New LANCE® Ultra Reagents for the Development and Optimization of Kinase Assays

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1 Introduction

Protein kinases regulate several important functions within cells such as proliferation, apoptosis, and cell adhesion. Dysregulation of kinase activity plays causal roles in many human diseases, making kinases important drug discovery targets. PerkinElmer has developed a new line of reagents for kinase assays. This new product line, called LANCE Ultra, is an improved Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) technology ideal for high-throughput screening (HTS). LANCE Ultra reagents include a series of europium chelate-labeled anti-phosphopeptide antibodies and several kinase peptide substrates labeled with the UltraLight™ dye, a small molecular weight fluorescent dye with a red-shifted emission. These new reagents enable simple and easy development of homogeneous assays for the detection of kinase activity by monitoring the phosphorylation of a substrate peptide. Assay development of a LANCE Ultra kinase assay includes optimization of the concentration of various assay components including the enzyme, labeled substrate, Eu-antibody and ATP. We present here guidelines for the optimization of an ERK1 kinase assay using a LANCE-labeled MBP peptide substrate and an europium-anti-phospho-MBP antibody, with a demonstration of assay performance and robustness in a HTS set-up.

2 Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
<th>Cat.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>UltraLight™ peptide</td>
<td>PerkinElmer LAS, Inc.</td>
<td>TRF0010-M</td>
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<td>Eu-anti-phospho-MBP</td>
<td>PerkinElmer LAS, Inc.</td>
<td>TRF0021-M</td>
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<td>LANCE Detection Buffer</td>
<td>PerkinElmer LAS, Inc.</td>
<td>CR95-100</td>
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<td>MAP kinase 1/ERK1</td>
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<td>Staurosporine</td>
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<td>ATP</td>
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<td>50 mM EDTA pH 8</td>
<td>Invitrogen Corp.</td>
<td>15575-020</td>
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<td>White Opal™/TRF</td>
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<td>TopSpin Ultra</td>
<td>PerkinElmer LAS, Inc.</td>
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<td>Eu-5 µM Multibead Reader</td>
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<td>2103-0010</td>
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<td>Mirror LANCE/DELFIA Dual</td>
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<td>2100-1410</td>
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<td>PerkinElmer LAS, Inc.</td>
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<td>Emission Filter</td>
<td>PerkinElmer LAS, Inc.</td>
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<tr>
<td>Emission Filter LANCE 685 nm</td>
<td>PerkinElmer LAS, Inc.</td>
<td>2100-5110</td>
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<tr>
<td>ERK1 Kinase Buffer</td>
<td>50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 2 mM DTT and 0.01% Tween-20</td>
<td></td>
</tr>
</tbody>
</table>

3 Methods

Optimized LANCE Ultra ERK1 assay protocol:
To the wells of a white Opal™/TRF 384:
- Add 5 µL of 2X ERK diluted in Kinase Buffer (final conc: 1 nM)
- Add 5 µL of 2X UltraLight™ ATP and ATP (final concentrations 50 nM and 4 µM respectively) diluted in Kinase Buffer
- Incubate 60 min at RT
- Add 5 µL of 40 mM EDTA in LANCE Detection Buffer to stop the reaction

4 Assay Principle

In LANCE Ultra kinase assays, the phosphorylation of a LANCE-labeled peptide substrate is detected with a specific anti-phosphopeptide antibody that is labeled with europium chelate molecules (Eu). The labeling of the Eu-antibody (donor) to the phosphorylated LANCE MBP peptide (acceptor) substrate brings both the donor and acceptor dye molecules into close proximity. Upon excitation at 320 nm, the excited europium chelate transfers its energy to the nearby UltraLight dye molecule that in turn emits light at 685 nm. The intensity of light emission is proportional to the level of the LANCE MBP phosphorylation.

