

1 **A Ponceau Staining based Dot-Blot Assay for reliable and cost-effective Protein**
2 **Quantification**

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44 **Abstract**

45 Reliable quantification of protein extracts from tissues can be a challenge e.g. due to
46 interference of the high fat content in tissues of the nervous system. Further problems like
47 under- or overestimation of protein concentrations in protein quantification kits like the
48 bicinchoninic acid (BCA) assay can occur. In addition, common lysis buffers such as RIPA
49 buffer are known to be unable to solubilize a large amount of proteins (~10-30%) leading to
50 unsatisfactory and unreliable experimental results with techniques such as immunoblotting.
51 In this work, we have developed a Ponceau S staining based protein quantification assay.
52 This assay is compatible with tissues or cells directly lysed in 2x SDS gel loading buffer,
53 containing bromophenolblue, leading to more complete protein extraction. Protein
54 concentrations of several samples can be determined in a fast and cost-effective manner and
55 subsequent experiments (e.g. Western blot) can be performed without loss of proteins. The
56 presented protein quantification method is highly reliable, fast and economical. Using this
57 method, it is possible to save between 2300 to 3200€ per 1000 lysates as compared to the
58 costs of a commercial BCA kit.

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60 **Key Words:** Dot-Blot; Ponceau S; protein quantification; cost-effectiveness; sustainability

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1. Introduction

A variety of different methods exist for quantification or estimation of total protein content in lysates from cells and tissues. However, the most common methods like the BCA(1), Lowry(2) or Bradford(3) assay are based on photospectrometry which has the disadvantage of fast saturation problem if the protein concentration in lysates is high and therefore outside the range of the standard. Additional problems may occur because a variety of chemical substances (e.g. SDS, although SDS-containing buffers are used for tissue lysis and subsequent quantification with BCA assay) commonly used for effective lysis of biological material and consequent solubilization of the extracted proteins are known to interfere at high concentrations with the chemical reactions underlying the aforementioned methods(1, 4). Furthermore, high concentrations of lipids (e.g. in nerve and brain lysates) are known to interfere with photolorimetric assays like the BCA assay(5). In addition, large volumes of lysate may be needed leading to a profound loss of the sample. This is an undesirable side-effect especially if handling small tissues with small protein yield like sciatic nerves. Thus, these methods are not only costly, likely unreliable and require large sample volumes. Here, we demonstrate a (compared to the commercial BCA kit) time- and money-saving method for protein quantifications with the use of small sample volumes. We achieved this by using a Ponceau S based Dot blot method ("PDB-assay"). Ponceau S staining is normally used as a loading control for protein loaded membranes during Western blotting(6). Our assay gives completely linear standard curves and shows no saturation even in the range of very high protein concentrations of BSA (around 8 $\mu\text{g}/\mu\text{l}$) and also when testing samples with high protein content (e.g. spleen, brain).

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94 **2. Materials and Methods**

95 **2.1 Reagents**

96 Ponceau S (Merck KGaA Darmstadt, Germany, #P3504-10G)

97 Pierce Bovine Serum Albumin Standard (BSA) ampules Thermo Fisher Scientific Inc.,

98 Waltham, MA, USA, #23209)

99 2x SDS Gel Loading buffer (=”2x SDS LB”):

100 100mM Tris-HCl (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, #9090.3)

101 4% SDS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, #1057.1)

102 20% Glycerol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, #3783.1)

103 0,2% Bromophenolblue (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, #A512.1)

104 **2.2 Experimental animals**

105 All animals used in this study were housed under constant temperature on a 12h light/dark

106 cycle and had access to food and water *ad libitum* and were on a C57/BL6-J background. All

107 mice were handled in strict adherence to local governmental and institutional animal care

108 regulations.

109 **2.3 Lysate Preparation**

110 Cell lysates were prepared by direct lysis of cells in 2x SDS LB on the plate using a cell

111 culture scraper and subsequent pulse-vortexing for 10 seconds. Sciatic nerves of either the

112 right or the left side from two different mice were pooled and snap frozen in liquid nitrogen

113 immediately after isolation. The harvested brain was divided into two pieces and lysates were

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114 prepared. Spleens were homogenized and shown data represent organs from four different
115 mice (animal numbers 379, 383, 384 and 387). Tissues were homogenized using ceramic
116 beads in a Precellys® 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France)
117 in either Pierce RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with
118 cOmplete protease inhibitor and phosSTOP phosphatase inhibitor (Roche Diagnostics
119 GmbH, Mannheim, Germany) or in 2x SDS LB.

120 2.4 BCA Assay

121 The microscale BCA assay (Micro BCA Protein assay kit (Thermo Fisher Scientific Inc.,
122 Waltham, MA, USA, #23225) was performed according to the manufacturers instructions and
123 a linear equation based on the linear trendline of the standard curves was generated with
124 Microsoft Excel and used for the determination of protein concentrations.

125 2.5 Dot blot and Ponceau S staining

126 Protein lysates or purified BSA were applied point wise to dry nitrocellulose membranes.
127 Lysates in 2x SDS LB were boiled for 8 minutes at 98°C before applying them to the
128 membrane. The lysates were allowed to dry on the membrane for 15 minutes and were
129 either directly used for Ponceau S staining or concerning the samples in 2x SDS LB, the
130 membranes were washed 3x 5min in deionized (DI) water on a shaker. Afterwards Ponceau
131 S solution (0.1% Ponceau S in 5% acetic acid) was applied for one minute on loaded
132 membranes and equal distribution was ensured. Afterwards the membrane was briefly
133 washed with DI water until background staining was removed and membranes were placed
134 into a plastic foil and scanned with a Epson Perfection V750 Pro scanner using the
135 professional mode and the reflective document type in the scanning software.

136 2.6 Protein quantification with Fiji and Microsoft Excel

137 After creating a greyscale 8 bit image in the free, open-source Fiji software, the rectangle tool
138 and ROI manager were used to define the different dots as regions of interest. The rectangle
139 was always left at the same size for all dots to avoid variation in the “area” variable of the

140 formula for the integrated density. After selection of all dots the pre-selected integrated
141 density was measured and used for quantifications. Values were averaged from technical
142 dupli-or triplicates and divided by 10^5 for easy handling. A standard curve was generated
143 using a linear “scatter chart” in Microsoft Excel and a linear trendline was inserted. The
144 corresponding linear equation was used for the calculation of protein concentrations.

