Western blotting of dystrophin: Quantification and correlations

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Disclosures

- Site principal investigator for PTC Therapeutics, Prosensa, Akashi, and the NIH FOR-DMD study; site co-investigator for Sarepta
- Advisory boards for Sarepta, PTC, Audentes, Eli Lilly, and Italafarmico
- None of the work presented today is directly related to these relationships

Quantification of dystrophin

- Immunofluorescence
- Immunoblot

- Challenges of Western blotting include:
  - Standardization of electrophoresis and blotting
  - Choice of antibodies
  - Methods of imaging
  - Choices for normalization

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Quantification of dystrophin immunofluorescence in dystrophinopathy muscle specimens

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Table 1. Summary clinical and genetic features of dystrophinopathy patients from whom archived biopsy tissue was analysed

ID	*Diagnosis*	*Mutation*	*Age (years) at loss of ambulation*	*Age (years) at biopsy*	*Steroids*
1	DMD	deletion exon 6	Ambulant as of 5	1.5	Prednisone, twice/week (1 year)
2	DMD	duplication exons 10–17	9	16	Never
3	DMD	duplication exons 29–43	11.5	6	Never
4	DMD	duplication exon 7	Ambulant as of 7	5.5	Never
5	DMD	deletion exon 43	Ambulant as of 9	9	Never
6	IMD	Pseudoexon (c.6614 + 3310G>T)	Ambulant as of 14	9	Deflazacort, daily (since 2003)
7	IMD	duplication exons 3–4	15	14	Deflazacort, daily (since 2004)
8	BMD	Pseudoexon (c.1331 + 17770C>G)	23	11	Never
9	BMD	deletion exons 3–27	Ambulant as of 10	8.5	Prednisone, daily (3 years)
10	BMD	deletion exons 10–44	Ambulant as of 3	2	Never
11	BMD	deletion exons 45–51	Ambulant as of 14	12	Never
12	BMD	Nonsense (c.5404C>T)	Ambulant as of 45	42	Never
NC	WT	None	N/A	6	N/A

BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; IMD, intermediate muscular dystrophy; N/A, not applicable; NC, normal control tissue; WT, wild type.

Method of quantifying dystrophin intensity at the muscle membrane using spectrin masking

Serial 10 micron muscle sections

Primary antibodies:

- Mouse monoclonal spectrin (NCL-SPEC1, Leica Microsystems Inc.), 1:100
- Rabbit polyclonal C-terminal dystrophin (ab15277, Abcam, Cambridge, Massachusetts, USA), 1:400

Secondary antibodies (both 1:500):

- Alexa Fluor® 488 F(ab')2 fragment goat anti-mouse IgG (H+L) (A11017, Molecular Probes, Eugene, Oregon, USA)
- Alexa Fluor® 568 goat antirabbit IgG (A11036, Molecular Probes)

Mounted in ProLong® Gold antifade reagent (P36934, Molecular Probes).

Method of quantifying dystrophin intensity at the muscle membrane using spectrin masking

12-bit .tiff images are obtained using the confocal microscope.

(**A**) the spectrin image and (**B**) the dystrophin image files are both opened.

(**C**) A threshold picked by the user is applied to the spectrin image to determine contiguous regions that will create the spectrin mask.

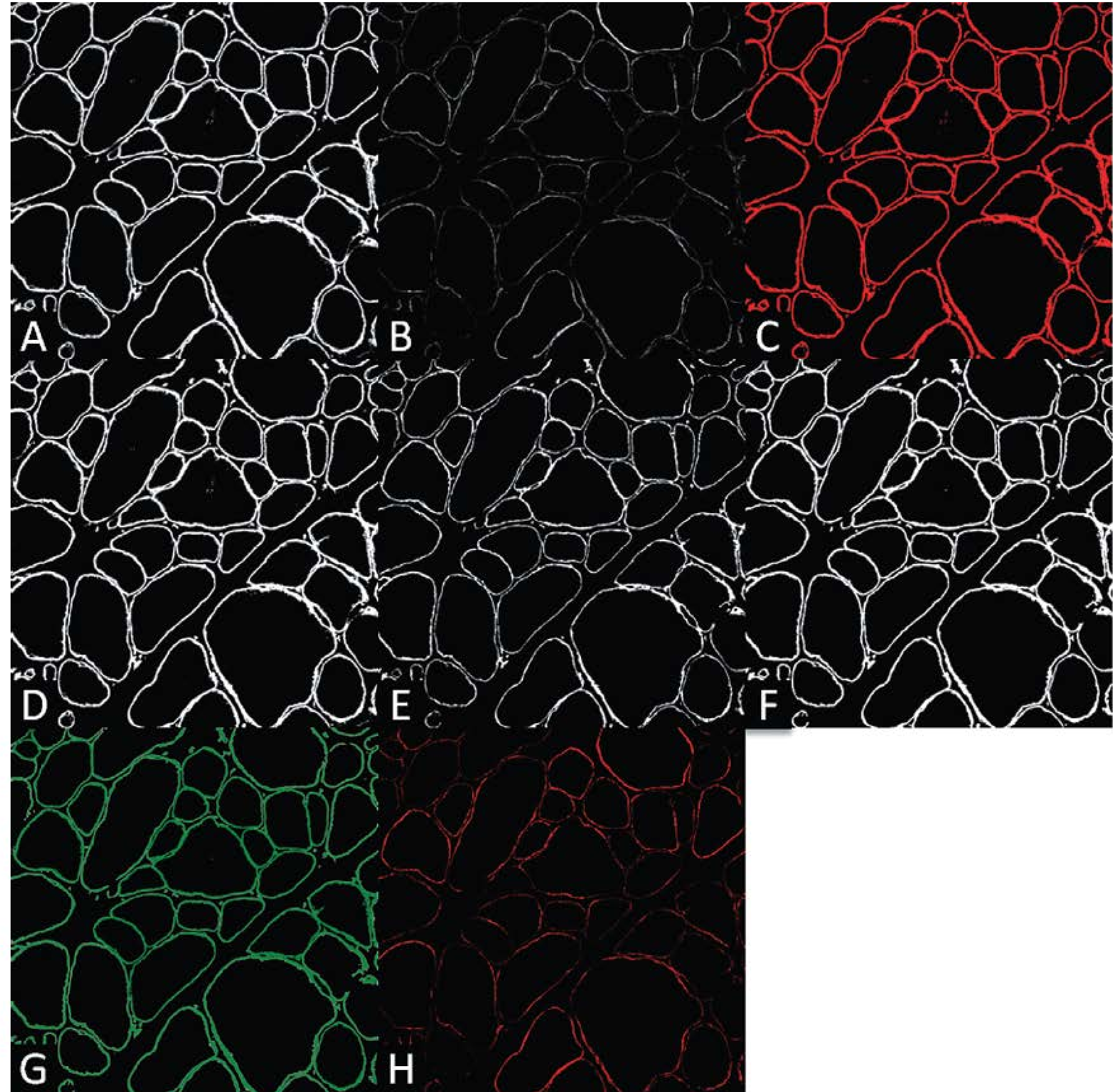
(**D**) The thresholded spectrin image is converted into a binary image where all white pixels have a value of 1 and all black pixels have a value of 0.

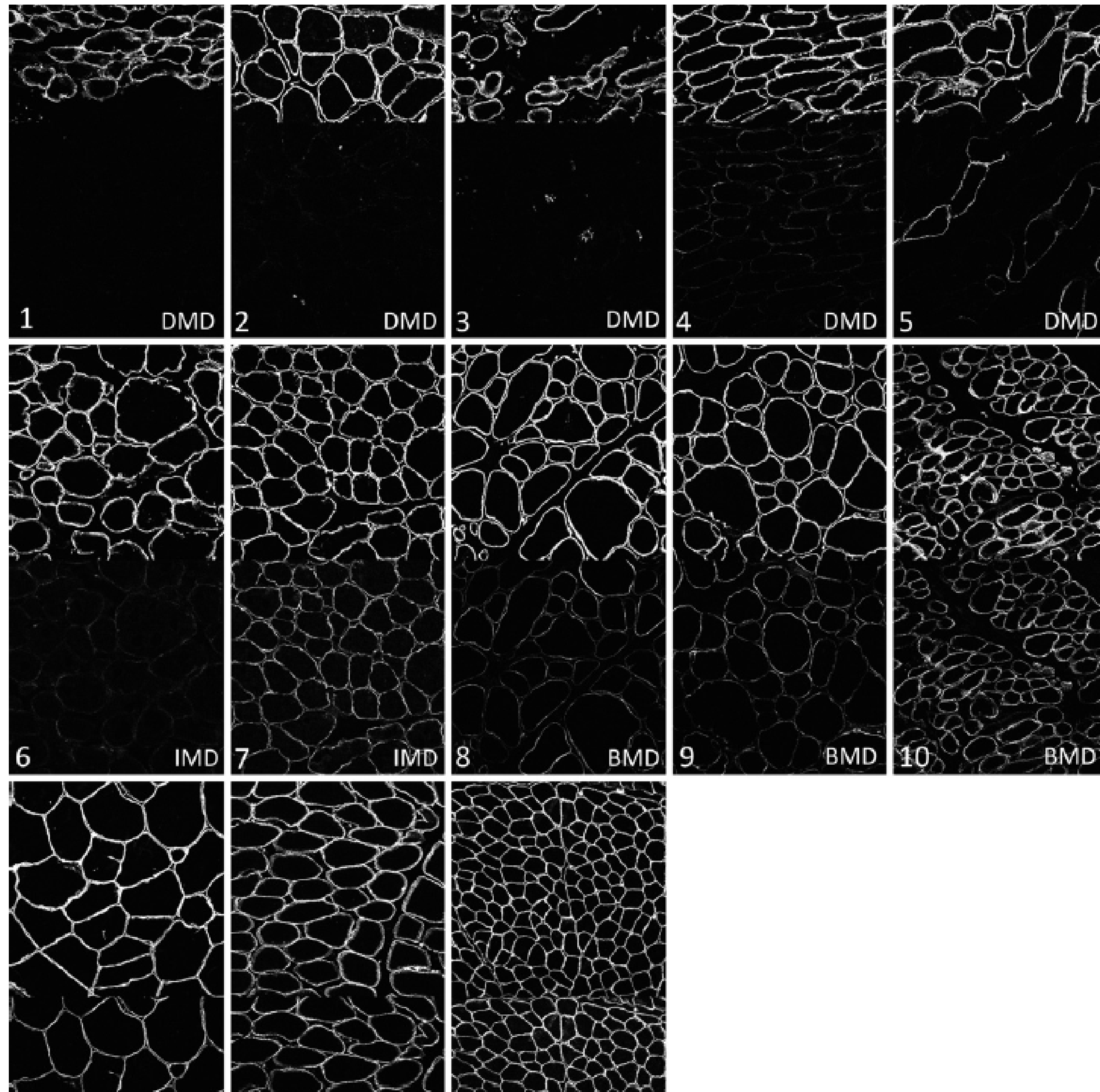
(**E**) The binary image is eroded by converting pixels with a value of 1 to 0, if the surrounding pixels are less than or equal to the Neighbourhood value of 3.

