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Application of high-throughput IEF-PAGE for EPO-doping testing

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Since its first publication in 2000, the isoelectric focusing (IEF) method of erythropoietin (EPO) used in doping control has been considered a procedure with relatively small sample number capacity. To overcome this limitation, a variation of the current protocol was evaluated, which uses double-sized gels with 48–120 wells plus three electrodes and hence multiplies the capacity of the electrophoretic chamber. With this modification up to 120 samples and standards can be run on a single gel – thus, making IEF-PAGE of EPO a high-throughput method. The protocol is ideally suited for large-scale screening purposes.

Keywords: erythropoietin; high-throughput screening; isoelectric focusing; IEF-PAGE; doping control

Introduction

The EPO IEF-polyacrylamide gel electrophoresis (PAGE) method used in doping control[1] has been criticized by several authors as being a low-throughput method due to the 'laborious procedure' of the test.[2,3] Typically, up to 24 samples and standards are run on a single IEF-gel and for higher throughput two or more IEF chambers are used in parallel. However, the employed electrophoretic chamber (Multiphor II) allows the use of two anodes in combination with a single cathode, but this option has not been exploited in anti-doping control so far. Together with the frequently practiced well-casting technique[4] this modification would significantly increase sample throughput. Gels for running up to 120 samples in parallel were cast and tested with the EPO IEF-PAGE protocol aiming to provide a high-throughput alternative of the currently practiced method.

Experimental

Materials

Glass plates (200 x 260 mm with 1.0 mm rubber U-frame, and 200 x 260 x 4 mm without frame), gel support film (GelBond PAG film, 203 x 260 mm), and clamps (FlexiClamps) for assembling the polyacrylamide gel casting moulds were bought from GE Healthcare (Uppsala, Sweden). The electric isolation tape (polyvinylchloride (PVC), *ca*. 15 mm width) was from Schuller Eh'klar GmbH (St Florian, Austria). Acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne ReadySol IEF), urea (PlusOne), ammonium peroxidisulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), as well as carrier ampholytes (Servalytes 2-4, 4-6, 6-8) were received from GE Healthcare and Serva (Heidelberg, Germany). Electrode strips were either bought ready-made (GE Healthcare) or prepared from blotting paper (GB 005; central electrode strip) (Whatman GmbH; Dassel, Germany). Isoelectric focusing (IEF)-polyacrylamide gel electrophoresis (PAGE) was performed on a Multiphor II flat-bed electrophoresis system together with an EPS 3500 XL power supply, both from GE Healthcare.

Erythropoietin (EPO) reference standards were from the European Directorate for the Quality of Medicines (Strasbourg, France) (human recombinant EPO; BRP-EPO batch 3) and the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK) (human urinary EPO, uhEPO; second international reference preparation), respectively. Urine samples were received from a male volunteer and were collected daily over a period of several weeks. All filtration devices (Steriflip; Amicon Ultra-0.5 and Ultra-15, both with a nominal molecular weight limit (NMWL) of 30 kDa) were obtained from Millipore (Billerica, MA, USA). For EPO-purification an ELISA kit from StemCell Technologies (Vancouver, Canada) was used. Western blots were performed on a semi-dry blotter (Trans-Blot SD) from BioRad (Hercules, CA, USA) with blotting papers from GE Healthcare (NovaBlot, and Blotting Paper 21 x 26 cm) and membranes (Durapore, Immobilon-P) from Millipore. For preparing blocking and washing solutions non-fat dry milk (NFM) from Bio-Rad was used. Additional chemicals were supplied by Sigma-Aldrich (St Louis, MO, USA) (sucrose (electrophoresis grade), bovine serum albumin (BSA; ELISA grade), methyl red, DL-dithiothreitol (DTT), phosphate buffered saline (PBS) tablets, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)), Merck (Darmstadt, Germany) (phosphoric acid (p.a.), methanol (LiChrosolv, gradient grade), glacial acetic acid (p.a.)), Thermo/Pierce (Rockford, IL) (Tween-80 (Surfact-Amps; 10%)), and GE Healthcare (tris(hydroxymethyl)aminomethane (Tris; PlusOne), glycine (PlusOne)). MilliQ (MQ) water (Millipore) was used for preparing all buffers and solutions. Thermomixer and microcentrifuge were from Eppendorf (Hamburg, Germany).

