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Robust comparison of protein levels across tissues and throughout development using standardized quantitative western blotting

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1   **TITLE:**
2   Robust comparison of protein levels across tissues and throughout development using
3   standardized quantitative western blotting
4
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26 **KEYWORDS:**
27 Western blotting; protein analysis, fluorescence, development, whole tissue analysis,
28 quantitative biology, loading control
29
30 **SUMMARY:**
31 This method describes a robust and reproducible approach for the comparison of protein levels
32 in different tissues and at different developmental timepoints using a standardized quantitative
33 western blotting approach.
34
35 **ABSTRACT:**
36 Western blotting is a technique that is commonly used to detect and quantify protein expression.
37 Over the years, this technique has led to many advances in both basic and clinical research.
38 However, as with many similar experimental techniques, the outcome of Western blot analyses
39 is easily influenced by choices made in the design and execution of the experiment. Specific
40 housekeeping proteins have traditionally been used to normalize protein levels for
41 quantification, however, these have a number of limitations and have therefore been
42 increasingly criticized over the past few years. Here, we describe a detailed protocol that we have
43 developed to allow us to undertake complex comparisons of protein expression variation across
44 different tissues, mouse models (including disease models), and developmental timepoints. By

45 using a fluorescent total protein stain and introducing the use of an internal loading standard, it
46 is possible to overcome existing limitations in the number of samples that can be compared
47 within experiments and systematically compare protein levels across a range of experimental
48 conditions. This approach expands the use of traditional western blot techniques, thereby
49 allowing researchers to better explore protein expression across different tissues and samples.
50
51 **INTRODUCTION:**
52 Western blotting is a technique that is commonly used to detect and quantify protein expression,
53 including in tissue homogenates or extracts. Over the years, this technique has led to many
54 advances in both basic and clinical research, where it can be used as a diagnostic tool to identify
55 the presence of disease[1,2]. Western blotting was first described in 1979 as a method to transfer
56 proteins from polyacrylamide gels to nitrocellulose sheets and subsequently visualize proteins
57 using secondary antibodies that were either radioactively labelled or conjugated to fluorescein
58 or peroxidase[3]. Through the development of commercially available kits and equipment, Western
59 blotting methods have been increasingly standardized and simplified over the years. Indeed, the
60 technique is now readily performed by scientists with varying backgrounds and levels of
61 experience. However, as with many similar experimental techniques, the outcome of Western
62 blot analyses is easily influenced by choices made in the design and execution of the experiment.
63 It is important, therefore, that the accessibility of standardized Western blotting methods does
64 not obscure the need for careful experimental planning and design. Experimental considerations
65 include, but are not limited to, sample preparation and handling, selection and validation of
66 antibodies for protein detection, and gel-to-membrane transfer efficiency of particularly small or
67 large (<10 or >140 kDa) proteins[4-9]. Protein quality of the original sample plays a significant role
68 in determining the outcome of the subsequent Western blot analysis. As protein can be extracted
69 from a wide variety of samples and sources, including cell lines, tissues from animal models, and
70 post-mortem human tissues, consistency in handling and processing is required to obtain
71 reproducible results. For example, when long-term storage of samples for protein extraction is
72 required, it is important to realize that, although protein is generally stable at -80 °C, differences
73 in protein stability between extracted proteins and intact tissues at -80 °C have been reported[10].
74 Moreover, to obtain reproducible estimates of protein quantities, consistent homogenization of
75 samples is crucial. Optimizing different lysis buffers and homogenization methods (e.g., manual
76 homogenization compared to automated methods) may be required before starting a large-scale
77 quantitative experiment.
78
79 Normalization strategies to correct for protein loading and quantification variability are essential
80 to obtain robust, quantitative results of protein expression. Housekeeping proteins such as β-
81 actin, α-tubulin, β-tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have
82 traditionally been used to normalize protein levels for quantification. However, normalization to
83 specific housekeeping proteins for quantification purposes has been increasingly criticized over
84 the past few years[11,12]. For example, the expression of housekeeping proteins can change across
85 different developmental stages[13,14], across tissues from the same animal[4], and under various
86 disease conditions[4,15-17]. Therefore, the use of specific housekeeping proteins limits the
87 possibilities of making more complex comparisons between protein expression from different
88 tissues, at different timepoints and under varying experimental conditions. An alternative to

89   housekeeping proteins to control for protein loading variation is the use of a total protein stain
90   (TPS) that labels and visualizes all proteins present in a sample. TPS allows signal normalization
91   based on total protein load rather than levels of one specific protein and therefore quantification
92   of TPS signal should be comparable and reproducible regardless of experimental condition,
93   sample type or developmental timepoint. Examples of total protein stains include Ponceau S,
94   stain-free gels, Coomassie R-350, Sypro-Ruby, Epicocconone, Amydo Black, and Cy5 (reviewed in
95   ref. 18). Each of these methods has specific advantages and limitations and method selection
96   depends on the time and tools available as well as the experimental setup[4,18].

