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Improvement in the Background Correction of Epo IEF Images

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Introduction

The correct evaluation of the chemiluminescent images obtained as result of the erythropoietin (Epo) doping control test [1] is crucial to the application of classification criteria proposed in the respective technical documents [2]. In particular, inappropriate methods of image background subtraction may affect the identification and confirmation of recombinant Epo abuse. Currently, Epo image evaluation is mostly performed by either commercial image analysis software (e.g. AIDA Biopackage, Raytest, Germany) or the GASepo software package [3, ARC Seibersdorf research, Austria / WADA]. While the AIDA package is based on a simple 2D profile analysis paradigm involving data reduction, GASepo introduced a 3D concept using the full image information for background correction.

In the 2D case, background subtraction is commonly performed by the 'valley-to-valley' technique: The valley points of the integral profile curve are connected to each other, thus forming a baseline which is regarded to cover the non-Epo contributions of the band intensities. In GASepo, the marginal profiles of the lanes are used to generate a 3D background surface, and the intensity of the bands is calculated as a true 3D difference of pixel volumes.

In this paper we demonstrate that, in comparison to the GASepo approach, the simple 2D valley-to-valley background correction results in exposition time dependent changes in the sequence of the band intensities. This is due to the fact that valley-to-valley integration leads - from the statistical point of view - to a stronger weighting of lower abundant bands. On the contrary, the GASepo method is only using background intensity data originating from the lane margins and thus delivering the same 3D background surface independent of the exposition time. Besides, valley-to-valley integration also leads to a significantly higher variability in the analysis results. We support our findings by demonstrating results of a series of IEF runs with different gel lengths and pH gradients, and compare these results as evaluated by both the AIDA and GASepo software tools.

Experimental

Background correction methods

Two background correction methods were used for calculating corrected pixel volumes of bands representing endogenous urinary erythropoietin (uhEpo) isoforms, namely two-dimensional (2D) valley-to-valley correction and three-dimensional (3D) background surface correction. The former was done using the AIDA image analysis software package (AIDA Biopackage, Raytest, Germany), the latter by employing the GASepo software tool (ARC Seibersdorf research, Austria / WADA), which was specifically designed for Epo analysis.

The valley-to-valley background correction method [4] uses a two-dimensional lane density profile representing the Epo isoform (band) distribution and then defines a background profile line (baseline) by piecewise linear connection of the valley points between each band (Figure 1). The useful signal (background corrected pixel volumes) is obtained by subtracting this baseline from the measured lane profile, assuming that the signal is additively composed of Epo and background contributions.

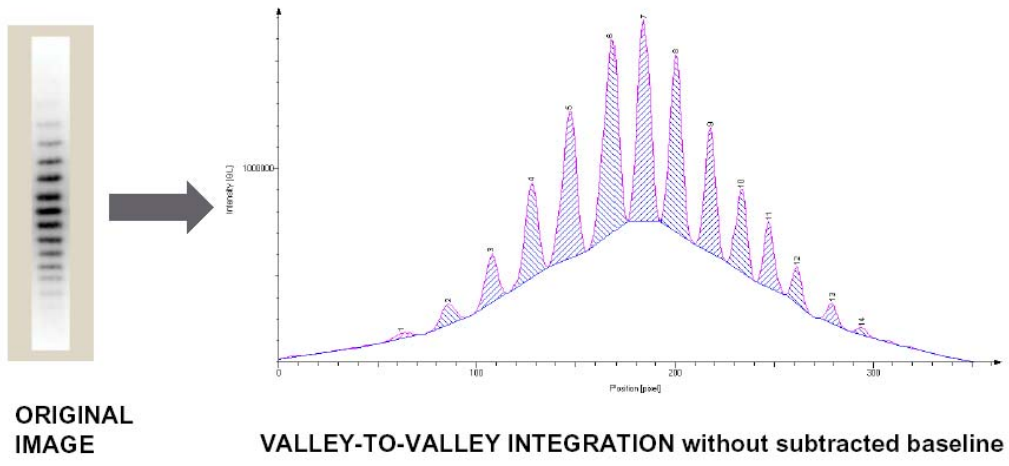


Figure 1. Valley-to-valley integration. The pixel volumes of individual Epo isoforms are calculated by subtracting the baseline from the two-dimensional lane density profile.

The GASepo background correction model (Figure 2) is also based on this assumption but considers the useful part of the signal to be variable along the lane width. Therefore, a three-dimensional approach is required in order to eliminate the influencing factor of the membrane background.

