

Cell-free RNA Biomarker Detection from Liquid Biopsy and Urine

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ABSTRACT

Cell-free nucleic acids can be used as robust biomarkers to determine disease development and progression. In general, use of cell free RNA (cfRNA) is ideal for biomarker identification for use in screening, diagnostics, treatment monitoring, and tumor tissue of origin prediction for oncology samples. Applications vary widely with liquid biopsy being an efficient method to detect specific cfRNA for a wide range of conditions including several neurodegenerative disorders like Amyotrophic lateral sclerosis (ALS). Alternatively, urine is an ultra-non-invasive analyte ideal for urogenital cancer detection, including prostate cancer. Here we describe cfRNA extraction and functional RNA-Seq analysis on two ideal minimally/non-invasive sample types, liquid biopsy and urine.

Preliminary testing of the cfRNA isolation method was first applied to a series of liquid biopsy control samples spiked with miRNA. Traditional methods of cfRNA isolation may be unable to achieve sufficient yields to detect the low allele frequency rates required for liquid biopsy testing. Several extraction kits were tested ,including a novel cfRNA isolation technique that has highly efficient recovery rates of cfRNA with variable input amounts of 1-100 mL of sample.

Following successful pilot studies with control samples, cfRNA was isolated from 5 liquid biopsy samples: 4 diagnosed ALS and one healthy control. The cfRNA was then subjected to highly sensitive RNA-Seq to evaluate a series of ALS-associated biomarkers, including cryptic introns: an intron that is not spliced out and is released into the blood stream in patients with ALS.

