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MICROSCOPY & IMAGE ANALYSIS — **APPLICATION NOTE**

Automated imaging of Western blots & clonogenicity assays

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One of the world's top five pharmaceutical companies, AstraZeneca, currently spends over £5 million each working day on global research and the development of new therapeutics for a range of diseases.

One of the company's major research targets is to deliver more than 15 candidate drugs annually by 2003. To reach this target, the company needs to constantly find ways to improve the speed and accuracy of its preclinical research processes. An example of this is the company's current work in one of its Cancer Research Groups at Alderley Park (Cheshire, UK).

The Cancer Research Group is working on a range of cancers, including breast, colorectal, lung and prostate, and it is studying how small molecules can regulate the proteins involved in apoptosis (programmed cell death). Studying molecular functions of the protein involved in the mechanisms of apoptosis could lead to insights into how to design molecules that eliminate these types of tumour cells through apoptosis induction. Broadly, there are two mechanisms by which cells die due to apoptosis; one is generated by signals arising within the cell and the other mechanism is triggered by death activator proteins that bind to receptors at the cell surface.

The group has been looking at ways to improve the accuracy and speed of Western blot and clonogenicity assay endpoints to measure the effectiveness of small molecules and antisense oligonucleotide as potential lead compounds.

Western Blot Analysis

The group treated cells with antisense oligonucleotides (synthetic pieces of single-stranded DNA that bind to specific sequences of mRNA in gene targets) targeted at oncogenes, which are believed to negatively regulate apoptosis in tumour cells. Cultured human cell lines such as A549 and SJSA-1 over-expressing the oncogene were used for the assays.

Antisense oligonucleotides were added to these cells to determine if they could down-regulate the oncogenes and whether this translated into effects on signalling pathways. To visualise such apoptosis-regulating proteins, cell lysates were harvested and run out on polyacrylamide gels. The proteins were transferred onto nitrocellulose or nylon membranes by standard Western blotting and down-regulation of target oncogene product or altered signalling was measured using a range of polyclonal and monoclonal antibodies. Some antibodies used were proprietary and specific to AstraZeneca.

The primary antibodies directed at the oncogene product were detected using secondary anti-sheep or anti-rabbit antibodies conjugated to horseradish peroxidase enzymes. The blots were stained with a chemiluminescent substrate, either ECL Plus (**Amersham-Pharmacia,** Bucks, UK) or, more commonly, Super Signal (**Pierce Chemical Co.,** Rockford, IL, USA) because the ChemiGenius image analysis system (**Syngene,** Cambridge, UK) was optimised for use with this reagent.

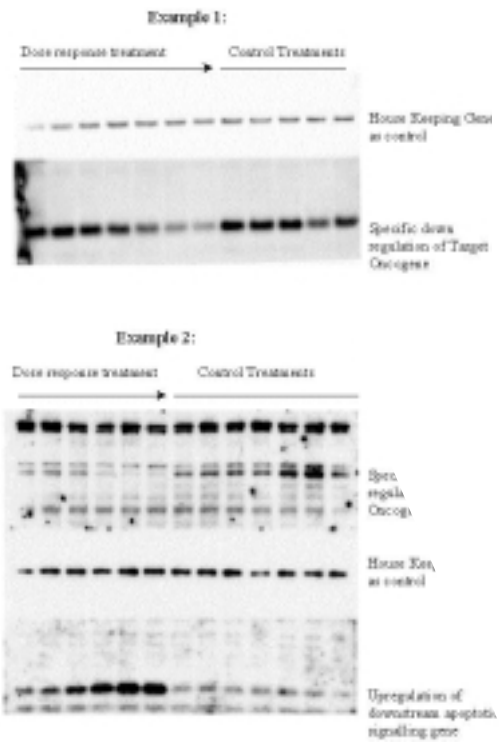

Figure 1

Example of a Western blot stained with Super Signal obtained using the ChemiGenius system.

In initial experiments, blots were exposed using autoradiography film and a developer in a dark room for 5–10 min and the amounts of protein produced were compared, by eye, with protein standards made from proprietary AstraZeneca in-house gene controls.

Automating Western Blot Analysis

Visualising blots does not allow for accurate protein quantification. In addition, due to variability between assays, the group found it difficult to judge the time needed to obtain the correct exposure. Consequently, researchers had to use several films to obtain an optimum exposure and were not able to determine when the film was over-exposed.

In an attempt to bypass these limitations, the group evaluated systems with sophisticated charge-coupled device (CCD) cameras with reduced noise and longer integration abilities, enabling them to automate their chemiluminescence imaging.

