

Research Article

Significance of Using SYPRO Ruby against CBB R-250 for Visualizing Haematoxylin Stained Proteins in Gels

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Abstract

Laser Capture Micro-dissection (LCM) is a technique that is used to isolate specific tumor cells from a heterogeneous tumor tissue sample. To aid in identifying and dissecting pure tumor cells from other parts of the tissues such as the stroma, tissues are stained with Haematoxylin and Eosin. The cells are then used for protein, RNA or DNA extraction. However, the effect of Haematoxylin and Eosin or other different stains routinely used in the laboratories on the recovery, quantity and quality of proteins especially for down stream application such as 2-dimensional gel electrophoresis (2-DE) and MS not known. This study, determined the effect of Haematoxylin staining on the detection methods used in 1-D SDS-PAGE for protein quantification. A series of concentration of proteins were obtained from human pancreatic whole tissue and was run on a SDS-PAGE parallel with the proteins obtained from Haematoxylin stained and unstained tissues. The protein band intensities were measured with a densitometer after separately stained with SYPRO Ruby or CBB R-250. The protein band intensity ratios of the whole tissue and Haematoxylin stain/ Haematoxylin unstained tissue were calculated. According to the ratios, there was an intensity loss in the Haematoxylin stained proteins when detecting through CBB R-250 but not from SYPRO Ruby. This was due to the structure and reactivity of these two stains towards proteins in the presence of Haematoxylin. The study recommends the use of SYPRO Ruby instead of CBB R-250 to visualize proteins in 2-DE gels when tissues were stained with Haematoxylin.

Keywords: CBB R-250; Haematoxylin; Intensity; Laser Capture Micro Dissection; Protein; Staining; Syproruby; Tissue

Introduction

Tissue heterogeneity is the major problem in the analysis of cancer tissues and presents a main difficulty in identifying specific cancer biomarkers. Many techniques have been developed to overcome this problem and enrich for the cells of interest. One of these techniques is Laser Capture Microdissection (LCM) [1,2]. The technique consists of 3 steps: (I) preparation of tissue section, (II) tissue sections staining with a suitable stain, and (III) manual or automated dissection using system such as ArcturusXT (Molecular device, CA) and Leica (Leica, Milton Keynes, UK).

In cancer research, LCM in conjunction with many other techniques such as genomic and proteomic has been used to determine the gene or protein expression profiles of tumor cells without the effect of normal or surrounding stroma cells. In the

field of proteomic, it has been coupled with reverse phase array, antibody array surface enhanced laser desorption/ionization-time of flight mass spectrometry, and two-dimensional gel electrophoresis (2D-PAGE). 2D-PAGE allows hundreds to thousands of proteins to be analyzed simultaneously. These proteins can be isolated in pure form from the resultant spots which later can be quantified and further analyzed by mass spectrometry.

Although LCM in combination with 2D-PAGE analysis of cancer tissue is promising technology especially in cancer biomarkers research, there are certain limitations to this technology. Firstly, collecting the adequate number of cells to obtain enough protein for 2D-PAGE analysis is time-consuming. Secondly, there is a limited sensitivity of different stains to visualize the protein spots in the gel. From our experience, we found that even though enough cells were collected and there was enough protein for 2D-PAGE analysis; the number of spots in the gel was less than that obtained with pure E. coli proteins when using Coomassie blue

stain (the commonly used stain in laboratory due to its affordable cost compared to other stain). However, these differences were not seen with SYPRO® Ruby stain. Although, SYPRO Ruby is a very sensitive fluorescent stain which has many advantages over other stains [3,4] and is relatively expensive for daily laboratory use [3]. These advantages could not exclusively explain the huge differences seen between the two stains. Thus, in this study we have examined the effect of Haematoxylin (the commonly used histological stain for tissue preparation for LCM) on second stains such as Coomassie blue and SYPRO Ruby, which are used to visualize protein spots in 2D-PAGE.