A secondary series of samples derived from patients with urogenital cancers and healthy controls were evaluated for cfRNA extraction and RNA-Seq. Extracted cfRNA underwent both quantitative and qualitative quality control, followed by functional evaluation using RNA-Seq to assess yield, transcriptome complexity, and detection of known cancer-associated biomarkers. RNA-Seq results demonstrated detection of rare transcripts and cancer-associated biomarkers, confirming this is a reliable method for biomarker detection.

As these results show, cfRNA can be extracted from both plasma and urine, with the detection of rare biomarkers associated with disease. The high-efficiency extraction method enables sensitive detection of low-frequency transcripts, addressing limitations of conventional techniques.

METHODS

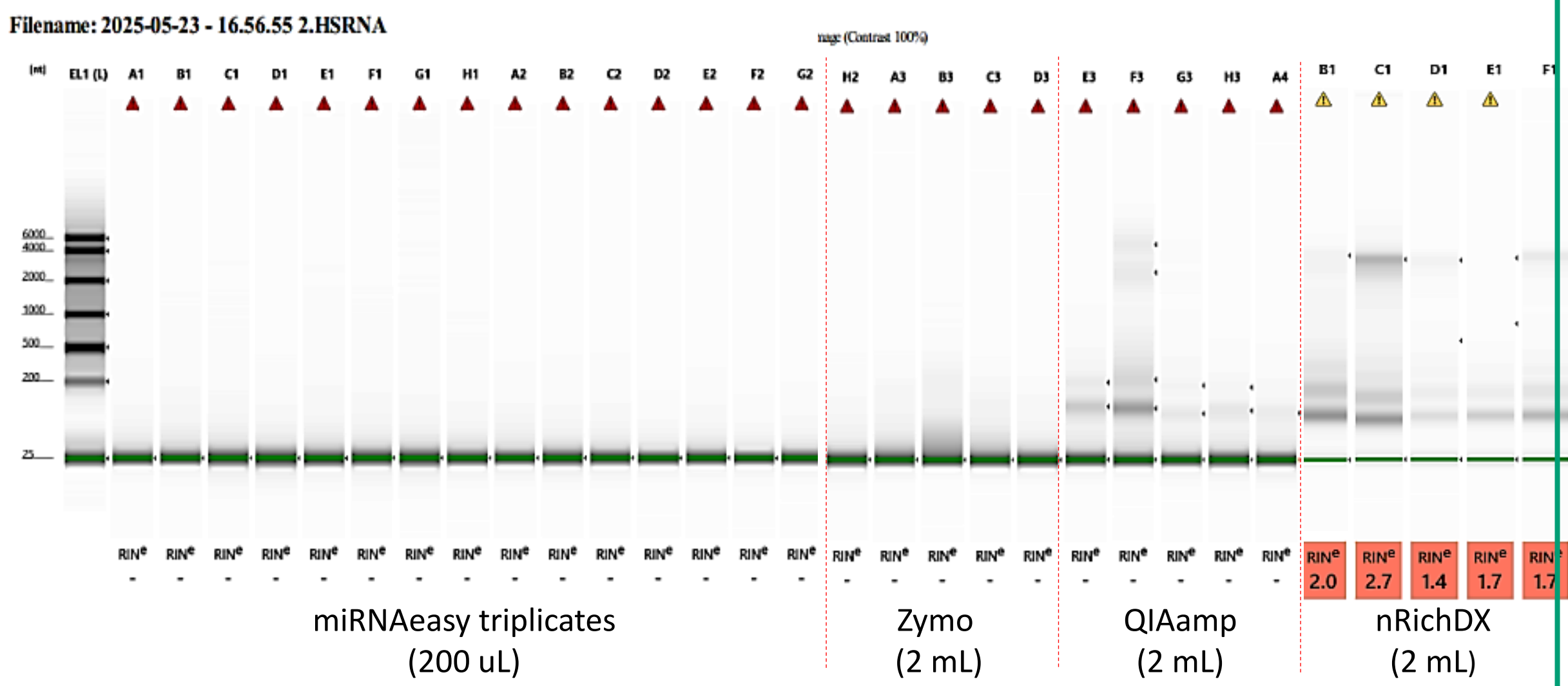
Workflow for cell-free RNA sequencing from plasma (Left). Five plasma samples (four ALS cases and one healthy control) were evaluated using four RNA extraction kits to identify the optimal method. MiRNeasy and QIAamp yielded no detectable RNA, Zymo produced detectable RNA levels, and nRichDX achieved 2- to 5-fold higher yields with consistently measurable RNA. Samples extracted with nRichDX, along with a universal human reference control (UHC), underwent library preparation using either the Takara SMART-Seq Total RNA kit or the Thermo Fisher Ion AmpliSeq Transcriptome panel. Libraries were sequenced to approximately 20 million reads per sample and analyzed for quality metrics and detection of ALS-associated cryptic introns.

