Targeted Quantification of Dystrophin by Mass Spectrometry and Comparison to Antibody Based Assays.

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Targeted Mass spectrometry Assay

Total protein extract

↓

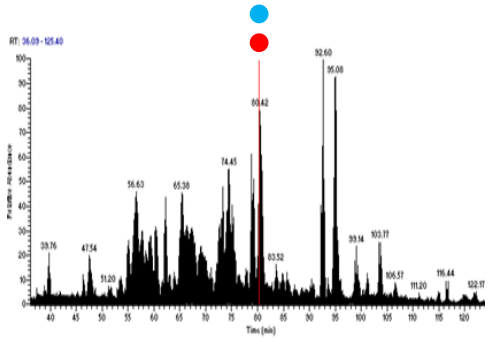
tryptic digest

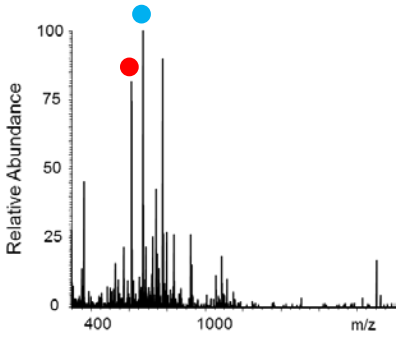
↓

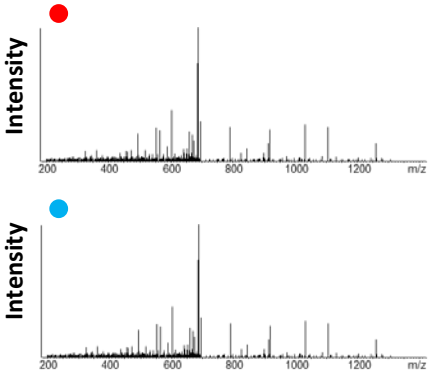
Spike with stable isotope labeled target protein

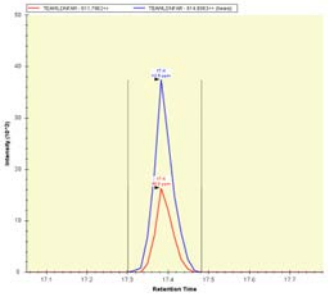
Spike with stable isotope labeled target peptides

LC → MS → MS/MS → Extracted ion intensity









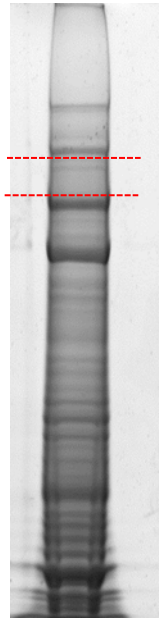
Identification

Quantification

Method

Previously

Human muscle extract
+
$^{13}C_6$, $^{15}N_2$-Lys labeled mouse muscle extract



In gel digestion with trypsin

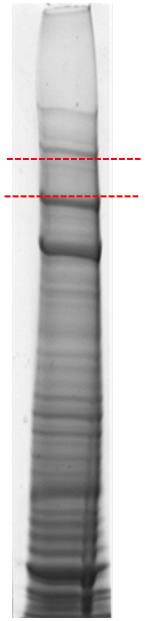
Extract peptides

LC-MS/MS

5 peptides used for quantification

Method improvement

Human muscle extract
+
$^{13}C_6$,$^{15}N_2$-Lys & $^{13}C_6$-Arg labeled human myotube extract



In gel digestion with trypsin

Extract peptides

LC-MS/MS

15 peptides used for quantification

Brown et al. J Bioanal. Biomed. 2013, S7

Peptide used for quantification and their position in dystrophin sequence

SILAM mouse strategy (5 peptides)

