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TECHNICAL BRIEF

Epicocconone staining: A powerful loading control for Western blots

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Western blot analysis is routinely employed for quantifying differences in protein levels between samples. To control equal loading and to arithmetically compensate loading differences, immunodetection of housekeeping proteins is commonly used. Due to potential biases, this approach has been criticized. Here, we evaluate epicocconone-based total protein staining (E-ToPS) as an alternative. We compared it with two other total protein stainings (Coomassie and Sypro Ruby) and with immunodetection of housekeeping proteins (β-tubulin and glyceraldehyde 3-phosphate dehydrogenase). Evaluation comprised both the natural and the synthetic epicocconone compound. Both compounds produced highly congruent results and showed more sensitive ($\leq$ 1 μg) and less variable staining properties than the other variants. The high sensitivity of E-ToPS, covering minute protein amounts, makes it a powerful loading control, especially for precious samples. Regarding biological and technical variances, E-ToPS outperformed immunostaining against β-tubulin and glyceraldehyde 3-phosphate dehydrogenase. Furthermore, E-ToPS had no impact on subsequent immunodetection, allowing for an early control of proper loading prior to immunodetection. In contrast to earlier studies, we found logarithmic staining properties for E-ToPS, which should be considered when using it for arithmetic normalization. In conclusion, we demonstrate the superior power of E-ToPS as a loading control for Western blots.

Keywords:
β-Tubulin / GAPDH / Loading control / Technology / Total protein staining / Western blot

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When comparing protein profiles from various samples in proteomics (e.g. different disease states, ages, or knockout conditions), Western blotting, followed by immunodetection, is a well-established and routinely used (semi-)quantitative technique to validate any intensity differences [1–5]. Important requirements for unbiased Western blot results are a uniform gel loading and an equal protein transfer to the blotting membrane. To control these steps or to arithmetically compensate possible inequalities, immunodetection of housekeeping proteins, such as β-tubulin, β-actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is standard practice, and many journals require this loading control for publication. This approach relies on the assumption that housekeeping proteins are equally expressed, but this is not generally warranted. Rather, the viability of housekeeping proteins as internal controls (e.g. β-actin, GAPDH) has become under some doubt because of potential biases and limitations to distinguish loading differences [6–11].

A recently introduced alternative to housekeeping proteins as internal control employs the visualization of the protein lanes via Coomassie blot staining [6]. Major advantages of total protein staining over quantifying single housekeeping proteins are (i) a more reliable assessment of the real loading amount and (ii) a more comprehensive control of

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Abbreviations: **BR**, biological replicate; **$E^{n/s}$-ToPS**, natural/synthetic epicocconone-based total protein staining; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase

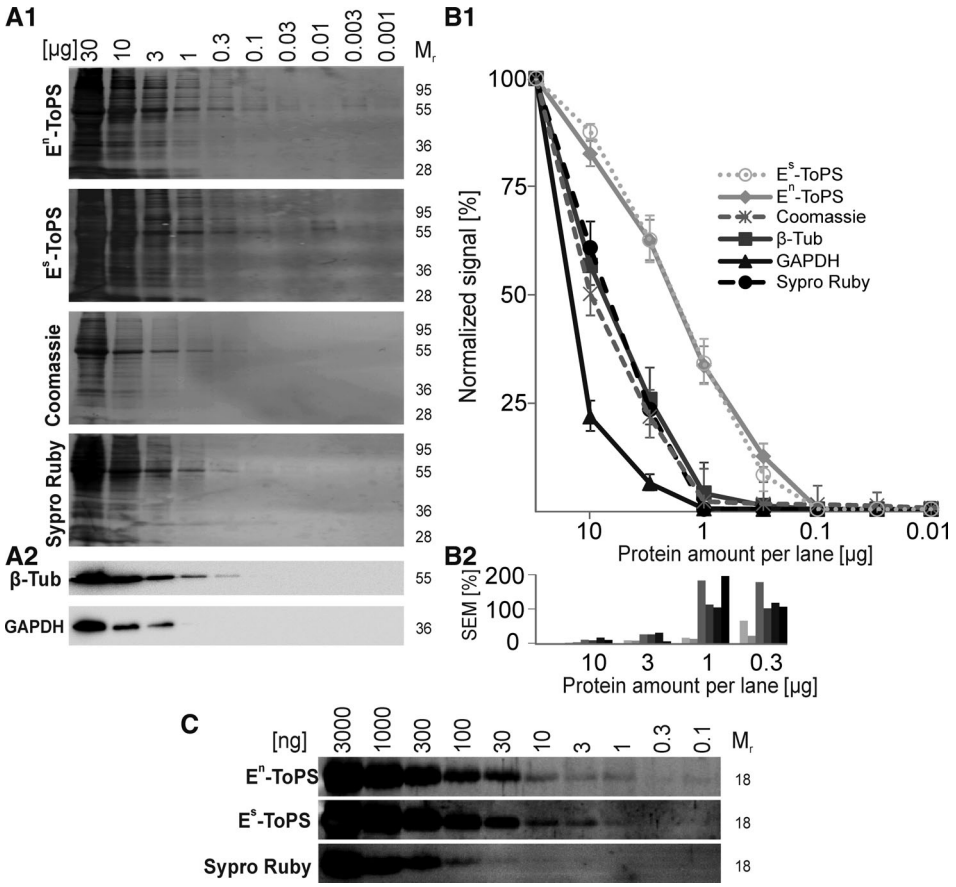

Figure 1. Calibration curves and sensitivity of E-ToPS compared with Coomassie and Sypro Ruby staining as well as β-tubulin and GAPDH immunostaining. (A1) E^n-ToPS, E^s-ToPS, Coomassie, and Sypro Ruby staining from a dilution series of 30–0.001 µg (Rest brain). M_r = molecular weight marker [kDa]. (A2) Same dilution series as in A1, depicting immunosignals for β-tubulin (β-Tub) and GAPDH. (B1) Background-subtracted and normalized signal volumes of all variants used in A, plotted against protein amounts (10–11 replicates each). Error bars depict SEM. (B2) SEM% values of the six staining variants at the relevant protein amounts. (C) Dilution series (3000–0.1 ng) of ß-lactoglobulin visualized with three staining variants.

homogeneous transfer. However, Coomassie staining also has its pitfalls. In particular, it precludes subsequent antibody analysis. Consequently, protein loading and transfer must be controlled after the final immunodetection step. Thus, a potential rejection of the blot, because of its low quality, is time-consuming and associated with the waste of precious antibodies. Furthermore, prior treatment of the blotting membrane with blocking solution and antibodies will affect the Coomassie results. In order to circumvent these caveats, we here evaluate an alternative total protein staining method, namely *e*picocconone-based *to*tal *p*rotein *s*taining (E-ToPS). It makes use of the natural fluorescent compound epicocconone [12, 13], commercially distributed as Deep Purple™, LavaPurple™ [14], or Lightning fast™ [15]. Aside from the natural compound, a synthetic epicocconone analogue has been introduced in 2011 [16] and is distributed as Serva Purple™. We assessed this analogue as a loading control in addition to the natural compound. We also compared the two E-ToPS variants with two other total protein stainings (Coomassie [6] and Sypro Ruby [17–21]) and immunodetection of two housekeeping proteins (β-tubulin and GAPDH). Thus, our analysis comprised a total of six staining variants: four total protein stainings and two immunostainings.

In a first set of experiments, we assessed the sensitivity of the six staining variants (Fig. 1). Brain tissue from 8- to 9-week-old Sprague-Dawley rats was homogenized (see Supporting Information for details). Dilution series of 30–0.001 µg total protein were loaded onto acrylamide gels ($n = 36$) and transferred to PVDF membranes [22]. Eleven of these membranes were used for natural epicocconone-based total protein staining (E^n-ToPS; Deep Purple™ Total Protein Stain (GE Healthcare, Uppsala, Sweden) and LavaPurple™ (Serva Electrophoresis, Heidelberg, Germany; Fig. 1A1)). Four membranes were used for *s*ynthetic epicocconone-based total protein staining (E^s-ToPS; Serva Purple™, Serva Electrophoresis). The staining protocols followed the manufacturer's instructions, with marginal modifications (see Supporting Information). Ten membranes were stained with Coomassie (R–250, Carl Roth Karlsruhe, Germany) as described [23]. Finally, 11 membranes were stained with Sypro Ruby (SYPRO® Ruby protein blot stain, Bio-Rad, Munich, Germany) following the manufacturer's instructions. The 11 E^n-ToPS membranes subsequently served for immunostaining with antibodies against β-tubulin (mouse, 1:1000, T5201, Sigma-Aldrich, Munich, Germany) and GAPDH (mouse, clone 6C5, 1:500, MAB374, Millipore, Schwalbach, Germany; Fig. 1A2; see Supporting Information). E-ToPS/Sypro Ruby signals and immunosignals were visualized by a VersaDoc 3000 documentation system, and Coomassie-stained blots were scanned with an EPSON Transparency Unit (Model

EU-35, Seiko Epson, Meerbusch, Germany). All stainings were quantified via Quantity One software (Bio-Rad; for details, see Supporting Information). For normalization, the 30 μg signal volumes for all six assays were set to 100%.