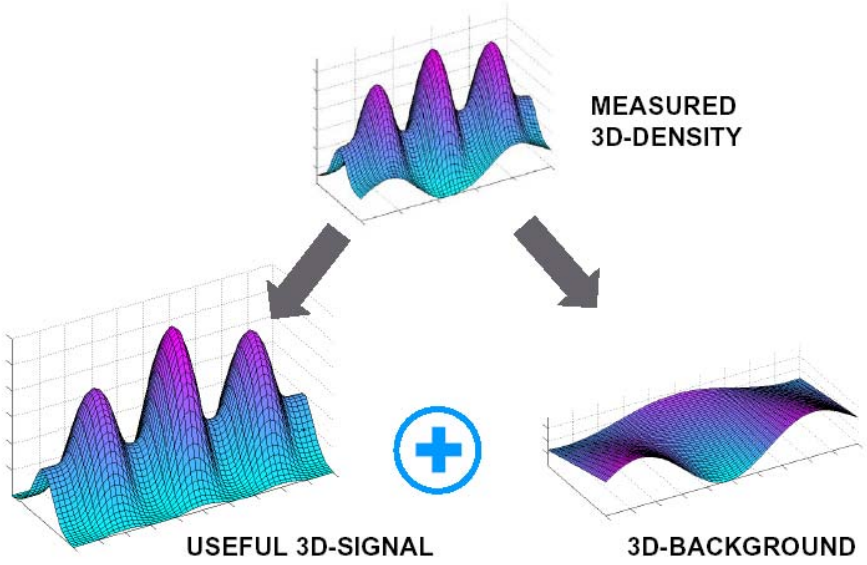


Figure 2. The GASepo background model. The measured signal is decomposed into a useful signal and a three-dimensional background surface signal. This background signal is then subtracted from the 3D raw data.

GASepo assumes that the lane margins are essentially free of useful Epo-related signals and uses them for defining a background surface. The region inside the margins is filled up with linearly interpolated values – thus a background *surface* (as opposed to a background *curve*) is obtained. This background surface is then subtracted from the originally measured total signal.

Separation of erythropoietin isoforms

Isoelectric focusing (IEF) in carrier ampholyte (CA) slab gels was used for separating erythropoietin isoforms. Two different pH-gradients were employed, namely pH 2 to 6 and pH 3 to 5. The method was essentially performed as described by Lasne [5, 6]. Briefly, a 1:1 mixture of Servalyt carrier ampholytes (Servalytes 2-4 and 4-6 or Servalytes 3-4 and 4-5, Serva, Germany) and a 5 % T and 3 % C polyacrylamide gel matrix were used for establishing the pH-gradient. Urinary erythropoietin (uhEpo) was obtained from the National Institute for Biological Standards and Control (NIBSC, United Kingdom). After prefocusing for 1 h a total amount of 0.02 IU (ca. 0.16 ng) uhEpo was applied per lane onto the gel. For the pH 2 to 6 carrier ampholyte gel an interelectrode distance of 10 cm was used with limiting factors being voltage (2000 V), current (1 mA / cm) and power (1 W / cm^2, 1 mm gel thickness). Duration for the main focusing step was 4000 Vh. For high resolution IEF (pH 3 to 5 CA-IEF extra large (XL) slab gels) the interelectrode distance was increased to 17 cm and the electrophoretic

conditions were adjusted accordingly (Table 1). In both cases, the temperature of the cooling device was set to 10° C.

Table 1. Focusing conditions for low and high resolution IEF slab gels.

Step	pH 2 to 6 IEF gel (24 x 10 cm)	pH 3 to 5 IEF XL gel (24 x 17 cm)
Prefocusing	250 V / 24 mA / 24 W / 60 min.	3500 V / 40 mA / 34 W / 60 min.
Focusing	2000 V / 131 mA / 24 W / 4000 Vh	3500 V / 30 mA / 34 W / 75 min.
		3500 V / 12 mA / 200 W / 75 min.
		3500 V / 14 mA / 200 W / 40 min.
		3500 V / 16 mA / 200 W / 40 min.
		3500 V / 30 mA / 200 W / 120 min.

