Bio-Plex™ suspension array system tech note 5312

Feasibility of Multiplexing Bio-Plex Total Target and Phosphoprotein Assays

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Introduction

Bio-Plex assays allow detection of mutiple target proteins in a small volume of sample. The assays are based on detection of signal from tagged fluorescent beads (xMAP technology) that have bound to each target protein. Typically, each type of bead is conjugated with distinct antibodies that recognize one of the target proteins. Appropriate sets of beads are then selected that enable simultaneous, or multiplex, assays of the proteins of interest in the sample. Bio-Plex phosphoprotein assays report the level of a protein that is phosphorylated at a specific site or sites, while total target assays report the level of the protein regardless of its phosphorylation state.

Typically, because of epitope binding competition for the same target protein, a total target assay should not be multiplexed with the corresponding phosphoprotein assay. Also, because the pair of antibodies used in the two assays differ, the signal from a total target assay may not be comparable to that of its corresponding phosphoprotein assay, so it is not appropriate to calculate the percentage of phosphorylated target protein using the results from a total and a phosphoprotein assay in the same reaction. However, because maximizing the number of assays in a particular reaction is desirable to conserve sample, in this note, we present results from a case study testing the feasibility of multiplexing a Bio-Plex total target assay with Bio-Plex phosphoprotein assays for different target proteins. This study was designed to answer two questions:

- Can a total target assay be multiplexed with phosphoprotein assays?
- Can a total target assay detect differences in protein concentration among samples?

Methods

We designed the study using HEK 293 cells, which respond to UV treatment by increasing the level of phosphorylation of several proteins. All proteins selected for the phosphoprotein assays in this study are activated by this treatment. The multiplex run (6-plex) included the following targets:

- Total ERK2
- Phospho-p53 (Ser^{15})
- Phospho-p70 S6 kinase (Thr^{421}/Ser^{424})
- Phospho-ATF-2 (Thr^{71})
- Phospho-JNK (Thr^{183}/Tyr^{185})
- Phospho-c-Jun (Ser^{63})

Untreated and UV-treated HEK 293 cell lysates were prepared according to methods detailed in the Bio-Plex phosphoprotein assay instruction manual. Both untreated HEK 293 and UV-treated HEK 293 cell lysates were tested at three different protein concentrations to test dose-response characteristics of the assays. Blank wells were included in the run, with cell lysis/assay buffer used to replace the cell lysate. Duplicates were run for each sample.

Results and Discussion

Total Target Assays Can Be Multiplexed With Phosphoprotein Assays for Distinct Targets

Data from a multiplex run of five phosphoprotein assays with a total ERK2 assay are presented in Figure 1. To show the results clearly, each phosphoprotein signal was plotted together with the total ERK2 assay signal. Although the phosphorylation levels found between UV-treated and untreated cell lysates varied, the total ERK2 signal level at the same protein concentration was very consistent between treatments, indicating that none of the phosphoprotein assays interfered with the total target assay in this multiplex run.

For all tested phosphoprotein targets, the level of detected phosphorylation increased in HEK 293 cells in response to UV treatment, with the extent of increase depending on the phosphoprotein, as expected. The most dramatic signal increase was detected for phospho-c-Jun (Ser^{63}) (Figure 1E).