97
98   In addition to using a TPS to correct for within-membrane loading and quantification variability,
99   it may be necessary to compare samples between different membranes, particularly when
100 performing large-scale protein expression analysis. However, variability in factors such as
101 antibody binding efficiency and total protein stain intensity may introduce further variability
102 between protein samples that are analyzed on separate gels and membranes. For robust
103 quantification in this situation, it is therefore necessary to introduce a further normalization step
104 to account for between-membrane variability. This can be achieved by including an internal
105 loading standard on each of the separately analyzed membranes that is kept constant across
106 experiments. This standard can take the form of any protein lysate that can be obtained in
107 sufficient quantities to be used across all membranes included in the experiment. Here, we use
108 a lysate of mouse brain (obtained from 5 day old control mice), as brain is readily homogenized
109 and the obtained protein lysate contains a significant amount of protein at a high concentration.
110 Loading an internal standard in triplicate allows samples on separate membranes to be
111 normalized and compared directly.

112
113 Here, we describe a detailed protocol that we have developed to allow us to undertake complex
114 comparisons of protein expression variation across different tissues, mouse models (including
115 disease models), and developmental timepoints[19]. By combining a fluorescent TPS with the use
116 of an internal loading standard, we were able to overcome existing limitations in the number of
117 samples and experimental conditions that can be compared within a single experiment. This
118 approach expands the use of traditional Western blot techniques, thereby allowing researchers
119 to better explore protein expression across different tissues and samples.

120
121 **PROTOCOL:**
122 Tissues for this procedure were obtained from animal studies that were approved by the internal
123 ethics committee at the University of Edinburgh and were performed in concordance with
124 institutional and UK Home Office regulations under the authority of relevant personal and project
125 licenses.

126
127 NOTE: This protocol has been optimized using standardized, commercially available kits and
128 reagents in order to increase reproducibility (see **Table of Materials**).

129
130 **1.   Preparation of samples**

131
132 1.1. **Protein extraction**

133

134   1.1.1. Transfer snap-frozen cell or tissue samples from -80 °C on dry ice, thaw on ice, and wash
135   as required with ice cold 1x phosphate-buffered saline (PBS) (for details on tissues and PBS
136   washes, see **Table 1**). Avoid unnecessary freeze-thaw cycles as this will affect protein quality.

137

138   1.1.2. Add radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl,
139   1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1x protease inhibitor to each sample,
140   using the optimal amount per tissue weight (see **Table 1** for recommendations).

141

142   NOTE: Depending on the application, the type and amount of homogenization buffer may need
143   further optimization.

144

145   1.1.3. Use a hand-held electric homogenizer with a polypropylene pestle to homogenize tissue
146   samples. Between each sample, wash the pestle in double-distilled water and dry with a clean
147   tissue. Change the pestle between different experimental conditions and tissues.

148

149   1.1.4. Leave the samples on ice for 10 min after homogenization. Centrifuge the samples at
150   >10,000 x g at 4 °C for 10 min.

151

152   1.1.5. Transfer the supernatant to a new tube on ice without disturbing the pellet. The
153   supernatant is the protein sample. Store the extracted protein at -80 °C or directly proceed to
154   measuring the protein concentration.

155

156   1.2.   **Quantification and normalization of protein concentration**

157

158   1.2.1. Measure the protein concentration using a bicinchoninic acid (BCA) assay. Prepare a BCA
159   assay mix according to the manufacturer's instructions in a 96-well optical plate using 200 µL of
160   BCA mix per well.

161

162   NOTE: Other quantification methods such as Lowry and Bradford assays can also be used to
163   determine protein concentration as long as protein concentration is quantified consistently
164   across experiments.

165

166   1.2.2. Prepare bovine serum albumin (BSA) standards at increasing concentrations in triplicate
167   and add 1 µL of each protein sample in duplicate. Incubate the 96-well plate in a preheated heat
168   block at 60 °C for 10 min or longer if the protein concentration is expected to be low.

169

170   1.2.3. After incubation, measure the absorption at 560 nm using a plate reader.

171

172   1.2.4. Export the plate reader measurements and calculate the protein concentration by
173   comparing the average absorbance values of each sample to a standard curve obtained using the
174   protein standard. The R-squared value for the standard curve should be greater than or equal to
175   0.98 to accurately estimate sample protein concentration.

176

177 1.2.5. Normalize the amount of protein by preparing dilutions of protein samples in sample
178 buffer and ultrapure water. The total volume can be adjusted depending on the type of gel used.
179 Loading 30 µg of protein per lane as a starting amount is recommended. Add reducing agent such
180 as dithiothreitol (DTT; final concentration 5 mM) or beta-mercaptoethanol (final concentration
181 200 mM) to each sample as required. Pipette undiluted beta-mercaptoethanol in a fume hood.

183 1.2.6. Incubate the samples in a heat block at 70 °C for 10 min. Put the samples on ice, vortex
184 and spin down briefly to collect. Keep on ice until loading the gel.

186 **2.   Gel electrophoresis of protein samples**

188 2.1. **Device and gel set up**

190 2.1.1. Setup a precast 4–12% Bis-Tris gradient gel (see **Table of Materials**) in the gel
191 electrophoresis chamber system. Rinse the gels using double-distilled water before use.

193 NOTE: Depending on the size, interactions and abundance of the protein of interest, gels with a
194 different gradient, buffering agent or well size and number can be used.

196 2.1.2. Add 500 mL of 1x MES SDS running buffer diluted in double-distilled water per tank.
197 Carefully remove the comb from the gels after adding the running buffer without disturbing the
198 wells in the stacking gel.