The group chose the ChemiGenius image analysis system, which has its own light-tight cabinet with transilluminated UV and white light sources and three filters for fluorescent applications. It has a camera that allows real-time image capture, but shows such low noise levels that it can acquire images over long periods, making it well suited for capturing the group's chemiluminescent Western blot images (*Figure 1*).

For the group's chemiluminescent applications, the ChemiGenius offers the advantage of being a genuine 16-bit system. The bit number represents how the CCD camera output signal is converted to a readable signal. This measurement is the number of grey levels that the camera sensor can resolve; for example, an 8-bit camera is $2^8 = 256$, and a 16-bit camera is $2^{16} = 65,536$.

The higher the number of grey levels, the greater the dynamic range of the camera. In simple terms, this means that when quantifying a chemiluminescent protein band image, the Cancer Group could distinguish between faint bands and brighter ones.

The system's dynamic range is also important for the group's Western blot work because its 16-bit images provide 65,536 grey levels, which translates to a dynamic range of 4.8 orders, more than double that of photographic film. This higher level of accuracy gives the group greater confidence when performing quantitative studies to visualise the amounts of protein produced. Another positive aspect that the group found was that because of the ChemiGenius's wider dynamic range, the researchers were able to tell when they had reached the top end of the dynamic range, whereas when they used film, they found this difficult with potential for (unknown) over-exposures.

Another difficulty the group faced in working up the assay was accurately measuring the amount of protein on a blot. Assessing protein quantities by simple visual comparison of the film of an unknown protein with protein standards produced a very rough approximation.

The group uses quantitative Western blot data as the basis of measuring the IC_{50} (the concentration at which the treatment results in 50% down-regulation of the target protein or cell signalling) of the molecule being screened. These IC_{50} results are then used by AstraZeneca's medicinal chemists to alter the design of the molecule to increase potency. Therefore, they need protein quantification results that are as accurate as possible.

To overcome this problem, the group uses the ChemiGenius's GeneTools software to analyse their Western blots. The software automatically compares the amount of protein in standards with specified protein bands to produce quantified protein results from the captured images. The software produces accurate protein data, which are stored in a **Microsoft** Excel (Redmond, WA, USA) spreadsheet and can be used to calculate IC_{50} results.

Clonogenicity Assays

The Cancer Group tested the effectiveness of antisense oligonucleotides and small molecule inhibitors in cell clonogenicity assays. Tumour cells that had been treated with antisense molecules were plated out on selective media and, following a number of days of growth, colonies were stained with crystal violet. The colonies were counted manually and compared to control treatments to determine the antisense oligonucleotide's potency.

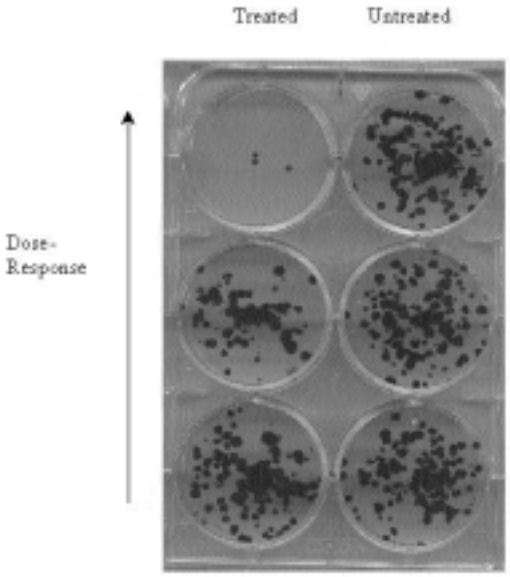

Figure 2

Example of a clonogenicity assay showing colonies stained with crystal violet obtained using the ChemiGenius system.

Automating Clonogenicity Assays

The Cancer Group's main problem with the manual colony counting method was that it was very time consuming. The group now uses the ChemiGenius for automated colony counting because its one-click image capture technology means that it can quantify the number of colonies on a plate in seconds and store the images of the assays for future reference (*Figure 2*).

Conclusion

The Cancer Group chose the multi-purpose ChemiGenius system because it could handle both fluorescent and white light imaging applications from DNA gels, protein gels and colony plates, making it good value compared to alternative imaging systems.

The system has improved certainty in the group's research results. The dynamic range of the camera, coupled with automated software analysis, has ensured that quantification of protein on Western blots is more precise. This in turn has meant that the group has more confidence in its IC_{50} results, which helps the chemists to modify these molecules. In addition, the automated image capture has produced more rapid colony counting with its clonogenicity assays. The group recognises the system's advantages and versatility, which are reflected in its widespread application in other cancer projects.

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