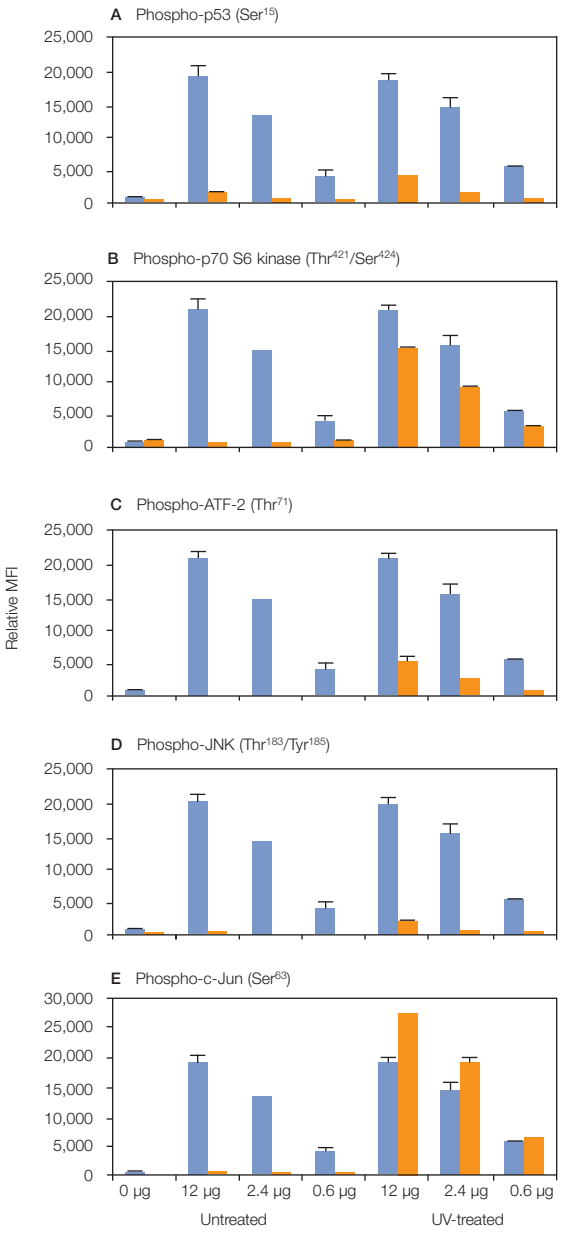

Fig. 1. Bio-Plex phosphoprotein and total ERK2 assays in lysates from untreated and UV-treated HEK 293 cells. Signal is plotted as relative mean fluorescent intensity (MFI) values for various protein loads. Error bars indicate SD. Lavender bars, ERK2 signal (data shown in all panels for ease of comparison); orange bars, phosphoprotein signal.

At 0.6 µg/ml protein, there was more than a 40-fold increase in phospho-c-Jun signal detected in UV-treated HEK 293 compared to untreated cell lysate. Notably higher levels of phosphorylation were also detected for phosphorylated p70 S6 kinase and phospho-ATF-2 assays in UV-treated cell lysates. The signals for untreated cell lysates were consistently low across all diluted samples. The low signals, around 60–70 MFI, in untreated cell lysates made the ratio of UV-treated to untreated as high as 76 at 12 µg of total protein concentration. Lower phosphorylation levels were detected for phospho-p53 (Ser^{15}) and phospho-JNK (Thr^{183}/Tyr^{185}), but the signal differences between untreated and UV-treated cell lysates were still significant at all tested protein concentrations.

Data from this 6-plex run (one total target assay multiplexed with five phosphoprotein assays) suggest that it is practical to mutiplex Bio-Plex total target assays with Bio-Plex phosphoprotein assays, provided that the targets are not the same.

Total ERK2 Assay Results Correlated With Protein Concentration

The total ERK2 assay showed consistent results in both untreated and UV-treated HEK 293 cell lysates. The signal for total ERK2, though not linear, showed a good dose-response over the range from 12 to 0.6 µg/ml. The same was true for the phosphoproteins in each of the treated samples. These results emphasize the importance of establishing the dose-response relationship between the concentration of each protein target and the corresponding signal when a specific cell lysate sample is tested. These results demonstrate that multiplexing a total assay with five phosphoprotein assays can detect differences among samples with different protein concentrations.

Conclusions

The results of this study successfully answered the questions posed, demonstrating that:

- A total target assay can be multiplexed with phosphoprotein assays, provided the target proteins are different
- A total target assay can detect differences in protein concentrations among samples

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