(**F**) The eroded image is dilated to restore positive regions lost during the erosion step. Black pixels with a value of 0 are converted to white pixels with a value of 1 if the surrounding white pixels are greater than or equal to the Neighbourhood value of 3. Erode and Dilate used in combination help remove noise. The resulting image is used as the spectrin mask.

The spectrin mask is applied to both the (**G**) spectrin image and (**H**) the dystrophin image.

Colours are used by the Metamorph program to show the mask being applied to the original image. Area and intensity values within the mask are automatically recorded in an Excel spreadsheet.




1
DMD
2
DMD
3
DMD
4
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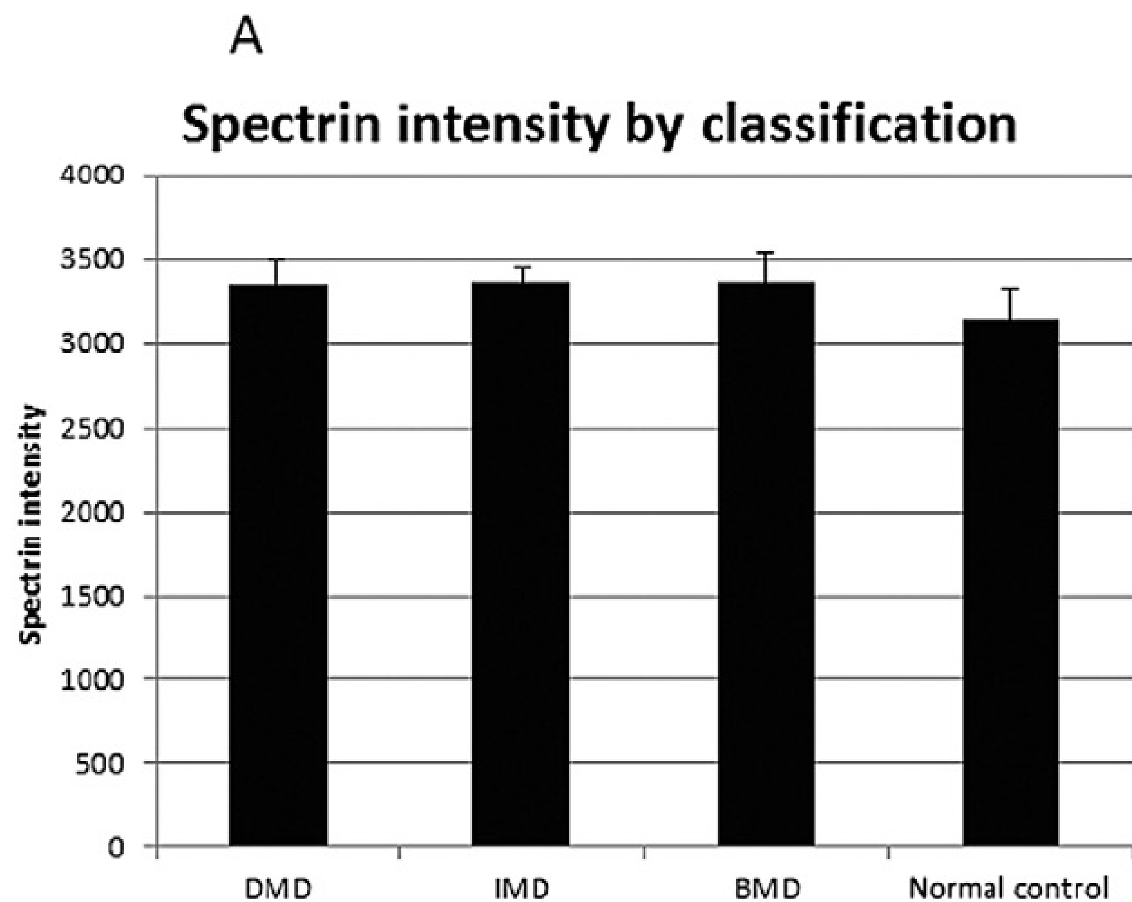
5
DMD
6
IMD
7
IMD
8
BMD
9
BMD
10
BMD



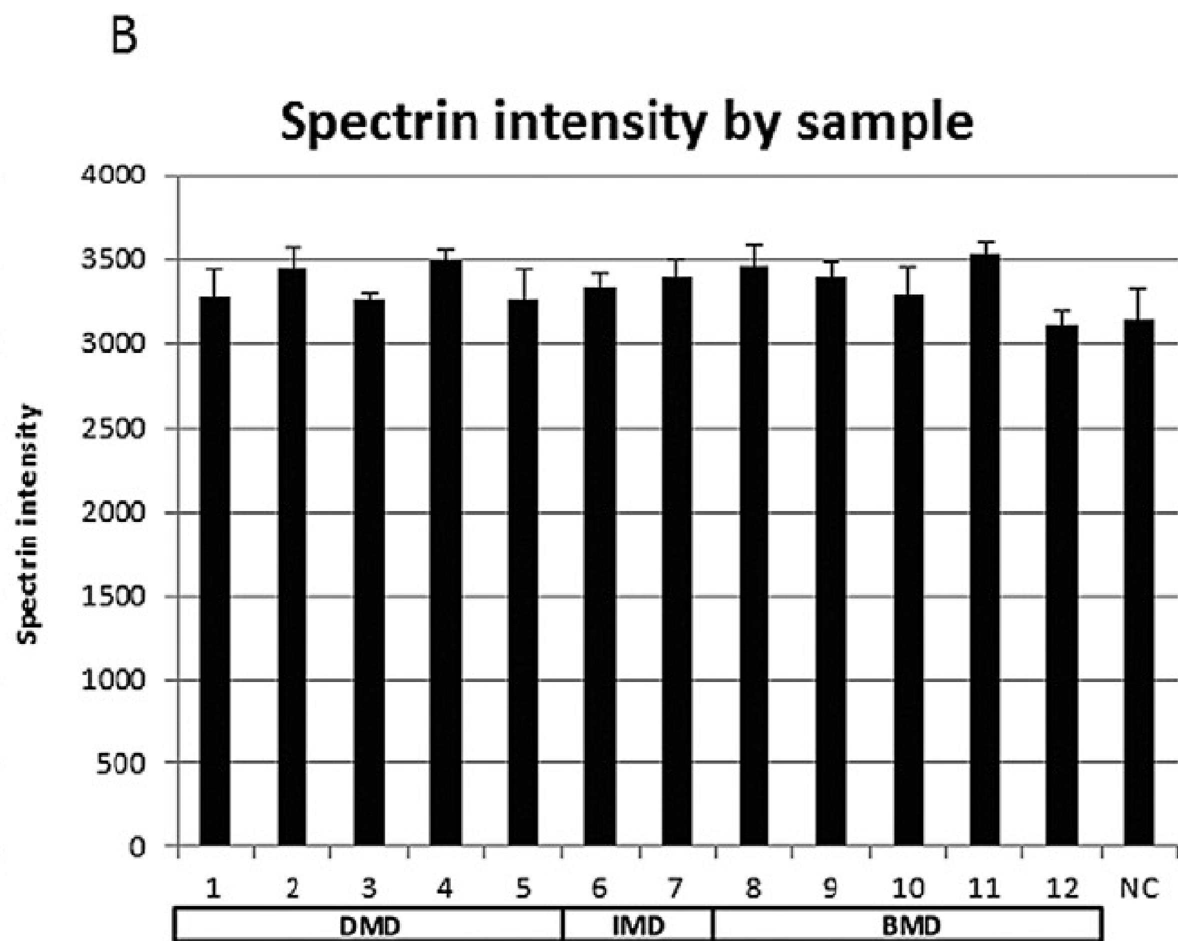


Figure 3. Mean spectrin intensity values. (A) Spectrin intensity for each phenotype subclass. There is no significant difference between Duchenne muscular dystrophy (DMD), intermediate muscular dystrophy (IMD) or Becker muscular dystrophy (BMD) groups and normal control (*P*-values: DMD *vs.* normal 0.182, IMD *vs.* normal 0.209, and BMD *vs.* normal 0.163). (B) Spectrin intensity among 12 dystrophinopathy samples and wild-type control. Values are arbitrary fluorescent units on a 12-bit scale (in which a saturated pixel registers at 4095), using a mean value from two independent staining and imaging events on separate days. NC, normal control tissue.

