PerkinElmer Life and Analytical Sciences, Inc.

LANCE® *Ultra*
KINASELECT™ SER/THR KIT
(5 X 250 ASSAY POINTS)

CATALOG NUMBER:
TRF0300-C

For Laboratory Use Only

Research Chemicals for Research Purposes Only

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PRECAUTIONS

- Spin tubes for a few seconds to improve recovery of content.
- Avoid multiple freezing and thawing of frozen reagents.
- Mix gently solutions containing Eu-labeled antibodies. Do not vortex vigorously.
- The type of plate used is critical to the assay. White OptiPlate-384 microplates are strongly recommended. Black plates will produce less signal but acceptable S/B ratios.
- Small volumes used in the assay may be prone to evaporation. It is recommended to cover microplates with TopSeal-A adhesive sealing film to reduce evaporation during incubation.
- **TopSeal-A film must be removed before reading the plate.**
- Reagents contain sodium azide (NaN_3) as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Disposal of all waste should be in accordance with local regulations.

I. BEFORE STARTING

Receiving the LANCE *Ultra* KinaSelect Ser/Thr Kit

Upon receiving the KinaSelect Kit, ensure that the product is on dry ice. Verify that you received all kit components listed in the table below. Store at the recommended temperature. Kit components should be stable for at least three months when stored as recommended.

Table 1. Kit Contents

Reagent	Item Number	Storage Temperature
U*Light*-CREBtide (Ser133)	TRF0107-C	-20°C
U*Light*-Myelin Basic Protein Peptide	TRF0109-C	-20°C
U*Light*-PLK (Ser137) Peptide	TRF0110-C	-20°C
U*Light*-Histone H3 (Thr3/Ser10) Peptide	TRF0125-C	-20°C
U*Light*-p70 S6K (Thr389) Peptide	TRF0126-C	-20°C
Eu-anti-phospho-CREBtide (Ser133)	TRF0200-C	4°C
Eu-anti-phospho-Myelin Basic Protein	TRF0201-C	4°C
Eu-anti-phospho-PLK (Ser137)	TRF0203-C	4°C
Eu-anti-phospho-Histone H3 (Thr3)	**TRF0211-C**	**-20°C**
Eu-anti-phospho-p70 S6K (Thr389)	TRF0214-C	4°C
LANCE Detection Buffer, 10X, 1.5 mL	CR97-100C	4°C

Note: For storage after thawing, we recommend snap-freezing the U*Light*-p70 S6K (Thr389) Peptide on dry ice to prevent peptide precipitation.

Note: The Eu-anti-phospho-Histone H3 (Thr3) antibody should be kept at -20°C for long term storage.

Description of Kit Components

U*Light*-Peptides: A quantity of **0.125 nmole** of each peptide is supplied in 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% BSA and 0.05% sodium azide as preservative. This quantity is enough for 250 assay points, using 0.5 pmole per assay point (50 nM in a 10-µL kinase reaction).

Europium-anti-phospho antibodies: A quantity of **1.6 µg** (10 pmoles) of each antibody is supplied in 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% BSA and 0.05% sodium azide as preservative. This quantity is sufficient for 250 assay points, using 40 fmoles per assay point (2 nM in a 20-µL detection reaction).

The **LANCE Detection Buffer, 10X,** should be diluted to 1X with ultrapure water prior to use.

LANCE *Ultra* Product Offering

Please consult Appendix B and our website (**www.perkinelmer.com**) for our complete LANCE *Ultra* product offering.

Required Reagents and Materials

The following reagents and instruments are required but not included in the kit. Equivalent sources can be substituted.

