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Quantitation of Dystrophin Expression in Patients with Duchenne Muscular Dystrophy by Western Blot Analysis Adjusted for Muscle Content

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Background

- Dystrophin-restoring therapies, including exon-skipping and gene transfer approaches, require reliable dystrophin protein quantitation to support assessment of therapeutic response in DMD
- Western blotting has been widely applied for this purpose; however, methodological differences across development programs limit direct comparability of dystrophin expression results
- Variations in choice of dystrophin standards, loading controls, normalization strategies, and approaches to account for muscle content can contribute to differences in reported dystrophin levels[1,2]
- Because non-muscle tissues contribute to the total protein loaded in a western blot, muscle fiber dystrophin protein may be underestimated
  - Complementary strategies to assess muscle fiber content, including histological and biochemical approaches, can further support consistent interpretation of dystrophin quantitation

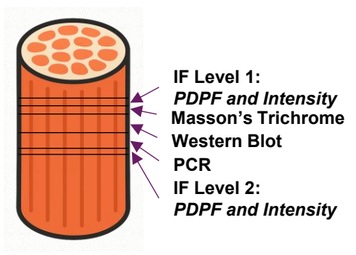
Objective

To describe quantitation of exon-skipped dystrophin and delandistrogene moxeparvovec micro-dystrophin at baseline and following treatment with dystrophin-restoring therapies, incorporating muscle content adjustment into the western blot assay

Methods

Figure 1 *Western Blot with Muscle Content Adjustment Workflow*

Muscle Biopsy	• Open or probe-based biopsy collection
Process Biopsy	• Freezing for protein and tissue integrity and cryosectioning
Lysis of tissue	• Urea-based for maximal extraction of dystrophin
Total Protein Quantitation	• Quantitation to load samples based upon protein concentration
Gel electrophoresis; membrane transfer	• Exon-skipped: standard curve is NC pool diluted in DMD (null) pool • µ-dys: standard curve is a recombinant protein diluted in DMD (null) pool
Immunoblot	• Primary antibodies: DYS1 (exon-skipped) or DYS3 (µ-dys) • α-actinin detection as an internal control
Dystrophin Analysis	• Chemiluminescent detection (digital imager) • Linear regression of log-transformed standard curve • Dystrophin expressed as %NC
Muscle Content Adjustment	• The %NC dystrophin may be adjusted to the %muscle, an area-based measurement from a MTri-stained adjacent tissue section



Graphical representation of biopsy allocation for outcome measures. The muscle content measurement is from the same biopsy as the western blot. Not to scale.

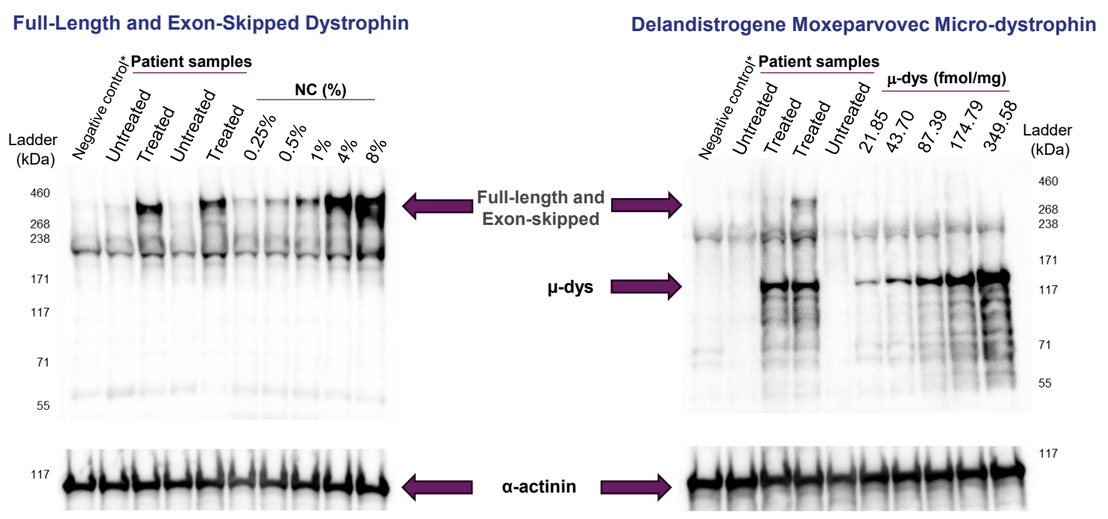
- **Figure 1** shows an overview of the validated western blot with muscle content adjustment workflow; western blot parameters are shown in **Table 1**
  - Full-length and exon-skipped dystrophin: the standard curve is the normal control (NC) pool (prepared by pooling healthy muscle biopsy samples to mitigate interindividual variability)
  - Delandistrogene moxeparvovec micro-dystrophin: standard curve is a recombinant protein diluted in DMD (null) pool
  - α-actinin serves as an internal protein loading control but is not used for normalization
  - Masson's trichrome (Mtri)-stained cryosections cut concurrently with sections used for western blotting (**Figure 1, right panel**) undergo color-segmentation-based digital image analysis of the whole slide to determine MTri %muscle

