TEMPUS

xR IVD

TECHNICAL INFORMATION

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Intended Use

The Tempus xR IVD assay is a qualitative next generation sequencing-based in vitro diagnostic device that uses targeted high throughput hybridization-based capture technology for detection of rearrangements in two genes using RNA isolated from formalin-fixed paraffin embedded (FFPE) tumor tissue specimens from patients with solid malignant neoplasms.

Information provided by xR IVD is intended to be used by qualified health care professionals in accordance with professional guidelines in oncology for patients with previously diagnosed solid malignant neoplasms. Results from xR IVD are not intended to be prescriptive or conclusive for labeled use of any specific therapeutic product.

Special Condition for Use Statement(s):

For in vitro diagnostic use only. For prescription use only.

Contraindications

There are no known contraindications.

Limitations

- For in vitro diagnostic use.
- For prescription use only. This test must be ordered by a qualified medical professional in accordance with clinical laboratory regulations.
- The acceptable preparation method for xR IVD tumor specimens is formalin-fixation and paraffin-embedding (FFPE). Other preparations have not been evaluated.

- xR IVD requires a minimum tumor percentage of 20% for detection of alterations, with tumor content enrichment recommended for specimens with tumor percentage lower than 20%. This assay may not detect alterations if the proportion of tumor cells in the sample is less than 20%.
- Genomic findings are not prescriptive or conclusive for labeled use of any specific therapeutic product.
- A negative result does not rule out the presence of a mutation below the limits of detection of the assay.
- xR IVD is only approved for use with Tempus pre-qualified Illumina NovaSeq 6000 instruments.
- The test is intended to be performed on specific serial number-controlled instruments by Tempus.
- 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.
- For the mutations listed as Genomic Findings with Potential Clinical Significance, the clinical significance has not been demonstrated with this test.
- This device is designed to report out somatic variants and is not intended to report germline variants. This device is designed to accept tumor-only samples (as opposed to tumor-normal pairs).
- This assay reports rearrangements in BRAF and RET. A negative test result from this device does not eliminate other alterations in these genes or any alterations in other genes.
- Due to tumor temporal and spatial heterogeneity, tumor samples collected at different time points, from different regions of the tumor, or from different anatomical locations on the patient may display distinct mutational profiles, even if the samples were collected from the same patient.
- Overall pass rates for xR IVD decrease with increasing age of FFPE blocks.

Test Principle

The xR IVD assay is a next generation sequencing (NGS) assay that includes reagents, software, instruments, and procedures for testing RNA extracted from formalin-fixed, paraffin embedded (FFPE) tumor specimens. The assay employs RNA extraction methods from routinely obtained FFPE tumor tissue samples. Extracted RNA undergoes conversion to double stranded cDNA and library construction followed by hybridization-based capture using a whole-exome targeting probe set with supplemental custom Tempus-designed probes. Hybrid-capture-selected libraries are sequenced on Illumina NovaSeq 6000 platform, qualified at Tempus. Sequence data is processed using a customized analysis pipeline designed to detect gene rearrangements, including rearrangements in BRAF and RET.

Summary and Explanation

xR IVD identifies gene rearrangements that may inform patient management in accordance with professional guidelines. The output of xR IVD includes classification of oncologically relevant events based on FDA Fact Sheet describing the CDRH's Approach to Tumor Profiling for NGS tests[1]. xR IVD results are presented in two categories:

Level 2: Genomic Findings with Evidence of Clinical Significance

Level 3: Genomic Findings with Potential Clinical Significance

xR IVD includes four critical quality control checks (QC) conducted across the assay workflow to monitor assay performance and ensure that only high-quality data are generated and used for biomarker detection. These checks operate at each step of the assay as follows:

1. RNA Extraction (QC1)
2. Library Preparation (QC2)
3. Hybridization Capture (QC3)
4. Sequencing (QC4)

Test Kit Contents

xR IVD includes a Specimen Collection Box for the specimen type used with the assay. The collection box includes specimen preparation and shipping instructions, and a return shipping label.

All other reagents, materials and equipment needed to perform the assay are used exclusively in the Tempus Laboratory.

Sample Collection and Test Ordering

To order the xR IVD, a test requisition form must be fully completed and signed by an ordering physician or authorized medical professional. Specimen preparation and mailing instructions are provided in the Specimen Collection Box.

