Spotlight

Biotechnology & Microtechnology

Two-dimensional electrophoresis is a critical technique for proteomic research; many researchers believe two-dimensional (2D) electrophoresis can only be accomplished using a combination of 2D gel electrophoresis to separate proteins, followed by visualisation through either (a) mass spectrometry (MS) or (b) the use of a cooled CCD imaging system combined with image analysis software for protein identification. Although these techniques are powerful and sensitive, proteins can be identified easily and quickly by staining a 2D gel with either Coomassie blue or silver stain. 2D gels were used to determine a quick method to differentiate human alpha-NAGAL protein from IGG antibodies (with similar molecular weights) contaminates obtained during fractionation.

"An impressive feature of the IEF100 unit is its unique ability to monitor each IPG strip individually in real time and maintain a record of each strip's focusing data for later review and publishing."

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A Quick and Easy Method to Separate Proteins of Similar Molecular Weights Using 2D Electrophoresis

MATERIALS AND METHODS

IPG BlueStrips were from Serva Electrophoresis GmbH (Heidelberg, Germany). CodeBlue stain was from Pierce (Rockford, IL). All other reagents used were from Hoefer, Inc (Holliston, MA).

Preparation

Samples of Human alpha-NAGAL protein, IGG antibodies, and a mixture of both were loaded onto 7cm (3-10NL) immobilized pH gradient (IPG) strips using overnight rehydration. Three samples were prepared; (a)10µg of pure alpha-NAGAL protein (b)10µg of rabbit IGG (c)5µg of pure alpha-NAGAL protein and 5µg of rabbit IGG. Each sample was mixed in 130µls of fresh rehydration buffer (Table 1) and then 130µl of rehydration buffer was added to each channel of the rehydration tray (*Table 2*).

Each sample was placed on different IPG strips, the IPG strips were placed gel side down in the rehydration buffer and then overlaid with mineral oil to prevent evaporation during the overnight rehydration at room temperature.

Note - It is important not to place the IPG strips into a refrigerator or the urea will crystallise.

First Dimension Electrophoresis - Running the IPG Strips in the Isoelectric Focusing Unit

The 6 rehydrated 7cm IPG strips were removed from the rehydration tray and inserted gel side up into the channels of the focusing tray with the anodic (+) end to the left side of the Hoefer IEF100 Isoelectric focusing unit.

A filter paper wick was placed on both the anodic and cathodic end of each IPG strips. Each wick was moistened with distilled de-ionised water and lightly blot dry with a paper towel. The electrodes were placed onto the filter paper wicks and secured by clipping into place on the focusing tray. After making sure the electrodes were secured, each focusing tray channel was filled with 10mls of mineral oil.

To separate the proteins on the IPG strips, pre-programmed protocol 2 was used: 7cm Constant Wattage (Table 3). During the run, four 10% 8 X 10cm Tris-Glycine SDS-PAGE gels were cast with the Hoefer SE245 Dual Gel Casters.

A 1cm space was left at the top of each gel to accommodate the IPG strip. At the end of the run, the electrodes were removed and, using forceps, the strips were placed into a clean rehydration tray and equilibrated for 15 minutes in EBI (*Table 4*), then 15 minutes in EBII (*Table 5*). At the end of the equilibration steps, the three strips were stained with CodeBlue stain to observe the banding (*Figure 1*).

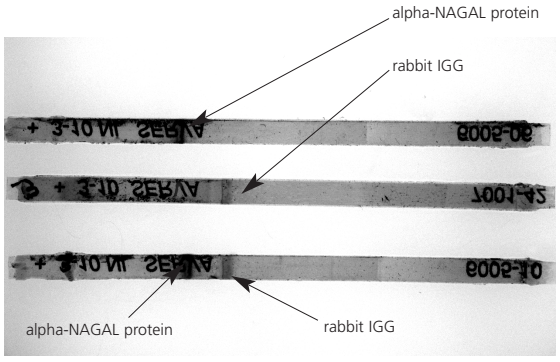


Figure 1. IPG Strips 3-10NL stained with CodeBlue; note the banding on the strips. Top strip is pure alpha-NAGAL protein; middle strip is rabbit IGG; bottom strip is a mixture of rabbit IGG and pure alpha-NAGAL protein.