145 2.7 Cost calculations

146 We calculated the costs regarding each the PDB-method and the micro BCA assay for a
147 reaction with 12 biological samples and standards. Both the commercial BCA assay and the
148 selfmade variant (Reagent A: 1% sodium bicinchoninate, 2% sodium carbonate, 0.16%
149 sodium tartrate, 0.4% NaOH, 0.95% sodium bicarbonate, 10M NaOH, pH 11.25, Reagent B:
150 4% cupric sulfate) were taken into the comparison. We provide a range of possible total
151 costs, which depends on the distributor providing the ingredients.

152 We calculated the costs for the RIPA buffer and the SDS Gel loading buffer per 1ml. We
153 compared commercial RIPA buffer (RIPA Lysis and Extraction Buffer, #89900, Thermo
154 Fisher Scientific Inc., Waltham, MA, USA) and selfmade RIPA buffer (25mM Tris-HCl, pH
155 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with selfmade SDS Gel
156 loading buffer (100 mM Tris-HCl, 4% SDS, 0.2% bromophenol blue, 20% glycerol). We
157 generally chose high quality distributors, and the smallest available packing size of each
158 product served as the basis for our calculations. Everyday lab chemicals such as Tris-HCl or
159 NaCl were not included into the calculations. Included in the final costs for both commercial
160 and selfmade RIPA buffer were the relative costs for a protease (c0mplete Protease Inhibitor,
161 CO-RO Roche, Merck KGaA, Darmstadt, Germany) and phosphatase inhibitor (PhosStop,
162 Phoss-Ro Roche, Merck KGaA, Darmstadt, Germany).

163 2.8 Immunoblotting

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164 Immunoblotting was performed as previously described(7). The used antibodies are listed in
165 Table 1. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Fisher
166 Scientific Inc., Waltham, MA, USA)

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Antibody	Source	Supplier	Number	Dilution
Erk 1/2	Mouse	Cell Signaling	4696	1:2000
GAP-43	Rabbit	Santa Cruz	10786	1:500
GAPDH	Mouse	Santa Cruz	32233	1:5000
Histone H1	Mouse	Santa Cruz	8030	1:500
LC3A/B	Rabbit	Cell Signaling	4108	1:1000
MBP	Rat	Novus Biologicals	NB600-717	1:1000
Mek 1/2	Rabbit	Cell Signaling	8727	1:2000
Merlin	Rabbit	Cell Signaling	12896	1:1000
NF-M	Mouse	Santa Cruz	16143	1:500
P0	Chicken	Abcam	39375	1:2000
P-Erk 1/2	Rabbit	Cell Signaling	4370	1:2000
P-Mek1/2	Mouse	Abcam	91545	1:2000
Anti-chicken HRP	Goat	Abcam	97135	1:5000

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Anti-Rabbit HRP	Goat	Agilent Dako	P0448	1:2000
Anti-Mouse HRP	Goat	Agilent Dako	P0447	1:2000
Anti-rat HRP	Rabbit	Invitrogen	61-9520	1:2000
Anti-Goat HRP	Rabbit	Agilent Dako	P0449	1:1000

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170 2.9 Statistical procedures and Figure preparation

171 Two-tailed, unpaired Student's t-tests for calculation of p-values and F -tests to check the
172 normal distribution of datasets were performed using Graphpad Prism 7.0. Statistical
173 significance was accepted at $p \leq 0.05$. All data are presented as mean \pm SD. All figures were
174 either made with Graphpad Prism 7.0 or Microsoft PowerPoint and were assembled in Adobe
175 Photoshop CS6.

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189 **3. Results and Discussion**

190 To test whether Ponceau Staining of Dot blots can be used as a protein quantification
191 method we spotted undiluted, commercially available BSA solution at a concentration of 2
192 mg/ml in a range of 0.25 µg to 4 µg to nitrocellulose membranes. Membranes were stained
193 with a 0.1% Ponceau S solution in 5% acetic acid for one minute and scanned to quantify the
194 stained dots (**Fig. 1A**).

195 **Figure 1**

196 **A** Representative Ponceau S stained dot blot of undiluted BSA spots of indicated protein
197 amounts (n=1). Different amounts of BSA were applied onto the membrane in triplicates.

198 **B** Linear standard curves of different Ponceau S stained dot blots from BSA standards
199 generated with either PDB assay or BCA assay (n=3, each). Replicates are defined by usage
200 of BSA from three different ampules.

201 **C** Comparison of correlation coefficients from either BCA or PDB assay (n=3, ns=p>0.05,
202 unpaired, two-sided student's t-test).

203

204 Because the sizes of the resulting dots were unequal we used the “Integrated Density” of
205 each dot which is the product of the selected area and its mean grey value over the
206 measured area. By using the same rectangle size when analysing the dots in *Fiji* only the
207 grey values decrease as the protein amount increases in a linear manner (**Fig. 1B, left**). The
208 resulting linear standard curve revealed high consistency/low variability within the three
209 different standards. A correlation coefficient R^2 of 0,9925 also indicated high linearity of the
210 standard curve generated with our PDB-assay. To avoid the necessity of spotting different
211 volumes, which could be a reason of variation, we diluted the BSA in either ddH₂O or the

212 same RIPA buffer in which we lysed the different organs used in this study. Dilution of the
213 BSA in ddH₂O still gives a clear dot, but unfortunately the dilution of BSA in RIPA buffer
214 resulted in the distribution of the BSA in form of circles(**Fig.S1**).

215 **Figure S1**

216 Ponceau S stained dot blot of BSA diluted either in ddH₂O or RIPA buffer. Diluted samples
217 were applied onto the membrane in duplicates.

218 This is known in the literature as “coffee rings” (8). This could not be prevented by washing
219 the membrane after spotting the BSA onto it, to remove potentially interfering SDS. It has
220 been shown that the formation of such a “coffee rings” depends on the evaporation speed of
221 the liquid and the particle movement. We can only speculate about the cause of this
222 observation but we assume that the low concentration of SDS inside the RIPA buffer
223 decreases the speed of the particle movement, as it has been shown that lower amounts of
224 SDS decrease the diffusion coefficient of ovalbumin (9).The dilution of BSA in ddH₂O also
225 resulted in a non-linear standard curve, making it inappropriate or unsuitable for proper
226 quantification of lysates (**Fig. S2**).

227 **Figure S2**

228 **A** Representative Ponceau S stained dot blot of in ddH₂O diluted BSA of indicated amounts
229 (n=1). Different amounts of BSA were applied onto the membrane in triplicates.

230 **B** Linear standard curve of different BSA standards diluted 1:1 in ddH₂O (n=3).