Workflow for cell-free RNA sequencing from urine (Right). Twenty-four urine samples (four positive conditions analyzed in triplicate and 12 matched negative controls) were processed using the nRichDX extraction method, selected based on its superior performance in plasma cfRNA extraction. Extracted RNA was prepared using three library construction methods: Takara SMART-Seq Total RNA, Thermo Fisher Ion AmpliSeq Transcriptome, and Twist RNA Exome. Libraries were sequenced to approximately 20 million reads per sample and analyzed for quality metrics and detection of prostate cancer-associated transcripts.

In collaboration with:

CELL-FREE RNA DETECTION FROM PLASMA

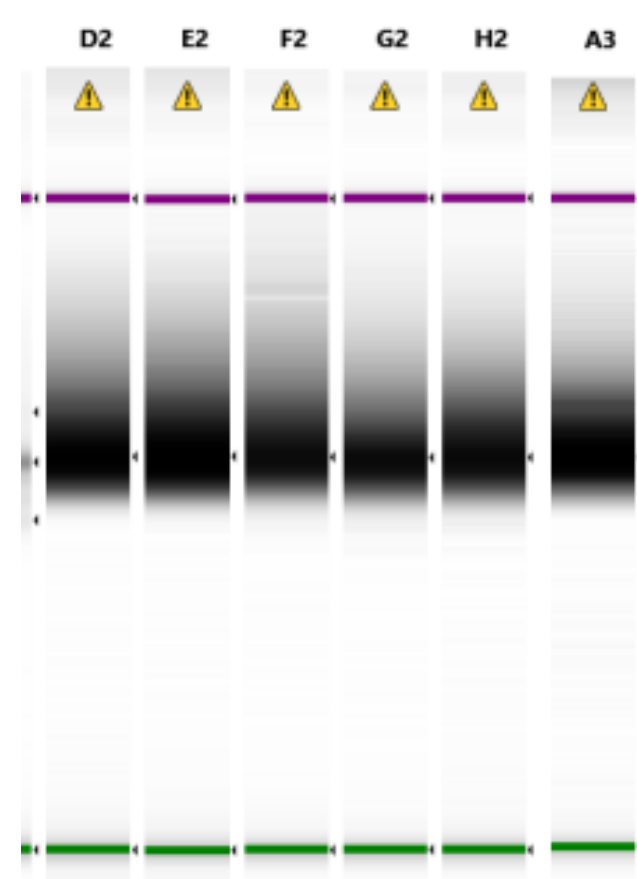
EXTRACTION CHARACTERIZATION				
High Sensitivity TapeStation (pg/uL)				
	miRNeasy	Zymo	QIAamp	nRichDX
ALS-1	38.7	30.1	86.0	491.0
ALS-2	37.4	31.1	255.0	582.0
ALS-3	35.2	62.5	55.3	174.0
ALS-4	25.5	37.1	46.5	189.0
Healthy-1	22.6	22.1	43.7	250.0



