

A Sensitive LC-HRMS Method for the Quantitation of Dystrophin in Human Muscle Tissue

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INTRODUCTION

Quantitation of candidate biomarkers requires specific bioanalytical assays to selectively detect and quantitate target proteins with high sensitivity in biological fluids and tissues. For Duchenne Muscular Dystrophy (DMD) therapy, dystrophin protein levels have been typically determined using immunohistochemistry and western blotting. However, recent advances in high precision mass spectrometry analysis in combination with stable isotope spike-in strategy have made this technique well suited for the detection and quantitation of low abundant biomarkers. In this research, we present a mass spectrometry-based approach to accurately quantitate low levels of dystrophin protein in a total protein extract from human muscle biopsies. The approach uses a combination of stable isotope labeled dystrophin as a spike-in standard, gel electrophoresis and high resolution mass spectrometry. The fit-for-purpose validated method was used to support a Phase 2 dose finding study for NS-065/NCNP-01 (NCT02740972), a novel anti-sense oligonucleotide for the treatment of DMD.

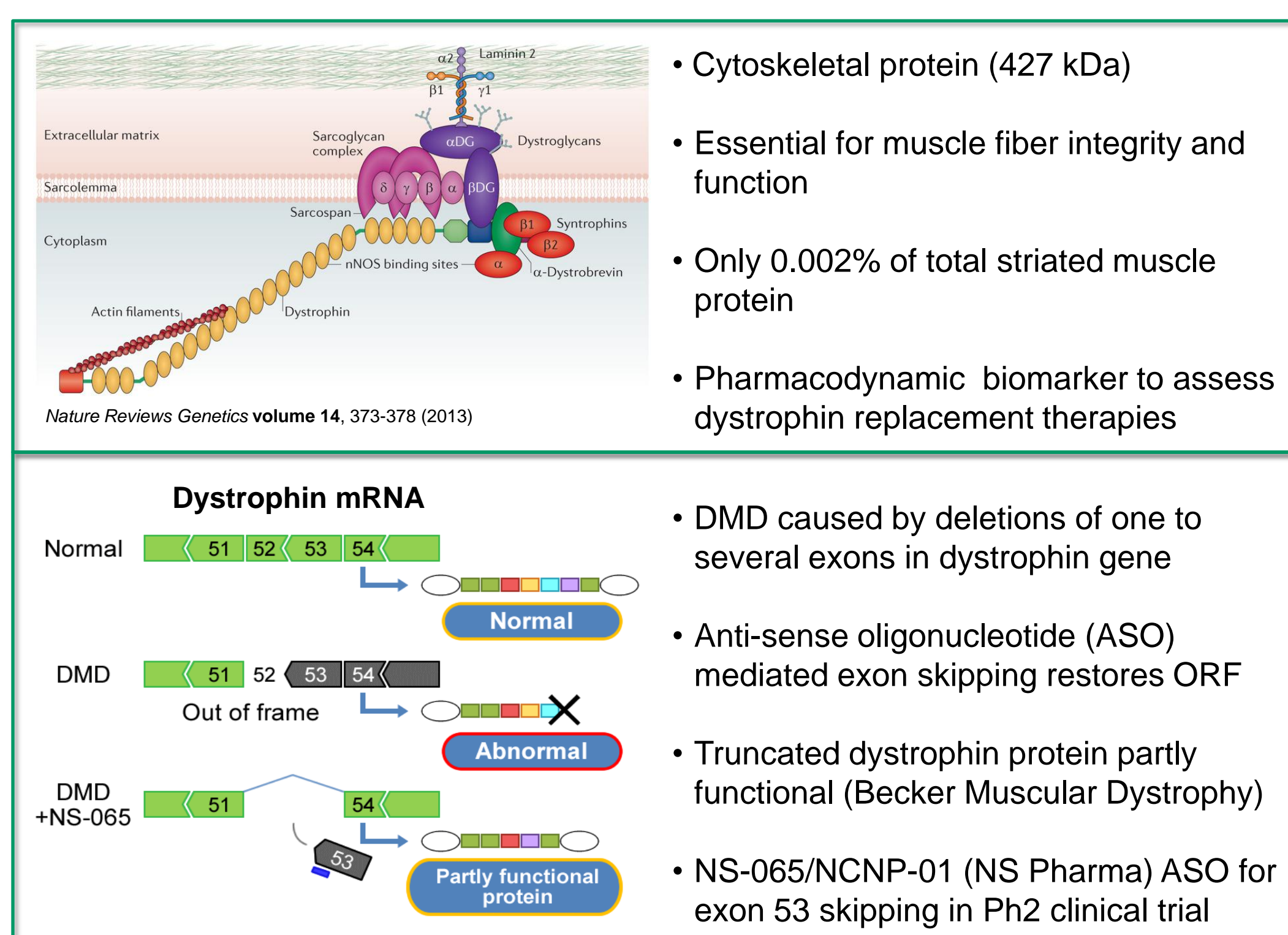
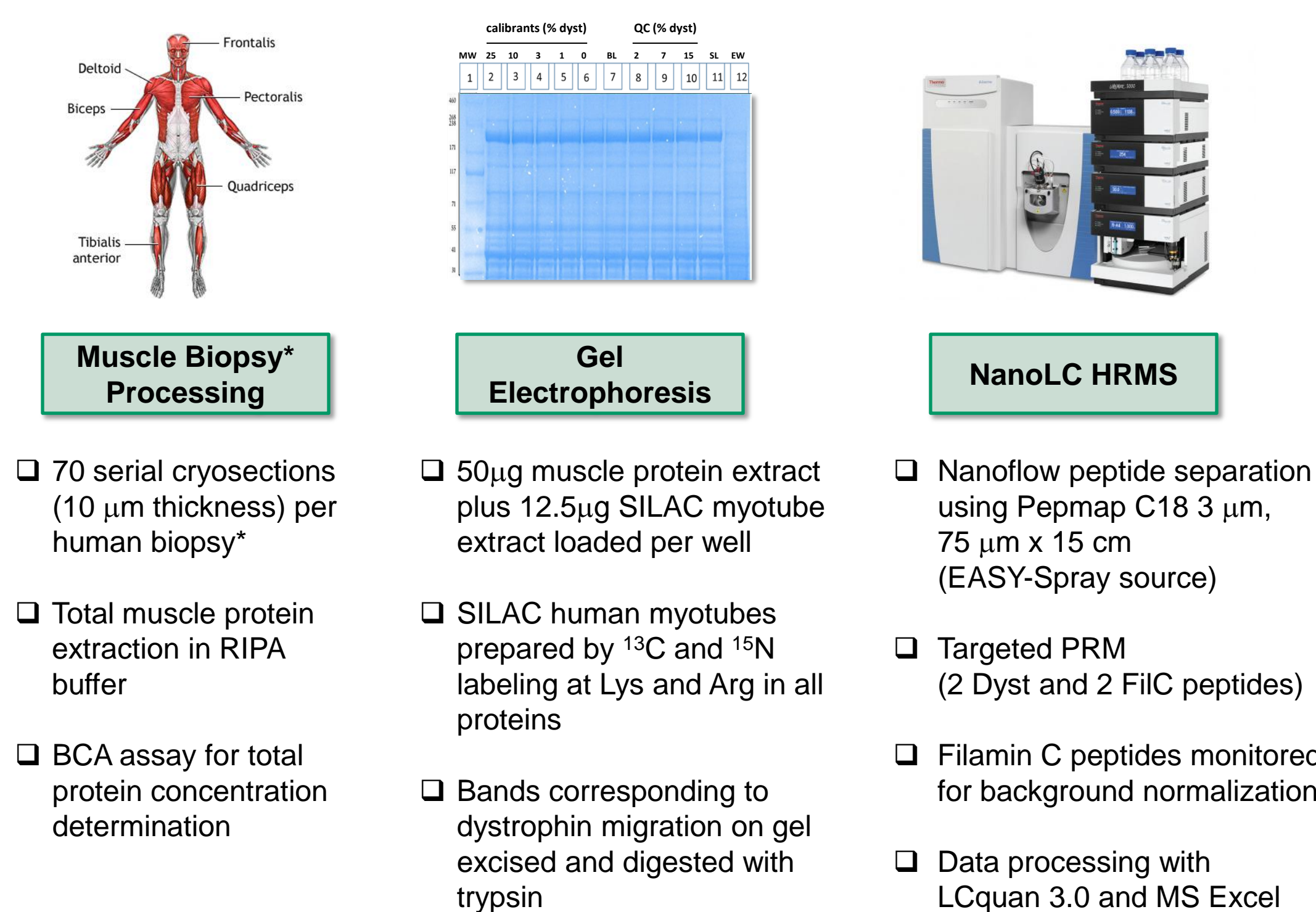


Figure 1. Schematic diagram of the skeletal muscle dystrophin-associated protein complex (top panel). Anti-sense mediated exon skipping strategy to reframe DMD transcripts (bottom panel).