231

232 This is probably the result of loss of diluted BSA during serial dilution of the standard on the
233 walls of pipette tips and tubes (10). Therefore, we used undiluted BSA to generate standard
234 curves. For comparison, we also generated the standard curve by bicinchoninic acid (BCA)
235 assay, a well established method. The BCA assay also showed a good linearity within a
236 range of 125 ng to 2 µg.(**Fig. 1B, right**). Comparison of the correlation coefficients
237 demonstrates that the PDB assay is in performance completely equal to a BCA assay (**Fig.**
238 **1C**).

239 However, the commonly used microscale BCA assay is known to display saturation of the
240 photolorimetric reaction at protein concentrations outside the range of the standard,
241 meaning above 2 µg/µl. The PDB has the big advantage of being linear also in the range of
242 higher amounts of protein (in this experiment up to 4 µg).

243 To test the applicability of our PDB method with tissue lysates, we collected spleens as
244 protein-rich organs from four different mice, lysed them in 1 ml RIPA buffer and used 1 µl per
245 dot of this lysate for quantification (**Fig. 2A**).

246 **Figure 2**

247 **A** Membrane with spotted dots of either BSA standard (range from 0,25µg to 4µg as indicated
248 handwritten) and spleen lysates from four different mice (#379, #383, #384, #387). Different
249 samples/amounts of protein were applied onto the membrane in duplicates. Dots crossed
250 out with an "x" were excluded due to accidental application of unequal BSA amounts.

251 **B** Table displaying protein concentrations of spleen lysates determined by either PDB or BCA
252 assay.

253 **C** Membrane with 5µg or 2,5µg dots of spleen lysates or BSA (lower two rows). For the
254 determination of the protein concentrations inside lysates the PDB assay was used for the dots
255 in the first two rows and the BCA assay in row three and four. Different samples/amounts of
256 protein were applied onto the membrane in duplicates.

257 **D** Diagrams depicting integrated densities of dots from the membrane shown in **C** (n=4,
258 ****=p<0.0001, two-sided, unpaired student's t-test).

259

260 In parallel, the same lysates were quantified by BCA assay for comparison. Due to the high
261 protein content in the spleen lysates, the BCA assay showed values around the upper border
262 of the standard range, between 1,7 µg/µl and 2,4 µg/µl. In contrast, with the PDB assay the
263 protein concentrations were determined between ~6 and 9 µg/µl, three to four times higher
264 than the values given by the BCA method (**Fig. 2B**). To validate the concentrations
265 determined by PDB, we calculated the required lysate volume for 5 and 2,5 µg with the
266 concentrations from BCA and PDB and applied these amounts together with 5 and 2,5 µg
267 BSA onto membranes (**Fig. 2C**). Strikingly, the staining of dots of lysates which
268 concentrations were determined with the BCA assay were much stronger than those which
269 have been quantified by PDB. This is reflected in **Fig.2D**: Dots of BCA quantified lysates

270 displayed much lower integrated densities meaning higher protein content which can be
271 explained by saturation of the BCA assay in working ranges above 2 µg. Integrated densities
272 of PDB quantified dots were equal to those from BSA, indicating reliable performance of our
273 method.

274 Although widely used RIPA buffer has the disadvantage that around 10-30 % of all proteins
275 are lost during lysis due to the insolubility of some proteins in RIPA buffer(11). Hence, we
276 tested another commonly used lysis buffer, 2x SDS Gel loading buffer, containing 4% SDS
277 for more efficient solubilization of test tissues (11). We first applied BSA, diluted 1:1 in 2x
278 SDS LB, to a membrane and compared it to the staining of BSA diluted in ddH₂O. A nearly
279 invisible circular shape of the applied dot was observed which was in comparison to the
280 strong signal of the same amount of BSA diluted in ddH₂O nearly nothing (**Fig. S3**).

281 **Figure S3**

282 Ponceau S stained dot blot of BSA diluted either in ddH₂O or 2x SDS LB which was not washed
283 in DI-tap water before Ponceau S staining. Diluted samples were applied onto the membrane
284 in duplicates.

285

286 We hypothesized that the decreased staining effectivity might be due to the high
287 concentration (2%) of SDS in the 1:1 diluted sample which could interfere with the binding of
288 the Ponceau S dye to proteins. Therefore, we washed the membrane after drying three times
289 for five minutes in DI-tap water before staining. This led to effective staining of the dots which
290 were, compared to dots of undiluted BSA, weaker in their intensities but spread over a larger
291 area. This was probably due to the fact that two times the volume of undiluted BSA was used
292 to achieve equal protein amounts (**Fig. 3A**). Interestingly, we did not again observe the
293 “coffee ring”-phenomena as we did when we diluted the BSA in RIPA buffer. Again we can
294 only speculate and explain this by the reported observation that higher amounts of SDS
295 normalize the diffusion coefficient of ovalbumin which was decreased by low amounts of
296 SDS(9). Therefore, the speed of evaporation of the lysate droplet is again equal to the speed
297 of the particle movement within the droplet. As before, we used the same BSA standards as

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298 in **Fig.1** to produce a standard curve of BSA diluted in 2x SDS LB. This mean standard curve
299 also again displayed good linearity, reflected by a mean R^2 of 0,9945 (**Fig. 3B**). Compared to
300 the other correlation coefficients shown in **Fig. 1C**, there were no changes between all three
301 different methods in linearity of prepared standards as addressed by the correlation
302 coefficients (**Fig. 3C**).

303 **Figure 3**

304 **A** Representative Ponceau S stained dot blot of in 2x SDS LB diluted BSA of indicated amounts
305 ($n=1$). Different amounts of BSA were applied onto the membrane in duplicates.

306 **B** Linear standard curve of different Ponceau S stained dot blots from BSA standards diluted
307 1:1 in 2x SDS LB ($n=3$). Replicates are defined by usage of BSA from three different ampules.

308 **C** Comparison of correlation coefficients from standard curves of BCA assay, PDB assay with
309 undiluted BSA or PDB assay with BSA diluted 1:1 in 2x SDS LB ($n=3$, $ns=p>0.05$, unpaired, two-
310 sided student's t-test). Please note that the data for the BCA assay and PDB assay with
311 undiluted BSA are the same as shown in **Fig.1C**.

312 **D** Membranes with stained undiluted BSA standard curves (ranging from 8 μ g to 0,25 μ g), 1 μ l
313 spots of sciatic nerve ("l.l."), brain ("l.H.") lysates in RIPA buffer (upper membrane) or in 2x SDS
314 LB 1:1 diluted BSA (ranging from 8 μ g to 0,25 μ g), 2 μ l spots of sciatic nerve ("r.l.") and brain
315 ("r.H.") lysates in 2x SDS LB buffer diluted 1:1 in ddH₂O (lower membrane). Different
316 samples/amounts of protein were applied onto the membrane in duplicates.

