APPLICATION NOTE

LANCE *ULTRA* TR-FRET TECHNOLOGY

Development and Optimization of Kinase Assays using New LANCE *Ultra* TR-FRET Reagents

Introduction

Dysregulation of kinase activity has been shown to be associated with several human diseases including cancer, diabetes and morphological disorders. Because of their function, kinases are crucial targets for drug discovery. Analysis of the human genome has revealed the existence of nearly 520 genes encoding kinases. The abundance of these potential therapeutic targets provides a compelling impetus for developing efficient and robust high throughput screening (HTS) platforms for the discovery of kinase modulators.

Time-resolved fluorescence resonance energy transfer (TR-FRET) assays are homogeneous proximity assays where the interaction of two labeled binding partners is detected by the energy transfer from an excited donor to an acceptor dye, and measurement of the subsequent light emission by the acceptor dye. A number of TR-FRET platforms are currently available. They differ principally in the nature of the donor and acceptor dyes used for the energy transfer. The LANCE® technology uses an europium-based chelate (Eu chelate) as the donor dye. Eu chelates have a high quantum yield, large Stokes' shift and a narrow-banded emission at ~615 nm. The lifetime of their light emission is exceptionally long, allowing for time-delayed measurements. These unique fluorescence properties make Eu chelates ideal energy donors in TR-FRET assays. In classical LANCE assays, the acceptor dye is allophycocyanin (APC). APC receives the energy from irradiated Eu chelate molecules in close proximity and in turn emits light at 665 nm. Although APC allows for the efficient capture and reemission of the transferred energy, it has some disadvantages. Since it is a large protein (~100 kDa), small molecules such as peptides and oligonucleotides cannot be labeled directly and an additional labeled component, such as APC-streptavidin (APC-SA), must be introduced in the assay set-up. Also, the bulkiness of APC can potentially create steric hindrance in some assay configurations. Finally, APC is light sensitive, which requires special precautions during assay set-up and incubation. To overcome these limitations, PerkinElmer has developed the new LANCE *Ultra* HTS platform in which the APC acceptor dye has been replaced by a new U*Light*™ acceptor dye.

Authors
Mireille Caron, Anja Rodenbrock, Philippe Roby, Nathalie Bouchard, Jaime Padrós, Valérie Paquet, Claire Normand, Marjolaine Roy, Véronique Brechler and Lucille Beaudet

PerkinElmer Life and Analytical Sciences

PerkinElmer® precisely.

U*Light* is a small acceptor dye with spectral characteristics similar to APC but with two distinct advantages. First, its low molecular weight makes it suitable for the direct labeling of peptides and other small molecules; second, it is light resistant, which simplifies the handling of assay components and plates. LANCE *Ultra* reagents include generic assay components such as U*Light*-labeled Protein A, Streptavidin, anti-His tag and anti-GST tag antibodies as well as a series of specific Eu-labeled anti-phospho-substrate antibodies with the corresponding U*Light*-labeled peptide substrates for HTS kinase assays.

A LANCE *Ultra* kinase assay is illustrated in Figure 1. In the presence of kinase and ATP, the U*Light* peptide substrate is phosphorylated. It is then captured by a Eu-anti-phospho-substrate antibody, which brings the Eu chelate donor and U*Light* acceptor dyes into close proximity. Upon excitation at 320 or 340 nm, the Eu chelate transfers its energy to the U*Light* dye, resulting in a fluorescent light emission at 665 nm.

This application note provides detailed guidelines for the development and validation of a LANCE *Ultra* kinase assay. It demonstrates how to optimize the concentration of assay components (enzyme, U*Light*-labeled substrate, ATP, Eu-anti-phospho-substrate antibody) as well as the kinase assay reaction and antibody detection times. It also shows how to validate the performance of a kinase assay by titration of a known kinase inhibitor and determination of the Z'-factor. A LANCE *Ultra* ERK1 kinase assay developed using the U*Light*-labeled Myelin Basic Protein peptide substrate (U*Light*-MBP) and Eu-anti-phospho-MBP antibody will be presented as an example.

