TECHNICAL NOTE

U-TRF #11

LANCE *Ultra* PKC Kinase Assay

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LANCE® *Ultra* TR-FRET Technology

This LANCE *Ultra* kinase assay measures the phosphorylation of a PKC peptide substrate at Ser25.

Europium-anti-phospho-PKC (Ala25Ser) Peptide Antibody:

- TRF0207-D: 10 µg, 1,562 assay points*
- TRF0207-M: 100 µg, 15,625 assay points*

**40 fmol/assay point*

U*Light*™-PKC Peptide:

- TRF0108-D: 0.5 nmole, 1,000 assay points*
- TRF0108-M: 5 nmoles, 10,000 assay points*

**0.5 pmol/assay point*

Recognized Motif:

RFARKG**pS**LRQKNV

Europium-labeled mouse monoclonal antibody recognizing phospho-Ser25 in human PKC in which Ala25 is mutated to Ser and phosphorylated.

Peptide Sequence:

CRFARKG**S**LRQKNV

Synthetic peptide derived from amino acids 19-31 of PKC-α; phosphorylation site: Ser25.

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 PKC kinase assay using a U*Light*-labeled peptide substrate. The binding of a Eu-labeled antibody directed against Ser25 phosphorylation of the PKC 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.

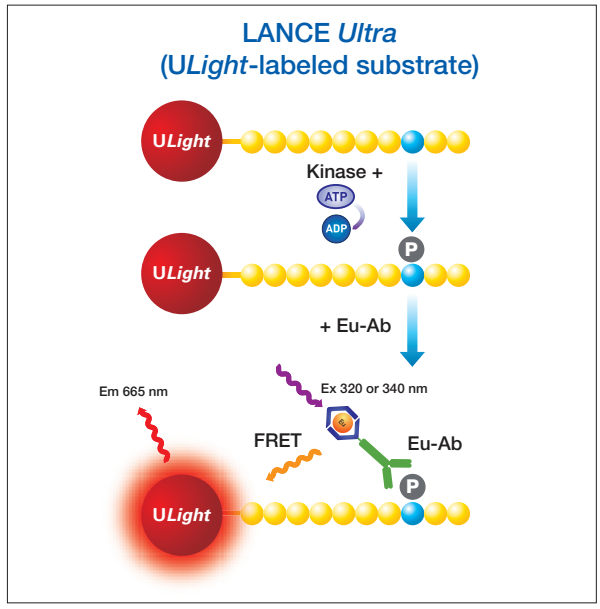

Figure 1. Schematic representation of the LANCE *Ultra* detection of a phosphorylated peptide substrate.

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Development of a PKC Kinase Assay

Reagents needed for this assay:

Europium-labeled anti-phospho-PKC (Ala25Ser) Peptide Antibody	PerkinElmer # TRF0207
U*Light*-PKC Peptide	PerkinElmer # TRF0108
PKC-α	Invitrogen # P2227
LANCE® Detection Buffer, 10X	PerkinElmer # CR97-100
OptiPlate™-384, white	PerkinElmer # 6007299
TopSeal™-A film	PerkinElmer # 6050195

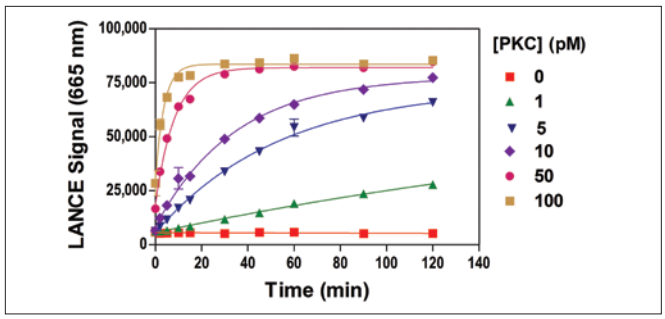
Kinase Buffer: 50 mM HEPES pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT and 0.01% Tween-20.