317 **E** Ponceau S stained membrane with different protein amounts of sciatic nerve lysates in
318 either RIPA buffer or 2x SDS LB.

319

320 To test the suitability of direct tissue lysis in 2x SDS LB and to compare the extraction ability
321 with RIPA, we used sciatic nerves and a brain from C57/BL6 mice. These tissues are
322 normally hard to lyse due to their high content of fatty myelin. We pooled the sciatic nerves
323 from the left and right side of two different mice (left sciatic nerves were lysed in RIPA buffer
324 and right sciatic nerves were lysed in 2x SDS LB) and also used one mechanically disrupted
325 mouse brain which we divided into two halves and subsequently lysed either in RIPA buffer
326 or 2x SDS LB. Since we expected very high concentrations for the lysed brain, we also
327 included 8 μ g of BSA into the range of our standard. The resulting standard curve still
328 maintained a good linearity (**Fig. S4**), supporting the suitability of Ponceau S dye for the
329 quantification of tissue lysates with high protein content.

330 **Figure S4**

331 Linear standard curves of Ponceau S stained dot blots from BSA standard shown in **Fig.3D**
332 (n=1).

333 With both buffers we could lyse and determine protein concentrations effectively of brain
334 pieces and nerves (**Fig. 3D**). To test whether quantification of protein concentrations inside
335 lysates, in which proteins were differentially extracted, gave us in both cases true values, we
336 subjected the lysates of 50, 25 and 15 ug protein content to a SDS-PAGE followed by protein
337 transfer to the membrane, subsequent Ponceau S staining and immunoblotting .

338 The Ponceau S staining showed overall equal loading between the two extraction methods if
339 one compares only sciatic nerves or brain lysates among themselves (**Fig. 3E**). This proves
340 similar performance of the PDB assay with either lysates prepared in in RIPA buffer or in 2x
341 SDS LB (**Fig. 3E**). Intriguingly, there was a general difference between the loading of sciatic
342 nerves and brain lysates (**Fig. 3E**). We suppose that this is due to the high abundance of
343 albumin (strong band below 70 kDa) and IgG heavy (strong band slightly above 55 kDa) and
344 light (strong band between 25 and 35 kDa) chain in the PNS which are absent in the CNS
345 due to the blood brain barrier (12, 13). The presence of these highly abundant serum
346 proteins would lead to overestimation of the real protein content of the sciatic nerve itself and
347 therefore leads to unequal loading compared to both brain lysates. This is an important point
348 if researchers attempt to compare expression of different proteins between CNS and PNS.

349 As expected, subsequent immunoblotting revealed better extraction of different proteins by
350 2x SDS LB. It has been described that e.g. cytoskeleton associated proteins and
351 extracellular matrix components are to a certain degree insoluble in RIPA buffer(11). The
352 tumor suppressor protein merlin as a cytoskeleton associated protein was extracted more in
353 2x SDS LB in both sciatic nerve and brain lysates as described before (14)(**Fig.4**).

354 Cytoplasmic proteins like MEK 1/2, ERK 1/2, GAPDH and GAP-43 were present to the same
355 extent in both lysates. The nuclear protein Histone H1 and the autophagic vesicle membrane
356 proteins LC3A/B were slightly less abundant in the RIPA buffer extractions. Since we
357 included phosphatase inhibitors in the RIPA buffer, we were surprised as we detected slightly

358 higher P-ERK1/2 but massively higher P-MEK1/2 signals suggesting more efficient
359 phosphatase inhibition in 2x SDS LB, probably due to the strong denaturing effect of SDS.
360 This finding is important for researchers studying fast-changing signaling processes e.g.
361 during nervous system regeneration and degeneration (15, 16). Lastly, we observed a
362 slightly enhanced ability of 2x SDS LB to extract the axonal intermediate filament
363 neurofilament-M and obviously enhanced ability to solubilise the extracellular matrix
364 associated myelin proteins myelin basic protein (MBP), which is present in both the PNS and
365 CNS, and the PNS specific myelin protein zero (P0; **Fig. 4**).

366

367 **Figure 4**

368 Immunoblots for indicated target proteins of the membrane shown in **Fig.3E** (n=1).

369

370 Throughout the course of our experiments we realized that the PDB assay, especially if
371 combined with the use of 2x SDS LB as lysis buffer instead of RIPA buffer, must be relatively
372 cheap if compared to the established workflow in our laboratory from tissue harvesting to
373 immunoblotting. Therefore, we estimated the possible amount of money a laboratory could
374 save with the usage of our method. First we calculated the costs for one reaction with
375 quantification of 12 samples. While a “selfmade” BCA kit would cost between 15,29€ and
376 24,91€ and a commercial BCA kit 13,47€ our PDB assay only costs 2,05€ per reaction.
377 Based on our laboratory experience we know that a prepared Ponceau S solution can be
378 used at least 20 times to stain membranes. One bottle with 10g of Ponceau S powder at a
379 final dilution of 0,1% in 5% acetic acid is enough to stain 800 membranes with 12 samples
380 per membrane. If we calculate the costs for measuring 1000 samples and compare these
381 with the costs of 1000 BCA reactions, it turns out that with our PDB method a laboratory
382 would save around 951,67€ (**Tab.1**).

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Method	Materials	Price (for 12 samples)
Ponceau Dot Blot	<ul style="list-style-type: none"> • Ponceau powder • Nitrocellulose membrane (84cm²) • Bovine serum albumin 	2,05€
Commercial BCA Kit	<ul style="list-style-type: none"> • Kit Materials • Pierce 96-wellplate 	13,47€
Selfmade BCA Kit	<ul style="list-style-type: none"> • Kit Materials • Pierce 96-wellplate 	Ranging from 15,29€ to 24,91€ (depending on producer)
	<p>Price for 1000 samples Ponceau Dot Blot: 170,83€ Commercial BCA Kit: ~1122,50€ <u>$\Delta = \sim 951,67\text{€}$</u></p>	

386 **Table 1. Cost estimations for the PDB assay as well as commercial and selfmade BCA**
387 **kits.**

388 Since we have shown that direct lysis of tissues in 2x SDS LB is not only compatible with our
389 method but rather even more recommended because it extracts and solubilises different
390 proteins better (**Fig.4**), as it has been reported previously(11), we also calculated how much
391 money could be saved with the usage of 2x SDS LB instead of RIPA buffer with addition of
392 phosphatase and protease inhibitors. One ml RIPA lysis buffer with phosphatase and
393 protease inhibitors routinely used in our laboratory costs around 2,41€ while the same
394 volume of selfmade 2x SDS LB only costs 0,1€. One ml of selfmade RIPA lysis buffer with
395 phosphatase and protease inhibitors would still cost 1,48€. If we project this to 1000
396 prepared lysates, a laboratory would save between 1380 and 2310€ (**Tab.2**). Adding up
397 these amounts shows that the laboratory could save 2331,67 and 3261,67€ per 1000 lysates
398 by using 2x SDS LB for lysis of tissues and the PDB assay for quantification of these lysates
399 (**Tab.2**).