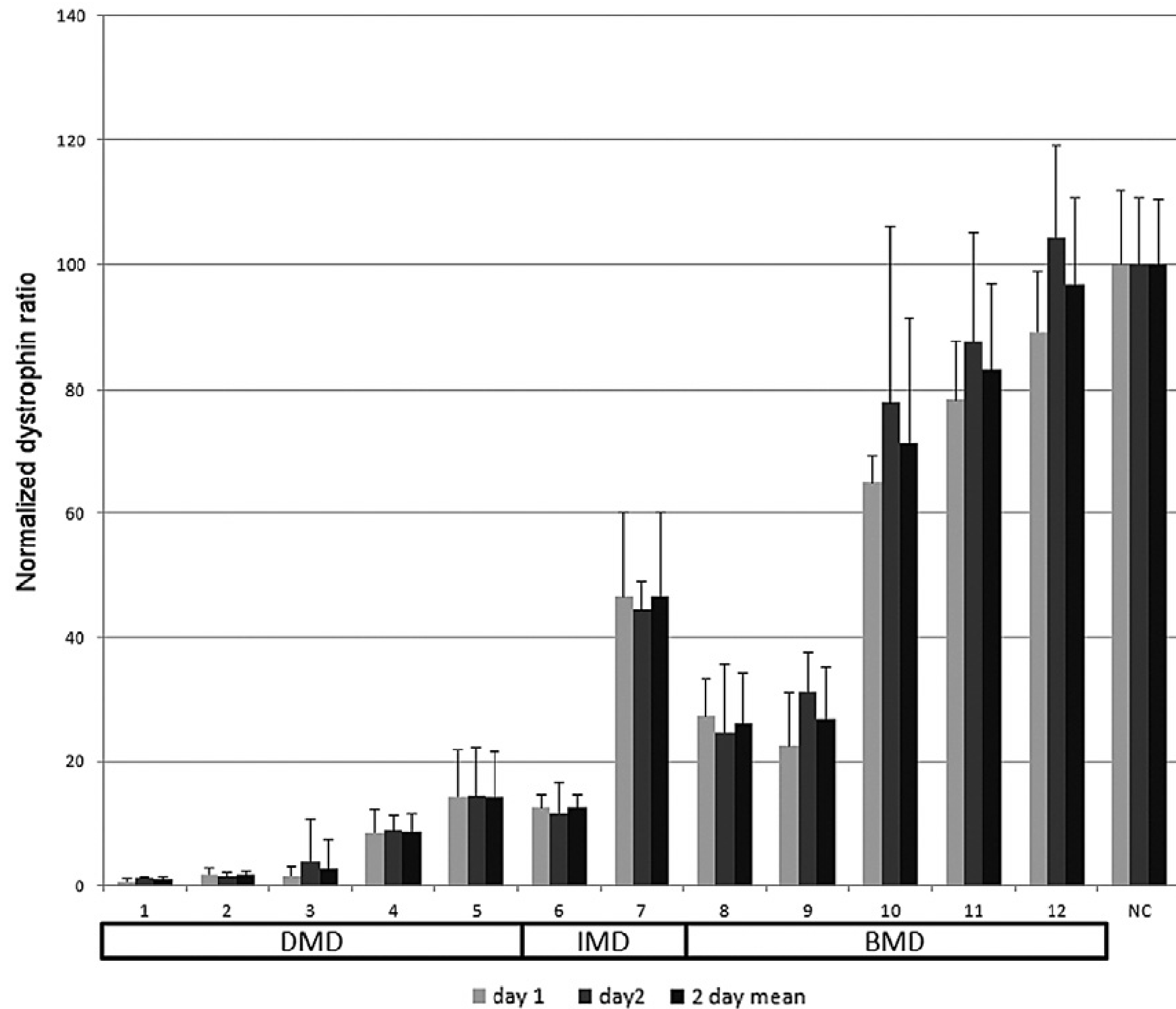


Figure 4. Dystrophin : spectrin ratios, normalized to wild-type muscle, from images acquired on two separate days of staining and imaging. There is no significant difference in the ratios between the two days (P-value = 0.124). BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; IMD, intermediate muscular dystrophy; NC, normal control tissue.

Western blot method

- 10 muscle sections (10 micron) solubilized in 400 ml lysis buffer on Tissuelyzer II (30 s X 3)
    - 4.4 mM Tris, pH, 9% SDS, 4% glycerol, 5% b-Mercaptoethanol)
- 25 mg total protein 2–8% Tris-Acetate precast gel (Invitrogen)
- Run at 30 V (constant) for 5 h and 30 min, at which time the voltage was increased to 100 V for 1 hour
- Transfer to nitrocellulose at 300 mAmps for 18 h at 4°C
- Blocked in 5% non-fat dry milk diluted in TBST (0.1% Tween20) for 1.5 h at room temperature
- Primary antibodies for 19 hours at 4 deg C:
    - dystrophin (Ab15277) at 1 mg/ml
    - Pan-actin (Neomarkers, Kalamazoo, Michigan, USA) at 0.5 mg/ml) for 19 h at 4°
- Room temp incubation for 30 min with the HRP-conjugated secondary antibodies:
    - Goat anti-rabbit (1:15000)
    - Goat anti-mouse IgG (H+L) (1:500000)
- SuperSignal West Femto Maximum Sensitive Substrate (Thermo Scientific) on Kodak Biomax Light film
- Bands quantified using the ImageJ gel analyser function.
- Dystrophin band intensity was normalized to pan-actin in each lane
- Expressed as a percentage of the control specimen dystrophin signal.

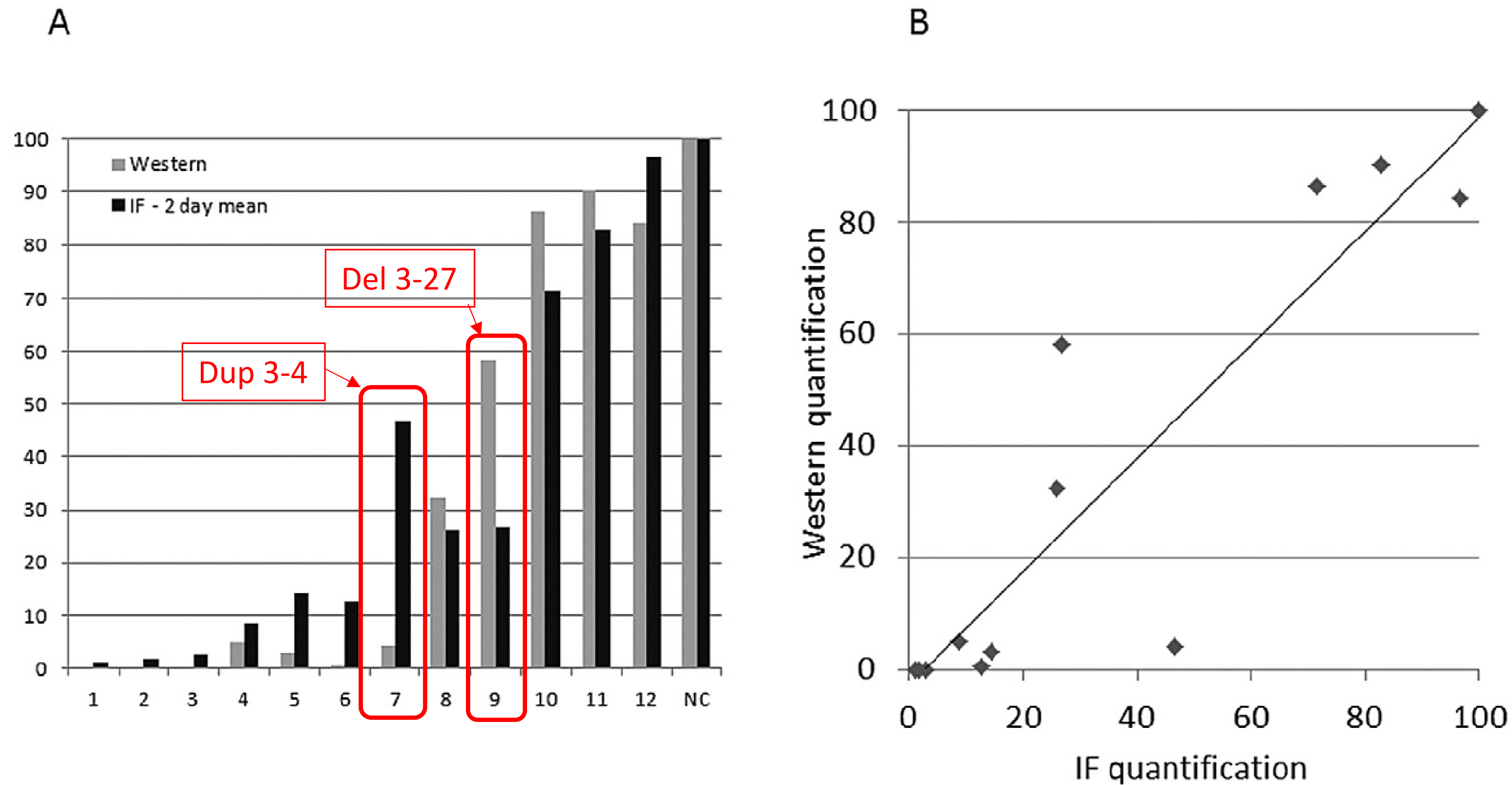


Figure 7. Comparison of dystrophin expression by two different methods. (A) Quantification results using immunoblot (light bars) and immunofluorescence (dark bars) (mean of two different experiments) (B) Linear regression performed with the paired observations indicates strong correlation between the two methods (Pearson correlation coefficient = 0.91 for all samples). A *t*-test of the paired two sample means suggests no significant difference of the averaged dystrophin percent between the Western and immunofluorescent (IF) methods (*P*-value = 0.666).

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Dystrophin quantification

Biological and translational research implications

OPEN

ABSTRACT

Objective: We formed a multi-institution collaboration in order to compare dystrophin quantification methods, reach a consensus on the most reliable method, and report its biological significance in the context of clinical trials.

Methods: Five laboratories with expertise in dystrophin quantification performed a data-driven comparative analysis of a single reference set of normal and dystrophinopathy muscle biopsies using quantitative immunohistochemistry and Western blotting. We developed standardized protocols and assessed inter- and intralaboratory variability over a wide range of dystrophin expression levels.

Results: Results from the different laboratories were highly concordant with minimal inter- and intralaboratory variability, particularly with quantitative immunohistochemistry. There was a good level of agreement between data generated by immunohistochemistry and Western blotting, although immunohistochemistry was more sensitive. Furthermore, mean dystrophin levels determined by alternative quantitative immunohistochemistry methods were highly comparable.