Materials and Methods

Study Design

In this study, we have used pancreatic cancer tissue to examine the effect of Haematoxylin stain on other stains as mentioned above. Also, 1D-PAGE was used instead of 2D-PAGE analysis, due to the ease of determining the bands intensities. Tissues from pancreatic cancer without sectioning or staining were used as control and compared to tissues sections that were stained with Haematoxylin or unstained.

Tissue and Protein Preparation

(a) preparation from whole pancreatic tissues

Pancreatic cancer tissues were homogenized using lysis buffer (7M urea, 2M thiourea, 1% MEGA-10, 1% Octyl- β -D-glucopyranoside, 40mM Tris, 50mM DTT, 2mM tributylphosphine). The homogenate was centrifuged (1000rpm for 5 min); and protein concentration in the supernatant was determined using Amido black protein assay (sample # 1).

(b) Protein preparation from stained and unstained tissue section

Sections (8 μ M thick) were prepared from pancreatic tissues using a cryostat and mounted in a clean glass slides. Slides were stained with hematoxylin (Sample # 2) then scraped or scraped without staining (Sample # 3) in Eppendorf tubes for protein preparation. The tissue sections for sample #2 were stained with Haematoxylin before scraping as follow: 100% acetone- (15s), Haematoxylin(15s), protease inhibitor containing water (15s), 75% ethanol (15s), 95% ethanol (15s), absolute alcohol(15s), Xylene(15s). All solutions were prepared in water containing protease inhibitor. Both scraped stained and unstained tissue sections were mixed in lysing buffer, homogenized and vortexed at 37°C for 30min. The protein concentrations in the supernatants were determined with Amido black assay.

Gel Electrophoresis

The proteins extracted from whole, not sectioned or stained tissues (sample # 1); sectioned and stained with hematoxylin tissues (sample # 2); or section but not stained tissues (sample # 3) were separated into a 10% SDS-PAGE. Twenty micrograms of the Bovine Serum Albumin (Pierce Chemical Company, Rockford, IL, USA) were used as the standard marker. The gels were run at 200V and stained with CBB R-250 for 2hrs and then de-stained for 4-6 hrs or were stained with SYPRO® Ruby. They were visualized through Kodak-Digital Science Image Station 440-CF (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY, USA) and the intensities of some of the clear bands were measured (by selecting the band area). A graph was drawn by using the concentrations of whole tissue extracted protein series as the x-axis and the bands intensities as the y-axis. The bands' intensities obtained from Haematoxylin stained and unstained gels were plotted in the graph for the comparison [5].

Results

Effect of Hematoxylin in Protein Detection Using CBB R-250

The band's intensities of the Haematoxylin stained tissue and the unstained tissue sections with respect to the whole tissue standard series are shown in Figure 1. The proteins were stained with CBB R-250. Thirty micrograms of Haematoxylin stained tissue gave lower intensity than the 30 μ g of the whole tissue. The calibration curve for the intensity of the bands of the protein from whole tissue vs. concentration series of the proteins from whole tissue is shown in Figure 2. The intensities of the 30 μ g of proteins obtained from Haematoxylin stained and unstained tissues (10 μ g) were compared with the calibration curve. Proteins from the Haematoxylin stained tissue (30 μ g) gave a lower intensity compared to the unstained and whole tissues.

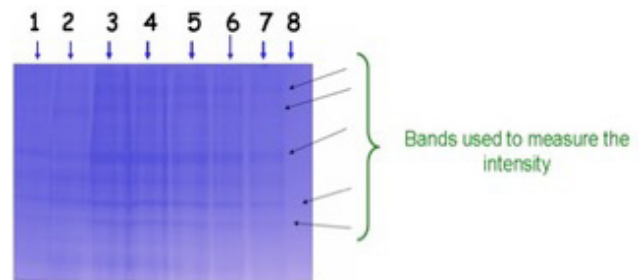


Figure 1: 1-DE SDS-PAGE of proteins extracted by different protocols using CBB R-250 as the protein staining. Lane 1: 20 μ g from whole tissue, lane 2: 30 μ g from whole tissue, lane 3: 60 μ g from whole tissue, lane 4: 20 μ g from Haematoxylin stained tissue slide sections, lane 5: 20 μ g from unstained tissue slide sections: lane 6: 20 μ g of standard BSA.