MLWWEEVEDCYEREDVQKKTFTKWVNAQFSKFGKQHIENLFSDLQDGRRLLDLLEGLTGQ
KLPKEKGSTRVHALNNVNKALRVLQNNNVDLVNIGSTDIVDGNHKLTLGLIWNIILHWQV
KNVMKNIMAGLQQTNSEKILLSWVRQSTRNYPQVNVINFTTSWSDGLALNALIHSHRPDL
FDWNSVVCQQSATQRLEHAFNIARYQLGIEKLLDPEDVDTTYPDKKSILMYITSLFQVLP
QQVSIEAIQEVEMLPRPPKVTKEEHFQLHHQMHYSQQITVSLAQGYERTSSPKPRFKSYA
YTQAAYVTTSDPTRSPFPSQHLEAPEDKSFGSSLMESEVNLDRYQTALEEVLSWLLSAED
TLQAQGEISNDVEVVKDQFHTHEGYMMDLTAHQGRVGNILQLGSKLIGTGKLSEDEETEV
QEQMNLLNSRWECLRVASMEKQSNLHRVLMDLQNQKLKELNDWLTKTEERTRKMEEEPLG
PDLEDLKRQVQQHKVLQEDLEQEQVRVNSLTHMVVVVDESSGDHATAALEEQLKVLGDRW
ANICRWTEDRWVLLQDILLKWQRLTEEQCLFSAWLSEKEDAVNKIHTTGFKDQNEMLSSL
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SEILKKQLKQCRLLVSDIQTIQPSLNSVNEGGQKIKNEAEPEFASRLETELKELNTQWDH
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KRAKEEAQQKEAKVKLLTESVNSVIAQAPPVAQEALKKELETLTTNYQWLCTRLNGKCKT
LEEVWACWHELLSYLEKANKWLNEVEFKLKTTENIPGGAEEISEVLDSLENLMRHSEDNP
NQIRILAQTLTDGGVMDELINEELETFNSRWRELHEEAVRRQKLLEQSIQSAQETEKSLH
LIQESLTFIDKQLAAYIADKVDAAQMPQEAQKIQSDLTSHEISLEEMKKHNQGKEAAQRV
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QLNHCVNLYKSLSEVKSEVEMVIKTGRQIVQKKQTENPKELDERVTALKLHYNELGAKVT
ERKQQLEKCLKLSRKMRKEMNVLTEWLAATDMELTKRSAVEGMPSNLDSEVAWGKATQKE
IEKQKVHLKSITEVGEALKTVLGKKETLVEDKLSLLSNSNWIAVTSRAEEWLNLLLEYQKH
METFDQNVDHITKWIIQADTLLDESEKKKPQQKEDVLKRLKAELNDIRPKVDSTRDQAAN
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NDLALKLLRDYSADDTRKVHMITENINASWRSIHKRVSEREAALEETHRLLQQFPLDLEK
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LQLKDDELSRQAPIGGDFPAVQKQNDVHRAFKRELKTKEPVIMSTLETVRIFLTEQPLEG
LEKLYQEPRELPPEERAQNVTRLLRKQAEEVNTEWEKLNLHSADWQRKIDETLERLQELQ
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QAEAKVNGTTVSSPSTSLQRSDSSQPMLLRVVGSQTSDSMGEEDLLSPPQDTSTGLEEVM
EQLNNSFPSSRGRNTPGKPMREDTM

SILAC myotube strategy (15 peptides)

