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

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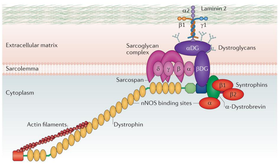
INTRODUCTION

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.



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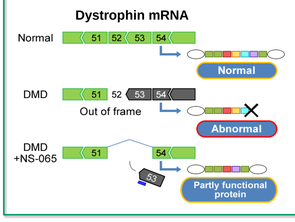
- Cytoskeletal protein (427 kDa)
- Essential for muscle fiber integrity and function
- Only 0.002% of total striated muscle protein
- Pharmacodynamic biomarker to assess dystrophin replacement therapies



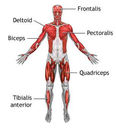
- DMD caused by deletions of one to several exons in dystrophin gene
- Anti-sense oligonucleotide (ASO) mediated exon skipping restores ORF
- Truncated dystrophin protein partly functional (Becker Muscular Dystrophy)
- NS-065/NCNP-01 (NS Pharma) ASO for exon 53 skipping in Ph2 clinical trial

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

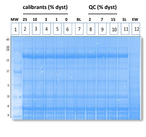
SAMPLE PREPARATION AND ANALYSIS

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.



Muscle Biopsy* Processing

- ❑ 70 serial cryosections (10 μm thickness) per human biopsy*
- ❑ Total muscle protein extraction in RIPA buffer
- ❑ BCA assay for total protein concentration determination



Gel Electrophoresis

- ❑ 50μg muscle protein extract plus 12.5μg SILAC myotube extract loaded per well
- ❑ SILAC human myotubes prepared by ^{13}C and ^{15}N labeling at Lys and Arg in all proteins
- ❑ Bands corresponding to dystrophin migration on gel excised and digested with trypsin

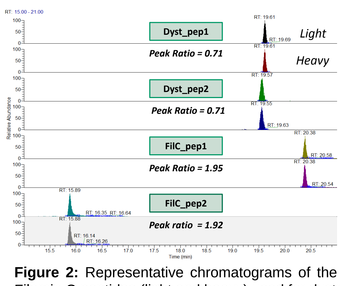
NanoLC HRMS

- ❑ Nanoflow peptide separation using Pepmap C18 3 μm, 75 μm x 15 cm (EASY-Spray source)
- ❑ Targeted PRM (2 Dyst and 2 FilC peptides)
- ❑ Filamin C peptides monitored for background normalization
- ❑ Data processing with LCquan 3.0 and MS Excel

* Biopsies were pre-existing and de-identified archive from the Binghamton University tissue bank or were purchased from Cureline (CA, USA). Ethical approval for use of the de-identified biopsies was provided by an independent ethics institutional review board (Veritas, Quebec, Canada). None of the biopsies were treated with NS-065/NCNP-01.

RESULTS

DYSTROPHIN QUANTITATION



Dystrophin protein ratio
equals

$$\frac{\text{(Dyst pep1 ratio + Dyst pep2 ratio)}}{\text{(FilC pep1 ratio + FilC pep2 ratio)}}$$

peptide ratio = Peak area of Light/Heavy

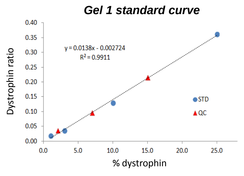
Figure 2: Representative chromatograms of the two dystrophin peptides and two Filamin C peptides (light and heavy) used for dystrophin quantitation.

Table 1. Dystrophin and Filamin C surrogate peptides

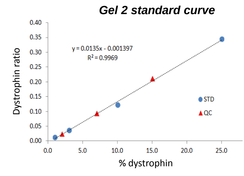
Dystrophin	Peptide Sequence	MS1 (m/z)	Summed Product Ions
Dyst_pep1	LLQQFPLDLEK	672.3821	y6, y7,y9$^{(2+)}$
Dyst_pep2	IFLTEQPLEGLEK	758.9165	y7, y9,y10,y11
Filamin C	**Peptide Sequence**	**MS1 (m/z)**	**Summed Product Ions**
FilC_pep1	VAVGGEQAFSVNTR	753.3890	y6, y7,y8,y9
FilC_pep2	SPFVVNVAPPLDLSK	791.9456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

METHOD VALIDATION

Two Coomasie 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.



Sample	Calc Conc (%)	% deviation
STD 1%	1.2	21.8%
STD 3%	2.4	-18.8%
STD 10%	9.3	-7.3%
STD 25%	26.1	4.3%
QC 2%	2.5	23.6%
QC 7%	6.8	-3.1%
QC 15%	15.4	2.9%



Sample	Calc Conc (%)	% deviation
STD 1%	1.1	8.0%
STD 3%	2.9	-3.8%
STD 10%	9.2	-7.3%
STD 25%	25.8	3.1%
QC 2%	1.9	-2.1%
QC 7%	7.1	1.5%
QC 15%	15.9	5.6%

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 ±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 ±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).

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