Note: for symbols used in the Specimen Collection Box please refer to Tempus' Symbols Glossary at https://www.tempus.com/resources/iso-symbol-glossary/.

Instruments

xR IVD uses Illumina NovaSeq 6000 Sequencers qualified by Tempus; high throughput sequencing systems employing sequencing-by-synthesis chemistry.

[1] https://www.fda.gov/media/109050/download.

Performance Characteristics

Performance characteristics were established using RNA derived from a wide range of FFPE tissue types. Studies included representative gene rearrangements in various genomic contexts.

1. Precision

Two studies were conducted to evaluate the precision for xR IVD. Study 1 evaluated precision of multiple gene rearrangements to provide totality of supporting data for xR IVD and Study 2 evaluated gene rearrangements in two select genes, RET and BRAF.

Study 1: Precision for xR IVD Panel

Precision between runs was based on repeated measurements of 25 patient specimens and 2 commercially available control materials representing 8 different tumor types (including colorectal cancer, non-small cell lung cancer, and ovarian cancer). Replicates (n=6) of each specimen were measured across 3 non-consecutive days, with multiple operators, reagent lots, and instruments. A total of 162 replicates contributed to the evaluation of precision. The distribution of tumor types is provided in **Table 1**, below.

Table 1. Distribution of Tumor Types for Characterization of Inter-Assay (between run) PrecisionSummary and Explanation

Tumor Type	Number of Specimens
Colorectal Cancer	1
Glioblastoma	6
Melanoma	1
Non-Small Cell Lung Cancer	8
Ovarian Cancer	2
Prostate Cancer	3
Sarcoma	3
Skin Cancer	1
Control	2

Among the specimens evaluated, there were 29 gene rearrangements. The overall positive percent agreement (PPA) across all precision conditions (days, operators, reagent lots, and instruments) for all specimens and replicates was 98.3% (95% CI 0.9795, 0.9984) for gene rearrangements. Overall NPA was 99.9% (95% CI 0.9989, 0.9997) for gene rearrangements. Results are provided in **Table 2**.

Table 2. Concordance of Gene Rearrangement Calls Between Runs

Alteration	Pairs with a Positive Call	Both Calls Positive	PPA	Two-Sided 95% CI	Pairs with a Negative Call	Both Calls Negative	NPA	Two-Sided 95% CI
Gene Rearrangements	356	350	98.3%	(0.9795, 0.9984)	16762	16752	99.9%	(0.9989, 0.9997)

Study 2: Precision for RET and BRAF Gene Rearrangements

Precision was evaluated for RET and BRAF fusions in 12 FFPE samples from 7 tumor types, including glioblastoma, bladder cancer, non-small cell lung cancer, thyroid and colorectal cancer. Specimens evaluated for reproducibility were run using 3 different library preparation reagent lot combinations, 2 different operators, and 3 different instrument (sequencer) combinations in duplicate on non-consecutive days, for a total of 36 replicates per sample. Specimens were evaluated for within or intra-run precision by running replicates using a single reagent lot and operator throughout the workflow.

Precision for RET rearrangements was determined by calculating the percent agreements, PPA and NPA, at the gene level and the sample level and across all measurements against the majority call or the most frequently occurring observation. The PPA was 98.61% for RET gene rearrangement detection (**Table 3**). Similarly, the PPA at the sample level was 100% for 3 of the 4 RET-positive samples evaluated (**Table 4**). A single sample expected to be a RET-positive sample had 2 false negative replicate results (of 36 total) for a PPA of 94.44%. The NPA for all conditions was 100% since there were no false positives observed.

Table 3. PPA and NPA at the Gene Level for RET

Driver Gene	Total	TP*	FP	TN	FN	PPA	Two Sided 95% CI	NPA	Two Sided 95% CI
RET	432	142	0	288	2	98.61%	[0.951-0.998]	100%	[0.987-1]

**Example: 142 calculated as 4 samples positive for gene rearrangement in RET x 36 replicates - 2 FN*

Table 4. PPA and NPA at the Sample Level for RET

Sample ID	Fusion	FDA Level*	Total Number of Replicates	Positive Replicates Observed	Negative Replicates Observed	Relative LOD	PPA (Two Sided 95% CI)	NPA (Two Sided 95% CI)
Sample 01	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 02	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 03	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 04	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 05	CCDC6_RET	Level 2	36	34	2	1.98	94.44% (81.34%, 99.32%)	-
Sample 06	CCDC6_RET	Level 2	36	36	0	4.06	100% (90.26%, 100%)	-

