Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 345 (2005) 158–160
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Notes & Tips

Comparative advantages of imidazole–sodium dodecyl sulfate–zinc reverse staining in polyacrylamide gels

Amanda S. Gillespie, Edith Elliott *

School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu–Natal, Pietermaritzburg, South Africa

Received 16 February 2005
Available online 29 June 2005

Polyacrylamide gel electrophoresis (PAGE), which is widely used to assess protein purity and molecular weight [1], is best combined with a highly sensitive, reproducible, inexpensive, rapid, and easy to perform protein staining/detection method. Coomassie brilliant blue (CBB)[1] staining is a popular method, known for its simplicity, economy, and compatibility with downstream analysis but relatively poor sensitivity (about 50 ng protein/band), low affinity for acidic proteins, variability in background staining, and lack of reproducibility. The silver staining procedure is sensitive but relatively tedious and is not specific for proteins [2,3]. Ruthenium-complex-based SYPRO Ruby fluorescent staining is protein specific and as sensitive as silver staining. It has a broad linear dynamic range and a simple stain/destain protocol and is compatible with subsequent mass spectrometry or Edman sequencing [4,5]. Imidazole–SDS–zinc reverse staining is reportedly more sensitive than CBB staining [6] and approximately as sensitive as silver staining [2]. In this method a white, insoluble imidazole–zinc complex forms on the surface of gels as a background against which proteins, complexed with SDS during pretreatment, appear as unstained, transparent bands [7]. In the study reported here, a comparison of the imidazole–SDS–zinc, SYPRO Ruby, silver, and CBB staining methods was made. Imidazole–SDS–zinc reverse staining, with minor modifications of the original method [6,8], was shown to best meet all the optimal criteria (as stated above), while allowing sensitive, reproducible detection and sample recovery.

To compare the sensitivity, reproducibility, and simplicity of staining methods, four Tris–glycine SDS–PAGE gels [9] were equally loaded, run under identical conditions, rinsed with distilled water, stained, and photographed using a VersaDoc 4000 Imager (Bio-Rad, California, USA). The gels were loaded with 500–1 ng/protein, from serial twofold dilutions of 1 mg/ml of phosphorylase b, bovine serum albumin (BSA), ovalbumin, and carbonic anhydrase. Additionally, three homogenates were applied, a purified neutrophil homogenate of ~170,000 cells [10], a monocyte homogenate of ~50,000 cells, and whole blood homogenate (~0.25 µl blood) [11].

CBB staining was carried out with some modifications of the method of Fernandez-Patron et al. [6]. Overnight staining [0.125% (m/v) CBB R–250, 50% (v/v) methanol, 10% (v/v) acetic acid] was followed by destaining for several hours in multiple changes of destain [50% (v/v) methanol, 10% (v/v) acetic acid], followed by rehydration [5% (v/v) methanol, 7% (v/v) acetic acid]. Gels were illuminated from below with white light and photographed without a filter.

SYPRO Ruby fluorescent staining was conducted according to the manufacturer's instructions (Molecular Probes, Oregon, USA). Gels were fixed, stained overnight, rinsed, placed on a UV-transilluminator (312 nm), and photographed using a 610-nm longpass emission filter.

Imidazole–SDS–zinc reverse staining was effected by pretreating gels in imidazole–SDS solution [200 mM imidazole, 0.1% (m/v) SDS, 10–15 min] and rinsing briefly (distilled water, 30 s) before developing (200 mM zinc sulfate) until the gel background turned intensely white with transparent protein bands (15–60 s). The extent of development was best monitored during manual agitation of

* Corresponding author. Fax: +27 33 2505462.
E-mail address: elliott@ukzn.ac.za (E. Elliott).

[1] *Abbreviations used:* CBB, coomassie brilliant blue; BSA, bovine serum albumin.

doi:10.1016/j.ab.2005.06.018

gels over a dark surface and was stopped by discarding the developer and rapidly rinsing with running distilled water for 10–15 s (instead of the originally described 3 × 5-s washes [8]). As development of background continues for a few seconds after the developer is discarded, the reaction was best stopped just as the bands of interest became visible. Photography of gels was best achieved using epiwhite light (instead of epifluorescent light [8]) on a dark background. No filter was required but bands in the low-nanogram range (<5 ng) were best photographed using a 610-nm longpass filter.

Silver staining was performed as previously described [12,13]. Gels were fixed [50% (v/v) methanol, 10% (v/v) acetic acid, 2× 15 min or overnight], washed [10% (v/v) ethanol, 5% (v/v) acetic acid, 6 min], rinsed thoroughly in ultrapure water (2 × 9 min), pretreated with sodium dithionite [20 μg/ml, 9 min], impregnated with silver nitrate/formaldehyde [0.1% (m/v) silver nitrate, 0.03% (v/v) formaldehyde, 9 min], and briefly rinsed (ultrapure water, 30 s) before developing in a carbonate solution [3% (m/v) sodium bicarbonate, 0.04% (v/v) formaldehyde, 0.002% (m/v) sodium thiosulfate] until bands were sufficiently stained (usually less than 10 min). Thiosulfate was included in the developing solution as a silver chelator to prevent silver carbonate precipitation. The reaction was stopped using 5% (m/v) Tris containing 2.5% (v/v) acetic acid (20 min). Silver-stained gels were illuminated from below with white light and photographed without a filter.