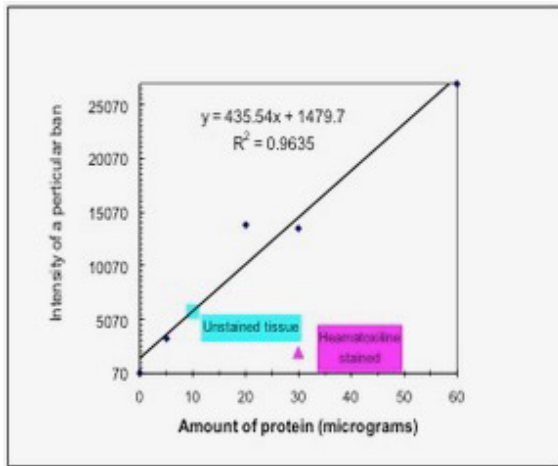


Figure 2: Comparison of the band intensities of proteins from whole tissue, stained and unstained Haematoxylin tissue sections using CBB R-250. 1-DE SDS-PAGE of proteins extracted by different protocols using CBB R-250 as the protein staining. Lane 1: 10 µg of protein from unstained tissue sections, lane 2: 30 µg of protein from haematoxyline stained tissue sections, lane 3: 60 µg of protein from whole tissue, lane 4: 30 µg of protein from whole tissue, lane 5: 20 µg of protein from whole tissue, lane 6: 10 µg of protein from whole tissue, lane 7: 5 µg of protein from whole tissue, lane 8: 0 µg protein.

Effect of Haematoxylin in Protein Detection Using SYPRO Ruby

Figure 3 shows a comparison between the intensities of the proteins from Haematoxylin stained tissues (lane 7) and the proteins from unstained tissues (lane 8) with respect to proteins from whole tissue standard series (lane 1-6). There was no significant difference between the band's intensities of the lanes 5, 7 and 8 (each lane was loaded with 30 µg of protein). The calibration curve of the intensity of the protein bands vs. concentrations of the whole homogenized tissue when stained with Syproruby is shown in Figure 4. The intensities of the proteins (30 µg) from the homogenized Haematoxylin stained and the homogenized unstained tissues were compared with the calibration curve of the homogenized whole tissue concentration series. There was no significant difference between the intensities of the whole tissue homogenized proteins, homogenized Haematoxylin stained tissue and the homogenized unstained tissue sections [6].

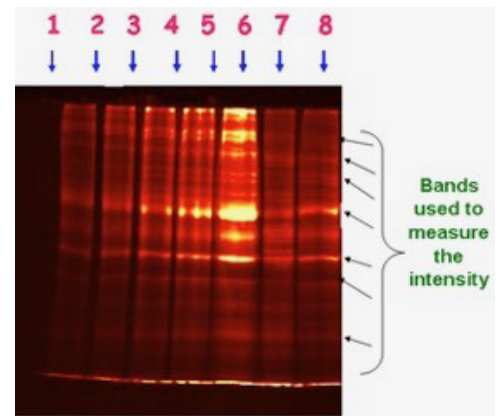


Figure 3: Calibration curve of the intensity of a particular band for the homogenized whole tissue series vs. the loaded whole tissue proteins to the gel. ■-intensity for the 10 µg unstained tissue, ▲- intensity for the 30 µg Haematoxylin stained tissue, (stained with CBB R -250.).