5 Enzyme and Substrate Titrations

As a first step, a cross-titration of the ERK1 kinase and UltraLight™ MBP peptide was performed to determine minimal concentrations of enzyme and substrate giving high signal to background (S/B) ratio while not saturating the detection by the antibody.

The ERK1 enzyme, was diluted from 0.25 to 4 nM and the UltraLight™ peptide substrate from 25 to 500 nM in Kinase Buffer supplemented with 20 µM ATP. Enzymatic reaction was incubated for 60 min at RT.

6 ATP Titration

When optimizing a LANCE Ultra kinase assay for screening purposes, the concentration of ATP in the reaction 0.2 µM to 4 µM must be optimized in order to maximize the effect of potential ATP competitors. ATP is usually used at a concentration at or near the apparent Kᵢ value of the enzyme for ATP.

ATP was serially diluted in assay reactions containing 1 nM ERK1 kinase and 50 nM UltraLight™ peptide substrate. Reactions were incubated for 60 min at RT. The apparent Kᵢ value for ATP was 4 µM for phosphorylating kinase assays near the apparent Kᵢ of the enzyme for ATP, the effect of ATP competitors is maximized while maintaining a good S/B ratio.

7 Time Course of Phosphorylation

A time course of substrate phosphorylation was performed to select a reaction incubation time in the linear zone of the enzymatic reaction.

The ERK1 enzyme was incubated for up to 2 h with the UltraLight™ peptide substrate in Kinase Buffer containing 4 µM ATP. Reactions were stopped by the addition of 10 µL EDTA. The LANCE signal was linear up to 90 min, with a signal of 50 000 counts at 60 min and a S/B ratio of 8.4

8 Titration of the Eu-Labeled Anti-Phospho-MBP Antibody

The Eu-labeled anti-phospho-MBP antibody was titrated to determine the optimal concentration of antibody to be added in the detection reaction.

Concentrations of Eu-labeled anti-phospho-MBP were diluted from 0.2 µM to 30 nM, S/B ratio indicated above linear was reached a maximum of 0.7 ± 2 nM. Above 2 nM, specific signal was higher, but with increased backgrounds and lower S/B ratios. Therefore, the selected Eu-labeled anti-phospho-MBP concentration was 2 nM, giving the highest S/B ratio in the assay, while maintaining acceptable background levels.

9 DMSO Tolerance

The tolerance of the assay to DMSO was evaluated. The antibody was incubated with a UltraLight-labeled phospho-MBP peptide in kinase buffer with increasing DMSO concentrations.

10 Staurosporine Inhibition

Staurosporine is a broad spectrum kinase inhibitor that blocks activity by interacting with the ATP cleft of the ERK1 kinase, thereby rendering it relatively insensitive to staurosporine, with an IC₅₀ value in the micromolar range.

11 Assay Performance

The performance of the optimized LANCE Ultra kinase assay was evaluated by setting up a four-parameter curve for the determination of ATP, 1) Staurosporine (50 µM) and 3) basal signal and 2) signal, and data were analyzed. DMSO at 2% was added to buffer in fluorescent screening conditions. The stability of the LANCE Ultra signal was also evaluated over time, 50, 60 and Z’ factors were calculated.

12 Conclusions

PerkinElmer’s new LANCE Ultra reagents were used for the development of a robust and sensitive ERK1 kinase assay.

- Careful optimization of the assay components’ concentration allows minimizing enzyme consumption while maintaining high S/B ratio.
- Working with ATP at a concentration near the apparent Kᵢ value (4 µM) maximizes the effect of staurosporine, which inhibits ERK1 activity by competing for the binding of ATP.
- UltraLight™ substrate and the Eu-anti-phospho-MBP antibody are resistant to concentrations of DMSO up to 10%.
- The optimized assay developed using LANCE Ultra reagents shows robust performance suitable for HTS, as demonstrated by a Z’ value of 0.83.
- Simplicity of use and minimal interference with screening compounds makes the UltraLight dye the reagent of choice for HTS kinase assays.

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