200 2.2. **Protein loading**

202 2.2.1. Load 3.5 µL of a protein standard into the well. Depending on the sample layout, loading a
203 protein ladder on both sides of the gel can aid in more accurately estimating protein size. Use
204 fine-tipped gel loading tips for more accurate sample loading.

206 2.2.2. When using an internal standard for between-membrane normalization (see step 5 below
207 and discussion), load an amount that is equal to the other samples into the first 3 wells next to
208 the protein ladder.

210 2.2.3. Load 30 µg of each sample in the remaining wells. Add 1x sample buffer to all empty wells.

212 2.3. **Electrophoresis**

214 2.3.1. Assemble the gel tank after loading the samples. Run the samples through the stacking gel
215 at 80 V for 10 min followed by 150 V for an additional 45-60 min.

217 **3.   Protein transfer**

219 NOTE: Protein transfer in this protocol is performed using a commercially available semi-dry
220 blotting system (see **Table of Materials**) for fast and consistent outcomes.

221

222   3.1. Prepare the protein transfer by pre-soaking filter paper in double-distilled water and
223   ensuring the gel knife, plastic Pasteur pipette, blotting roller and forceps are ready to use.

224

225   3.2. Open the transfer stack by carefully removing all wrapping foil. Remove the top from the
226   bottom stack and set it aside. Quickly moisten the membrane on the bottom stack with several
227   drops of electrophoresis running buffer (2-3 mL). Once the transfer stack is open, it is important
228   to prevent the PVDF membrane from drying out.

229

230   3.3. After stopping the electrophoresis, open the pre-cast gel using the gel knife and cut off the
231   stacking gel. Cut the gel around its edges to free it from the plastic cast. Keep the gel knife wet
232   to prevent damage to the gel.

233

234   3.4. Assemble the transfer stack from bottom to top: bottom stack (containing the PVDF
235   membrane), protein gel, filter paper. Use the blotting roller to remove all air bubbles. Place the
236   top stack on top of the filter paper and roll the stack again to remove air bubbles. Do not push
237   too strongly as this may cause the gel to deform during protein transfer.

238

239   3.5. Transfer the whole stack into the transfer device with the electrode on the left side of the
240   device and place the gel sponge on top of the stack so that it is aligned with the corresponding
241   electrical contacts on the device. Close the lid, select and start the appropriate program (20 V for
242   7 min is a recommended starting point).

243

244   3.6. When finished, leave the lid closed for 2 min to allow the stack to cool down and to prevent
245   the membrane from drying out. Remove the transfer stack and cut the membrane to the gel size.
246   Wash the cut membrane quickly with double-distilled water before continuing with the total
247   protein stain.

248

249   **4.   Total protein staining**

250

251   NOTE: Using fluorescent detection provides a substantial benefit over more traditional
252   approaches (e.g., ECL detection), as the linear range and sensitivity can be much better
253   controlled[4]. Therefore, in steps **4 and 5,** a fluorescent TPS and fluorescent secondary antibodies
254   are used (see **Table of Materials**).

255

256   4.1. Roll the membrane into a 50-mL tube with the protein side facing inwards. Because of light
257   sensitivity of the fluorescent TPS and secondary antibodies, all subsequent steps are carried out
258   in the dark.

259

260   4.2. Add 5 mL of protein stain solution (see **Table of Materials**) and incubate on a roller for 5
261   min at room temperature. Because TPS and wash buffer contain methanol, carry these steps out
262   in a fume hood.

263

264   4.3. Discard the staining solution and wash twice quickly with the 5 mL of wash solution (6.3%

265 acetic acid in 30% methanol). Place the tube briefly back on the roller between wash steps. Rinse
266 the membrane briefly with ultrapure water before continuing.
267
268 **5.   Blocking, antibody incubation and detection**
269
270 5.1.   **Blocking the membrane**: Add 3 mL of blocking buffer (see **Table of Materials**) to the 50 mL
271 tube containing the membrane. Incubate the membrane on a roller for 30 min at room
272 temperature. Depending on the choice of antibody, the type of blocking buffer used may require
273 optimization.
274
275 5.2.   **Primary antibody incubation**
276
277 5.2.1. Discard the blocking buffer and replace with the primary antibody at the appropriate,
278 optimized concentration (**Figure 1** and **Figure 2**: mouse-anti-SMN, at 1:1000, diluted in blocking
279 buffer).
280
281 NOTE: Adequate optimization of primary antibodies should include confirmation that the
282 antibody detects a protein product of the right size whilst showing no or minimal binding to
283 other, unspecific protein products. If possible, test and compare multiple antibodies against the
284 protein of interest.
285
286 5.2.2. Incubate the membrane on a roller overnight at 4 °C. The next day, remove the antibody
287 solution and wash 6 times for 5 min with 1x PBS on a roller at room temperature (RT).
288
289 5.3.   **Secondary antibody incubation**
290
291 5.3.1. Prepare the specific secondary antibody at 1:5,000 against the host of the primary
292 antibody in 5 mL blocking buffer. Other secondary antibodies may require other dilutions or the
293 use of alternative blocking buffers.
294
295 5.3.2. Incubate the membrane with the secondary antibody solution on a roller for 1 h at RT.
296 After incubation, wash the membrane three times 30 min with 1x PBS on a roller.
297
298 5.3.3. Dry the membrane and keep the membrane protected from light using aluminum foil until
299 detection. Membranes can be kept at 4 °C for long term storage.
300
301 5.4.   **Image acquisition**
302
303 5.4.1. Login to the computer attached to the scanner. Place the membrane on the scanner with
304 the protein side facing down and select the scanning area in the software.
305
306 5.4.2. Optimize the laser intensity for both (700 nm and 800 nm) channels, by confirming no
307 saturation occurs. Acquire the images in both channels and export the images for further analysis
308 (step 6).

