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RNA Sequencing Analytical Validation

Version 1.0
This report describes the analytical validation parameters for RNA sequencing assay performed at MD Anderson Cancer Center CIMAC. The assay is intended for use in PACT and CIMAC approved clinical trials as a research platform for retrospective gene expression profiling of specimens that have undergone immunotherapy treatment. The sequencing workflow utilizes IDT xGen Library prepation with xGen Exome Hyb Panel V2 for exome capture targeting a 34 megabase (Mb) region that covers 19,435 genes of the human genome.

RNA Sequencing	
Analytical sensitivity	Seraseq® FFPE Tumor Fusion RNA v4 reference material was tested in triplicate using RNA input amounts of 50 ng, 100 ng, and 250 ng. An input of 100 ng was found to be optimal, based on the lowest percentage of genes falling within the noise threshold at that level.
Analytical Specificity	The ability to distinguish between tumor and normal samples was assessed via clustering analysis of healthy donor samples and patient tumor samples.
Accuracy	Accuracy was assessed by comparing gene expression profiles from the 54 sequenced samples to corresponding Nanostring results. Spearman correlation coefficients were greater than 0.7 for 87% of the samples, indicating strong concordance.
Precision: Intra and Inter-assay	Precision was evaluated within runs and across runs using Seraseq® reference materials and patient samples. Clustering analysis was performed to show reproducibility of the RNAseq data. Pearson's correlation coefficient was also determined.
Any other performance characteristics required for assay performance	All the required equipment, have annual service contracts with regular Preventive Maintenance performed to maintain optimal calibration and performance. All other small equipment such as multi-channel pipettes and laboratory material have calibration performed by certified vendors.

1. ***Samples:*** Fresh Frozen (FF), Formalin Fixed Paraffin Embedded (FFPE) tissue samples and Peripheral Blood Mononucleated Cells (PBMCs) from cancer patients, healthy donors and Seraseq® reference materials (Seracare) were selected for the validation (See Appendix, **Supplemental Table 1** for details on sample cohort).

2. ***RNA Extraction: RNA was extracted by the following methods:***
   i. RNA was extracted from PBMCs and FF tissues using the Qiagen RNeasy Mini Kit (cat # 74104)
   ii. RNA was extracted from FFPE tissues using Qiagen AllPrep DNA/RNA FFPE kit (cat #80234)
   RNA Quality Control (QC): Total RNA was assessed using the qubit fluorometer, RNA quality was assessed using the Tapestation 4200 to determine the RNA integrity number and the DV200. DV200 values ranged from 23% -74% for FFPE, over 80% for FF and 50-70% for PBMC samples (**Supplemental Table 1**).

   Grading criteria for RNA samples based on DV200 values were derived from initial familiarization studies using low-quality RNA. Although DV200 values below 30 are widely regarded as indicative of poor sample quality, our data showed that libraries could still be generated when DV200 exceeded 20, particularly with increased RNA input. In contrast, samples with DV200 values below 20 rarely produced viable libraries, even under optimized input conditions. Accordingly, a DV200 threshold of greater than 20 was established as the minimum requirement for library preparation. Using the grading criteria shown in **Table 1**, thirty-seven (37) FFPE samples were classified as excellent quality, for RNA sequencing, fourteen (14) as good, six (6) as poor and none as inapplicable.

   Table 1. Grading criteria of RNA samples based on DV200

Grade	DV200 (Percentage of RNA >200 nt)
Excellent FFPE RNA	RNA >50%
Good FFPE RNA	30% ≤ RNA ≤ 50%
Poor FFPE RNA	20% ≤ RNA ≤ 30%
Inapplicable FFPE RNA	<20%

3. ***Technical platform(s):***
   This report outlines the analytical validation parameters—including analytical sensitivity (RNA input), specificity, accuracy, and precision—of the IDT RNA exome chemistry and workflow implemented at the Department of Translational Molecular Pathology (TMP) at MD Anderson Cancer Center.
   The workflow utilizes IDT xGen chemistry and the xGen Exome Hyb Panel V2, which comprises 415,115 probes targeting a 34 megabase (Mb) region encompassing 19,435 genes of the human genome.

4. Reagents and Equipment

xGen Broad-Range RNA Library Prep Kit
xGen Normalzae UDI Primer Plates
SPRI select beads
Nuclease Free Water
IDTE, pH 8.0
xGen Hyb Panels
xGEn Hybridiation and wash v2 kit
Human Cot DNA
xGen Universal Blockers for TruSeq libraries
xGen Library Amplification Primer Mix
Thermal Cyclers
Ice bucket
Pipettes
Illumina Nextseq 550 sequencer
Illumina Novaseq 6000 sequencer
Agilent 4200 Tapestation
Qubit Fluorometer
Microcentrifuge
Vacuum concentrator
Vortex mixer
DynaMag-PCR Magnet
Dynamag-2 Magnet

5. Study Design

Details of the IDT RNA exome sequencing workflow performed at MDACC is provided in **Figure 1**. RNA sequencing was performed on RNA extracted from FF, FFPE, PBMC, and reference controls. Determination of the ideal input needed for successful library generation and sequencing were performed with Seraseq® reference materials (seracare controls) and repeated with selected patient samples. Assay specificity was determined by running approximately 20 healthy donor controls to distinguish between tumor and normal samples. Accuracy was determined from sequencing approximately 54 FFPE samples previously assayed with Nanostring's nCounter® PanCancer Immune Profiling Panel. Finally, precision was determined by running seracare FF, FFPE and WT FFPE control samples in triplicate and sequencing each set separately. Details of the study design is shown in **Figure 2.**

Figure 1: IDT RNA Exome Workflow

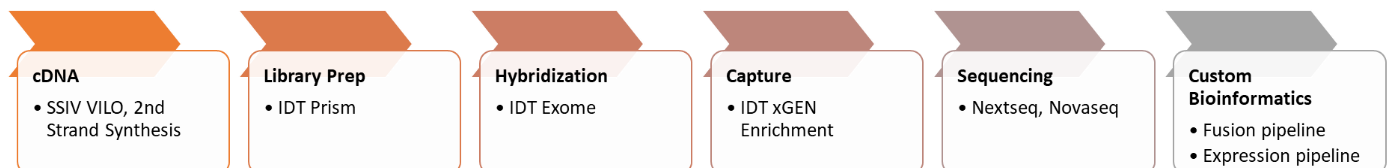
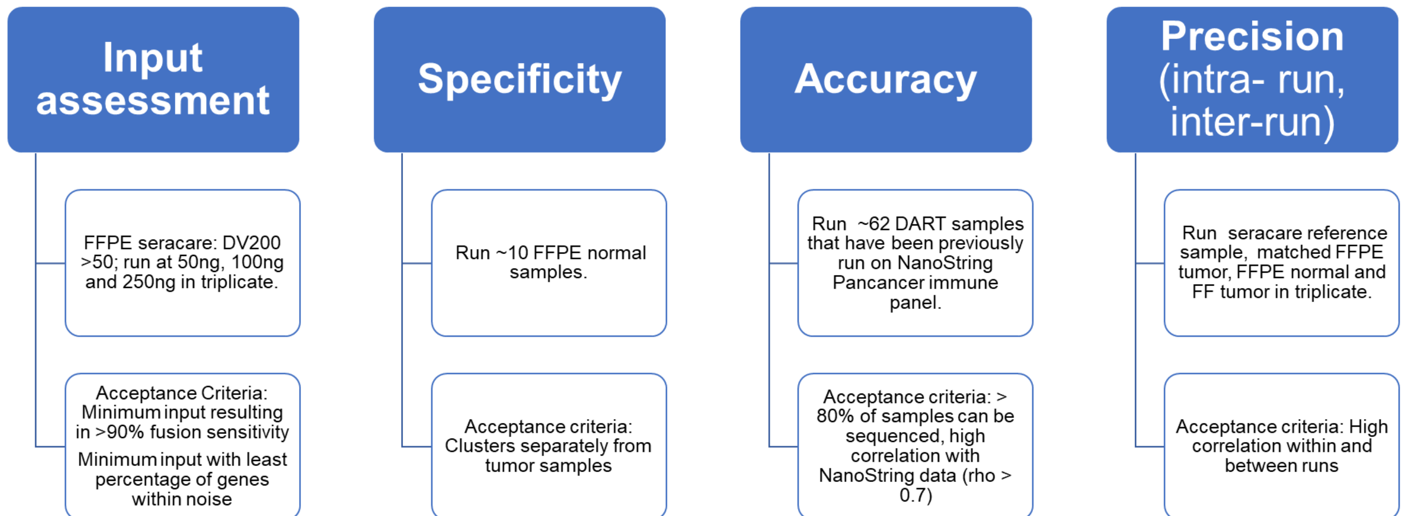


Figure 2: Analytical Validation Study Design



6. ***Library Preparation and sequencing:*** All RNA samples underwent quality control (QC) to assess quantity and quality using the Qubit Fluorometer and Agilent Tapestation 4200. RNA input ranged from 50 ng to 250 ng for analytical sensitivity testing. All other samples were processed with a standardized input of 100ng and only samples with DV200 >20% proceeded. Libraries were prepared using the IDT xGen chemistry, incorporating xGen exome Hyb Panel V2 for hybridization capture. Sequencing was performed on the Novaseq 6000 or Next 550 platform using paired-end 150 bp reads at a depth of >50M. Raw data met standard quality metrics established for Novaseq and Nextseq output.

