pGL2 vector series, which all contain the same multiple cloning site, in part composed of MluI and NheI sites. In consequence, these vectors may contribute by themselves to background luciferase activity and confound the analysis of results obtained in co-transfection experiments using transcription factors such as the SREBP or other transcription factors of the basic helix-loop-helix family. Therefore, the use of the unmodified pGL3 and pGL reporter vectors should be avoided (or carefully controlled) in studies aimed at exploring the activity of these transcription factors.

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Generalized 96-Well Format for Quantitative and Qualitative Monitoring of Altered Protein Expression and Posttranslational Modification in Cells

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A cornerstone of analysis in cell biology is monitoring changes in the level or posttranslational state of specific proteins. Virtually all techniques that do so rely on the specificity of immunoglobulins to bind an antigen of interest, while subsequent detection steps allow for the indirect visualization of the target molecule. Western blotting is the most sensitive and accurate assay to determine the abundance and size of a given antigen. However, this method is laborious since it requires protein extraction, size fractionation, transfer, and detection. Consequently, it can only be used with a modest number of samples. In addition, a good deal of valuable information is lost by western blot analysis, such as determining which particular subset of cells within the population express the antigen and where within the cell it localizes. A complementary approach is to employ immunohistochemistry to detect antigen expression in situ using horseradish peroxidase (HRP)-mediated oxidation of a precipitating chromagen, typically 3,3′-diaminobenzidine (DAB). This produces a dark brown precipitate that can be used to identify expressing cells. While this approach allows for the acquisition of the types of data lost by western blotting, quantification relies on labor-intensive manual counting techniques that are susceptible to researcher bias (i.e., what constitutes a “positive” cell? How strong is the staining relative to control cells? Etc.). Given the number of genes that may represent therapeutic targets for pharmacological research, the technical constraints associated with both of these approaches represent real limitations.

Here we describe an easy, sensitive, and quantitative high-throughput assay that can be employed with any antisera to monitor changes in the absolute levels or conformational state of specific cellular proteins. In addition, an optional step allows visualization of cells containing the antigen. This allows for both the quantification of the number of antigen-positive cells and the level and subcellular distribution of the antigen.

This assay is based on standard immunochromatographic techniques utilizing an appropriate primary antibody, followed by detection with an HRP-labeled secondary antibody using 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (1) as the chromagen. In the
To demonstrate the utility of this method, we monitored the levels of three different proteins in the mammalian myoblast cell line C2C12: sarcolemmal myosin heavy chain (MHC) (new gene expression); caspase-3 activation (change in protein conformation); and Sug1 26S proteasome subunit expression (constitutive protein expression). When cultured in growth medium (DMEM + 10% FBS), C2C12 myoblasts readily cycle (5). Following transfer to differentiation medium (DM) (DMEM + 5 µg/mL each of insulin and holo-transferrin), some cells cease cycling, fuse, form multinucleated myotubes, and upregulate muscle-specific genes, such as MHC. Other cells undergo apoptosis, a process that is mediated by the proteolytic activation of endogenous pro-caspase-3 (2).

C2C12 cells were seeded at a density of 8000/well in 96-well plates, resulting in approximately 80% confluency. At different times after transfer to DM (described in Figure 1 legend), cells were fixed for 1 h at 4°C in 3% paraformaldehyde, washed three times with PBS containing 0.1% Tween® 20 (PBST), and then blocked for 30 min in PBST with 10% normal serum of the species used to generate the secondary antisera (carrier solution). Cells were then incubated overnight at 4°C with (i) a 1:100 dilution of a rabbit polyclonal antiserum against activated caspase-3 (Cell Signaling Technology, Beverly, MA, USA), (ii) a 1:100 dilution of a mouse monoclonal antiserum against MHC (MF20; Developmental Studies Hybridoma Bank, University of Iowa), or (iii) a 1:200 dilution of a rabbit polyclonal antiserum directed against the mouse Sug1 26S proteasomal ATPase (Cell Signaling Technology, Beverly, MA, USA) until the desired intensity was observed. As expected, MHC expression in -myotubes was restricted to multinucleated myotubes, activated caspase-3 was seen in condensed mononucleated cells, and Sug1 was equally expressed in all cells (data not shown).

