In-Cell Western™ Assay Quality Assessment Using Z'-factor

I. Introduction

During ICW assay optimization, it is important to assess the overall quality and reliability of the assay. The Z'-factor statistic provides a way to evaluate whether or not assay conditions (reagents, protocols, instrumentation, kinetics, and other conditions not directly related to the test compounds) are optimized. Z'-factor, introduced by Zhang et al., is a dimensionless value that represents both the variability and the dynamic range between two sets of sample control data.

Z'-factor experiments are performed on one or more ICW assay plates containing replicate wells designated for background subtraction, negative control samples, and positive control samples. Typically, negative control wells are those in which the cells receive an appropriate treatment to elicit the lowest desired percent response (usually untreated cells); positive control wells are those in which the cells receive an appropriate treatment to elicit the maximum desired percent response; background wells are treated the same as negative control wells, except primary antibody incubation is excluded.

Here we briefly describe how to set up, analyze, and interpret an ICW Z'-factor experiment. For further information on Z'-factor analysis and cell-based assay validation in general, refer to the following publications:

J.H. Zhang, T.D.Y. Chung, K.R. Oldenburg, *A simple statistical parameter for use in evaluation and validation of high throughput screening assays*, J. Biomol. Screen. 4 (1999) 67-73.

V. Boveia, K. Ambroz, D.M. Olive, *Using the Z'-Factor coefficient to monitor quality of near-infrared fluorescent cell-based assays*, LI-COR Application Note (2009).

P.W. Iversen, B.J. Eastwood, G.S. Sittampalam, K.L. Cox, *A comparison of assay performance measures in screening assays: signal window, Z' factor, and assay variability ratio*, J. Biomol. Screen. 11 (2006) 247-252.

B.J. Eastwood, M.W. Farmen, P.W. Iversen, T.J. Craft, J.K. Smallwood, K.E. Garbison, N.W. Delapp, G.F. Smith, *The Minimum Significant Ratio: A Statistical Parameter to Characterize the Reproducibility of Potency Estimates from Concentration-Response Assays and Estimation by Replicate-Experiment Studies*, J. Biomol. Screen.11 (2006) 253-261.

II. EXPERIMENTAL DESIGN

As with any statistical parameter, the accuracy of the Z'-factor value improves with larger data sets used for the calculation. We recommend running at least one full 96-well ICW plate with background wells and replicate negative and positive control wells in order to obtain a meaningful Z'-factor value. For a better assay quality assessment, 3-4 plates should be run on different days. Wells can be arranged as desired, but a typical arrangement may look like this:
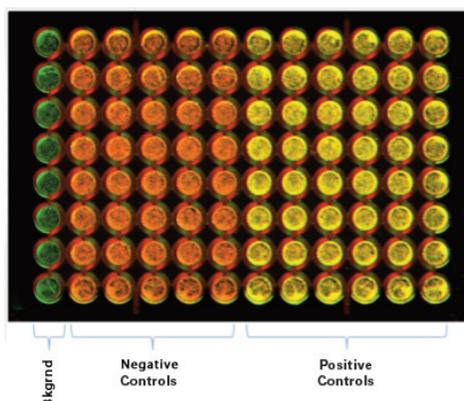
Follow these guidelines when you set up the ICW Z'-factor experiment:

- All reagents (apart from test compounds and reagents), antibodies, materials, and assay conditions should be the same as those that you intend to use for the final ICW assay.

- Background wells must be included on the assay plate to account for nonspecific secondary antibody binding and plate autoflourescence. Background wells are treated the same as the negative control wells, except that primary antibody is excluded; only antibody diluent (i.e. blocking buffer) should be applied to background wells during this step.

  Important: If DNA stain or NHS ester protein labeling is used for normalization, these reagents should also be excluded from the background wells.

- Be sure to correct for well-to-well variation by normalizing against a second primary antibody, a DNA stain, or by total protein labeling. Refer to the section on normalization for more information.

- When choosing treatment compounds to be used for Z'-factor analysis (i.e. stimulants, inhibitors, drugs, etc.), select well-characterized agents which give the best known response in an appropriate cell line.

III. Analysis

The Z'-factor value can be calculated manually using normalized intensity values, or by enabling Z'-factor calculations within the Odyssey application software (v.3.0 or later).

Manual Calculation

1. Export the 700 and 800 channel intensity values Grid Sheet for each well of the microplate to a spreadsheet software application (e.g. Microsoft Excel). Values should not be background-subtracted. Refer to the Odyssey User Guide or the Odyssey Tutorial Manual for detailed instructions.

2. Calculate the mean background intensity in each channel for the background wells.

3. In each channel, subtract the appropriate mean background intensity value from each sample well (negative and positive controls).

4. In the channel used for normalization only, determine the maximum intensity value from the sample wells (negative and positive controls). Divide each intensity value by this maximum value. The resulting relative intensity values will normally be between 0.0 and 1.0, though negative numbers are possible.