MLWWEEVEDCYEREDVQKKTFTKWVNAQFSKFGKQHIENLFSDLQDGRRLLDLLEGLTGQ
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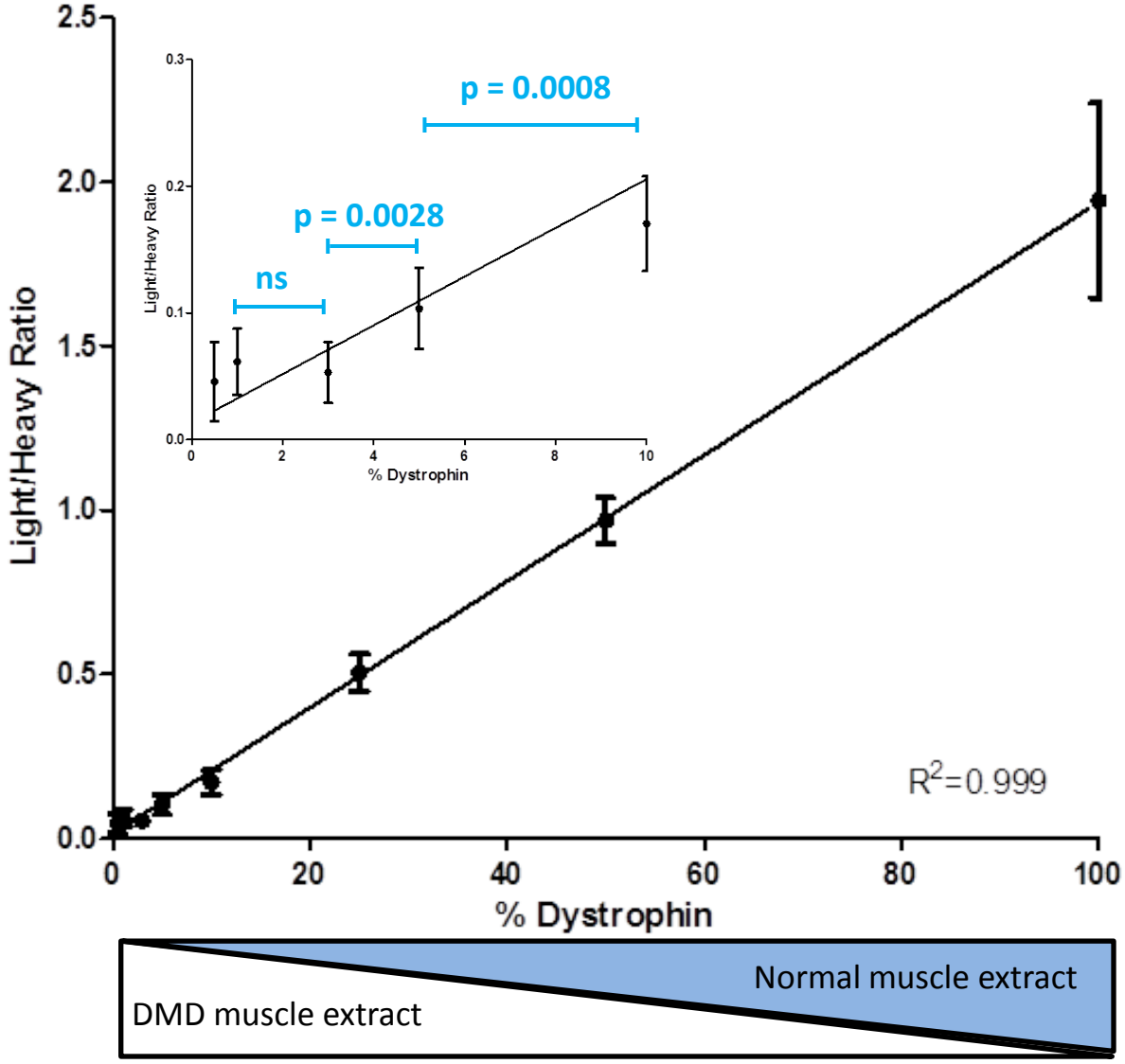
QLTTLGIQLSPYNLSTLEDLNTRWKLLQVAVEDRVRQLHEAHRDFGPASQHFLSTSVQGP
WERAISPNKVPYYINHETQTTCWDHPKMTELYQSLADLNNVRFSAYRTAMKLRRLQKALC
LDLLSLSAACDALDQHNLKQNDQPMDILQIINCLTTIYDRLEQEHNNLVNVPLCVDMCLN
WLLNVYDTGRTGRIRVLSFKTGIISLCKAHLEDKYRYLFKQVASSTGFCDQRRLGLLLHD
SIQIPRQLGEVASFGGSNIEPSVRSCFQFANNKPEIEAALFLDWMRLEPQSMVWLPVLHR
VAAAETAKHQAKCNICKECPIIGFRYRSLKHFNYDICQSCFFSGRVAKGHKMHYPMVEYC
TPTTSGEDVRDFAKVLKNKFRTKRYFAKHPRMGYLPVQTVLEGDNMETPVTLINFWPVDS
APASSPQLSHDDTHSRIEHYASRLAEMENSNGSYLNDSISPNESIDDEHLLIQHYCQSLN
QDSPLSQPRSPAQILISLESEERGELERILADLEEENRNLQAEYDRLKQQHEHKGLSPLP
SPPEMMPTSPQSPRDAELIAEAKLLRQHKGRLEARMQILEDHNKQLESQLHRLRQLLEQP
QAEAKVNGTTVSSPSTSLQRSDSSQPMLLRVVGSQTSDSMGEEDLLSPPQDTSTGLEEVM
EQLNNSFPSSRGRNTPGKPMREDTM