   Library Quality Control (QC)

   Prehybridization library QC: RNA with DV200 >20% was converted to cDNA using SSIV VILO followed by 2nd strand synthesis. Libraries were prepared using the IDT Prism kit and analyzed on the Agilent TapeStation 4200. For libraries to be considered viable for hybridization it required at least 150ng of DNA and peak fragment size of approximately 400bp. Samples failing to meet these thresholds did not proceed to hybridization or sequencing. The ideal input for hybridization is 500ng.

   Post hybridization library QC: After hybridization capture, washes and PCR amplification, libraries were re-evaluated using the TapeStation 4200. To be sequenced, samples had to meet the following criteria: a minimum concentration of 1nM; a peak fragment size between 200bp - 400 bp. Samples that did not meet these standards were excluded from sequencing. The table below shows the general QC metrics for samples undergoing RNAseq.

QC Metrics values for RNA-seq (FFPE)

Metric	Reference
DV200 [%]	≥20
RIN	≥1.3
Input [ng]	≥100
Library Yield [ng]	≥500
Library Peak [bp]	≥200 and <400
Million reads	≥50,000,000
Percentage Exonic Reads	≥35
Percentage Intronic Reads	<40
Percentage Intergenic Reads	<5

7. *Data Analyses and Bioinformatics*

(i) BCL files (raw output of Illumina Instruments) were processed using Illumina's bcl2fastq (v2.20.0.422) tool for de-multiplexing/conversion to FASTQ format, which is the standard input for most aligners and downstream analytic tools.
(ii) The FASTQ files were trimmed to remove the synthetic adapter sequences introduced during the library preparation. The trimmed reads were aligned to the human reference genome (GRCh37) using STAR, and transcriptome-mapped alignments were generated for downstream analysis.
(iii) The generated BAM files are subject to quantification of gene expression using Salmon (v1.10.2), and the fusion detection is performed by STAR alignment (v2.7.11) followed by three variant callers subworkflows: Arriba (v2.5.0), STAR-Fusion (v1.15.1), and FusionCatcher (v1.15.1).

8. *Data Summary*

Gene expression and fusion detection were assessed for the IDT exome RNA sequencing validation. Two predefined open-source nf-core pipelines executed through Nextflow (v23.10.0): rnaseq (v3.14) for gene expression analysis and rnafusion (v.3.0.2) for fusion detection in all the libraries generated. Post-sequencing quality control (QC) metrics were evaluated, including raw read quality and post-alignment QC using RSeQC (v5.0.4) and Qualimap (v2.3), also used to examine the read distribution. The gene expression pipeline provided raw counts and transcripts per million (TPM), while the gene fusion pipeline reported annotated fusions from multiple variant callers. These results were subsequently parsed to Fusion-report (v4.0.1), incorporating and weighing the findings from COSMIC, Mitelman, and Fusion GDB2 databases.
Gene expression data were normalized and transformed for further statistical analyses and visualization. Cluster heatmap analyses were performed using a pairwise Euclidean distance between samples through DESeq2 (v1.48.1) and utilizing the Pearson correlation coefficient

for sample comparisons. Pearson correlation was used for evaluating intra- and inter-assay experiments, while Spearman's correlation was implemented for accuracy experiments.

Sample distribution across sequencing runs was strategically designed to support distinct analytical validation objectives.

- Analytical sensitivity: To determine the optimal RNA input amount, SeraCare FFPE control samples run in triplicate at input levels of 50 ng, 100 ng, and 250 ng.
- Analytical specificity: Approximately 20 healthy donor PBMC samples were analyzed to assess the pipeline's ability to distinguish between normal and tumor samples using clustering analysis.
- Intra-assay precision: SeraCare FF_T, FFPE_T, and FFPE_WT controls were run in triplicate at both 50 ng and 100 ng input levels. Additionally, two patient samples were run in duplicate at the same input levels.
- Inter-assay precision: The same SeraCare controls (FF_T, FFPE_T, and FFPE_WT) were run in triplicate and sequenced across three independent runs to evaluate consistency across assays.
- Accuracy: A total of 54 patient samples previously analyzed using the Nanostring nCounter platform, were sequenced at 100ng input. Gene expression profiles were then compared between the RNA-seq and Nanostring datasets to assess concordance.

9. *Analytical Sensitivity: RNA input*

To evaluate the optimal RNA input for reproducible results and to assess the impact of suboptimal input levels on assay performance, SeraCare FFPE control samples were sequenced in triplicate using input amounts of 50 ng, 100 ng, and 250 ng. 2 patient samples were also sequenced in duplicate at 50 ng and 100 ng input as well. **Table 2** summarizes the input characteristics of the tested samples. Based on input quantification, both the assay's limit of detection and the optimal RNA input for consistent performance were determined.

Table 2. Analytical Sensitivity Sample Information.

Tumor Type	Input	Replication
Seracare FFPE control	50ng	3
Seracare FFPE control	100ng	3
Seracare FFPE control	250ng	3
Patient sample (C3RX0K3JC.01)	50ng	2
Patient sample (C3RX0K3JC.01)	100ng	2
Patient sample (C3RX2Z958.01)	50ng	2
Patient sample (C3RX2Z958.01)	100ng	2

Results showing total number of reads, reads by genomic origin for all 3 inputs and replicates for the seracare FFPE control and patient samples are shown in **Figure 3a and Figure 3b** respectively. The samples were analyzed and compared to known fusions in the seracare FFPE fusion control reference sample at all 3 inputs. All 16 known fusions in the control were found at all 3 inputs as shown in **Table 3.**