Second Dimension Electrophoresis - Running the SDS-PAGE Gel

One IPG strip was placed onto a 8 x 10cm Tris-Glycine SDS-PAGE gel using forceps. The protein marker was placed in a 90°C water bath for 3 minutes before applying 200ng to a 5mm x 5mm piece of filter paper. The IPG strip and filter paper with marker were held in place with a molten 0.5% agarose solution overlay ensuring contact between the IPG strip and the gel.

The Hoefer SE250 Vertical electrophoresis unit was run at 200 volts for 40 minutes. After the run, the gel was removed and stained with CodeBlue (*Figure 3*).

1D separation – Running the SDS-PAGE gel

A 10% PAGE resolving gel with 4% stacking gel was cast with the Hoefer SE245 Dual Gel Caster and then placed into a Hoefer SE250 mini vertical electrophoresis unit. Protein markers were placed in a 90°C water bath for 3 minutes, after which 200 ng was added to the first well of the 8 x 10 cm Tris-Glycine SDS-PAGE gel.

10µg of purified Human a-NAGAL was placed in the second well. The mini vertical electrophoresis unit was run at 200 volts for 40 minutes. After the run, the gel was removed and stained with CodeBlue (*Figure 2*).

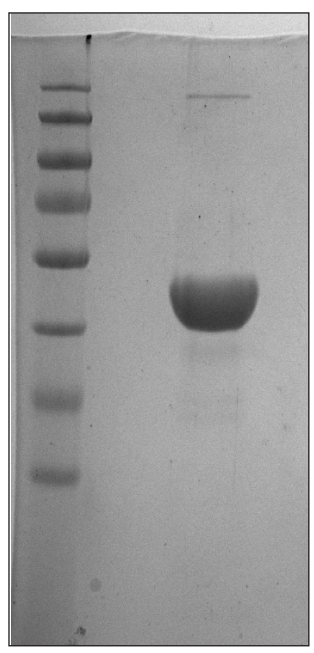


Figure 2. Shows a 1D lane of purified human alpha-NAGAL protein.

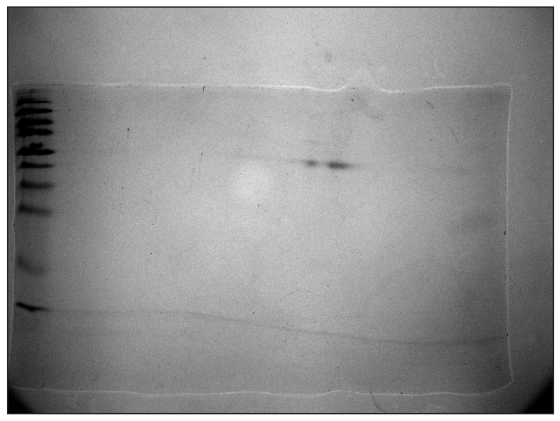
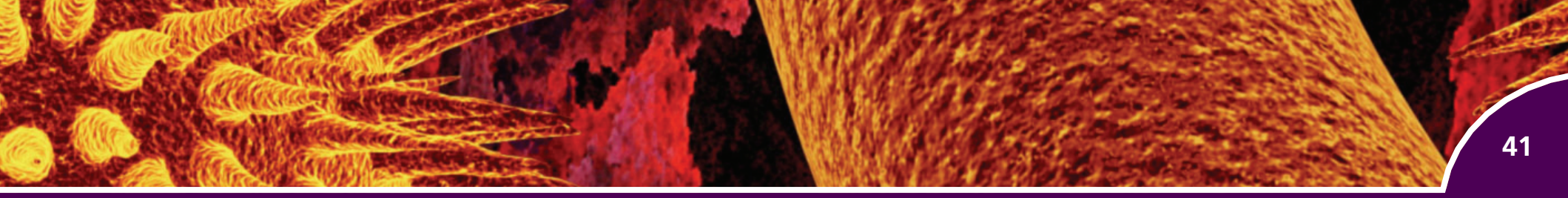


Figure 3. is the 2D gel of the purified human alpha-NAGAL protein. Note the two bands are revealed by the 2D experiment, which are not apparent in the 1D gel (Figure 2).



CONCLUSION

The Hoefer IEF100 offers a simple and fast means of separating proteins. No prior experience running 2D equipment is needed to operate the IEF100.