Sample ID	Fusion	FDA Level*	Total Number of Replicates	Positive Replicates Observed	Negative Replicates Observed	Relative LOD	PPA (Two Sided 95% CI)	NPA (Two Sided 95% CI)
Sample 07	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 08	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 09	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 10	RET Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 11	KIF5B_RET	Level 2	36	36	0	3.92	100% (90.26%, 100%)	-
Sample 12	KIF5B_RET	Level 2	36	36	0	3.42	100% (90.26%, 100%)	-

**N/A = criteria are not applicable for negative samples.*

Precision for BRAF rearrangements was also determined by calculating the percent agreements, PPA and NPA, at the gene level and the sample level and across all measurements against the majority call or the most frequently occurring observation. The PPA was 100% for BRAF gene rearrangement detection at the gene (**Table 5**) and sample level (**Table 6**). The NPA for all conditions was 100% since there were no false positives observed.

Table 5. PPA and NPA at the Gene Level for BRAF

Driver Gene	Total	TP*	FP	TN	FN	PPA	Two Sided 95% CI	NPA	Two Sided 95% CI
BRAF	432	144	0	288	0	100%	[0.975-1]	100%	[0.987-1]

**Example: 144 calculated as 4 samples positive for gene rearrangement in BRAF x 36 replicates - 0 FN*

Table 6. PPA and NPA at the Sample Level for BRAF

Sample ID	Fusion	FDA Level*	Total Number of Replicates	Positive Replicates Observed	Negative Replicates Observed	Relative LOD	PPA (Two Sided 95% CI)	NPA (Two Sided 95% CI)
Sample 01	AGAP3_BRAF	Level 3	36	36	0	1.71	100% (90.26%, 100%)	-
Sample 02	RRBP1_BRAF	Level 3	36	36	0	6.13	100% (90.26%, 100%)	-
Sample 03	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)

Sample ID	Fusion	FDA Level*	Total Number of Replicates	Positive Replicates Observed	Negative Replicates Observed	Relative LOD	PPA (Two Sided 95% CI)	NPA (Two Sided 95% CI)
Sample 04	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 05	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 06	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 07	KDM7A_BRAF	Level 3	36	36	0	1.86	100% (90.26%, 100%)	-
Sample 08	KIAA1549_BRAF	Level 2	36	36	0	2.32	100% (90.26%, 100%)	-
Sample 09	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 10	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 11	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)
Sample 12	BRAF Negative	N/A	36	0	36	0	-	100% (90.26%, 100%)

**N/A = criteria are not applicable for negative samples.*

2. Sensitivity / Limit of Detection for Tumor Purity

The sensitivity of the assay for the detection of gene rearrangements was evaluated by establishing an initial tumor purity limit of detection (LoD) followed up by confirmation with additional samples at the anticipated LoD level.

The initial LoD for gene rearrangements was estimated by testing 12 FFPE clinical specimens (from 8 different cancer types) containing 12 gene rearrangements at five dilution levels ranging from 5% to >40% tumor purities and an undiluted sample. A commercially available control material was also tested containing an additional 15 gene rearrangements. LoD for gene rearrangements was confirmed by testing an additional 21 FFPE clinical specimens (representing 11 tumor types) in duplicate and using 2 reagent lots for a total of 58 replicates (measurements). Among the specimens evaluated, 55 of 58 gene rearrangements were detected. The PPA between the test result and the expected positive result was 94.8% (95% CI 0.8586, 0.9823) at 20% tumor purity. The high PPA at 20% tumor purity confirmed a limit of detection of 20% tumor purity for gene rearrangements.

3. Sensitivity / Limit of Detection for Supporting Reads

The read-based LoD of gene rearrangements was determined per gene using a probit regression model that estimated the hit-rate (detection of target variants) as a function of read support. The LoD was defined as the point-estimate of read support from the probit model at which a 95% hit rate is achieved. Samples were diluted to 6 different dilution levels with 12-14 replicates per dilution level. Dilutions were evaluated by 2 different operators, with at least 6 replicates per dilution level per operator, on different days using different manufacturer reagent lots and sequencing instruments.