Figure 3a: Reads by Genomic Origin- Seracare control

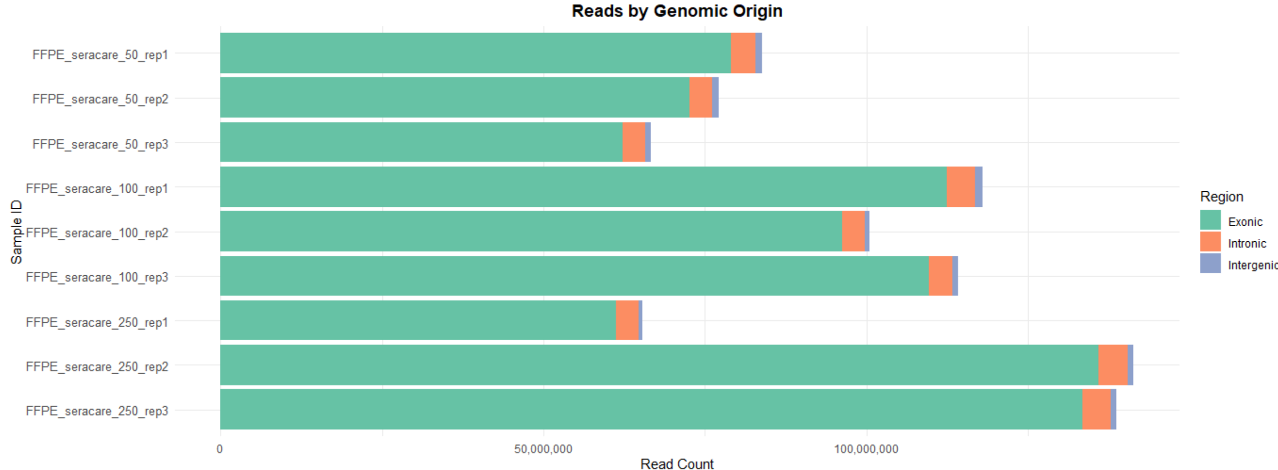


Figure 3b: Reads by Genomic Origin- Patient samples

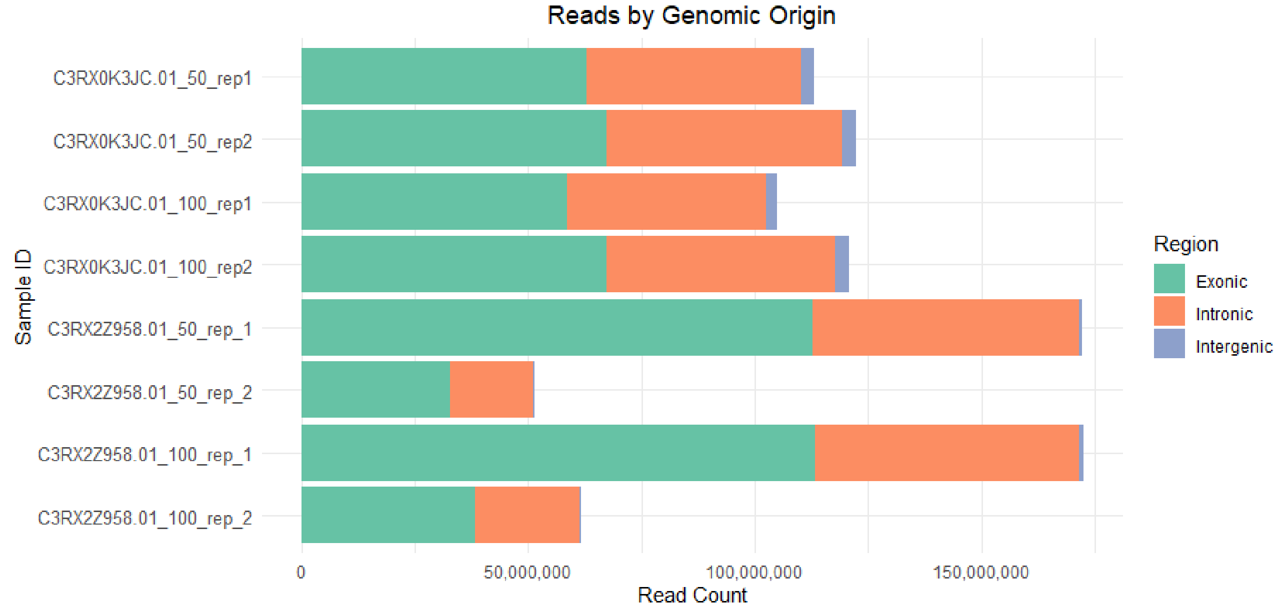


Table 3. Gene Fusion Sensitivity

Expected Fusion	Average per control FFPE_seracare_50 Found	FFPE_seracare_100 Found	FFPE_seracare_250 Found
CCDC6--RET	Yes	Yes	Yes
CD74--ROS1	Yes	Yes	Yes
EGFR--SEPTIN14	Yes	Yes	Yes
EML4--ALK	Yes	Yes	Yes
ETV6--NTRK3	Yes	Yes	Yes
FGFR3--BAIAP2L1	Yes	Yes	Yes
FGFR3--TACC3	Yes	Yes	Yes
KIF5B--RET	Yes	Yes	Yes
LMNA--NTRK1	Yes	Yes	Yes
NCOA4--RET	Yes	Yes	Yes
PAX8--PPARG	Yes	Yes	Yes
SLC34A2--ROS1	Yes	Yes	Yes
SLC45A3--BRAF	Yes	Yes	Yes
TFG--NTRK1	Yes	Yes	Yes
TMPRSS2--ERG	Yes	Yes	Yes
TPM3--NTRK1	Yes	Yes	Yes
Found mutations (Overall)	16	16	16
Sensitivity (Overall)	100%	100%	100%

As seen in **Figure 4a,** with increasing RNA input, the total number of genes expressed increased up to the 250ng run. Additional higher input runs were not performed to determine a plateau since the differences in gene counts among the 3 inputs were negligible. **Figure 4b** shows the total number of genes expressed for the 2 patient samples at 50ng and 100ng. The total number of genes was similar for the same patient sample at 50ng input or 100ng input. Of note is less number of genes observed for the patient sample with DV200 at 26 compared to patient sample with DV200 at 30 at both inputs. The limit of detection based on input quantification for gene expression was assessed by the percentage of data that was within the noise using the genes that are included in the NanoString immune panel used for accuracy study. Noise was defined as TPM below 0.24 (**Table 4).** Based on percentage of the genes within the noise, 100ng was selected as the optimal input for subsequent experiments. Note that one of the replicates for the 250ng input had a technical issue during library preparation procedure so was excluded from the data in **Figure 4a** and **Table 4.**

