Comparison of LANCE® *Ultra* with Other TR-FRET Platforms for a Src Tyrosine Kinase Assay

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1 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 drug discovery. Analysis of the human genome has revealed the existence of nearly 520 genes encoding kinases. The abundance of these potential therapeutic targets strengthens the need for developing efficient and robust high throughput screening (HTS) platforms for the discovery of kinase modulators.

LANCE® *Ultra* is PerkinElmer's improved TR-FRET platform enabling the rapid development of robust and sensitive kinase assays. LANCE *Ultra* reagents include a series of europium (Eu) chelate-labeled anti-phospho-substrate antibodies and several kinase substrates labeled with the new U*Light*™ acceptor dye. The U*Light* dye is a small molecular weight fluorescent dye with a red-shifted emission.

This study shows the performance of two LANCE *Ultra* assay set-ups (U*Light*-poly GT-based and U*Light*-streptavidin-based), and compare it to classical LANCE (APC-based), and to an alternative fluorescein/terbium-based TR-FRET platform. Phosphorylation of poly Glu-Tyr (poly GT) by the Src kinase was used as a model system.

2 Materials

Item	Supplier	Cat. Number
➢ U*Light*™-poly GT	PerkinElmer LAS, Inc.	TRF0100-M
➢ U*Light*™ -streptavidin	PerkinElmer LAS, Inc.	TRF0102-M
➢ SureLight™ APC-SA	PerkinElmer LAS, Inc.	CR130-100
➢ LANCE EU-PY20 Antibody	PerkinElmer LAS, Inc.	AD0066
➢ Biotinylated-poly GT	PerkinElmer LAS, Inc.	custom product
➢ LANCE Detection Buffer 10X	PerkinElmer LAS, Inc.	CR97-100
➢ Fluorescein-Poly GT	Invitrogen Corp.	PV3610
➢ Tb-PY20 antibody	Invitrogen Corp.	PV3552
➢ TR-FRET dilution buffer	Invitrogen Corp.	PV3574
➢ Src, active	Millipore Corp.	14-326
➢ White OptiPlate™-384	PerkinElmer LAS, Inc.	6007290
➢ TopSeal™-A	PerkinElmer LAS, Inc.	6005250
➢ EnVision® Multilabel Reader	PerkinElmer LAS, Inc.	2103-0010
➢ Mirror: LANCE/DELFIA Dual	PerkinElmer LAS, Inc.	2100-4160
➢ Excitation Filter: UV2(TRF) 320 nm	PerkinElmer LAS, Inc.	2100-5060
➢ Emission Filter: Eu 615 nm	PerkinElmer LAS, Inc.	2100-5090
➢ Emission Filter: LANCE 665 nm	PerkinElmer LAS, Inc.	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

3 Methods

LANCE *Ultra* (U*Light*-poly GT-based):
In the optimized assay, 1 nM c-Src, 100 nM U*Light*-poly GT, and ATP (concentrations indicated in text) were pre-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 RT and kinase reactions were stopped by the addition of 5 µL of EDTA at 40 mM. After 5 min, 5 µL of Eu-labeled PY20 antibody at 8 nM was added for the detection of the phospho-substrate. Detection reactions were incubated for 1 h at RT. The LANCE *Ultra* signal was detected using the EnVision Multilabel Reader. Excitation wavelength was set at 320 nm and emission was recorded at 665 nm.

LANCE *Ultra* (U*Light*-streptavidin-based):
U*Light*-streptavidin was used in combination with biotinylated poly GT. Optimized kinase assay conditions were similar to the ones for the assay with U*Light* poly-GT except that for the capture of the biotinylated substrate. U*Light*-streptavidin was added at a final concentration of 100 nM together with the Eu-PY20 antibody.

Classical LANCE (APC-streptavidin-based):
SureLight APC-streptavidin was used in combination with biotinylated poly GT. Optimized kinase assay conditions were similar to the ones described above except that for the capture of the biotinylated substrate. SureLight, APC-streptavidin was added at a final concentration of 100 nM together with the Eu-PY20 antibody.

TR-FRET (terbium, fluorescein-poly GT-based):
Optimized assay procedures were identical to the ones developed for the LANCE *Ultra* U*Light* poly-GT assay except that fluorescein-poly GT and terbium (Tb)-labeled PY20 antibodies were used in place of the U*Light* substrate and Eu-PY20 antibodies. The fluorescein substrate was diluted in buffer provided by the supplier and Tb-PY20 antibodies were diluted in TR-FRET dilution buffer. The excitation wavelength was set at 320 nm and emission was recorded at 520 nm.