Table 1 *Current Western Blot Analysis Parameters*

Parameter	Full-length and exon-skipped	µ-dys
Standard Curve	NC Pool	Recombinant µ-dys
Standard Curve Range	5-point NC 8%, 4%, 1%, 0.5%, 0.25% NC	5-point standard curve: 349.6 -21.9 fmol/mg (*equates to 54.8%, 27.4%, 13.7%, 6.8%, and 3.4% NC)*
Null Matrix	Dystrophin-negative DMD Pool	Dystrophin-negative DMD Pool
NC Pool	Non-dystrophic muscle tissues; bridging lots	Same as exon-skipped; used in qualifying recombinant protein to establish %NC equivalency
Gel	3-8% Tris-acetate gradient gel	3-8% Tris-acetate gradient gel
LLOQ	0.25%	21.9 fmol/mg
Total Protein	20 µg	20 µg
Primary Antibody	DYS1 monoclonal (rod domain)	DYS3 monoclonal (N-terminal)
Chemiluminescent Detection	Imager	Imager
Loading Control*	α-actinin	α-actinin

DMD, Duchenne muscular dystrophy; LLOQ, Lower Limit of Quantification; NC, normal control; µ-dys, delandistrogene moxeparvovec micro-dystrophin
α-actinin is a loading control but not used in normalization

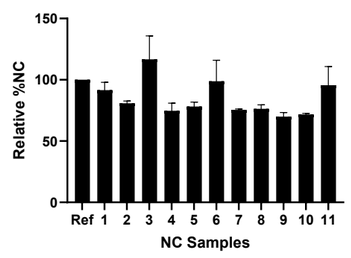
Figure 2 *Example western blots*



*Negative controls consist of a pooled DMD negative control

- Although exon-skipped dystrophin cannot be differentiated from full-length protein by western blot, µ-dys is differentiated by protein size (**Figure 2**)
- Given the limited nature of individual non-dystrophic NC samples, a pool of multiple NC samples was prepared, and a strong bridging protocol[3,4] is in place to ensure consistency between the NC pool lots prepared and used over time
  - Bridging between NC pools is critical as higher dystrophin expression in a NC pool would lead to a lower %normal, and a low expressing pool will lead to higher %normal values
  - Analysis of 11 NC samples relative to a single NC (Ref) shows relative variance in dystrophin expression (**Figure 3**) consistent with other reports[1]
  - An initial NC pool was prepared from these 11 samples, and met lot bridging acceptance criteria of within 20%[3,4]
  - NC pools remained stable for up to 4.5 years at -80°C and through four freeze-thaw cycles

