APPLICATION NOTE

LANCE *ULTRA* TR-FRET TECHNOLOGY

Comparison of LANCE *Ultra* TR-FRET to PerkinElmer's Classical LANCE TR-FRET Platform for Kinase Applications

Introduction

Protein kinases play a major role in normal cellular functions such as cell proliferation, angiogenesis, and cell adhesion. Dysregulation of kinase activity has been shown to be associated with several human diseases including cancer, diabetes and morphological disorders. This makes kinases crucial targets for the discovery and development of new drugs. 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) assay platforms for the discovery of kinase modulators.

Time-resolved fluorescence resonance energy transfer (TR-FRET) assays are homogeneous proximity assays in which energy is transferred from a donor to an acceptor molecule. A number of TR-FRET platforms are currently available that differ principally in the nature of the donor and acceptor dyes. The LANCE® technology, for example, uses an europium chelate (Eu) as donor dye, which offers a number of advantages, including a high quantum yield, large Stokes' shift and a narrow-banded emission at around 615 nm. Furthermore, the lifetime of emitted light from Eu chelate dyes is exceptionally long, allowing for time-delayed measurements. The unique fluorescence properties of Eu chelates make them 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 re-emission of the transferred energy, there are some disadvantages. APC is a bulky and light-sensitive protein prone to generating potential steric hindrances in some assay configurations.

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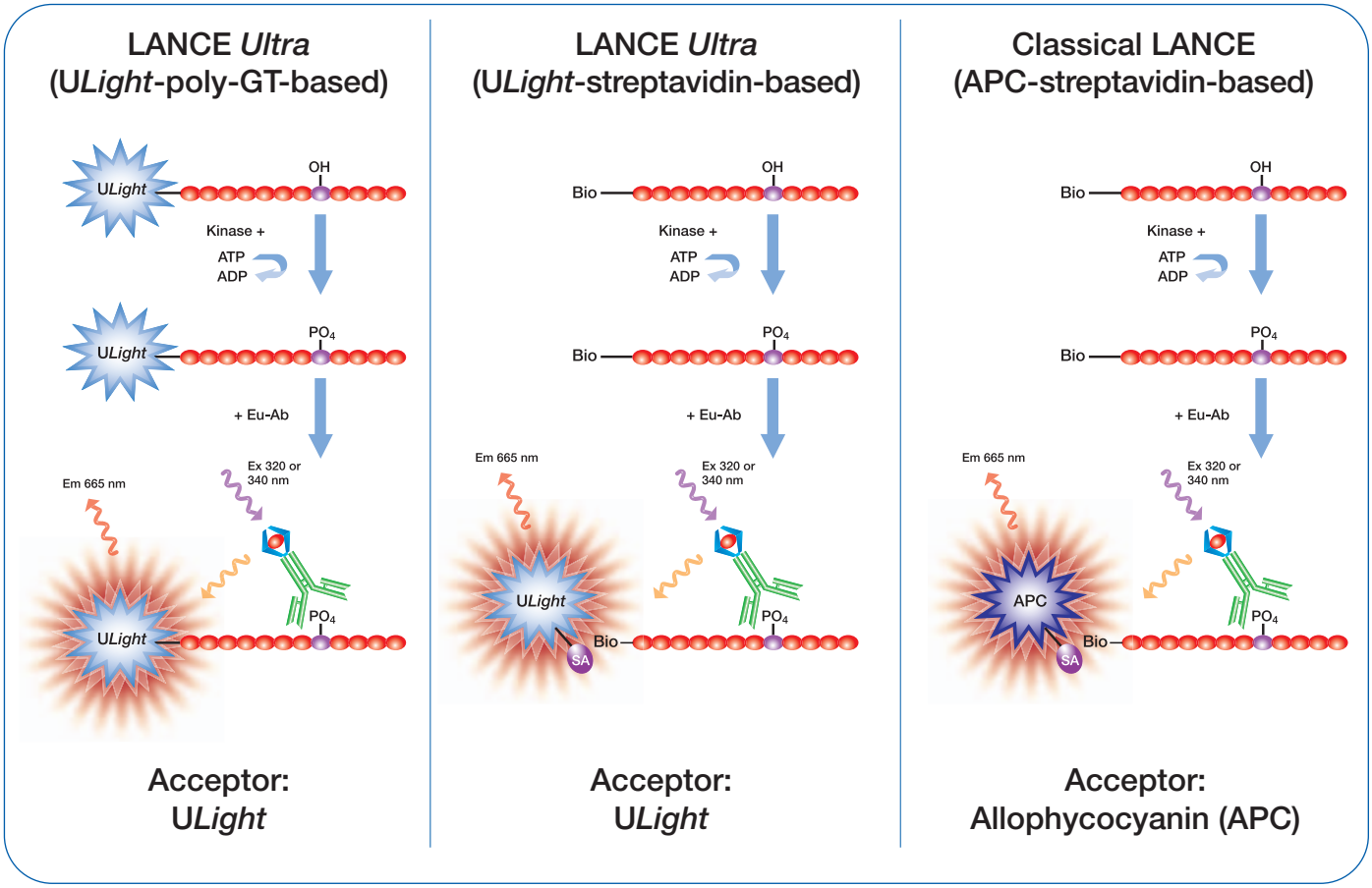

