTRK* (expression imbalance caller)	*NTRK* fusion
Detected	Detected	Detected	Detected (*NTRK1* imbalance)	Detected
Detected	Detected	Detected	Not detected	Not detected (missing from panel)
Failed QC (detected)	Detected	Detected	Detected (*NTRK3* imbalance)	Detected
Failed QC (detected)	Failed QC (detected)	Detected	Detected (*NTRK3* imbalance)	Detected
Detected	Detected	Detected	Detected (*NTRK3* imbalance)	Detected
Detected	Detected	Detected	Not detected	Failed QC (contaminated with neighboring sample[†])
Detected	Failed QC (detected)	Detected	Detected (*NTRK1* imbalance)	Detected (contaminated with neighboring sample[†])
Detected	Detected	Detected	Detected (*NTRK1* imbalance)	Not detected (missing from panel)[‡]
Detected	Detected	Detected	Not detected	Detected
Detected	Detected	Detected	Not detected	Detected
Detected	Detected	Detected	Detected (*NTRK1* imbalance)	Detected
Detected	Failed sequencing pipeline	Detected	Detected (*NTRK3* imbalance)	Failed QC (not detected)
10/12 Passed (83%) 12 Samples (100%)	9/12 Passed (75%) 11 Samples (100%)	12/12 Passed (100%) 12 Samples (100%)	12/12 Passed (100%) 8 Samples (67%)	8/10 Passed (80%) 8/8 Samples (100%)

validating local quality standards when implementing distributed NGS kits as laboratory-developed tests. However, caution should be used when quality metrics fail and an unknown sample shows as *NTRK* fusion positive, as rare false positives cannot be ruled out with any technology.

The tested SeraSeq *NTRK* reference material showed variable results across and within lots, as well as extraction kits. The SeraSeq *NTRK* standards may therefore be more suitable for qualitative (ie, detected versus not detected) uses and additional characterization may be needed if a quantitative measure is required. The SeraSeq reference materials also yielded markedly fewer fusion copies/ng RNA than the SeraCare CofA; it is unclear whether this was due to the different extraction kit being used for QC release by the manufacturer. Because of this variability, the CofA cannot be used reliably to determine expected fusion copy numbers in this type of study.

A further technical challenge that arose was QC failure of several RNA samples during the pilot phase; these were likely due to poor sample quality and were resolved by reducing the amount of sample loaded into the extraction columns. In clinical practice, sample conditions, such as age, may have had an additional impact on RNA quality/quantity received after extraction. Generally, following the manufacturer's instructions regarding sample input and QC process helped to resolve technical issues associated with RNA extraction of contrived samples.

Unexpectedly, the OFA and OPA had a limit of detection about 10 times lower than the value published by Thermo Fisher (approximately 1 to 28 total fusion copies for both assays versus the published 255 for OFA and 10 to 377 for OPA) (Thermo Fisher white paper: An Approach for Establishing Oncomine Focus Assay Performance and Thermo Fisher: Oncomine Precision Assay Performance Report). This may be explained by a conservative limit of detection provided by the manufacturer and the fact that sensitivity experiments were performed using H2228 cells expressing an *EML4:ALK* fusion, for which the assay sensitivity may differ compared with *NTRK* fusions. The limit of detection for the TSO500 assay was as expected on the basis of information published by Illumina (approximately 30 to 290 versus published 200 total fusion copies) (Illumina data

Table 7 Summary of the Analytical Performance of the AFL, TSO500, OPA, and OFA Assays for the Detection of *NTRK* Fusions in Preclinical and Clinical Samples