Figure 4a: Gene Expression Counts for input assessment

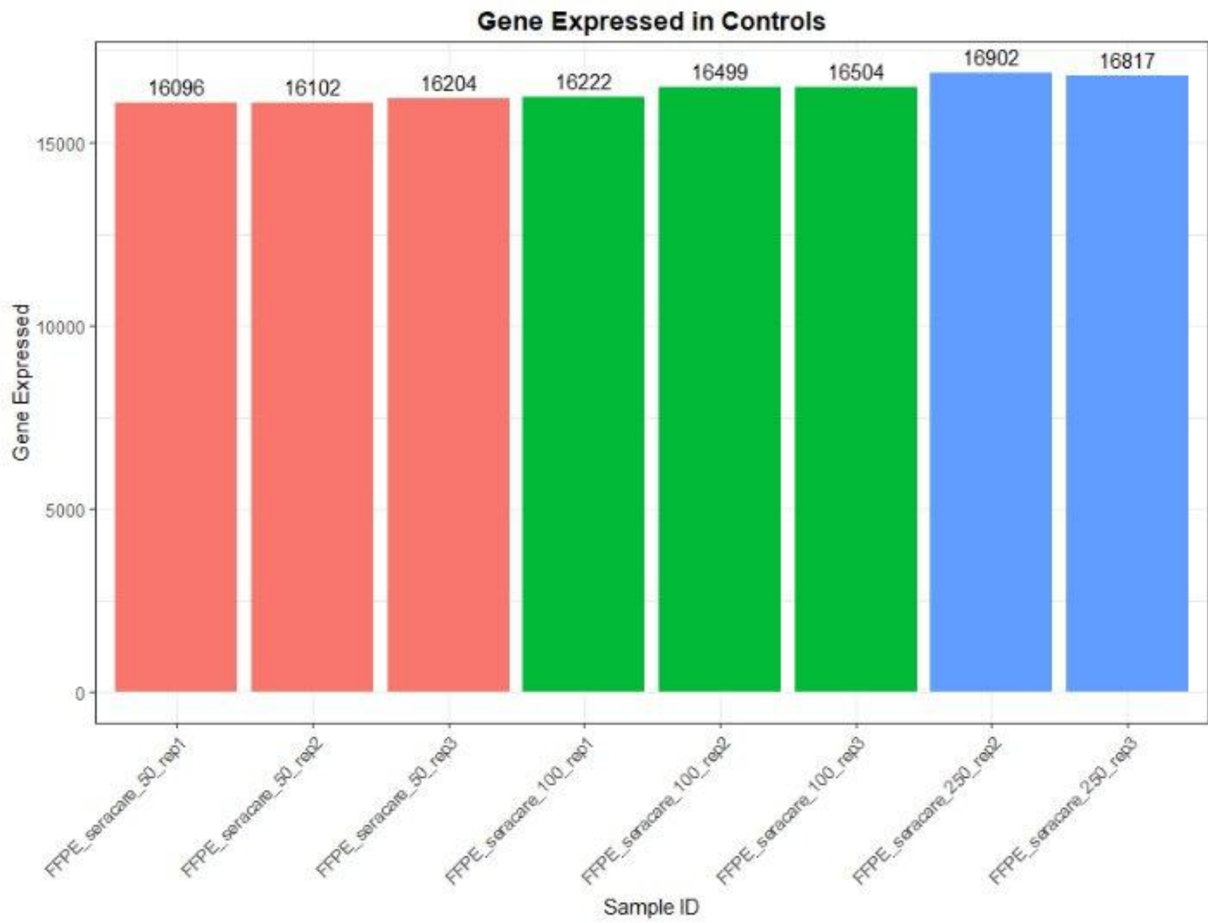


Figure 4b: Gene Expression Counts for input assessment in patient samples

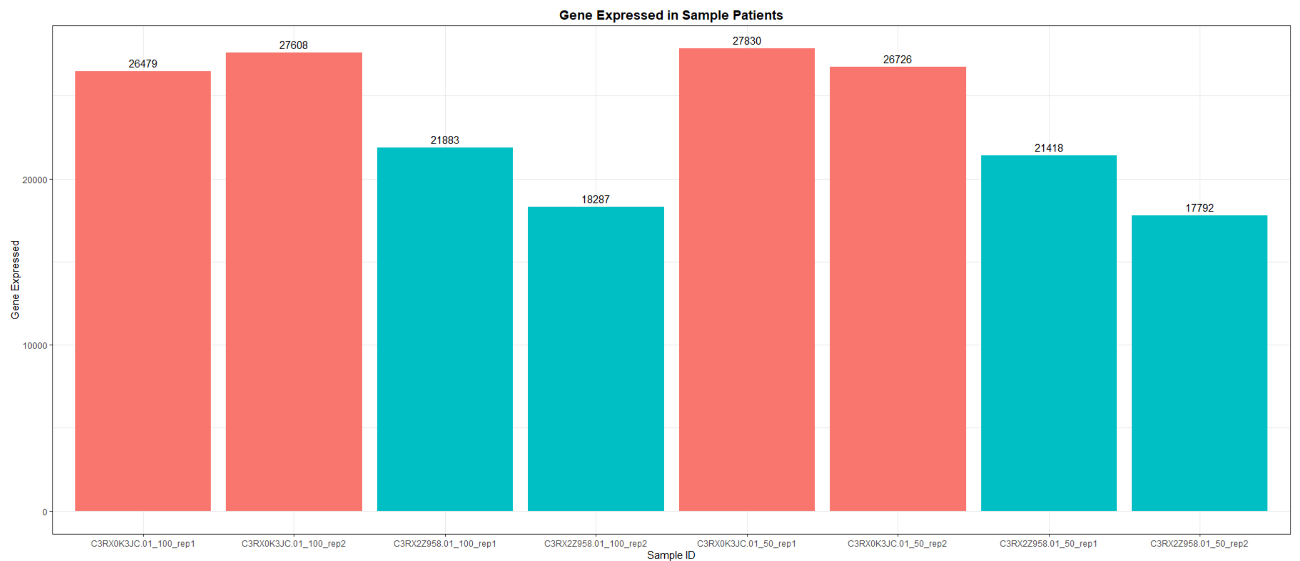


Table 4. Percentage of genes within noise

Sample ID	NanoString Genes in Noise	NanoString Genes Above Noise	Total NanoString Genes detected	% NanoString Genes in Noise
FFPE_seracare_50_rep1	91	527	612	15%
FFPE_seracare_50_rep2	99	536	629	16%
FFPE_seracare_50_rep3	86	533	614	14%
FFPE_seracare_100_rep1	101	535	632	16%
FFPE_seracare_100_rep2	80	542	619	13%
FFPE_seracare_100_rep3	79	551	630	13%
FFPE_seracare_250_rep2	135	551	674	20%
FFPE_seracare_250_rep3	121	553	664	18%

10. *Analytical Specificity: Healthy donor vs control FFPE tumor samples*

To evaluate the analytical specificity of the IDT chemistry and the nf-core bioinformatics pipeline in distinguishing normal from tumor samples, FFPE tumor samples were compared to 20 healthy donor samples, all processed using 100 ng of input RNA.

Figure 5a presents a heatmap of correlation coefficients based on the gene counts for each sample, showing clear clustering of the healthy donor samples, separate from the tumor samples. This distinct separation demonstrates the pipeline's ability to differentiate between normal PBMCs and tumor tissues. **Figure 5b** displays a heatmap of correlation coefficients derived from gene counts across samples, illustrating that samples from the same tumor type generally cluster together, with a few notable exceptions. Tumor type was indicated based on the primary tumor site; however, the samples included a mix of both primary and metastatic sites, thus the tissue type does not always correspond to the designated primary site. This explains why some samples with the same primary tissue designation did not cluster together.

Figure 5a: Clustering of healthy donors compared to patient tumor samples

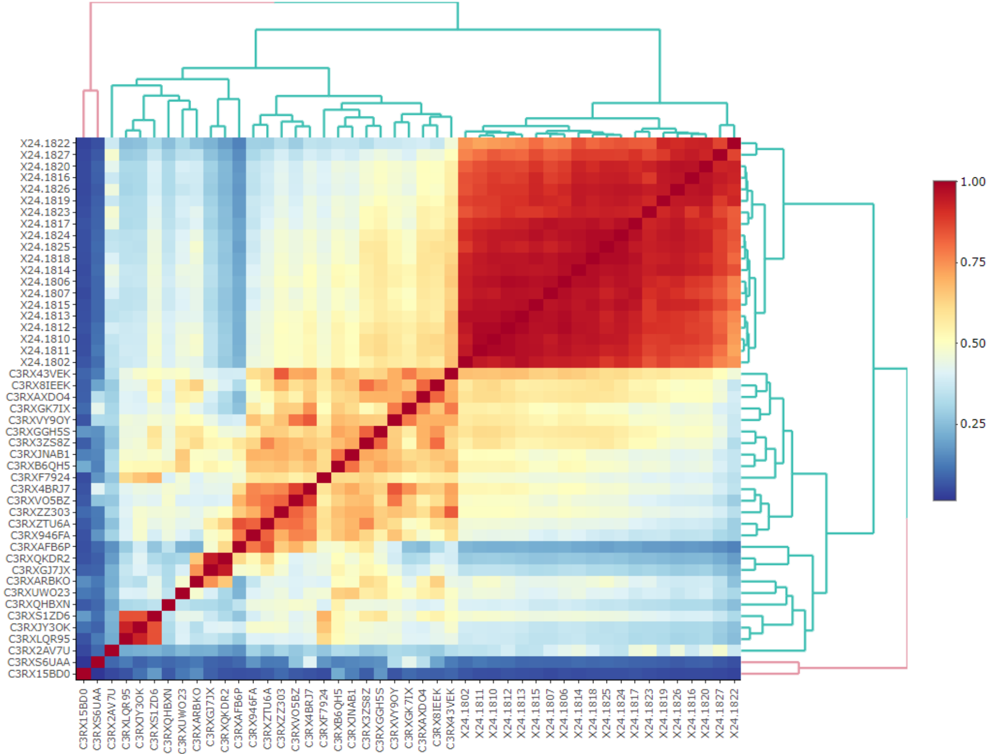
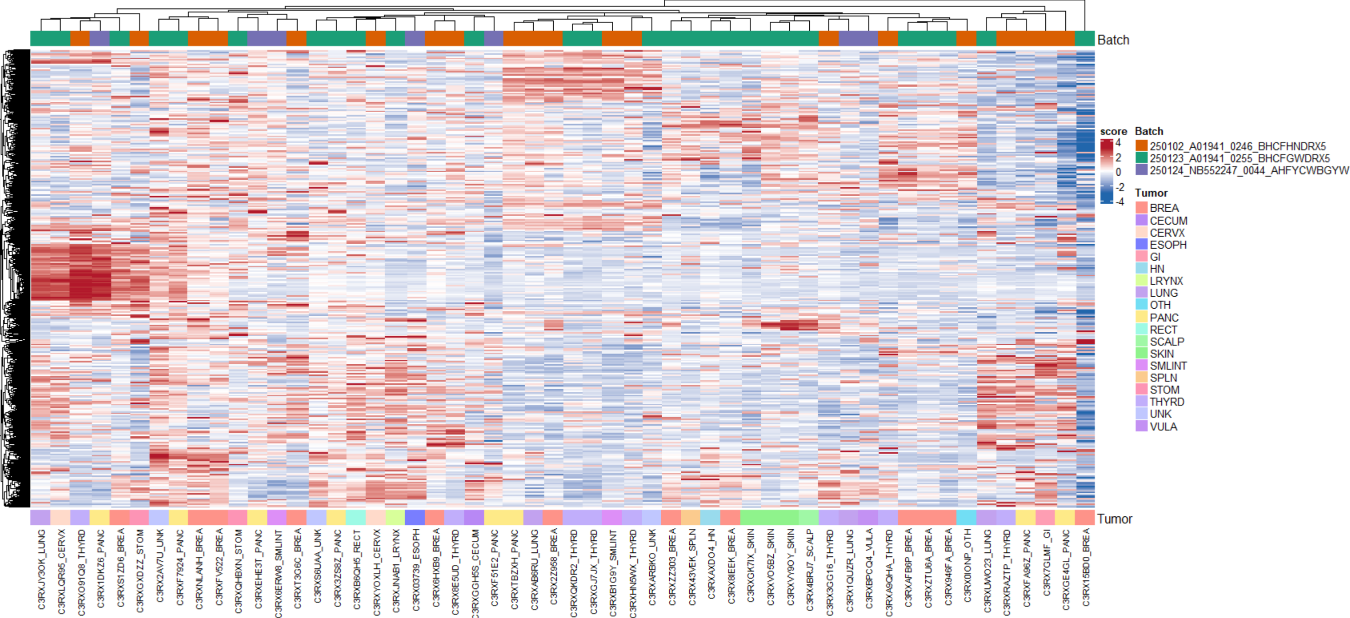


Figure 5b: Clustering of patient tumor samples by tumor type for top 3000 variable genes



11. *Accuracy:*

To assess the accuracy of the IDT RNAseq exome panel and associated bioinformatics pipeline (BIP), 54 tumor RNA samples were sequenced and compared to corresponding NanoString gene expression results. Each sample was sequenced to a target depth of over 50 million reads. Sample distribution by DV200 and total read counts is shown in **Figure 6.** Eight samples fell below the 50 million read threshold, three of which had RNA input amounts below 500 ng for hybridization capture. Spearman correlation coefficients between RNAseq and NanoString data exceeded 0.7 for 47 of the 54 samples, indicating strong concordance. **Figure 7** displays a bar graph showing the distribution of Spearman correlation values across all samples. Detailed correlation plots and coefficients for each sample are provided in **Supplementary Figure 1** in the Appendix. Note that while DV200 is a useful quality metric, other technical and biological factors can also influence cross-platform correlation as seen with samples with spearman correlation <0.7 but with DV200>40.

