TECHNICAL NOTE

*U*-TRF #27

# LANCE *Ultra* EphA4 Kinase Assay

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## LANCE® *Ultra* TR-FRET Technology

This LANCE *Ultra* kinase assay measures the phosphorylation of the U*Light*™-TK peptide substrate.

- TRF0127-D: 0.5 nmole, 1,000 assay points*
- TRF0127-M: 5 moles, 10,000 assay points*

**0.5 pmol/assay point*

### Europium-anti-phosphotyrosine (PT66):

- AD0068: 50 µg, 7,800 assay points*
- AD0069: 1 mg, 156,000 assay points*

**40 fmol/assay point*

### Recognized Motif:

Phosphorylated tyrosine residues

### LANCE *Ultra* 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 EphA4 kinase assay using a U*Light*-labeled peptide substrate. The binding of the Eu-labeled PT66 antibody to the phosphorylated U*Light*-TK peptide 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.

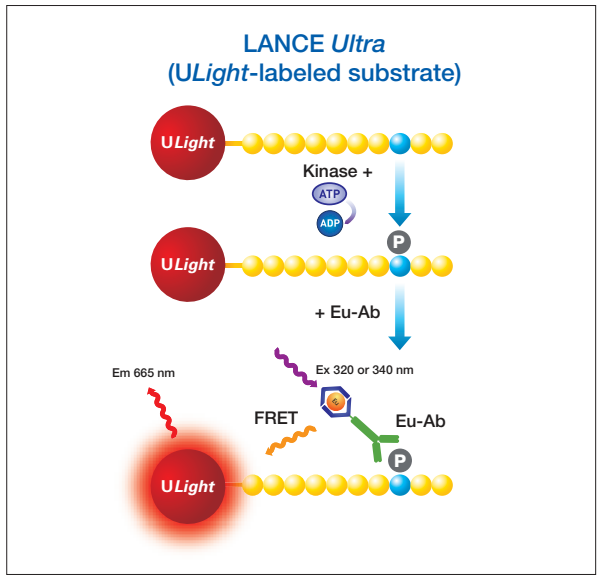


*Figure 1.* Schematic representation of the LANCE *Ultra* detection of a phosphorylated peptide substrate.

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## Development of an Eph4A Kinase Assay

### Reagents needed for this assay:

| | |
|---|---|
| Europium-labeled anti-phospho-tyrosine Antibody | PerkinElmer # AD0068/AD0069 |
| U*Light*-TK Peptide | PerkinElmer # TRF0127 |
| EphA4 | Carna # 08-123 |
| LANCE Detection Buffer, 10X | PerkinElmer # CR97-100 |
| White opaque OptiPlate™-384 | PerkinElmer # 6007290 |
| TopSeal™-A Film | PerkinElmer # 6050195 |

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

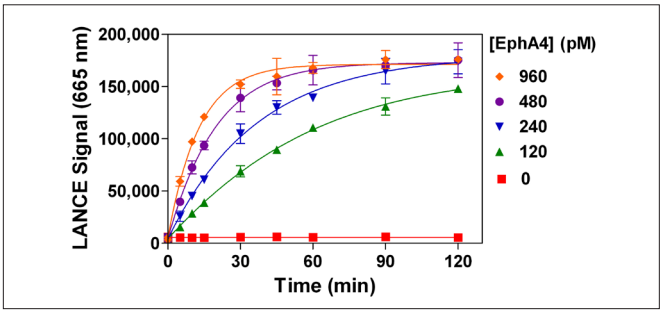
### Standard Protocol

- Dilute the EphA4 enzyme, ATP, inhibitors and U*Light*-TK Peptide in Kinase Buffer.
- Prepare a 4X Detection Mix by diluting the Eu-anti-phosphotyrosine Antibody to 8 nM in 1X LANCE Detection Buffer.
- Add to the wells of a white OptiPlate-384:
  – 5 µL of EphA4 enzyme
  – 2.5 µL of inhibitor or Kinase Buffer
  – 2.5 µL of U*Light*-TK Peptide/ATP mix (for ATP titration, ATP dilutions are added separately in Kinase Buffer).
- Cover the plate with TopSeal-A film and incubate at RT.
- Stop kinase reactions by adding 5 µL of 40 mM EDTA prepared in 1X LANCE Detection Buffer (Stop Solution). Leave for 5 min at RT.
- Add 5 µL of Detection Mix (Eu-anti-phosphotyrosine Antibody at a final concentration of 2 nM).
- Cover with TopSeal-A film and incubate 1 h at RT.
- Remove the TopSeal-A film and read signal with the EnVision® Multilabel Plate 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.

