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A novel strategy for quantitative isoform detection directly performed from culture supernatant

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Abstract

Currently, one of the most used techniques for the determination of isoform pattern analysis is isoelectric focusing. Routinely, this is performed by immunoblotting. Blotting of proteins after isoelectric focusing on IPG gels may cause several problems, such as protein loss by the blotting itself and band broadening, in some cases the immunostaining with antibodies might be problematic. In the present study, an alternative isoform prestaining method with CyDye fluors is presented. For this approach, a highly glycosylated fusion protein, Epo-Fc, was used consisting of two recombinant human erythropoietin attached to the Fc part of a human IgG_1 molecule. By using CyDye fluors, up to three samples can be focused on the same lane under identical electrophoretic conditions. A fundamental benefit of this technique is the ability to perform quantitative isoform pattern analysis directly from serum-free culture supernatant.

Keywords: Fusion protein Epo-Fc; CHO cells; Isoelectric focusing; Isoform pattern; CyDye fluors

1. Introduction

Routinely, visualization of isoform pattern from culture supernatant and purified protein were performed on IPG gels by immunoblotting [1,2]. However, some drawbacks are inherent to these techniques. Besides the technical deficiencies, such as protein loss by the blotting itself and band broadening, in some cases the immunostaining with antibodies might be problematic. Even though specific antibodies are available, greater or lesser extent of background staining and insufficient linearity prevent quantification. Due to these drawbacks, immunoblotting is a time-consuming qualitative method. However, with the development of new imaging systems, protein detection and quantitation methods based on fluorescent staining and/or labeling provide a promising alternative. Depending on the chemical and physical nature of the fluorophore, they have a comparatively wide linear dynamic range (>10^3) [3]. CyDye fluors, mainly used for 2D difference gel electrophoresis (2D DIGE), allow more accurate and sensitive quantitative protein studies. Currently, three different CyDye fluors (Cy 5, Cy 3 and Cy 2) are available from GE Healthcare (Sweden). The fluorophores are structurally similar and undergo nucleophilic substitutions with the ε-amino group of lysine residues forming an amide. The fluorescent dyes have very similar molecular masses and are positively charged to match the charge that is replaced on the lysine residue. The charge and mass matching ensure that all samples essentially co-migrate to the same point during electrophoresis. In the labeling reaction, the dye:protein ratio is low. This ensures that protein molecules are only labeled with a single dye molecule [4]. In fact, only 1–2% of lysine residues in the proteins are fluorescently modified, so that the solubility of the labeled proteins is maintained during electrophoresis [5]. Due to the availability of three different CyDye fluors with similar attributes, up to three different samples can be analyzed on the same lane. Thus, by

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this technique spiking experiments with reference standards or other samples can be performed simultaneously.

To evaluate the suitability of this prestaining technique for one-dimensional IEF, all experiments were performed with our fusion protein Epo-Fc, which consists of two recombinant human erythropoietin (rHuEpo) attached to the Fc part of a human IgG_1 molecule. Epo-Fc comprises of 798 amino acids and has a molecular weight of about 112 kDa, of which about 89 kDa is contributed by the polypeptide chain itself. For the characterization of posttranslational modifications, respectively, the analysis of isoforms, the sialylated glycan structures presented by the Epo molecules are primarily responsible. HuEpo is a 30.4 kDa glycoprotein hormone produced by the kidney in adult humans [6,7]. It consists of a 165 amino acid single polypeptide chain containing two disulphide bonds [8,9] and has three N-linked (Asn-24, Asn-38, Asn-83) and one O-linked (Ser-126) sugar chain [10,11]. Its microheterogeneity is related to the charged carbohydrate moiety of the protein and is studied extensively [12,13]. The microheterogeneity of HuEpo is seen on the N-linked carbohydrate chains, where the oligosaccharide may contain bi-, tri- and tetra-antennae, each of which is typically terminated with the negatively charged sialic acid molecule. With the exception of sialic acid, all sugar moieties are neutral. As a consequence of the variability of sugar structures, the number of sialic acids molecules varies and give rise to HuEpo isoforms with differences in charge. Therefore, the electrophoretic mobility and the isoelectric point (p*I*) or pattern are strongly influenced by the degree of sialylation of polysaccharide chains. Due to this, microheterogeneity not only determines the biochemical properties of this molecule, but also its biological activity, respectively, half life time. For instance, the amount of terminal sialylated tetraantennary structures prevents rapid clearance of HuEpo by the liver in vivo [14,15]. Furthermore, the molecule itself is stabilized by the carbohydrate structure, whereas the non-glycosylated form is less stable than all other asialic or sialic variants [16]. Therefore, analysis of the isoform pattern is an indispensable part in quality control. As described above, our fusion protein consists of two rHuEpo attached to the Fc part of a human IgG_1 molecule. Thus, the microheterogeneity of the fusion protein Epo-Fc is manifested in a complex isoform pattern.