PVDF membranes (Immobilon-P, Millipore, France) were preactivated in methanol, rinsed with water and then equilibrated in blotting buffer (25 mM Tris, 192 mM glycine) ca. 30 min. before focusing was finished. Another set of PVDF membranes (Durapore, Millipore, France) acting as filtering and separating layers between the gel surfaces and the Immobilon-P membranes were equilibrated solely in blotting buffer. Sandwiches consisting of two filter paper stacks, the gels and the two membranes (facing towards the anode) were transferred to a semidry blotting unit (TE 77, GE Healthcare, Germany). Western blotting was done at constant current (1.0 mA / cm^2) and for 1 h. Subsequently, the membranes were incubated in a solution of DTT in PBS (5 mM) for 1 h and then blocked for another hour with 5% low fat milk in PBS. Afterwards they were incubated with the primary (anti-Epo) antibody (1 µg / mL in 1% low fat milk in PBS, clone AE7A5, RnD Systems, USA). After several washing steps (0.5 % low fat milk in PBS) the bound primary antibody was transferred under acidic conditions (0.7 % acetic acid) to a second PVDF membrane (double blotting technique), which was again blocked with 5% low fat milk in PBS. After incubation with a biotinylated secondary antibody (Pierce, USA) followed by washing steps (0.5 % low fat milk in PBS) and a streptavidin-HRP incubation step (Pierce, USA) the membranes were washed in PBS. Chemiluminescent detection was done by incubation in a luminol based substrate solution (West Pico, Pierce, USA) and image acquisition with a CCD camera (epoCAM, ARC Seibersdorf research, Austria) at variable exposition times (1 min., 2 min., 5 min., and 10 min.).

Method comparisons

Valley-to-valley background correction as performed by the AIDA software tool and the 3D-background surface method (GASepo) were compared to each other. The evaluation protocol consisted of comparisons between different IEF methods (low resolution pH 2-6 gels versus high resolution pH 3-5 XL gels) and different exposition times (1min., 2 min., 5 min., and 10 min.). Band volumes were normalized against the most intense band. All bands were checked for pixel saturation. All CCD camera images were 16 bit digital images. Experiments were repeated ten times. For evaluating the exposition time dependent variability in the background corrected analysis results residuals were calculated for each band (differences between mean and single pixel volumes of each band) and plotted graphically against the band number. Due to the fact that the band intensities in the basic region of the IEF gel play an essential role in Epo doping control analysis the sequence of the background corrected fourteen uhEpo isoforms was also studied in an exposition time dependent manner.

Results

Background correction methods

Valley-to-valley background correction was prone to overweighting inter-band spaces. Due to the fact that the IEF sum signal is additively composed of contributions ascribable to the individual isoforms and the membrane background, the pixel intensity *between* the bands was not devoid of Epo signals (Figure 3). As a consequence, lower abundant Epo isoforms were statistically stronger weighted than higher abundant ones, which resulted in data misinterpretation (refer to *Method comparisons*, this chapter).

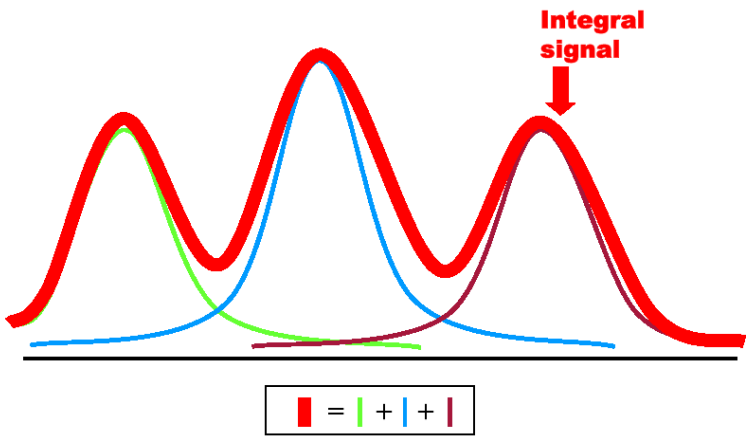


Figure 3. Composition of the Epo IEF signal. Because of its additive nature inter-band signals also contained valuable information for Epo quantification.

By applying valley-to-valley background correction this inter-band information was lost. Contrary to this, the GASepo 3D algorithm led to a lower background subtraction rate and diminished losses in Epo-related chemiluminescent signals.