Figure 6: Distribution of tumor samples based on DV200

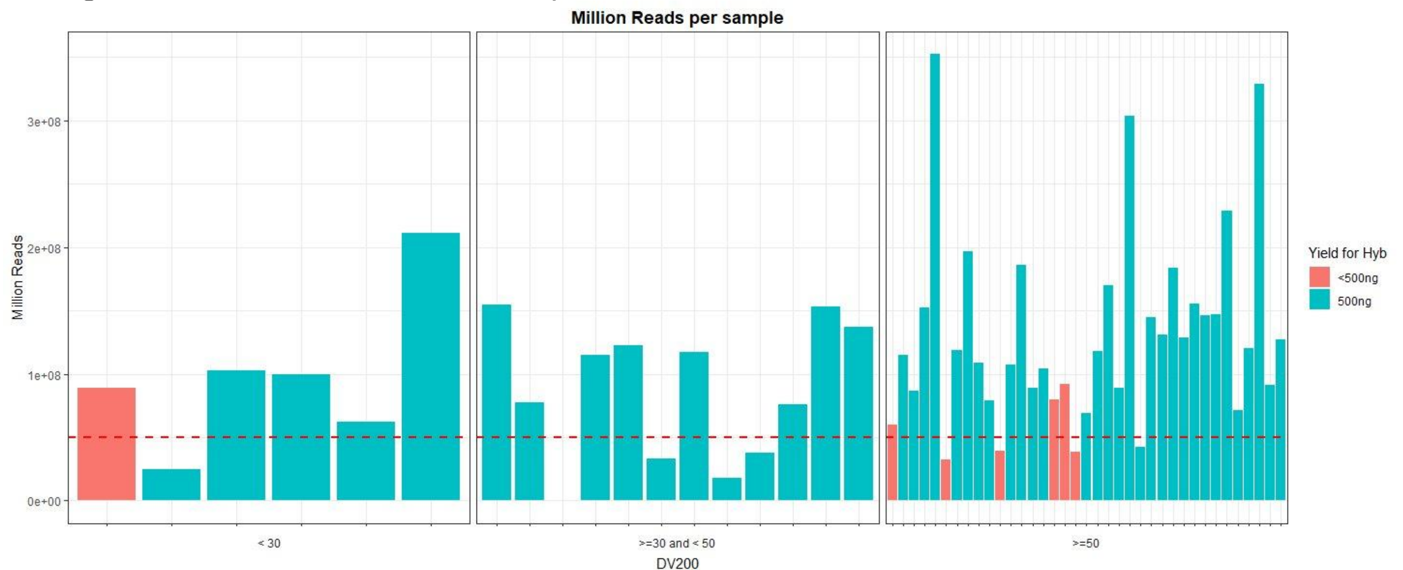
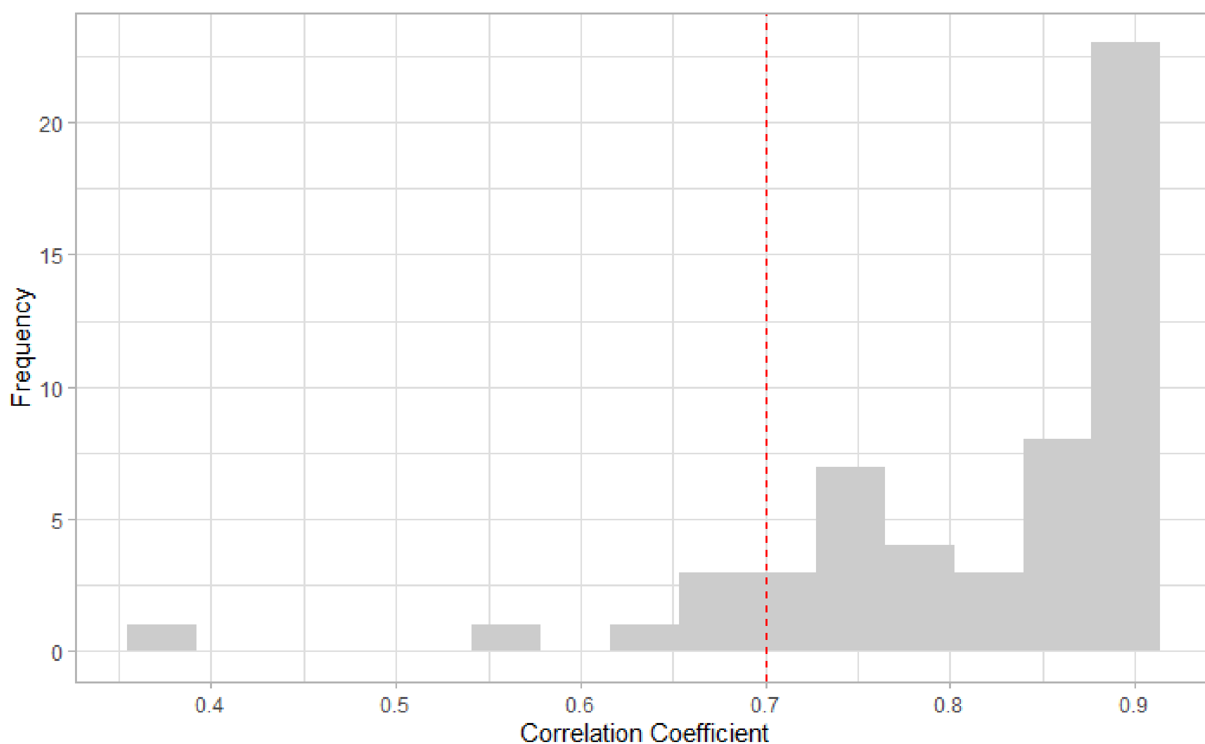


Figure 7: Spearman correlation Between NanoString and RNAseq for 54 patient samples.



12. *Precision: Reproducibility*

Two measures of precision were determined, intra-assay precision and inter assay precision.

<u>Intra assay precision:</u> To determine intra-assay precision, SeraCare FF_T, FFPE_T, and FFPE_WT controls (Seraseq® reference materials) were run in triplicate with RNA inputs of 100 ng for FFPE and 50 ng for FF. Additionally, two patient samples were run in duplicate at both 50ng and 100ng inputs. The total number of genes for the patient samples in 2 runs at 100ng input are shown in **Table 5.** Intra-assay precision for the patient samples, assessed by Pearson's correlation coefficient, was high (>0.95) for duplicates run within the same sequencing run, as shown in **Figure 8**. **Figure 9** shows the total reads and gene counts by genomic region for the SeraCare reference samples run in triplicate per run. The fraction of reads mapping to intronic, exonic and intergenic regions across the 3 runs are detailed in **Supplementary Table 3, Supplementary Table 4 and Supplementary Table 5** respectively. The number of genes detected for the SeraCare reference samples is presented in **Table 6**. Intra-assay precision for the SeraCare reference samples, measured across triplicates within the same run, was similarly high, with Pearson's correlation coefficients exceeding 0.95, as shown in **Figure 10.**

Table 5: The total number of genes for the patient samples

Input (ng)	Sample ID	Replicate	Run 1	Run 2	Mean	Sample Mean
100	C3RX0K3JC.01	1	29,005.00	26,479.00	27,742.00	27,675.00
		2	-	27,608.00	27,608.00	
	C3RX2Z958.01	1	21,822.00	21,883.00	21,852.50	20,069.75
		2	-	18,287.00	18,287.00	