Immunological detection of EPO on Western blots was achieved with antibodies from R&D Systems (Minneapolis, MN; anti-EPO antibody cone AE7A5) and Thermo/Pierce (ImmunoPure biotinylated

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goat anti-mouse IgG (H + L)) in combination with a streptavidin horseradish peroxidase (HRP) complex from Biospa (Milano, Italy) and substrate for enhanced chemiluminescence detection (West Femto; Thermo/Pierce). Images were acquired on a LAS-4000 CCD-camera (Fujifilm; Tokyo, Japan) and analysed with GASepo image analysis software (version 2.1; Seibersdorf Laboratories, Austria).

Methods

Preparation of gel casting moulds for high-throughput (HT) applications

The casting moulds were prepared as described previously.[4,5] Briefly, two parallel lines were drawn with a waterproof pen on the outer face of the U-framed glass plate and in *ca*. 88 and 104 mm distance from the upper edge of the lower rubber gasket. Along these lines and on the inner face of the glass plate 2 x 4 layers of electric isolation tape were fixed above each other (each layer *ca*. 25 cm in length). Special care was taken to avoid inclusion of air bubbles between layers and glass plate. Depending on the preferred number of wells, slot-formers were prepared by vertically cutting each of the two stacks of tape layers with a scalpel or carpet knife. A 48 well slot-former plate was prepared for example by cutting vertically every 5 mm and starting *ca*. 7 mm from the left or right edge of the rubber frame, and 80 and 120 well plates were made by cutting every 3 and 2 mm, respectively, starting *ca*. 6 mm from the edges. Subsequently, both stacks were reduced in width by cutting them horizontally, e.g. widths of 6, 8, and 12 mm were used for the 48, 80, and 120 well moulds. The optimum width was defined by the desired well volume, which was typically between 20 and 30 µL (e.g. a 2 x 12 mm well held about 22 µL of liquid). Finally, every second of the small rectangular tape stacks was removed with tweezers resulting in two 'combs' of slot-formers in the middle of the U-framed glass plate (Figure 1). The final casting mould consisted of the slot-former plate, a second non-U-framed glass plate with attached GelBond PAG film, and six FlexiClamps.

Casting high-throughput IEF-PAGE gels

Carrier ampholyte gels (pH 2 to 6) were prepared as described elsewhere (5% T/3% C acrylamide-bisacrylamide, 7 M urea, Servalytes 2-4 and 4-6 at a final concentration of 2% (*w/v*) each).[1] A typical composition of the gel solution is shown in Table 1.

Table 1. Composition of the solution used for casting double-sized gels for high-throughput IEF-PAGE applications (pH 2–6) with optimized volumes of APS and TEMED

Acrylamide/bisacrylamide solution (40% T, 3% C)	9.48 mL
Urea	29.42 g
Sucrose	3.5 g
Servalyt 2-4	3.78 mL
Servalyt 4-6	3.78 mL
MQ water	31.5 mL
APS (10%)	600 µL
TEMED	60 µL

After dissolution of urea the solution was degassed for 15 min under stirring, supplemented with TEMED and APS and then transferred into the casting mould using a 50 ml glass pipette. During the filling procedure special care was taken to not introduce any air bubbles into the polymerizing solution. Gels were polymerized overnight at room temperature and in a wet-chamber.