The aim of this study was to evaluate the potency of this method using our complex model protein Epo-Fc. Optimization for optimal protein amount, reproducibility and sample preparation was performed. Thereafter, the method was evaluated for serum-free culture supernatants. Therefore, evaluation was accomplished with three different purified protein samples and their corresponding culture supernatant followed by simultaneous analysis of an in-house standard and selected samples.

2. Materials and methods

2.1. Materials

All reagents used were electrophoresis grade. CyDye DIGE Fluors (minimal dyes) for Ettan DIGE, dimethylformamide (DMF), 2D Clean-Up Kit, Pharmalyte (pH 3–10), dithiothreitol (DTT), GelBond-PAG film and Protein A Sepharose FF were purchased from GE Healthcare (Sweden). Proto Gel Ultrapure was purchased from National Diagnostics (USA). Tetramethylethylenediamine (TEMED), acrylamido buffer solution "Immobiline" p*K* 3.6, 4.6, 6.2, 7.0, 8.5 and 9.3, lysine, Tris, glycine, bromophenole blue, ammoniumperoxodisulfate (APS) and 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS) were from Fluka (Switzerland). Glycerol and urea were from Merck (Germany).

2.2. Cell line, culture medium and cultivation

Dihydrofolate reductase-deficient Chinese Hamster Ovary cells (DUKX-B11, ATCC CRL-9096) were co-transfected with genes for fusion protein Epo-Fc and dihydrofolate reductase (DHFR). Transfected cells were selected for growth in the presence of 0.096 μM MTX. After revitalization from a research cell bank, cells were cultivated in suspension in Dulbecco's Modified Eagle's Medium DMEM/HAM'S F-12 (1:1 mixture), supplemented with 0.58 g/l L-glutamine, an in-house developed protein-free supplement (proprietary formulation), 0.25% soy peptone, 0.1% Pluronic F68 and 0.096 μM MTX. Within 2 weeks the CHO cells were adapted in spinner flasks (TECHNE, UK) to an in-house developed production medium (Polymun Scientific GmbH, Austria) of proprietary formulation. Repeated batch cultivation at different pH and temperature levels were performed in a Sixfors multireactor system (Infors, Switzerland).

2.3. Purification of Epo-Fc

The secreted Epo-Fc from CHO cells was purified by immunoaffinity on Protein A Sepharose FF (GE Healthcare, Sweden). Cell-free culture supernatant samples containing Epo-Fc were adjusted to pH 8.5 with Tris (pH 9.0; 1 M) and loaded onto a 314 μl HR 5 column at a flow-rate of 0.2 ml/min (60 cm/h). After extensive washing of the column with Tris (0.025 M)–NaCl (0.15 M) (pH^* 8.5), Epo-Fc was eluted with glycine–HCl (pH 3.5; 0.1 M) with a flow-rate adjusted to 0.1 ml/min (30 cm/h). The eluted fraction was rapidly neutralized with Tris (pH 9.0; 1 M). After elution, the column was regenerated with glycine–HCl (pH 2.5; 0.1 M).

2.4. Isoelectric focusing of Epo-Fc

2.4.1. Sample preparation and labeling

During IEF the salt concentration should always be kept below 0.05 M. For that purpose, purified and non-purified Epo-Fc samples were treated with 2D Clean Up Kit according to the kit instructions and resuspended in Tris (0.03 M), urea (3 M), CHAPS (4% (w/v)). Thereafter, 4 pmol/8 μl of Epo-Fc sample was minimally labeled with 1 μl of Cy 5 and Cy 3 (3.5 pmol/μl) on ice for 30 min in the dark. The labeling reaction was quenched by the addition of 1 μl lysine (0.01 M) and subsequently incubated on ice for 10 min in the dark. Following the labeling reaction, the Cy 5 labeled sample was mixed with the Cy 3 labeled sample and adjusted with CHAPS (4% (w/v)) to the

appropriate volume (40 μl), or 20 μl if just one sample (Cy 5 or Cy 3) was focused.

