LANCE Ultra EphA4 Kinase Assay

This LANCE Ultra kinase assay measures the phosphorylation of the ULight™-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 ULight™, a small molecular weight acceptor dye with a red-shifted fluorescent emission.

In this technical note, we present the optimization of an EphA4 kinase assay using a ULight-labeled peptide substrate. The binding of the Eu-labeled PT66 antibody to the phosphorylated ULight-TK peptide substrate brings the Eu donor and ULight acceptor dye molecules into close proximity. Upon irradiation at 320 or 340 nm, the energy from the Eu donor is transferred to the ULight acceptor dye which, in turn, generates light at 665 nm. The intensity of the light emission is proportional to the level of ULight substrate phosphorylation.

Figure 1. Schematic representation of the LANCE Ultra detection of a phosphorylated peptide substrate.
Development of an EphA4 Kinase Assay

Reagents needed for this assay:
- Europium-labeled anti-phosphotyrosine Antibody: PerkinElmer # AD0068/AD0069
- ULight-TK Peptide: PerkinElmer # TRF0127
- EphA4: Carma # 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 MgCl₂, 2 mM DTT and 0.01% Tween-20.

Standard Protocol
- Dilute the EphA4 enzyme, ATP, inhibitors and ULight-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 ULight-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 ULight-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 ULight-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 ULight-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 ULight-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.