Sample preparation

After centrifugation (2000 *x g* / 10 min) and microfiltration (Steriflip) urine samples (typically between 10 and 20 ml) were concentrated by sequential ultrafiltration (Amicon Ultra-15 (2000 *x g* / 20 min) and Ultra-0.5 (14000 *x g* / 15 min)).[1] Retentates were subsequently immunoaffinity purified using a recently published ELISA-plate protocol.[6] In brief, retentates were transferred into the wells of a commercial EPO-ELISA plate, mixed with 20 µL PBS, sealed with PCR film, and incubated overnight under cold room conditions (4–8 °C). After five washes with PBS (300 µl each), wells were tapped dry and eluted with 25 µl of 4.4% (*w/v*) CHAPS in MQ water (90 °C, 5 min, 650 rpm; Thermomixer with microtiter plate heating block; wells resealed with PCR film). Subsequently, wells were cooled down to room temperature and 2.5 µl of Tween-80 solution were added to the eluate.

IEF-PAGE and Western blot

After releasing the gel from the casting mould it was placed on the cooling plate of the Multiphor II unit (set to 10 °C) with MQ

Figure 1. Rubber U-framed glass plate with 120 slot-formers. The plate was used for casting high-throughput IEF-PAGE gels with 2 x 12 mm sample wells.

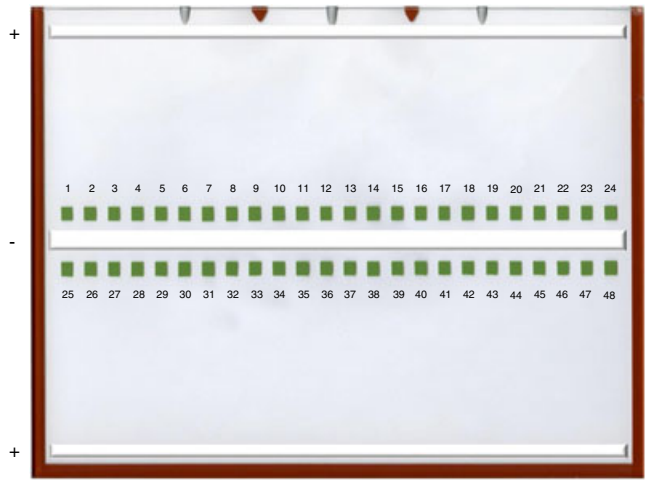

Figure 2. Principle of using three (2 anodes, 1 cathode) instead of two electrodes for HT-IEF-PAGE. Two electric circuits are generated, which can be used to simultaneously run two electrophoretic separations on one gel.

water as contact fluid between cooling plate and GelBond PAG film. A second anode was mounted on electrode holder with the cathode in-between.[7] Two electrode strips (GE Healthcare; *ca*. 243 x 6 mm) were soaked with 0.5 M phosphoric acid (anolyte) and placed on the upper and lower long edge of the gel. The electrode strip for the central cathode was slightly broader than the ready-made strips (*ca*. 243 x 8 mm) and was cut from thick Whatman blotting paper (Figure 2). Catholyte was a 2% (w/v) dilution of Servalyte 6–8 in MQ water, which contained 10 µl of methyl red solution (1 mg/mL methanol) per 2 ml electrolyte. Prefocusing was performed at constant voltage (250 V) for 30 min (25 W maximum power). After application of the eluates into the gel-wells, focusing continued at 40 W constant power for 3200 volt-hours.

For semi-dry Western blotting the gel was cut into two halves. All subsequent steps were more or less identical to the protocol already described elsewhere.[8] The same blotter (Trans-Blot SD unit) was used for both the first (25 mM Tris, 192 mM glycin; 1 mA/cm^2, 30 min) and second (0.7% acetic acid; 0.8 mA/cm^2, 10 min) transfers (Immobilon-P as acceptor, Durapore as separator membranes). Incubation steps in reducing (5 mM DTT/PBS) and blocking (5% (*w/v*) NFM/PBS) solutions were typically between 45 and 60 min. All washing procedures in 0.5% NFM/PBS and PBS were shortened to 3 x 7 min using *ca*. 500 ml buffer for each step. Incubations in primary antibody (clone AE7A5, 1:1000 (*v/v*) in 1% NFM/PBS) were overnight under cold room conditions; all other incubations were at room temperature and for 60 min each (biotinylated goat anti-mouse secondary antibody, 1:2000 (*v/v*); streptavidin HRP, 1:2000 (*v/v*)). Chemiluminescent detection was achieved by placing the membrane on a methanol-cleaned glass plate and directly exposing the blot together with the substrate (West femto) in a CCD-camera (LAS-4000).