METHODS

SAMPLE PREPARATION AND ANALYSIS

Muscle biopsy processing and separation of muscle extracts by SDS-PAGE were carried out at AGADA Biosciences (Halifax, NS). Analysis of gel-extracted peptides by nanoLC HRMS (Q Exactive Plus) was carried out at Altasciences.



RESULTS

DYSTROPHIN QUANTITATION

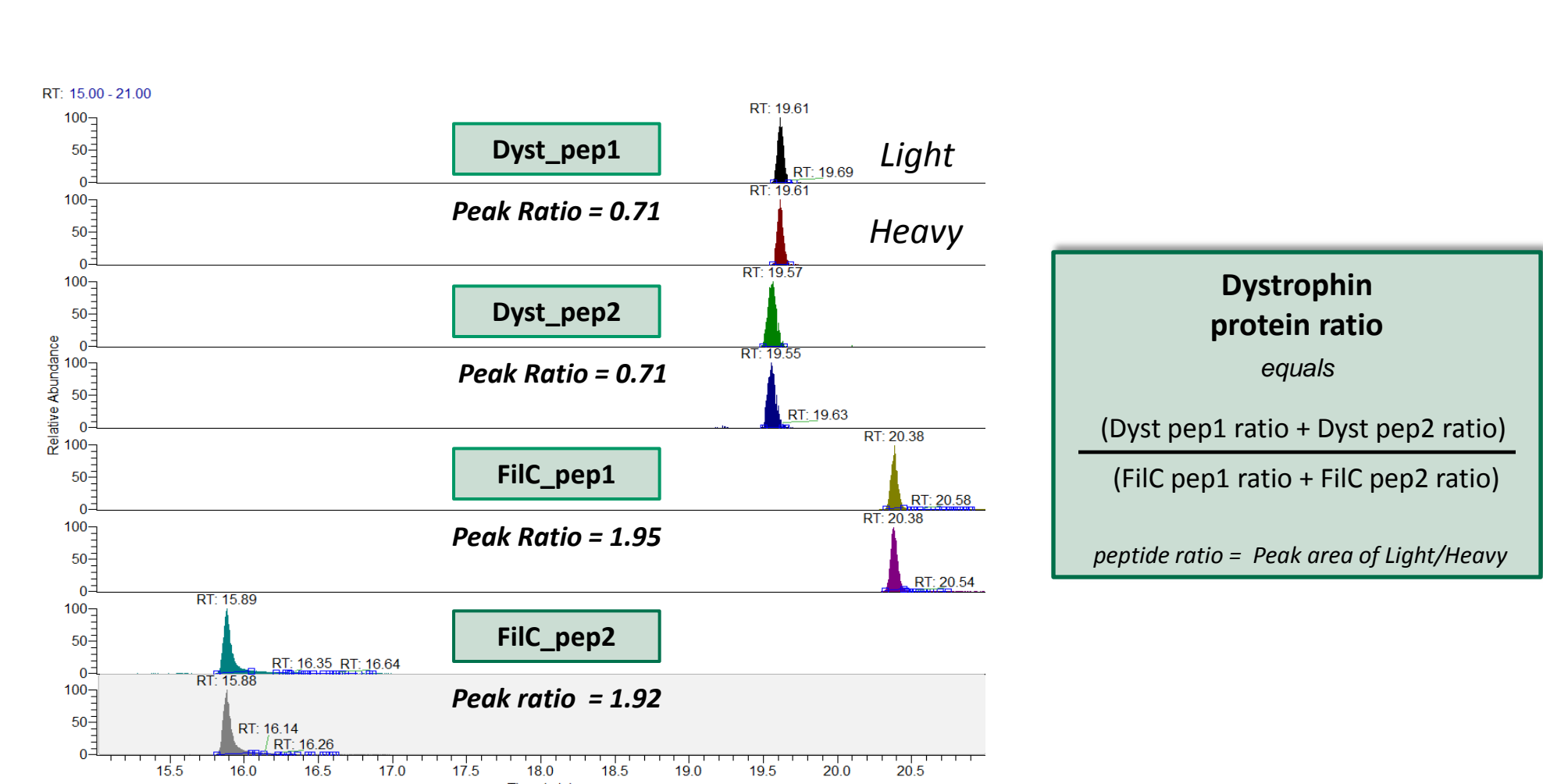


Figure 2: Representative chromatograms of the two dystrophin peptides and two Filamin C peptides (light and heavy) used for dystrophin quantitation.