Table 2. Required Reagents and Materials

Reagent or Material	Recommended Source	Catalog number
Kinases	Various suppliers	
HEPES	Sigma-Aldrich Co.	H3375
ATP	Sigma-Aldrich Co.	A7699
DTT	Sigma-Aldrich Co.	D0632
EGTA	Sigma-Aldrich Co.	E4378
$MgCl_2$	Sigma-Aldrich Co.	M9272
$MnCl_2$	Sigma-Aldrich Co.	M3634
Calmodulin	Millipore	14-368A
$CaCl_2$	Sigma-Aldrich Co.	C4901
EDTA	Invitrogen Corp.	15575-020
Tween-20	Pierce/ThermoFisher Scientific Inc.	28320
Ultra-Pure water (18 meg ohms /cm)	Various suppliers	
OptiPlate™-384, white	PerkinElmer Inc.	6007290
TopSeal™-A 384	PerkinElmer Inc.	6005185
TRF detection reader (ViewLux®, EnVision® VICTOR™, or equivalent)	PerkinElmer Inc.	

II. INTRODUCTION

The LANCE® *Ultra* KinaSelect™ Ser/Thr kit is intended for selecting the optimal peptide substrate for serine and threonine (Ser/Thr) kinases. Kinase activity is measured in a LANCE time-resolved fluorescence resonance energy transfer (TR-FRET) assay using five different U*Light*-labeled peptide substrates with their corresponding europium (Eu)-labeled anti-phospho-antibodies. Substrate/antibody pairs giving the best performance can then be used for further assay development and optimization.

The five U*Light*-peptides selected for the KinaSelect kit were found to generate signal with over 80% of a panel of 184 Ser/Thr kinases. The core motif of the phosphorylation site of each substrate is indicated in the table below.

Table 3. U*Light*-Peptide Phosphorylation Motifs

Substrate	Core Motif[1]
U*Light*-CREBtide (Ser133) Peptide	RRP**S**YRK
U*Light*-Myelin Basic Protein Peptide	VTPR**T**PPP
U*Light*-PLK (Ser137) Peptide	RRR**S**LLE
U*Light*-Histone H3 (Thr3/Ser10) Peptide	AR**T**KQTA
U*Light*-p70 S6K (Thr389) Peptide	FLGF**T**YVAP

[1]*Phosphorylation site is underlined*

The LANCE *Ultra* KinaSelect kinase kit is an ideal tool when the specific substrate of a kinase is either not known or not available. In KinaSelect assays, kinase reactions are performed in different wells with the five substrates using non-limiting concentrations of ATP and enzyme. Once one or more U*Light*-substrates are identified, assay development and optimization can then be completed using larger sizes of standalone reagents of the selected LANCE *Ultra* product pair. Consult Appendix B for larger size formats of KinaSelect kit reagents.

Note: Many kinases from the MAP kinase pathway do not phosphorylate peptides efficiently. Better results can be obtained with protein substrates or in a cascade assay.

Assay Principle

LANCE *Ultra* TR-FRET assays use the proprietary W1024 europium chelate (Eu) donor dye with U*Light*, a low molecular weight acceptor dye with a red-shifted fluorescent emission. In a typical LANCE *Ultra* kinase assay (Fig. 1), the phosphorylation of a U*Light*-Peptide substrate is detected with a specific anti-phospho-peptide antibody labeled with Eu. The binding of the Eu labeled anti-phospho peptide antibody to the phosphorylated U*Light* labeled peptide brings both donor and acceptor molecules into proximity. Upon irradiation of the kinase reaction at 320 or 340 nm, energy emitted by the excited Eu donor is transferred to nearby U*Light* acceptors, which then emit a light signal detected at 665 nm. The intensity of the light emission is proportional to the level of U*Light*-substrate phosphorylation.

