Improved stability of the LANCE *Ultra* signal in kinase assays.

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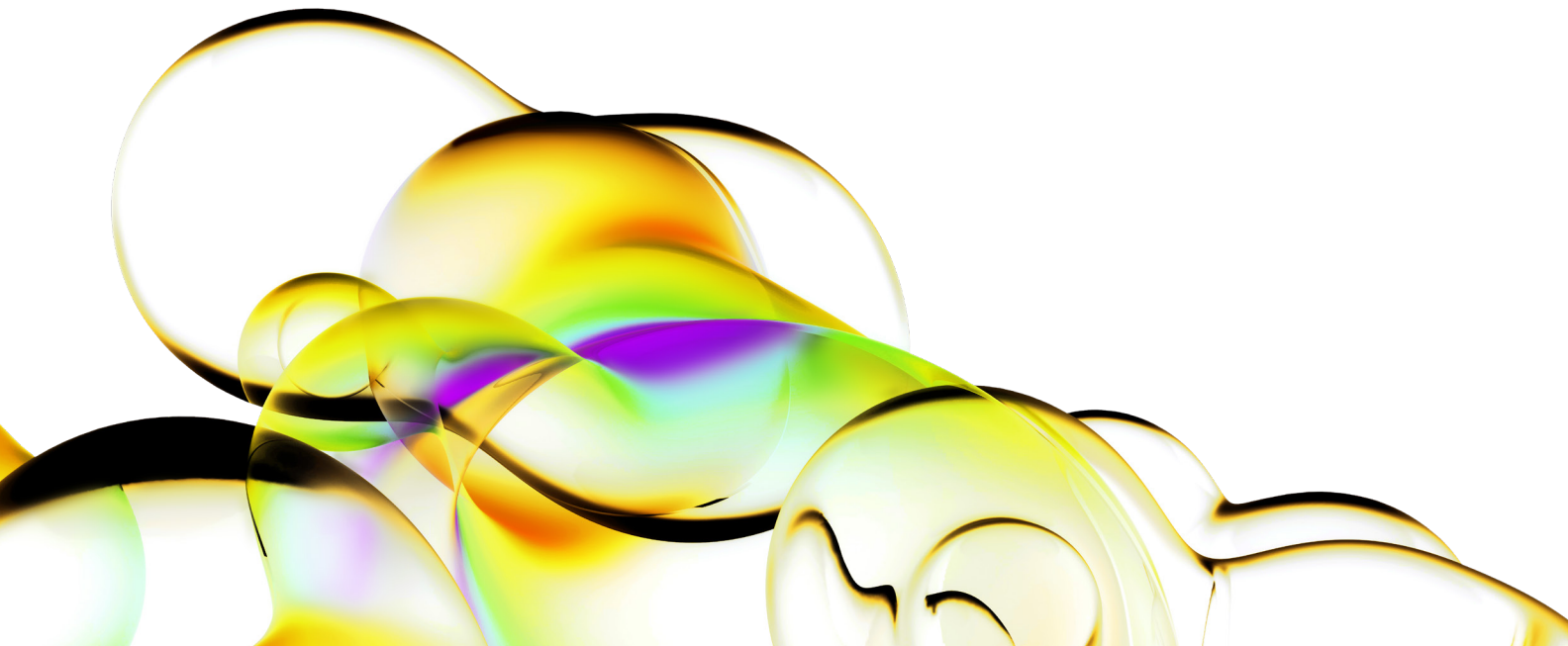
Revvity

LANCE™ *Ultra* is a high throughput screening (HTS) technology platform optimized for homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) kinase assays. The LANCE *Ultra* donor dye is a Europium (Eu) chelate, and the acceptor dye is a proprietary small-molecular weight molecule called U*Light*™, a fluorescent dye with a red-shifted light emission. In typical LANCE *Ultra* kinase assays (Figure 1), a peptide directly labeled with the U*Light* dye is phosphorylated in the presence of kinase and ATP. At completion, kinase reactions are stopped with EDTA, and phosphorylated peptides are captured by Eu-labeled antiphosphopeptide antibodies. The proximity between the donor and acceptor dyes in the peptideantibody complex allows for an efficient TR-FRET: irradiation of the samples at 320 or 340 nm results in the excitation of the Eu-chelate, which transfers its energy to the U*Light* dye that, in turn,will fluoresce at 665 nm with an intensity proportional to the level of peptide phosphorylation.

In HTS campaigns, assay plates are sometimes read several hours after kinase reactions are stopped. It has been observed on these occasions that the LANCE signal of some assays is reduced. Although neither assay robustness (Z′ factor) nor pharmacology (IC_{50}) are affected by this signal decrease, raw counts might differ between plates read at different incubation times. In this application note, we characterize the cause of the decrease of the LANCE signal over time in kinase assays and demonstrate how to prevent it by reducing the EDTA concentration used to terminate the kinase reactions.