309

6.   Western blot analysis and quantification

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NOTE: These recommendations are based on the freely available Image Studio software. However, comparable analyses can also be done using other software packages, such as ImageJ.

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6.1. **Import the file**: Create a local workspace on the computer used for analysis. This generates a database of image files for acquired western blots. Import files obtained on the scanner and select the image for analysis.

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6.2. **TPS analysis**

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320

6.2.1. Display the 700 nm channel to show the total protein staining result. An example of a TPS image is included in **Figure 1** in which different tissues from neonatal (P5) (**Figure 1A-B**) and 10-week old mice (**Figure 1C-D**) are compared directly. Similarly, **Figure 2** shows an example of a direct comparison of brain tissue from mice of different ages.

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6.2.2. Select **Analysis** tab from top right corner and select **Add Rectangle** to define the area of interest for normalization (**Figure 1B**, **D**). Copy and paste the first rectangle area onto each individual sample to ensure the defined region is at the same size for all analyzed lanes.

326
327
328
329

6.2.3. Copy the result from the **Shapes** tab in the bottom left corner of the software.

330
331

6.3. **Quantification**

332
333

NOTE: The optimal approach for quantifying samples depends on the experimental design. We will here provide an illustrative example of the detection of the survival motor neuron protein (Smn; a key protein involved in the neuromuscular disease spinal muscular atrophy[20,21]), that is known to decrease over time[19] and how normalization of Smn signal intensity to TPS provides reliable estimates of protein expression development.

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6.3.1. **Figure 2** shows a decrease of Smn expression with increasing age of the animal with TPS as protein loading control. Repeat Step 6.2.1-6.2.3 to quantify protein loading (**Figure 2B**). Repeat Step 6.2.1-6.2.3 in the 800 nm channel (**Figure 2A**) to analyze the protein of interest.

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343

6.3.2. Copy the results from both TPS and protein of interest to a spreadsheet program. On the spreadsheet, first normalize the protein loading by determining the highest TPS signal and dividing each TPS signal value by this value to obtain the normalized protein loading value.

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6.3.3. Divide the 800 nm signal value from each individual sample by its corresponding normalized protein value to calculate the relative protein expression ratio in different samples.

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350

6.3.4. After the first normalization, compare various time points or tissues to the average value of the internal standard to allow direct comparisons across different membranes and

351
352

353   experiments.
354
355   **REPRESENTATIVE RESULTS:**
356   To determine any statistically significant differences in protein expression across complex and
357   large groups of samples, appropriate statistical methodology is required. Although a detailed
358   discussion of statistical background goes beyond the scope of this paper, we want to highlight
359   several considerations and detail a successful approach that we have used previously[19].
360
361   As for many experiments, protein quantification measurements do not represent completely
362   independent data. Here, for example, multiple tissues are generally obtained from single animals
363   to determine protein levels across multiple organs at a single experimental time-point. Therefore,
364   we used a mixed effects models to analyze differences in protein expression over time, and
365   between tissues. In general, mixed effects models provide an effective means to deal with non-
366   independence and thereby avoid pseudo-replication[22,23]. In the present case, mixed effects
367   models increase statistical power by accounting for repeated measurements among tissues,
368   within individuals. We use the statistical software package R to perform these analyses, as this is
369   freely available and versatile. However, other commercially available packages may be able to
370   perform similar analyses.
371
372   The current experimental design involves a "split plot" design because each mouse belonged to
373   only one age group. Therefore, we modeled individual mice (mouse ID) as a random effect with
374   a unique identifier; we also accounted for tissue, age, and their interaction as fixed effects. We
375   modeled the data using the function lmer in the R library, lme4. As a quality control step, we
376   visualized residuals to assess the assumptions of equal variance and a normal distribution and
377   transformed the data where necessary to meet these assumptions. To test for a significant
378   interaction between tissue and age, we fit an identical model that lacked this interaction, and
379   compared the models using parametric bootstrapping (R function PBmodcomp in the library,
380   pbkrtest). Where significant interactions arose, we used the function emmeans (in the emmeans
381   R library) to determine the cause of the interaction. For example, we compared mean expression
382   among age classes within each tissue; a significant interaction may arise between age and tissue
383   if, say, two given age classes differed significantly for one tissue but not another. In summary,
384   these approaches provide a way to extend the robustness of conclusions from western blot
385   experiments by providing statistical support to these results.
386
387   We include examples of the use of TPS and an internal standard to facilitate comparisons of
388   protein levels across tissues and time points. **Figure 1** shows results from Western blotting on
389   protein extracted from tissues obtained from neonatal (postnatal day 5) in comparison to adult
390   mice (10-week old). TPS and Smn immunoblot are shown in **Figure 1A, C**. Quantification of
391   fluorescence intensity of the TPS was achieved by measuring the fluorescence intensity inside
392   the rectangle box on each lane and its results are shown in the tables in **Figure 1B** and **1D**. Note
393   that samples from different tissues are characterized by different TPS protein band patterns and
394   therefore it is necessary to use the whole lane for normalization purposes. Indeed, when whole
395   lanes are analyzed, the fluorescence intensity remains relatively similar across samples,
396   indicating TPS for normalization is suitable for this purpose. An internal standard consisting of a