Figure 8: Intra-assay Precision for patient samples

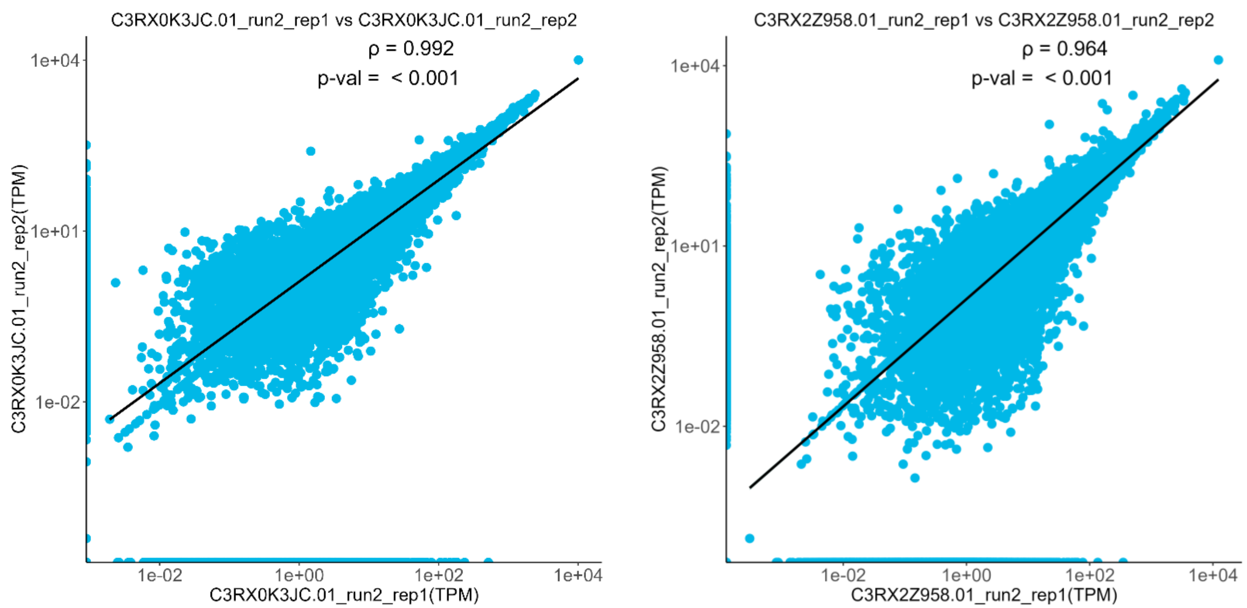


Figure 9: SeraCare ref samples genes by genomic region and reads per run

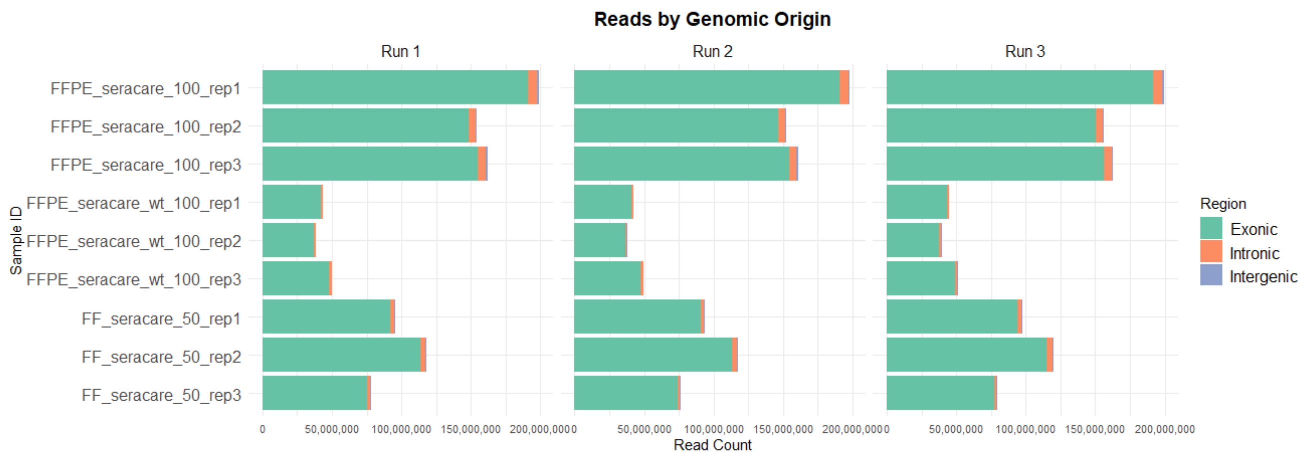
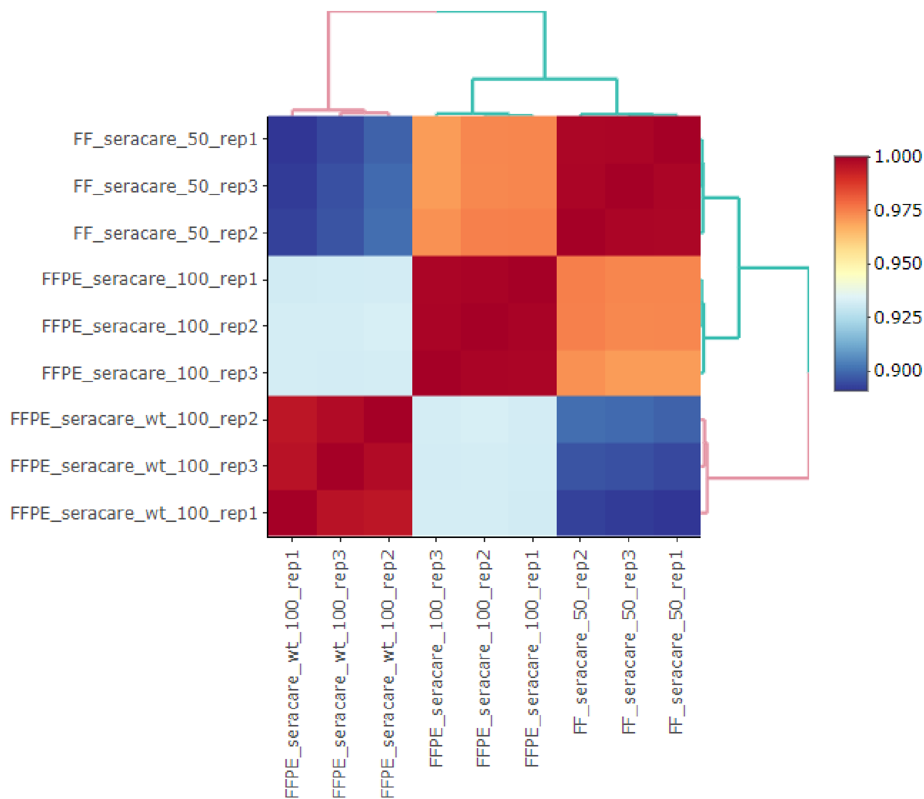


Table 6: Total number of genes for Seracare reference samples

Input (ng)	Type	Replicate	Run 1	Run 2	Run 3	Mean	Sample Mean
100	FFPE Seracare Tumor	1	22,248.00	21,888.00	22,294.00	22,143.33	21,794.00
		2	21,800.00	21,483.00	21,845.00	21,709.33	
		3	21,597.00	21,286.00	21,705.00	21,529.33	
	FFPE Seracare WT	1	20,202.00	20,025.00	20,435.00	20,220.67	20,344.67
		2	20,462.00	20,201.00	20,577.00	20,413.33	
		3	20,421.00	20,208.00	20,571.00	20,400.00	
50	FF Seracare Tumor	1	20,137.00	19,862.00	20,268.00	20,089.00	20,142.00
		2	20,941.00	20,559.00	21,007.00	20,835.67	
		3	19,529.00	19,320.00	19,655.00	19,501.33	

Figure 10: Intra-assay Precision for SeraCare Reference Samples



Inter-assay precision: Building on the previously demonstrated high intra-assay precision, inter-assay precision was evaluated by running the same SeraCare controls (FF_T, FFPE_T, and FFPE_WT) in triplicate across three independent sequencing runs to assess consistency between runs. Additionally, two patient samples were run in duplicate at 50 ng and 100 ng input across two separate runs.

The total number of genes for the patient samples in 2 runs at 100ng input are shown in **Table 5.** Inter-assay precision across patient samples remained high. For samples with DV200 values greater than 30, Pearson's correlation coefficients exceeded 0.9.The sample with DV200 = 30 showed a coefficient of 0.785 (**Figures 11a and 11b**).

