Application Note

Using the Z'-Factor Coefficient to Monitor Quality of Near-Infrared Fluorescent Cell-Based Assays

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The Z'-factor coefficient was adapted to monitor the quality of immunocytochemical assays such as the In-Cell WesternTM assay. Z'-factor experiments were performed throughout the development of three different assays and factors that significantly affected the signal dynamic range or variability in replicate positive and negative control samples were easily detected. The effect of changes made to three different key assay factors was demonstrated: choice of primary antibody, method of cell permeabilization, and method of cell number normalization. Z'-factor was also used to meas- ure the effect of external factors such as day to day assay execution.

Keywords: In-Cell Western, Z'-factor, near infrared

Introduction

Many factors affect the quality and reproducibility of microplate-based immunocytochemical assays like the In-Cell WesternTM (ICW) assay. Consquently, an efficient, reliable performance measurement for detection of changes in assay quality is desirable. The Z'-factor coefficient is a simple statistical parameter that is commonly used to assess the quality of high throughput screening (HTS) assays. The Z'-factor is unitless and sensitive to factors which affect either the dynamic range or variation in measured signal between a group of positive controls and a group of negative controls [1]; therefore, Z'-factor can be used for direct comparison of individual ICW experiments.

The ICW assay is a near-infrared (NIR) immunocytochemical assay which enables in situ analysis of protein signaling events in cultured cells. Though based on conventional immunocytochemical methods, ICW utilizes near-infrared chemistry and a dual laser fluorescence excitation instrument for signal detection. The result is higher sensitivity and reduced interference from cells, microplates, and drug compounds. Two spectrally distinct detection chemistries permit normalization of target signal against total protein amount in a single well, thereby providing for accurate quantitation [2]. Here we demonstrate how Z'-factor, together with fold-response calculations and graphical examination of data, can be utilized to monitor the quality of ICW assays during optimization and after finalization.

Materials and Methods

Nunc™ 96-well tissue culture microplates were obtained from Thermo Fisher Scientific (Rochester, NY). Odyssey® Blocking Buffer, infrared labeled secondary antibodies, Sapphire700™ cell stain, the Odyssey Infrared Imager, and the Aerius® Infrared Imager were obtained from LI-COR Biosciences (Lincoln, NE). DRAQ5TM DNA stain was obtained from Biostatus Limited (Shepshed, Leicestershire, UK).

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Primary antibodies were selected based on their performance on Western blots against appropriately stimulated cell lysates using infrared-labeled secondary antibodies and the Odyssey instrument for fluorescent detection. In cases where many antibodies to the desired target were available, those that produced the highest signal to background ratio were chosen for further use in ICW Z'-factor studies. Because there is no protein separation as with traditional Western blotting, antibody specificity is critical for ICW; thus, only antibodies that yielded a single band on Western blot were used for testing.

A-431, NIH/3T3 and HeLa cells, and fetal bovine serum, were obtained from American Type Culture Collection (Rockville, MD). DMEM Cell culture medium was obtained from Sigma-Aldrich (St. Louis, MO). Cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were seeded in 96-well microplates at approximately 1.5 X 104 cells per well and allowed to grow to 85-90% confluency. For serum starvation, medium was removed by aspiration, replaced with serum-free DMEM, and incubated at 37˚C for 4-16 hours.

Epidermal Growth Factor (EGF) was obtained from R&D Systems (Minneapolis, MN). Anisomycin was obtained from Sigma-Aldrich (St. Louis, MO). Platelet Derived Growth Factor (PDGF) was obtained from Millipore (Bellerica, MA). Cell culture microplates were incubated with the appropriate stimulatory substance and incubated at the predetermined optimal concentration and time interval. Following incubation, the medium was discarded and the cells were fixed with 3.7% formaldehyde in PBS for 20 min. The fixative was then discarded and the cells were permeabilized either with five washes with 0.1% Triton® X-100 in PBS (5 min each), or with one 10 min wash using ice-cold methanol. Permeabilization was carried out at room temperature with gentle shaking. Next, Odyssey Blocking Buffer was added and the cells were incubated for 1.5 h at room temperature with gentle shaking.

Normalization for well-to-well cell number variability was performed by ratiometric analysis between the phosphorylation antibody in one wavelength, and total protein in the second wavelength. Measurement of total protein content was accomplished using either mouse anti-ERK2 pan antibody from Santa Cruz Biotechnology (Santa Cruz, CA), or a combination of Sapphire700 and DRAQ5 stains.