2.4.2. Gel preparation

Preparation of the linear IPG gel (pH 4–8) was performed as previously described [17,18]. The IPG gel was rehydrated for 2 h in a solution containing urea (6 M), DTT (0.01 M), CHAPS (2% (w/w)), bromophenole blue 0.1% (0.4% (w/w)) and Pharmalyte 3–10 (2% (w/w)). After rehydration, the linear IPG gel was scratched into 2 cm wide strips to prevent sample interfering during focusing.

2.4.3. Isoelectric focusing

IEF of labeled samples was performed on a Multiphor II flat bed electrophoresis unit (GE Healthcare, Sweden) [17] with the following settings: 15 °C, 2.5 mA, 5 W, 0.075 kVh (0.75 h, 100 V, step-n-hold), 0.375 kVh (0.75 h, 500 V, step-n-hold), 0.500 kVh (0.5 h, 1000 V, step-n-hold) and 72 kVh (18 h, 4000 V, step-n-hold). After focusing, the gel was washed two times with water for 5 min each in order to remove paraffin. Thereafter, the gel was immediately scanned using the Typhoon 9400^{TM} variable mode imager (GE Healthcare, Sweden). Gels were placed gel side-down on the imager for optimal detection.

2.4.4. Image acquisition

The Cy 5 images were scanned using a 633 nm laser and an emission filter of 670 nm bandpass (BP) 30. Cy 3 images were scanned using a 532 nm laser and an emission filter of 580 nm BP 30. The photomultiplier tube (PMT) voltage was adjusted for each channel (Cy 5 and Cy 3) in preliminary low-resolution scans to give maximum pixel values but keep the signals below the saturation level. These settings were subsequently used for high resolution (100 μm) scans for all gels. Image analysis was carried out with the Image Quant TL v2003 software (GE Healthcare, Sweden).

3. Results

The microheterogeneity of Epo-Fc is mainly dependent on the presence of terminal sialic acid residues on glycan structures. To determine the complex isoform pattern of Epo-Fc, IEF was performed with prelabeled samples on IPG gels (pH 4–8).

3.1. Reproducibility

Due to the fact that a certified reference standard was not available for our fusion protein we used a representative purified Epo-Fc sample as internal reference standard (IRS). To evaluate the suitability, respectively, reproducibility of the prestaining method, we were focusing the IRS on four independent lanes under the same electrophoretic conditions. Therefore, the IRS was treated with 2D Clean Up Kit, labeled with Cy 3 and applied onto the gel. After scanning, isoform interpretation was performed by Image Quant TL v2003 software (GE Healthcare, Sweden). The mean values of the percentage distribution of the IRS isoforms are shown in Fig. 1. Based on these results, we analyzed different Epo-Fc samples.

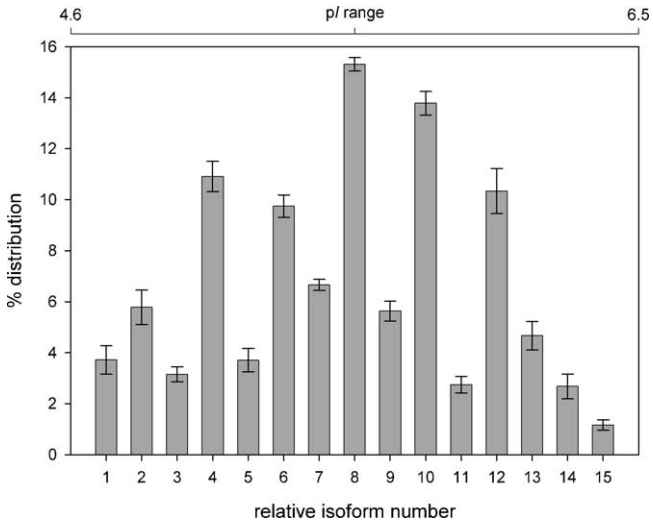

Fig. 1. Reproducibility of the isoform pattern of the IRS. Epo-Fc concentration was 4 pmol. The maximum S.D. of four independent analyses of several corresponding isoforms amounted ±0.9%.