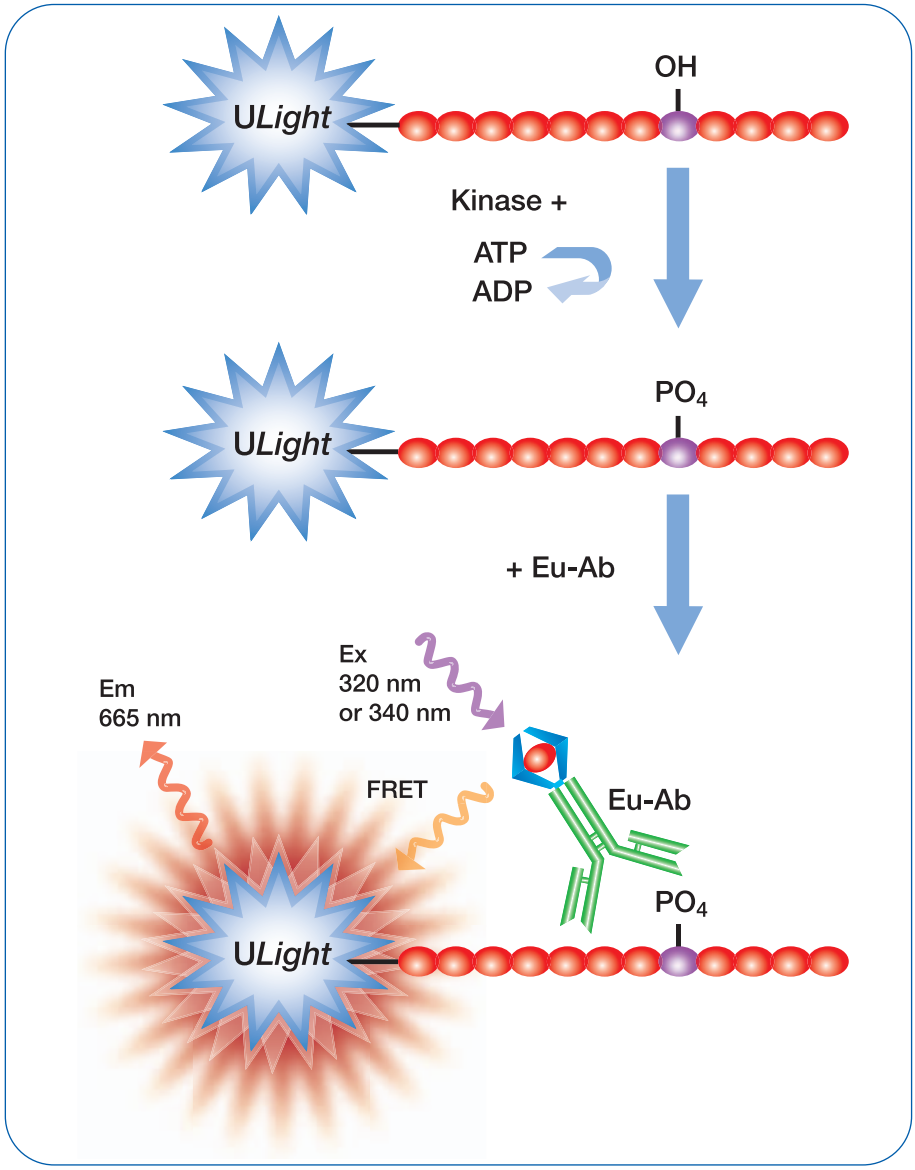


Figure 1. **Principle of the LANCE *Ultra* assay**

Materials and Methods

Materials

Table 1 lists the materials used for this study, including suppliers and product numbers.