Separation of erythropoietin isoforms

The high resolution pH 3-5 XL IEF gel (25 x 17 cm) resulted in almost baseline separated Epo isoforms. In consequence of the superior inter-band separation, the contribution of the individual chemiluminescent light emitting bands to the total lane background was minimized (i.e. the "valleys" on the sideplot became extremely deep). Thus, the 2D valley-to-valley integration resulted in less of the valuable isoform information being subtracted from the raw data. The following figure shows how strongly the individual light emitting Epo isoform bands contributed to the valley-to-valley "background" signal and how vastly reduced this contribution is in the high resolution pH 3-5 XL IEF gel.

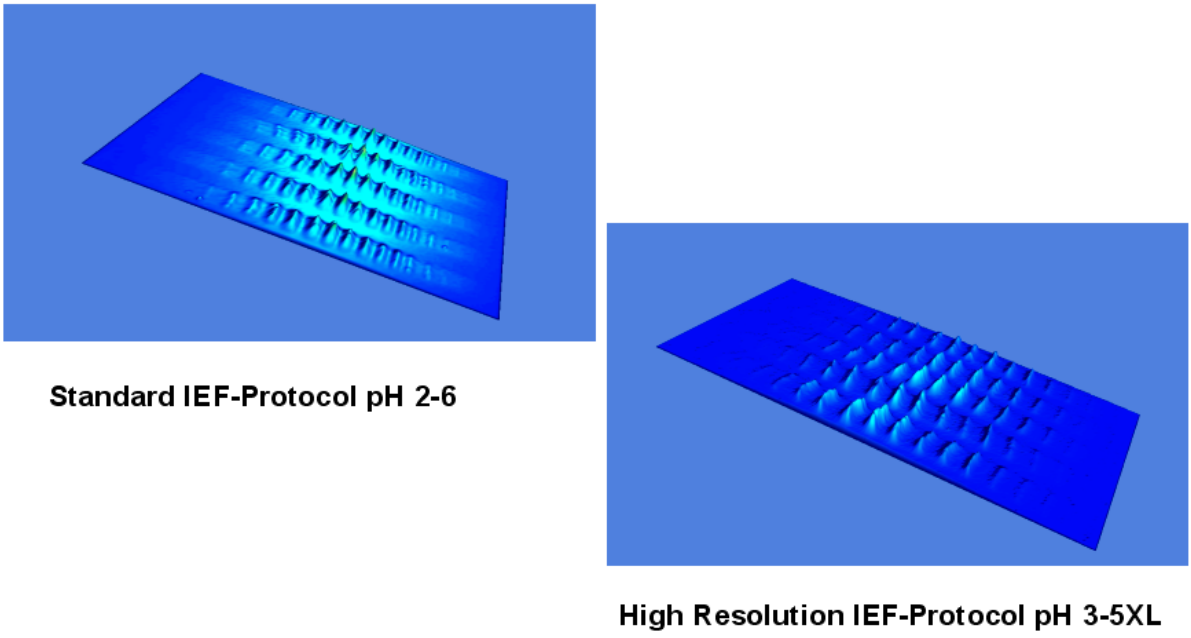


Figure 4. Due to the low resolution of the pH 2-6 IEF gel the individual Epo isoforms strongly and differently contributed to the valley-to-valley background. Subtracting this information led to highly variable and exposition time dependent analysis results.

Method comparisons

Comparisons of the normalized band volumes of each Epo isoform after background correction employing the valley-to-valley integration method and the GASepo algorithm revealed that valley-to-valley integration led to an increased variability in the corrected band intensities. While for the high resolution pH 3-5 XL gel both methods resulted in very low and almost comparable exposition time dependent variabilities (however, the GASepo algorithm still led to a lower variability), the effect was dramatically increased for the pH 2-6 low resolution gel – leading to strongly exposition time dependent variabilities when applying the valley-to-valley integration method (Figures 5 and 6).