To normalize the phosphorylation signal against pan antibody, the phosphospecific and pan antibodies were diluted together in blocking buffer and added to the cells simultaneously. To account for background caused by secondary antibody binding to cells nonspecifically, eight microplate wells received diluent only (no primary antibody). The cells were incubated for 2 h at room temperature with gentle shaking. The next day, the primary antibody solution was discarded and the plate was washed four times for 5 min each with gentle shaking, using PBS wash buffer containing 0.1% Tween® 20. The appropriate NIR dye labeled secondary antibodies were diluted together in blocking buffer containing 0.2% Tween 20 and added to all of the cells simultaneously. The cells were incubated for 1 h at room temperature with gentle shaking. The secondary antibody solution was discarded and the plate was washed four times for 5 min each with gentle shaking, using PBS wash buffer containing 0.1% Tween 20. The plate was imaged by scanning with the Odyssey or the Aerius Imager in both the 700 and 800 nm channels simultaneously.

To normalize the phosphorylation signal against cell stains, the phospho antibody was added and incubated as described above. Sapphire700 diluted 1:1000 in PBS and DRAQ5 diluted 1:2000 in PBS were added to the secondary antibody solution. Sapphire700 and DRAQ5 stains were not included in background wells. Washes, secondary antibody solution incubation, and imaging were carried out as described above.

Stimulation conditions were predetermined by adding EGF, PDGF or anisomycin to plated cells at increasing concentrations and varying time intervals. Treatment conditions that exhibited a maximum fluorescent signal response were used to generate the positive controls in subsequent Z'-factor experiments. Cells that received no stimulatory treatment were designated as negative controls in Z'-factor experiments. Each Z'-factor plate contained 40 replicate negative controls and 48 replicate positive controls. Eight wells were used for fluorescent background subtraction, in which neither primary antibody nor stain was added. Plates were scanned on the Odyssey or Aerius Imager, and fluorescent signal intensity values were measured. Mean and standard deviation of intensity were calculated for each set of controls. Z'-factor was then calculated for each plate:

$$\text{Z'-factor} = 1 - \frac{3\text{SD of pos controls} + 3\text{SD of neg controls}}{|\text{mean of pos controls} - \text{mean of neg controls}|}$$

ICW assays which consistently produced Z'-factor values ≥ 0.5 were considered to be high-quality assays. Z'-factor experiments were performed to discern changes in quality with each major change to the assay. After assay finalization, three 96-well plates were run on different days to obtain an average performance value.