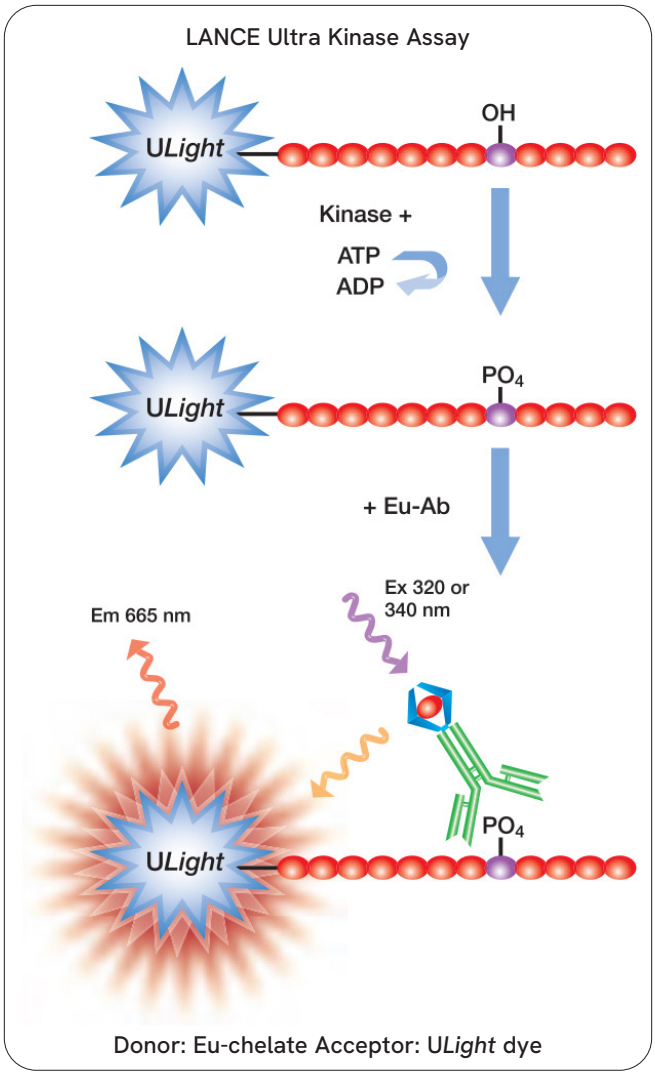


Figure 1. Schematic representation of a LANCE *Ultra* kinase assay

Materials and method

Materials

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

Method (Illustrated in Figure 2)

- Kinases, ATP, staurosporine and U*Light*-peptides were diluted in kinase assay buffer at concentra- tions optimized previously for each kinase and were added to a 384-well OptiPlate-384 in a volume of 10 µL.
- Kinase reactions were incubated at room temperature for up to 2 h depending on the kinase and then stopped by the addition of 5 µL EDTA.
- A volume of 5 µL of the specific Eu-labeled-anti-phosphopeptide antibody diluted in LANCE Detection Buffer was then added to a final concentration of 2 nM. Plates were incubated at 23 °C and the LANCE signal was measured on an EnVision™ multilabel plate reader at the indicated time. Excitation wavelength was set at 320 nm andemission recorded at 665 nm.

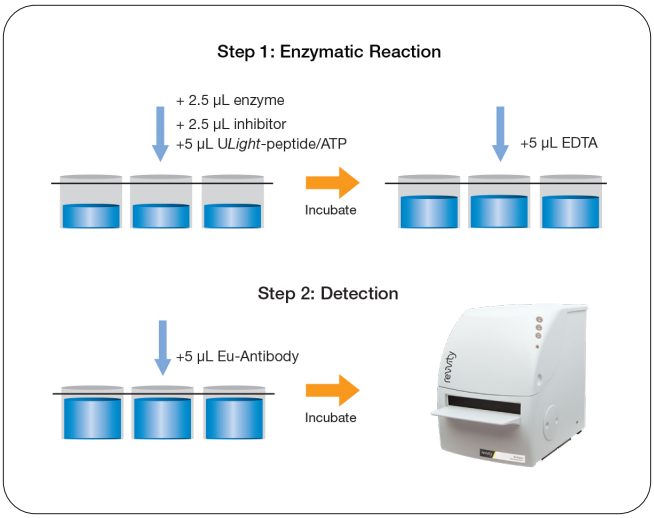


Figure 2. General assay procedure for LANCE *Ultra* kinase assays.