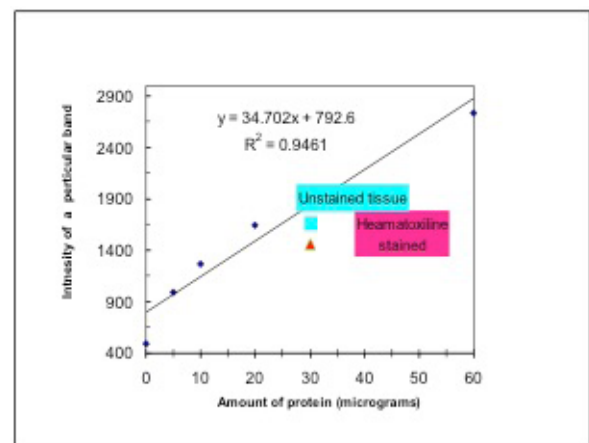


Figure 4: Comparison of the band intensities of proteins from whole tissue, stained and unstained Haematoxylin tissue sections using syproruby. 1-DE of the proteins obtained from homogenized tissue, Haematoxylin stained tissue and unstained tissue sections using syproruby as the staining method. Lane 1: 0 µg of protein, lane 2: 5 µg of protein from whole tissue, lane 3: 10 µg of protein from whole tissue, lane 4: 20 µg of protein from whole tissue, lane 5: 30 µg of protein from whole tissue, lane 6: 60 µg of protein from whole tissue, lane 7: 30 µg of protein from Haematoxylin stained tissue sections, lane 8: 10 µg of protein from unstained tissue sections

Table 1 gives the mean value of the ratios between the band's intensities taken from the two gels (CBB R-250 and Sypro ruby). This value was calculated to determine the effect of Haematoxylin on the band intensities of the proteins with respect to CBB R-250 and syproruby. The mean ratio between the intensities of the protein bands obtained from the whole tissue and the Haematoxylin stained tissue was close to 4. The ratio between the homogenized unstained and the homogenized Haematoxylin tissue was 2.55. But when syproruby was used, the mean ratio for both was around 1. The intensity ratio between the unstained/whole tissue with respect to the CBB R-250 and syproruby was around 1.

Intensity ratio of the protein bands	CBB R-250	Syproruby
Whole tissue/Haematoxlin stained tissue sections	3.81 ± 1.38	1.17 ± 0.25
Unstained tissue sections/ Haematoxlin stained tissue sections	2.55 ± 0.59	0.99 ± 0.14
Whole tissue /unstained tissue sections	1.49 ± 0.50	1.19 ± 0.26

Table 1: Intensity ratio of the protein bands using CBB R -250 and syproruby for different tissue sections.

Discussion

The data from this study showed that Haematoxylin stain affects proteins when it was visualized in gel using CBB R-250 stain. However, this effect was not seen with SYPRO Ruby. We hypothesized that the observed effect of Haematoxylin may be due to structure and reactivity of these stains towards [7,6].

Pure Haematoxylin is a colorless solution [8] and it is activated to Haematein by a mordant. The Haematoxylin-mordant complexes are positively charged and behave as a cationic dye at low pH. The most common mordants are aluminum, iron or tungsten. The cationic stain-mordant complexes are attracted to negatively charged sites in the staining process [9]. Under suitable conditions, the complexes will stain cationic proteins such as histones associated with nucleic acids [10-13] and nucleoproteins that carry lysine and arginine residues [12,14-16].

Anions of CBB formed in acidic conditions combine with the protonated amino groups of the proteins by electrostatic attraction (arginine, lysine, tyrosine, and histidine residues) [17,18]. Sypro ruby dye interacts strongly with lysine, arginine, and histidine residues. It closely resembles CBB staining through basic amino acids side chains [19]. Although the binding of sypro ruby and CBB are the same, the sypro ruby has a significant sensitivity towards protein staining against the CBB because of its structure. Syproruby has a ruthenium atom. This can react with amino acids such as cysteine through ligand exchange. This atom is also able

to form η⁶-complexes with derivatives of tryptophan amino acids (Figure 1)[18].