The highest sensitivity was obtained with the two E-ToPS (Fig. 1). Both staining variants revealed detectable signals down to a total protein amount of 0.1 μg, whereas Coomassie and Sypro Ruby displayed signals down to only 1 μg (Fig. 1A1 and 1B1). Similarly, immunosignals for β-tubulin and GAPDH were detectable down to protein amounts of 0.3 μg and 3 μg, respectively (Fig. 1A2 and 1B1). Together, the results demonstrate that the detection limit for the two E-ToPS variants is three- to tenfold lower than that of the other four staining variants. E-ToPS appears to be especially suitable for controlling the loading of protein amounts <1 μg. This is of special interest if Western blots are performed with highly precious samples, such as microdissected tissue comprising only some thousands cells [24, 25].

The calibration curves of the two E-ToPS variants were almost identical (Fig. 1B1), demonstrating equal staining properties of the natural and the synthetic variant. Interestingly, for the three fluorescence stainings, the best fit between 30 μg and their detection limit was obtained with a logarithmic model (E^n-ToPS: $R^2 = 0.99$; E^s-ToPS: $R^2 = 0.98$; Sypro Ruby: $R^2 = 0.99$, see also Supporting Information Fig. 1A). In contrast, a linear model was best for the three other staining variants (Coomassie: $R^2 = 0.96$; β-tubulin: $R^2 = 0.93$; GAPDH: $R^2 = 0.99$).

The GAPDH curve illustrated in Fig. 1B1 differed from the one described earlier [6], where no signal increase was detected above 5 μg. The discrepancy may be explained by a different antiserum and points to the general problem of availability of adequate antibodies. Further, the different slopes of the calibration curves clearly demonstrate the widely discussed necessity of applying calibration curves for an unbiased relative quantification of labeling signals [8, 19, 26, 27]. Otherwise, the detected fold changes may be overestimated or underestimated.

The lowest SEM% values, namely 2.1–65.7%, were obtained for the two E-ToPS variants from 10 to 0.3 μg (Fig. 1B2). Sypro Ruby reached a similarly low value only at 3 μg. In general, however, the values for E-ToPS were 2.4- to 15.3-fold lower than those of the other variants. As each variation distorts the process of compensating loading differences, E-ToPS thus emerges again as most suitable. Together, the results from the calibration analyses show that E-ToPS displays a broader and, likewise, a less variable dynamic range than the other four variants.

In a next series of experiments, we further evaluated the suitability of E-ToPS concerning sensitivity and variation. In a first step, the sensitivity was addressed for a one-protein sample, including Sypro Ruby staining for comparison. To do so, we used ß-lactoglobulin (Sigma-Aldrich) (3000–0.1 ng; 10 membranes; 3, 2, 5 replicates for E^n-ToPS, E^s-ToPS, and Sypro Ruby, respectively). To guarantee appropriate and comparable settings, we consistently used exposure times leading