Standard Protocol

- Dilute the PKC-α kinase, ATP, inhibitors and U*Light*-PKC peptide in Kinase Buffer.
- Prepare a 4X Detection Mix by diluting the Eu-anti-phospho PKC (Ala25Ser) antibody to 8 nM in 1X LANCE Detection Buffer.
- Add to the wells of a white OptiPlate-384:
  - 5 µL of PKC-α enzyme
  - 2.5 µL of inhibitor or Kinase Buffer
  - 2.5 µL of U*Light*-PKC substrate/ATP mix (for ATP titration, ATP dilutions are added separately in Kinase Buffer).
- Cover the plate with TopSeal-A film and incubate for 30 min at room temperature (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 4X Detection Mix (Eu-anti-phospho-PKC (Ala25Ser) Antibody at a final concentration of 2 nM).
- 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).

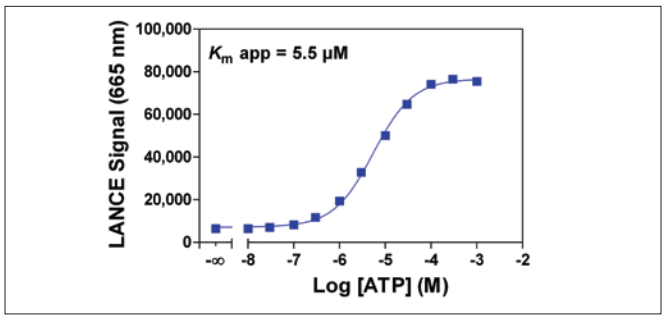
NOTE: *Eu-labeled antibodies and EDTA can be premixed just before use as a 2X concentrated Stop Solution/Detection Mix to minimize the number of liquid handling steps.*

Experiment 1: Enzymatic Titration and Time Course



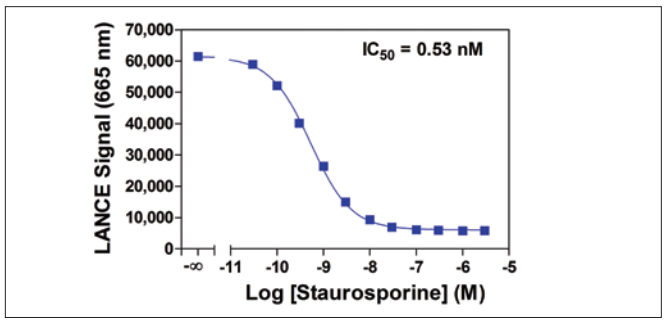
Enzymatic progress curves were produced by incubating PKC-α enzyme at concentrations ranging from 1 to 100 pM with 50 nM U*Light*-PKC substrate and 10 µM ATP. Kinase reactions were terminated at the indicated times by the addition of EDTA. Detection mix was added and signal read after 60 minutes.

Experiment 2: ATP Titration



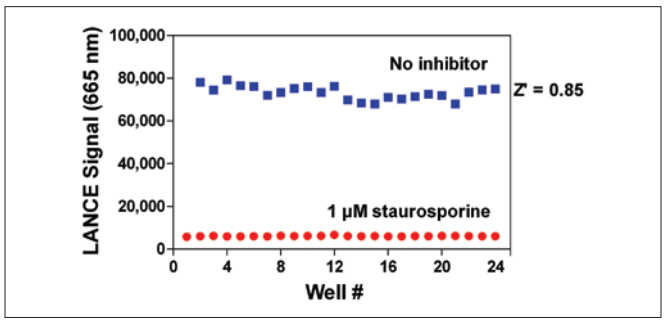
Serial dilutions of ATP ranging from 10 nM to 1 mM were added to 10 pM PKC-α and 50 nM U*Light*-PKC substrate. Kinase reactions were terminated after 30 min by the addition of EDTA.

Experiment 3: Enzyme Inhibition Curve



Serial dilutions of staurosporine ranging from 30 pM to 3 µM (final concentrations in 1% DMSO) were incubated with 10 pM PKC-α, 50 nM U*Light*-PKC substrate and 10 µM ATP. Kinase reactions were terminated after 30 min by the addition of EDTA.

Experiment 4: Z'-factor Determination



PKC-α enzyme at 10 pM was incubated with 50 nM U*Light*-PKC substrate and 10 µM ATP, with or without 1 µM staurosporine (final concentrations in 1% DMSO). Kinase reactions were terminated after 30 min by the addition of EDTA.

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