For RET, 3 unique FFPE samples, each containing 1 RET gene rearrangement, were evaluated in this study and were run at 6 different dilution levels each with 12 replicates per dilution level. Samples originated from 3 different tumor types, including, Colorectal, Thyroid, and Non Small Cell Lung Carcinoma.

Using a probit regression model, the LoD estimate was 6.67 reads for CCDC6-RET, with a P-value of 1. **Table 7** shows the read support LoD from the probit model in the second to last column. A chi-square goodness of fit test was used to assess probit model fit to the data, results for which are shown in the last column. The RET LoD per sample and tumor type was determined to be 5-7 reads based on a sample-level probit analysis as shown in **Table 8**.

Table 7. Probit Regression Model for LoD Estimation per Gene Rearrangement for RET

Gene Rearrangement	Dilution Level (targeted reads)	N of observations	Hit-rate	Hit-rate 95% CI	LoD Estimates (reads)	Goodness of fit P-Value
CCDC6-RET	1.875	37	0.541	0.369 - 0.705	6.667	1
	3.75	38	0.868	0.719 - 0.956		
	5.63	24	0.958	0.789 - 0.999		
	7.5	36	1	0.903 - 1		
	15	36	1	0.903 - 1		
	30	36	1	0.903 - 1		

Table 8. Probit Regression Model for LoD Estimation per Sample and Tumor Type for RET

Gene	Sample ID - Tumor Type	Fusion	Sample Level LoD (reads)	LoD Range (reads)
RET	LOD 06 - Thyroid Cancer	CCDC6-RET	5	5 - 7
	LOD - 08 Non-Small Cell Lung Cancer	CCDC6-RET	5	
	LOD - 02 Colorectal Cancer	CCDC6-RET	7	

For BRAF, 3 unique FFPE samples, each containing 1 BRAF gene rearrangement, were evaluated in this study and were run at 6 different dilution levels each with 12 replicates per dilution level. Samples originated from breast, prostate, and pancreatic cancer tumor types.

Using a probit regression model, the LoD estimate was 8.91 reads for CCNY-BRAF, with a P-value of 0.987 and 6.54 reads for SND1-BRAF, with a P-value of 1. **Table 9** shows the read support LoD from the probit model in the second to last column. A chi-square goodness of fit test was used to assess probit model fit to the data, results for which are shown in the last column. The BRAF LoD per sample and tumor type was determined to be 6-9 reads based on a sample-level probit analysis as shown in **Table 10**.

Table 9. Probit Regression Model for LoD Estimation per BRAF Rearrangement

Gene Rearrangement	Dilution Level (targeted reads)	N of observations	Hit-rate	Hit-rate 95% CI	LoD Estimates (reads)	Goodness of fit P-Value
CCNY-BRAF	1.875	12	0.333	0.099 - 0.651	8.91	0.987
	3.75	12	0.583	0.277 - 0.848		
	5.63	12	1	0.735 - 1		
	7.5	12	0.917	0.615 - 0.998		
	15	12	1	0.735 - 1		
	30	12	1	0.735 - 1		
SND1-BRAF	1.875	24	0.542	0.328 - 0.744	6.54	1
	3.75	24	0.917	0.73 - 0.99		
	5.63	12	0.917	0.615 - 0.998		
	7.5	23	1	0.852 - 1		
	15	24	1	0.858 - 1		
	30	24	1	0.858 - 1		

Table 10. xR IVD LoD for BRAF per Sample and Tumor Type

Gene	Sample ID - Tumor Type	Fusion	Sample Level LoD (reads)	LoD Range (reads)
BRAF	LOD 01 - Breast Cancer	SND1-BRAF	8	6 - 9
	LOD 07 - Prostate Cancer	SND1-BRAF	6	
	LOD 09 - Pancreatic Cancer	CCNY-BRAF	9	

4. Specificity / Interference

The robustness of the xR IVD workflow was assessed while evaluating tumor FFPE samples in the presence of interfering substances. Samples were evaluated per interferent and tested with no interferent (control), low level interferent and high level interferent (listed in **Table 11**) at relevant steps of the xR IVD workflow. xR IVD robustness was evaluated by determining the concordance of variant calls in samples with interferents when compared to the control samples. Study results showed a high PPA (100%) and NPA (≥98%) for gene rearrangement detection.