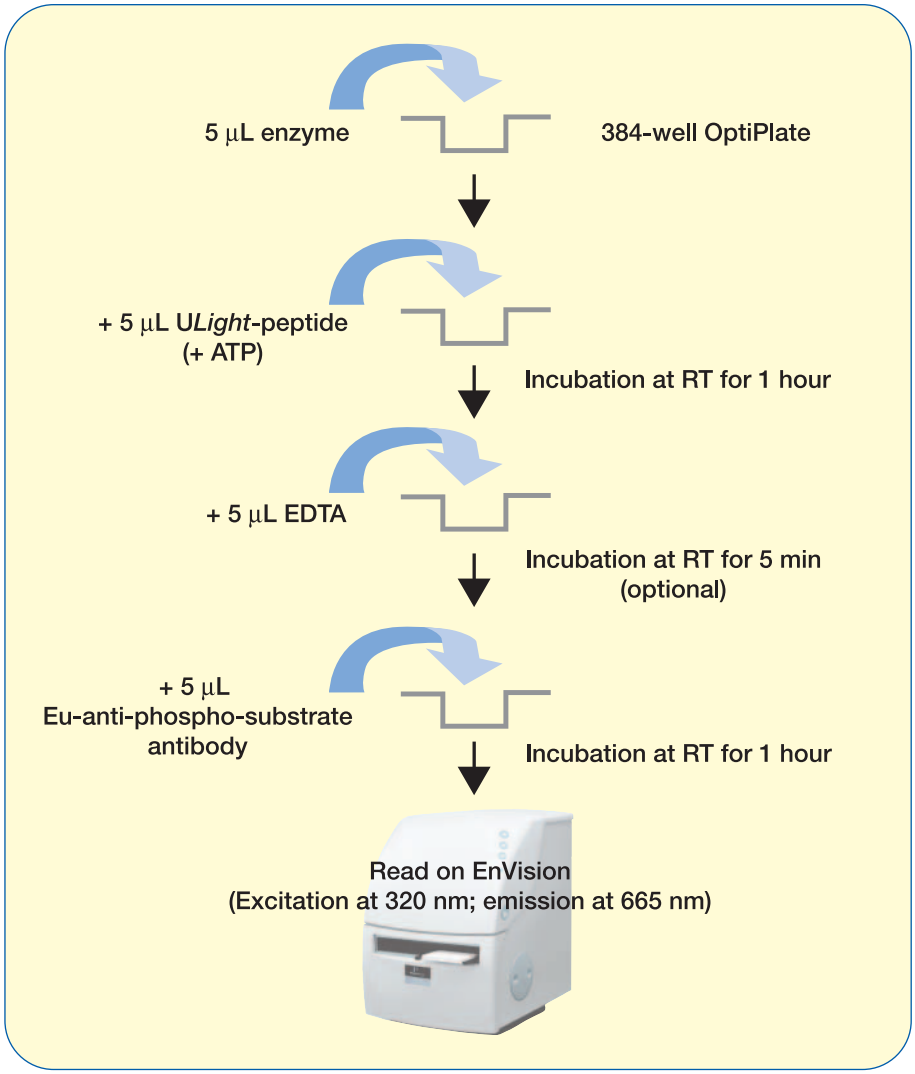
Table 1. Reagents and Consumables

Item	Supplier	Product No.
U*Light*-MBP peptide	PerkinElmer	TRF0109
Eu-anti-phospho-MBP	PerkinElmer	TRF0201
LANCE Detection Buffer 10X	PerkinElmer	CR97-100
White OptiPlates-384	PerkinElmer	6007290
MAP kinase 1/ERK1, active	Millipore Corp.	14-439
Staurosporine	Sigma-Aldrich	S4400
ATP	Sigma-Aldrich	A2383
TopSeal-A™	PerkinElmer	6005250
EnVision Multilabel Reader	PerkinElmer	2103-0010
Mirror: LANCE/DELFIA™ Dual	PerkinElmer	2100-4160
Excitation Filter: UV2(TRF) 320 nm	PerkinElmer	2100-5060
Emission Filter: Eu 615 nm	PerkinElmer	2100-5090
Emission Filter: LANCE 665 nm	PerkinElmer	2100-5110