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Lysis buffer	Price per sample in 1 ml lysis buffer	Cat.Nr./Supplier
cOmplete Protease Inhibitor Cocktail	0,28€	000000011697498001/Roche
PhosStop	1,20€	000000004906845001/Roche
RIPA buffer (commercial)	0,93€	89900/Thermo Scientific
RIPA buffer, final solution	2,41€	
cOmplete Protease Inhibitor Cocktail	0,28€	000000011697498001/Roche
PhostStop	1,20€	000000004906845001/Roche
RIPA buffer (selfmade)	0,0006€	Various suppliers
RIPA buffer, final solution	1,48€	
SDS gel loading buffer (selfmade)	0,1€	Various suppliers
	Price for 1000 lysates RIPA buffer: 2410€ RIPA buffer (selfmade): 1480€ 2x SDS LB: 100€ $\Delta=1380$ to 2310€	
	Price for 1000 lysates+quantification Commercial BCA kit with lysates in either selfmade or commercial RIPA buffer: ~2602,5 to 3532,5€ Ponceau Dot Blot Assay with lysates in 2x SDS LB: 270,83 € $\Delta= 2331,67$ to $3261,67\text{€}$	

400 **Table 2. Cost estimations for different lysis buffers used with either our PDB assay or**
 401 **a BCA assay.**

402 Although a similar principle was described previously (17), our study highlights some more
 403 critical points and adds a new improvement: If using RIPA buffer as lysis buffer it is extremely
 404 important not to dilute the BSA which is used for preparation of a standard curve. This also
 405 leads to a faster workflow of our method compared to the other one reported. If BSA is

406 diluted in ddH₂O, a non-linear standard curve will be the result and if diluted in RIPA buffer, a
407 circle instead of a dot will form. Furthermore, we could show that tissues can directly be
408 lysed in 2x SDS Gel loading buffer which is faster and superior to lysis in RIPA buffer and the
409 preferable method since losing a lot of protein during extraction and lysis could be avoided.
410 The fact that Ponceau S staining of dot blots is suitable for accurate quantification of tissue
411 lysates is another important improvement compared to the publication from Morcol et al.,
412 who only used different purified proteins but no tissue lysates(17).

413 **4. Conclusion**

414 We describe a rapid, low-budget and highly reliable technique for quantification of protein
415 lysates as an alternative to more common established methods like the BCA assay. Our
416 method is a considerable improvement of the method described previously (17), based on
417 the aforementioned points.

418 Different protein extractions and lysis protocols could also be the reason for contradicting
419 reports in the literature. Since Western blotting with subsequent immunodetection of different
420 target proteins is one of the most widespread methods in biomedical research, laboratories
421 working on the same model system/organ or topic of interest could standardize the
422 obtainment of results by using the same strategies/protocols which would lead to the
423 publication of more reliable results. With the money saved by the usage of our technique, the
424 research in every laboratory could be highly improved by the contingency to purchase more
425 antibodies, chemicals, biological materials, etc.

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433 **Conflict of Interest**

434 DLH, LB, YC and HM applied for the here described method for a patent at the german
435 patent and trade mark office. LKS has no conflict of interests to declare.