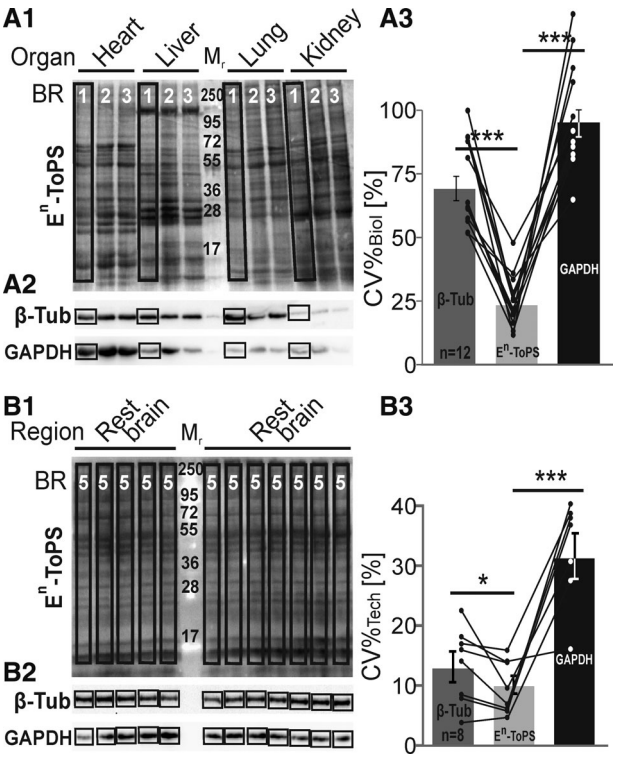

Figure 2. Biological (A) and technical (B) variation of E^n-ToPS compared with β-tubulin and GAPDH immunosignals. (A1) E^n-ToPS of a blot membrane, depicting 12 samples from four organs (heart, liver, lung, kidney), each organ being represented by three BRs (BRs 1–3). Each lane was loaded with 10 μg protein. (A2) Same blot as in A1, depicting immunosignals for β-tubulin (β-Tub) and GAPDH. (A3) Mean coefficients of biological variation ($CV\%_{Biol}$) for β-tubulin, E^n-ToPS, and GAPDH signals across the four organs. Lines connect CV% values of the 12 individual samples. (B1) Like in A1, but all 12 lanes loaded with 10 μg from the same sample (BR 5). (B2) Same blot as in B1, depicting immunosignals for β-tubulin and GAPDH. (B3) $CV\%_{Tech}$ across the 12 samples of β-tubulin, E^n-ToPS, and GAPDH signals. Lines connect CV% values of the eight individual replicates. Framed rectangles in A1, A2, B1, B2 exemplarily depict areas analyzed for signal volume measurements. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, paired Student's *t*-test. Error bars depict SEM.

to signal volumes just below saturation. The two E-ToPS variants reached their detection limit at 1–3 ng, while Sypro Ruby staining revealed a protein band only down to 10 ng (Fig. 1C). The calibration curves of the two E-ToPS variants again run very similarly and behaved rather logarithmic (E^n-ToPS: $R^2 = 0.98$; E^s-ToPS: $R^2 = 0.97$) than linear (E^n-ToPS: $R^2 = 0.73$; E^s-ToPS: $R^2 = 0.72$; range: 1000–3 ng; Supporting Information Fig. 1B and C). Together, the results from the one-protein sample experiments validate the superiority of the two E-ToPS variants over Sypro Ruby regarding sensitivity.

In a second step, the biological and technical variation was addressed (Fig. 2). In these experiments, only E^n-ToPS was employed, as it had performed like E^s-ToPS. For comparison,

we used immunostaining against β-tubulin and GAPDH. The biological variation was analyzed across various organs (Fig. 2A). To do so, the samples from heart, liver, lung, and kidney from a total of 12 biological replicates (BRs 1–12) were prepared as described above and loaded onto four gels (BRs 1–3 on the 1st gel, BRs 4–6 on the 2nd, etc.). Figure 2A1 exemplarily shows the results achieved with E^n-ToPS for BRs 1–3, whereas Fig. 2A2 illustrates the results from the same blot after immunostaining of β-tubulin and GAPDH. In order to quantify the biological variation, we calculated the coefficients of variation as a percent value (CV%, standard deviation × 100/mean) across the four organs obtained from the same BR ($CV\%_{Biol}$, e.g. framed rectangles in Fig. 2A1 and A2). The average CV% values of the 12 BRs were determined by calculating the geometrical mean (Fig. 2A3). The lowest $CV\%_{Biol}$ values were obtained for E-ToPS (mean 23.1%, range 11.7–47.8%; β-tubulin: mean 69.0%, range 51.6–99.7%, $p = 4.6 \times 10^{-6}$; GAPDH: mean 95.1%, range 64.6–137.3%, $p = 1.7 \times 10^{-6}$; Fig. 2A3). The threefold and fourfold lower values for E-ToPS compared with β-tubulin and GAPDH, respectively, demonstrate the superiority of E-ToPS. Remarkably, GAPDH displayed a distinctly higher immunosignal in the heart than in the other organs, whereas the β-tubulin immunosignal was lower in the kidney (Fig. 2A2), confirming that housekeeping proteins are not uniformly expressed.

