TECHNICAL NOTE

U-TRF #24

LANCE *Ultra* COT (MAP3K8) Kinase Assay

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LANCE® *Ultra* TR-FRET Technology

This LANCE *Ultra* kinase assay measures the phosphorylation of a p70 S6K peptide substrate at Thr389.

U*Light*™-p70 S6K (Thr389) Peptide:

- TRF0126-D: 0.5 nmole, 1,000 assay points*
- TRF0126-M: 5 nmoles, 10,000 assay points*

**0.5 pmol/assay point*

Europium-anti-phospho-p70 S6K (Thr389) Antibody:

- TRF0214-D: 10 µg, 1,562 assay points*
- TRF0214-M: 100 µg, 15,625 assay points*

**40 fmol/assay point*

Recognized Motif:

FLGF**T**YVAP

Europium-labeled mouse monoclonal antibody recognizing phospho-Thr389 in human p70 S6K.

Peptide Motif:

FLGF**T**YVAP

Synthetic peptide containing the residues surrounding Thr389 of human p70 S6K; phosphorylation site: Thr389.

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 COT (MAP3K8) kinase assay using a U*Light*-labeled peptide substrate. The binding of the Eu-labeled anti-phospho-p70 S6K antibody to the p70 S6K peptide substrate phosphorylated at Thr389 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.

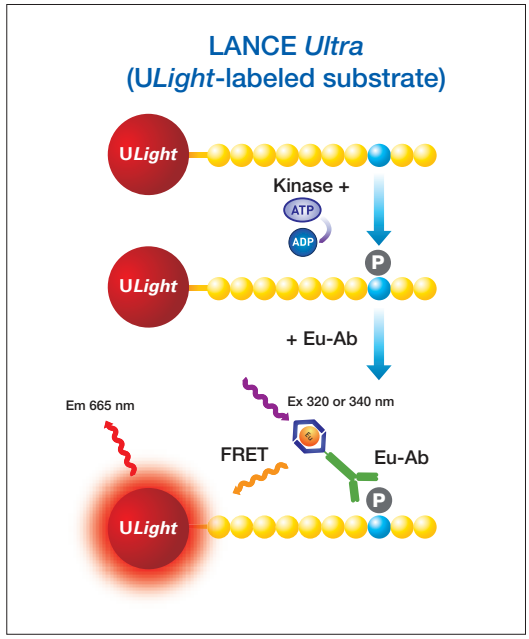

Figure 1. Schematic representation of the LANCE *Ultra* detection of a phosphorylated peptide substrate.

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Development of a COT (MAP3K8) Kinase Assay

Reagents needed for this assay:

Europium-labeled anti-phospho-p70 S6K (Thr389) Antibody	PerkinElmer # TRF0214
U*Light*-p70 S6K (Thr389)	PerkinElmer # TRF0126
COT (MAP3K8)	Carna # 07-301
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, 3 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM DTT and 0.01% Tween-20.	

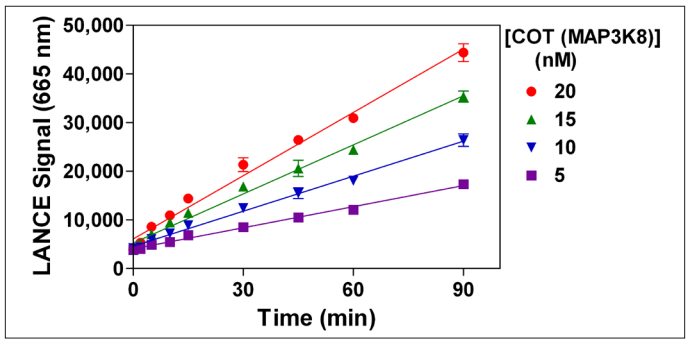
Note: $MnCl_2$ might not be required for other kinases.

Standard Protocol

- Dilute the COT (MAP3K8) enzyme, ATP, inhibitors and U*Light*-p70 S6K Peptide in Kinase Buffer.
- Prepare a 4X Detection Mix by diluting the Eu-anti-phospho-p70 S6K Antibody to 8 nM in 1X LANCE Detection Buffer.
- Add to the wells of a white OptiPlate-384:
  - 5 µL of COT (MAP3K8) enzyme
  - 2.5 µL of inhibitor or Kinase Buffer
  - 2.5 µL of U*Light*-p70 S6K Peptide/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 kinase reaction 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-phospho-p70 S6K Antibody at 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 Plate Reader in TR-FRET mode (excitation at 320 or 340 nm and emission at 665 nm).