When staining, CBB R-250 binds through arginine, lysine, tyrosine, and histidine residues in proteins [17,18]. Meanwhile, sypro ruby binds through the same amino acids [19]. When staining with Haematoxylin, it binds to nucleoproteins with lysine, arginine residues or other parts having suitably spaced carboxyl and hydroxyl groups [11,16]. Haematoxylin masked these binding sites; hence, the results presented in this paper show that the band's intensities for the Haematoxylin stained homogenized tissue and the unstained homogenized tissue are not similar (Figures 2&3). According to Table 1, the ratio between the whole tissue homogenized proteins and the homogenized Haematoxylin stained tissue band's intensities was around 4. The ratio between the protein band's intensities of the homogenized unstained tissue sections and the homogenized Haematoxylin stained tissue sections was 2.55. When detecting with CBB R-250, the Haematoxylin stained tissue had a low intensity compared to the whole homogenized tissue and the homogenized unstained tissue. However, syproruby had no effect on the Haematoxylin stain tissue. The ratio between the intensities of the protein bands of the homogenized whole tissue and the homogenized Haematoxylin stained tissue was close to 1. The ratio between the homogenized unstained tissue sections and the Haematoxylin stain tissue was also close to 1 (Table 1). This may be due to the special type of interaction between the syproruby and the amino acids through metal-ligand interaction. These interactions did not interfere with the Haematoxylin staining. Thus, the effect of Haematoxylin on syproruby detection was much lower than the CBB detection. Finally, it should be emphasized that when the tissues were stained with Haematoxylin before running the gels, it was better to use sypro ruby to visualize the proteins instead of CBB because it allowed the visualization of more proteins and trace amounts of proteins in 1-D.

The work from Craven et al. and De Souza et al. showed that there was no effect on the protein recovery when stained with Haematoxylin & Eosin. Nevertheless, there was an effect on the proteins' quality [1,20]. This finding contradicts our results and may be due to several reasons. In our work we focused on the proteins' intensity instead of the proteins' recovery. Craven et al. and De Souza et al. also used silver staining instead of sypro ruby to detect the proteins and the stain differentiation was longer (Craven et al. - 70% ethanol for 30s, 100% ethanol for 1min, xylene for 2 X 5 min), (De Souza- 70% ethanol for 30s, 100% ethanol for 1min, xylene for 5min). In our protocol, we used a shorter stain differentiation time (75% ethanol 15s, 95% ethanol 15s, absolute alcohol 15s, xylene 15s). It is better to use a short differentiation time in LCM for the visualization of proteins.

Mouledous et al. work indicates that the fixing of the tissue had no effect on the recovery of protein. However, syproruby

affects Haematoxylin and eosin. This contradicts our results, which indicates that Syproruby does not influence the intensity of the proteins stained with Hematoxylin. Although both works concluded that there was no effect on the fixation, they had different fixation procedures [21].

Ahram et al obtained results like ours showing that Haematoxylin did not influence the qualitative and quantitative determination of proteins. Eosin however had an effect on the proteins' separation. The detection was done by aminoacyl silver staining [22]. Our work is the first work looking at the effect of Haematoxylin on the detection of syproruby & CBB R-250.

In the future, it would be appropriate to test the effect of other types of tissue staining methods like Haematoxylin, eosin, methylene blue, Wright-Giemsa and toluidine blue on the detection methods of syproruby and CBB R-250. Because syproruby is expensive it using other types of staining methods instead of Haematoxylin such as CBB R-250 may give better results. This will be more economically advantage. Furthermore, it is important to assess the effect of these stains on the recovery and quantity of proteins.

Conclusion

Tumor tissues staining for LCM with Haematoxylin resulted in the proteins' bands intensity drop when later visualized through CBB R-250 in gels. However, there was no intensity drop in the proteins' bands when visualized through SYPRO Ruby. SYPRO Ruby should therefore be used in conjunction with Haematoxylin. This is more important when using LCM because the amount of proteins that could be visualized in 1-D and 2-D gel will be higher, especially in the presence of trace amounts of proteins in the sample. Therefore, the study recommends assessing the effect of tissue stains on the recovery and quantity of proteins for downstream applications such as protein extraction for 2-D analysis.