Next, we assessed whether the lower CV% values achieved with E-ToPS may also be obtained with less diverse samples, namely various brain regions (Supporting Information Fig. 2). To do so, we prepared protein samples as described above from four brain regions: the cochlear nuclear complex, the superior olivary complex, the inferior colliculus, and residual parts of the brain (Rest brain). Twelve animals were used and samples from two animals were pooled, resulting in six BRs. Upon gel treatment and staining, the lowest $CV\%_{Biol}$ values were again detected for E-ToPS (mean 13.1%, range 10.2–16.6%, β-tubulin: mean 51.7%, range 13.2–74.7%, $p = 0.0042$; GAPDH: mean 80.3%, range 64.3–120.0%, $p = 0.0008$; Supporting Information Fig. 2A3). The fourfold and sixfold lower values for E-ToPS compared with β-tubulin and GAPDH, respectively, confirm the superiority of E-ToPS. Noticeably, β-tubulin exhibited a distinctly higher immunosignal in the inferior colliculus and the Rest brain (Supporting Information Fig. 2A2), again refuting the idea that housekeeping proteins are equally expressed.

Next, we assessed the technical variation ($CV\%_{Tech}$) of E-ToPS signals and immunosignals. To do so, we loaded 12 Rest brain samples of the same BR onto one gel and determined their CV% values. In total, eight gels were run accordingly. A representative example is illustrated in Fig. 2B1 and B2. Like in the analyses before, the lowest $CV\%_{Tech}$ values were obtained for E-ToPS (Fig. 2B3; mean 9.9%; range 6.2–16.7%). The mean values for β-tubulin and GAPDH were significantly higher (12.9%, range 5.4–22.9%, $p = 0.0333$ and 31.3%, range 16.9–39.6%, $p = 0.0002$, respectively) and thus less reproducible, further demonstrating the benefit of E-ToPS. Our results also showed a twofold higher $CV\%_{Tech}$

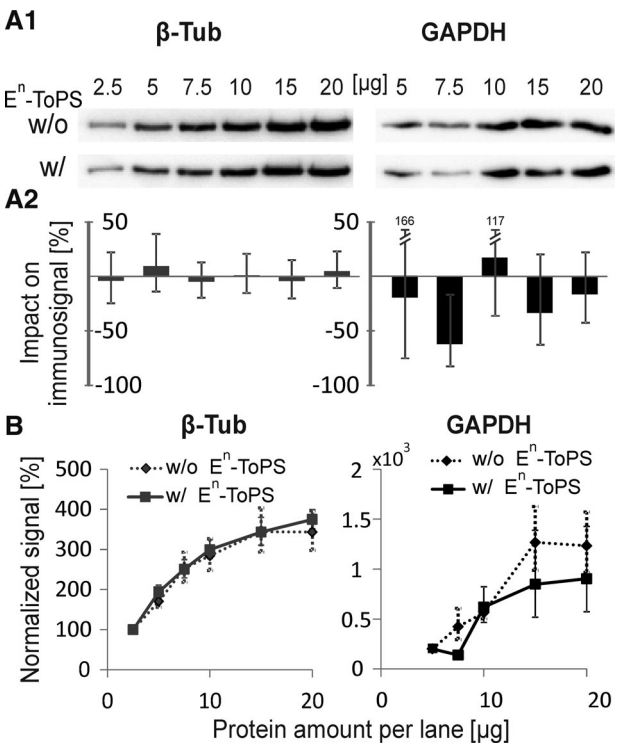

Figure 3. Assessment of the influence of E^n-ToPS on subsequent immunosignals. (A1) Antibody signals for β-tubulin (β-Tub) and GAPDH obtained from two dilution series with protein samples (2.5/5–20 µg) obtained from Rest brain. Immunosignals obtained without (w/o) and with (w/) prior E^n-ToPS are juxtaposed. (A2) Statistical summary of eight analyses like the one exemplified in A1. A value of zero represents no influence (for details, see Supporting Information). (B) Background-subtracted and normalized β-tubulin and GAPDH signal volumes without and with E^n-ToPS. Error bars depict SEM.

for the GAPDH signal than for the β-tubulin signal. This is in contrast to previous reports on several cell and tissue types where GAPDH was found to be a more reliable loading control than other housekeeping proteins [8, 9, 28]. It adds an additional caveat to the analysis of housekeeping protein immunosignals, in that the reliability depends on the specific cell or tissue type.