Conclusions: Considering the biological function of dystrophin at the sarcolemma, our data indicate that the combined use of quantitative immunohistochemistry and Western blotting are reliable biochemical outcome measures for Duchenne muscular dystrophy clinical trials, and that standardized protocols can be comparable between competent laboratories. The methodology validated in our study will facilitate the development of experimental therapies focused on dystrophin production and their regulatory approval. *Neurology*® 2014;83:2062–2069

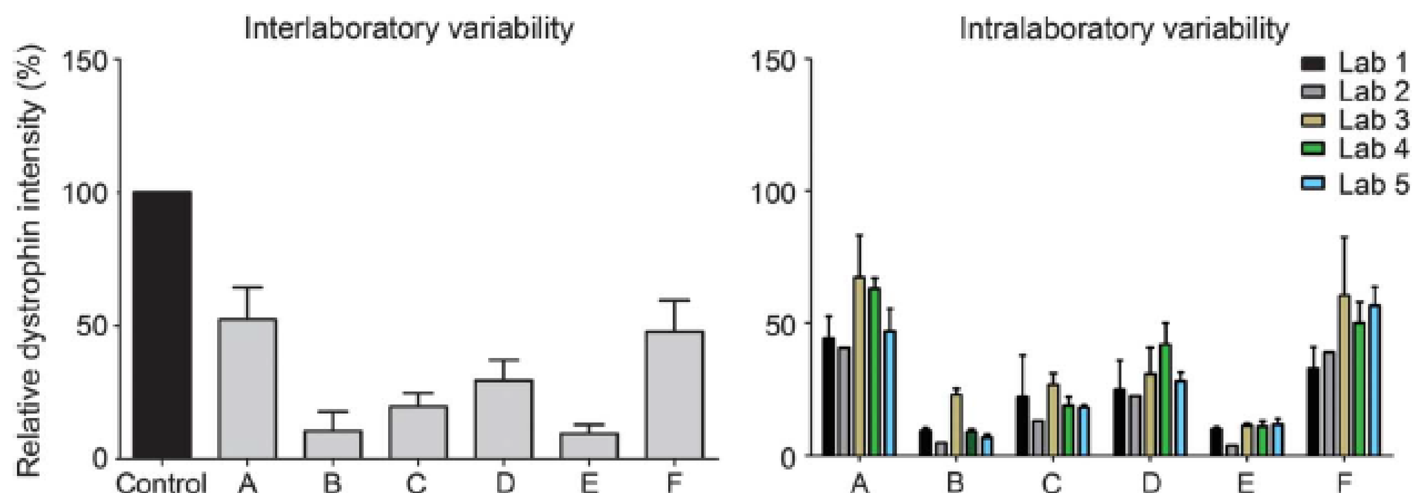
Good concordance for ranking of samples in order or dystrophin expression

Table 1 Sample ranking order by laboratory

Immunohistochemistry						Western blotting					
Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
A (BMD)	1	1	1	1	2	F (BMD)	1	1	1	1	1
F (BMD)	2	2	2	2	1	D (BMD)	2	3	2	3	2
D (BMD)	3	3	3	3	3	A (BMD)	3	2	3	2	3
C (DMD)	4	4	4	4	4	C (DMD)	4	4	4	4	4
E (DMD)	5	6	6	5	5	E (DMD)	5/6	5/6	5	5	5/6
B (DMD)	6	5	5	6	6	B (DMD)	5/6	5/6	6	6	5/6

Abbreviations: BMD = Becker muscular dystrophy; del = deletion; DMD = Duchenne muscular dystrophy; dup = duplication; ex = exon.
Samples: A = c.40_41delGA; B = dup ex 10–17; C = dup ex 7; D = del ex 3–27; E = del ex 6; F = del ex 10–44.

Figure 1 **Inter- and intralaboratory variability of dystrophin quantification using immunohistochemistry**

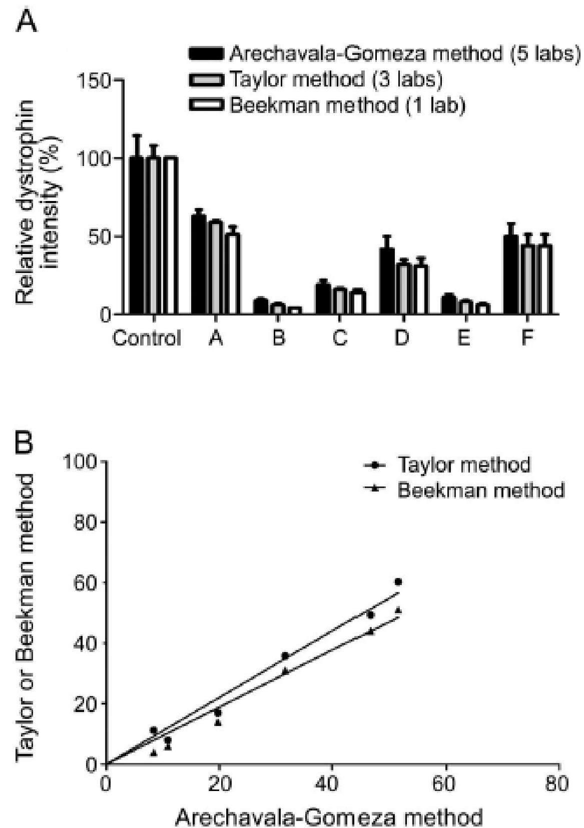


Interlaboratory variability				Intralaboratory variability	
Sample	n	Mean ± SD	CV	Lab	Average CV
A	5	52.42 ± 11.93	22.76	1	29.83
B	5	10.6 ± 7.13	67.26	2	n/a
C	5	19.79 ± 5.04	25.47	3	20.9
D	5	29.64 ± 7.6	25.64	4	14.41
E	5	9.56 ± 3.33	34.83	5	13.71
F	5	47.85 ± 11.65	24.35	—	—

Five laboratories each quantified the level of dystrophin expression in the same 6 biopsies using a standardized immunohistochemistry protocol; data were analyzed using the Arechavala-Gomeza method.[19] To assess interlaboratory variability, the mean ± SD for each biopsy was calculated as well as the coefficient of variation (CV). Note how this variation is higher for those samples containing less dystrophin (E and B). To assess intraassay precision within each laboratory, the mean ± SD for each laboratory per sample was calculated as well as the average CV per laboratory. Laboratories are unidentified.

Concordance between the Taylor and Arechavala-Gomeza methods of IF quantification

Figure 2 **Assessing the agreement between different methods of immunohistochemical dystrophin measurement**



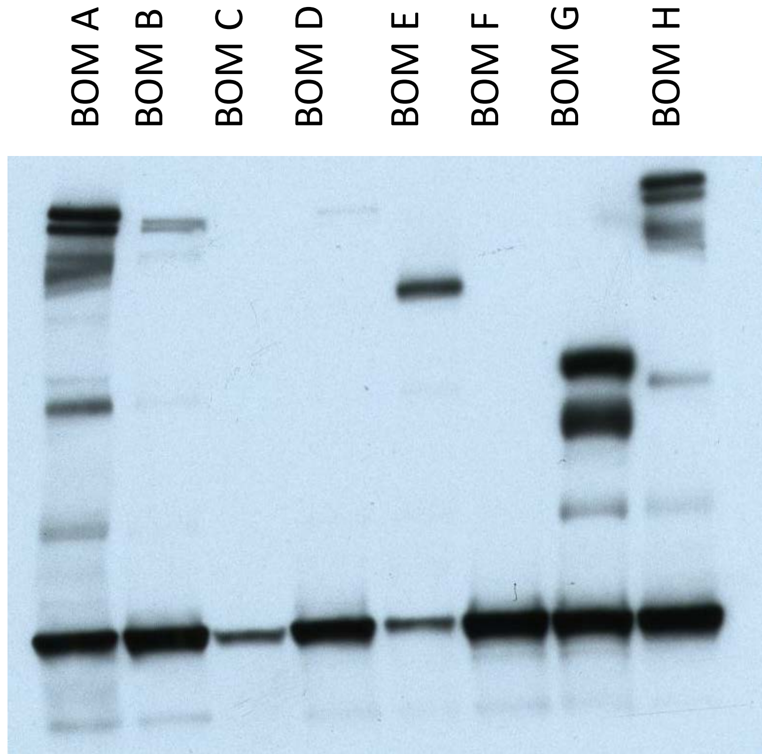
C
20
0
-20
Difference
Upper 95%
Bias
Lower 95%
0
20
40
60
Mean

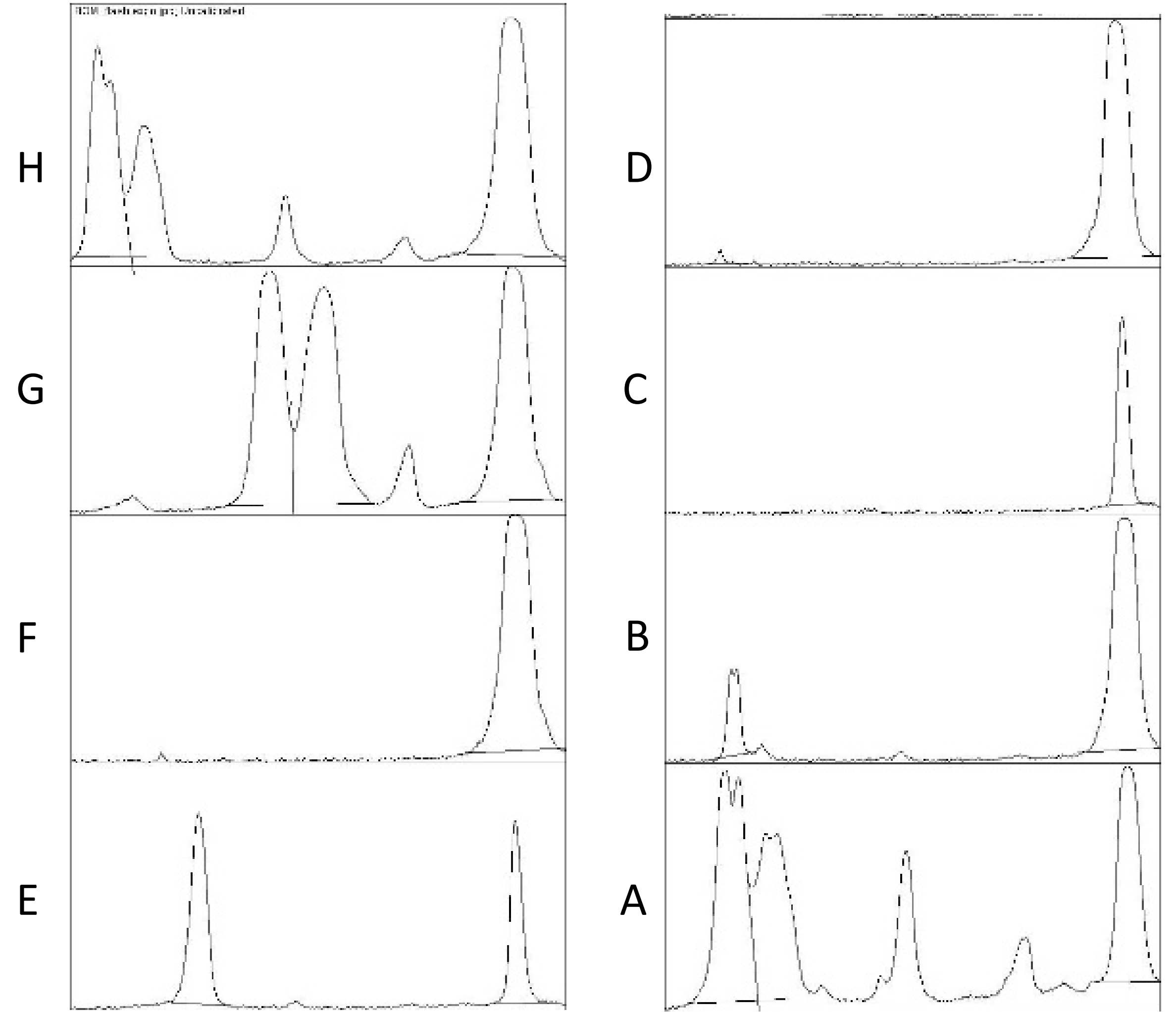
The mean data from each method were compared in a bar chart ± SD (A) and plotted with a regression line (B). The difference between the Arechavala-Gomeza and Taylor methods was plotted against their mean in a Bland-Altman plot (C) where the mean of the differences between the methods represents the bias (i.e., the value determined by one method minus the value determined by the other method) and the upper and lower 95% confidence limits represent the upper and lower limits of agreement, respectively (the difference between the 2 methods should lie within these bounds on 95% of occasions).

