

Investigations of EGF Receptors in Ovarian Cancer Cell Lines Using Chemiluminescence Detection for Gel Electrophoresis

The use of chemiluminescence indicators in gel electrophoresis applications has become increasingly popular in recent years. Chemiluminescence indicators can provide a useful alternative to radioactive indicators and do not suffer from the health and safety concerns associated with the use of radioactive material. Chemiluminescence output, however, is inherently weak in intensity and is most frequently detected by exposure to film over an extended period of time. This application note introduces ChemiGenius, a specially developed gel documentation and analysis system which combines the use of an automatic analysis system and a high sensitivity cooled CCD camera for direct imaging of chemiluminescence emissions.

The Oncology Research Laboratory at Derby City General Hospital has a special interest in the way ovarian cancer cells respond to Epidermal Growth Factor (EGF), expression of EGF receptor (EGF-R) and how this may be affected by tyrosine kinase inhibitors. The Department has 10 human ovarian cancer cell lines which are being test screened for sensitivity to clinically used anti-cancer drugs and have potential for identifying new drugs with therapeutic activity against ovarian cancer. Cellular response to EGF is dependent on specific binding of EGF to its receptor, which in turn activates receptor-associated tyrosine kinase. This then leads to activation of other kinases involved in the cascade mechanism which lead to mitosis. Tyrosine kinase phosphorylation may be a result of EGF binding or autophosphorylation. Antibodies to phosphotyrosine provide one of the best tools for the detection and characterisation of phosphotyrosine-containing proteins. Whereas traditional phosphotyrosine analysis required radiolabelling of proteins with ³²P and phosphoamino acid analysis, antibodies to phosphotyrosine provide a fast, safe and easy way to detect phosphotyrosine even in complex cellular systems. The use of a peroxidase linked secondary antibody allows the chemiluminescence agent ECL to be bound to the phosphotyrosine antibodies.

Direct imaging of chemiluminescence

The traditional method of detecting chemiluminescence using film has a number of disadvantages. Firstly, it has only two orders of magnitude of dynamic range, making quantitative analysis unreliable. In addition, the development process requires expensive equipment and involves the use of toxic chemicals. This makes the entire process extremely costly for groups running only a few gels per week. Cooled CCD cameras offer the sensitivity and dynamic range required for the extremely low light emissions from such samples, and have been used with varying degrees of success.

However, the ChemiGenius system has heralded a new era in the analysis of chemiluminescent samples. ChemiGenius is a complete analysis system which features a darkroom cabinet containing a Peltier cooled CCD camera with a motorised zoom lens, linked to a PC for control and analysis using a fully integrated image acquisition, archiving and analysis package running in a Windows™ 95, Windows™ 98 or Windows™ NT environment.

Using this system, the exposure time of the camera can be adjusted and a digital image of the gel automatically acquired and saved. Not only that, a completely automatic analysis of the saved image can be carried out within seconds of the image being acquired, with a

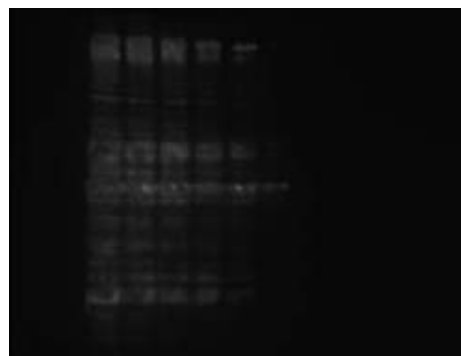
report conforming to Good Laboratory Practice containing a full analysis of the gel automatically generated. Newly developed detection algorithms will also automatically follow grimages allowing the software to follow the band shape. By calibrating a marker lane, the molecular weights of all of the other bands on the gel are immediately and automatically calculated and displayed. Calibration can be carried out using a library of marker peak numbers or a known marker of the user's choice. Quantitative analysis of the molecular components can also be important and the area under the histogram peak for each band is proportional to the concentration of the material there. By using a marker of known concentration, the quantity, or volume, of the unknown can be automatically calculated.

Why cooled CCD cameras?

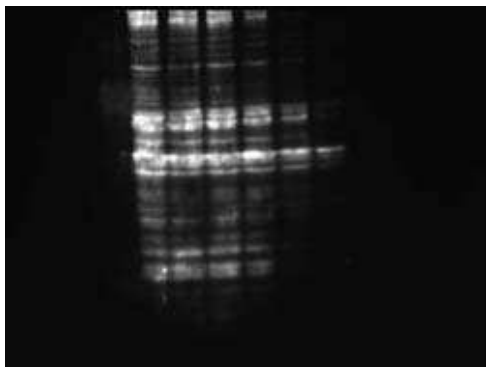
In many applications for gel documentation the normal CCD camera frame rate of 1/30th second is suitable to capture high quality images of fluorescent or non-fluorescent samples. However, when using extended exposures with these types of cameras the inherent dark current will contribute an increasing level of noise to the signal resulting in a degradation of the image although with the normal SYNGENE camera this is not such a problem as with many other competitive systems. When working with Chemiluminescence applications individual frames may be integrated for several seconds or even minutes to achieve the final high sensitivity image. To operate in this way the CCD detector must be cooled to reduce the inherent dark current to a level which does not effect the signal.