ThermoFisher AmpliSeq



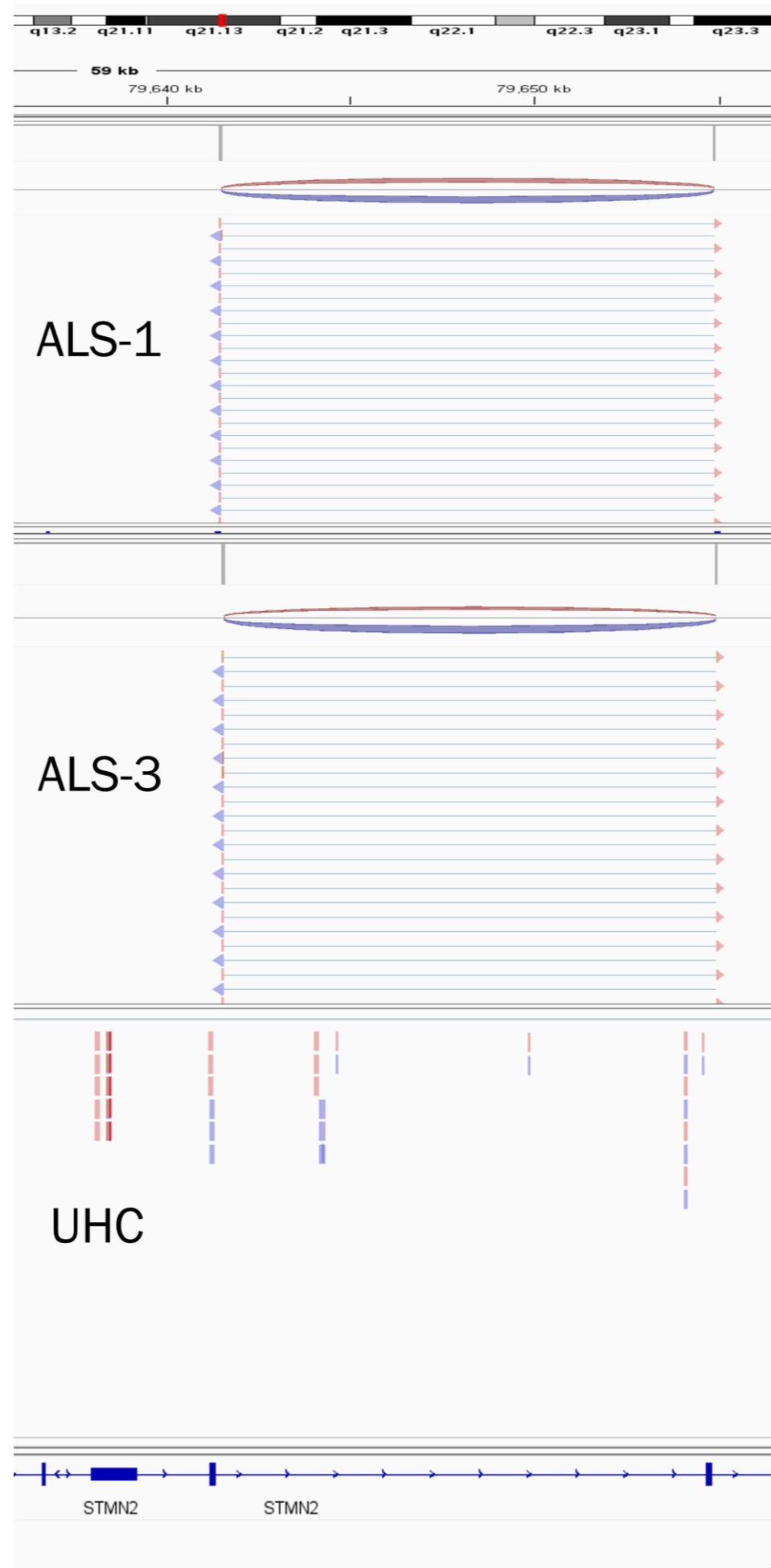
Takara Pico



AmpliSeq		STMN2	HDGFL2
	ALS-1	0.4061	6.444
	ALS-2	0	6.0459
	ALS-3	0.3992	3.3923
	ALS-4	0	3.5663
	Healthy-1	0	10.5178
	UHC	0	1.7699
	NTC	0	0

Takara			
	ALS-1	0	9.124
	ALS-2	0	5.5833
	ALS-3	0	19.0656
	ALS-4	0	8.9622
	Healthy-1	0	7.6992
	UHC	0	1.7364
	NTC	0	0