Figure 1. **Schematic representation of LANCE *Ultra* and LANCE for Src kinase assays.** Following Src kinase reactions, labeled phospho-poly-GT is detected using three different LANCE assay formats. A) Poly-GT is labeled with the small U*Light* dye. B) Poly-GT is biotinylated and captured by U*Light*-labeled streptavidin. C) Poly-GT is biotinylated and captured by APC-SA. In all three assays, the phosphorylated amino acid is recognized by an anti-phospho-tyrosine antibody (PY20) labeled with Eu. Upon excitation of the Eu-labeled antibody, energy is transferred to the acceptor dye resulting in emission of light at 665 nm.

In addition, small molecules such as peptides and oligonucleotides cannot be labeled directly with APC, resulting in the need to use a biotinylated substrate combined with APC-streptavidin (APC-SA). 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 (Figure 1).

U*Light* is a small, light resistant acceptor dye with spectral characteristics similar to APC. Its red-shifted emission is less sensitive to quenching by colored compounds, which eliminates the need for ratiometric data analysis. Importantly, the U*Light* dye's low molecular weight allows direct labeling of molecules of any size. In kinase assays, the dye can be coupled directly to kinase substrates or to streptavidin for the capture of biotinylated substrates.

This application note presents a comparison of LANCE *Ultra* with the classical LANCE platform. The development of a Src tyrosine kinase assay measuring the phosphorylation of a poly Glu-Tyr (poly-GT) substrate was used as a model system.

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*-poly-GT	PerkinElmer	TRF0100-M
U*Light*-Streptavidin	PerkinElmer	TRF0102-M
SureLight™ Allophycocyanin-Streptavidin	PerkinElmer	CR130-100
Biotinylated-poly-GT	PerkinElmer	Custom product
LANCE Eu-PY20 Antibody	PerkinElmer	AD0066
LANCE Detection Buffer 10X	PerkinElmer	CR97-100
Src Kinase, active	Millipore Corp.	14-326
ATP (adenosine 5'-triphosphate disodium salt)	Sigma-Aldrich, Inc.	A2383
Staurosporine	Sigma-Aldrich, Inc.	S4400
White OptiPlate-384	PerkinElmer	6007290
TopSeal-A™	PerkinElmer	6005250
EnVision Multilabel Reader	PerkinElmer	2102-0010
Excitation Filter UV2(TRF) 320 nm	PerkinElmer	2100-5060
Mirror LANCE/DELFIA™ Dual	PerkinElmer	2100-4160
Emission Filter: Eu 615 nm	PerkinElmer	2100-5090
Emission Filter: LANCE 665 nm	PerkinElmer	2100-5110
Src Kinase Buffer: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT and 0.01% Tween-20		

Kinase Assays

Assay conditions were optimized independently for each of the three TR-FRET platforms. All concentrations listed below are final concentrations in either the kinase or detection reaction. LANCE *Ultra* and classical LANCE signals were detected using the same settings on the EnVision™ Multilabel Reader (excitation at 320 nm and emission at 665 nm).