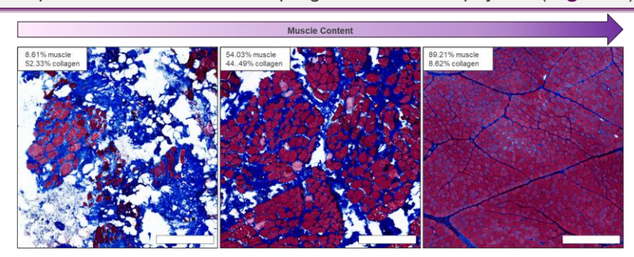
Figure 3 *Production of a NC pool that captures the relative variance in dystrophin*



NC, normal control; Ref, one NC reference sample from initial studies

- For MTri histological staining, a tissue section from adjacent sections within the same muscle biopsy as the western blot and any additional analyses (e.g., immunofluorescence [IF]) is used (**Figure 1**, **right panel**)
  - MTri histological staining distinguishes muscle fibers from collagen-containing tissues using Biebrich Scarlet-Acid Fuchsin and Aniline Blue staining, respectively
- Whole-slide scanning of MTri-stained slides followed by a digital image analysis algorithm quantifies the area of muscle within the total area of biopsy tissue to determine the %muscle for muscle content adjustment
  - The image analysis method, developed using AI-based technology, is sensitive enough to measure low muscle quantities in a biopsy
  - Areas not discernable by the algorithm (e.g., folds or tissue damage) are excluded from analysis
  - The image analysis method has been validated,[3,4] and pathologist review of algorithm classification accuracy indicated that the false negative and false positive rates for muscle identification were <2.2% and <1.5%, respectively
  - Two separately developed and validated MTri staining/imaging analysis platforms showed significant positive correlation when applied to samples from the same biopsy sample (Pearson, 0.8842; 95% CI, 0.6662 to 0.9630; P<0.001)
  - The algorithm is applied in a blinded manner
- Muscle and collagen composition can vary substantially between patients and with disease progression and biopsy site (**Figure 4**)

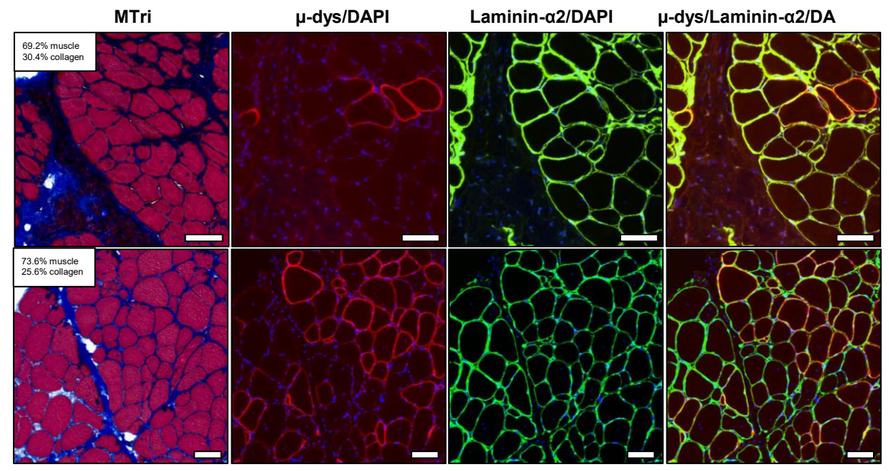
Figure 4 *MTri staining clearly differentiates skeletal myofibers (red staining) from collagenous connective tissue (blue staining) to allow accurate quantification of relative muscle content via image analysis in DMD biopsies*



Digital images of MTri-stained slides acquired at 2X (white ruler bar = 500 µm) in HALO Link.