STMN2 Cryptic Intron Expression



PATIENT SAMPLES



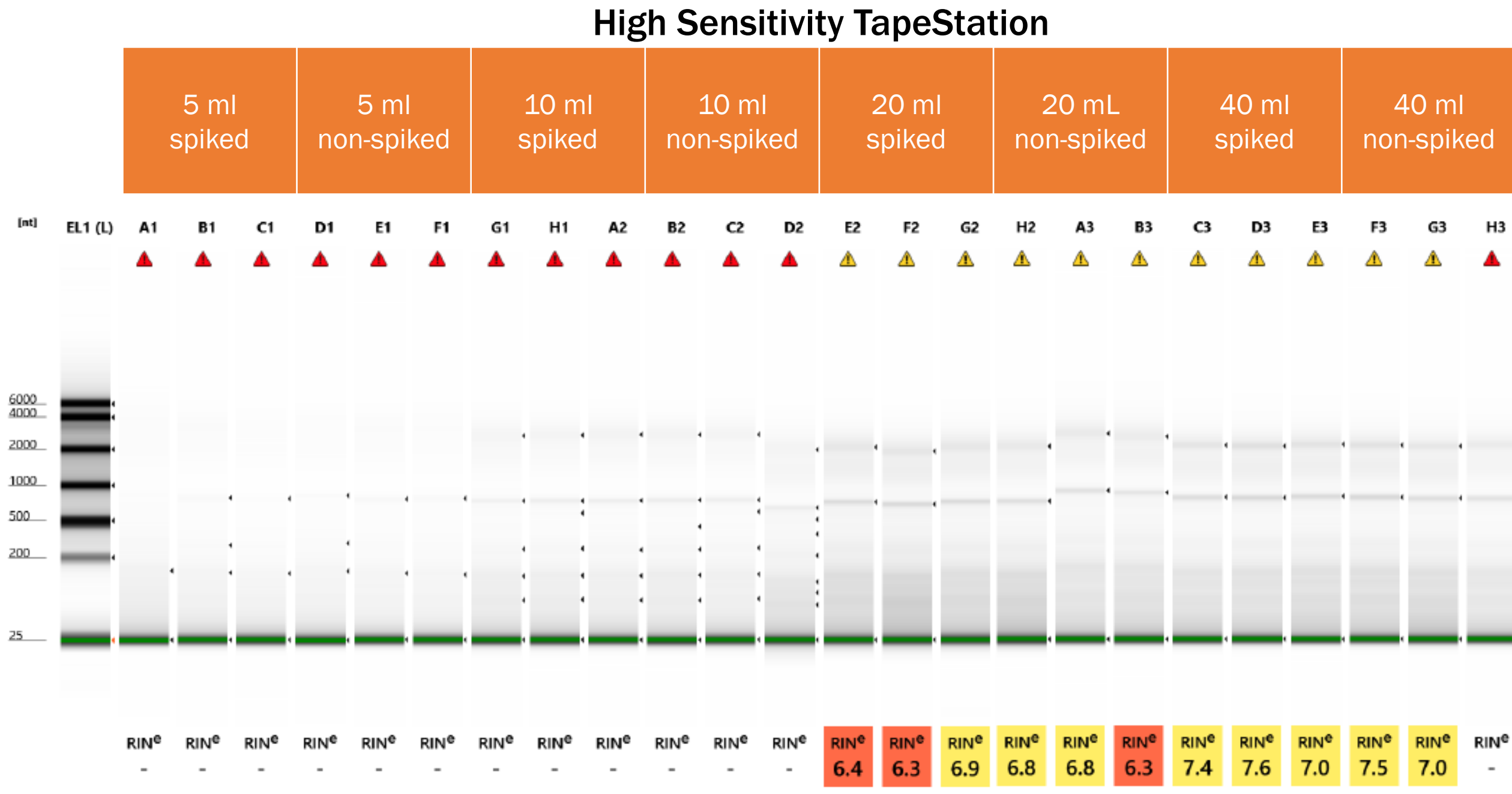
Liquid Biopsy
Extraction
& QC

Library Prep
& QC

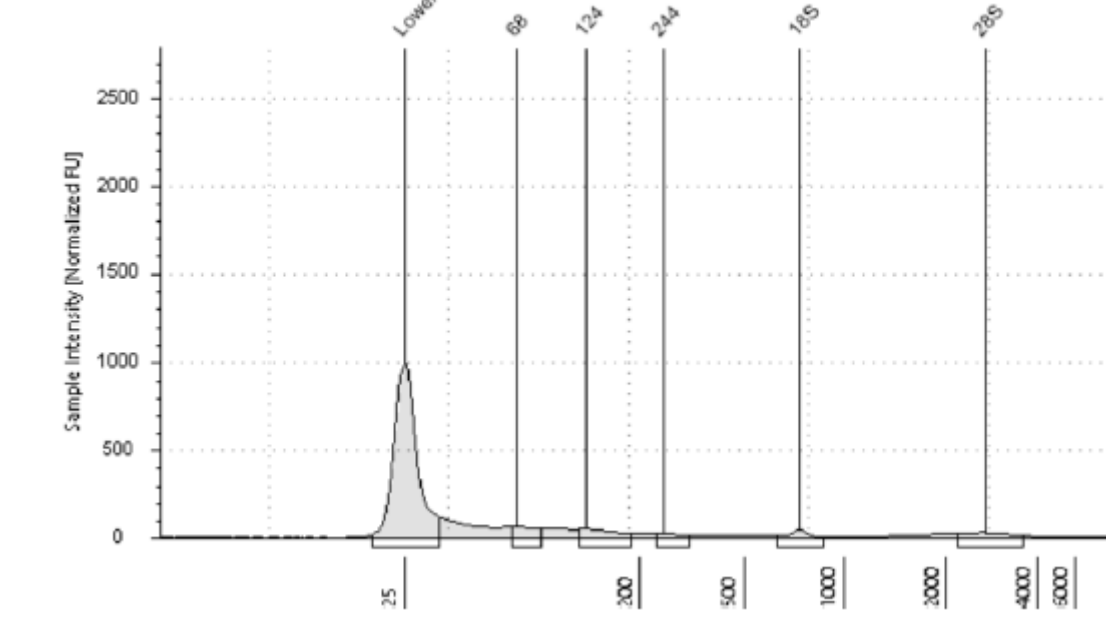