Linearity, dynamic range and Limit of quantification of the MS assay

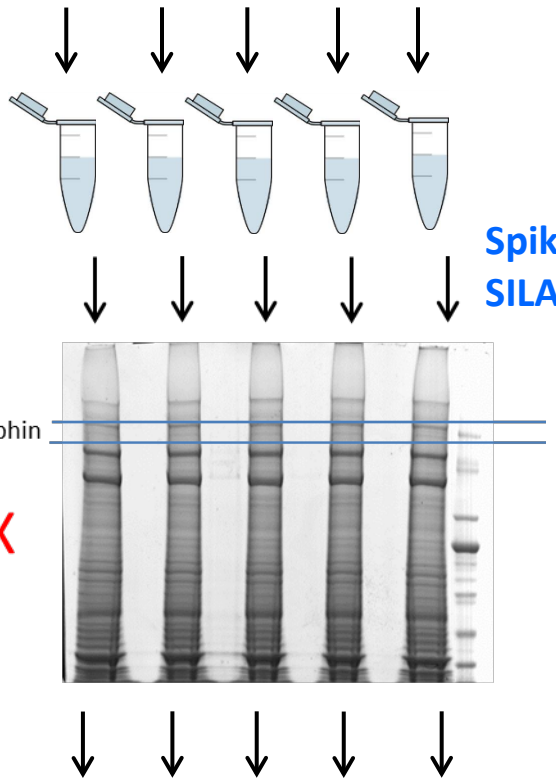


Replicate experiments to evaluate precision of MS based assay

3 Muscles

Normal, BMD mid & BMD low

5 X Sectioning



Spike with SILAC_MT extract

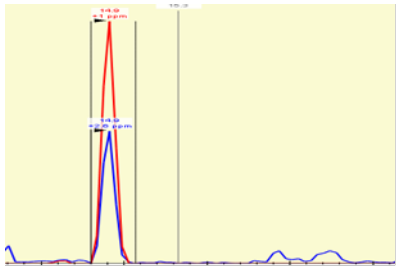
In-Gel Digest Dystrophin area

Inject each digest 3 times on the mass spectrometer

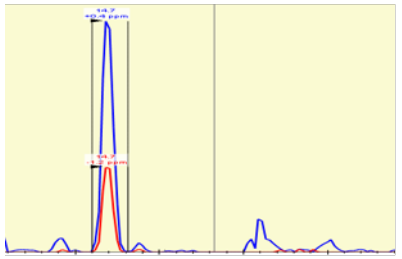
SLEGSDDAVLLQR (1401.725 Da)
SLEGSDDAVLLQR (1407.745 Da)