- The MTri method of adjustment is a calculation from the two separately validated methods,[3,4] one determining the dystrophin content (%NC) by western blot and the second determining the %muscle as measured by MTri staining of an adjacent section
  - The formula for adjustment is Dystrophin expression (%NC) / Percent Muscle Content *100
- Comparison of the MTri and µ-dys expression by IF demonstrate the localized expression to the muscle fibers
  - In these examples, where the unadjusted dystrophin expression by western blot is 9.3%; with a muscle content of 69.2% muscle, the adjusted dystrophin expression is 13.5% (**Figure 5, top panel**)
  - Similarly, where the unadjusted dystrophin expression by western blot is 34.1%; with a muscle content of 73.6% muscle, the adjusted dystrophin expression is 46.3% (**Figure 5, bottom panel**)

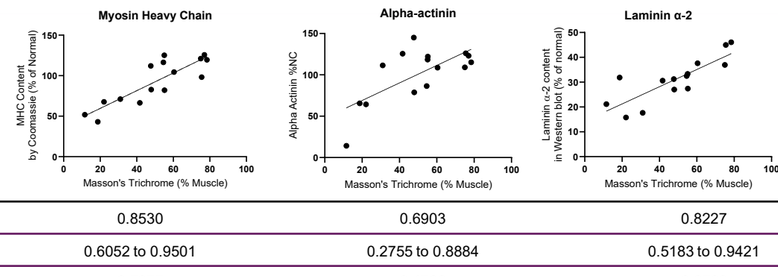
Figure 5 *Example of MTri muscle content adjusted of percent of NC µ-dys by western blot.*



Digital images of MTri histochemical stained and fluorescent-labeled (µ-dys, laminin-α2, and DAPI) slides acquired at 8X (white ruler bar = 100 µm) in HALO Link (Indica Labs).

- Alternative methods for muscle content adjustment/loading control normalization include MHC, α-actinin, and laminin α2 analyses[1,5]
- Control and DMD muscle biopsies spanning a range of muscle content were compared to %NC α-actinin (western blot), MHC (Coomassie), and laminin α-2 (western blot) (**Figure 6**)
- Positive correlations indicate that all methods adjust for muscle content
  - The %NC for both MCH and α-actinin were generally calculated as >50% of the NC and do not capture the lower range of muscle content
  - MHC quantification by Coomassie and alpha-actinin by western blot, when performed within the same gel, are not quantitative independently due to lack of a standard curve.
  - Due to abundance of MHC and alpha-actinin proteins, signal saturation and lack of dilutional integrity, are key challenges to validating these measures.
  - Using both MHC and α-actinin to normalize could overestimate the amount of dystrophin
- MTri is most sensitive to detect the full dynamic range of dystrophic muscle content
  - Laminin α-2 correlated with MTri without signal saturation and may serve as an independently validated method

Figure 6 *MTri sensitively detects the range of muscle content in biopsies*



	Myosin Heavy Chain	Alpha-actinin	Laminin α-2
	Masson's Trichrome (% Muscle)	Masson's Trichrome (% Muscle)	Masson's Trichrome (% Muscle)
Pearson r	0.8530	0.6903	0.8227
95% CI	0.6052 to 0.9501	0.2755 to 0.8884	0.5183 to 0.9421
R^2	0.7277	0.4766	0.6768
P-value	<0.0001	0.0044	0.0003

CI, confidence interval.

Conclusions

- In conditions with progressive muscle loss and fibrosis like DMD, it is important that biopsied muscle content is precisely measured for reliable dystrophin quantitation; however, the methods used by most developmental programs are largely unknown
- Western blot methods for exon-skipped dystrophin and micro-dystrophin are validated procedures[3,4] and incorporate the bridging of critical reagents, including NC pool lots, to ensure consistent, robust performance
- MTri staining/analysis represents an appropriate approach to determine muscle content and adjust the amount of dystrophin detected by western blotting due to heterogeneity of muscle biopsy
- Without an established commercial reference standard, transparency on methodology used for dystrophin quantitation, and defined regulatory guidelines, comparison of dystrophin restoration therapies cannot be made
- Appropriate dystrophin quantitation methods are needed to evaluate biological efficacy of exon-skipping and gene transfer therapies, as part of the totality of evidence, including additional biological biomarkers, long-term functional outcomes, and delay of DMD progression milestones

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Abbreviations

CI, confidence interval; DMD, Duchenne muscular dystrophy; IF, immunofluorescence; MHC, myosin heavy chain; MTri, Masson's trichrome; NC, normal control; PDPF, percent dystrophin-positive fibers..

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