Table 11: Final Reaction Concentrations of Interfering Substances Assessed

Interfering Substance	Step Added	Low Concentration	High Concentration
Axygen MAG PCR Clean-Up Beads (Axygen)	Hybridization	0.5%	1%
Ethanol	Library Preparation	5%	10%
Melanin	Library Preparation	0.05 µg/mL	0.1 µg/mL
gDNA	Library Preparation	0.1 ng/µL	2.5 ng/µL

Interfering Substance	Step Added	Low Concentration	High Concentration
Universal Molecular Identifier (UMI) Adapters	Library Preparation	+15%	+30%
RNA XP Clean Beads (XP)	Library Preparation	5%	10%
Proteinase K	RNA Isolation	0.002 mg/mL	0.02 mg/mL
Xylene	RNA Isolation	0.000025%	0.000050%
Tissue Necrosis	TNA Extraction	10% - 30%	40% - 60%

5. Assay Cut-off / Limit of Blank

The limit of blank (LoB) was established by assessing the frequency of false-positive calls in clinical samples known to be wild-type (alteration-negative) for gene rearrangements. A total of 24 FFPE tumor specimens representing 12 different tumor types were tested in duplicate (at the maximum RNA input of 300 ng) and using 2 reagent lots for a total of 96 measurements (4 measurements per sample). 24 of 24 FFPE samples passed QC metrics and were included in the false positive rate calculations. xR IVD detected a gene rearrangement (VOPP1-EGFR) with 3 supporting reads in 1 replicate of 1 sample (1/96 measurement). This false positive rate of 1.04% was used to set the xR IVD LoB threshold of 3 total supporting reads for gene rearrangement calling. xR IVD does not report gene rearrangements below 4 total supporting reads.

6. Stability

6.1 Reagent

The stability of reagents used in the RNA isolation, library preparation, and hybrid capture steps for xR IVD were evaluated using 3 lots of reagents for each assay step, tested at defined time points. Results support the stability of the RNA isolation, library preparation, and hybrid capture reagents up to 180 days (6 months) when stored under manufacturer recommended conditions.

6.2 Sample

6.2.1 Extracted Nucleic Acid

Stability of extracted total nucleic acid (TNA) and RNA was evaluated by assessing the agreement for gene rearrangement detection between freshly extracted nucleic acid and after storage at < -70oC at different timepoints and up to 377 days or after storage at < -70oC at different freeze-thaw cycles and up to 15 cycles.

TNA real-time stability and freeze-thaw stability was established by testing 3 clinical specimens and 2 control materials. PPA and NPA across all samples for the real-time stability for the three genes was found to be 100%. These data found that xR IVD performance was not impacted when using extracted TNA stored at < -70oC for up to 377 days after extraction. Thus, TNA stability for use with xR IVD was determined to be up to 365 days when stored at < -70°C. PPA and NPA across all samples for freeze-thaw stability was found to be 100%. Thus, the stability of TNA as material used for testing with xR IVD for gene rearrangements was determined to be 13.5 freeze-thaw cycles.

RNA real-time stability and freeze-thaw stability was established by testing 8 clinical specimens and 2 control materials. PPA and NPA across all samples for the real-time stability was found to be 100%. Thus, RNA stability for use with xR IVD was determined to be up to 365 days when stored at < -70°C. PPA and NPA across all samples for freeze-thaw stability was

found to be 100%. Thus, the stability of RNA as material used for testing with xR IVD for gene rearrangements was determined to be 13.5 freeze-thaw cycles.

6.2.2 FFPE Slides

Slide stability was determined by evaluating the xR IVD positive percent agreement (PPA) in gene rearrangement calls between freshly prepared and aged slides. Freshly prepared slides were stored at ambient temperature at Tempus and mimicked the expected conditions for clinical slide storage. Testing occurred at 4 different timepoints and up to 377 days.

Testing included 3 clinical specimens and 1 control material. The PPA and NPA for gene rearrangements evaluated at all time points compared to freshly prepared slides was 100% at all time points evaluated. These data demonstrated that FFPE slides are stable for use with xR IVD up to 365 days when stored under expected conditions for clinical slide storage (ambient temperature).

6.2.3 FFPE Scrolls

The stability of RNA within FFPE scrolls over a period of 30 days under standard storage conditions within the Tempus laboratory environment was evaluated. Testing included 2 clinical samples and 2 control materials. Across all samples tested in this study, the PPA and NPA for gene rearrangement detection between T = 0 and all other timepoints was 100%. The stability of FFPE scrolls as material used for testing with xR IVD for gene rearrangements was determined to be 30 days when stored at 4°C.

