

LANCE *Ultra* Akt1/PKB α Kinase Assay

U-TRF #9

LANCE[®] *Ultra* TR-FRET Technology

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This LANCE *Ultra* kinase assay measures the phosphorylation of a Crosstide peptide substrate at Ser21.

Europium-anti-phospho-Crosstide (GSK-3 α Ser21) Antibody:

- TRF0202-D: 10 μ g, 1,562 assay points*
- TRF0202-M: 100 μ g, 15,625 assay points*

*40 fmol/assay point

- RECOGNIZED MOTIF: CGSGSGRPRTSpSFAEG
- Europium-labeled mouse monoclonal antibody recognizing phospho-Ser21 in peptides derived from glycogen synthase kinase-3 alpha (GSK-3 α)

ULight[™]-Crosstide (GSK-3 α Ser21) Peptide:

- TRF0106-D: 0.5 nmole, 1,000 assay points*
- TRF0106-M: 5 nmoles, 10,000 assay points*

*0.5 pmol/assay point

- PEPTIDE SEQUENCE: CGSGSGRPRTSpSFAEG
 - Synthetic peptide derived from glycogen synthase kinase-3 alpha (GSK-3 α)
 - Phosphorylation site: Ser21

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 ULight[™], a small molecular weight acceptor dye with a red-shifted fluorescent emission.

In this technical note, we present the optimization of an Akt1/PKB α kinase assay using a ULight-labeled peptide substrate. The binding of an Eu-labeled antibody directed against Ser21 phosphorylation of the Crosstide 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.

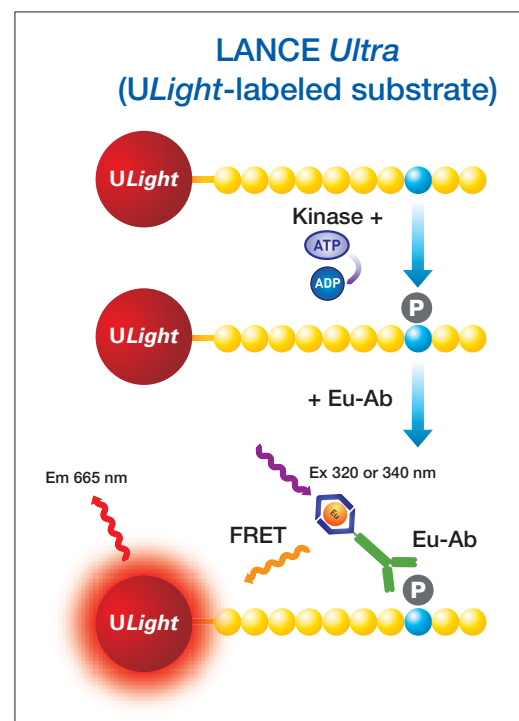


Figure 1. Schematic representation of the LANCE *Ultra* detection of a phosphorylated peptide substrate.

Development of a Akt1/PKB α Kinase Assay:

Reagents needed for this assay:

Europium-labeled anti-phospho-Crosstide (GSK-3 α Ser21) Antibody	PerkinElmer # TRF0202
ULight-Crosstide Peptide	PerkinElmer # TRF0106
Akt1/PKB α , active	Millipore # 14-276
LANCE Detection Buffer, 10X	PerkinElmer # CR97-100
OptiPlate™-384, white	PerkinElmer # 6007299
TopSeal-A™ film	PerkinElmer # 6050195

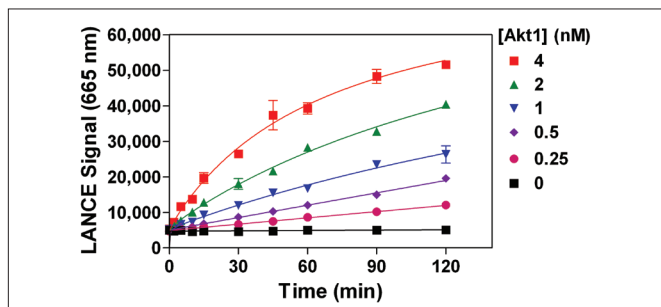
Kinase Buffer: 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM MgCl₂, 2 mM DTT and 0.01% Tween-20