397   P5 brain lysate mixture was also included to illustrate how it can be used for further comparisons
398   between different membranes. Furthermore, in **Figure 2,** we show how a fluorescent TPS can be
399   used to compare protein levels at different developmental time points. Here, we show Smn levels
400   in brain lysates from neonatal (P5), weaning age (P20) and adult (10W) mice (**Figure 2A**).
401   Although Smn levels clearly decrease with age, TPS quantification remains constant as illustrated
402   in **Figure 2B**.
403
404   **FIGURE AND TABLE LEGENDS:**
405   **Figure 1**. **Western blots showing TPS and Smn protein levels in mouse tissues at two different**
406   **ages.** TPS and Smn protein for P5 (**A**) and 10 week-old (**C**) mice. (**B, D**) The fluorescence intensity
407   of whole-lane TPS was calculated and is indicated (in arbitrary units). M: marker/protein standard;
408   kDa: kilodalton; a.u.: arbitrary unit; P5: postnatal day 5; 10W: 10 weeks.
409
410   **Figure 2**. **Analysis of Smn expression in mouse tissues at different developmental time points.**
411   (**A**) Brain lysates from tissue obtained from P5, P20 and 10 week-old mice was analyzed using TPS
412   (top panel) and SMN (bottom panel). (**B**) The fluorescence intensity of the TPS was calculated and
413   is indicated in arbitrary units. M: marker/protein standard; kDa: kilodalton; a.u.: arbitrary unit;
414   P5: postnatal day 5; P20: postnatal day 20; 10W: 10 weeks.
415
416   **Table 1. Overview of expected tissues weights and corresponding recommendations for PBS**
417   **washes and lysis buffer volume to be used for homogenization.** The weights are indications for
418   tissue obtained from postnatal day 8 (P8) mice. PBS and lysis buffer volumes can be scaled up
419   and down according to experimental needs.
420
421   **DISCUSSION:**
422   With the appropriate experimental design, control measures and statistical analysis, western
423   blotting can be used to make reliable quantitative estimates of protein expression within and
424   between a varied range of biological samples. The protocol we describe in the current manuscript
425   aims to serve as a guideline for researchers looking to use Western blotting to undertake
426   quantitative analysis across larger and more complex groups of samples, by using a combination
427   of fluorescence-based detection methods, total protein loading normalization and internal
428   standards. Although the focus here is on determining and comparing protein expression from
429   different mouse tissues and at different ages, this approach can also be extended to compare
430   protein expression in other experimental conditions.
431
432   A central step in our current protocol is the normalization of proteins of interest to total protein
433   loaded by quantifying a fluorescent total protein stain. TPS normalization corrects for variation
434   in sample loading and error margins in protein quantification methods. However, because the
435   number of protein samples that can be analyzed on a single membrane is often limited, further
436   normalization may be required to compare multiple membranes. Indeed, variability between
437   how proteins are detected on different membranes (due to for example antibody incubation time
438   or temperature variation) may cause variation beyond that introduced through loading and
439   quantification steps of the protocol. Here, we use an internal standard that consists of a single
440   protein lysate mix that is loaded in triplicate on each gel and allows for comparison and

441 normalization between gels/membranes. Theoretically, this could be any protein sample, as long
442 as it can be made in sufficient quantities so that the same sample can be used for all experiments.
443 In our experiments, we use a mixture of brain protein lysates, as brain homogenates contain large
444 quantities of protein and are typically obtained at a high concentration. Averaging quantification
445 of triplicate standard should further increase the accuracy of quantifications across membranes
446 and contribute to the reproducibility of the experiment.

447

448 Protein levels can be determined by a number of different techniques and the preferred method
449 depends on the sample type being analyzed and the goal of the experiment. Reproducible
450 quantification of Western blot works best in situations where experimental conditions can be
451 well-controlled, such as when using mouse or other animal models of a defined genetic
452 background. In contrast, in many experiments using human patient samples, this may be less
453 feasible as age, genetic variability and tissue sampling times are much harder to control than in
454 (animal) model systems. Plate-based techniques such as ELISA might be more suitable for these
455 analyses, although the careful validation of antibody specificity is crucial. For example, in fragile
456 X syndrome research, antibodies have been shown to detect different isoforms and when used
457 in ELISA this would lead to overestimation of the total amount of protein as ELISA determines a
458 signal for all isoforms combined[24]. Optimal choices for methods to determine protein expression
459 are therefore depending on context, sample type and the research question that is being
460 investigated.

