Analytical Biochemistry 467 (2014) 1–3

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting

B. Rivero-Gutiérrez [a,1], A. Anzola [a,1], O. Martínez-Augustin [b], F. Sánchez de Medina [a,*]

[a] Department of Pharmacology, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), School of Pharmacy, Instituto de Investigación Biosanitaria (ibs.GRANADA), University of Granada, 18071 Granada, Spain
[b] Department of Biochemistry and Molecular Biology II, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), School of Pharmacy, Instituto de Investigación Biosanitaria (ibs.GRANADA), University of Granada, 18071 Granada, Spain

ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 13 August 2014
Accepted 22 August 2014
Available online 1 September 2014

Keywords:
Western blot
Loading control
Ponceau staining
Housekeeping proteins
TGX Stain-Free gels

ABSTRACT

It is currently a routine practice to require a measurement of a housekeeping reference, including actin, glyceraldehyde-3-phosphate dehydrogenase, β-tubulin, among others, in Western blots, as it is the rule in RNA blots. Reversible Ponceau staining has been applied successfully to check equal loading of gels. Here we test a new technique, with the Stain-Free gels from Bio-Rad, against both Ponceau staining and housekeeping protein immunodetection under different conditions. Our results show that Stain-Free gels outperform Ponceau staining and that both are more consistent than housekeeping proteins as a loading control.

The use of so called 'loading controls' is standard practice in RNA gels [1]. Despite the name, it is not equal loading but the absence of RNA degradation that is sought by this approach. This is based on the well-known liability of RNA, but it has gradually extended also to the technical validation of Western blots, despite the fact that proteins are much more stable, especially after processed for electrophoresis. Even if this requirement may be not justified, it has become a common practice. Following again the Northern blot path, most laboratories measure a reference protein to ensure equal loading of different lanes in the protein gel, in effect performing a second parallel Western, preferably in the same blot but sometimes using a duplicate [2]. To this end, proteins with a substantial expression level that is considered to be unaffected by the experimental conditions in the study (i.e. 'housekeeping' proteins) are typically used, usually actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[2] or β-tubulin, but also including a variety of other proteins such as COX-IV (mitochondrial loading control protein), phosphoribosyltransferase, or cyclophilin, among others. Apart from the obvious waste of time and resources, a major caveat of this approach is that the absence of changes in housekeeping protein expression is normally assumed rather than established. In some cases these proteins are indeed affected by the experimental conditions, for instance GAPDH, β-actin and β_2-microglobulin in normal vs. inflamed intestine, GAPDH in Von Hippel Lindau defective or transfected cell lines, and again GAPDH in melanoma vs. control patients, to mention a few documented examples [2–5]. Because of the relatively high expression level of these proteins and the limited dynamic range of antibodies used in immunodetection, the signal is prone to saturate the detection system, so that effective changes in expression may not be observed.

Tracking the total protein level after gel loading may be a more suitable approach to assess technical correctness [5]. We previously applied reversible Ponceau staining of protein blots to track the total protein level in the blot lanes and compared this technique with housekeeping protein normalization [6]. Ponceau staining was found to be a low cost, valid alternative in this setting. Here we compared both approaches to a new technology introduced by Bio-Rad, through TGX Stain-Free gels [7]. The principle is based on the fluorescent detection of tryptophan residues contained in the protein sequence which are previously modified by a trihalo compound included in the electrophoresis gel after separation. Therefore it requires no extra addition of reagents, provided that the trihalo compound containing gels is used.

Female Wistar rats and Zucker obese and lean control rats were used in this study, in addition to Caco-2 cells cultured in a standard fashion. Liver samples and Caco-2 cells were homogenized in RIPA buffer and processed for Western blot [6]. We ran a series of protein

* Corresponding author at: Department of Pharmacology, CIBERehd, School of Pharmacy, Instituto de Investigación Biosanitaria (ibs.GRANADA), University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain. Fax: +34 958 248964.
E-mail address: fsanchez@ugr.es (F.S. de Medina).

[1] Both of these authors contributed equally to this work.