Western protocol standardized among all six labs

- Solubilized in lysis buffer
- Loading 25 mg of protein
- Each laboratory used their preferred gel electrophoresis methods/equipment
  - Typically 3%–8% tris-acetate gradient gels
- **Dystrophin** C-terminal primary antibody: **Abcam ab15277**
  - 1 mg/mL overnight at 4°C in 5% milk TBS-T (TRIS buffered saline, 0.1% Tween20).
- Sarcomeric **a-actinin** primary antibody (**Clone EA-53**; Sigma, St. Louis, MO) 1:3,000 in 5% milk
- TBS-T was added and membranes were incubated for 1 hour at room temperature.
- Membranes were washed (3X) for 10 minutes each in PBS-T.
- Secondary antibodies compatible with the laboratories' imaging equipment
- Each laboratory used their preferred image acquisition equipment (e.g., Image J–based software, Odyssey infrared imaging system)
- Data were normalized to a-actinin and presented relative to an average of the 2 controls.

Quantification by Image J

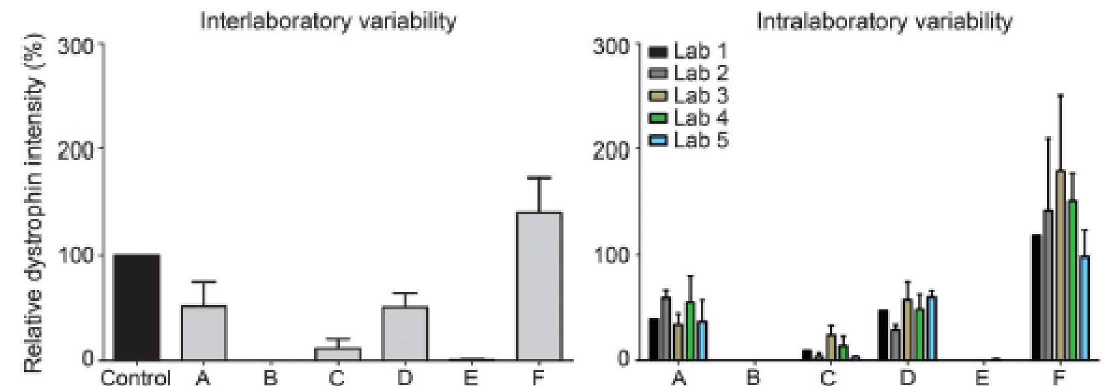




Higher variability in WB than in IF among labs

- Mean SD of 15.95 (ranging between 0.89 for sample E and 33.09 for sample F)
- CV values for Western blotting averaged 80% (ranging between 23% for sample F and 223% for sample E)
- **Inter**laboratory variability improves as the level of dystrophin increases.
- **Intra**laboratory variability was also more pronounced than for immunohistochemistry.
  - Only laboratory 1 had an optimal CV value of 0.3%; laboratory 3 had the highest at 119% (figure 3).

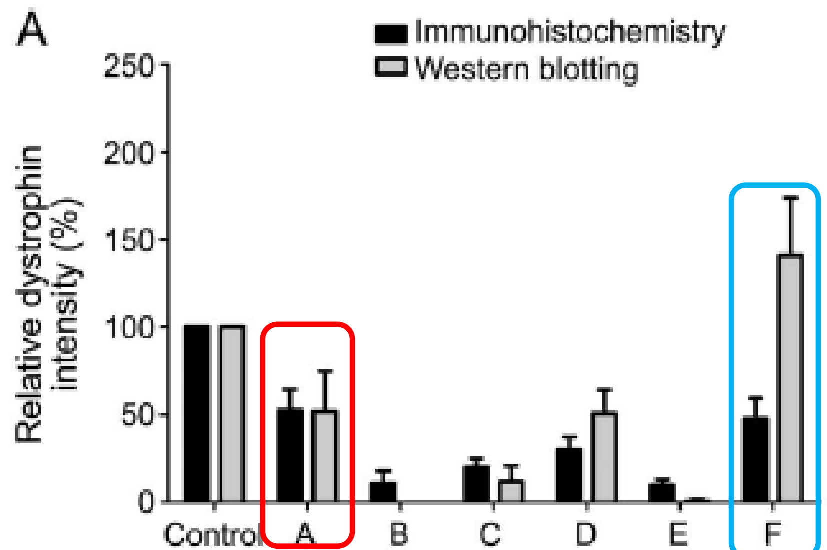
Figure 3 **Inter- and intralaboratory variability of dystrophin quantification using Western blotting**

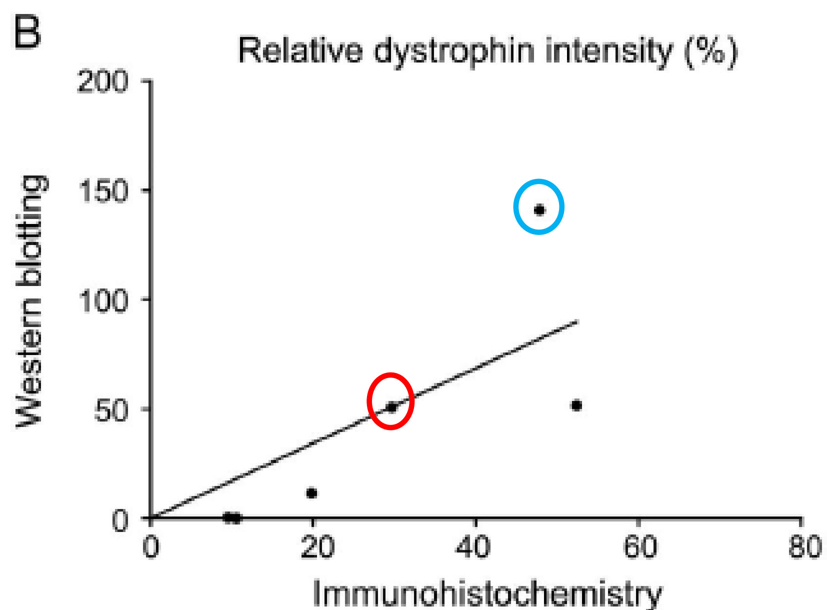


	Interlaboratory variability			Intralaboratory variability	
Sample	n	Mean ± SD	CV	Lab	Average CV
A	5	51.62 ± 23.17	44.89	1	0.3
B	5	0 ± 0	n/a	2	37.76
C	5	11.41 ± 9.22	80.81	3	118.88
D	5	50.67 ± 13.39	26.43	4	86.86
E	5	0.4 ± 0.89	222.5	5	32.98
F	5	140.85 ± 33.09	23.49	—	—

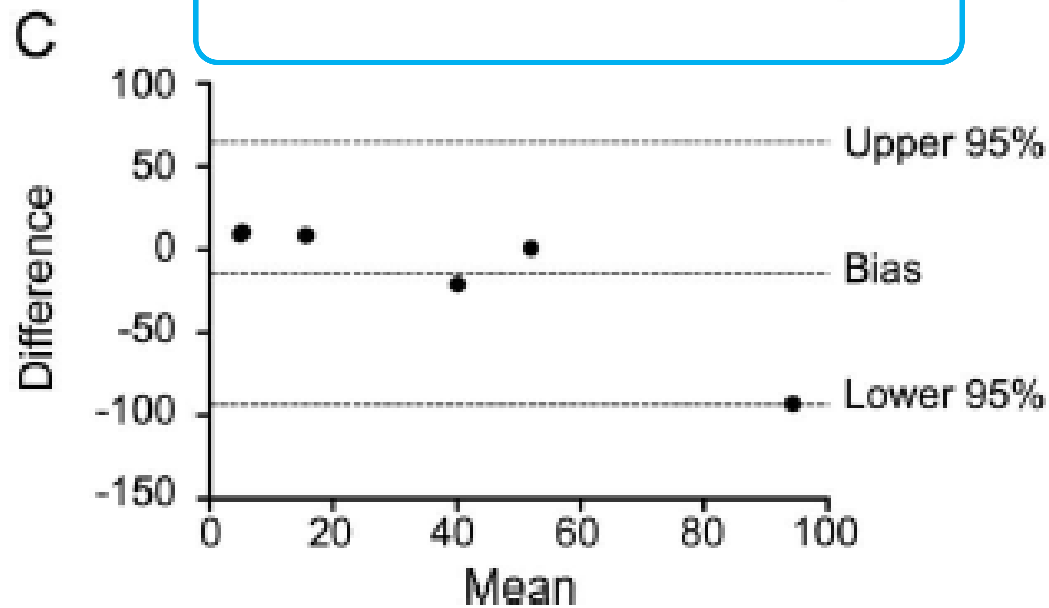
Five laboratories each quantified the level of dystrophin expression in the same 6 biopsies using a standardized Western blotting protocol. To assess interlaboratory variability, the mean ± SD for each laboratory and biopsy was plotted on a bar chart and the average coefficient of variation (CV) per laboratory calculated. To assess intralaboratory variation, the mean ± SD for each laboratory per sample was calculated as well as the average CV per laboratory. Laboratories are unidentified.