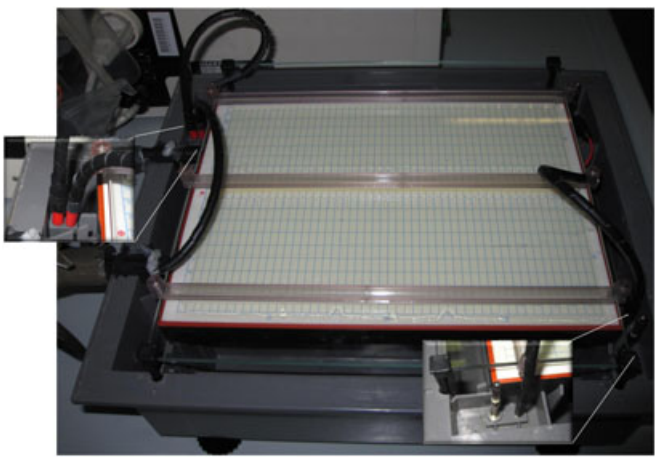

Figure 3. Electrophoretic chamber (Multiphor II) with the three electrodes correctly plugged in. Note the connection of the two anodes to the socket.

Results and discussion

The Multiphor II electrophoresis chamber is equipped with one male plug and a socket with two female plugs for connecting one cathode and up to two anodes to the power supply (Figure 3).[7] So far, in anti-doping control IEF-PAGE gels have been run using only one anode and cathode and the high-throughput option with two anodes has not been exploited. We hence tested the applicability of this option for EPO testing in urine. The aim was (1) to determine the maximum number of samples and standards, which can be simultaneously analyzed on a single gel and with a single Multiphor II chamber; (2) to elucidate possible limits of this methodology as observed in daily practice; and (3) to offer solutions for these limitations.

Casting high-throughput gels with wells

High-throughput IEF-PAGE gels use the same molar composition of ingredients as regular IEF-PAGE gels. In practice, the risk of entrapping air bubbles during the casting process increases with the number of wells. Hence, the amounts of APS and TEMED were slightly reduced in order to delay polymerization (Table 1). Consequently, the gel-solution stayed in a low viscosity state during casting and entrapped air bubbles could be easily removed by slightly tilting the casting mould.

The importance of a uniformly thick contact fluid film

Usually, contact between the gel-supporting GelBond sheet and the cooling plate of the Multiphor II is established with fluids like kerosene, decane, or MQ-water. Regardless which fluid is taken it is of utmost importance to completely remove the excess fluid before electrophoresis is started. For this purpose strips of thin blotting paper (e.g. NovaBlot) can be used. If the excess liquid has not been completely removed, the thickness of the resulting contact fluid film is not entirely uniform. This leads to curved lanes. Typically, the first and/or last few lanes of the gel are affected, where the excess liquid collects during the electrophoretic run. Hence, it is not only important to horizontally level

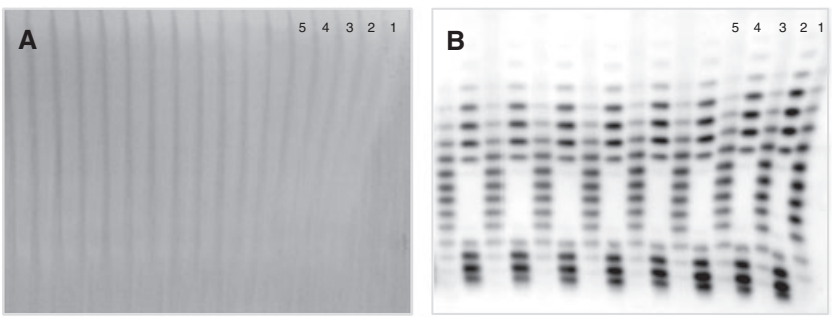

Figure 4. Effect of incomplete removal of contact fluid as observed (A) after the first Western blot during air-drying of the Durapore separator membrane, and (B) after chemiluminescent EPO detection on the second blot. Note the increase in lane curving towards the edge of the gel (lanes 1 to 5). IEF-PAGE gel with pH 2–6.