[2] *Abbreviations used:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; CV, coefficient of variation.

http://dx.doi.org/10.1016/j.ab.2014.08.027
0003-2697/

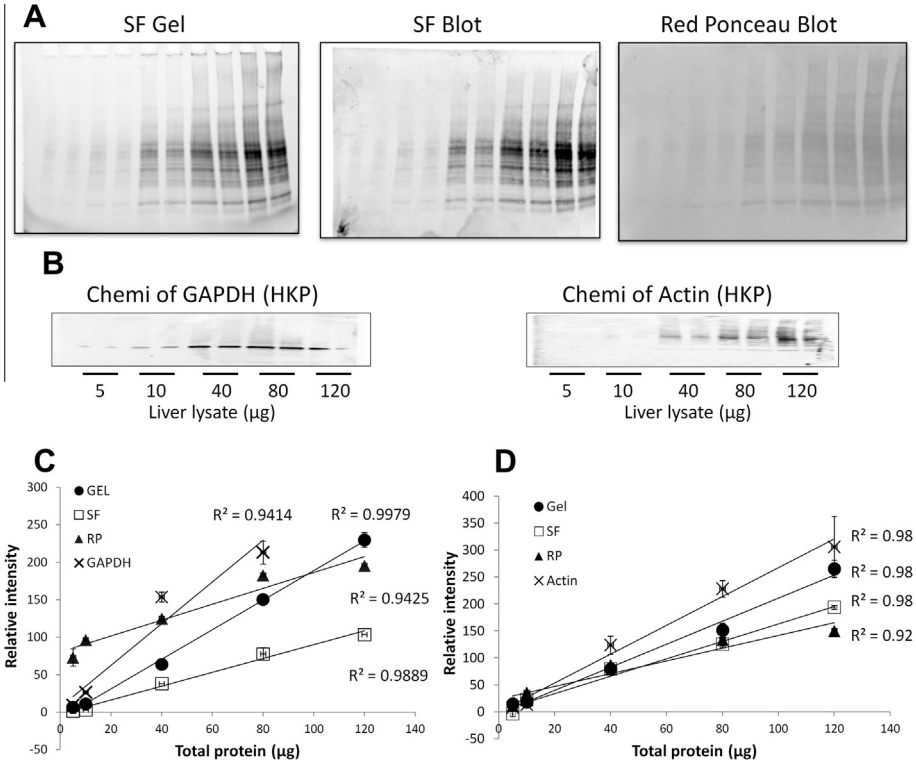

Fig.1. Assessment of protein quantity using increasing amounts of rat liver lysate. (A) Total protein detected by Stain-Free (SF) technology in the gel (left) and after transfer (center), and detected by Ponceau Red staining (right). (B) Immunoblot of the housekeeping proteins GAPDH and actin (images shown in A correspond to the GAPDH blot). (C, D) Protein quantitation.

blots using TGX Stain-Free gradient precast gels (Bio-Rad). Gel loading control was done by Ponceau S staining (right after transfer), by Stain Free detection (in the gel after the electrophoresis and on the blotting membrane after transfer) and by actin or GAPDH immunodetection using JLA-20 and GA1R monoclonal antibodies, respectively. A ChemiDoc™ MP imager (Bio-Rad) was used for gel/blot documentation in all cases. Antibodies were supplied by the Development Studies Hybridoma Bank at the University of Iowa (actin), Epitope Biotech Inc. (GAPDH), Cell Signaling Technology (phospho-specific AKT), BD Biosciences (PCNA) and Sigma (secondary peroxidase conjugated antibody). We used Bio-Rad electrophoresis and transfer units and Bio-Rad PVDF Turbo transfer packs, with 10 min transfer time (25 V and 2.5 A, Trans-Blot Turbo system). After transfer, a Stain-Free image was taken and the PVDF membranes were rinsed briefly in distilled water and incubated in Ponceau S solution (0.5% w/v in 1% v/v acetic acid) for 2 min [3], followed by a brief rinse in distilled water, so that the lanes and bands were clearly visible. After scanning, the membranes were rinsed once more in distilled water for 2–3 min, until the staining was completely eliminated, and we proceeded with the blocking and antibody incubation steps. Antibody bound peroxidase was detected by enhanced chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad), documented and quantitated (ImageLab™ software, Bio-Rad).

In order to assess internal reproducibility, the variation of signal generated by a single sample (rat liver) loaded in equal amounts in different lanes of a gel was measured as the coefficient of variation (%CV), defined as the percent standard deviation:mean ratio. It is important to note that Ponceau staining is carried out on the protein blot as a direct, one step procedure, while immunostaining involves a number of washing and antibody incubation steps, plus the detection itself, in this case by ECL. In contrast, the Stain-Free signal can be detected not only in the blot but also in the gel itself. Predictably, the variability increases as the number of steps before detection accumulates. Thus the Stain-Free showed an internal reproducibility of about 8% (8.5 ± 0.4%, $n = 7$) in the gel post electrophoresis. This was fairly preserved after transfer (9.7 ± 2.3%, $n = 7$), but was occasionally higher (up to ~20%) depending on the homogeneity of the transfer across the blot membrane. Red Ponceau staining includes a manual step by rinsing the excess of stain before taking an image. This step is critical as it may generate heterogeneity of staining across the surface of the membrane. In our hands this produced a variability of 13.0 ± 2.9% ($n = 7$), which was the result of increased heterogeneity among blots rather than of higher signal spread in each blot. Immunodetection of actin and GAPDH in parallel blots showed a %CV of 15.3% and 20.3%, respectively. Thus a variability of 15–20% is expected with either of the 3 techniques but tends to be lower with Stain Free.