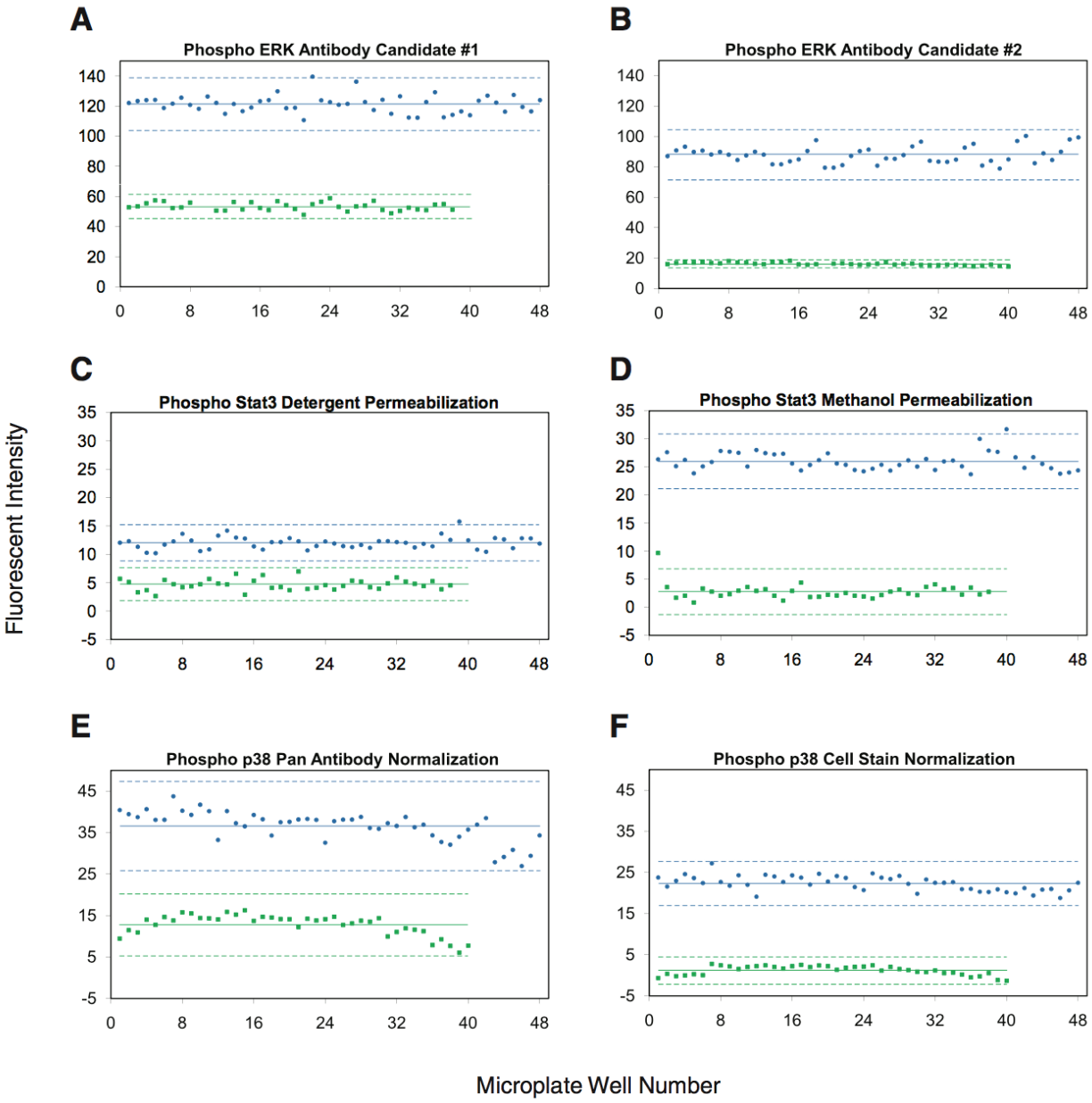

Figure 1. Graphical comparison examples of three different optimization conditions for three different ICW assays. Each scatter plot represents positive control data (blue, round data points) and negative control data (green, square data points) for a specific experimental condition in a given assay. Solid lines represent mean fluorescence, and dashed lines represent three standard deviations above and below each set of control data points. (A, B) Comparison of two different primary antibody candidates for detection of phospho ERK in A-431 cells stimulated with EGF. (C, D) Comparison of two different permeabilization methods for detection of phospho Stat3 in A-431 cells stimulated with EGF. (E, F) Comparison of two different normalization methods for detection of phospho p38 in anisomycin-treated HeLa cells.

Results

Throughout the ICW assay development process, many factors were considered in order to maximize quality, including microplate type and manufacturer, antibody concentrations, antibody incubation conditions, cell fixatives and fixation conditions, and instrument settings. In three separate cases the following factors elicited a distinct effect on Z′-factor values: choice of primary antibody, method for cell permeabilization, and method of cell number normalization. We present here assay optimization examples of these three factors to illustrate the importance of statistical quality measurement and visual inspection of data during assay optimization.

The mean Z′-factor value for two different phospho ERK antibody candidates was ≥ 0.5, signifying a good quality assay for both candidates. However, the Z′-factor for candi-

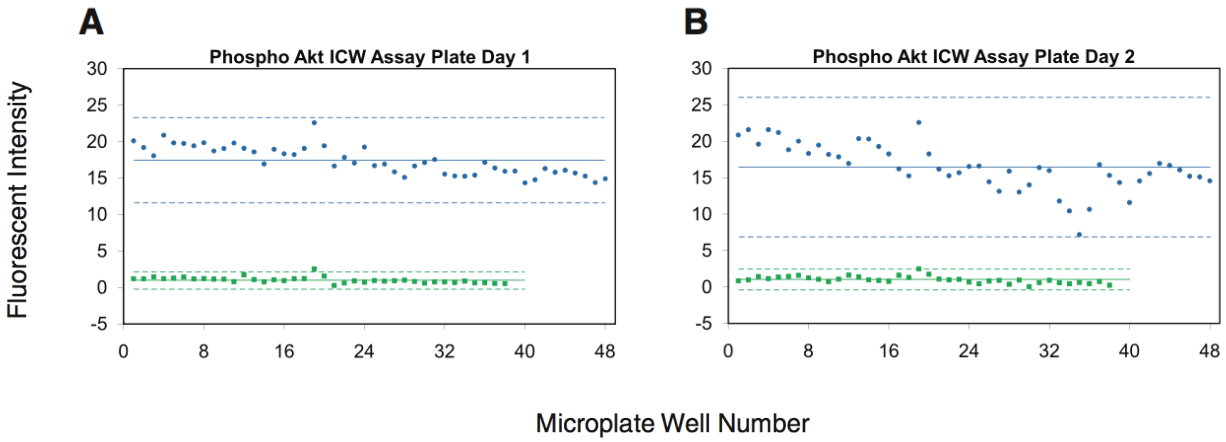

Figure 2. Graphical comparison example of an ICW assay run on two different days. Detection of phospho Akt in PDGF-stimulated NIH/3T3 cells was performed on day 1 (A), and repeated on day 2 (B). Each scatter plot represents positive control data (blue, round data points) and negative control data (green, square data points). Solid lines represent mean fluorescence, and dashed lines represent three standard deviations above and below each set of control data points.

date #2 was consistently higher from plate to plate than candidate #1. Candidate #1 gave a mean Z'-factor value of 0.52 (from three plates), with a corresponding mean fold-activation of 2.0. Candidate #2 gave a mean Z'-factor value of 0.73 (from three plates), with a corresponding mean fold-activation of 8.0. Figure 1, panels A and B, provide a graphical representation of the fluorescent data points for one of the three plates for each antibody. In this case, the higher Z'-factor value, resulting from both a higher fold-activation and from very low well-to-well fluorescence variability, provided sufficient reasoning for choosing candidate #2 over candidate #1.