Figure 4 Assessing the agreement between immunohistochemistry and Western blotting for dystrophin quantification





- In some samples, IF and WB compared quite well
  - Sample A, BMD: c.40_41del GA
- In others, WB results were much higher.
  - Sample F, BMD: large



Our conclusions (1)

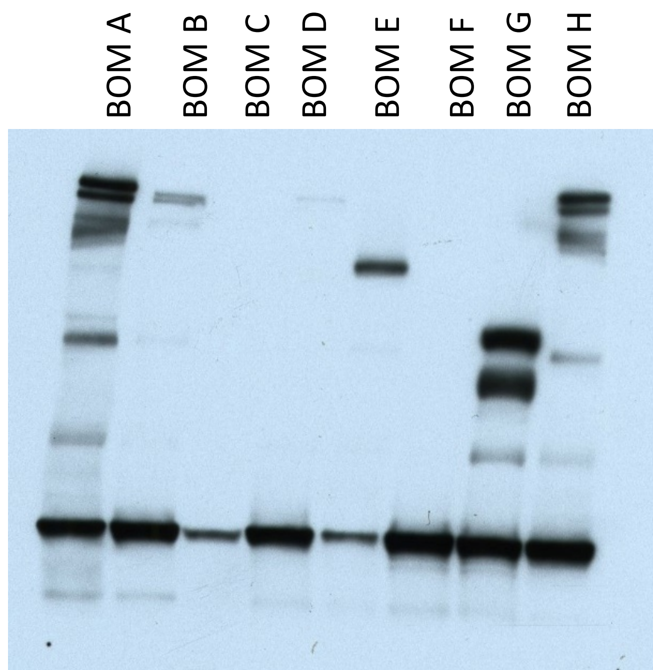
- Many BMD mutations (and presumably, the equivalent DMD mutations after exon skipping) affect the 3-dimensional structure and actin-binding properties of dystrophin
- Capturing both the total amount of dystrophin in the homogenate as well as its localization at the sarcolemma is clearly important

Can we minimize sources of variability in WB?

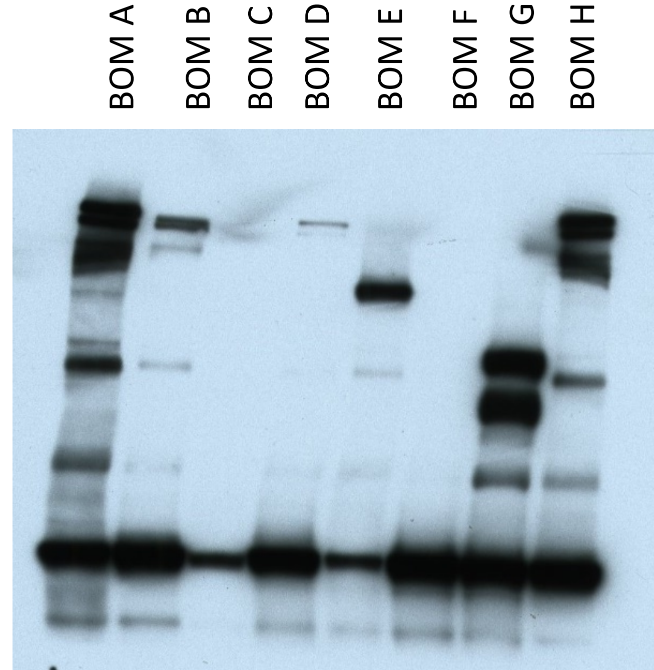
- ECL detection is sensitive (low pg detection)
- Limitations of ECL detection
  - Indirect signal (enzymatic reaction)
  - Timing of exposure; saturation of signal
  - Challenges to co-probing (often need to strip/re-probe)
  - Variability in response

Dys C-term
(Ab15277,
Thermo)
1:200

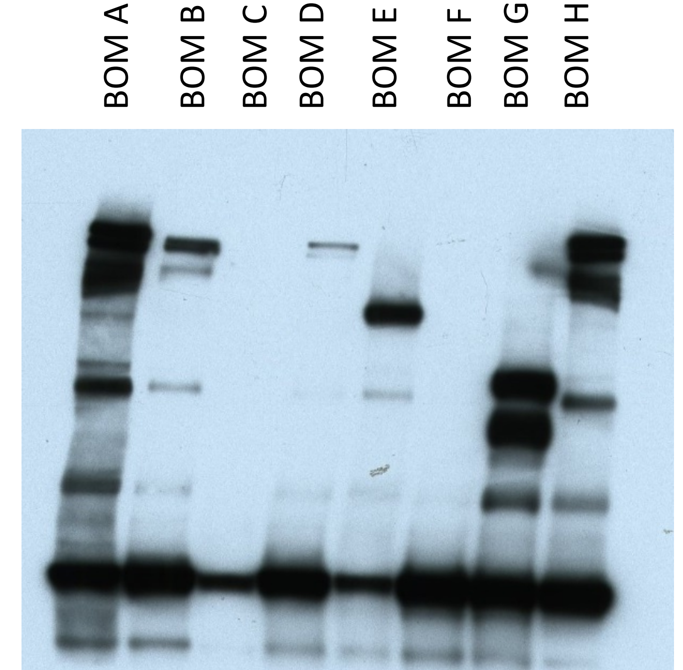
α-actinin
(EA-53, Sigma)
1:3000



Flash



10s

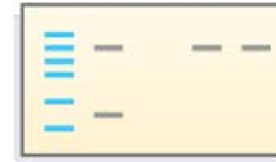


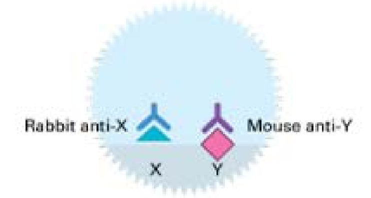
20s

Dual color infrared dye imaging (LiCor Odyssey)

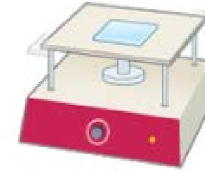
- Linearity of the signal
- Controlled for saturation
- Multiplex detection
- Improved normalization on same blot

Step 4:
Incubate with Primary Antibodies

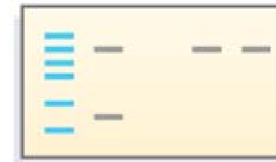




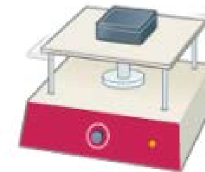
Step 5:
Wash



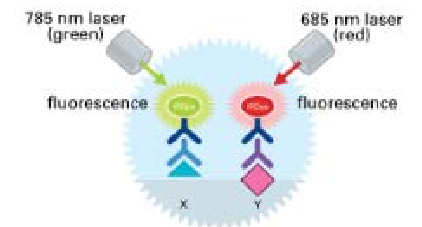
Step 6:
Incubate with Secondary Antibodies



Step 7:
Wash



Step 8:
Image with an Odyssey Imager



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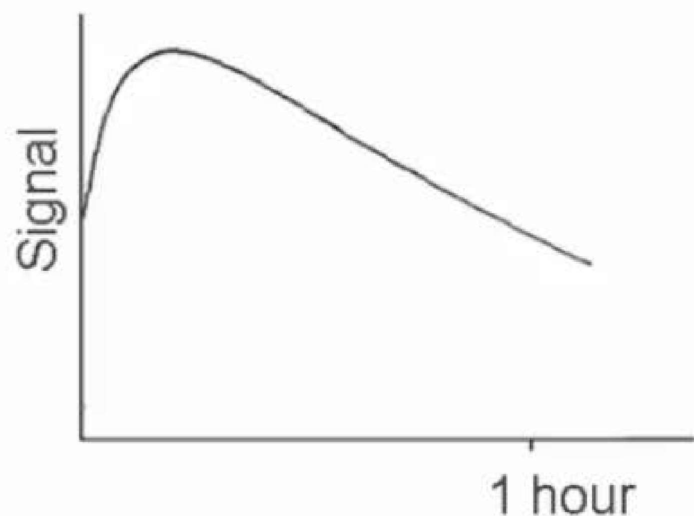
Detection system

Signal stability is critical for accurate quantitation



Chemiluminescence

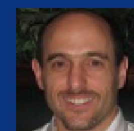
- Unstable signal declining within minutes
- High variation between blots
- Skills and controlled handling needed for accurate quantitation
- *Good choice for confirmatory Westerns*

Fluorescence

- Stable signal for months
- High reproducibility
- Accurate quantitation
- *First choice for quantitative Westerns*

Speaker: Tibor Harkany, Ph.D.
Medical University of Vienna
Vienna, Austria
View Bio

Speaker: Åsa Hagner McWhirter, Ph.D.
GE Healthcare Life Sciences
Uppsala, Sweden
View Bio