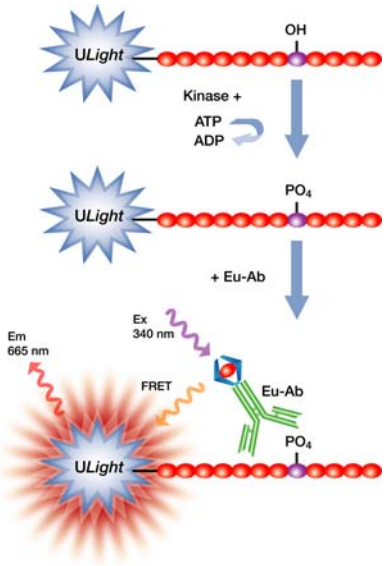

Figure 1. *Schematic representation of a LANCE Ultra kinase assay.*

III. KINASELECT SUBSTRATE SELECTION

For selecting the optimal U*Light*-peptide substrate for a given kinase, we recommend evaluating each of the five U*Light*-Peptide/Eu-anti-phospho-peptide antibody pairs provided with the KinaSelect kit by performing initially a single-point selection experiment using a high concentration of the kinase (e.g., 10-20 nM) with a non-limiting concentration of ATP (e.g., 100 µM). This should be done following the general assay protocol proposed on pages 12 and 13. The substrate(s) giving superior assay performance (i.e., highest S/B ratio using +ATP/-ATP data) will be selected for further optimization. If more than one substrate gives comparable assay performance, an enzyme titration experiment can be conducted in the presence of a non-limiting concentration of ATP (e.g., 100 µM). The optimal substrate can then be selected based on the enzyme requirements for the assay. For the Aurora A kinase assay shown in Figure 2, the U*Light*-PLK (Ser137) Peptide and Eu-anti-phospho-PLK (Ser137) antibody gave the highest S/B ratio and were therefore selected for further assay optimization.

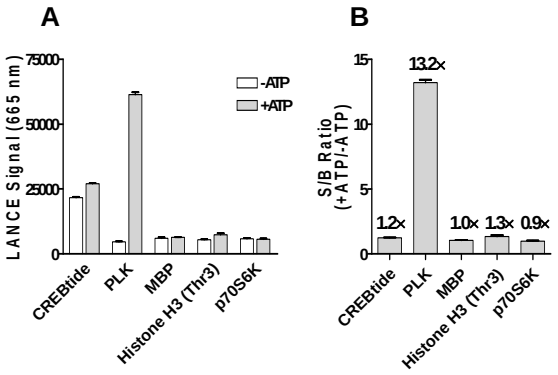

Figure 2. *Selection of the optimal substrate for the Aurora A kinase.* The Aurora A kinase (Carna Biosciences) at 20 nM was incubated with either U*Light*-CREBtide (Ser133), U*Light*-PLK (Ser137), UL*ight*-MBP, U*Light*-Histone H3 (Thr3) or U*Light*-p70 S6K (Thr389) Peptide in the absence or presence of 200 µM ATP. Kinase reactions were terminated after 1 hour by the addition of EDTA followed by the addition of 1X LANCE Detection Buffer containing the corresponding Eu-labeled antibody at a final concentration of 2 nM. Signal was read after 1 hour. A) LANCE signal at 665 nm. B) S/B ratio: +ATP/-ATP.

IV GENERAL KINASE ASSAY PROTOCOL

Table 4. Reagent Preparation

Kinase Reaction Buffer	Recommended reaction buffer composition is 50 mM HEPES (pH 7.5), 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT and 0.01% Tween 20. Add any essential kinase supplements (e.g., $MnCl_2$, $CaCl_2$, calmodulin, cGMP, lipids, etc.) at the appropriate concentrations.
1X LANCE Detection Buffer	Dilute 1 volume of LANCE Detection Buffer 10X with 9 volumes of ultrapure H_2O.
2X Enzyme solution	Dilute the enzyme in the kinase reaction buffer to prepare a solution that has 2X the final concentration needed in the 10 µL enzymatic step. Keep on ice.
4X U*Light*-Peptide solution	Dilute the U*Light*-Peptide in the kinase reaction buffer to a concentration of 200 nM.
4X ATP solution	Dilute the ATP in kinase reaction buffer to prepare a solution that has 4X the final concentration needed in the 10 µL enzymatic step. Keep on ice.
4X Stop solution	Dilute EDTA in 1X LANCE Detection Buffer to a concentration of 40 mM .
4X Detection Mix	Dilute the Europium-anti-phospho-peptide antibody in 1X LANCE Detection Buffer to a concentration of 8 nM.