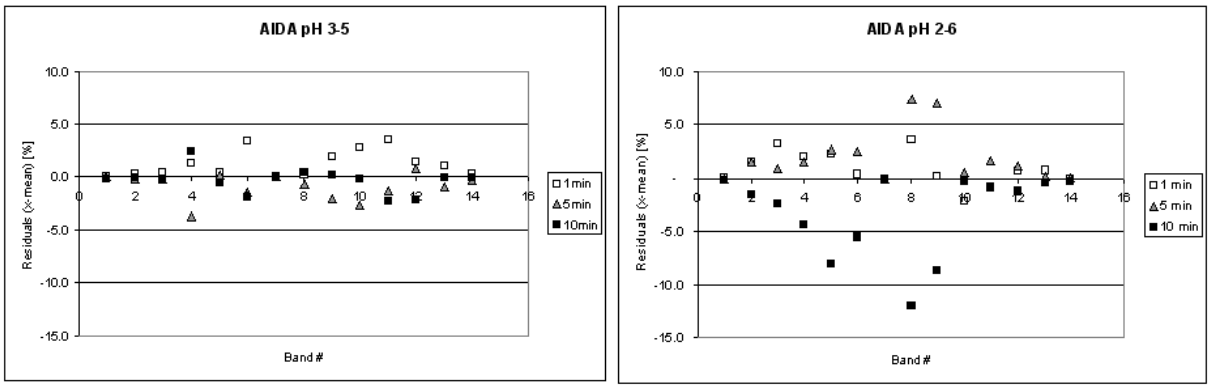


Figure 5. Comparison of identical samples (0.02 IU uhEpo standard) run on high resolution pH 3-5 XL (left) and low resolution pH 2-6 (right) IEF gels. Valley-to-valley background correction (performed by the AIDA software) led to increased exposition time dependent variability in the analysis results. Exposition time: 1 min., 2 min., 5 min., and 10 min.

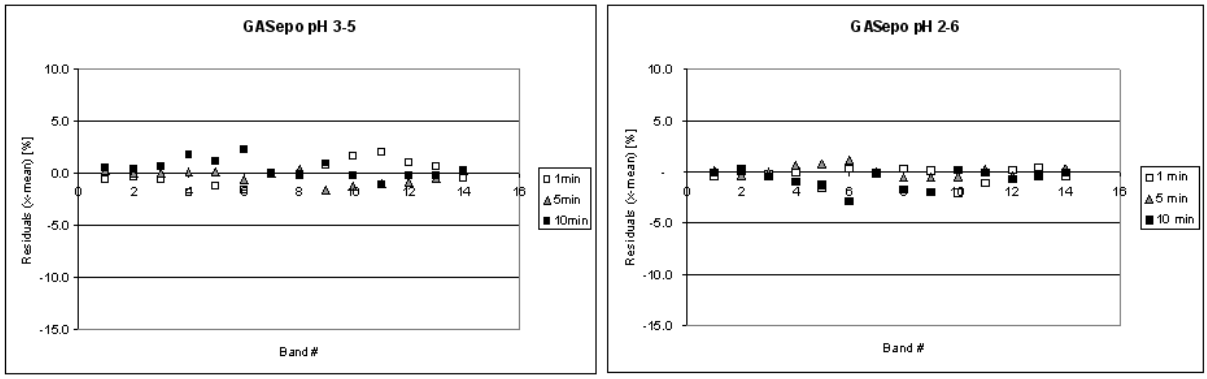


Figure 6. Comparison of identical samples (0.02 IU uhEpo standard) run on high resolution pH 3-5 XL (left) and low resolution pH 2-6 (right) IEF gels. 3D background correction performed by the GASepo algorithm led to almost no exposition time dependent variability in the analysis results. Exposition time: 1 min., 2 min., 5 min., and 10 min.

Contrary to this, the GASepo background correction showed no exposition time dependent band intensity variability (Figure 7).