Cameras can be cooled either cryogenically or by Peltier elements. The use of liquids and in particular nitrogen is often not convenient in many laboratories and so the better method is by using electronic Peltier cooling. There are many different ways in which the level of cooling can be expressed with cameras being expressed as operating from anywhere between -10C and -80C. It is important here to note whether this is an absolute temperature or simply a below ambient temperature.

With ChemiGenius we have found that at -40C the NEW cooled camera integrated into the system performs as well as those cameras quoted to operate at much higher levels simply because the "chip" used produces considerably less dark current than other brands of device. Another factor which should also be taken into consideration is the dynamic range of the camera. Again the new Syngene camera has a very large range, about 15-20 times that of film.



(image without cooling)



(image with cooling)

A typical experiment

Lysates were prepared from a panel of seven continuous human ovarian cell lines from ascites and tumour tissue. For four of the cell lines (OAW28, D13, 180D and 200D) lysates were prepared from both untreated cells and cells which had been treated with EGF (100 ng/ml for 10 minutes). Two human mesothelial cell lines, D739 and D742 were grown on media with or without EGF and hydrocortisone before lysate preparation. All lysates (40 µg/lane) were then loaded onto sodium dodecylsulphate polyacrimide gels (7.5%) using a Pharmacia SE600 unit. A431 (human epidermoid tumour cell line with high levels of EGF receptor) was used as a positive control for the antibody. The gel was electrophoresed overnight (10 mA) and then blotted onto a supported nitrocellulose membrane (Optitran Schleicher and Schuell) using a Transphor unit (Pharmacia) for 4 hours (370 mA). A rocking platform was used throughout the following procedure for all washings. The membrane was washed for 10 minutes at room temperature in Tris-Tween (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) and then blocked for 1 hour at room temperature in blocking buffer (Tris-Tween with 3% BSA). The membrane was then immunostained using PY20 antibody which detects tyrosine phosphorylation. PY20 mouse monoclonal antibody (Transduction Laboratories) was added at a dilution of 1:1000 in blocking buffer. The membrane was placed in a sealed bag and incubated overnight at 4°C. The PY20 was removed and the membrane washed with Tris-Tween for 30 minutes, changing the Tris-Tween every 5 minutes. Secondary antibody (sheep anti mouse Ig peroxidase linked whole antibody, Amersham) was added at a dilution of 1:2000 in blocking buffer (again in a sealed bag) and incubated at room temperature for 2 hours. The membrane was then washed in Tris-Tween for at least 1 hour, changing the Tris-Tween every 15 minutes. This was followed by treatment of the membrane with ECL (Amersham) for 1 minute, washing in 100 mM Tris pH 7.5 followed by further treatment with the ECL solution for 1 minute. The membrane was wrapped in Saran wrap and then examined in the ChemiGenius unit.

Results

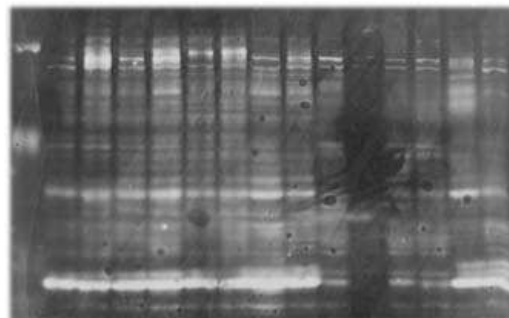


Figure 1. The image shown in Figure 1 resulted from imaging the gel for two minutes using ChemiGenius.

The ChemiGenius system was then used to conduct the analysis, as shown in Figure 2a.

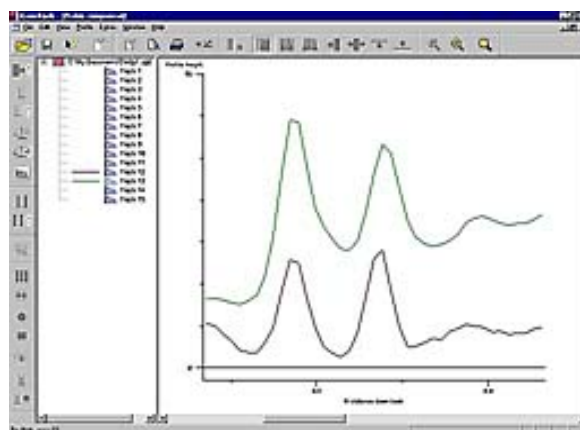


Figure 2a.

Track 12				Track 13			
Number	Mol weight	Height	Raw vol.	Number	Mol weight	Height	Raw vol.
1	224.99	12.330	10403.37	1	221.35	18.080	11752.57
2	180.49	25.788	5036.53	2	180.49	59.120	18496.75
3	165.00	28.181	4743.95	3	165.00	53.259	15785.91
4	115.44	22.179	20877.26	4	112.76	59.732	143226.23
5	73.83	19.324	6741.07	5	73.83	70.351	52021.30
6	66.68	12.357	3635.43	6	67.73	69.071	36400.16
				7	58.36	49.650	19512.42

Figure 2b.

The band of interest has a MW of around 180KD. The results show the presence and absence of the 180KD band and the relative concentration of treated and untreated samples.

The tracks are paired with 2-9 and 15 being epithelial, tracks 10, 11, 12 and 13 are mesothelial, track 14 is the A431 control and track 1 is the BioRad Kaleidoscope molecular weight marker.

The results shown are for the tracks 12 and 13. The histograms are overlaid for a full comparison.

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