Method

ERK1 kinase LANCE *Ultra* assay

All assay components were titrated individually (see Results and Discussion). Optimized concentrations of reagents for the LANCE *Ultra* ERK1 kinase assay were 1 nM ERK1 kinase, 50 nM U*Light*-MBP and 4 µM ATP. Kinase Assay Buffer consisted of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT and 0.01% Tween-20. Kinase assay components were prepared as concentrated pre-mixes in Kinase Assay Buffer and added to the wells of a 384-well OptiPlate™. The total volume of the kinase reaction was 10 µL. In the optimized assay, kinase reactions were incubated for 60 min at 23 °C and stopped by the addition of 10 mM EDTA. The Eu-anti-phospho-MBP antibody diluted in Detection Buffer was then added to a final concentration of 2 nM. Detection reactions were incubated for 1 h at 23 °C. The LANCE signal was measured on an EnVision™ Multilabel Microplate Reader. Excitation wavelength was set at 320 nm and emission recorded at 665 nm.



Results and Discussion

Cross-titration of ERK1 kinase and U*Light* labeled MBP substrate

A cross-titration of ERK1 kinase and U*Light*-MBP substrate was performed at a non-limiting ATP concentration (20 µM) in order to determine optimal concentrations of enzyme and substrate for the assay. Figure 2 shows that increasing amounts of enzyme leads to an increased LANCE signal, which reflects the higher level of phosphorylation of the U*Light*-MBP substrate. A signal to background (S/B) ratio of 8.5 was obtained using as little as 0.25 nM of ERK1 enzyme and 50 nM of U*Light*-MBP. However, since a significantly higher S/B ratio was obtained using 1 nM of ERK1 and 50 nM of U*Light*-MBP (S/B ratio of 31), these enzyme and substrate concentrations were selected for further experiments.

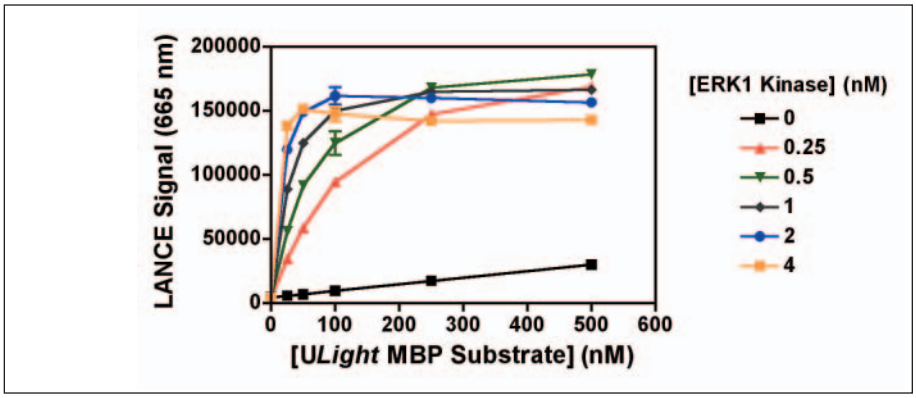


Figure 2. **Optimization of ERK1 enzyme and U*Light*-MBP substrate concentrations.** ERK1 enzyme was titrated from 0.25 to 4 nM and U*Light*-MBP substrate from 25 to 500 nM in Kinase Assay Buffer supplemented with 20 µM ATP (final concentrations in kinase reactions). Reactions were terminated after 90 min by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min on the EnVision Multilabel Reader.

ATP Titration

In order to identify competitive inhibitors for ATP, the concentration of ATP in the assay should be near the K_m value of the enzyme for ATP. In LANCE *Ultra* assays, the EC_{50} value for ATP can be used as an apparent K_m of the enzyme for ATP. ATP titration was performed using the optimized ERK1 enzyme and U*Light*-MBP substrate concentrations (Figure 3). An apparent K_m value of 4.2 µM was obtained. Consequently, all subsequent experiments were performed using 4 µM ATP in the kinase reaction.