Of particular interest is the notion that we performed all E-ToPS prior to immunostaining. This is in contrast to Coomassie staining, which is incompatible with subsequent immunodetection methods [29] and therefore performed as the final step [6] or in a replicate blot/gel [30]. A last question concerning us was whether E-ToPS affects the fidelity of the immunosignals. In order to address this question, we performed a final series of experiments in which 10 blots were handled in parallel and completely identically, except that five blots were subjected to E-ToPS prior to immunostaining, whereas the others were not. In addition, the protein amount was varied in a dilution series to assess possible

influences on the calibration curves. An example is illustrated in Fig. 3A1, showing no obvious effect of E-ToPS on β-tubulin or GAPDH immunosignals. We objectified our observation by calculating the impact (see Supporting Information) and found no influence of E-ToPS, i.e. the impact of E-ToPS on the immunosignals did not differ significantly from zero, independent of the protein amount (p-values > 0.250 and > 0.126, respectively; Fig. 3A2). Consistent with the results reported above (cf. Fig. 2), GAPDH immunosignals displayed a higher variability than those of β-tubulin. Finally, the calibration curves obtained with and without E-ToPS were almost equal in case of ß-tubulin and similar in case of GAPDH (Fig. 3B). No significant difference was detected between the two staining procedures. Hence, the advantage of applying E-ToPS prior to immunostaining does not go along with negative consequences.

Aside from the total protein stainings assessed here (E-ToPS, Coomassie, and Sypro Ruby), several other dyes have been used for loading control, such as Sypro Rose [31], Amido Black [19], Flamingo [17], Direct blue 71 [32], and Ponceau [33]. Concerning the sensitivity of these dyes, the following ranking was described so far: E-ToPS (Deep Purple™) > Sypro Ruby [13], present study > Direct blue 71 [32] > Coomassie [18] > Amido Black [34] > Ponceau S [34] (review: Miller et al. [35]). Thus, E-ToPS appears to employ the most sensitive dye.

Surprisingly, our results differed from literature regarding the regression model of the calibration curve of E-ToPS. Linearity for the epicocconone-based dye was described from 1 μg to 60 pg [36] and from 1 μg to 100 pg [13]. In contrast, we here describe logarithmic calibration curves from 30 μg to 100 ng for a total-protein sample (cf. Fig. 1A1 and B1) and from 1 μg to 3 ng for a one-protein sample (cf. Fig. 1C, Supporting Information Fig. 1B and C). In an attempt to solve the discrepancy, we re-evaluated the staining signals shown in Fig. 1A by Svensson and colleagues [36] (Supporting Information Fig. 3). In contrast to these authors, we did not logarithmically transform the data prior to the regression analysis, because this process is not statistically valid [37]. Interestingly, we found that the curves were logarithmic ($R^2 = 0.94$ in the range of 1 μg to 0.1 ng; $R^2 = 0.99$ in the range of 1 μg to 1 ng) instead of linear ($R^2 = 0.56$; Supporting Information Fig. 3B and C). Based on this re-evaluation and our own studies, a logarithmic calibration curve appears to be most appropriate for E-ToPS. Consequently, E-ToPS values should be converted by exponentiation before applying them for data normalization.

A further characteristic of E-ToPS is its reversible nature, which implies easy removal under conditions used for tryptic protein digestion [38] and does not tend to cause speckles, in contrast to Sypro Ruby [14]. Thus, E-ToPS is compatible with subsequent MS analysis via peptide mass fingerprinting [12, 13], and it is not based on a potentially toxic heavy metal component [13], adding further advantages to our method. Finally, regarding the costs of the staining compounds, E-ToPS (< 2 €/blot membrane) is 5- to 60-fold cheaper than all tested alternatives (> 7 €/blot membrane), except for Coomassie (< 0.10 €/blot membrane; Supporting Information Table 1).

In summary, we have compared different methods toward their ability to control loading and to compensate possible differences in Western blots. Notably, we included a new staining method based on a synthetic epicocconone (E^s-ToPS) besides the classical epicocconone staining method with the natural compound (E^n-ToPS). Our results show a high congruence between the two E-ToPS variants. Furthermore, E-ToPS outperforms other total protein stainings (Coomassie, Sypro Ruby) as well as immunostaining of housekeeping proteins (β-tubulin, GAPDH) because of its broader and less variable dynamic range down to loading amounts < 1 μg. This makes it a powerful loading control especially for precious samples. A further advantage is that E-ToPS, if performed prior to immunostaining, provides the possibility to control the quality of protein separation and transfer efficiency at an early stage. Low-quality blots can be discarded early, thus saving time and money spent for immunostaining. This is in contrast to Coomassie staining, which is not compatible with subsequent immunostaining and, therefore, must be performed after immunodetection. In contrast to earlier studies [19, 20, 33], we have quantified the influence of prior total protein staining on subsequent immunosignaling and found no significant impact. Altogether, we conclude a superiority of E-ToPS over other loading controls for Western blots, especially when using precious samples.

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The authors have declared no conflict of interest.

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