Extracted elution profile

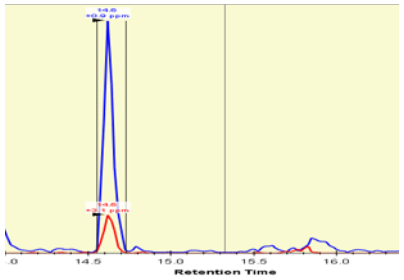
Normal



BMD mid



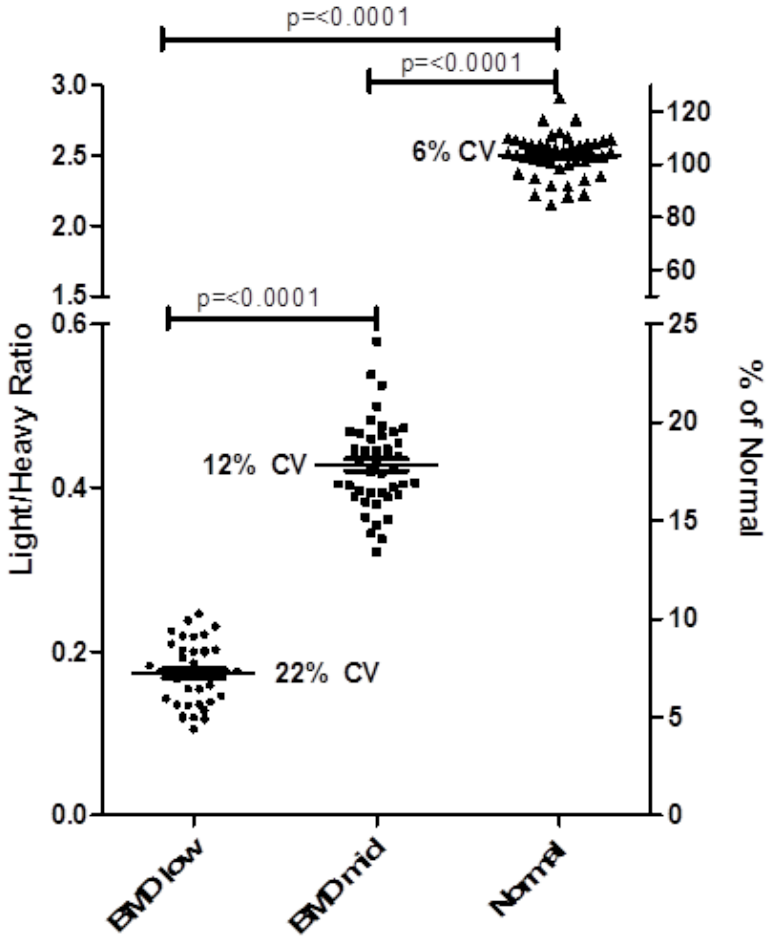
BMD low



14.7 min ± 0.3 min stable across all analyzed samples
Mass error ≤ 2 ppm

Entire procedure performed 3 times over 2 months, 135 runs total, 45 each

Coefficient of variation (% CV) for dystrophin quantification in three biological samples with low, medium and normal levels of dystrophin



For each sample:

- ✓ 5 independent muscle sectioning
- ✓ 5 independent RIPA extracts
- ✓ 3 different gels run at different times
- ✓ 5 lanes per gels
- ✓ 15 in gel digestion of the dystrophin band done at different times with different trypsin batches.
- ✓ 45 LC-MS/MS runs done at different times

Statistics: nonparametric Mann-Whitney, two-tailed, 95% confidence interval, corrections for multiple testing as needed.

What is Normal levels of dystrophin?

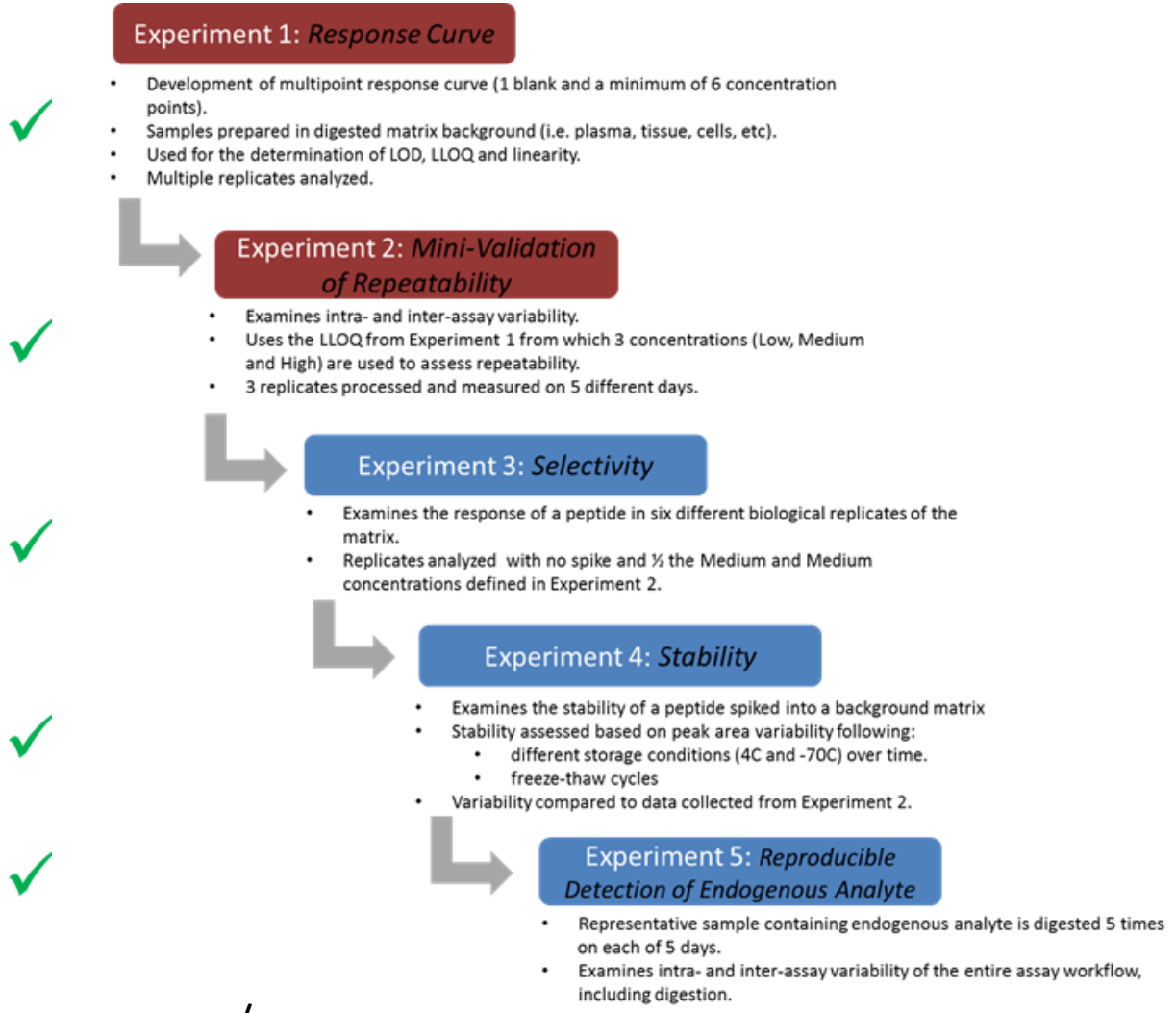
Sample ID	Age	Immunoblot against dystrophin	MS ratio to IS	% to normal mean level
4994	7	normal	2.79	106
4633	10	normal	2.79	106
3949	16	normal	2.84	94
*5106	19	normal	2.64	100
4462	33	normal	2.45	95
€5077	1 (infant)	normal	3.87	147