3.2. Isoform pattern of non-purified and corresponding purified Epo-Fc samples

To evaluate the suitability for the analysis of cell-free culture supernatants we analyzed various serum-free culture supernatants compared to corresponding purified samples. Cells were cultivated under different temperature and pH values. All samples were treated with 2D Clean Up Kit, labeled with Cy 5 and applied onto the gel. After scanning, isoform interpretation was performed by Image Quant TL v2003 (GE Healthcare, Sweden).

Cells cultivated at 30 °C, pH 6.9 in a Sixfors multireactor system were prepared as described above. The percentage distribution of the isoforms obtained with serum-free culture supernatant and the corresponding purified protein are shown in Fig. 2A. The isoform pattern of both samples resulted in similar distributions. Samples were analyzed in triplicates and the standard deviation of each isoform was calculated. As shown in Fig. 2A–C, the standard deviations are randomly distributed and not dependent on the magnitude of the measurements. The maximum difference between the corresponding isoforms, purified versus non-purified, was less than ±0.8%.

To verify the methodology itself, analyses were performed as described above with two other samples. Thereby, serum-free culture supernatants cultivated under different conditions were used.

In Fig. 2B and C the percentage distributions of the isoforms of the serum-free culture supernatant and the corresponding purified protein of two other samples, which were cultivated at 30 °C, pH 7.1 and 37 °C, pH 6.9 in a Sixfors multireactor system, are shown. Samples were analyzed in triplicates and the standard deviation of each isoform was calculated. The isoform pattern of both corresponding samples resulted in similar distributions. The maximum difference between the corresponding