Variable	AFL	TSO500	OPA	OFA
Technology	Anchored multiplex PCR	Hybrid capture	Amplicon-based enrichment	Amplicon-based enrichment
RNA sample input, ng	20—250 (200 used for this study)	40	10	10
Turnaround time, days	~5 Manual library preparation	~5 Manual library preparation Automated option available	~1 Fully automated with minimal hands-on time	~5 Manual library preparation Automated option available
Samples per run, *n*	48 (MiSeq)	8 DNA + 8 RNA (NextSeq High Output)	4—16	24 DNA + 24 RNA (530 chip)
Sequencing system compatibility	Illumina and Ion Torrent	Illumina only	Ion Torrent only	Ion Torrent only
Pilot study				
Cell lines	All *NTRK* fusions detected	All *NTRK* fusions detected	All *NTRK* fusions detected	All *NTRK* fusions detected
Reference materials	All *NTRK* fusions detected	16/18 *NTRK* fusions reported: 2 *ETV6:NTRK3* variants not reported, although they were present in sequencing data	All *NTRK* fusions detected	15/18 *NTRK* fusions detected: 3 missing were not included in panel
NTRK fusion-negative samples	No false-positive result	No false-positive result	No false-positive result	No false-positive result
Sensitivity				
Total fusion copies at estimated LoD	Cell lines: 30—620 Reference materials: 710—5200	All samples: ~30—290	Fusion Caller, all samples: ~1—28 Imbalance Caller, all samples: 12—28	All samples: ~1—28
Reproducibility at estimated LoD	Good: 3/5 to 5/5	Very good: 4/5 to 5/5	Very good: 4/5 to 5/5	Very good: 4/5 to 5/5
Specificity				
Specificity (% samples pass), %	100 (43)	100 (77)	100 (by internal validation, data not shown)	100 (83)
Clinical performance				
Sensitivity (% passing success rate), %	100 (83)	100 (75)	Fusion Caller: 100 (100) Imbalance Caller: 67 (100)	100 (80)

AFL, Archer's FusionPlex Lung panel; LoD, limit of detection; *NTRK*, neurotrophic tyrosine receptor kinase; OFA, Oncomine Focus Assay; OPA, Oncomine Precision Assay; TSO500, TruSight Oncology 500.

sheet: TruSight Oncology 500 and TruSight Oncology 500 High-Throughput. San Diego, CA). As no expected fusion copy numbers have been published by Archer for the AFL assay, the values from the present study could not be compared with those reported by the manufacturer.

The four assays were able to confirm the results from local testing, with on-panel *NTRK* fusion detection rates of 100%, except for the expression imbalance component of the OPA that confirmed 67% of *NTRK* fusions. When performing ddPCR on some clinical samples, on average five fusion copies/ng RNA (0.1 to 20 fusion copies/ng RNA) were detected. The reasons why not all *NTRK* fusions were detected by the assays are unclear, although, as explained above, high basal expression of *NTRK* in the tissues could be a partial explanation. In cases such as the *ASIP:NTRK2* fusion in rectal cancer and *TPM3:NTRK1* fusion in sigmoid colon cancer, in which none of the assays were able to confirm with high confidence, the local test may have been a false positive; these two samples were thus excluded from the calculation of clinical sample sensitivity. In other cases, the lack of *NTRK* fusion detected in these experiments could be due to a low sample quality, including aged FFPE samples, as explained above, or heterogeneity of the tumor tissue in different FFPE sections. This study utilized archival samples, which can represent additional pre-analytic challenges, particularly for RNA-based

approaches; similar analyses using newly derived biopsies would thus be helpful to evaluate methods for *NTRK* fusion detection. However, such studies would be impractical to run.

These data are intended to provide users with assay-to-assay comparisons in a setting closely approximating clinical practice. It is essential to be able to identify *NTRK* fusions in patients with solid tumors to determine the most appropriate treatment, as these patients often have distinct disease profiles to those with the same tumor type but without a gene fusion. The *NTRK* fusion-positive cell lines were relevant samples for this study but had low availability; similarly, commercial reference materials for *NTRK* fusions were difficult to source. Easily accessible contrived samples are thus an important tool to determine NGS assay performance for *NTRK* fusion detection.

In summary, all four tested NGS assays are able to detect *NTRK* fusions, although each assay had its own strengths and limitations (Table 7). The AFL assay requires a high RNA input, but is able to detect novel gene fusions at moderate sensitivity. The TSO500 assay requires a medium RNA input, has moderate sensitivity, and can detect novel fusions. Finally, the OPA and OFA only need small quantities of RNA and yield results at a high sensitivity, but they may miss novel fusions that are not covered on the amplicon-based panel. Overall, the OPA covers more *NTRK* fusions than the OFA, is fully automated for low throughput, has a short turnaround time, and can detect some novel partners through the 5′/3′ imbalance, although the sensitivity and specificity of this imbalance caller should be studied further.