* Same biopsy used as Normal in previous study, here fresh cryostat sectioning, new SILAC extract for spike-in and the obtained ratio is consistent.

€ the 1 year old infant has higher levels of dystrophin probably due to higher surface to volume ratio of the muscle fibers.

CPTAC: Clinical Proteomic Tumor Analysis Consortium

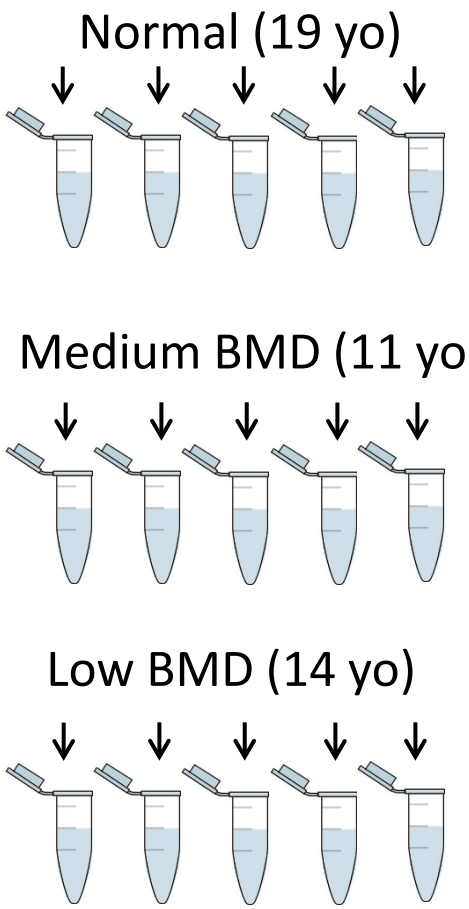
Assay Development Working Group - Experiments for Assay Characterization