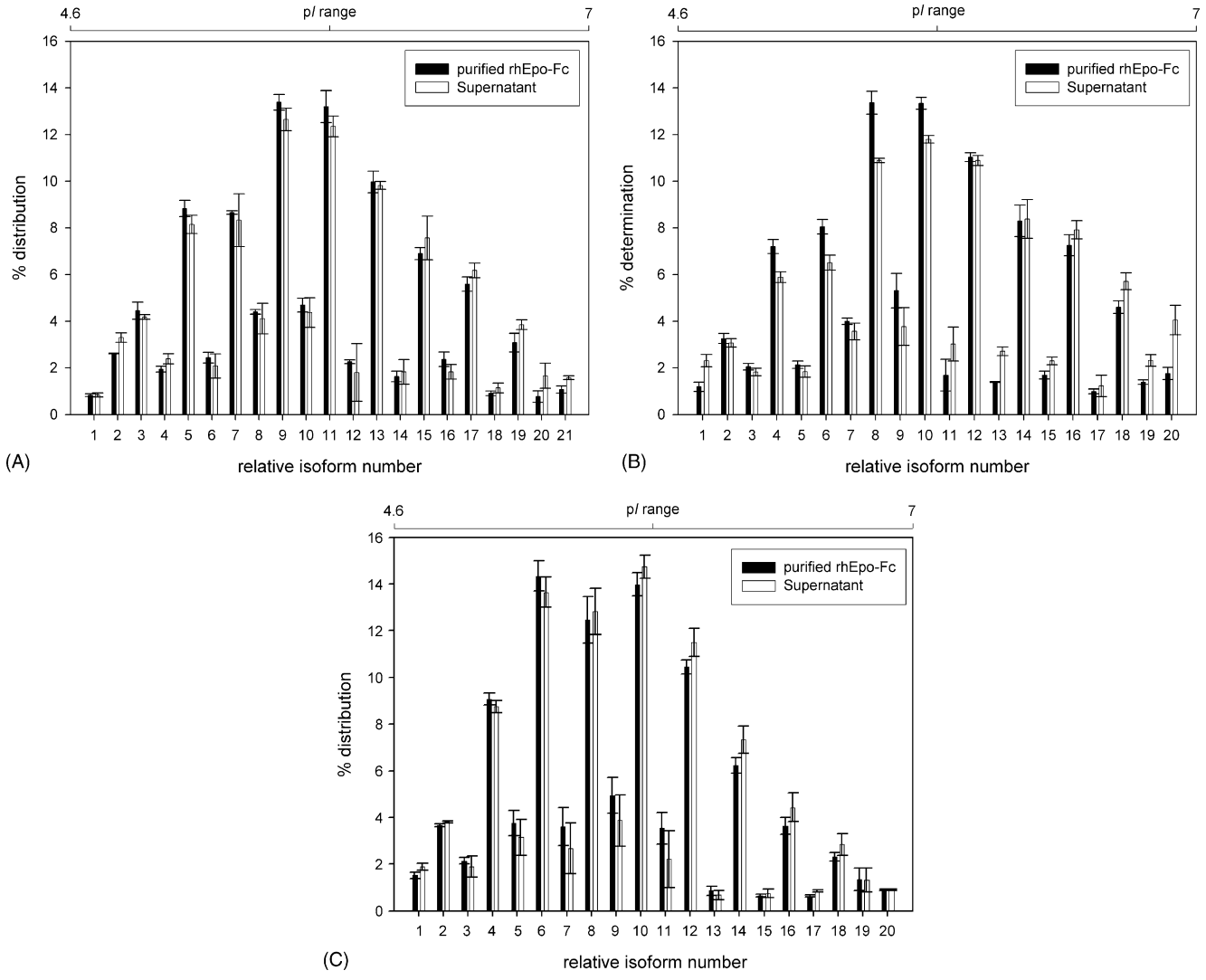

Fig. 2. Isoform pattern of a non-purified and the corresponding purified Epo-Fc sample under different culture conditions (A) 30 °C, pH 6.9, (B) 30 °C, pH 7.1, (C) 37 °C, pH 6.9, directly performed in the serum-free culture supernatant compared to the purified protein. The Epo-Fc concentration was 4 pmol. S.D. is shown with error bars of each isoform, calculated from triplicate analysis.

isoforms, purified versus non-purified, was less than ±2.4% for the sample cultivated at 30 °C, pH 7.1 and less than ±1.4% for the sample cultivated at 37 °C, pH 6.9.

Additionally, IRS and serum-free culture supernatant were prestained with different CyDye fluors. The IRS was labeled with Cy 3 and the serum-free culture supernatant was labeled with Cy 5. After labeling, both samples were mixed, applied onto the gel and separated on the same lane under identical electrophoretic conditions. After focusing, each sample was scanned at the appropriate wavelengths of the respective CyDye fluor. The isoform patterns of IRS, serum-free culture supernatant and the overlay are shown in Fig. 3. Both samples showed similar isoforms, which is represented by the yellow colored bands of the overlay. Additional, green or red bands in the overlay would demonstrate that the samples consisted of varying isoforms. In our case, the IRS and the sample showed similar isoforms except of the basic isoforms of the sample, which is shown by the appearance of red bands at the basic side of the gel. By this matching technique, each sample can be quantitatively compared to the selected IRS.

4. Discussion

Isoelectric focusing, to estimate biochemical properties of proteins, determined by the amino acid sequence and additional charges of posttranslational modifications, is a state of the art technology for many therapeutically used recombinant proteins. In parallel, the electrophoresis technology with high resolution gels and more efficient staining methods were established. Particularly, the establishment of 2D electrophoresis for entire protein separation necessitates more sensitive staining. For this purpose, fluorescent dyes with appropriate quality, such as the CyDyes, were developed. Due to their characteristics, CyDye fluors afford a sensitive prestaining of proteins. By the minimal labeling, the mobility of proteins and peptides during IEF is not affected and broad linear range quantification is feasi-