Characterizing the ability of commercially available NGS assays to reliably detect rare gene fusions, such as *NTRK*, *ROS1*, or *RET*, across tumor types will allow pathologists and physicians to make the most informed choice of relevant and high-quality, validated testing methods for this purpose. This will support broader access to high-quality molecular testing and identification of patients with solid tumors harboring gene rearrangements, who may benefit from treatment tailored to the genomic profile of their individual tumor.

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C.B.C. is the guarantor of this work and, as such, oversaw the execution of all experiments in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.09.008*.

References

1. Amatu A, Sartore-Bianchi A, Bencardino K, Pizzutilo EG, Tosi F, Siena S: Tropomyosin receptor kinase (TRK) biology and the role of *NTRK* gene fusions in cancer. Ann Oncol 2019, 30:viii5−viii15
2. Rolfo C: *NTRK* gene fusions: a rough diamond ready to sparkle. Lancet Oncol 2020, 21:472−474
3. Lassen U: How I treat *NTRK* gene fusion-positive cancers. ESMO Open 2019, 4:e000612
4. Cocco E, Scaltriti M, Drilon A: *NTRK* fusion-positive cancers and TRK inhibitor therapy. Nat Rev Clin Oncol 2018, 15:731−747
5. Garrido P, Hladun R, de Alava E, Alvarez R, Bautista F, Lopez-Rios F, Colomer R, Rojo F: Multidisciplinary consensus on optimising the detection of *NTRK* gene alterations in tumours. Clin Transl Oncol 2021, 23:1529−1541
6. Rolfo CD, De Braud FG, Doebele RC, Drilon AE, Siena S, Patel M, Cho BC, Liu SV, Ahn M-J, Chiu C-H, Farago AF, Goto K, Lee J, Bazhenova L, John T, Fakih M, Simmons BP, Pitcher B, Huang X, Demetri GD: Efficacy and safety of entrectinib in patients (pts) with *NTRK*-fusion positive (*NTRK*-fp) solid tumors: an updated integrated analysis. J Clin Oncol 2020, 38:3605
7. McDermott R, van Tilburg CM, Farago AF, Kummar S, Tan DSW, Albert CM, Berlin J, Lassen UN, Doz F, Geoerger B, Mascarenhas L, Federman N, Reeves JA, Dima L, Brega N, De La Cuesta E, Laetsch TW, Hong DS, Drilon A: Survival benefits of larotrectinib in an integrated dataset of patients with TRK fusion cancer. Ann Oncol 2020, 31(1955P):S1101−S1102
8. Cho BC, Doebele RC, Lin JJ, Nagasaka M, Baik C, van der Wekken AJ, Velcheti V, Lee KH, Liu SV, Solomon B, Kao S, Krebs MG, Zhu V, Stopatschinskaja S, Camidge DR, Drilon A: Phase 1/2 TRIDENT 1 study of repotrectinib in patients with *ROS1*+ or *NTRK*+ advanced solid tumors. Singapore, World Conference on Lung Cancer 2020, 2021
9. Jiang T, Wang G, Liu Y, Feng L, Wang M, Liu J, Chen Y, Ouyang L: Development of small-molecule tropomyosin receptor kinase (TRK) inhibitors for *NTRK* fusion cancers. Acta Pharm Sin B 2021, 11:355−372
10. Kirchner M, Neumann O, Volckmar AL, Stogbauer F, Allgauer M, Kazdal D, Budczies J, Rempel E, Brandt R, Talla SB, von Winterfeld M, Leichsenring J, Bochtler T, Kramer A, Springfeld C, Schirmacher P, Penzel R, Endris V, Stenzinger A: RNA-based detection of gene fusions in formalin-fixed and paraffin-embedded solid cancer samples. Cancers (Basel) 2019, 11:1309
11. Beadling C, Wald AI, Warrick A, Neff TL, Zhong S, Nikiforov YE, Corless CL, Nikiforova MN: A multiplexed amplicon approach for detecting gene fusions by next-generation sequencing. J Mol Diagn 2016, 18:165−175
12. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, Gooding WE, Hodak SP, LeBeau SO, Ohori NP, Seethala RR, Tublin ME, Yip L, Nikiforova MN: Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer 2014, 120:3627−3634
13. Marchio C, Scaltriti M, Ladanyi M, Iafrate AJ, Bibeau F, Dietel M, Hechtman JF, Troiani T, Lopez-Rios F, Douillard JY, Andre F, Reis-Filho JS: ESMO recommendations on the standard methods to detect *NTRK* fusions in daily practice and clinical research. Ann Oncol 2019, 30:1417−1427
14. Penault-Llorca F, Rudzinski ER, Sepulveda AR: Testing algorithm for identification of patients with TRK fusion cancer. J Clin Pathol 2019, 72:460−467
15. Samorodnitsky E, Jewell BM, Hagopian R, Miya J, Wing MR, Lyon E, Damodaran S, Bhatt D, Reeser JW, Datta J, Roychowdhury S: Evaluation of hybridization capture versus amplicon-based methods for whole-exome sequencing. Hum Mutat 2015, 36:903−914
16. Pfarr N, Kirchner M, Lehmann U, Leichsenring J, Merkelbach-Bruse S, Glade J, Hummel M, Stogbauer F, Lehmann A, Trautmann M, Kumbrink J, Jung A, Dietmaier W, Endris V, Kazdal D, Evert M,