461

462 Performing adequate statistical analysis is a prerequisite for the reliability of any conclusions
463 drawn from the quantification of biological data. Statistically analyzing complex data as
464 generated by comparing different tissues, time points or other experimental conditions and
465 combinations thereof may require more advanced statistical modelling than ANOVA with post-
466 hoc testing can deliver. For more complex statistical modelling, such as the mixed-effects model
467 approach, we describe in the current manuscript, it may be advisable to seek further advise from
468 biostatisticians. Adequate statistical analysis of large-scale protein expression can greatly
469 improve the robustness of the outcomes and the reliability and reproducibility of results.

470

471 In summary, the experimental approach we describe here provides a robust and reproducible
472 method for researchers that want to determine protein expression using western blot in complex
473 samples allowing to answer new and exciting research questions.

474

480

481 **DISCLOSURES:**
482 The authors have no competing interests to disclose.

483

484 **REFERENCES:**

Page 10 of 13

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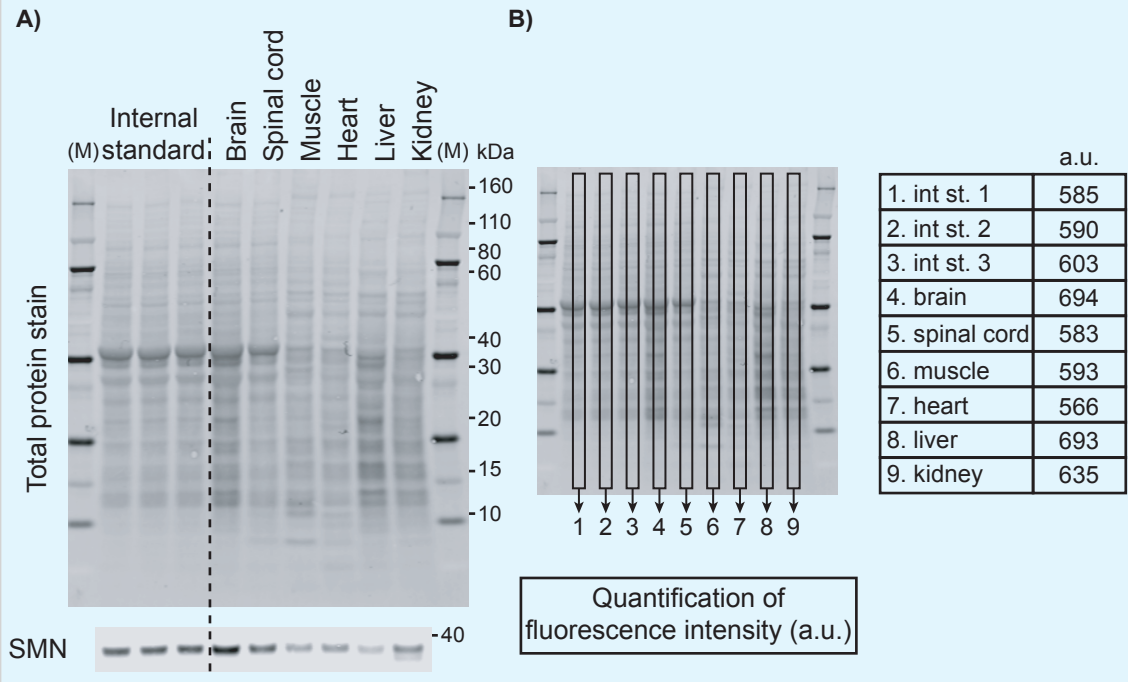547 doi:10.1038/nrneurol.2018.4, (2018).
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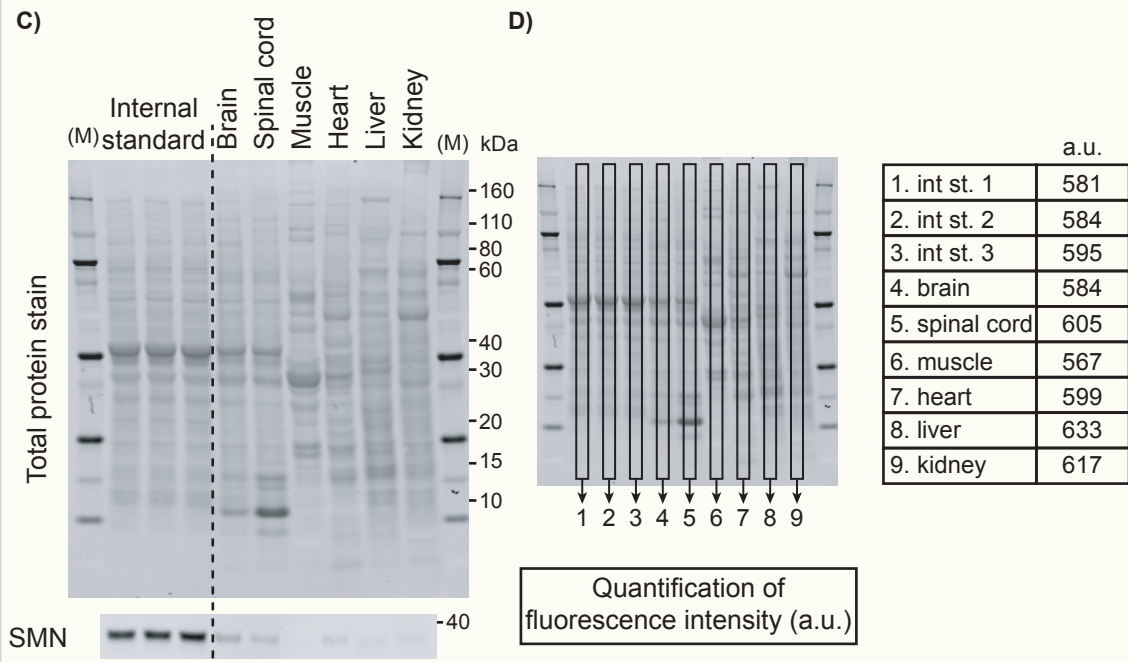554 (2016).
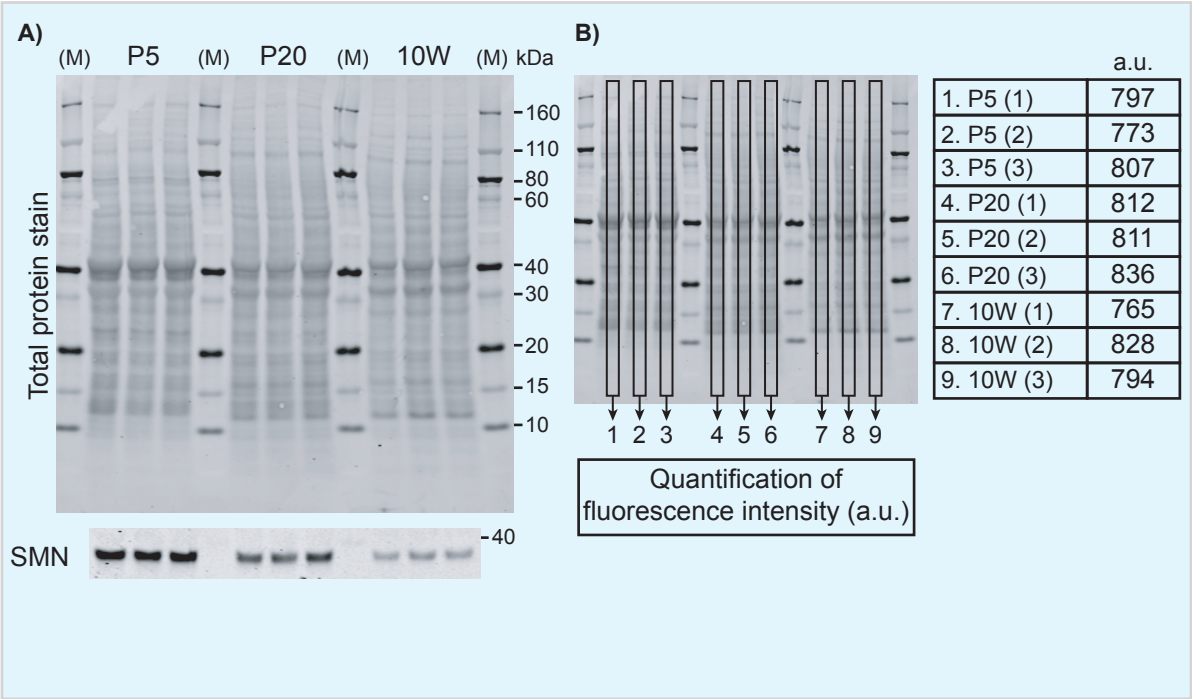555