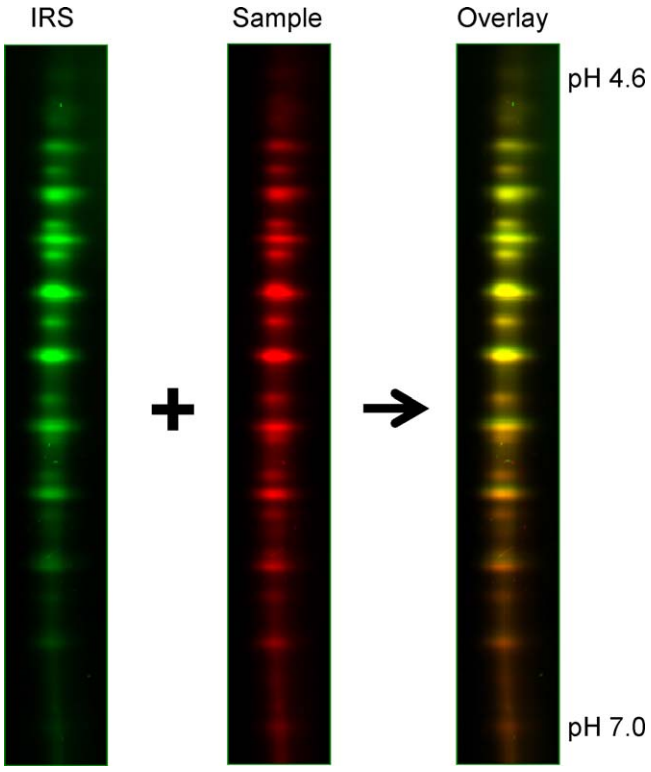

Fig. 3. Imaging. Isoform pattern of two Epo-Fc samples, directly performed from the serum-free culture supernatant by CyDye fluors. The IRS was labeled with Cy 3 and the serum-free culture supernatant was labeled with Cy 5, mixed and separated on the same lane under identical electrophoretic conditions. The isoform pattern at the right represented the overlay of the IRS and the serum-free culture supernatant. The Epo-Fc concentration of each sample was 4 pmol.

ble. High sensitivity (low detection limit), high linear dynamic range (excellent quantitative accuracy) and reproducibility feature them as eligible candidates for all electrophoretic methods [3].

In our approach, we evaluated the use of Cy 5 and Cy 3 CyDyes regarding reproducibility, suitability for crude cell-free culture supernatants and overlaying method. Therefore, we started to prove the reproducibility and suitability of the prestaining method exemplified on the complex protein Epo-Fc. Our results, obtained with four independent analyses, showed a maximum S.D. of ±0.9% corresponding isoforms. Based on these results, we analyzed different Epo-Fc samples. In this regard, purified as well as protein in culture supernatants were analyzed. For this approach, different culture supernatants containing Epo-Fc were harvested and a part of it was purified by affinity chromatography. All different culture supernatants were generated in serum-free media but under different culture conditions. The isoform pattern of the serum-free culture supernatant and the corresponding purified protein of all three analyzed samples showed comparable results. The maximum differences between the corresponding isoforms of all three samples ranged between ±0.8 and ±2.4%. Based on these results, we could demonstrate that this technique, including 2D Clean Up Kit and labeling with CyDye fluors, is suitable for protein characterization directly from culture supernatants. However, supernatants contain greater or lesser extent of various host proteins. Therefore, characterization of the protein of interest is only possible when these proteins are presented in small amounts and/or the protein of interest dominates. Nevertheless, CyDye fluors are very sensitive, detection limits of about 0.25–0.95 ng are published for 1D SDS-PAGE using protein standards [4]. Different to 1D SDS-PAGE, heterogeneous proteins, separated on IPG gels, do not migrate to one single spot, but were separated in various isoforms with individual amounts. In the case of Epo-Fc, 15–21 isoforms are detectable on IPG gels with a relative distribution in the range of about 0.5–14%, thus the total protein amount loaded onto the gel must be adjusted accordingly.

By the use of non-purified protein solutions in combination with fluorescence techniques faster analysis of protein isoforms in cell screening and fermentation is possible, avoiding prior time-consuming protein purification.

Furthermore, we directly compared the serum-free culture supernatant and the IRS by labeling them with Cy 5 and Cy 3 which were separated on the same lane and scanned at the appropriate wavelengths. With this technique up to three samples can be focused on the same lane under identical electrophoretic conditions. A fundamental benefit of this overlaying technique is the ability to co-detect and compare each sample in-gel with an internal standard. This internal standard can be used for normalization of the isoforms across all gels. With this approach, the experimental variation is further reduced and the accuracy of quantification is increased.

5. Conclusion

In summary, IEF in combination with 2D Clean Up Kit and CyDye fluors can be directly performed with protein/serum-free culture supernatants as well as purified proteins. Therefore, this technology provides a useful tool for clone screening and product quality analysis. Apart from our studies, it is advisable for optimization to analyze culture supernatants in comparison with the purified protein of interest to prove matrix influences. In the present study, 4 pmol Epo-Fc was loaded to quantify the relative isoform distribution, whereas the individual isoforms were in the range of 0.5–14%. Depending on the characteristics of the protein of interest, for instance the number of isoforms, the protein amount must be adjusted accordingly. Therefore, the protein concentration, corresponding with the detection limit, respectively, quantification limit, need to be optimized. However, it should be emphasized that depending on the linear dynamic range of a dye, there is always a compromise between the detection of minor and saturated isoforms.

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