5. In the channel used to analyze response, divide all values from the sample wells (negative and positive controls) by the corresponding relative intensity value calculated in step 4. This is the normalized intensity value that will be used to calculate the Z'-factor.

6. Calculate the mean and the standard deviation for each sample set (negative and positive controls) using the normalized intensity values for each of the sample sets.

7. Plug in the mean and standard deviation values calculated in step 6 into the formula below to obtain the unitless Z'-factor value:

$$Z' = 1 - \frac{3(\sigma_{c+} + \sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

Where:

σ_{c+} = Standard deviation of positive controls

σ_{c-} = Standard deviation of negative controls

μ_{c+} = Mean of positive controls

μ_{c-} = Mean of negative controls

1. Z'-factor calculations are enabled in the Odyssey application software (v.3.0 or later) by choosing **In-Cell Western > Change ICW Parameters** and switching to the **Calculations** tab. At the bottom of the window select **Calculations are for Z-Factor.**

   Note: *When **Calculations are for Z-Factor** is selected, In-Cell Western calculations are not performed, and any well links are ignored if links are defined on the **Links** tab of the **Change ICW Parameters** window.*

2. Make sure Subtract Background on All Channels is selected.

3. Make sure Calculate Relative Intensity in Channel is selected and the correct channel for normalization is chosen.

4. Switch to the **Well Types** tab in the **Change ICW Parameters** window. Each well should be designated as a positive control, negative control, background well, or not used. To change well designations, begin by selecting the type of well you want to mark. Move the cursor over a well on the well assignment grid and click to assign the selected well type to that well. Multiple well assignments can be made at once by clicking and dragging through a range of wells. When the mouse button is released, all wells within the selection rectangle change to the color assigned to the chosen well type. If **Assign Remaining** is clicked, any wells that are **Not Used** will be assigned the currently selected well type. If you make a mistake and need to start over, click **Clear Rows** to change all wells to unused.

5. View the calculated Z'-factor value by choosing **In-Cell Western > View ICW Analysis**. The Z'factor is listed in red above the data table in the View ICW Analysis window.

IV. Interpretation

The Z'-factor formula can also be written as follows:

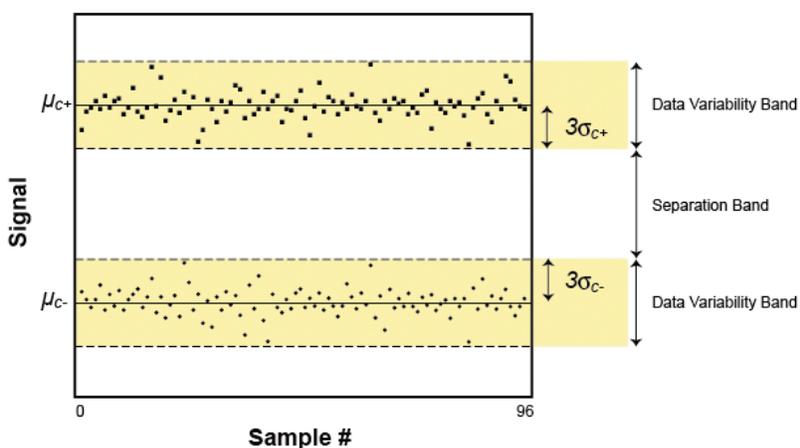$$Z' = \frac{|\mu_{c+} - \mu_{c-}| - (3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

In this formula,

$|\mu_{c+} - \mu_{c-}|$ represents the assay dynamic range, and

$|\mu_{c+} - \mu_{c-}| - (3\sigma_{c+} + 3\sigma_{c-})$ is the separation band between positive and negative control signals.

These variables are represented in the illustration below.

Dimensionless Z'-factor values are always 1 or less as described following:

Z′ = 1 Indicates an ideal assay. As standard deviations become very small or the difference between signals for positive and negative controls approaches infinity, Z′-factor approaches 1.

1 > Z′ ≥ 0.5 Indicates a high-quality assay exhibiting a wide separation between signal and background, and low data variability.

0.5 > Z′ > 0 Indicates a poor quality assay with marginal distinction between signal and background, and higher data variability.

Z′ ≤ 0 Indicates unreliable data. Assay conditions are not optimized or the assay is not capable of generating meaningful data.

Z′ = -1 There is no distinguishable difference between background signal and sample signal. If lower limit of detection (LLD) is expressed as the mean of the background signal plus three SD of the background signal, then the measured signal is equal to the LLD of the instrument.

The Z′-factor experimental guidelines described above can be utilized to evaluate ICW assay quality at any time throughout assay development, or near completion of assay optimization. Z′-factor measurement is also useful for monitoring assay quality over time, from user to user, or when conditions or reagents within the assay must be changed.