Typical results (Fig. 1) are arranged in order of overall sensitivity, judged not by the intensity of the bands but by the greatest number of protein bands just visible at the highest dilution (apparent detection limit) (Figs. 1A–D, respectively).

Variability in staining and detection limit of various proteins is apparent (Figs. 1A–D). Silver staining, though apparently giving rise to more intensely stained bands, is known to suffer from interprotein staining variability (generally attributed to variable numbers of Cys residues). This is evident as BSA (66 kDa), with 35 Cys residues, is detectable down to at least 4 ng, while ovalbumin (45 kDa), with 5 Cys residues, is visible at 8 ng, and carbonic anhydrase (31 kDa), with no Cys residues, is visible down to at least 31 ng (Fig. 1C, lanes 8, 7, and 5, respectively). CBB staining also shows variability in staining, with BSA detectable down to 8 ng, ovalbumin to 16 ng, and carbonic anhydrase to 125 ng (Fig. 1D, lanes 7, 6 and 3, respectively). This suggests that CBB staining may be more sensitive than previously reported [6] but is not as sensitive as silver staining (Fig. 1D vs C).

SYPRO Ruby (Fig. 1B) and imidazole–SDS–zinc staining (Fig. 1A) also show variability but this is much less marked than that in silver stained– (Fig. 1C, lanes 6–9) and CBB–stained (Fig. 1D, lanes 3–7) gels. Variation in the SYPRO Ruby- and imidazole–SDS–zinc-stained gels was seen only in the 1- to 8-ng range (Figs. 1B and A, lanes 7–10). In contrast to previously published observations [14], in this study SYPRO Ruby staining appears slightly more sensitive than silver staining (Figs. 1B and C, lanes 6–9, respectively). Imidazole–SDS–zinc reverse staining appears as sensitive as SYPRO Ruby and more sensitive than both silver stain and CBB for overall detection of most proteins (Figs. 1A–D). The limited solubility of phosphorylase b (97.4 kDa) restricted its use in the determination of maximum detection limits with respect to the other proteins in this study but nevertheless allowed comparison of different staining methods,

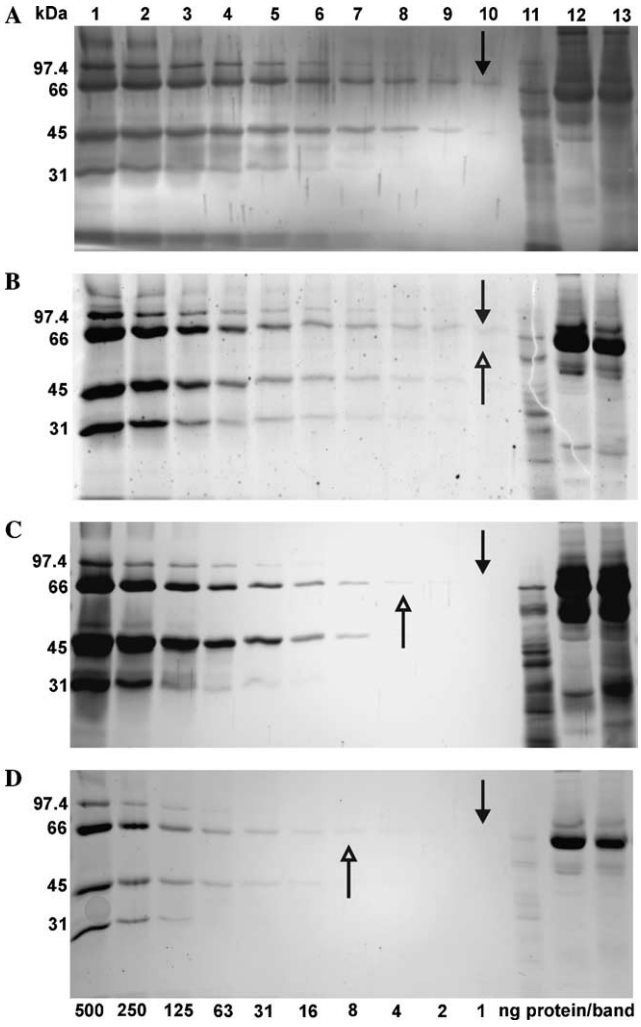

Fig. 1. Evaluation of the sensitivity of imidazole–SDS–zinc reverse staining (A) compared to SYPRO Ruby (B), silver stain (C), and CBB (D). The 12.5% polyacrylamide gels (1.5 mm thick) were equally loaded to give a final loading concentration of 500–1 ng/protein, with serial twofold dilutions of a single protein mixture containing 1 mg/ml phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa), (lanes 1–10). Lanes 11, 12, and 13 contained a neutrophil homogenate (~170,000 cells), a monocyte homogenate (~50,000 cells), and whole blood homogenate (0.25 μl), respectively. The solid arrow (A) indicates the lowest dilution of BSA detected by imidazole–SDS–zinc staining and is included in B–D for easy comparison. Open arrows indicate the lowest dilution of BSA detected by each other stain [SYPRO Ruby (B), silver stain (C), and CBB (D), respectively].