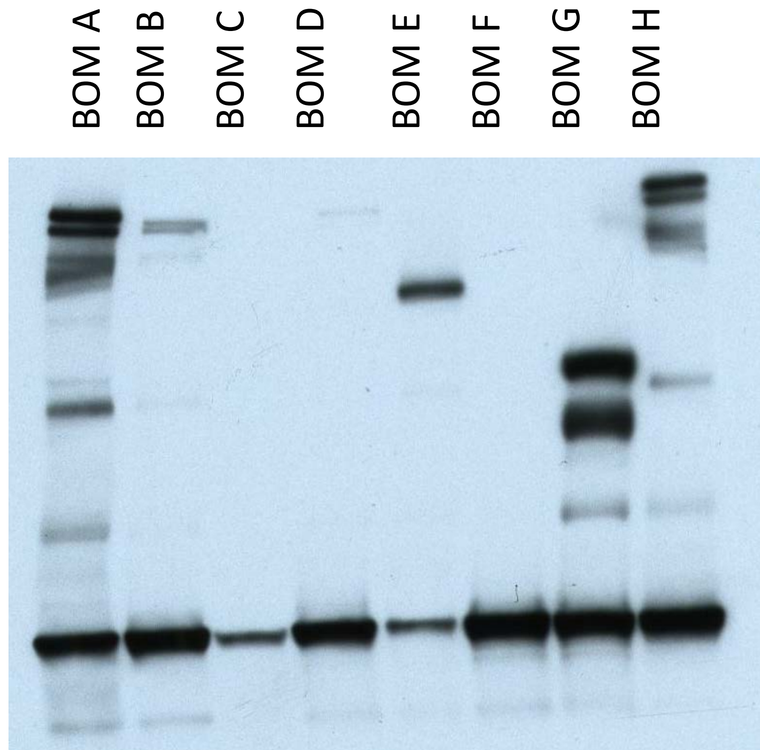


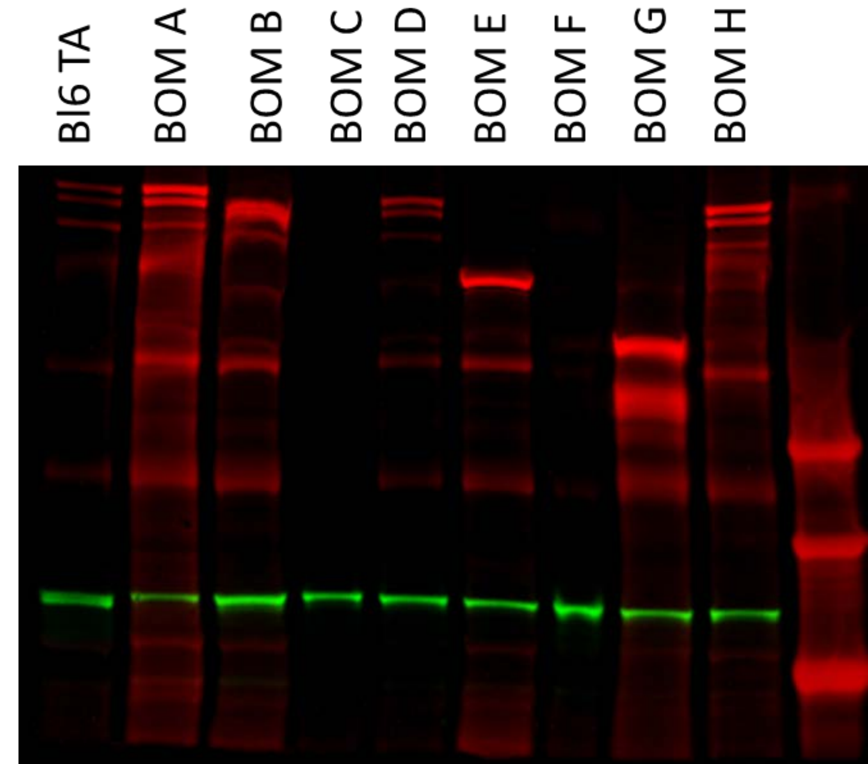
Moderator: Sean Sanders, Ph.D.
Science/AAAS
Washington, DC
View Moderator Bio

Click here to view the test stream

http://webinar.sciencemag.org/webinar/archive/quantitative-western-blotting (March 3, 2015)

BOM Samples with fluorometric detection (LiCor)