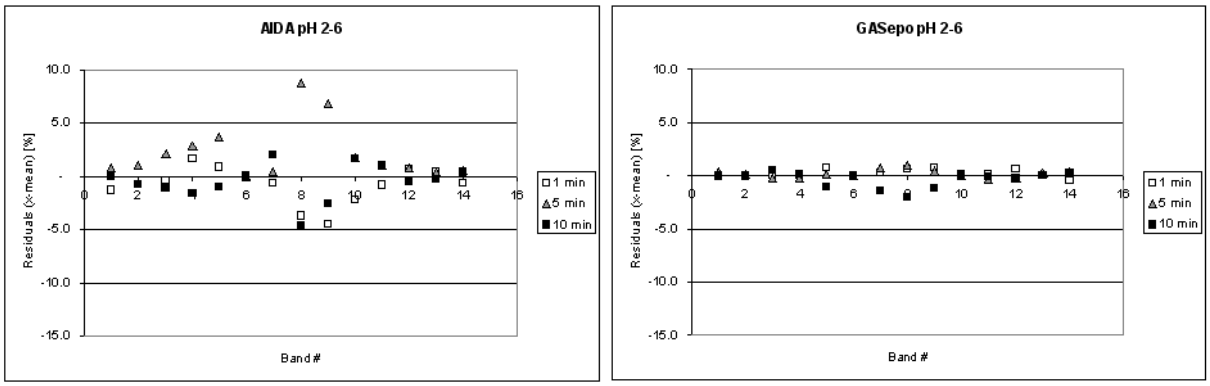
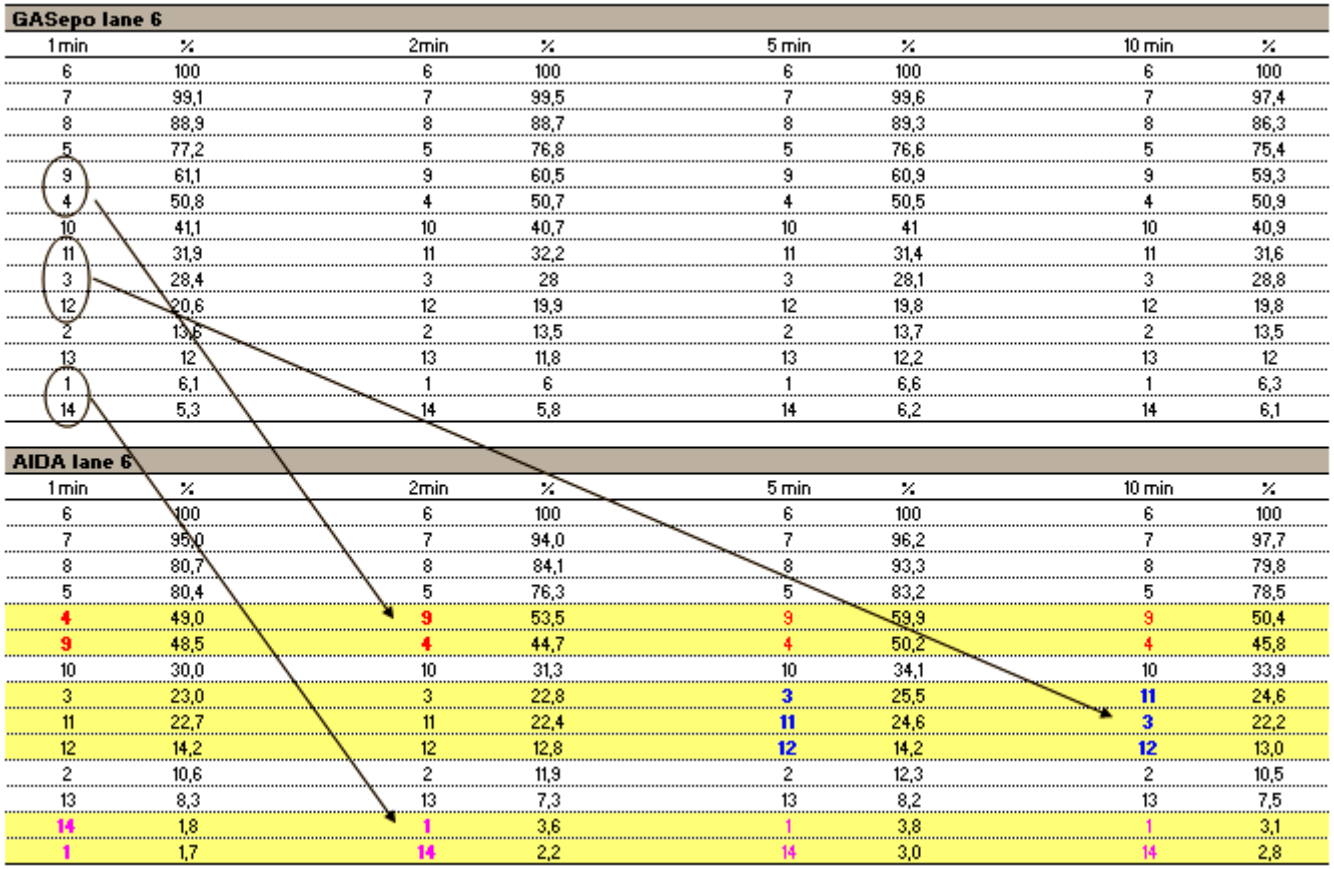


Figure 7. Comparison of one and the same lane of a low resolution pH 2-6 IEF gel (0.02 IU uhEpo standard) exposed for 1 min., 2 min., 5 min., and 10 min. The resulting four images were either valley-to-valley (AIDA software, left) or 3D (GASepo software, right) background corrected. The GASepo algorithm led to hardly any exposition time dependent band variability.