Table 1. Dystrophin and Filamin C surrogate peptides

Dystrophin	Peptide Sequence	MS1 (m/z)	Summed Product Ions
Dyst_pep1	LLQQFPLDLEK	672.3821	y6, y7, y9 ⁽²⁺⁾
Dyst_pep2	IFLTEQPLEGLEK	758.9165	y7, y9, y10, y11
Filamin C	Peptide Sequence	MS1 (m/z)	Summed Product Ions
FilC_pep1	VAVGQEQAFSVNTR	753.3890	y6, y7, y8, y9
FilC_pep2	SPFVVNVAPPLDLSK	791.9456	y7, y8, y10, y11

METHOD VALIDATION

Two Coomassie Blue gels were prepared on separate days. Each gel was comprised of a calibration curve (0, 1, 3, 10 and 25% dystrophin) and QC samples (2, 7 and 15% dystrophin) prepared by mixing non-DMD control samples (mix of 5 non-DMD biopsies) and a DMD sample (mix of two DMD biopsies). Calibration curves were fitted using a weighted 1/x linear regression. 0% standard was subtracted from all calibrants and QCs.

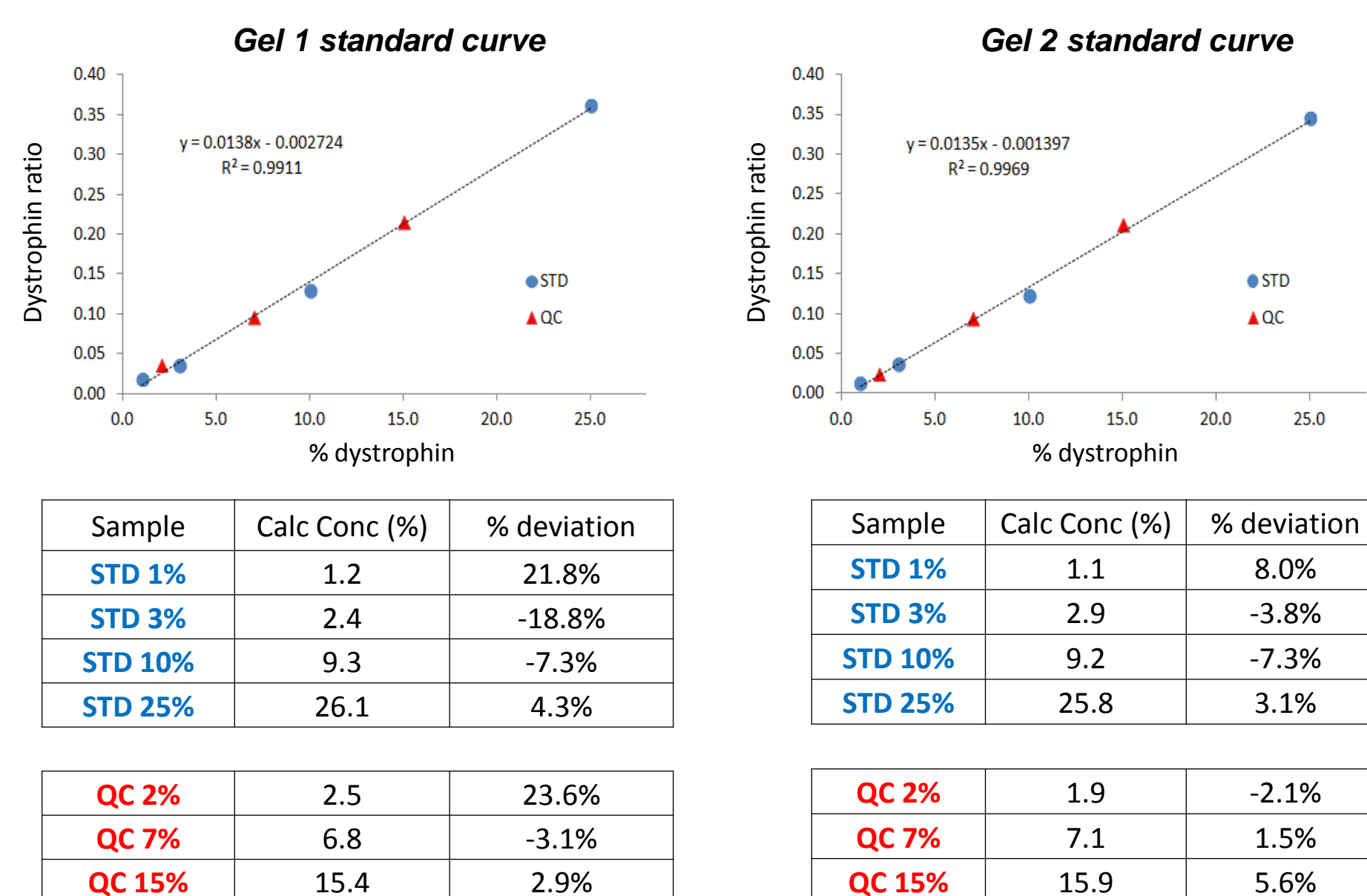


Figure 3. Dystrophin standard curves and QC plots for gels 1 and 2.

Table 2. Summary of dystrophin method validation evaluations and results*

Evaluation	Results
Linearity	Curve fitted to 1/x linear regression ($R^2 > 0.99$)
Intra-Assay Accuracy	Low QC: Gel 1: 23.6%; Gel 2: -2.1% Mid QC: Gel 1: -3.1%; Gel 2: 1.5% High QC: Gel 1: 2.9%; Gel 2: 5.6%
Inter-Assay Accuracy	Low QC: 10.8%; Mid QC: -0.8%; High QC: 4.2%
Sensitivity	LLOQ: 1% of normal
Selectivity	No significant interferences in blank samples (DMD only) at dystrophin peptide retention times
Carryover	Carryover controlled by injecting samples in concentration order and injecting blank samples between unknowns
Injection medium integrity	Confirmed up to a combined 20.4 hours at 10 °C (autosampler) and 14 days at 4 °C

* Acceptance criteria for precision and accuracy were set at $\pm 30\%$ for all STDs and QCs

CLINICAL BIOPSY ANALYSIS