Figure 1

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Figure 1.

Neonatal (P5)

A)

Total protein stain

(M) | Internal standard | Brain | Spinal cord | Muscle | Heart | Liver | Kidney | (M) kDa

- 160
- 110
- 80
- 60
- 40
- 30
- 20
- 15
- 10

SMN -40

B)

Quantification of fluorescence intensity (a.u.)

1 2 3 4 5 6 7 8 9

	a.u.
1. int st. 1	585
2. int st. 2	590
3. int st. 3	603
4. brain	694
5. spinal cord	583
6. muscle	593
7. heart	566
8. liver	693
9. kidney	635

Adult (10W)

C)

Total protein stain

(M) | Internal standard | Brain | Spinal cord | Muscle | Heart | Liver | Kidney | (M) kDa

- 160
- 110
- 80
- 60
- 40
- 30
- 20
- 15
- 10

SMN -40

D)

Quantification of fluorescence intensity (a.u.)

1 2 3 4 5 6 7 8 9

	a.u.
1. int st. 1	581
2. int st. 2	584
3. int st. 3	595
4. brain	584
5. spinal cord	605
6. muscle	567
7. heart	599
8. liver	633
9. kidney	617

Figure 2

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Figure 2.

A)

Total protein stain

(M) P5 (M) P20 (M) 10W (M) kDa

- 160
- 110
- 80
- 60
- 40
- 30
- 20
- 15
- 10

SMN — 40

B)

Quantification of fluorescence intensity (a.u.)

1 2 3   4 5 6   7 8 9

	a.u.
1. P5 (1)	797
2. P5 (2)	773
3. P5 (3)	807
4. P20 (1)	812
5. P20 (2)	811
6. P20 (3)	836
7. 10W (1)	765
8. 10W (2)	828
9. 10W (3)	794

Table 1

Tissue	Approx. weight*	1xPBS wash (4 °C)	Repeat wash
Spinal cord	40 mg	150 μL	3x
Muscle (GC)	20 mg	150 μL	3x
Brain	240 mg	400 μL	4x
Heart	60 mg	400 μL	4x
Liver	130 mg	400 μL	4x
Kidney	45 mg	400 μL	4x

* These weights are indicative values for tissue obtained from P8 mice.