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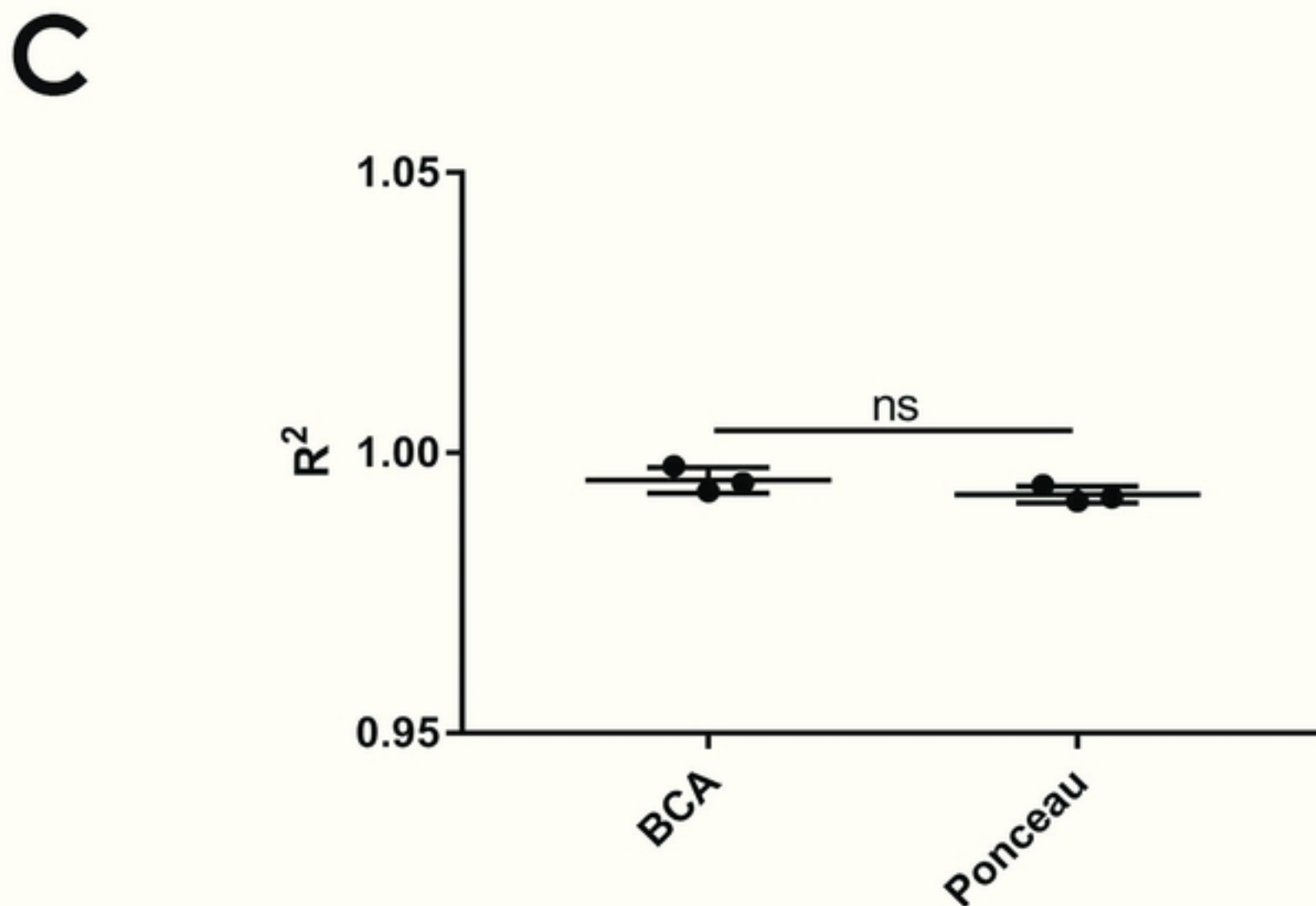
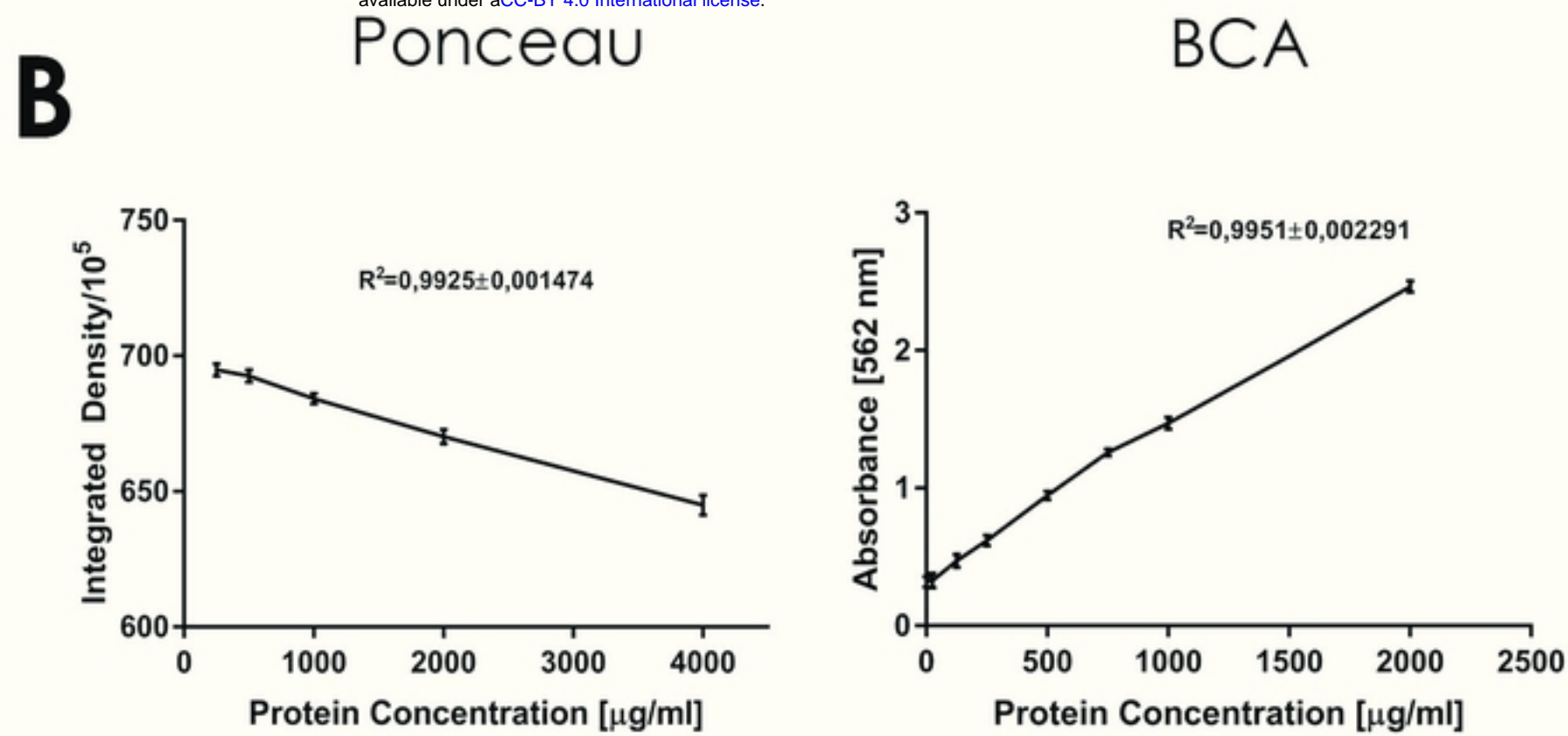