Illumina
Sequencing
& Analysis

CELL-FREE RNA DETECTION FROM URINE

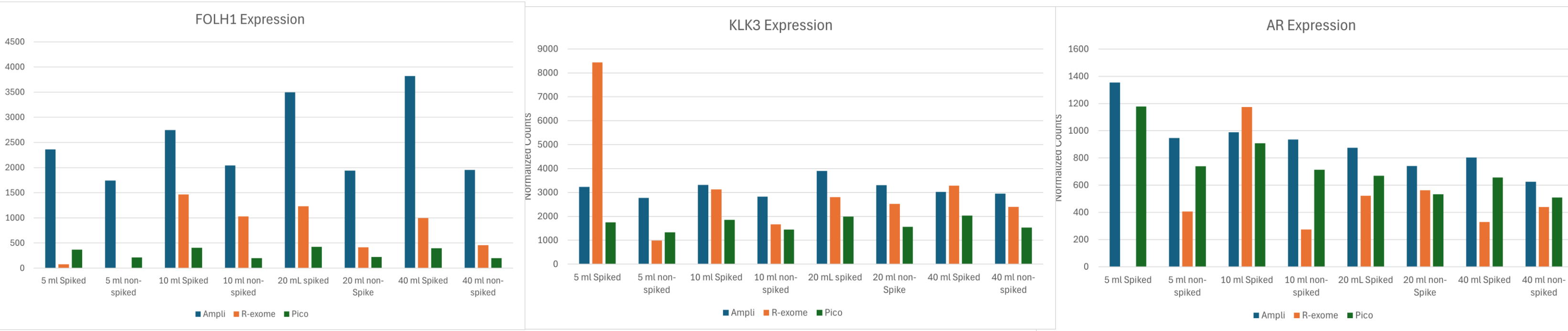
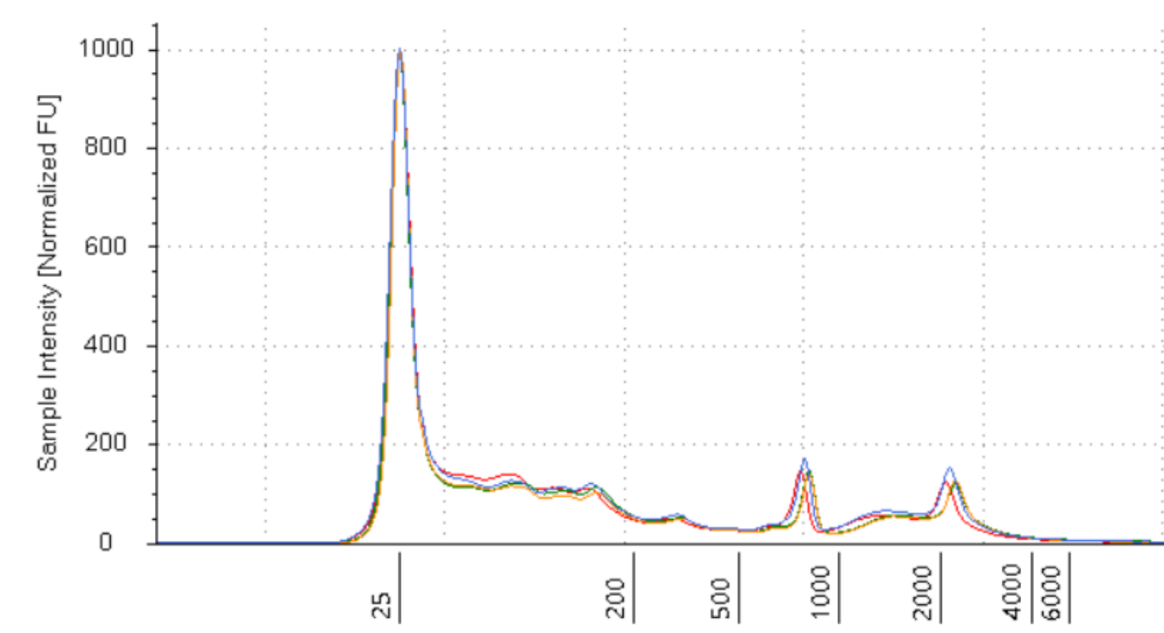
EXTRACTION CHARACTERIZATION