6.2.4 FFPE Blocks

Stability was assessed by testing FFPE blocks after 0-2 years, 2-5 years, and 5-10 years of storage since block preparation. 94 total samples representing 18 different tumor types were tested as part of the 3 storage ranges (or bins): 21 samples at 0-2 years, 37 samples at 2-5 years, and 36 samples at 5-10 years. 97% of the blocks in the 0-2 age group passed quality metrics (3% invalid rate) demonstrating that blocks that have been stored for up to 2 years are stable for use with xR IVD. The invalid rate of the blocks in the 2-5 years bin and the 5-10 year bins was 16% and 43%, respectively at the library preparation step. For all passing samples, the invalid rate at subsequent steps of the xR IVD workflow drops to 0%.

Stability was also evaluated by comparing the performance of xR IVD in fresh blocks vs. blocks that had been stored under Tempus laboratory conditions for 0 (baseline condition) to 1 year. 7 FFPE clinical samples and 1 control material were tested that included rearrangements in BRAF and RET. Rearrangements in these genes were consistently detected in 1 year old blocks for a PPA of 100%.

7. Input Guardbanding

RNA input tolerance range at the library preparation, hybridization capture, and sequencing steps of the xR IVD workflow was evaluated by determining the number of samples passing quality metrics when varying the RNA input at each step of the workflow. The input ranges evaluated were 10% - 50% above or below the intended range of operation. The intended range of operation at each step of the workflow is in **Table 13**, bolded.

4 FFPE clinical specimens and 1 commercially available control material were evaluated at the library preparation step. 9 FFPE clinical specimens were evaluated at the hybridization capture step and 4 clinical FFPE samples and 2 commercially available control materials were evaluated at the sequencing step.

Across all samples tested in this study, the PPA and NPA for gene rearrangement detection between the intended input level and all other mass input levels was found to be 100%.

Table 13: Summary of Success (Passing) Rate per Process and Per Input Level

Workflow Step	Input Level
Library Capture	ng 40 - 0.8x minimum (-20%)
	ng 45 - 0.9x minimum (-10%)
	ng 50 - 1x minimum
	ng 300 - 1x maximum
	ng 360 - 1.2x maximum (+20%)
Hybridization Capture	ng 120 - 0.8x minimum (-20%)
	ng 135 - 0.9x minimum (-10%)
	ng 150 - 1x minimum
	ng 200 - 1x maximum
	ng 220 - 1.1x maximum (+10%)
	ng 240 - 1.2x maximum (+20%)
Sequencing	0.5x minimum (-50%)
	0.75x minimum (-25%)
	1x minimum
	1x maximum
	1.25x maximum (+25%)
	1.5x maximum (+50%)

8. Carryover / Cross-Contamination

RNA sample cross-contamination (within plates) and carryover (between plates) during sample processing in library preparation was assessed by calculating the false positive rate of gene rearrangements in expected negative samples. Positive and negative samples were prepared in a checkerboard pattern within one run and in an inverse checkerboard pattern relative to the previous run to assess between-run carryover, respectively.

No cross-contamination or carryover of reportable gene rearrangements were detected during library preparation runs or between sequencing runs as there were no false positive results in any of the expected negative samples within a run or between runs.

9. Nucleic Acid Extraction

TNA and RNA extraction was assessed by extraction of 104 tumor specimens (21 samples of 0-2 years of age, 47 samples >2-5 years of age, and 36 samples >5-10 years of age) representing 19 different tumor types (**Table 14**) using 2 extraction instruments and 3 extraction reagent lots over the course of the study.

Table 14: Tumor Types Evaluated

Tumor Type	Number of Specimens
Bladder Cancer	9
Brain Cancer	1
Breast Cancer	29
Cervical Cancer	5
Clear Cell Renal Cell Carcinoma	3
Colorectal Cancer	10
Endometrial Cancer	1
Gastric Cancer	5
Glioblastoma	5
Kidney Cancer	3
Liver Cancer	1
Lung Cancer	5
Ovarian Cancer	6
Pancreato-Biliary Cancer	1
Prostate Cancer	5
Skin Cancer	2
Thyroid Cancer	8
Uterine Cancer	2
Tumor of Unknown Origin	3

The invalid rate for specimens that are 0-2 years is 4.76%. The invalid rate for specimens that are >2-5 and >5-10 years is 14.89% and 42.86%, respectively. These invalid rates are within the predetermined rates for aged specimens. These data demonstrated that xR IVD is capable of successfully extracting TNA/RNA from FFPE specimens derived from varying tissues of origin across different ages.