The validated method was used to analyze in duplicate pre- and post-treatment biopsy samples from 16 patients treated with NS-065/NCNP-01 (40 and 80 mg/kg/week; 20 or 24-week period). Accuracy of standard curve calibrants as well as linearity (r^2) of standard curves for 15 separate gels are highlighted in Table 3.

Table 3. Precision and accuracy of standard curve calibrants used for the analysis of dystrophin clinical biopsy samples

Gel #	Calibrant Concentration (%)				Linearity (r^2)
	1.00	3.00	10.00	25.00	
1	1.13	2.92	8.31	26.64	0.9856
2	1.02	2.95	9.86	25.16	0.9999
3	1.15	2.64	9.40	25.81	0.9956
4	1.01	RC	9.82	25.17	0.9998
5	1.09	2.90	9.00	26.01	0.9946
6	1.12	2.78	9.17	25.93	0.9952
7	0.84	3.06	12.25	22.86	0.9753
8	RC	2.97	9.46	25.53	0.9985
9	1.13	2.61	9.81	25.45	0.9972
10	1.14	2.75	8.97	26.14	0.9930
11	1.11	2.66	9.96	25.28	0.9981
12	1.23	2.42	9.22	26.13	0.9904
13	1.09	2.87	9.26	25.78	0.9967
14	1.10	2.84	9.14	25.92	0.9954
15	1.05	2.86	9.86	25.23	0.9995
Mean	1.09	2.81	9.57	25.53	
S.D.	0.089	0.185	0.864	0.855	
N	14	14	15	15	
% C.V.	8.2	6.6	9.0	3.4	
(%) Nominal	108.7	93.7	95.7	102.1	

RC = Rejected Calibrant (outside $\pm 30\%$ accuracy)

CONCLUSION

- Novel nanoLC HRMS method was validated (fit-for-purpose) to detect the biomarker dystrophin in muscle biopsies from DMD patients. The method is orthogonal to antibody-based assays like western blotting and immunohistochemistry.
- The method used stable isotope-labeled dystrophin as internal standard, filamin C for protein content normalization and nanoflow HRMS to detect with high specificity low levels of dystrophin (1% of normal).
- The method was used to support Ph2 clinical trial of NS-065/NCNP-01 (NCT02740972, Sponsor: NS Pharma).

REFERENCES

- Brown K. Accurate Quantitation of Dystrophin Protein in Human Skeletal Muscle Using Mass Spectrometry *J. Bioanal. Biomed.* 2012
- Uaesoontrachoon K. Orthogonal analysis of dystrophin protein and mRNA as surrogate outcome for drug development *Biomark. Med.* 2019

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