Similarly, inter-assay precision for the SeraCare reference samples, run in triplicate across three separate runs, showed strong reproducibility with Pearson's correlation coefficients exceeding 0.9, as shown in **Figure 12.**

Figure 11a: Inter assay precision- patient samples

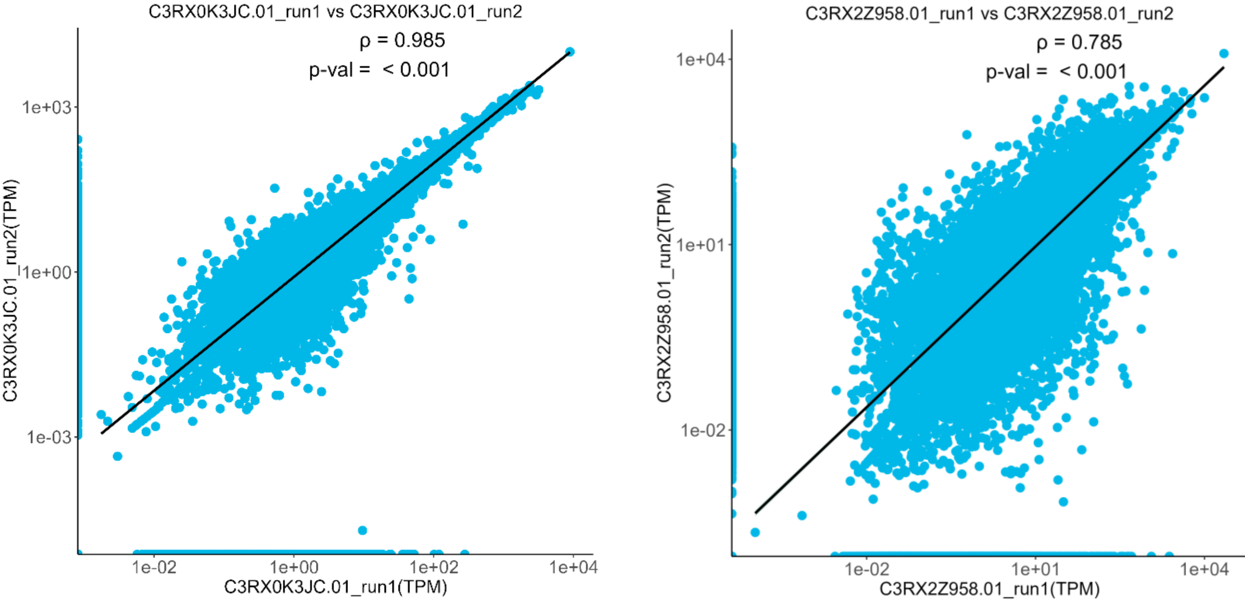


Figure 11b: Inter assay precision- additional patient samples

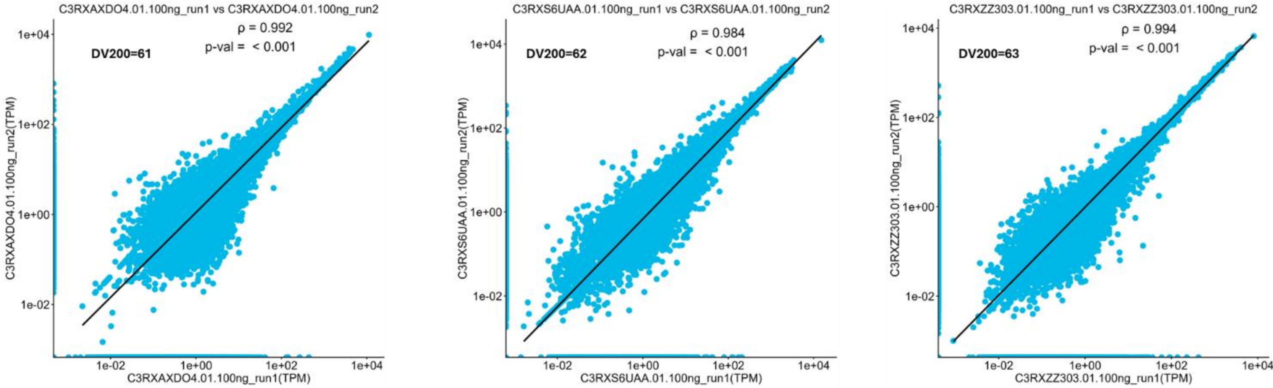
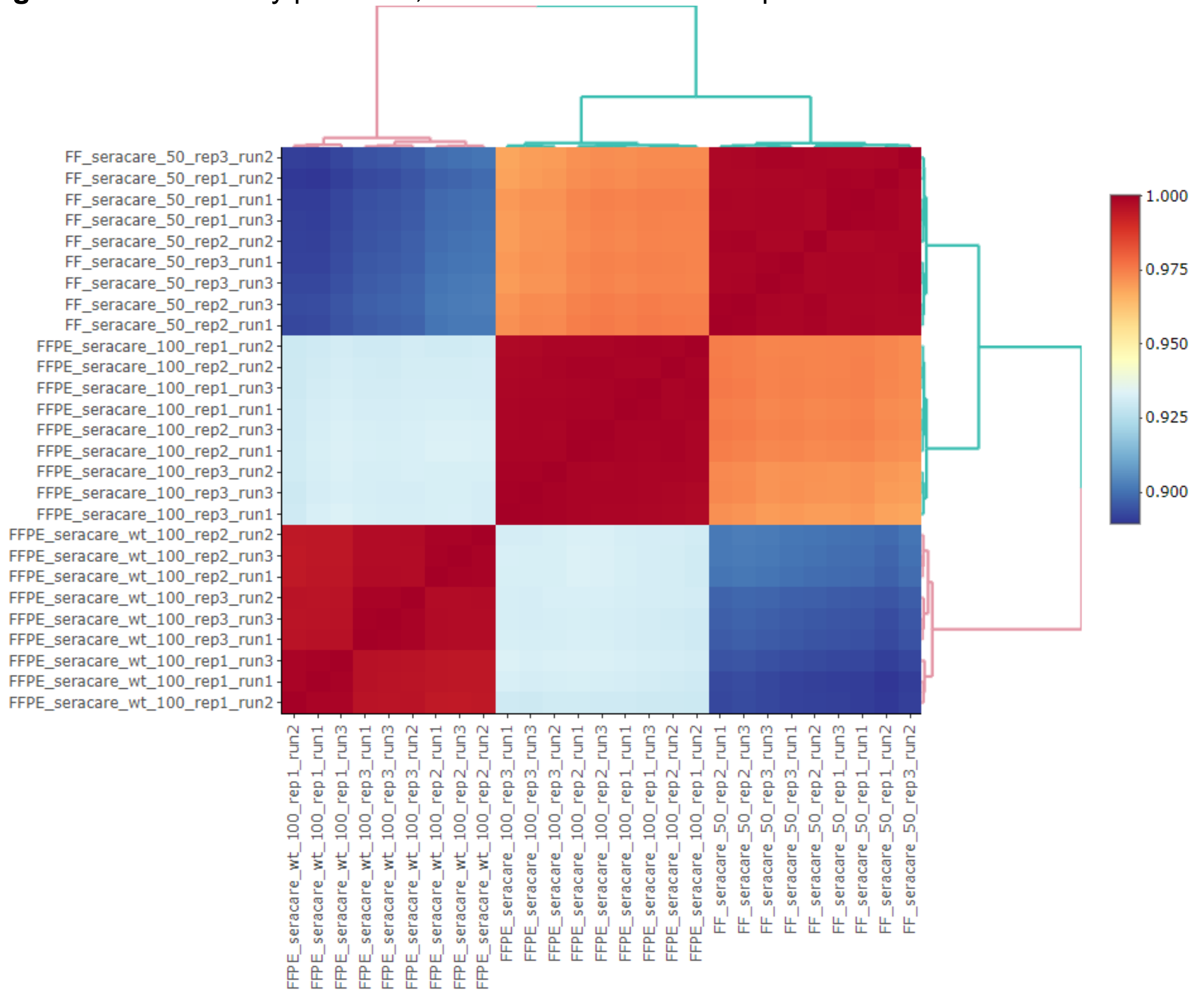


Figure 12: Inter assay precision, SeraCare reference samples



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[Signatures] and [dates]

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Name: *Dzifa Duose, PhD*
Title: *Research Group Leader*

Signature/date: ______________________________
Name: *Cara Haymaker, PhD*
Title: *Associate Professor*

