LANCE *Ultra* p60$^{c\text{-}src}$ kinase assay.

LANCE® *Ultra* TR-FRET technology

This LANCE *Ultra* kinase assay measures the phosphorylation of a poly GAT peptide substrate.

Europium-anti-phospho-tyrosine (PY20) antibody:

- AD0066: 50 µg, 7,800 assay points*
- AD0067: 1 mg, 150,000 assay points*

**40 fmol/assay point*

U*Light*™-poly GAT (1:1:1):

- TRF0101-D: 1 nmole, 1,000 assay points*
- TRF0101-M: 10 nmoles, 10,000 assay points*

**1 pmol/assay point*

Recognized motif:

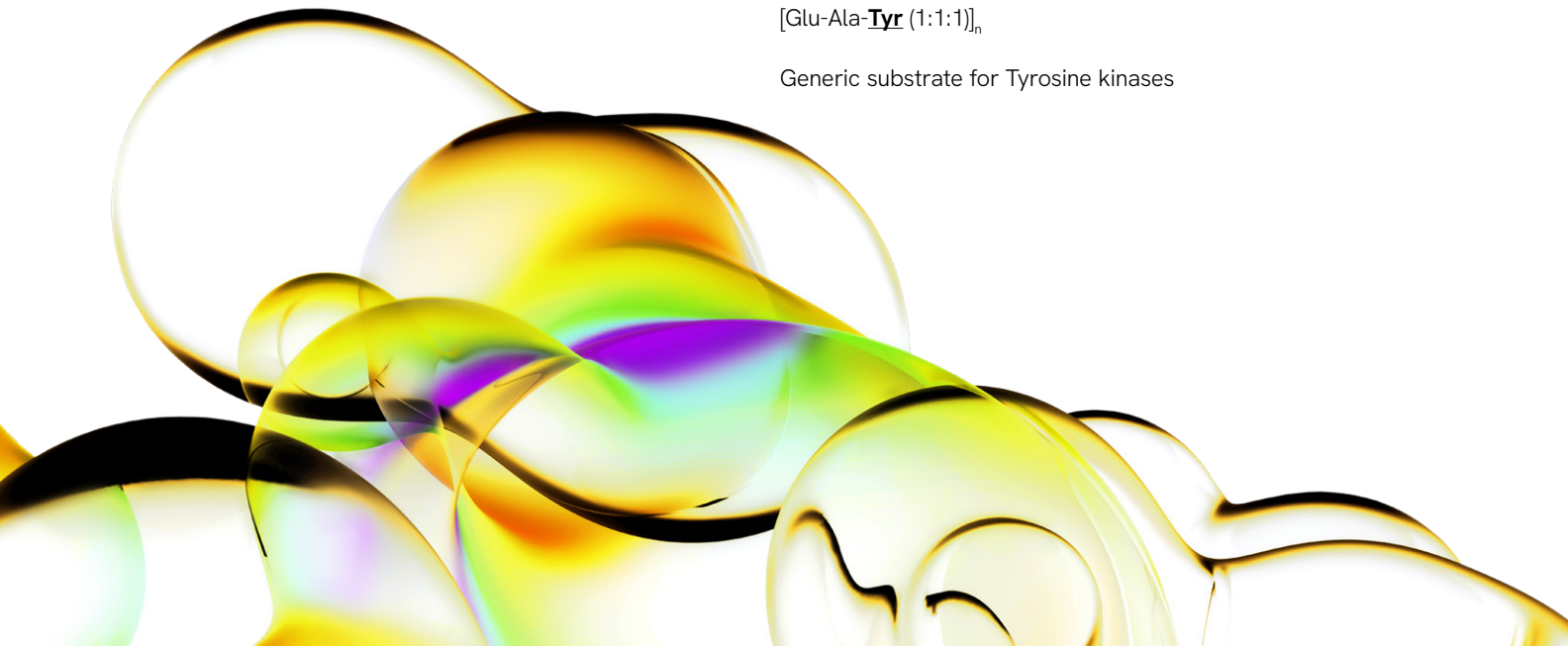
pTyr

Mouse monoclonal IgG^{2b} antibody directed against phospho-tyrosine

Peptide sequence:

[Glu-Ala-**Tyr** (1:1:1)]$_n$

Generic substrate for Tyrosine kinases



LANCE *Ultra* kinase assays:

LANCE *Ultra* time-resolved fluorescence resonance energy transfer (TR-FRET) assays use a proprietary europium chelate donor dye, W1024 (Eu), together with U*Light*™, a small molecular weight acceptor dye with a red-shifted fluorescent emission.