There are many commonly accepted methods for fixation and permeabilization of cells in immunocytochemistry applications. We routinely employ formaldehyde fixation in ICW assays, followed by permeabilization with a detergent such as Triton X-100. In some cases, harsh detergents can damage protein epitopes, so methanol is used as an alternative. As an example, detergent and methanol permeabilization methods were tested in an ICW assay for detection of phosphorylated Stat3 protein. The calculated mean Z'-factor value (from three plates) obtained from Triton X-100 permeabilization in this assay was 0.01, with a corresponding mean fold-activation of 2.6. The mean Z'-factor value (from three plates) obtained from methanol permeabilization was 0.63, with a corresponding mean fold-activation of 10.5. The higher Z'-factor value in this example resulted primarily from the higher fold-activation value. The graph in Figure 1, panels C and D, shows how the fluorescence data points for one of the three plates differed between the two permeabilization methods in this particular assay.

For normalization to be successful, the number of cells must be accurately represented within each well of the microplate. Both pan antibodies and total protein stains were tested to determine the effect on assay quality. For example, when phosphorylated p38 protein in anisomycin-treated HeLa cells was normalized against ERK2 pan antibody, the resulting mean Z'-factor (from three plates) was 0.24, with a corresponding mean fold-activation of 3.1. When the same phosphoprotein was normalized against Sapphire700 and DRAQ5 total protein stains, the resulting mean Z'-factor (from three plates) was 0.51, with a corresponding mean fold-activation of 16.6. In this instance, low signal variability in both the negative and positive controls contributed to attaining a higher mean Z'-factor value. The data for these experiments is shown in Figure 1, panels E and F.

Factors that affect the quality of an assay include not only the material components and experimental conditions, but also environmental and operational aspects such as day-to-day and user-to-user variation. Figure 2 shows that a single Z'-factor measurement is insufficient for making a final assessment of assay quality. In this case, the variability in phospho Akt positive control data changed significantly from day one to day two; so this assay was not considered reliable. Therefore, three or more Z'-factor measurements should be performed, preferably by different operators and on different days, to obtain an accurate assessment of overall assay quality. Z'-factor experiments can also be used to track changes in assay quality, or to determine the effect of unavoidable changes made to the assay, such as changes in reagent suppliers.

Discussion

The Z'-factor coefficient provides a good general assessment of the quality of an ICW assay; however, it does not offer sufficient information regarding the amount of variation or the difference in mean signal between negative and positive control data; this information is useful, particularly during assay optimization, to better understand possible causes of low (< 0.5) Z'-factor values. There can be instances where the Z'-factor is acceptable due primarily to very low data variability, but the percentage of signal increase between negative and positive controls is not suitable for the assay (Figure 1A, for example). Similarly, a wide dynamic signal range may result in an unaccept- able Z'-factor value if data variability is high (Figure 1E, for example).

Graphical representation of the data can also offer useful information for making a qualitative assessment of assay quality, and to better understand the cause for the calculated Z'-factor values. For example, the positive control data in Figure 1B appears to contain a certain amount of periodicity, in which the fluorescent intensity gradually increases and then decreases noticeably about every sixth well. This could be indicative of edge effects which are commonly observed in microplate-based assays.

Much of the fluorescent signal variation in ICW assays comes from the treated cells, which for Z'-factor calculation purposes are designated positive controls. This is because cell treatment conditions introduce another level of variability to the assay. Because of the normally low amount of variability in the untreated cells (negative controls), an alternative to the Z'-factor calculation may be used. An assay coefficient of variation (CVA), defined by Iverson et al, takes into account the variation in positive control data and the dynamic range between negative and positive controls, but not the variation in negative control data [3]. Hence, a greater number of assay conditions can be tested for quality using the same number of microplate wells, thereby reducing assay development time and cost.

Due to its sensitivity to the variability and dynamic range of negative and positive control data, Z'-factor is a good way to determine the robustness and reproducibility of ICW assays. When combined with fold-increase measurement and scatter plots of fluo- rescent signal data, Z'-factor provides a reliable method for monitoring development of ICW assays and tracking assay quality over time.

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