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References

1. Craven RA1, Totty N, Harnden P, Selby PJ, Banks RE (2002) Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: evaluation of tissue preparation and sample limitations. *Am J Pathol* 160: 815-822.
2. Espina V, Wulffkuhle JD, Calvert VS, VanMeter A, Zhou W, et al. (2006) Laser-capture microdissection. *Nat Protoc* 1: 586-603.
3. Berggren K1, Chernokalskaya E, Steinberg TH, Kemper C, Lopez MF, et al. (2000) Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex. *Electrophoresis* 21: 2509-2021.
4. Lopez MF, Berggren K, Chernokalskaya E, Lazarev A, Robinson M, et al. (2000) Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* 21: 3673-3683.
5. Wirth PJ1, Romano A (1995) Staining methods in gel electrophoresis, including the use of multiple detection methods. *J Chromatogr A* 698: 123-143.
6. Bettinger C1, Zimmermann HW (1991) New investigations on hematoxylin, hematein, and hematein-aluminium complexes. II. Hematein-aluminium complexes and hemalum staining. *Histochemistry* 96:215-228.
7. Arshid FM, Connelly RF, Desai JN, Fulton RG, Giles CH, et al. (1954) A Study of Certain Natural Dyes II-The Structure of the Metallic Lakes of the Brazilwood and Logwood Colouring Matters. *Journal of the Society of Dyers and Colourists* 70: 402-412.
8. Palmer RW, Lillie RD (1965) Spectroscopic and staining studies of the ripening and overripening of aluminum hematoxylin. *Histochemie* 5: 44-54.
9. Marshall PN, Horobin RW (1973) Measurements of the affinities of basic and "mordant" dyes for various tissue substrates. *Histochemie* 36: 303-312.
10. Baker JR (1962) Experiments on the action of mordants. 2. Aluminium-hematein. *Q. J Microsc Sci* 103: 493-517.
11. Puchtler H, Meloan SN, Waldrop FS (1986) Application of current chemical concepts to metal-hematein and -brazilein stains. *Histochemistry* 85: 353-364.
12. Hrapchak BB (1976) Selective staining with hematoxylin, applications and theory: a review. *Am J Med Technol* 42: 371-379.
13. Prentø P, Schulte E (1991) Staining Involving Metal Complex Dyes. In: Lyon H. (eds) *Theory and Strategy in Histochemistry*. Springer, Berlin, Heidelberg.
14. Baker JR (1960) Experiments on the action of mordants. 1. 'Single-bath' mordant dyeing. *Q. J. Microsc. Sci* 101: 255-272.
15. Scott JE, Willett IH (1966) Binding of cationic dyes to nucleic acids and their biological polyanions. *Nature* 209: 985-987.
16. Puchtler H, Sweat F (1964) On the mechanism of sequence iron-hematein stains. *Histochemie* 4: 197-208.
17. Srový I1, Hodný Z (1991) Staining and quantification of proteins separated by polyacrylamide gel electrophoresis. *J Chromatogr* 569: 175-196.
18. Ball MS, Karuso P (2009) Mass spectral compatibility of four proteomics stains. *J Proteome Res* 6: 4313-4120.
19. Steinberg TH, Chernokalskaya E, Berggren K, Lopez MF, Diwu Z, et al. (2000) Ultrasensitive fluorescence protein detection in isoelectric focusing gels using a ruthenium metal chelate stain. *Electrophoresis* 21: 486-496.
20. De Souza AI, McGregor E, Dunn MJ, Rose ML (2004) Preparation of human heart for laser microdissection and proteomics. *Proteomics* 4: 578-586.
21. Mouledous L, Hunt S, Harcourt R, Harry JL, Williams KL, et al. (2002) Lack of compatibility of histological staining methods with proteomic analysis of laser-capture microdissected brain samples. *J Biomol Tech* 13: 258-264.
22. Ahram M, Flaig MJ, Gillespie JW, Duray PH, Linehan WM, et al. (2003) Evaluation of ethanol-fixed, paraffin-embedded tissues for proteomic applications. *Proteomics* 3: 413-421.