LANCE *Ultra* (U*Light*-poly-GT)
Optimized conditions consisted of 1 nM Src, 100 nM U*Light*-poly-GT and ATP at the EC_{50} value. Assay components were diluted in Kinase Buffer and added to a white OptiPlate™-384 in a final volume of 10 µL. Plates were incubated for 90 min at room temperature (RT) and kinase reactions were stopped by the addition of 10 mM EDTA. Five minutes after the addition of EDTA, 2 nM of Eu-labeled PY20 antibody, diluted in detection buffer, was added for the detection of phospho-products. Reactions were incubated for 1 h at RT prior to signal reading.

LANCE *Ultra* (U*Light*-Streptavidin; biotinylated poly-GT)
Kinase reactions were performed as described for the U*Light*-poly-GT assay. Five minutes after the addition of EDTA, Eu-labeled PY20 (2 nM) antibody and U*Light*-Streptavidin (U*Light*-SA; 100 nM), diluted in Detection Buffer, were added for the detection of phospho-products. Reactions were incubated for 1 h at RT prior to signal reading.

Classical LANCE (APC-Streptavidin; biotinylated poly-GT)
Kinase reactions were performed as described for the U*Light*-poly-GT assay. Five minutes after the addition of EDTA, Eu-labeled PY20 (2 nM) antibody and APC-SA (APC-SA; 100 nM), diluted in Detection Buffer, were added for the detection of phospho-products. Reactions were incubated for 1 h at RT prior to signal reading.

Results

Assay Optimization

Assay optimization was performed in order to compare LANCE *Ultra* and classical LANCE assay formats. To determine the substrate concentration resulting in the best signal window, as well as to establish EC_{50} values, poly-GT was titrated. EC_{50} values in the low nanomolar range (0.6 nM to 4.7 nM) were obtained for all assay formats (Figure 2). Moreover, a specific signal could be detected using substrate concentrations as low as 300 pM. Due to saturation of the labeled streptavidin, a hook effect was observed at 100 nM substrate concentration in both the LANCE U*Light*-SA and the classical LANCE formats. For all assays, maximal signal window was generated at 100 nM poly-GT and this substrate concentration was used in all other experiments.