The dynamic range and linearity of the different techniques were evaluated by measuring the signal of duplicate lanes of the same sample (rat liver) loaded with increasing amounts of protein. The Stain-Free and Red Ponceau techniques showed similar linear range of detection from 5 to 80–120 µg of total proteins that is compatible with the charge required to detect common low abundant target protein, although linearity was more consistent with Stain-Free since some of the blots stained with Red Ponceau showed saturation (Fig. 1). Conversely, the signal corresponding to GAPDH immunodetection was saturated at 40 µg of protein loaded, due to the high sensitivity of this particular antibody. Actin immunodetection was fully linear though, owing to a less strong signal. Similar results were obtained with Caco-2 cells (human intestinal epithelial phenotype, Fig. 2).

Finally, the three techniques were applied to the study of different samples, namely rat liver from obese Zucker rats and their lean controls, run in duplicate in the same gel. In this case the intensity of the signal of the different samples was very similar using either Stain-Free or Red Ponceau detection, consistent with equal loading. However, GAPDH levels were markedly influenced by the type of sample, with higher levels being detected in the liver samples

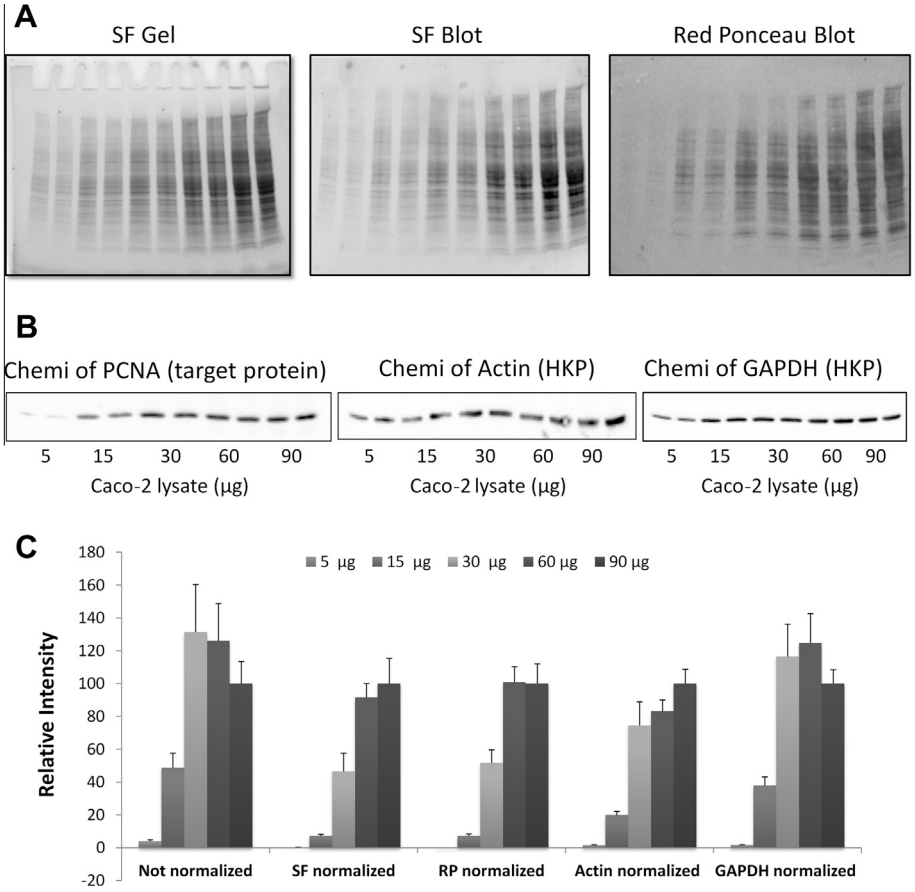

Fig.2. Putative housekeeping protein expression may be modified by experimental conditions. Duplicate samples of increasing amounts of Caco-2 lysate were run and analyzed by Stain-Free, Ponceau staining and PCNA, Actin or GAPDH immunoblotting. (A) Total protein detected by Stain-Free technology in the gel (left) and after transfer (center), and detected by Ponceau Red staining (right), $n = 6$. (B) Immunoblot of the putative housekeeping proteins Actin (center) and GAPDH (right), and of PCNA (left), $n = 2$ for each one (images shown in A correspond to the GAPDH blot). (C) Protein quantitation.