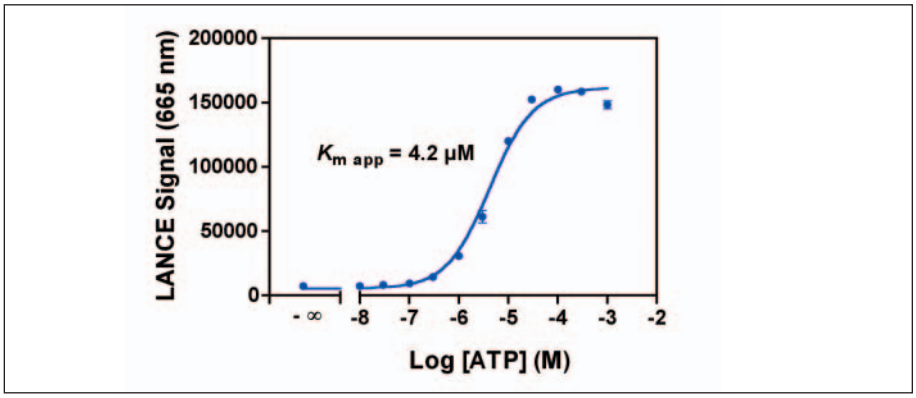


Figure 3. **ATP titration.** ATP was serially diluted in assay reactions containing 1 nM ERK1 kinase and 50 nM U*Light*-MBP substrate. Reactions were terminated after 90 min by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min incubation at RT on the EnVision Multilabel Reader.

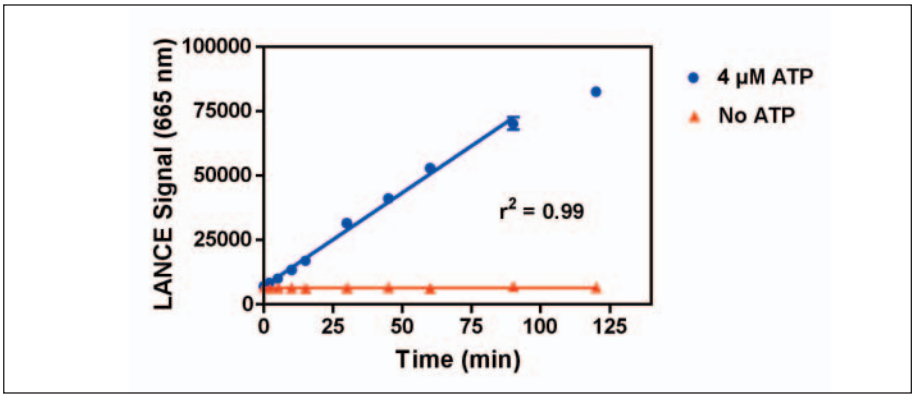


Figure 4. **Time course of ERK1 phosphorylation of U*Light*-MBP substrate.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in Kinase Assay Buffer supplemented with 4 µM ATP. Reactions were terminated at specific time points by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min incubation at RT on the EnVision Multilabel Reader.

Time course of U*Light*-MBP substrate phosphorylation

A time course of substrate phosphorylation was performed to ensure that the assay incubation time was in the linear range of the kinase reaction. As shown in Figure 4, signal generated by the LANCE *Ultra* ERK1 assay was linear for up to 90 min ($r^2 = 0.99$) with 53,000 counts measured at 60 min, corresponding to an S/B ratio of 8.4. Based on these results, incubation was reduced from 90 to 60 min, which permits a significant reduction of the LANCE *Ultra* assay time and obtaining results in just two hours.