10. Tissue Comparability

A retrospective analysis was conducted using 312 unique tumor specimens across 10 cancer types in order to establish the comparability of assay performance across tumor tissue types. The dataset for analysis consisted of FFPE clinical specimens tested in the xR IVD accuracy studies. xR IVD includes four QC checks conducted across the assay workflow to closely monitor performance at each step and ensure that only high-quality data are generated and used for variant detection. The QC checks are as follows: TNA/RNA extraction (QC1), library preparation (QC2), hybridization capture (QC3), and sequencing (QC4). The invalid rate for each of these QC steps for each cancer type is summarized in **Table 15**. The overall invalid rate did not exceed 6% for an overall passing rate of >94% for each tissue type. An overall >94% specimen pass rate at each assay step, and for each tissue type, demonstrated that xR IVD performance is independent of tissue type.

Table 15: Invalid Rate at Each Assay Step Across Cancer Types

Tissue Type	Total Replicates	Total Passing QC2	QC2 Invalid Rate	Total Passing QC3	QC3 Invalid Rate	Total Passing QC4	QC4 Invalid Rate	Overall Invalid Rate
Bladder	22	22	0%	22	0%	22	0%	0%
Brain	95	95	0%	95	0%	95	0%	0%
Breast	13	13	0%	13	0%	13	0%	0%
Colon	18	18	0%	18	0%	18	0%	0%
Liver	20	20	0%	20	0%	20	0%	0%
Lung	60	60	0%	60	0%	59	2%	2%
Lymph Node	24	24	0%	24	0%	24	0%	0%
Pleura	17	17	0%	17	0%	16	6%	6%
Prostate	32	32	0%	32	0%	32	0%	0%
Thyroid	11	11	0%	11	0%	11	0%	0%

11. Method Comparison / Accuracy

The detection of gene rearrangements was evaluated by comparing xR IVD results to results of an externally validated NGS-based orthogonal method (OM). The comparison between xR IVD and the OM included 290 unique FFPE clinical specimens representing 30 different tumor types. The distribution of tumor types is provided in **Table 16**.

Table 16: Tumor Specimens Evaluated for Gene Rearrangement Accuracy

Tumor Type	Number of Specimens
B Cell Lymphoma	1
Biliary Cancer	11
Bladder Cancer	19
Brain Cancer	4
Breast Cancer	23
Cervical Cancer	1
Clear Cell Renal Cell Carcinoma	1
Colorectal Cancer	34
Endocrine Tumor	1
Endometrial Cancer	9
Gastric Cancer	5
Gastrointestinal Stromal Tumor	2
Glioblastoma	12
Head and Neck Cancer	7

Tumor Type	Number of Specimens
Head and Neck Squamous Cell Carcinoma	9
Kidney Cancer	5
Low Grade Glioma	2
Melanoma	5
Meningioma	1
Non-Small Cell Lung Cancer	44
Oropharyngeal Cancer	1
Ovarian Cancer	11
Pancreatic Cancer	6
Prostate Cancer	51
Sarcoma	36
Skin Cancer	1
T Cell Lymphoma	1
Testicular Cancer	1
Thyroid Cancer	17
Tumor of Unknown Origin	8

There were 13 samples containing RET gene rearrangements and 277 RET gene rearrangement-negative samples in the study cohort (**Table 17**). xR IVD successfully detected 13 of 13 RET gene rearrangement events assessed for a PPA of 100% and 277 of 277 negative events for an NPA of 100% .

Table 17: RET Gene Rearrangement Detection Concordance

Gene	Total Samples	PPA (95% CI)	NPA (95% CI)
RET	290	100% (0.7719, 1)	100% (0.9863,1)

There were 13 BRAF rearrangement samples from 8 tumor types. xR IVD correctly identified 12 of the 13 BRAF rearrangement-positive samples for a PPA 92.3% and an NPA of 100% (**Table 18**).

Table 18: BRAF Gene Rearrangement Detection Concordance

Gene	Total Samples	PPA (95% CI)	NPA (95% CI)
BRAF	290	92.3% (0.6669, 0.9961)	100% (0.9863, 1)