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cfRNA Cryptic Intron Identification from Liquid Biopsy Samples

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rare, terminal neurodegenerative disorder that results in the progressive loss of both upper and lower motor neurons that normally control voluntary muscle contraction. The pathological hallmark of ALS is the presence of inclusion bodies (abnormal aggregations of protein) in the cytoplasm of motor neurons, with TDP-43, SOD1 and FUS proteins being most common. These protein aggregations can be due to a host of mutations to these protein encoding genes directly, or by mutations to genes associated with protein degradation, cytoskeleton and axonal transport. Over 40 genes have been identified as being associated with ALS. While some of these genes are more common than others, they collectively account for a significant portion of both familial and sporadic ALS cases. Cell-free nucleic acids can be used as robust biomarkers to determine disease development and progression, with liquid biopsy being an efficient method to detect specific cfRNA. Specifically, cfRNA can be utilized to detect cryptic introns: an intron that is not spliced out and is released into the bloodstream in patients with ALS.

Here we describe liquid biopsy cfRNA extraction and RNA-Seq analysis on a series of ALS affected and control samples. 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 additional 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. As these results show, cfRNA can be extracted from a limited amount of plasma, with detection of rare biomarkers associated with disease. This method is not limited to plasma and cfRNA, with the potential for application in other biofluids including urine, and other nucleic acid types including mitochondrial DNA.

In collaboration with: nRichDX POWERING LIQUID BIOPSY

PLASMA SAMPLES
4 ALS Patients + 1 Healthy Control

Extraction & QC

4 PLASMA RNA EXTRACTION KITS
- ✓ miRNeasy® Serum/Plasma Advanced
- ✓ QIAamp® ccfRNA
- ✓ Zymo® Quick cfRNA Serum & Plasma
- ✓ nRichDX® Revolution Plus System

Library Prep & QC

2 LIBRARY PREP KITS
- ✓ Takara SMARTer® Stranded Total RNA-Seq Kit v3 – Pico Mammalian (unbiased, whole transcriptome)
- ✓ ThermoFisher ION AmpliSeq™ RNA (target ~25k genes in transcriptome)

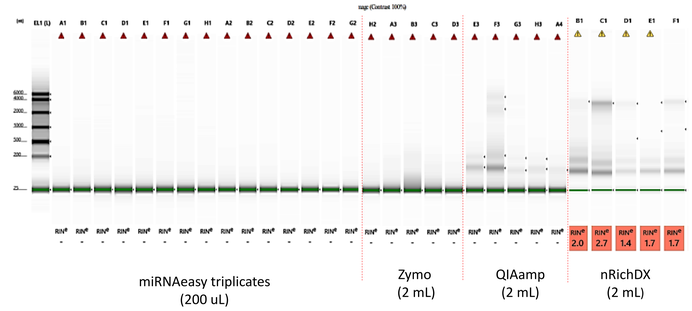
Sequencing & Analysis

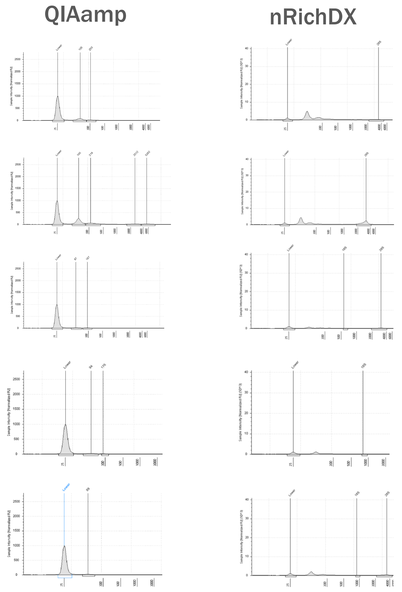
ILLUMINA® SEQUENCING & ANALYSIS
~1M reads/sample
- ✓ Read Quality & Mapping
- ✓ Functional Analysis of Cryptic Introns

Figure 1. Workflow for cell-free RNA sequencing from plasma. 5 plasma samples, 4 ALS affected and 1 normal control, were subjected to 4 different extraction kits to determine optimal method. MiRNeasy and QIAamp kits did not yield detectable RNA, Zymo had detectable levels, while nRichDX yielded 2-5x higher with measurable traces. The nRichDX extracted samples, along with a universal human reference control (UHC), were then subjected to two library prep methods: Takara RNA exome and ThermoFisher AmpliSeq custom targeted panel. All samples were sequenced to approximately 1M reads and analyzed for function QC of ALS-associated cryptic introns.

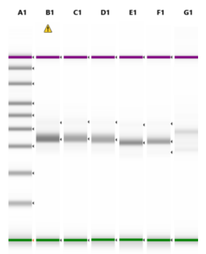
EXTRACTION CHARACTERIZATION
High Sensitivity TapeStation (pg/ul)

	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

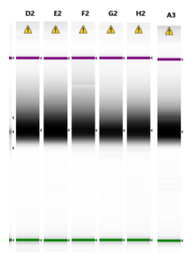




ThermoFisher AmpliSeq



Takara Pico



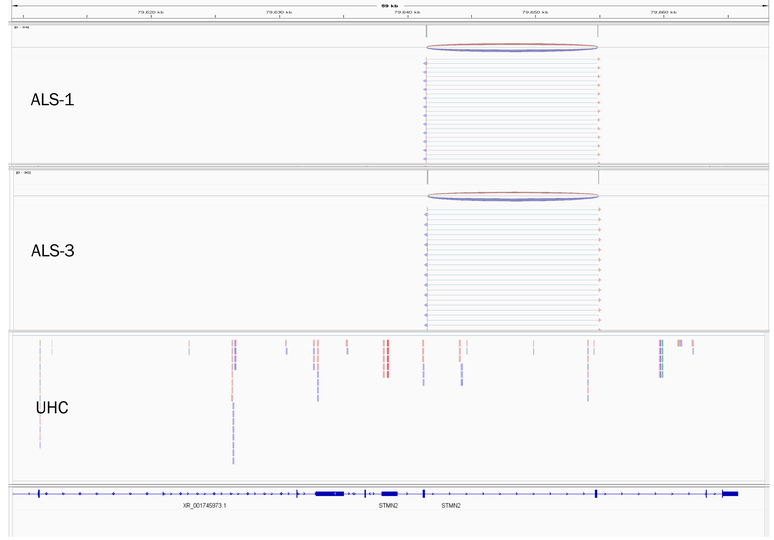
SIGNAL INTENSITY

	AmpliSeq	Takara Pico
ALS-1	+++	+++
ALS-2	++++	++++
ALS-3	++	++
ALS-4	++	++
Healthy-1	+	+
UHC	+++	+++

		STMN2	HDGFL2
AmpliSeq	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



Conclusions

- Extraction: MiRNeasy and QIAamp Plasma kit yield were below limits of detection. Zymo and nRichDX had measurable concentrations, with nRichDX 2-5x that of QIAamp. The nRichDX Revolution Plus System yielded more and higher-quality cfRNA, with measurable RIN values where other kits failed. This improved input enabled more sensitive detection of ALS-associated cryptic introns.
- Library Prep: Both approaches yielded similar library QC results, indicating successful preparation of all samples.
- Sequencing & Analysis: Post-sequencing QC was similar and unremarkable. Functional QC of cryptic introns revealed higher sensitivity with ThermoFisher ION AmpliSeq, However, this may be due to differences in design and coverage of assays.