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Initial Development of an Immobilised pH Gradient Gel for the EPO Urine Test

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Introduction

- The test for recombinant EPO in urine developed by Dr Francoise Lasne and her colleagues at Laboratoire National de Depistage du Dopage [1,2] separates the recombinant drug from the naturally occurring EPO on carrier ampholyte gels using isoelectric focusing.
- This method uses voltage to move proteins to their pI in a gradient made from carrier ampholytes. The problems with carrier ampholyte-based focusing include load limitations and unreproducible gels.
- Over the past decade, Immobilised pH Gradients (IPGs) have been produced which use immobilines embedded on a solid support to form the pH gradient [3]. This allows highly reproducible IPGs that can focus large amounts of protein.
- The availability of a suitable commercial IPG should assist in reducing both the inter and intra laboratory variation of the method
- At present there is no suitable IPG available and hence Proteome Systems are developing an IPG suitable for use in the detection of recombinant EPO in urine.

Method

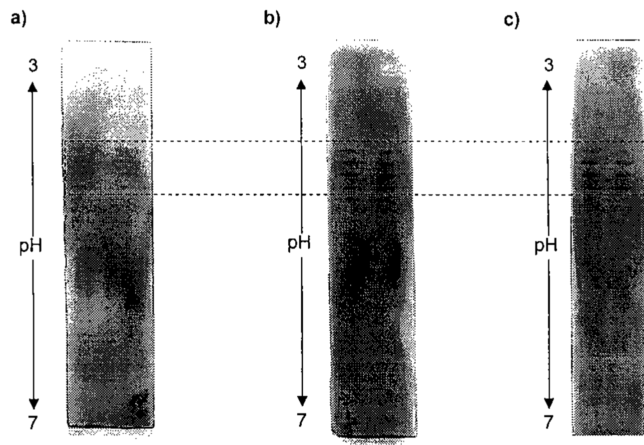
- In the initial stages the IPG gel slabs are being manually poured.
- An IPG slab with a pH range of pH 3-7 was poured using the following solutions:

pH 3	**pH 7**
Immobiline 3.1 112.4μL	Immobiline 4.6 252.2μL
Immobiline 4.6 383.2μL	Immobiline 6.2 351μL
Immobiline 6.2 22.5μL	Immobiline 10.3 197μL
1M Tris -	
40% T, 4% C: 1mL	40% T, 4% C: 1mL
MQ water to final volume of 8mL	MQ water to final volume of 8mL

- The casting plates were assembled as per the manufacturers' instructions, where Gelbond was used to bind the gradient gel to a solid support. Prior to pouring the gradient, 3.2μL of N,N,N',N'-tetramethylethylenediamine (TEMED) and 8μL of 40% Ammonium persulfate (APS) was added to each solution from the table above. Each solution was placed into either side of the gradient pourer and the gradient poured at a rate of 60 rpm. Once complete, the IPG slab was covered with water-saturated isobutanol and left to polymerise at 50°C for one hour. Once polymerisation was complete the IPG was removed from the casting plates and the gel washed with 10% methanol for 3 x 15 minutes, then a final wash in 5% glycerol, 10% methanol for 15 minutes. The IPG slab was allowed to dry overnight in a chemical fume hood. Once dried, the IPG was stored at -20°C.
- The pH 3-7 11cm slab was cut into smaller portions and rehydrated with 110% of their volume using 7M Urea, 2M Thiourea and 1% of the novel zwitterionic detergent C7BZ0 [4]. The isoelectric focusing program used was 100 Volts for 8 hours, 1000 Volts for 3 hours, 2500 Volts for 3 hours, and 5000 Volts for 5 hours. Total Volt hours reached was approximately 36000 Volt hours. The gels were stained with Colloidal Coomassie G250.

Results

The pictures below show the results of repeat experiments of isoelectric focussing using recombinant human EPO on the pH 3-7, 11cm IPG.



Approximately 2ug of recombinant EPO (Eprex) was cup loaded into each lane onto the IPG slab and focussed for 36000 Volt hours. Each isoelectric focussing experiment was undertaken on different days a) 21/1/02, b) 4/2/02 and c) 5/2/02. The positioning of the EPO bands are highly reproducible as shown by the horizontal lines. The anode is at the top of the image. Note that only 4 isoforms of EPO are evident after staining for 8 hours (a), whilst another two isoforms appear (one above and one below the four dark bands) after staining for 24 hours (b and c).

Mass spectrometry was conducted to confirm that the bands on the upper part of the gel were EPO. Protein bands were excised from the IPG Gelbond backing sheet and after washing the gel pieces were dried and then reswollen with 0.02μg/μL trypsin in 50mM NH_4HCO_3 buffer. The trypsin digestion was carried out overnight at 30°C. Peptides were extracted from each gel piece and the peptides were concentrated using Ziptips and

spotted onto a target plate. Alpha Cyano-4-Hydroxycinnamic Acid was used as the matrix for MALDI mass spectrometry. Mass Spectrometry was undertaken in an Axima Mass Spectrometer and spectra were acquired manually. The four bands were identified as coming from erythropoietin

Conclusions and Further Work

- An IPG gel has been produced that can reproducibly resolve the isoforms of recombinant EPO under the conditions of high urea content and high protein loading required for the EPO urine test.
- It is apparent that a gel with a pH range of 2.5 to 5.5 would be better suited to the optimum separation of recombinant EPO, urinary EPO and ARANESP.
- Blotting protocols are being developed so that the IPG gel can be used for the initial electrophoretic separation stage of the EPO urine test.

References

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