Figure 1

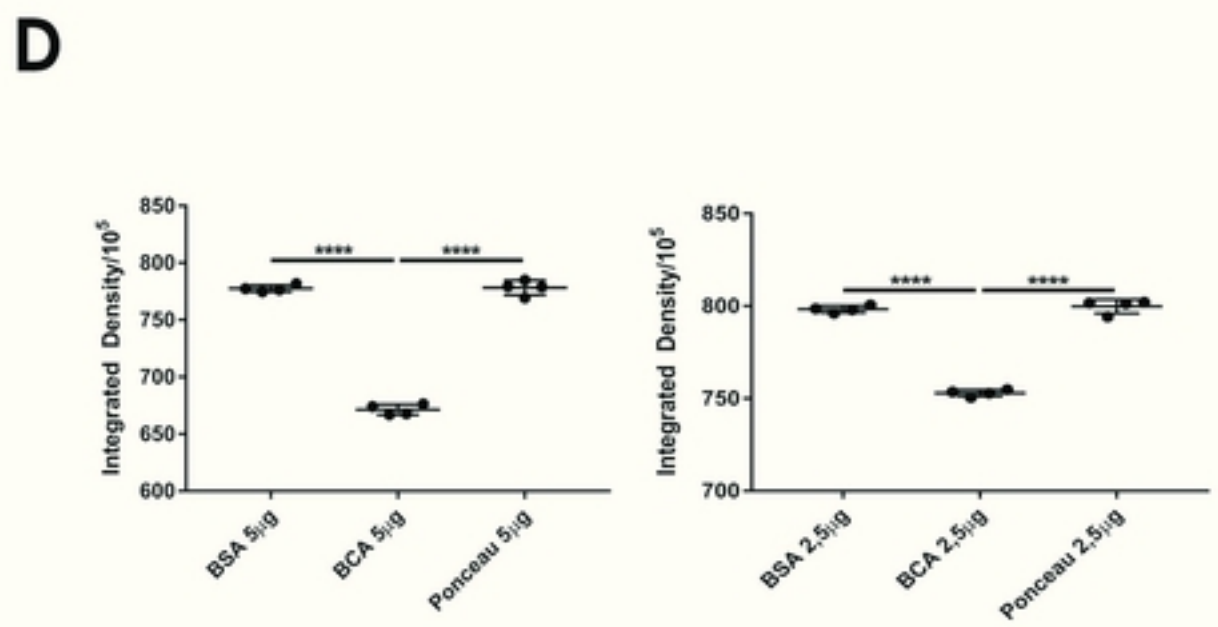
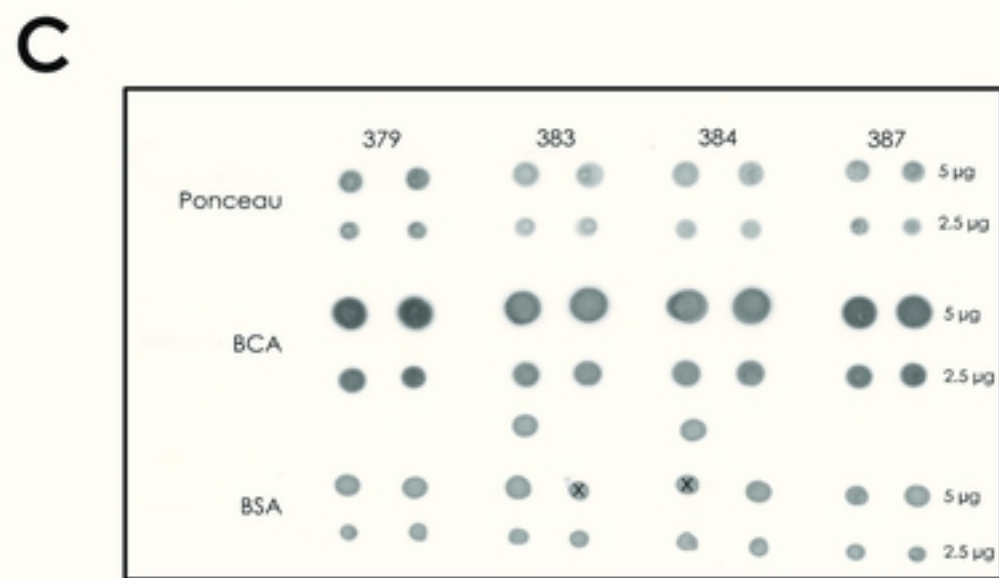
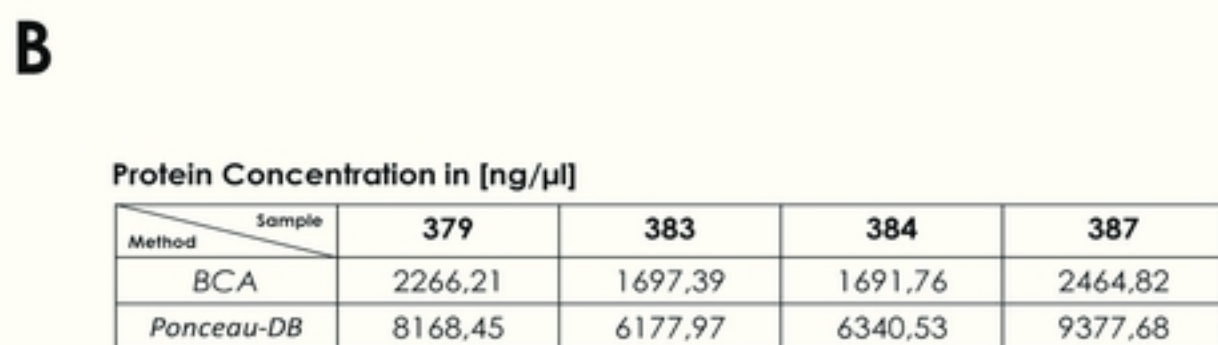
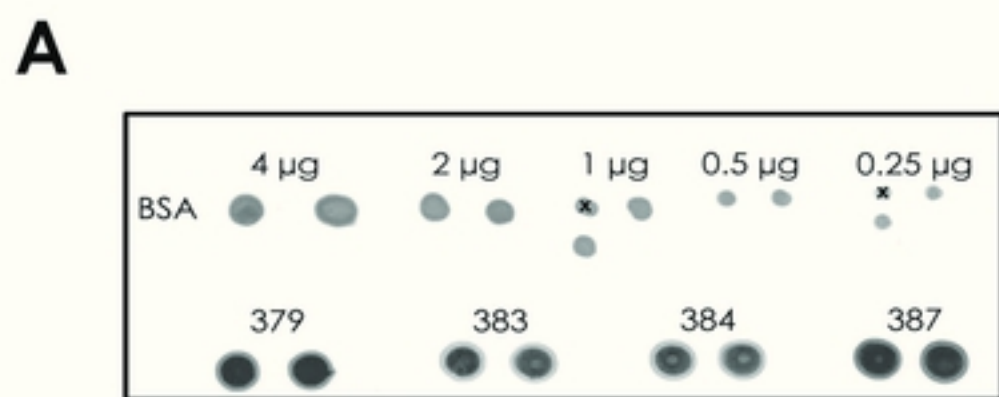