4 LANCE® *Ultra* Technology

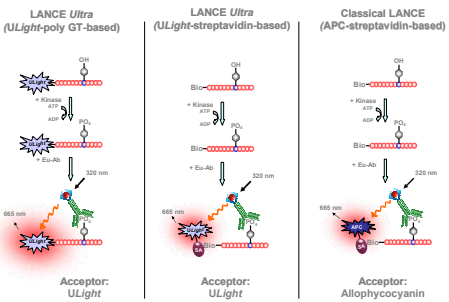
In LANCE TR-FRET assays, the donor dye is an europium-based chelate (Eu chelate). The life-time of the Eu light emission is exceptionally long, allowing for time-delayed measurements. The unique fluorescence properties of Eu chelates make them ideal energy donors in TR-FRET assays.

In classical LANCE assays, the energy emitted upon excitation of an Eu chelate is transferred to an allophycocyanin (APC) acceptor dye molecule which in turn emits light at 665 nm. Although APC allows for the efficient capture and reemission of the transferred energy, it has some disadvantages. APC is a large protein, whose bulkiness may create steric hindrance in some assay configurations. In addition, small molecules cannot be labeled directly with APC and a bridging assay component, such as streptavidin-APC (APC-SA), must be introduced in the assay set-up.

To overcome these limitations, PerkinElmer has developed the new LANCE *Ultra* HTS platform where the APC acceptor dye has been replaced by the new U*Light* dye. This small acceptor dye has spectral characteristics similar to APC and offers the advantage of not being light sensitive. Importantly, its low molecular weight makes it suitable for the direct labeling of molecules of any size. In kinase assays, the U*Light* dye is coupled either directly to the kinase substrate, or to streptavidin for the capture of a biotinylated substrate. In addition, the red-shifted emission of the U*Light* dye makes it resistant to color interference which eliminates the need for ratiometric analysis of the data.

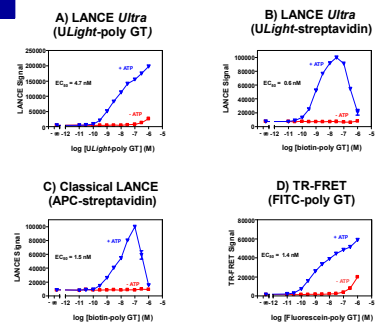
5 Assay Principle

Time-resolved fluorescence resonance energy transfer (TR-FRET) HTS assays are homogeneous proximity assays where the interaction of two dye-labeled binding partners is detected by the energy transfer between a donor and an acceptor dye, and the subsequent light emission by the acceptor dye. Currently, three LANCE TR-FRET platforms are available. They differ principally by the nature of the acceptor dye used for the energy transfer and by the substrate, which can be either directly labeled or biotinylated. Another type of TR-FRET platform currently available uses a terbium chelate as a donor and fluorescein as an acceptor dye.



In LANCE *Ultra*, U*Light*-poly GT-based, poly GT is directly labeled with the new U*Light* acceptor dye (left panel), while in LANCE *Ultra*, U*Light*-streptavidin-based, poly GT is biotinylated and captured by a U*Light*-streptavidin molecule (middle panel). In the classical LANCE APC-based assay, biotinylated poly GT is captured by an APC-SA molecule (right panel). In TR-FRET terbium assays (not shown), peptide substrates are labeled directly with fluorescein. In all four assays, the anti-phosphotyrosine PY20 antibody is labeled with the donor molecule (Eu or Tb chelate). Upon phosphorylation, the poly GT substrate is recognized by the anti-phosphotyrosine antibody bringing the donor and acceptor molecules into close proximity. Excitation of the Eu or Tb chelate leads to an energy transfer to the acceptor molecules resulting in a light emission proportional to the level of substrate phosphorylation.

6 Substrate Titration

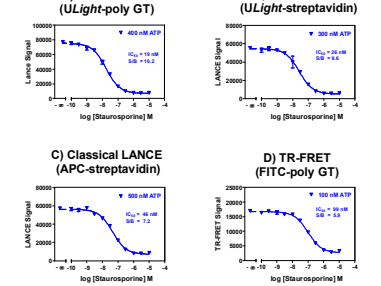


Dilutions of poly GT substrate ranging from 10 pM to 1 µM were incubated in the presence of fixed concentration of c-Src (4 nM) and ATP (20 µM). **A)** U*Light*-poly GT, **B) and C)** biotin-poly GT, **D)** Fluorescein-poly GT. Detection of the phosphorylated substrates was performed using **A)** 2 nM of Eu-PY20 antibody, **B)** 2 nM of Eu-PY20 antibody and 100 nM of U*Light*-streptavidin, **C)** 2 nM of Eu-PY20 antibody and 100 nM of APC-streptavidin and **D)** 2 nM of Tb-PY20 antibody. Note that signal in panels **B** and **C** decreases at substrate concentration higher than 100 nM due to the limiting concentration of labeled streptavidin in the assay (hook effect).