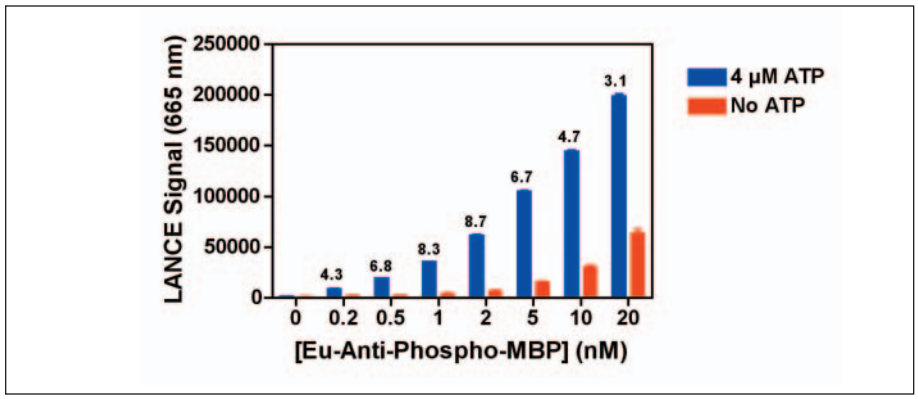


Figure 5. **Titration of Eu-anti-phospho-MBP.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in Kinase Assay Buffer supplemented with 4 µM ATP (final concentrations in kinase reactions). Reactions were terminated after 60 min by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of Eu-anti-phospho-MBP (dilutions from 0.2 to 20 nM in the detection reactions) and measured after 60 min incubation at RT on the EnVision Multilabel Reader.

Titration of the Eu-anti-phospho-MBP antibody

The Eu-anti-phospho-MBP used for the capture of the phosphorylated U*Light*-MBP substrate was titrated to optimize its concentration in the detection reaction. As shown in Figure 5, S/B ratios (indicated above bars) reached a maximum of 8.7 using 2 nM of Eu-labeled antibody. At higher antibody concentration, both specific (+ATP) and non-specific (-ATP) signals increased, which led to a significant decrease of S/B ratios. Therefore, 2 nM was kept as the Eu-anti-phospho-substrate antibody concentration in the detection reaction.

Antibody incubation time and signal stability

The Eu-anti-phospho-MBP antibody is used for the detection of phosphorylated products after the termination of the enzymatic reaction by EDTA. In order to determine 1) the equilibrium time for the detection step and 2) the stability of the LANCE *Ultra* signal

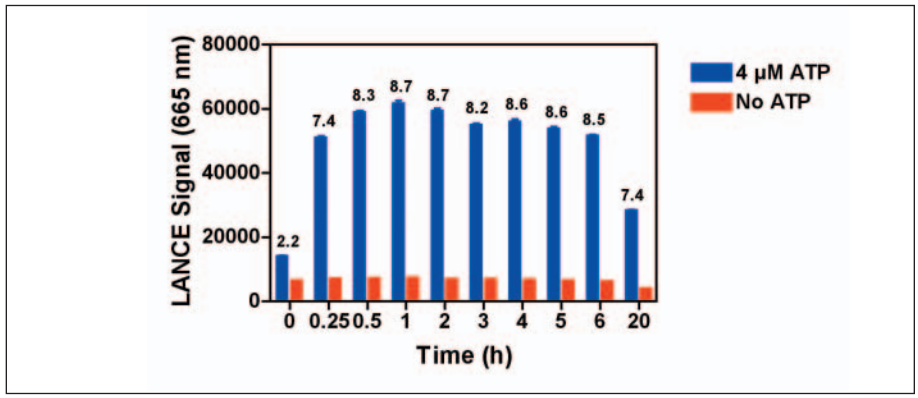


Figure 6. **Time course of detection.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in Kinase Assay Buffer supplemented with 4 µM ATP (final concentrations in the kinase reaction). Reactions were terminated after 60 min by the addition of 10 mM EDTA. U*Light*-MB peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after the indicated incubation times at RT on the EnVision Multilabel Reader.

over time, a time course of detection was conducted over a 20-hour period. As shown in Figure 6, equilibrium of the detection reaction was reached after 30 min, with an S/B ratio of 8.3. Signal was stable over at least 6 h. After overnight incubation, the signal decreased but the S/B ratio remained relatively stable (S/B ratio of 7.4). This time course experiment confirmed that the LANCE *Ultra* ERK1 assay is suitable for screening campaigns, where plates are often read offline.