the electrophoretic chamber and its cooling plate but also to meticulously remove this excess liquid (1) on all four edges of the GelBond and (2) by frequently changing the absorbing blotting paper strips. It is also a good idea to keep two of these strips (*ca.* 250 x 40 mm) in permanent contact with the long edges of the GelBond during the electrophoretic run. This has been the situation for the regular small sized (*ca.* 250 x 120 mm) IEF-PAGE gels used in EPO doping control. For the large sized high-troughput IEF-PAGE gels (*ca.* 250 x 190 mm) removal of excess contact fluid became even more essential (Figure 4).

The distortions were primarily caused by the fact that the large GelBond PAG film (203 x 260 mm) was slightly oversized for efficient removal of contact fluid. Hence, the non-gel-covered part of the PAG film was trimmed with scissors after releasing the gel from the mould in order to improve contact of blotting paper to the liquid. Figure 5 shows the performance characteristics of a HT-IEF gel after trimming the GelBond.

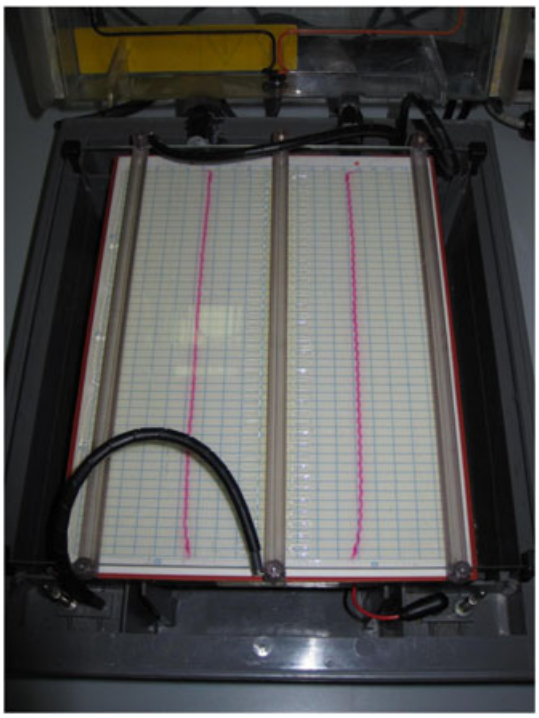

Figure 5. An 80 well HT-IEF-PAGE gel during the main focusing step. The migration behavior of the two methyl red lines can be used for monitoring the electrophoretic run and possible lane/band distortions due to e.g. insufficient removal of excess contact fluid.