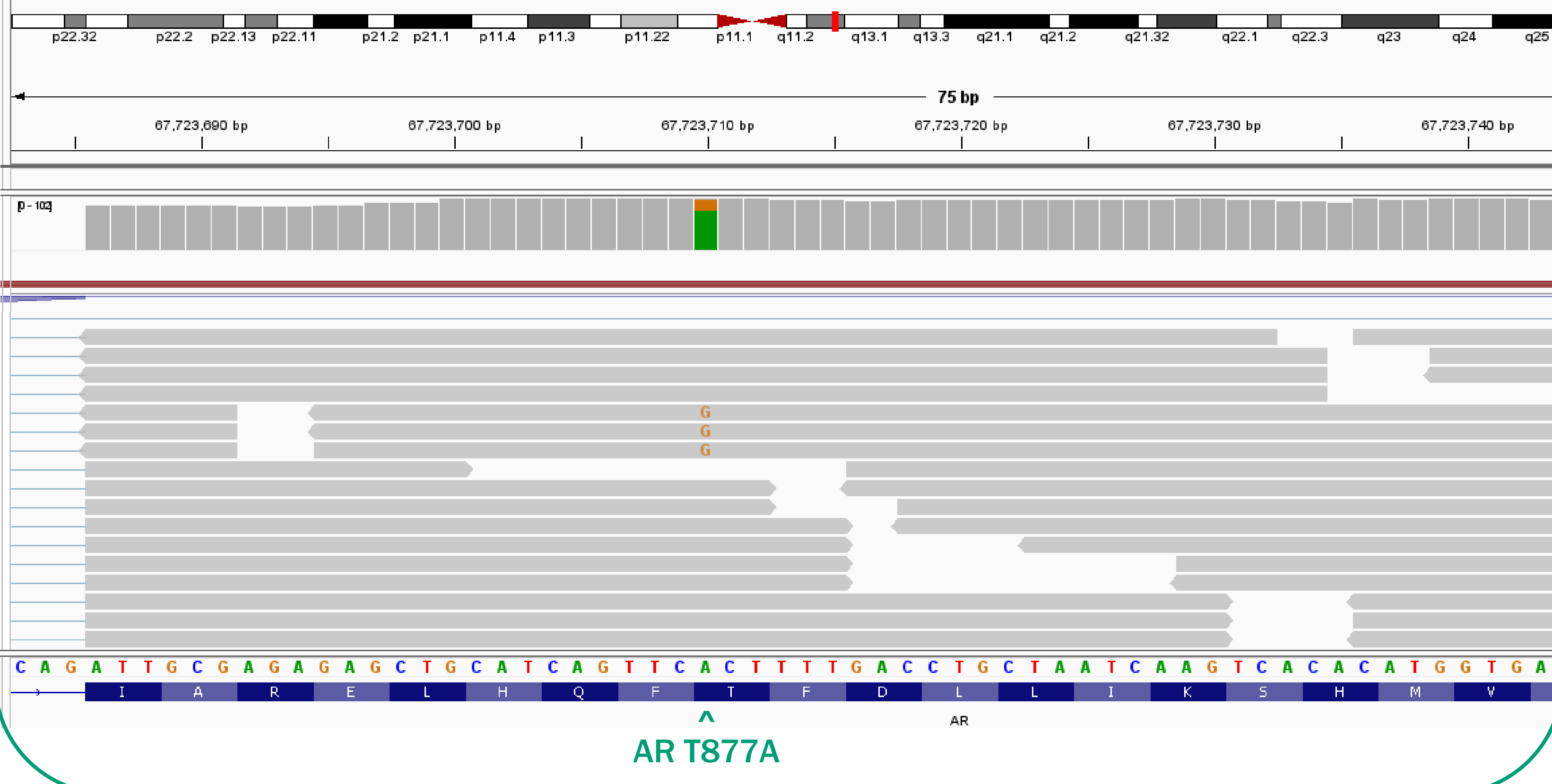
10 ml spiked = no RNA detected



40 ml spiked = RNA detected



Mutant AR T877A Expression



CONCLUSIONS

- cfRNA Extraction Performance:** The nRichDX platform demonstrated efficient cfRNA recovery from both plasma and urine. In plasma, MiRNeasy and QIAamp Plasma kits produced RNA yields below the limit of detection, while Zymo and nRichDX generated measurable RNA, with nRichDX achieving 2–5× higher yields. The nRichDX Revolution Plus System also produced higher-quality cfRNA, including measurable RIN values where other methods failed, enabling improved detection of ALS-associated cryptic introns. In urine, cfRNA yield increased proportionally with input volume, showing a clear linear relationship between 10 and 40 mL inputs. Samples with 5 mL input were below Qubit and TapeStation detection limits, whereas inputs ≥10 mL yielded robust and reproducible cfRNA recovery. All samples were advanced to library preparation regardless of measured yield.
- Library Preparation:** All samples were successfully converted into sequencing libraries, including those with cfRNA levels below Qubit and TapeStation detection thresholds.
- Sequencing and Analysis:** Post-sequencing quality control (QC) metrics were consistent across all samples and revealed no notable issues.
- Sequencing Outcomes and Functional QC:** All libraries were successfully sequenced, and post-sequencing QC metrics were comparable across all samples, including low-input specimens. In plasma, analysis of ALS-associated cryptic introns showed greater detection sensitivity with the Thermo Fisher Ion AmpliSeq platform, although this may reflect differences in assay design and transcript coverage. In urine, functional QC demonstrated elevated expression of key prostate cancer biomarkers, including FOLH1 (PSMA), KLK3 (PSA), and AR. Expression of the mutant AR T877A transcript was also confirmed in spike-in samples with input volumes of 10 mL or greater.