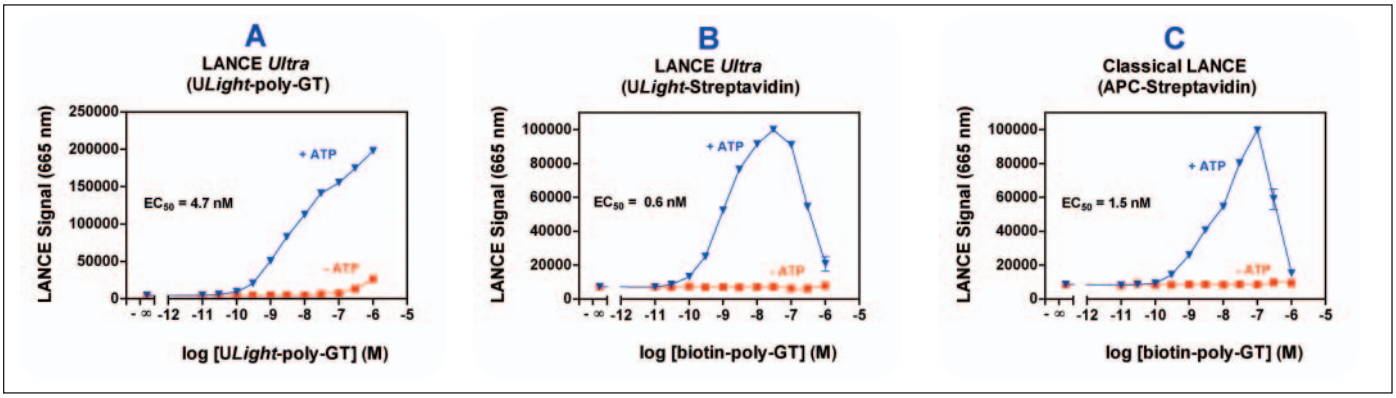

Figure 2. **Poly-GT substrate titration.** Dilutions of poly-GT substrate ranging from 10 pM to 1 µM were incubated with Src kinase (4 nM), ATP (20 µM), and A) U*Light*-poly-GT, or B) and C) biotin-poly-GT.

A Src enzyme titration was performed. Figure 3 shows that similar EC_{50} values were obtained in all three assays (U*Light*-poly-GT: 73 pM; U*Light*-SA: 37 pM; APC-SA: 65 pM). To limit assay variability and maximize the signal window, 1 nM Src kinase (a concentration in the assay plateau) was used for subsequent experiments.

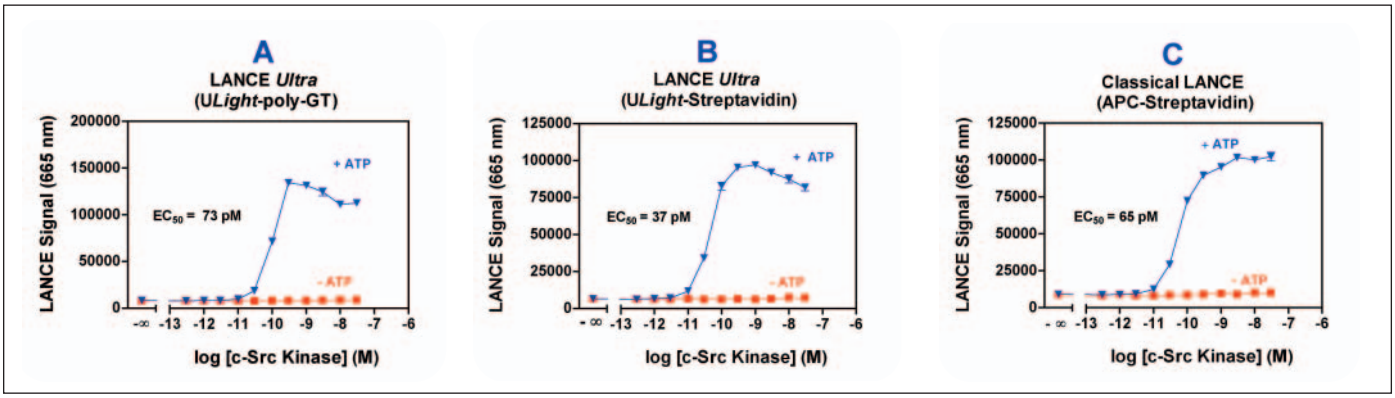

Figure 3. **Enzyme titration.** Dilutions of Src ranging from 0.3 pM to 30 nM were incubated with ATP (20 µM) and A) U*Light*-poly-GT (100 nM), or B) and C) biotin-poly-GT (100 nM).

In screening campaigns, the identification of ATP competitive inhibitors requires the use of ATP concentrations at or near the K_m value. ATP titration curves were performed to obtain EC_{50} values that were considered to be the apparent K_m values of the enzyme for ATP. Figure 4 shows similar values ranging from 250 to 500 nM for the three assays.