Standard Protocol

- Dilute Akt1/PKB α kinase, ATP, inhibitors and ULight-Crosstide Peptide in Kinase Buffer.
- Prepare a 4X Detection Mix by diluting the Eu-anti-phospho-Crosstide Antibody to 8 nM in 1X LANCE Detection Buffer.
- Add to the wells of a white OptiPlate-384:
 - 5 μ L of Akt1/PKB α enzyme
 - 2.5 μ L of inhibitor or Kinase Buffer
 - 2.5 μ L of ULight-Crosstide Peptide/ATP mix (for ATP titration, ATP dilutions are added separately in Kinase Buffer).
- Cover the plate with TopSeal-A film and incubate at room temperature (RT).
- Stop kinase reactions by adding 5 μ L of 40 mM EDTA prepared in 1X LANCE Detection Buffer. Leave for 5 min at RT.
- Add 5 μ L of Detection Mix (Eu-anti-phospho-Crosstide Antibody at a final concentration of 2 nM).
- Cover with the 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).

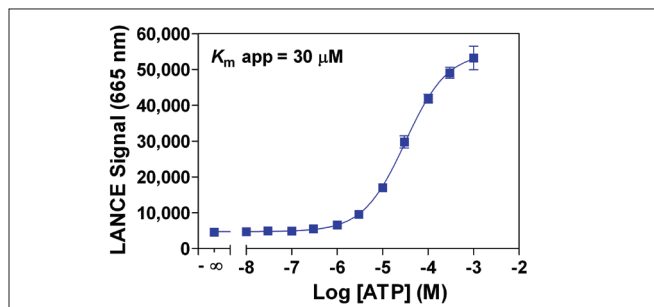
NOTE: Eu-labeled antibodies and EDTA can be premixed just before use as a 2X concentrated Stop/Detection mix to minimize the number of liquid handling steps.

Experiment 1: Enzymatic Titration and Time-Course



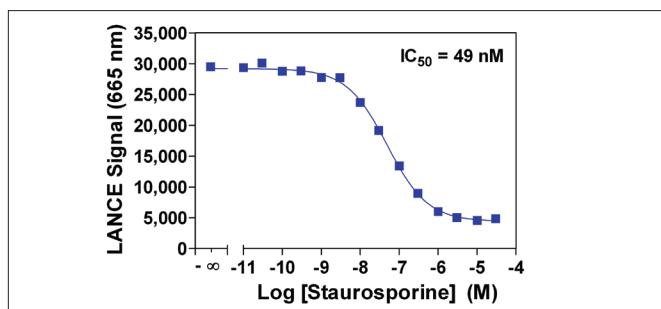
Enzymatic progress curves were produced by incubating Akt1/PKB α enzyme at concentrations ranging from 0.25 to 4 nM with 50 nM ULight-Crosstide Peptide and 20 μ M ATP. Kinase reactions were terminated at the indicated times by the addition of EDTA. Detection mix was added and signal read after 60 minutes.

Experiment 2: ATP Titration



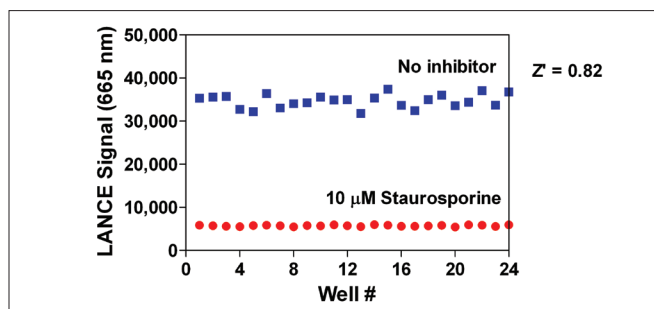
Serial dilutions of ATP ranging from 10 nM to 1 mM were added to 2 nM Akt1/PKB α enzyme and 50 nM of ULight-Crosstide Peptide. Kinase reactions were terminated after 60 min by the addition of EDTA.

Experiment 3: Enzyme Inhibition



Serial dilutions of staurosporine ranging from 10 pM to 30 μ M (final concentrations in 2% DMSO) were incubated with 2 nM Akt1/PKB α enzyme, 50 nM ULight-Crosstide Peptide and 30 μ M ATP. Kinase reactions were terminated after 60 min by the addition of EDTA.

Experiment 4: Z'-factor Determination



Akt1/PKB α enzyme at 2 nM was incubated with 50 nM ULight-Crosstide Peptide and 30 μ M ATP with or without 10 μ M staurosporine (final concentrations in 2% DMSO). Kinase reactions were terminated after 60 min by the addition of EDTA.

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