At the end of the assay, cells were washed once in PBS and incubated at room temperature in 1× SigmaFAST™ DAB/Urea H2O2 reagent (Sigma, St. Louis, MO, USA) until the desired intensity was observed. As expected, MHC expression was restricted to multinucleated myotubes, activated caspase-3 was seen in condensed mononucleated cells, and Sug1 was equally expressed in all cells (data not shown). The level and distribution of the DAB

**Figure 1.** Quantitative measurement of differentiative events in C2C12 myoblasts. Spectrophotometric measurement of antibody binding in C2C12 myoblasts following incubation in growth (GM) or differentiation (DM) media. Comparison for (A) induced sarcolemmal myosin heavy chain (GM and 3 days in DM), (B) activated caspase-3 (GM and 2 days in DM), and (C) constitutively expressed Sug1 (GM and 2 days in DM). Data are mean ± standard error for 8 replicate wells/group.

**Figure 2.** Visualization of myosin heavy chain expression in myotubes with and without prior protein quantification with ABST. C2C12 myoblasts were induced to differentiate in vitro and stained with a monoclonal antibody directed against myosin heavy chain. Cells were then processed as described in the text and stained with either DAB alone (A) or with ABST followed by DAB (B). Photography and processing were performed identically for both groups of cells. Scale: myotube diameters are approximately 30 µm.
staining was indistinguishable from that obtained without prior incubation with the ABTS reagent (Figure 2). Consequently, both quantitative levels and cellular distribution data can be readily obtained from the same cells in this assay.

Presumably, this assay can be extended to monitor the expression, conformational change, or posttranslational modification of any protein for which an appropriate antiserum exists. Obviously, it is necessary to optimize the assay with each different antiserum employed. It is worth varying antiserum concentration over a broad range to enhance the signal-to-noise ratio. A separate but important issue is normalizing the measured signals to the number of cells in the assay. One option is to employ a duplicate column of cells within each plate for separate staining and would loosen the limit for the maximum size of the PCR fragments. To achieve the DNA restoration, we have developed the following protocol.

We assumed that the poor quality of PET-derived DNA is at least to some extent attributed to the single-strand breaks (1). Therefore, the non-denatured DNA template can be partially restored by filling of the nicks in the DNA polymerase reaction (8). This would eventually result in better suitability of archival DNA for genetic investigations and would loosen the limit for the maximal size of the PCR fragments. To achieve the DNA restoration, we have developed the following protocol.

First, the 5 µm-thick, formalin-fixed, paraffin-embedded tissue sections are subjected to the standard deparaffinization procedure (incubation in xylene and ethanol for 30 min each).

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Partial Restoration of Degraded DNA from Archival Paraffin-Embedded Tissues

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The development of PCR methods for the analysis of archival paraffin-embedded tissues (PET) has significantly improved the efficiency of medical research and diagnostics. However, the utilization of PET has some limitations related to the partial degradation of DNA during the fixation procedure (7,10). Although several studies have reported the successful amplification of the DNA sequences up to 1 kbp long, it is generally agreed that the fragments below 180 bp seem to be the most suitable for routine, highly reproducible PCR analysis of PET (9,10). The investigations aimed to improve the yield and quality of PET-derived DNA have drawn a high level of attention. The proposed technical tips include the right choice of the fixative protocol (4), prolonged proteinase K digestion (5), use of Chelex® X-100 ion exchange resin (2), etc. Unfortunately, the actual efficiency of these modifications varies between different laboratories; therefore, the domestic optimization of DNA isolation may require an experimental comparison of the published approaches. Here, we present a novel supplementary manipulation that leads to a noticeable facilitation of PCR analysis of archival tissues.

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