The necessity of immunoaffinity purification for HT-IEF-PAGE

One of the advantages of IEF-PAGE as a separation method in protein chemistry is its relative robustness concerning higher protein loads. Depending on the matrix and its complexity in protein composition total protein amounts up to high μg-ranges are tolerated. Typical protein concentrations of urinary retentates used in EPO-doping testing and as obtained by ultrafiltration of *ca.* 20 ml urine are in the μg/μL range (e.g. 10–40 μg/μl, depending on the specific gravity of the urine and the obtained volume of retentate). About 10–20 μl of these retentates are used per sample lane for IEF-PAGE, which is tolerated in most cases. Immunoaffinity purification of urine or urinary retentate is one way to avoid possible band distortions due to protein overloading. Recently, several methods were developed and published for EPO-specific enrichment using either ELISA-plates or columns.[6,9,10] Another option is EPO-selective protein depletion by, for example, protein precipitation with perchloric acid, which enriches EPO in the supernatant.[11] Currently, several anti-doping control labs are using immunoaffinity purification already for EPO-testing with the regular lower-throughput standard IEF-PAGE method (e.g. 24 wells per gel). HT-IEF-PAGE achieves increased sample throughput by decreasing the well width and thus multiplying the number of wells per gel. The disadvantage of this strategy is that the total protein load per gel is multiplied accordingly, which eventually leads to protein overloading. For EPO HT-IEF-PAGE of urinary retentates band distortions, increased electroendosmotic flow, and gel damaging were observed with increasing the number of wells. The problem was aggravated due the fact that for EPO IEF-PAGE samples have to be applied cathodically, i.e. in the HT-case near the single central cathode. This led to an increased accumulation of liquid at the cathode over the course of focusing, resulting in well destruction and lane broadening in several cases. We hence used immunoaffinity purified samples for HT-IEF-PAGE of EPO and in particular for gels with 120 wells. The applied ELISA immunoafinity purification protocol was completely compatible

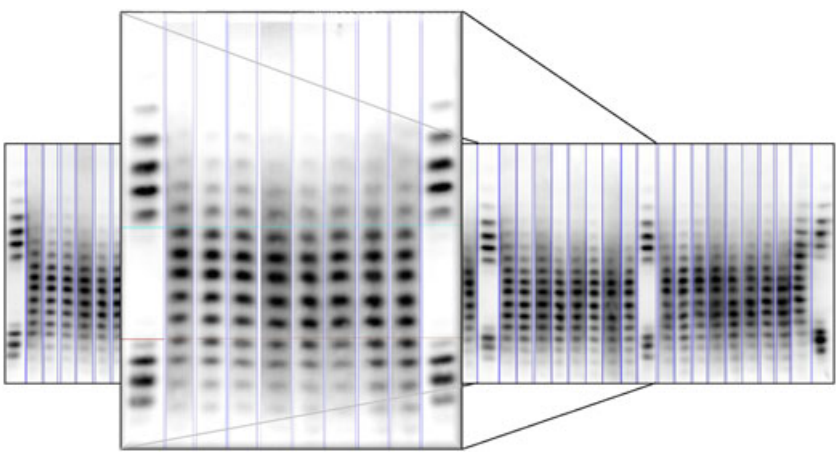

Figure 6. Result of a HT-IEF-PAGE gel (pH 2–6) with 120 wells after Western double-blot and chemiluminescence detection. The samples were from a single person and were collected over a period of several weeks. ELISA immunoaffinity purified urinary retentates were applied on the gel. Shown is one half of the gel.

with high-throughput isoelectric focusing of EPO. Figure 6 shows the results obtained with immunoaffinity purified urinary retentates and a 120 well gel.

Another option for circumventing the problem of cathodic sample application in combination with HT-IEF-PAGE of EPO on an instrument equipped with a single cathode and two anodes is reversion of polarity. By connecting the electrophoretic chamber with inverted plugs to the power supply, a system with two terminal cathodes and one central anode is obtained. However, we have not tested this option so far as excellent results were obtained with the described protocol.

The maximum number of samples per EPO IEF-PAGE run

HT-IEF-PAGE of EPO was tested with *ca*. 250 x 190 mm sized gels with 48, 80, 84, 96, and 120 wells. The dimension of the wells was decreased accordingly, i.e. from 5 x 6 mm (48 wells) down to 2 x 12 mm for the gel with 120 wells. However, the wells of this latter gel were separated from each other by only 2 mm wide gel bars. In our hands, this was technical limit - below 2 mm the bars became too fragile and partly broke.

Conclusion

Criticism of the EPO IEF-PAGE method for anti-doping testing using the 'low-throughput' argument is no longer justified. Evaluation of high-throughput IEF-PAGE showed that up to 120 samples and standards can be run on a single gel and electrophoretic chamber within the same time-frame as usual. Only minimal modifications of the established methodology were necessary, i.e. using a third electrode and casting a double-sized gel. The described protocol is ideally suited for large-scale screening purposes.

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