In this technical note, we present the optimization of a p60$^{c\text{-}src}$ kinase assay using a U*Light*-labeled poly GAT (1:1:1) substrate. The binding of a Eu-labeled antibody directed against tyrosine phosphorylation of the U*Light*-poly GAT (1:1:1) substrate brings the Eu donor and U*Light* acceptor dye molecules into close proximity. Upon irradiation at 320 or 340 nM, the energy from the Eu donor is transferred to the U*Light* acceptor dye which, in turn, generates light at 665 nM. The intensity of the light emission is proportional to the level of U*Light* substrate phosphorylation.

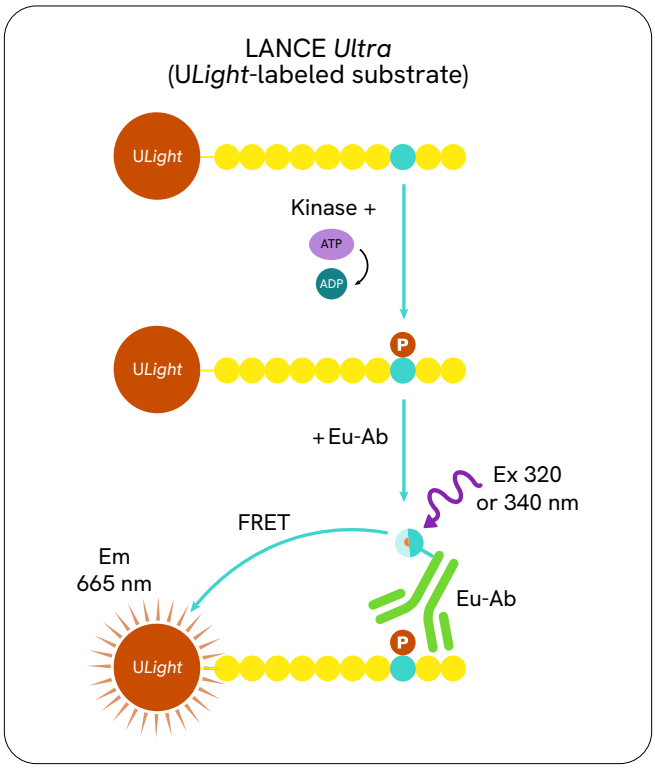


Figure 1: Schematic representation of the LANCE *Ultra* detection of a phosphorylated substrate.

Development of a p60$^{c\text{-}src}$ kinase assay

Reagents needed for this assay:

Europium-labeled Europium-anti-phospho-tyrosine (PY20) Antibody	Revvity # AD066 and AD067
U*Light*-poly GAT (1:1:1)	Revvity # TRF0101
p60$^{c\text{-}src}$, recombinant	Millipore # 14-326
LANCE® Detection Buffer, 10X	Revvity # CR97-100
OptiPlate™-384, white	Revvity # 6007299
TopSeal™-A film	Revvity # 6050195

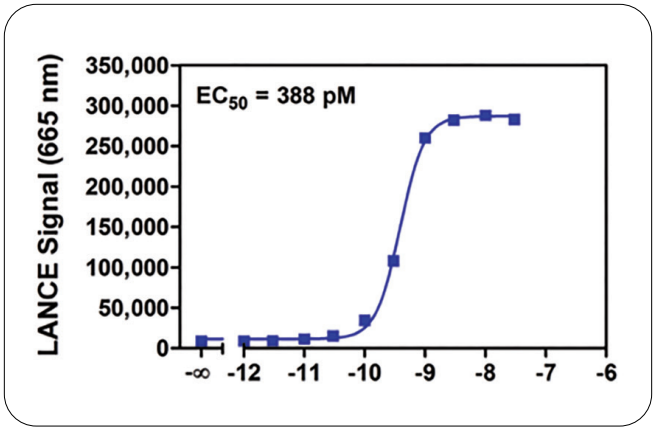
Kinase Buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT and 0.01% Tween-20

Standard protocol

- Dilute kinase, ATP, inhibitors and U*Light*-poly GAT (1:1:1) in Kinase Buffer.
- Dilute antibody (Ab) in 1X LANCE Detection Buffer to 8 nM.
- Add to the wells of a white OptiPlate-384:
  - 5 µL of p60$^{c\text{-}src}$ enzyme
  - 2.5 µL of inhibitor or Kinase Buffer
  - 2.5 µL of U*Light*-poly GAT (1:1:1)/ATP mix (for ATP titration, ATP dilutions are added separately in Kinase Buffer).
- Cover the plate with TopSeal-A film and incubate the enzymatic reaction at room temperature (RT).
- Stop the reaction by adding 5 µL of 40 mM EDTA in 1X LANCE Detection Buffer. Leave 5 min at RT.
- Add 5 µL of the antibody dilution (2 nM final concentration).
- Cover with TopSeal-A film and incubate for 1 h at RT.
- Remove the TopSeal-A film and read signal with the EnVision® Multilabel Reader in TR-FRET mode (excitation at 320 or 340 nM and emission at 665 nM).