** These volumes are indications and can be further adjusted according to the w

Homogenizing buffer **
100 μL
100 μL
400 μL
200 μL
400 μL
200 μL

eight of the tissue.

Name of Material/ Equipment	Company	Catalog Number
Fine Tipped Gel Loading Tips	Alpha Laboratories	GL20057SNTL
Halt Protease Inhibitor Cocktail, EDTA-free 100x 5mL	ThermoFisher Scientific	78437
Handheld homogeniser	VWR Collection	431-0100
iBlot 2 Gel Transfer Device	ThermoFisher Scientific	IB21001
iBlot Transfer Stack, PVDF, regular size	ThermoFisher Scientific	IB401031
Image Studio Lite	Licor	N/A
IRDye 800CW secondary antibodies	Licor	--
Micro BCA Protein Assay Kit	ThermoFisher Scientific	23235
Novex Sharp Pre-stained Protein Standard	ThermoFisher Scientific	LC5800
NuPAGE 4-12% Bis-Tris Protein Gels, 1.0 mm, 15-well	ThermoFisher Scientific	NP0323BOX
NuPAGE LDS Sample Buffer (4X)	ThermoFisher Scientific	NP0007
NuPAGE MOPS SDS Running Buffer (20X)	ThermoFisher Scientific	NP0001
Odyssey Blocking Buffer	Licor	927-40000
Purified Mouse anti-SMN (survival motor neuron) monoclonal antibody	BD Transduction Laboratories	610646
REVERT Total Protein Stain, 250 mL	Licor	926-11021
REVERT Wash Solution	Licor	926-11012
RIPA Lysis and Extraction Buffer	ThermoFisher Scientific	89900
XCell SureLock Mini-Cell	ThermoFisher Scientific	EI0001

Comments/Description
Free download from https://www.licor.com/bio/products/software/image_studio_lite/
Select appropriate secondary antibody that is specific against host of primary antibody.
Is used extensively in the SMN/SMA literature and gives consistent results regardsless of lot number

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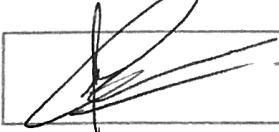Department: _School of Biomedical Sciences_

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These issues have all been addressed.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
We have highlighted the steps for filming in yellow, keeping in mind the recommendations in point 14 – 16. The length should be within the required 2.75 page of A4 limit.

17. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
18. Please upload Table 1 individually to your Editorial Manager account as an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
This has been updated.

Reviewers' comments:
We would like to thank the reviewers for these helpful and positive comments. We feel that both reviewers' and the editor's comments have further increased the quality and readability of our work.

Reviewer #1:
Manuscript Summary:
The group of Huang et al presents a precisely written and complete protocol of Western blotting. They highlight the importance of careful planned experiments, including thorough thoughts on standardization, homogenization and other potential pitfalls in protein analyses. Alternatives or additional steps of the protocol are mentioned and described.
This article is very helpful for everyone performing Western blot experiments with different tissues of humans and/or animals.

Major Concerns:
No major concerns.

Minor Concerns:

I have a few minor concerns that need to be addressed.
1) The authors specifically state that it is vital to keep all protein samples and reagents on ice to prevent protein degradation and extensively point out the other pitfalls in protein analysis. I think one point is lacking here; they should add the (extensive) effects of freeze-thaw on protein experiments to make their list of pitfalls complete.

We thank the reviewer for pointing out this issue and have added this comment to point 1.1.1 of the protocol.

2) There is an inconsistent use of abbreviations and brand names. Abbreviations should be spelled out at least one time (according to the journals format). They should also either consistently use the brand names (but then a list of different possible brand should be given as well) or the name of the material itself.

We apologize for these inconsistencies and have updated the manuscript to correct this. Moreover, we have updated the Table of Materials and refer more extensively to that where required.

Reviewer #2:

Manuscript Summary:
Huang and colleagues have addressed a major problem associated with Western blotting. Clearly, normalizing to housekeeping proteins, which may be expressed at level very different from the protein of interest, is not adequate. These workers have developed a new procedure using total protein staining. The procedure is described in a specific and step-wise manner.

Major Concerns:
1. For certain proteins, "on ice' may not be adequate. Perhaps the remainder of this paragraph makes this point.
2. Considering the considerable limitations of Microsoft Excel, I do not see recommending it specifically or solely. Or, the least other examples should be given. Maybe Prism could be added?

The reviewer is correct and in line with the editor's suggestions, we have removed specific commercial names -including Microsoft Excel- and instead now refer to 'spreadsheet' program.
3. I like the use of the 50 ml container. This will make this aspect of the procedure doable for everyone and likely more reproducible.
4. Have the authors used other image acquisition systems?
5. Do the authors think other scanners can be used?

In response to point 4 and 5, unfortunately we have no experience with other imaging systems suitable for western blot analysis. However, in line with point 2, we have removed specific references to the Licor system as indeed we agree that other scanners and imaging systems will be equally suitable for this type of experiments.

Minor Concerns:
1. There is not much instruction about the staking gel, but this will be obvious to everyone familiar with Western blotting.