Finally, the sequence of the band intensities of the fourteen uhEpo isoforms after background correction and normalization was compared in an exposition time dependent manner. It could be demonstrated that the valley-to-valley integration led to different band intensity sequences depending solely on the exposition time. Thus, different analysis results were obtained. GASepo's 3D background correction algorithm produced identical results independent of the exposition time (Figure 8).



GASepo lane 6

1 min	%	2min	%	5 min	%	10 min	%
6	100	6	100	6	100	6	100
7	99,1	7	99,5	7	99,6	7	97,4
8	88,9	8	88,7	8	89,3	8	86,3
5	77,2	5	76,8	5	76,6	5	75,4
9	61,1	9	60,5	9	60,9	9	59,3
4	50,8	4	50,7	4	50,5	4	50,9
10	41,1	10	40,7	10	41	10	40,9
11	31,9	11	32,2	11	31,4	11	31,6
3	28,4	3	28	3	28,1	3	28,8
12	20,6	12	19,9	12	19,8	12	19,8
2	13,6	2	13,5	2	13,7	2	13,5
13	12	13	11,8	13	12,2	13	12
1	6,1	1	6	1	6,6	1	6,3
14	5,3	14	5,8	14	6,2	14	6,1

AIDA lane 6

1 min	%	2min	%	5 min	%	10 min	%
6	100	6	100	6	100	6	100
7	95,0	7	94,0	7	96,2	7	97,7
8	80,7	8	84,1	8	93,3	8	79,8
5	80,4	5	76,3	5	83,2	5	78,5
4	49,0	9	53,5	9	59,9	9	50,4
9	48,5	4	44,7	4	50,2	4	45,8
10	30,0	10	31,3	10	34,1	10	33,9
3	23,0	3	22,8	3	25,5	11	24,6
11	22,7	11	22,4	11	24,6	3	22,2
12	14,2	12	12,8	12	14,2	12	13,0
2	10,6	2	11,9	2	12,3	2	10,5
13	8,3	13	7,3	13	8,2	13	7,5
14	1,8	1	3,6	1	3,8	1	3,1
1	1,7	14	2,2	14	3,0	14	2,8

Figure 8. Valley-to-valley integration led to exposition time dependent changes in the band intensity sequences and consequently exposition time dependent analysis results. The 3D background corrected analysis results (GASepo algorithm) were independent of the exposition time.

Conclusion

The 3D background correction method as implemented in the GASepo software was superior to the valley-to-valley integration method as performed by the AIDA software on Epo IEF profiles after Western double blotting and chemiluminescent detection.

It could be demonstrated that valley-to-valley integration led to increased variabilities in band intensities (pH 3-5 XL versus pH 2-6 IEF gels) and exposition time dependent changes in the sequence of band intensities (pH 2-6 IEF gel, e.g. 1 min. versus 10 min.) - and thus exposition time dependent analysis results. On the other hand, the 3D background correction algorithm of the GASepo software performed with extremely high robustness leading to no exposition time dependency and thus identical analysis results.

Acknowledgements

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References

[1] Lasne, F., and de Ceaurriz, J. (2000) Recombinant erythropoietin in urine. *Nature* **405**, 635

[2] Catlin, D., Howe, C., Lasne, F., Nissen-Lie, G., Pascual, J. A., and Saugy, M. (2004) Harmonization of the method for the identification of epoetin alfa and beta (Epo) and Darbepoetin alfa (NESP) by IEF-double blotting and chemiluminescent detection. WADA Technical Document TD2004EPO, 1-6

[3] Bajla I., Holländer, I., Minichmayr, M., Gmeiner, G., and Reichel, C. (2005) GASepo-a software solution for quantitative analysis of digital images in Epo doping control. *Comput Methods Programs Biomed.* **80**, 246-270

[4] Lasne, F., Martin L., Crepin N., and de Ceaurriz, J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. *Anal Biochem.* **311**, 119-126

[5] Lasne, F. (2001) Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures. *J Immunol Methods* **253**, 125-131

[6] Lasne, F. (2003) Double-blotting: a solution to the problem of nonspecific binding of secondary antibodies in immunoblotting procedures. *J Immunol Methods* **276**, 223-226