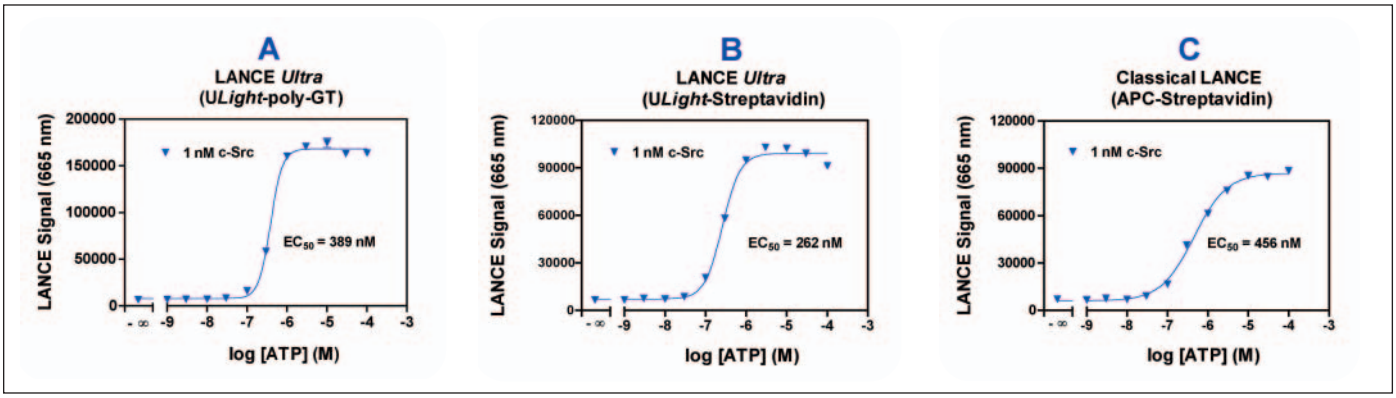

Figure 4. **ATP titration.** Dilutions of ATP ranging from 1 nM to 100 µM were incubated with Src kinase (1 nM) and A) U*Light*-poly-GT (100 nM), or B) and C) biotin-poly-GT (100 nM).

Evaluation of assay performance

Staurosporine is a broad spectrum kinase inhibitor competing for ATP binding. Staurosporine inhibition of the Src enzyme was evaluated to compare the performance of the three optimized LANCE assay formats. Competition curves performed at the apparent K_m for ATP show that the three TR-FRET platforms give virtually identical IC_{50} values for staurosporine (U*Light*-poly-GT: 19 nM; U*Light*-SA: 26 nM; APC-SA: 46 nM). These values are all within one log of published IC_{50} values for a staurosporine inhibition of Src[1].

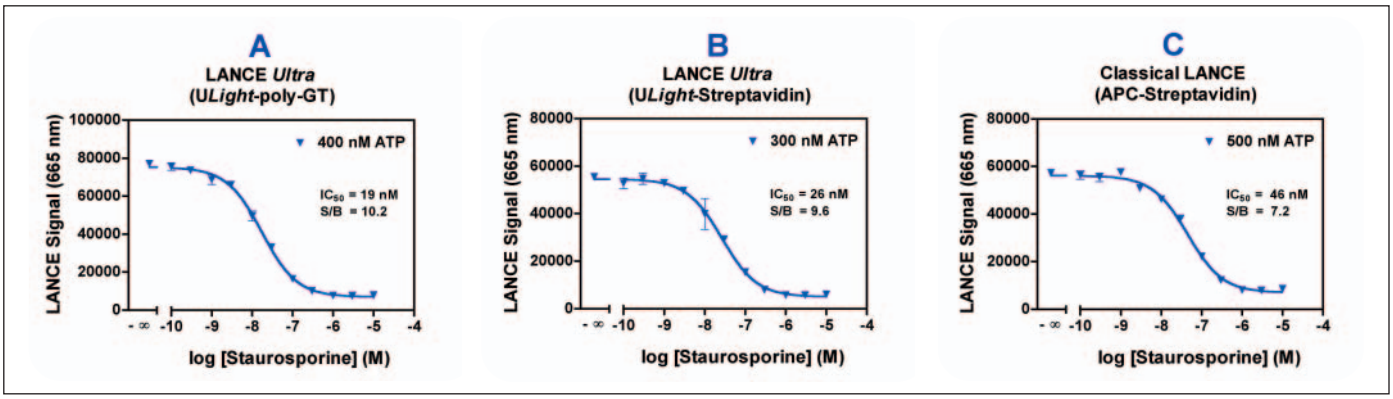

Figure 5. **Staurosporine inhibition curves.** Dilutions of staurosporine ranging from 100 pM to 10 µM were pre-incubated for 10 min at RT using optimized Src and substrate conditions. ATP concentrations at the EC_{50} value were used: A) 400 nM, B) 300 nM, and C) 500 nM.