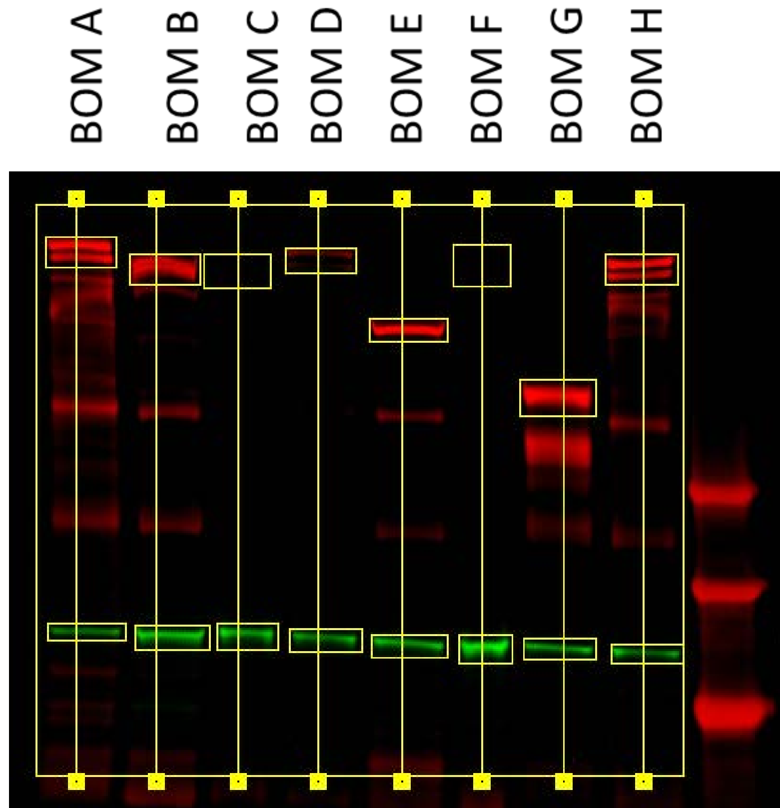


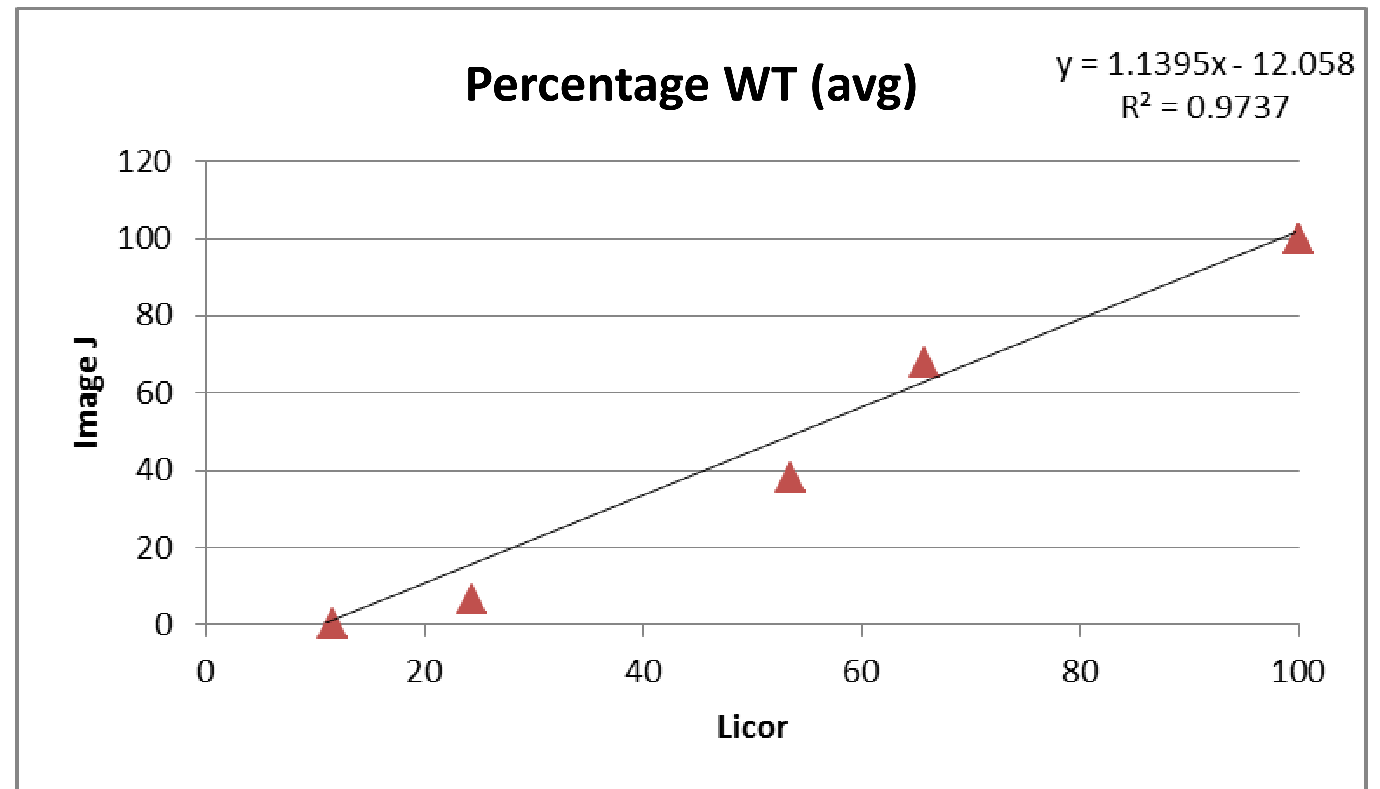


Dys C-term
(Dys, Thermo)
1:200

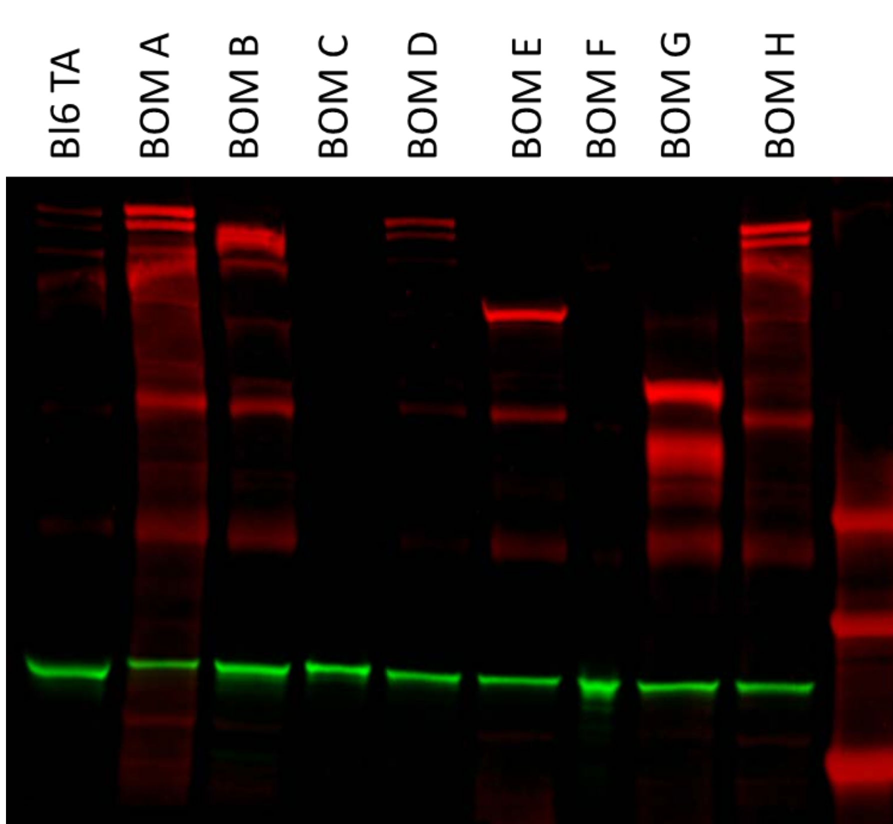
α-actinin
(EA-53, Sigma)
1:3000

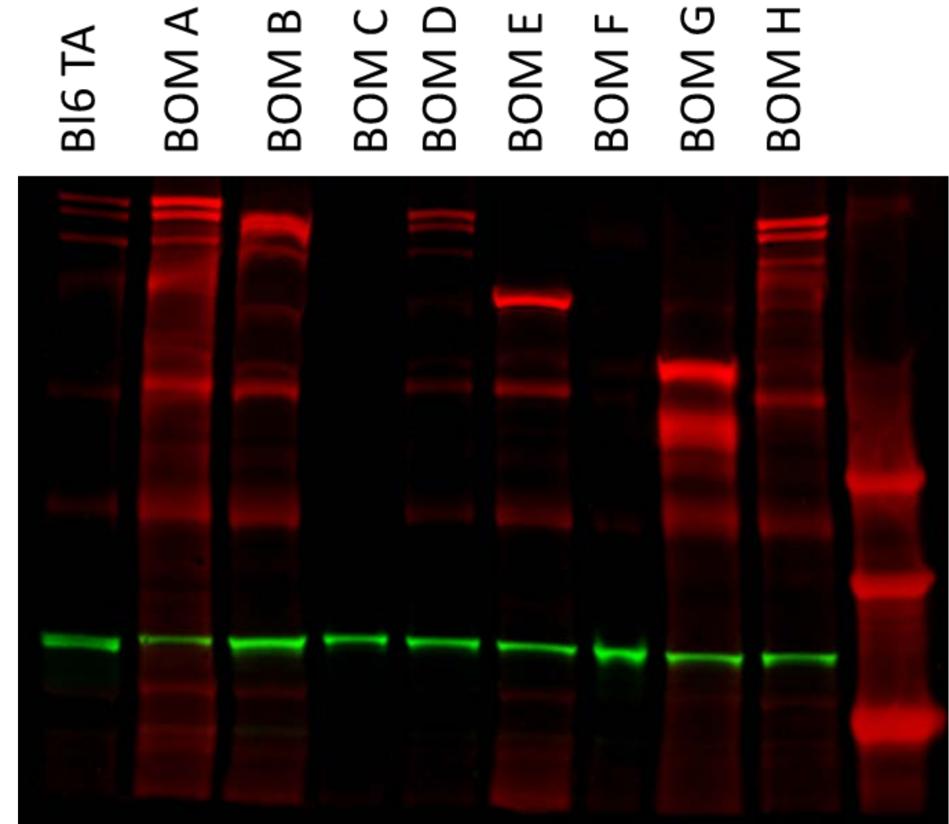
Excellent concordance between ECL and LiCor quantification





Blot-to-blot reproducibility is high

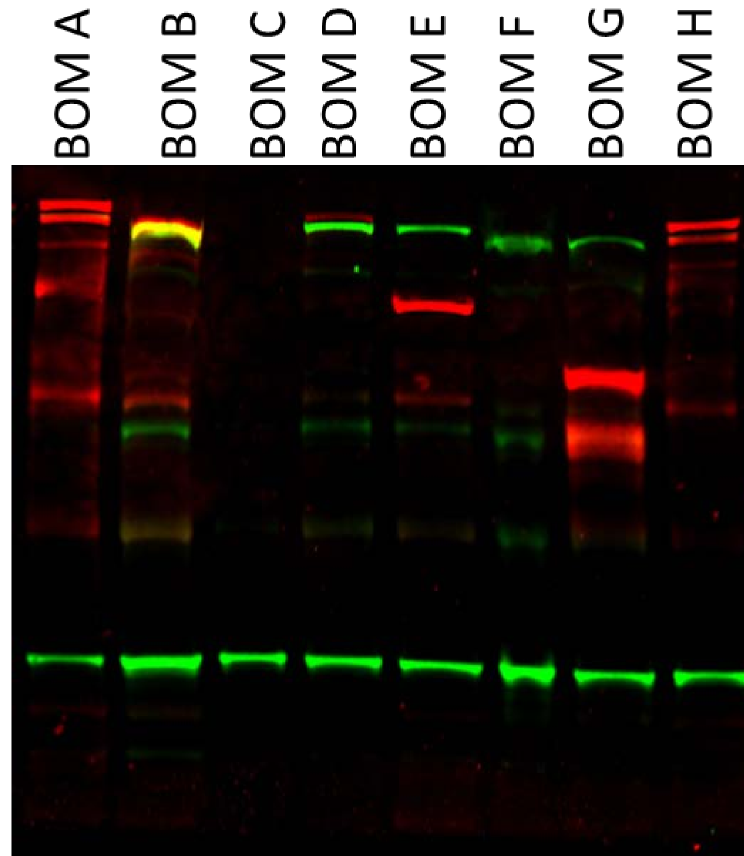




Further multiplexing is possible

Utrophin (green)
Mancho3

α-actinin (green)
(EA-53, Sigma)

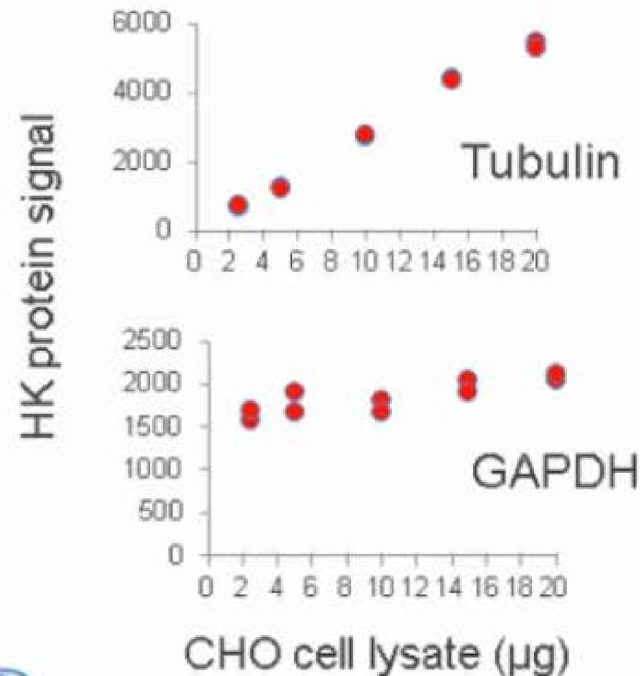


Dys C-term
(Dys, Thermo)

Validation of house-keeping proteins critical for accurate normalization results

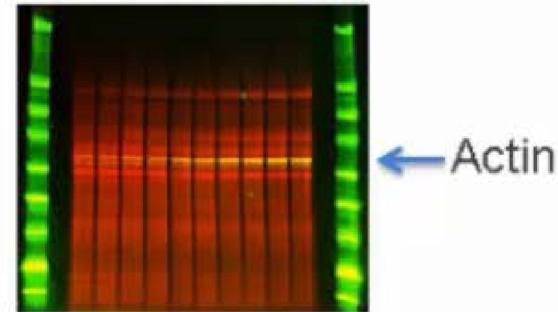
Speaker: Åsa Hagner McWhirter, Ph.D.
GE Healthcare Life Sciences
Uppsala, Sweden
View Bio

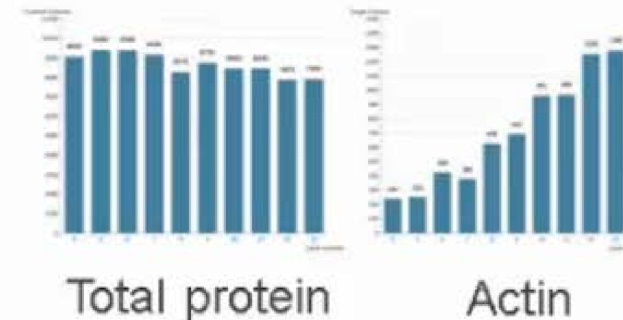
- Select house-keeping protein and probing conditions (antibody dilution) producing proportional response in the sample range to be used.



- Make sure the house-keeping protein is not affected by treatment

A431 cell lysate
EGF stimulation



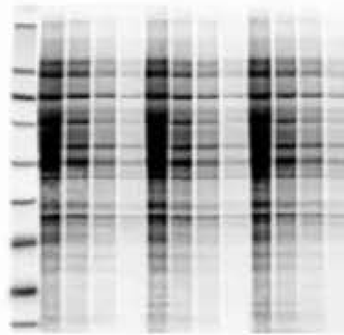


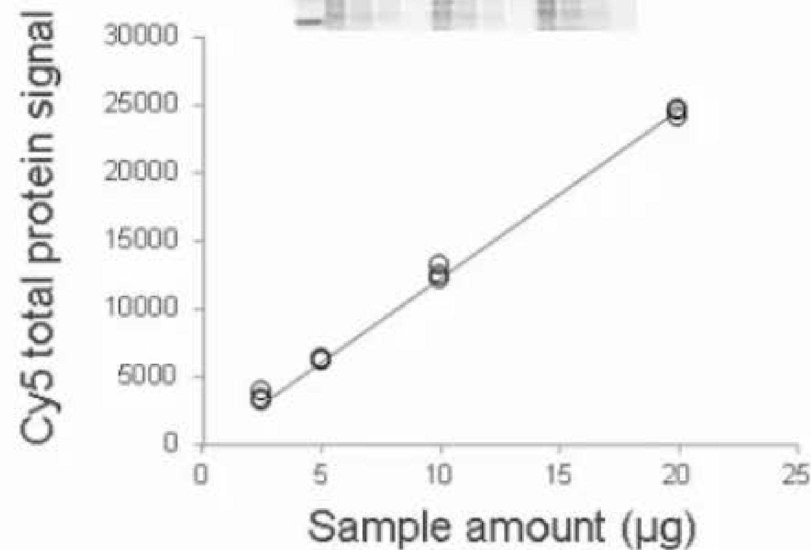
http://webinar.sciencemag.org/webinar/archive/quantitative-western-blotting (March 3, 2015)

Normalization using total protein

Speaker: Åsa Hagner McWhirter, Ph.D.
GE Healthcare Life Sciences
Uppsala, Sweden
View Bio

Cy™5 total protein pre-labeling





Reliable normalization method:

- Antibody independent
- Not affected by treatments
- Sum of many protein signals
- The whole lane or part of the lane can be used

http://webinar.sciencemag.org/webinar/archive/quantitative-western-blotting (March 3, 2015)

Conclusions

- Assessing dystrophin by both IF and WB is important, because a different pattern of expression can lead to differences in the functional outcome irrespective of the total amount of protein present
- Reference samples can be shared among laboratories – even internationally – for reproducible Western blotting
- A move to infrared dye imaging methods (LiCor, Amersham) will likely improve reproducibility further
- Normalization to total protein content (Cy5 labeling, for example) should be considered

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