LanthaScreen® TR-FRET tyrosine kinase and protein kinase C assay

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- Dual wavelength detection of terbium and fluorescein using dedicated optic settings
- Strong and long-lasting donor signal minimizes background
- Rapid and reliable detection of PKC’s and tyrosine kinase activity

Introduction

Protein Kinase C (PKC) enzymes are a diverse family of enzymes that under specific signaling conditions phosphorylate proteins on serine and threonine amino acids. PKCs are involved in many cellular functions including neuronal activity, cell growth, cell proliferation, and cell movement, as well as in several diseases including cardiovascular, cancer, diabetes, and Alzheimer’s. Therefore a TR-FRET screening assay, such as the one described here, can aid in further elucidating PKC’s function.

Tyrosine kinases (TKs) are a diverse family of enzymes that under specific signaling conditions phosphorylate proteins on the amino acid tyrosine. TKs are involved in many cellular functions such as cell division and cell proliferation, as well as in several diseases including cancer and diabetes. Tyrosine kinases play important roles in cell growth, thereby making them important drug targets in cancer therapy. For that reason a TR-FRET screening assay, such as the one described here, can aid in elucidating specific inhibitors for tyrosine kinases.

Assay Principle

A fluorescein labeled poly-GT or poly-GAT (Glu, Ala, Tyr) substrate is incubated with a tyrosine kinase and ATP. For protein kinase C assays a fluorescein labeled PKC substrate is incubated with PKC and ATP. Subsequently, a terbium labeled antibody that binds to the phosphorylated form of the substrate is added. When the antibody interacts with the phosphorylated substrate, FRET will occur between the terbium label (emits at 490 nm) and the fluorescein moiety (emits at 520 nm) (Figure 1).

PKC Titration

Multiple PKC isoforms were titrated to determine optimal kinase concentrations for screening. The following protocol is an example of the conditions used to determine the EC50 of PKCα and its substrate. A dilution series of PKCα, starting at a final concentration of 2.0 μg/ml, was added and mixed to create a final volume of 20 μl per well, a final 1X EDTA concentration of 10 μM ATP in a total volume of 10 μl in a microplate. After a 90-minute incubation at room temperature, 10 μl of TR-FRET dilution buffer containing 2X EDTA (20 mM) and 2X Tb-PKC antibody (1.0 nM) was added and mixed to create a final volume of 20 μl per well, a final 1X EDTA concentration of 10 μM and a final 1X antibody concentration of 0.5 μM. After incubating for 60 minutes at room temperature, the plate was read on the BMG LABTECH microplate reader. Each data point represents the average of three wells.

Tyrosine Kinase Titration

To demonstrate the functionality of a universal tyrosine kinase assay using LanthaScreen™, both fluorescein-poly-GAT (Glu, Ala, Tyr) and fluorescein-poly-GT (Glu, Tyr) substrates were used with terbium labeled PY20 antibody. A dilution series of kinase was incubated with 400 nM fluorescein labeled substrate and 200 μM ATP in a total volume of 10 μl in a microplate. After 60 mins of incubation at room temperature, 5 μl of TR-FRET dilution buffer containing 4X EDTA (60 mM) was added. Next, 5 μl of TR-FRET dilution buffer with 4X antibody (8 nM) was added and mixed to create a final volume of 20 μl per well, a final 1X EDTA concentration of 15 mM, and a final 1X antibody concentration of 2 nM. After 60 mins of incubation at room temperature, the plate was read in the microplate reader.

Measurements are taken at 490 and 520 nm and the 520/490 ratio is plotted versus the concentration of enzyme to determine an EC50 for the enzyme and its substrate.
Results & Discussion

Figure 2 shows representative kinase titration curves for PKCα and PKCζ.

Table 1 shows statistical data for all kinases tested.

Table 1: Lanthascreen™ on the PHERaStar: statistics for PKC isomform titrations.

<table>
<thead>
<tr>
<th>PKC α</th>
<th>PKC β-</th>
<th>PKC βII</th>
<th>PKC βIII</th>
<th>PKC δ</th>
<th>PKC ε</th>
<th>PKC ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (ng/ml)</td>
<td>0.63</td>
<td>0.27</td>
<td>0.22</td>
<td>0.07</td>
<td>0.03</td>
<td>0.16</td>
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<tr>
<td>Min. Value</td>
<td>0.64</td>
<td>0.63</td>
<td>0.79</td>
<td>0.55</td>
<td>0.73</td>
<td>1.02</td>
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<tr>
<td>Max. Value</td>
<td>2.69</td>
<td>2.66</td>
<td>2.61</td>
<td>2.64</td>
<td>2.63</td>
<td>2.63</td>
</tr>
<tr>
<td>Max-min</td>
<td>2.25</td>
<td>2.23</td>
<td>1.62</td>
<td>2.09</td>
<td>1.90</td>
<td>1.61</td>
</tr>
<tr>
<td>Hill Slope</td>
<td>1.1</td>
<td>0.89</td>
<td>1.01</td>
<td>0.70</td>
<td>0.93</td>
<td>0.96</td>
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<tr>
<td>R2</td>
<td>0.991</td>
<td>0.996</td>
<td>0.988</td>
<td>0.979</td>
<td>0.988</td>
<td>0.976</td>
</tr>
<tr>
<td>S:N</td>
<td>60</td>
<td>47</td>
<td>18</td>
<td>31</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Z’-factor value</td>
<td>0.94</td>
<td>0.91</td>
<td>0.78</td>
<td>0.87</td>
<td>0.85</td>
<td>0.74</td>
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</table>

All PKC isoenzymes were titrated in a similar manner as PKCα. Regardless of "fold difference" or signal-to-noise ratios, all assays provided Z’ values far greater than 0.5. This is due to the use of FLR-based, ratiometric data analysis, which leads to low standard deviations of the replicates. Both hill slope and R2 measurements fall within acceptable limits. R2 values approaching 1.0 indicate a perfect curve fit.

In figure 3 kinase titration curves for ZAP-70 are presented (Fig. 3A+B).

The results show that use of a fluorescein-labeled substrate common for many TKs (poly-GT or poly-GAT) allows for the development of a “universal” TK assay using the Lanthascreen™ format.

Conclusion
Invitrogen’s terbium-based Lanthascreen™ can easily be measured on BMG LABTECH instrumentation. The assay shows several advantages:

- The ratiometric nature of Lanthascreen™ assay eliminates well to well variation.
- The time-resolved nature allows for the use of fluorescein without the associated drawbacks of compound interference.
- The ability to use fluorescein simplifies assay development and costs.