The preprogrammed protocols make running the unit easy, furthermore, the programs are easily edited to support flexibility as needs evolve.

An impressive feature of the IEF100 unit is its unique ability to monitor each IPG strip individually in real time and maintain a record of each strip's focusing data for later review and publishing. This avoids wasting money and time on the second dimension separation of IPG strips that did not focus correctly in the first dimension.

The IEF100's ability to run at 12,000V, more than three times other competitive units, enables 2D separation in a single day.

Our assessment of the IEF100 is that it is extremely user friendly and supports easy and accurate separation of proteins of similar molecular weights.

Table 1. Rehydration Buffer

Reagent	Concentration Range	Amount
Urea*	8M (8-9M)	4.8g
CHAPS	1% (1-4%)	10mg
DTT	13mM (13-100mM)	20mg
Carrier Ampholytes, 40%	0.5% (0.25-2%)	125µl
Distilled De-Ionized Water		10ml

*Urea can be replaced with up to 25% Thiourea

When DTT is used, a second equilibration step is required. The Iodoacetamide prevents protein re-oxidation during electrophoresis and alkylates residual DTT to minimise vertical streaking.

Table 2. Rehydration of IPG Strips

IPG Strip Length	Volume per Strip (µl)
7cm	130
18cm	340
24cm	450

Table 3. Preset Program parameters in the Hoefer IEF100

Program 2
Name: 7cm Constant watt Delay 0:00, Delay temp 20°C, Run temp 20°C, 500 µA, 6000 V, 0.5 W
Step 1, Constant watt, 0.1W, 1:00 Hrs
Step 2, Constant watt, 0.5W, 8000 Vhrs
Step 3, Constant volt, 1000 V, 1:00 Hrs

Table 4. Equilibration Buffer I (EBI)

Prepare immediately prior to use		
Reagent	**Concentration Range**	**Amount**
6M Urea	3.6g/10ml	4.8g
2% SDS	0.2g/10ml	10mg
0.375M Tris-HCl	2.5ml 1.5M	20mg
20% Glycerol	2ml/10ml	125µl
130mM Iodoacetamide	200mg/10ml	10ml

pH 8.8 for the 10ml mixture

Table 5. Equilibration Buffer II (EBII)

Prepare immediately prior to use		
Reagent	**Concentration Range**	**Amount**
6M Urea	3.6g/10ml	4.8g
2% SDS	0.2g/10ml	10mg
0.375M Tris-HCl	2.5ml 1.5M	20mg
20% Glycerol	2ml/10ml	125µl
135mM Iodoacetamide	250mg/10ml	10ml

pH 8.8 for the 10ml mixture

Table 6. 10% Acrylamide solution resolving gel

	10% Acrylamide gel
Distilled Water	6.3ml
30% Stock Acrylamide Solution	5ml
4 X Tris-Glycine Solution pH 8.8/ 0.4% SDS	3.75ml
10% Ammonium Persulphate	150µl
TEMED	15µl

Table 7. 4% Acrylamide solution stacking gel

	4% Acrylamide gel
Distilled Water	6.1ml
30% Stock Acrylamide Solution	1.3ml
4 X Tris-Glycine Solution pH 8.8	2.5ml
10% SDS	100µl
10% Ammonium Persulphate	50µl
TEMED	10µl

New Validated Cell Colony Formation Assay

TTP LabTech has validated a new cell colony formation assay on the Acumen® eX3 fluorescence microplate cytometer in collaboration with ChemPartner. As an alternative to the traditional method this latest protocol uses fixed as opposed to live cell colonies for analysis.

The Acumen eX3 laser scanning cytometer is an ideal platform on which to analyse cell colony formation assays. It enables rapid and accurate enumeration of colony number and is more suitable for higher throughput compound assessment than current microscope based methods. This non-confocal system enables scanning of whole wells for fluorescent objects without the need for the nuclear stains as required by other platforms. This approach determines colony number through the application of a volume algorithm and permits the differentiation of cytostatic effects, where the number of colonies and size remains constant, from cytotoxic effects where the size and number may be reduced. Application of Acumen's microplate cytometric technology offers significant benefits over alternative methods in the search for novel chemotherapeutic agents and provides a simple high content and high throughput assay.