https://assays.cancer.gov/

Replicate sample preparation for MS, WB and IF

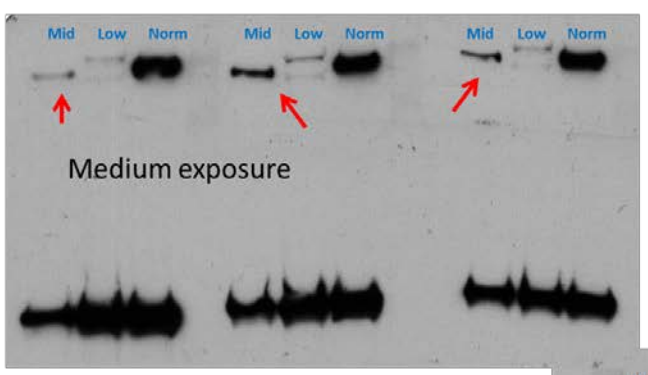
3 muscle biopsies

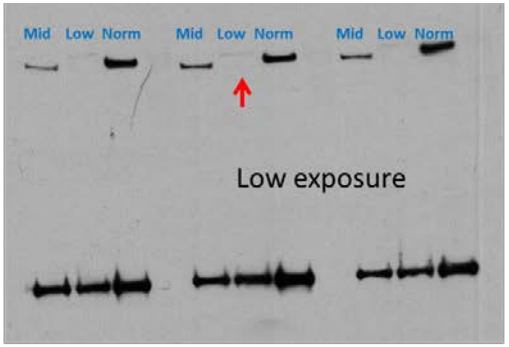


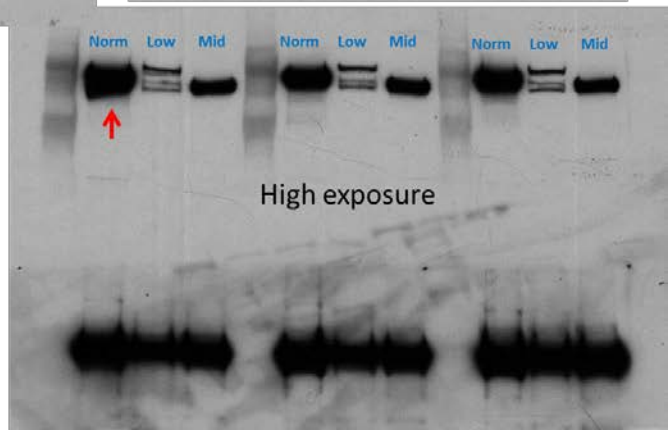
WB and IF followed the protocol described in Anthony K et al. Neurology. 2014; 83:2062-9

WB and IF on Same Samples

WB



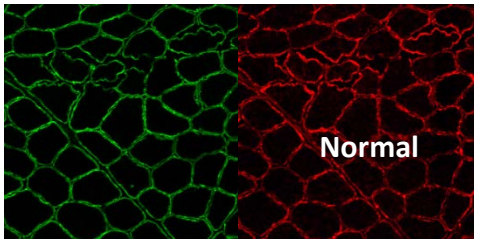


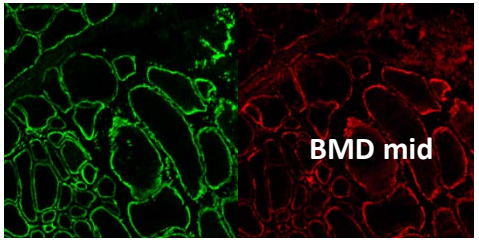


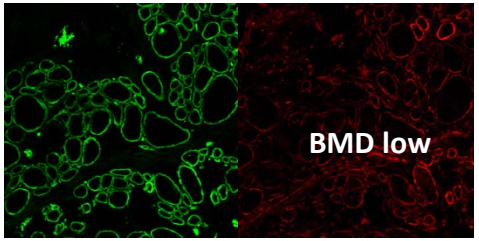
Red arrows indicate:

- Poor Transfer Issues
- Detection issues
- Poor reproducibility
- Saturation issues (poor dynamic range)

IF





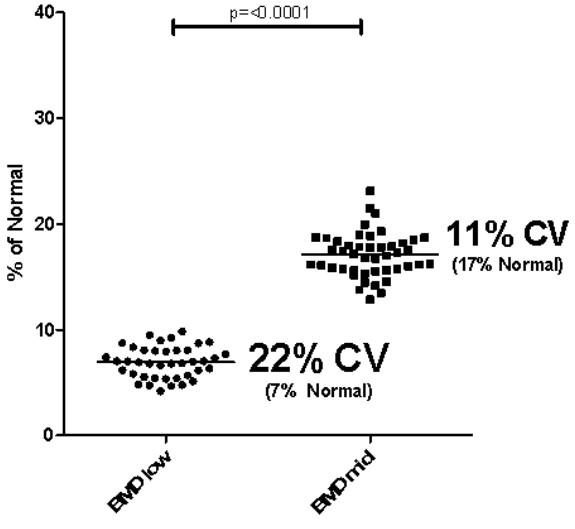


Must balance exposure and detection tug of war.