DMSO tolerance

As DMSO is routinely used as a carrier solvent for compound libraries, it is essential that its presence in the assay reaction does not impact significantly the assay performance. Figure 7 shows that addition of DMSO to ERK1 kinase reactions results in a concentration-dependent decrease of the LANCE signal. This effect is not due to a decrease in the performance of the LANCE *Ultra* reagents since the detection of an U*Light* phosphorylated MBP peptide by the Eu-anti-Phospho-MBP antibody was not

affected by DMSO concentrations up to 10% (data not shown). This result rather indicates that DMSO causes a reduction in the ERK1 enzyme activity. Since HTS assays might require the addition of DMSO at concentrations up to 2%, we decided to use 2% DMSO for the validation of the ERK1 assay in the presence of staurosporine.

Staurosporine inhibition

Staurosporine is a broad spectrum kinase inhibitor that interferes with ATP binding. To evaluate staurosporine inhibition of the LANCE *Ultra* ERK1 assay, the activity of the enzyme was measured in the presence of increasing concentrations of staurosporine (Figure 8). An IC_{50} value of 2.8 µM was obtained, consistent with values reported in the literature using other technologies[1] and showing that ERK1 is relatively resistant to staurosporine inhibition.

Assay robustness and performance

The robustness and performance of the LANCE *Ultra* ERK1 kinase assay was evaluated by manually conducting a Z' analysis[2] in 384-well format. Two series of 48 replicates were analyzed: in the absence (total signal) or presence (minimal signal) of 30 µM staurosporine (Figure 9). DMSO was added to all reactions to a final concentration of 2% to simulate screening conditions. Percent coefficient of variation (%CV), S/B ratio, and Z'-factor were calculated. A %CV of 3.2 and an S/B ratio of 4 were obtained for the total signal. These values, combined with a calculated Z'-factor of 0.83, clearly demonstrate the robustness of the optimized ERK1 LANCE *Ultra* assay and its suitability for HTS applications.

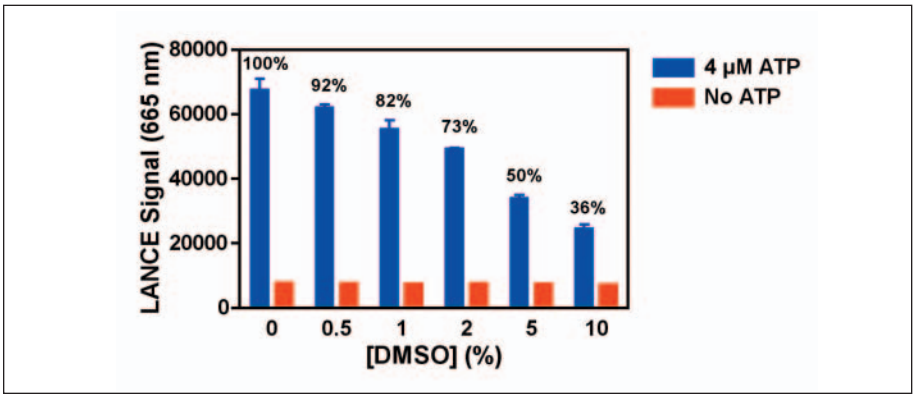


Figure 7. **DMSO Tolerance of the LANCE *Ultra* ERK1 assay.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in Kinase Assay Buffer supplemented with 4 µM ATP and increasing DMSO concentrations. Reactions were terminated after 60 min by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min incubation at RT on the EnVision Multilabel Reader.