Note: Alternatively, the Stop solution and Detection Mix can be premixed as a 2X concentrated mix and added together to the kinase reaction to minimize the number of liquid handling steps. <u>However, the combined Stop solution/Detection Mix must be used within two hours.</u>

Assays are performed in triplicate in 384-well white Opti-Plates. The final total volume of the reaction is 20 µL.

Table 5. Kinase Assay Steps

Step 1: Initiation of enzymatic reaction	a) Add 5 µL of 2X enzyme solution. b) Add 2.5 µL of 4X U*Light*-Peptide solution (50 nM final concentration in the 10 µL enzymatic reaction). c) Add 2.5 µL of 4X ATP solution. d) Cover plate with TopSeal-A and incubate 60 min at room temperature.
Step 2: Termination of enzymatic reaction	Add 5 µL of 4X Stop solution and incubate 5 min at room temperature (10 mM final concentration in the 20 µL detection reaction).
Step 3: Detection reaction	a) Add 5 µL of 4X Detection Mix (2 nM Europium-anti-phospho-peptide antibody final concentration in the 20 µL detection reaction). b) Cover plate with TopSeal-A and incubate 60 min at room temperature. c) **Remove TopSeal-A** and read signal in TR-FRET mode (see note below).

Note: Steps 2 and 3 can be combined in a single step by premixing the Stop solution and Detection Mix. However, the combined Stop solution/Detection Mix must be used within two hours.

Note: Recommended instrument settings are provided in Appendix A.

V. TROUBLESHOOTING GUIDE

A. Low signal

- The europium labeled antibody was premixed with EDTA for more than two hours. Make the Stop solution/Detection Mix just before using.
- EDTA is used at an excessively high concentration. Use an EDTA concentration equal to the concentration of free divalent cations or titrate EDTA to find the optimal concentration.
- Essential enzyme cofactor missing: see literature for additive kinase requirements such as Mn^{2+}, Ca^{2+}, calmodulin, cGMP, AMP or lipid activator.
- Low quality water. Contaminating heavy metal cations at high concentrations can interact with the europium chelate and quench the fluorescence. Only use ultrapure laboratory grade water for reagent preparation.

B. High background

- U*Light*-Peptides were used at too high concentrations. Use the recommended optimized concentration (50 nM final concentration in the 10 µL enzymatic reaction). Concentrations above 100 nM will increase the background signal and therefore will not necessarily improve assay performance.
- Instrument settings were not optimal for LANCE *Ultra*. Ensure appropriate instrument settings for your instrument are used (Appendix A).

C. No specific signal

- The selected kinase does not phosphorylate any of the U*Light*-Peptides efficiently. Ensure essential cofactors are included in the kinase reaction buffer. Look for your kinase in the LANCE *Ultra* Selection guide available from our website (www.perkinelmer.com).
- Many kinases from the MAP kinase pathway do not phosphorylate peptides efficiently. Better results can be obtained with protein substrates or in a cascade assay. As an example, inactive ERK1 can be used in a cascade assay with upstream kinases such as MEK1 and RAF1. Once activated, ERK1 will phosphorylate the U*Light*-MBP peptide substrate.