confirming the better or equal sensitivity of imidazole–SDS–zinc reverse staining compared with these of SYPRO Ruby, silver stain, and CBB. The homogenate samples of neutrophils, monocytes, and whole blood (Figs. 1A–D, lanes 11–13) appear to confirm these observations. Bands appear to be slightly smeared in the imidazole–SDS–zinc-stained gels, while the SYPRO Ruby- and silver-stained gels exhibit clear, sharp bands (Figs. 1B and C, lanes 11–13). Very few bands are visible in the CBB–stained gel (Fig. 1D, lanes 11–13). The smeared appearance may be a result of the increased sensitivity of the imidazole–SDS–zinc procedure and could represent faint background bands which may have eventually become visible with the silver stain procedure had the silver development been allowed to continue longer.

In summary, CBB, SYPRO Ruby, and imidazole–SDS–zinc staining are the simplest to perform, while silver staining is comparatively tedious. CBB and SYPRO Ruby staining, however, require overnight staining for maximal signal strength, while silver staining takes less than 2 h, and imidazole–SDS–zinc staining may be completed in less than 20 min for most one dimensional minigels. SYPRO Ruby and imidazole–SDS–zinc staining appear to be more sensitive than CBB and silver stains and manifest the least protein staining variability. Imidazole–SDS–zinc staining, unlike SYPRO Ruby and CBB, is rapid and, because no fixative is used, also completely reversible [7], making it compatible not only with downstream analysis by mass spectrometry and Edman sequencing but also with immunoelution/Western blotting techniques. Using the criteria of sensitivity, simplicity, rapidity, cost efficiency, interprotein variability, and compatibility with downstream analysis, imidazole–SDS–zinc reverse staining seems to have a number of advantages.

Acknowledgments

This study was supported by NRF Grant 2054195 and an NRF Prestigious Scholarship awarded to Amanda S. Gillespie. The authors are grateful to Clive Dennison for critical appraisal of the manuscript.

References

[1] Q. Shi, G. Jackowski, One-dimensional polyacrylamide gel electrophoresis, in: B.D. Hames (Ed.), Gel Electrophoresis of Proteins: A Practical Approach, Oxford University Press, Oxford, 1998, pp. 1–52.
[2] E. Hardy, L.R. Castellanos-Serra, "Reverse-staining" of biomolecules in electrophoresis gels: analytical and micropreparative applications, Anal. Biochem. 328 (2004) 1–13.
[3] W.F. Patton, Detection technologies in proteome analysis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 771 (2002) 3–31.
[4] T.H. Steinberg, R.P. Haugland, V.L. Singer, Applications of SYPRO orange and SYPRO red protein gel stains, Anal. Biochem. 239 (1996) 238–245.
[5] M.F. Lopez, K. Berggren, E. Chernokalskaya, A. Lazarev, M. Robinson, W.F. Patton, A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling, Electrophoresis 21 (2000) 3673–3683.
[6] C. Fernandez-Patron, E. Hardy, A. Sosa, J. Seoane, L. Castellanos, Double staining of coomassie blue-stained polyacrylamide gels by imidazole–sodium dodecyl sulfate–zinc reverse staining: Sensitive detection of coomassie blue-undetected proteins, Anal. Biochem. 224 (1995) 263–269.
[7] C. Fernandez-Patron, M. Calero, P.R. Collazo, J.R. Garcia, J. Madrazo, A. Musacchio, F. Soriano, R. Estrada, R. Frank, L.R. Castellanos-Serra, E. Mendez, Protein reverse staining: high-efficiency microanalysis of unmodified proteins detected on electrophoresis gels, Anal. Biochem. 224 (1995) 203–211.
[8] C. Fernandez-Patron, L. Castellanos-Serra, P. Rodriguez, Reverse staining of sodium dodecyl sulfate polyacrylamide gels by imidazole-zinc salts: sensitive detection of unmodified proteins, Biotechniques 12 (1992) 564–573.
[9] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[10] D.G. Wright, Human neutrophil degranulation, Methods Enzymol. 162 (1988) 538–551.
[11] G.S. Makowski, M.L. Ramsby, Calibrating gelatin zymograms with human gelatinase standards, Anal. Biochem. 236 (1996) 353.
[12] T. Rabilloud, Detecting proteins separated by 2-D gel electrophoresis, Anal. Chem. 72 (2000) A48–A55.
[13] J.H. Morrissey, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity, Anal. Biochem. 17 (1981) 307–310.
[14] K. Berger, D. Wissmann, C. Ihling, S. Kalkhof, A. Beck-Sickinger, A. Sinz, R. Paschke, D. Fuhrer, Quantitative proteome analysis in benign thyroid nodular disease using the fluorescent ruthenium II tris(bathophenanthroline disulfonate) stain, Mol. Cell. Endocrinol. 227 (2004) 21–30.