obtained from obese rats compared with their lean controls (not shown). Thus when the signal of a target immunodetected protein such as phospho-AKT was assessed in a parallel blot, appropriate normalization was obtained with either Red Ponceau or Stain-Free, but not with GAPDH. In short, GAPDH is not a valid housekeeping protein in this setting and its use can be misleading.

Based on our results, both Stain Free and Red Ponceau total protein signals outperform housekeeping protein immunodetection as loading controls since (1) there is no added advantage of using these compared to Stain-Free or Ponceau (which save extra money and time and gives reliable results); (2) antibodies giving a high signal predictably saturate at higher protein loads; and (3) individual loading control proteins are subject to variation in certain biological situations. Stain Free appears to have an increased linear range and less variability than Ponceau staining, the latter probably derived from the lack of the manual washing step. Conversely, Ponceau staining is cheaper although it is likely that Stain-Free technology will also become increasibly affordable over time. Increased range may be of importance when using high protein loads, in the vicinity of 70–80 µg or higher. These data are in agreement with the report by Colella et al. [8], which showed that Stain Free is superior to Sypro Ruby protein staining, and also with the more recent paper by Gilda and Gomes [9].

Acknowledgments

The authors want to thank the input of Arnaud Remy and Enrique Orozco of Bio-Rad Laboratories. This study was supported by the Ministry of Science and Innovation (SAF2011-22922 and SAF2011-22812), and by funds from Junta de Andalucía (CTS6736 and CTS164). B. Rivero and A. Anzola were supported by fellowships of the Ministry of Education and Science of Spain and Junta de Andalucía, respectively. CIBERehd is funded by the Instituto de Salud Carlos III.

References

[1] F. Herrera, V. Martin, I. Antolin, G. Garcia-Santos, J. Rodriguez-Blanco, C. Rodriguez, Standard curve for housekeeping and target genes: specific criteria for selection of loading control in Northern blot analysis, J. Biotechnol. 117 (2005) 337–341.
[2] R.E. Ferguson, H.P. Carroll, A. Harris, E.R. Maher, P.J. Selby, R.E. Banks, Housekeeping proteins: a preliminary study illustrating some limitations as useful references in protein expression studies, Proteomics 5 (2005) 566–571.
[3] G. Bamias, D. Goukos, E. Laoudi, I.G. Balla, S.I. Siakavellas, G.L. Daikos, S.D. Ladas, Comparative study of candidate housekeeping genes for quantification of target gene messenger RNA expression by real-time PCR in patients with inflammatory bowel disease, Inflamm. Bowel Dis. 19 (2013) 2840–2847.
[4] O. Giricz, J.L. Lauer-Fields, G.B. Fields, The normalization of gene expression data in melanoma: investigating the use of glyceraldehyde 3-phosphate dehydrogenase and 18S ribosomal RNA as internal reference genes for quantitative real-time PCR, Anal. Biochem. 380 (2008) 137–139.
[5] S.L. Eaton, S.L. Roche, M. Llavero Hurtado, K.J. Oldknow, C. Farquharson, T.H. Gillingwater, T.M. Wishart, Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting, PLoS ONE 8 (2013) e72457.
[6] I. Romero-Calvo, B. Ocon, P. Martinez-Moya, M.D. Suarez, A. Zarzuelo, O. Martinez-Augustin, F.S. de Medina, Reversible Ponceau staining as a loading control alternative to actin in Western blots, Anal. Biochem. 401 (2010) 318–320.
[7] A. Posch, J. Kohn, K. Oh, M. Hammond, N. Liu, V3 stain-free workflow for a practical, convenient, and reliable total protein loading control in Western Blotting, J. Vis. Exp. 30 (2013), http://dx.doi.org/10.3791/50948.
[8] A.D. Colella, N. Chegenii, M.N. Tea, I.L. Gibbins, K.A. Williams, T.K. Chataway, Comparison of stain-free gels with traditional immunoblot loading control methodology, Anal. Biochem. 430 (2012) 108–110.
[9] J.E. Gilda, A.V. Gomes, Stain-free total protein staining is a superior loading control to beta-actin for Western blots, Anal. Biochem. 440 (2013) 186–188.