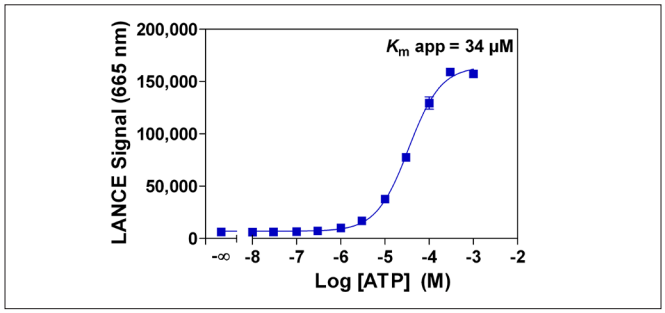
## Results

### Experiment 1: Enzyme Titration and Time-Course



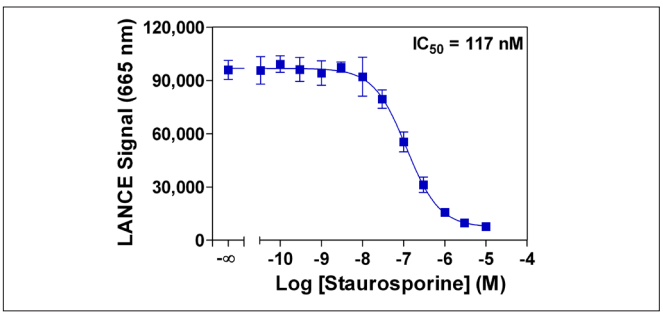

Enzymatic progress curves were produced by incubating the EphA4 enzyme at concentrations ranging from 120 to 960 pM with 50 nM U*Light*-TK Peptide and 200 µM ATP. Kinase reactions were terminated at the indicated times by the addition of EDTA. Detection mix was added and signal read after 60 minutes.

### Experiment 2: ATP Titration



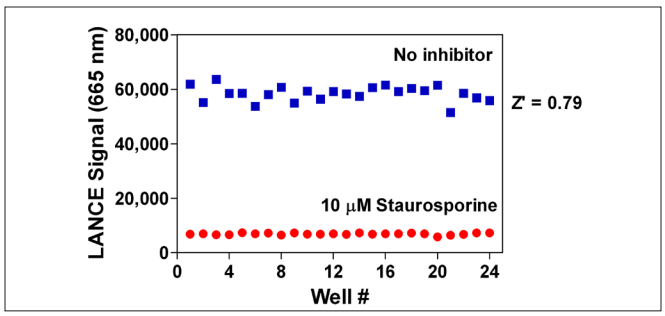

Increasing concentrations of ATP ranging from 10 nM to 1 mM were incubated with 120 pM EphA4 enzyme and 50 nM of U*Light*-TK Peptide. Kinase reactions were terminated after 60 min by the addition of EDTA.

### Experiment 3: Enzyme Inhibition Curve




Increasing concentrations of Staurosporine ranging from 30 pM to 10 µM (final concentrations in 2% DMSO) were incubated with 120 pM EphA4 enzyme, 50 nM U*Light*-TK Peptide and 35 µM ATP. Kinase reactions were terminated after 60 min by the addition of EDTA.

### Experiment 4: Z'-factor Determination




EphA4 enzyme at 120 pM was incubated with 50 nM U*Light*-TK Peptide in kinase assay buffer with 35 µM ATP, and with or without 10 µM staurosporine (final concentrations in 2% DMSO). Kinase reactions were terminated after 60 min by the addition of EDTA. A Z' value of 0.79 indicates that the assay is very robust and can be used for HTS.

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