Horst D, Kreipe H, Kirchner T, Wardelmann E, Lassen U, Buttner R, Weichert W, Dietel M, Schirmacher P, Stenzinger A: Testing *NTRK* testing: wet-lab and in silico comparison of RNA-based targeted sequencing assays. Genes Chromosomes Cancer 2020, 59:178−188

17. Bruno R, Fontanini G: Next generation sequencing for gene fusion analysis in lung cancer: a literature review. Diagnostics (Basel) 2020, 10:521
18. Heydt C, Wölwer CB, Velazquez Camacho O, Wagener-Ryczek S, Pappesch R, Siemanowski J, Rehker J, Haller F, Agaimy A, Worm K, Herold T, Pfarr N, Weichert W, Kirchner T, Jung A, Kumbrink J, Goering W, Esposito I, Buettner R, Hillmer AM, Merkelbach-Bruse S: Detection of gene fusions using targeted next-generation sequencing: a comparative evaluation. BMC Med Genomics 2021, 14:62
19. Benayed R, Offin M, Mullaney K, Sukhadia P, Rios K, Desmeules P, Ptashkin R, Won H, Chang J, Halpenny D, Schram AM, Rudin CM, Hyman DM, Arcila ME, Berger MF, Zehir A, Kris MG, Drilon A, Ladanyi M: High yield of RNA sequencing for targetable kinase fusions in lung adenocarcinomas with no mitogenic driver alteration detected by DNA sequencing and low tumor mutation burden. Clin Cancer Res 2019, 25:4712−4722
20. Davies KD, Le AT, Sheren J, Nijmeh H, Gowan K, Jones KL, Varella-Garcia M, Aisner DL, Doebele RC: Comparison of molecular testing modalities for detection of ROS1 rearrangements in a cohort of positive patient samples. J Thorac Oncol 2018, 13:1474−1482
21. Heydt C, Ruesseler V, Pappesch R, Wagener S, Haak A, Siebolts U, Riedel R, Michels S, Wolf J, Schultheis AM, Rehker J, Buettner R, Merkelbach-Bruse S: Comparison of in situ and extraction-based methods for the detection of ROS1 rearrangements in solid tumors. J Mol Diagn 2019, 21:971−984
22. Murphy DA, Ely HA, Shoemaker R, Boomer A, Culver BP, Hoskins I, Haimes JD, Walters RD, Fernandez D, Stahl JA, Lee J, Kim KM, Lamoureux J, Christiansen J: Detecting gene rearrangements in patient populations through a 2-step diagnostic test comprised of rapid IHC enrichment followed by sensitive next-generation sequencing. Appl Immunohistochem Mol Morphol 2017, 25:513−523