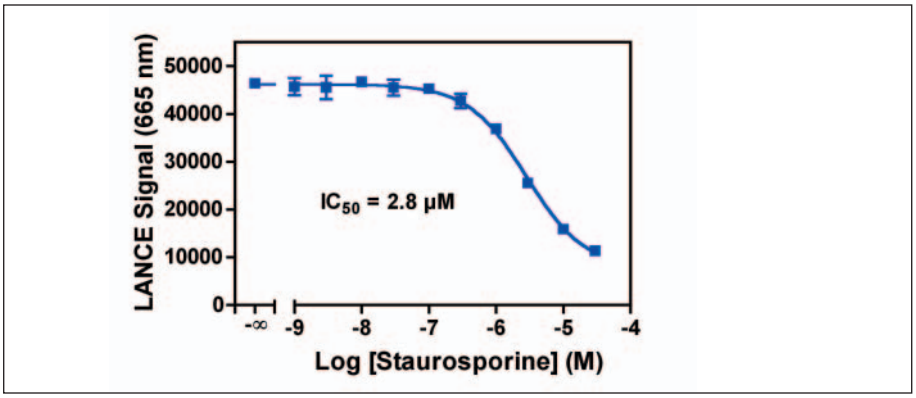
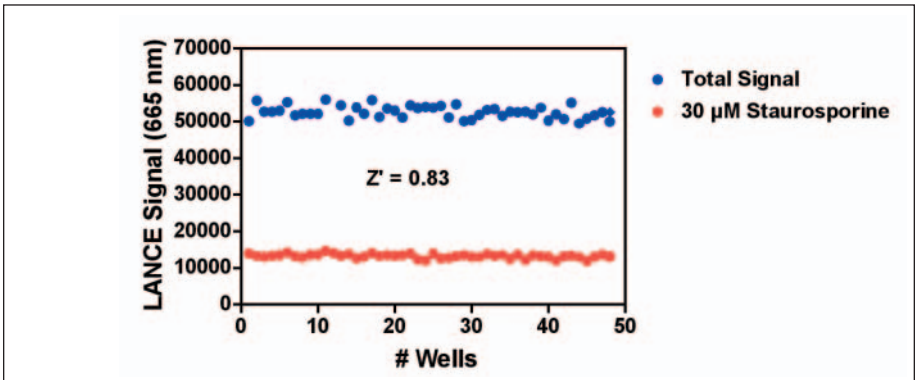


Figure 8. **Staurosporine inhibition.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in the presence of serial dilutions of staurosporine (1 nM to 30 µM) in Kinase Assay Buffer supplemented with 4 µM ATP, in the presence of 2% DMSO (final concentrations in the kinase reaction). Reactions were terminated after 60 min by the addition of 10 mM EDTA. U*Light*-MBP peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min incubation at RT on the EnVision Multilabel Reader.



Total Signal		Staurosporine-Inhibited Signal		Total/Staurosporine	
Average	%CV	Average	%CV	S/B	Z'
52,566	3.2	13,189	4.4	4.0	0.83

Figure 9. **Z' analysis for a 384-well manual ERK1 assay.** ERK1 enzyme (1 nM) was incubated with U*Light*-MBP substrate (50 nM) in Kinase Assay Buffer supplemented with 4 µM ATP, in the absence (total signal) or presence of 30 µM staurosporine (final concentration in the kinase reaction). Reactions were terminated after 60 min by the addition of 10 mM EDTA. Phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-MBP and measured after 60 min incubation at RT on the EnVision Multilabel Reader.

Conclusion

The new LANCE *Ultra* reagents, U*Light*-MBP and Eu-anti-phospho-MBP were used for the development of a sensitive and robust ERK1 kinase assay that allowed:

- Minimizing enzyme consumption while maintaining a high S/B ratio by careful optimization of the concentration of each assay component.
- Working at a concentration near the apparent K_m value for ATP (4 µM), which provides the most sensitive screen for detecting ATP competitive inhibitors.
- Developing a robust assay, suitable for HTS applications, as demonstrated by (1) the assay's signal stability over 6 h, (2) the accuracy of evaluating staurosporine potency and (3) a Z'-factor of 0.83.

References

1. Meggio, et al. (1995) *Eur. J. Biochem.* **234**: 317–322.
2. Zhang, et al. (1999) *J. Biomol. Screen.* **4**(2): 67–73.

PerkinElmer Life and
Analytical Sciences
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®
precisely.

For a complete listing of our global offices, visit www.perkinelmer.com/lasoffices

007755_02 Printed in USA