➡ EC_{50} values in the low nanomolar range (0.6 nM to 4.7 nM) were obtained for the poly GT substrate with all four platforms.

Titration of the Src enzyme was also performed for the four assay set-ups (data not shown). Again, similar EC_{50} values were obtained: 73 pM using U*Light*-poly GT, 37 pM using U*Light*-streptavidin, 65 pM using APC-SA and 37 pM using Fluorescein-poly GT.

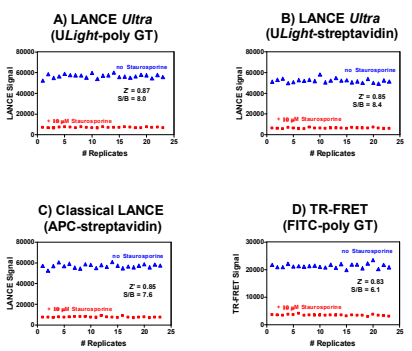
7 Staurosporine Inhibition



Dilutions of staurosporine ranging from 100 pM to 10 µM were pre-incubated for 10 min at RT in the presence of 1 nM of Src kinase. Kinase reactions included 100 nM of poly GT substrate with ATP concentrations at the EC_{50} value determined for each platform: **A)** 400 nM, **B)** 300 nM, **C)** 500 nM and **D)** 100 nM. Detection was performed using **A)** 2 nM of Eu-PY20 antibody, **B)** 2 nM of Eu-PY20 antibody and 100 nM of U*Light*-streptavidin, **C)** 2 nM of Eu-PY20 antibody and 100 nM of APC-streptavidin and **D)** 2 nM of Tb-PY20 antibody. Signal to background (S/B) ratio is superior in LANCE *Ultra* assays compared to classical LANCE and TR-FRET assays using a fluorescein-labeled substrate.

➡ IC_{50} values determined for staurosporine inhibition of the Src kinase were of the same order of magnitude for the four TR-FRET platforms, with values of 19 nM for LANCE *Ultra* (U*Light*-poly GT), 26 nM for LANCE *Ultra* (U*Light*-streptavidin), 46 nM for classical LANCE, and 99 nM for terbium-based TR-FRET.

8 Assay Performance



Assay performance and robustness were evaluated by calculating the Z' factor[1] with two sets of data: 1) total signal from a kinase reaction incubated in the absence of inhibitor (24 wells) and 2) signal obtained from kinase reactions incubated in the presence of 10 µM staurosporine (24 wells). Src enzyme (1 nM) was pre-incubated in Kinase Buffer in the presence or absence of staurosporine for 10 min at RT. Afterwards, poly GT substrates at a concentration of 100 nM and ATP at concentrations corresponding to the EC_{50} value (see Figure 7) were then added. Detection was performed using **A)** 2 nM of Eu-PY20 antibody, **B)** 2 nM of Eu-PY20 antibody and 100 nM of U*Light*-streptavidin, **C)** 2 nM of Eu-PY20 antibody and 100 nM of APC-streptavidin and **D)** 2 nM of Tb-PY20 antibody.

➡ Z' factors of 0.87 and 0.85 were calculated for the U*Light*-poly GT assay and U*Light*-streptavidin assay, respectively. Z' factors obtained using the other two TR-FRET platforms were 0.85 for classical LANCE and 0.83 for fluorescein-poly GT. These results show that excellent assay robustness is achieved with all four TR-FRET platforms.

[1]*Zhang et al., J Biomol Screen. 1999;4:67–73*

9 Conclusions

Optimization of an HTS Src kinase assay using PerkinElmer new LANCE *Ultra* reagents highlights the robust performance of the new U*Light* dye as an acceptor for TR-FRET assays.

- The Src assay developed using LANCE *Ultra* reagents results in performances comparable to classical LANCE, with similar EC_{50} and IC_{50} values for poly GT, ATP and staurosporine.
- LANCE *Ultra* compares favorably to terbium/fluorescein-based TR-FRET platform assays with higher signals, wider assay windows, and an assay robustness demonstrated by Z' factors of 0.85 and above.
- Our data show that, in addition to simplifying assay set-up, the direct labeling of kinase substrates with the smaller U*Light* dye brings improved assay performance. PerkinElmer also offers U*Light* custom labeling of proteins, peptides and other small molecules.
- The red-shifted emission properties of the new U*Light* dye provide minimal screening compound interference. This feature makes LANCE *Ultra* the platform of choice for HTS kinase assays.
- The new Lance *Ultra* reagents offer assay design flexibility with a choice of U*Light*-labeled kinase substrates and U*Light*-streptavidin for use with biotinylated substrates.

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