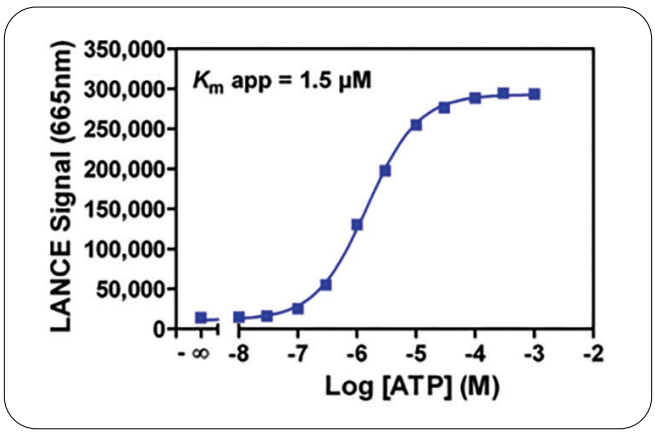
Note: Eu-labeled antibodies and EDTA can be premixed just before use as a 2X concentrated Stop Solution/Detection Mix to minimize the number of liquid handling steps.

Experiment 1: Enzymatic titration



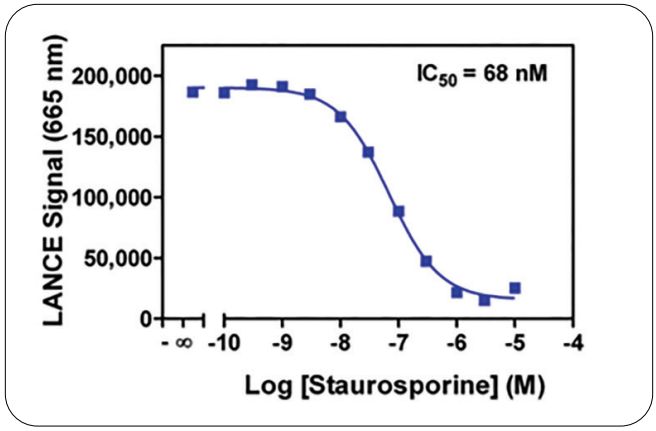
p60$^{c\text{-}src}$ enzyme was incubated at concentrations ranging from 1 pM to 30 nM with 100 nM U*Light*-poly GAT (1:1:1) and 20 µM ATP. Kinase reactions were terminated at the indicated times by the addition of EDTA. Detection mix was added and signal read after 60 min.

Experiment 2: ATP titration



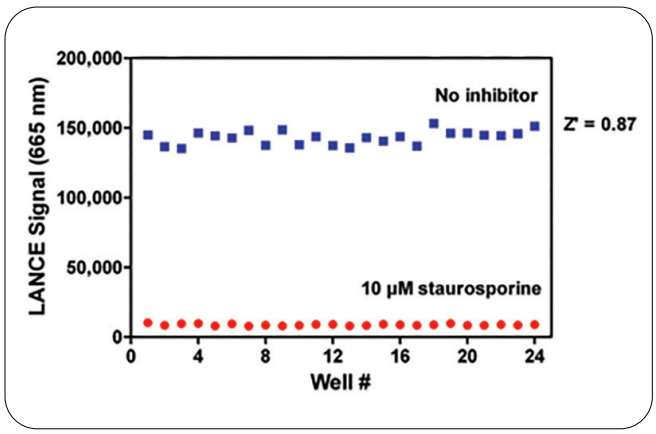
Serial dilutions of ATP ranging from 10 nM to 1 mM were added to 1 nM p60$^{c\text{-}src}$ kinase and 100 nM of U*Light*-poly GAT (1:1:1) substrate. Kinase reactions were terminated after 60 min by the addition of EDTA.

Experiment 3: Enzyme inhibition curve



Serial dilutions of staurosporine ranging from 100 pM to 10 µM (final concentrations in 2% DMSO) were pre-incubated for 5 min with the p60$^{c\text{-}src}$ enzyme (1 nM final concentration). Then 100 nM U*Light*-Poly GAT (1:1:1) and 1.5 µM ATP were added. Kinase reactions were terminated after 60 min by the addition of EDTA.

Experiment 4: Z′-factor determination



p60$^{c\text{-}src}$ enzyme at 1 nM was incubated with 100 nM U*Light*-poly GAT (1:1:1) substrate in kinase assay buffer with 1.5 µM ATP, and with or without 10 µM staurosporine. Reactions were terminated after 60 min by the addition of EDTA.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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