Spectrin(Green)
Dystrophin (Red)

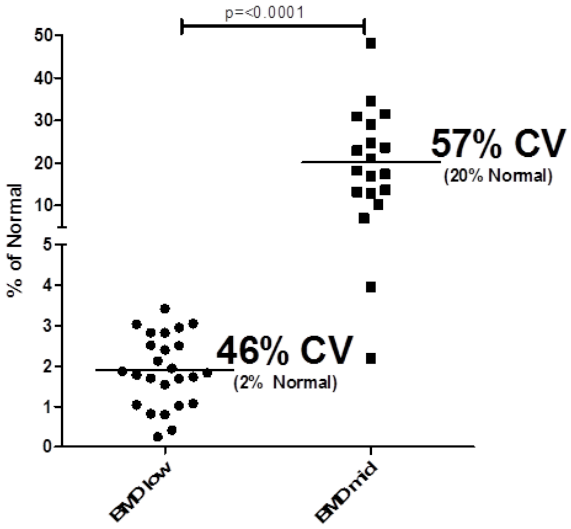
3 Assays, 3 Answers

MS



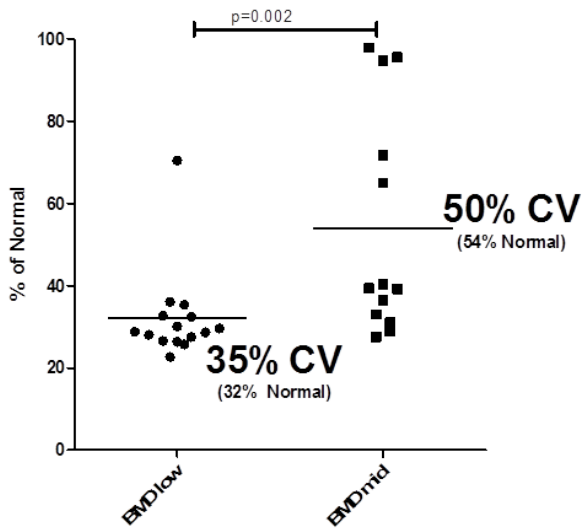
Calibration curve shows high accuracy

WB



Calibration curve shows limited accuracy

IF



Calibration curve not possible. Over estimates abundance.

Summary table

Assay	CV (%) at HLOQ *Ideal <15%	CV % at LLOQ *Ideal <20%	Linearity of the assay	LOQ	# BOM-SG Inter lab CVs
MS assay	6 % -11 %	22%	3 to 100%	3%	Not applicable
WB	57 %	46 %	3 to 50%	3%	23 % to 223%
IF	50%	35%	Not applicable	NA	23 % to 67%

* http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf

BOM-SG : biochemical outcome measures study group (Anthony K et al. Neurology. 2014; 83:2062-9)

Conclusions

Advantages of MS targeted quantitation of dystrophin

- ✓ Highly reproducible, replicate experiments and over time.
- ✓ Linear over wide dynamic range (can reliably quantify levels of dytrophin in the range 3% to 100% relative to normal).
- ✓ Limit of quantification (LOQ): Can accurately measure as low as 3% of the amount of dystrophin relative to normal.
- ✓ Overcome saturation effect when dealing with high and low levels of dystrophin.
- ✓ Reliable source of stable isotope labeled dystrophin that can be distributed.
- ✓ Work with small amount of muscle biopsy.
- ✓ Precise with CV that adheres FDA guidelines for bioanalytical assay.

Disadvantages

- ✓ Loss of spatial localization of dystrophin in muscle fiber (e.g. immunohistochemistry)
- ✓ Require state of the art mass spectrometry instrument and expertise

How can we improve the MS assay?

- Precision at low level of quantification by adjusting the amount of spiked in standard.
- Determine the absolute amount of dystrophin per mg of fresh muscle.
- Inter lab and inter instruments evaluation

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and

Skyline

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