APPENDIX

Supplementary Table 1. Study Sample Cohort and Quality Control

Sample Type	Sample IDs	GTDL DV200	GTDL ng/ul	RIN#	Tissue Type
Reference Material	Seracare ctrl FF fusion	92.62	25	9.9	FF RNA
Reference Material	Seracare ctrl FF fusion	92.62	25	9.9	FF RNA
Reference Material	Seracare ctrl FF fusion	92.62	25	9.9	FF RNA
Reference Material	Seracare ctrl FFPE fusion	74.81	30.3	2.8	FFPE RNA
Reference Material	Seracare ctrl FFPE fusion	74.81	30.3	2.8	FFPE RNA
Reference Material	Seracare ctrl FFPE fusion	74.81	30.3	2.8	FFPE RNA
Reference Material	Seracare ctrl FFPE WT	47.55	16.7	1.4	FFPE RNA
Reference Material	Seracare ctrl FFPE WT	47.55	16.7	1.4	FFPE RNA
Reference Material	Seracare ctrl FFPE WT	47.55	16.7	1.4	FFPE RNA
Patient sample	C3RXF51E2.01	53.2	32.8	NA	FFPE RNA
Patient sample	C3RX03739.01	64.3	20.2	NA	FFPE RNA
Patient sample	C3RXBPCQ4.01	58.6	26.4	NA	FFPE RNA
Patient sample	C3RXEHE3T.01	59.61	38.6	NA	FFPE RNA
Patient sample	C3RX1DKZ6.01	66.04	30.4	NA	FFPE RNA
Patient sample	C3RX1QUZR.01	58.61	32.8	NA	FFPE RNA
Patient sample	C3RX6ERW8.01	51.47	38.6	NA	FFPE RNA
Patient sample	C3RXJQGMM.01	40.58	35.2	NA	FFPE RNA
Patient sample	C3RXUWO23.01	64.24	35.2	NA	FFPE RNA
Patient sample	C3RXF7924.01	56	26.6	NA	FFPE RNA
Patient sample	C3RXLQR95.01	61.42	23.6	NA	FFPE RNA
Patient sample	C3RX3ZS8Z.01	70.4	53.8	NA	FFPE RNA
Patient sample	C3RXB6QH5.01	72.22	28	NA	FFPE RNA
Patient sample	C3RXJY3OK.01	65.16	37.2	NA	FFPE RNA
Patient sample	C3RXGGH5S.01	67.68	48.6	NA	FFPE RNA
Patient sample	C3RXJNAB1.01	69.36	32.4	NA	FFPE RNA
Patient sample	C3RXYOXLH.01	73.9	55	NA	FFPE RNA
Patient sample	C3RX7GLMF.01	69.52	30.4	NA	FFPE RNA
Patient sample	C3RXGE4GL.01	61.34	41.4	NA	FFPE RNA
Patient sample	C3RXGXDZZ.01	59.66	41.4	NA	FFPE RNA
Patient sample	C3RXFA96Z.01	22.97	41.2	NA	FFPE RNA
Patient sample	C3RXAB6RU.01	58.38	15.1	NA	FFPE RNA
Patient sample	C3RXTBZXH.01	69.51	16.4	NA	FFPE RNA
Patient sample	C3RX2Z958.01	26.00	42.8	NA	FFPE RNA
Patient sample	C3RXNLANH.01	26.00	58.8	NA	FFPE RNA
Patient sample	C3RX0IONP.01	43.78	67	NA	FFPE RNA
Patient sample	C3RX8E5UD.01	53.57	36.2	NA	FFPE RNA
Patient sample	C3RXB1G9Y.01	58.52	67.4	NA	FFPE RNA
Patient sample	C3RX3GG16.01	43.00	49.4	NA	FFPE RNA
Patient sample	C3RXA9QHA.01	39.00	50.6	NA	FFPE RNA
Patient sample	C3RX0K3JC.01	38.00	61	NA	FFPE RNA
Patient sample	C3RXRAZTP.01	52.00	64.2	NA	FFPE RNA

Patient sample	C3RXT3G6C.01	53.00	29.8	NA	FFPE RNA
Patient sample	C3RXHN5WX.01	46.00	52.4	NA	FFPE RNA
Patient sample	C3RXO91Q8.01	44.00	63.8	NA	FFPE RNA
Patient sample	C3RX6HXB9.01	57.00	53.8	NA	FFPE RNA
Patient sample	C3RXFV522.01	61.00	59.8	NA	FFPE RNA
Patient sample	C3RXZTU6A.01	26.00	58.8	NA	FFPE RNA
Patient sample	C3RXS6UAA.01	62.00	64.2	NA	FFPE RNA
Patient sample	C3RX15BD0.01	41.00	50.4	NA	FFPE RNA
Patient sample	C3RX2AV7U.01	40.00	14.8	NA	FFPE RNA
Patient sample	C3RX946FA.01	45.00	17.9	NA	FFPE RNA
Patient sample	C3RXLQ3JG.01	31	58	NA	FFPE RNA
Patient sample	C3RXGJ7JX.01	39	59.8	NA	FFPE RNA
Patient sample	C3RXQHBXN.01	62	64.6	NA	FFPE RNA
Patient sample	C3RXAFB6P.01	60.00	77	NA	FFPE RNA
Patient sample	C3RXGK7IX.01	67	39.6	NA	FFPE RNA
Patient sample	C3RX43VEK.01	59	58.2	NA	FFPE RNA
Patient sample	C3RXS1ZD6.01	38	58.8	NA	FFPE RNA
Patient sample	C3RXQKDR2.01	28.00	43.6	NA	FFPE RNA
Patient sample	C3RXARBKO.01	35.00	62.2	NA	FFPE RNA
Patient sample	C3RXVY9OY.01	58	27.6	NA	FFPE RNA
Patient sample	C3RX8IEEK.01	63	54.8	NA	FFPE RNA
Patient sample	C3RX4BRJ7.01	24	39.8	NA	FFPE RNA
Patient sample	C3RXAXDO4.01	61	60	NA	FFPE RNA
Patient sample	C3RXZZ303.01	70	62.8	NA	FFPE RNA
Patient sample	C3RXVO5BZ.01	57	15.7	NA	FFPE RNA
Healthy donor sample	24-1802	65.84	60	8.7	PBMC RNA
Healthy donor sample	24-1806	59.04	35.8	8.5	PBMC RNA
Healthy donor sample	24-1807	60.1	51	9.7	PBMC RNA
Healthy donor sample	24-1810	69.28	143	9.3	PBMC RNA
Healthy donor sample	24-1811	67.95	125	8.5	PBMC RNA
Healthy donor sample	24-1812	56.54	74	9.7	PBMC RNA
Healthy donor sample	24-1813	58.88	89	9.5	PBMC RNA
Healthy donor sample	24-1814	51.32	32.1	9.5	PBMC RNA
Healthy donor sample	24-1815	60.14	58	9.7	PBMC RNA
Healthy donor sample	24-1816	55.6	44.7	9.6	PBMC RNA
Healthy donor sample	24-1817	52.13	24.4	9.7	PBMC RNA
Healthy donor sample	24-1818	57.16	64.2	9.5	PBMC RNA
Healthy donor sample	24-1819	53.66	38.8	9.3	PBMC RNA
Healthy donor sample	24-1820	51.26	25.2	9.1	PBMC RNA
Healthy donor sample	24-1822	51	15.7	9.4	PBMC RNA
Healthy donor sample	24-1823	51.72	25.4	9.2	PBMC RNA
Healthy donor sample	24-1824	50.79	41.8	9.6	PBMC RNA
Healthy donor sample	24-1825	55.69	33.2	9.3	PBMC RNA
Healthy donor sample	24-1826	53.94	15.8	8.8	PBMC RNA
Healthy donor sample	24-1827	56.95	29	9.1	PBMC RNA

Supplementary Figure 1: Correlation Plots of NanoString versus RNAseq for 54 Samples

Supplementary Table 2: Fraction of Mapped Reads in Intronic Region

Input (ng)	Type	Replicate	Run 1	Run 2	Run 3	Mean	Sample Mean
100	FFPE Seracare Tumor	1	0.033	0.032	0.033	0.032	0.033
		2	0.034	0.033	0.033	0.033	
		3	0.035	0.034	0.035	0.035	
	FFPE Seracare WT	1	0.033	0.032	0.033	0.033	0.034
		2	0.035	0.034	0.036	0.035	
		3	0.036	0.035	0.036	0.035	
50	FF Seracare Tumor	1	0.030	0.028	0.030	0.029	0.030
		2	0.033	0.032	0.033	0.032	
		3	0.029	0.028	0.029	0.028	

Supplementary Table 3: Fraction of Mapped Reads in Exonic Region

Input (ng)	Type	Replicate	Run 1	Run 2	Run 3	Mean	Sample Mean
100	FFPE Seracare Tumor	1	0.961	0.962	0.961	0.961	0.960
		2	0.960	0.961	0.960	0.961	
		3	0.959	0.960	0.959	0.959	
	FFPE Seracare WT	1	0.960	0.961	0.960	0.961	0.958
		2	0.957	0.958	0.957	0.957	
		3	0.958	0.958	0.957	0.958	
50	FF Seracare Tumor	1	0.964	0.965	0.963	0.964	0.963
		2	0.960	0.961	0.960	0.960	
		3	0.965	0.965	0.965	0.965	

Supplementary Table 4: Fraction of Mapped Reads in Intergenic Region

Input (ng)	Type	Replicate	Run 1	Run 2	Run 3	Mean	Sample Mean
100	FFPE Seracare Tumor	1	0.006	0.006	0.006	0.006	0.006
		2	0.006	0.006	0.006	0.006	
		3	0.006	0.006	0.006	0.006	
	FFPE Seracare WT	1	0.007	0.007	0.007	0.007	0.007
		2	0.007	0.008	0.008	0.008	
		3	0.007	0.007	0.007	0.007	
50	FF Seracare Tumor	1	0.007	0.007	0.007	0.007	0.007
		2	0.007	0.007	0.007	0.007	
		3	0.007	0.007	0.007	0.007	