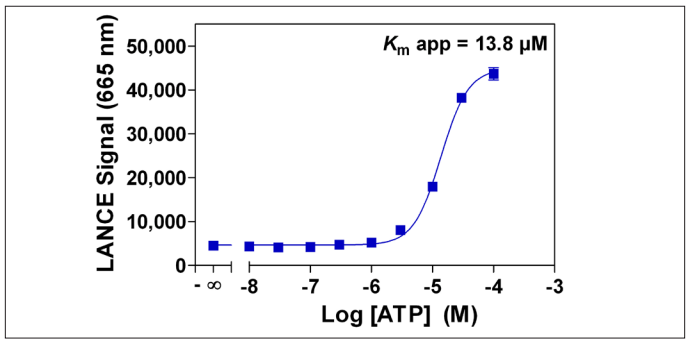
NOTE: *Eu-labeled antibodies and EDTA can be premixed just before use as a 2X concentrated Stop Solution/Detection Mix to minimize the number of liquid handling steps.*

Experiment 1: Enzymatic Titration and Time Course



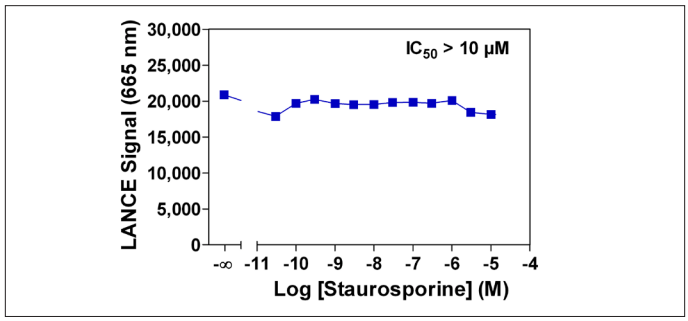
Enzymatic progress curves were produced by incubating COT (MAP3K8) enzyme at concentrations ranging from 5 to 20 nM with 50 nM U*Light*-p70 S6K Peptide and 200 µM ATP. Kinase reactions were terminated at the indicated times by the addition of EDTA. Detection mix was added and signal read after 60 minutes.

Experiment 2: ATP Titration



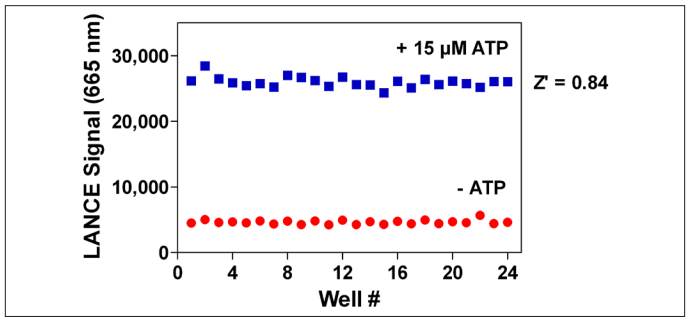
Serial dilutions of ATP ranging from 10 nM to 100 µM were added to 10 nM COT (MAP3K8) enzyme and 50 nM of U*Light*-p70 S6K Peptide. Kinase reactions were terminated after 90 min by the addition of EDTA.

Experiment 3: Enzyme Inhibition Curve



Serial dilutions of staurosporine ranging from 30 pM to 10 µM (final concentrations in 1% DMSO) were incubated with 10 nM COT (MAP3K8) enzyme, 50 nM U*Light*-p70 S6K Peptide and 15 µM ATP. Kinase reactions were terminated after 90 min by the addition of EDTA. Staurosporine does not inhibit COT activity at a concentration of 10 µM, consistent with literature data.

Experiment 4: Z'-factor Determination



COT (MAP3K8) enzyme at 10 nM was incubated in kinase asay buffer with 50 nM U*Light*-p70 S6K Peptide in the absence or presence of 15 µM ATP (final concentrations in 2% DMSO). Kinase reactions were terminated after 90 min by the addition of EDTA.

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