Figure 2

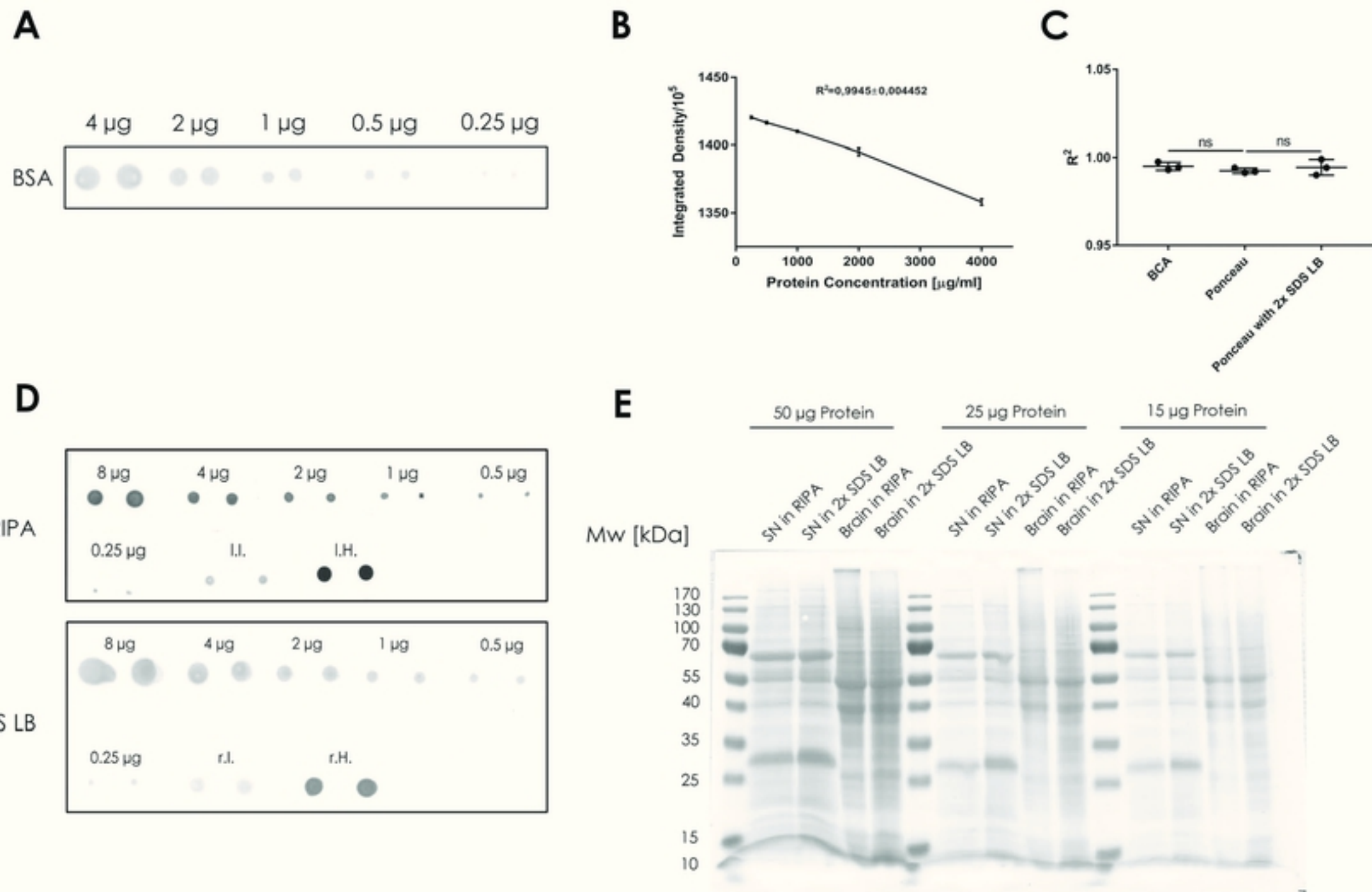


Figure 3

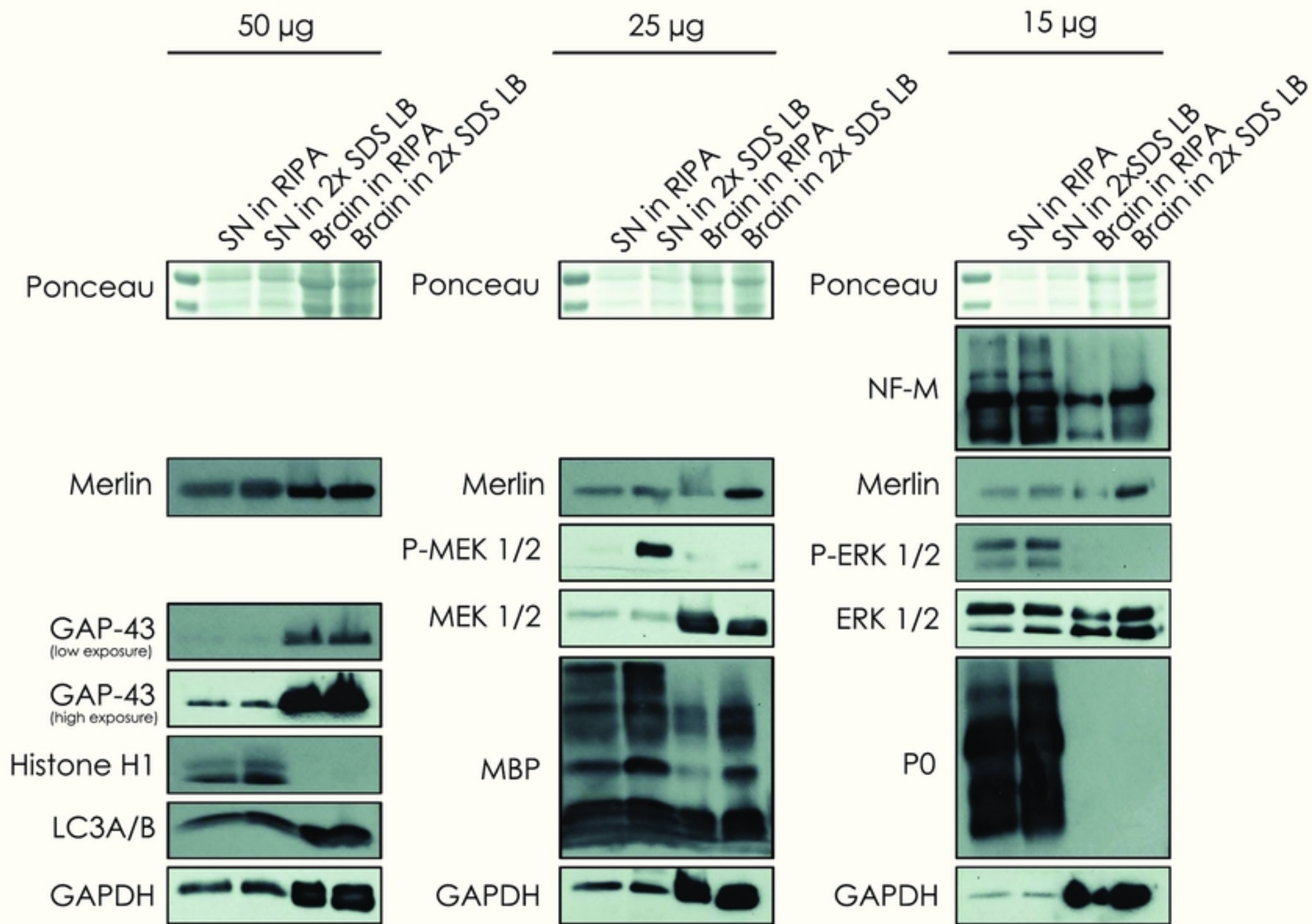


Figure 4