VI. APPENDIX A. INSTRUMENT SETTINGS AND CALIBRATION

It is critical to ensure that the instrument possesses the correct filters (excitation at 320 or 340 nm; emission at 615 and 665 nm). For the VICTOR and EnVision instruments, modifications to locked protocols according to the table below are recommended. Adjustments to the locked protocols can be made after copying them under a new name (e.g., Copy LANCE High Count 615 and 665 labels). To perform the flatfield calibration on the ViewLux instrument, we recommend using the LANCE positive control as the reference sample (LANCE Controls, PerkinElmer # AD0163). The LANCE positive control should be used diluted 1:5 in water. The volume of the sample should be the same as the assay sample volume. The flatfield calibration is performed using the calibration wizard for both 615 nm and 665 nm channels. Details of the protocol can be found in the ViewLux Reference Manual.

Table 6. Recommended Instrument Settings

Parameter	VICTOR™	EnVision®	ViewLux®*
Flash Energy Area	High	N/A	N/A
Flash Energy Level	150	100%	800,000
Excitation Filter	320 / 340	UV 320 / 340	DUG11 (UMB, AMC)
Integrator Cap	2 (or 3 **)	N/A	N/A
Integrator Level	2X the setting in LANCE High Count 615 label	N/A	N/A
Emission Filter	1) 615 2) 665	1) 203 - Eu 615 2) 205 - APC 665	1) 618/8 (Eu) 2) 671/8 (LANCE)
Delay Time	50 µs	90 µs	50 µs
Readout Speed, Gain and Binning	N/A	N/A	Medium, High, and 2X
Measurement time	N/A	100 (200**) flashes	20s exposure time
Window	100 µs (200-300 µs **)	100 µs (200-300 µs **)	354 µs
Mirror	N/A	402/412 (D400) or 452/462/662 (D400/D630)	Mirror 2 (UV dichroic)
Cycle	1000 µs	2000 µs	N/A

* ViewLux with flat field correction, bias correction, bias structure correction, cosmic ray detection, excitation energy compensation

** If signal too low with 2 or 100

VII. APPENDIX B. KINASELECT STANDALONE REAGENTS

Table 7. Larger Size Formats for Reagents Included in the KinaSelect Kit

Reagent	Item Number	Product Size	Assay Points*
U*Light*-CREBtide (Ser133)	TRF0107-D TRF0107-M	0.5 nmole 5 nmole	1,000 10,000
U*Light*-Myelin Basic Protein Peptide	TRF0109-D TRF0109-M	0.5 nmole 5 nmole	1,000 10,000
U*Light*-PLK (Ser137) Peptide	TRF0110-D TRF0110-M	0.5 nmole 5 nmole	1,000 10,000
U*Light*-Histone H3 (Thr3/ Ser10) Peptide	TRF0125-D TRF0125-M	0.5 nmole 5 nmole	1,000 10,000
U*Light*-p70 S6K (Thr389) Peptide	TRF0126-D TRF0126-M	0.5 nmole 5 nmole	1,000 10,000
Eu-anti-pospho-CREBtide (Ser133)	TRF0200-D TRF0200-M	10 µg 100 µg	1,500 15,000
Eu-anti-phospho-Myelin Basic Protein	TRF0201-D TRF0201-M	10 µg 100 µg	1,500 15,000
Eu-anti-pospho-PLK (Ser137)	TRF0203-D TRF0203-M	10 µg 100 µg	1,500 15,000
Eu-anti-pospho-Histone H3 (Thr3)	TRF0211-D TRF0211-M	10 µg 100 µg	1,500 15,000
Eu-anti-pospho-p70 S6K (Thr389)	TRF0214-D TRF0214-M	10 µg 100 µg	1,500 15,000
LANCE Detection Buffer, 10X	CR97-100	250 mL	250,000

**Based on a concentration of 0.5 pmole of peptide and 40 fmoles of antibody per well.*

Note: Large quantity bulk order quote is available upon request. Please inquire with your local PerkinElmer representative.

Note: For a complete list of our LANCE *Ultra* product offering, consult our website at:

http://las.perkinelmer.com/Catalog/default.htm?CategoryID=LANCE+Ultra

VIII. TRADEMARKS

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