The robustness of the three assay platforms was assessed by determining the Z'-factor[2] in the presence or absence of 10 µM staurosporine (Figure 6). Assays were performed using ATP concentrations at the apparent K_m. Reaction mixtures with or without staurosporine were dispensed into 24 wells of a 384-well OptiPlate. Z'-factors of 0.87 and 0.85 were obtained for the LANCE *Ultra* U*Light*-poly-GT and U*Light*-Streptavidin containing reactions, respectively. The Z'-factor calculated for the classical LANCE assay was similar, with a value of 0.85.

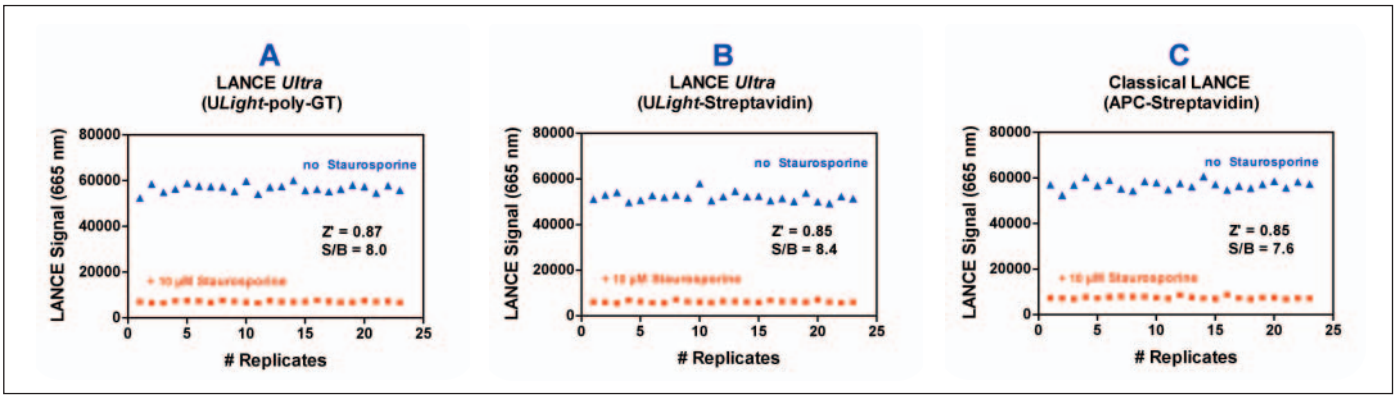

Figure 6. **Z'-factor determination for the Src kinase assay.** Optimized enzyme and substrate concentrations were used. ATP was added at the EC_{50} value. Kinase reactions were performed in the absence (24 wells) or presence of 10 µM staurosporine (24 wells).

Discussion

This application note describes the optimization of an HTS Src kinase assay using PerkinElmer's LANCE *Ultra* and classical LANCE platforms. When compared to classical LANCE, the new LANCE *Ultra* assays give similar EC_{50} values for the substrate, enzyme, and ATP. Additionally, the results obtained from the staurosporine inhibition curves and Z'-factor determinations illustrate the robust performance and HTS suitability of both LANCE *Ultra* assay formats.

The power of LANCE *Ultra* comes from the combination of PerkinElmer's Eu chelate donor dye and new U*Light* acceptor dye. The U*Light* dye offers distinct advantages over APC. It is light resistant and its small size allows for the direct labeling of molecules of any size. Moreover, the possibility of directly labeling kinase substrates with the U*Light* dye will greatly simplify assay development. For cases where a biotinylated substrate is preferred, the use of U*Light*-SA provides an ideal alternative.

References

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

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