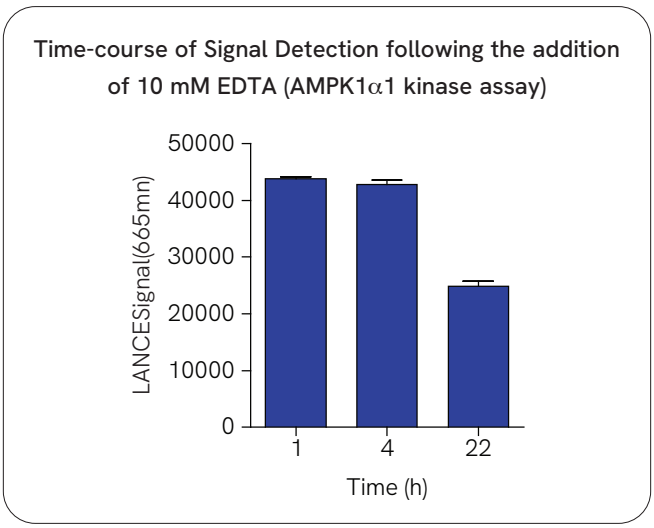


Figure 3. AMPKα1 enzyme (2 nM) was incubated with U*Light*-SAMS peptide (50 nM) in kinase assay buffer in the presence of 30 µM ATP. Reactions were stopped after 30 min by the addition of 10 mM EDTA. U*Light*-SAMS peptide phosphorylation was detected by the addition of 2 nM Eu-anti-phospho-Acetyl-CoA Carboxylase antibody and measured at the indicated incubation times on the EnVision multilabel plate reader.

Item	Supplier	Catalog number
AMPKα1	Carna Biosciences	02-113
ATP	Sigma-Aldrich	A2383
Aurora A, active	Millipore Corp.	14-511
CaMK1α	Carna Biosciences	02-104
Emission Filter: Eu 615 nm	Revvity	2100-5090
Emission Filter: LANCE 665 nm	Revvity	2100-5110
EnVision Multilabel Reader	Revvity	2103-0010
ERK1, active	Millipore Corp.	14-439
Eu-anti-phospho-Acetyl-CoA carboxylase (Ser79)	Revvity	TRF0208
Eu-anti-phospho-CREB (Ser133)	Revvity	TRF0200
Eu-anti-phospho-Crosstide	Revvity	TRF0202
Eu-anti-phospho-IkappaB-alpha (Ser32/36)	Revvity	TRF0206
Eu-anti-phospho-MBP (Thr232)	Revvity	TRF0201
Eu-anti-phospho-PKC (Ala25Ser)	Revvity	TRF0207
Eu-anti-phospho-PLK (Ser137)	Revvity	TRF0203
Eu-anti-phospho-tyrosine (PT66)	Revvity	AD0068
Excitation Filter: UV2 (TRF) 320 nm	Revvity	2100-5060
IKKβ, active	Millipore Corp.	14-485
JAK2	Carna Biosciences	08-045
JAK3	Carna Biosciences	08-046
LANCE Detection Buffer 10X	Revvity	CR97-100
Mirror: LANCE/DELFIA™ Dual	Revvity	2100-4160
PKA, catalytic subunit	Millipore Corp.	14-440
Staurosporine	LC Laboratories	S-9300
TopSeal™-A	Revvity	6050185
U*Light*-Acetyl-CoA Carboxylase (Ser 79) (SAMS) peptide	Revvity	TRF0118
U*Light*-CREBtide (Ser133) peptide	Revvity	TRF0107
U*Light*-Crosstide peptide	Revvity	TRF0106
U*Light*-IkappaB-alpha (Ser32/36) peptide	Revvity	TRF0113
U*Light*-IRS-1 (Tyr983) peptide	Revvity	TRF0120
U*Light*-JAK1 (Tyr1023) peptide	Revvity	TRF0121
U*Light*-MBP peptide	Revvity	TRF0109
U*Light*-PKC substrate	Revvity	TRF0108
U*Light*-PLK (Ser137) peptide	Revvity	TRF0110
White OptiPlate™ -384 Microtiter plates	Revvity	6007290

Results and discussion

Stability of the LANCE *Ultra* Signal

Stability of the LANCE signal over time was determined for the AMPKα1 kinase assay. Fluorescence measurements were made over a 22 h period after the addition of 10 mM EDTA and Eu-anti-phospho antibody. The AMPKα1 kinase phosphorylates the U*Light*-Acetyl-CoA Carboxylase (SAMS) peptide, which is then recognized by the Eu-anti-phospho-Acetyl-CoA Carboxylase antibody. As shown in Figure 3, the LANCE signal remained stable for the first four hours, but a 43% decrease was observed 22 h after addition of 10 mM EDTA.

EDTA chelates 2+ and 3+ metal ions with a stoichiometry of 1:1. It stops kinase reactions by complexing Mg^{2+} ions, which are essential catalysts for the enzymatic activity of kinases. We hypothesized that the decrease in signal over time could be due to an excess of free EDTA in the assay mixture, since other assay components had no effect on signal over time (data not shown). The free EDTA would extract Eu^{3+} ions from the Eu-chelate dye after prolonged incubation time with the Eu-labeled antibody. In standard LANCE Ultra kinase assays, final concentrations of Mg^{2+} and EDTA in the detection reaction are of 5 and 10 mM, respectively. In theory, there is 5 mM of un-complexed EDTA molecules left in the assay. We thus concentrated our efforts on characterizing the effect of EDTA on the LANCE signal and on finding ways to minimize the decrease in signal observed over time.

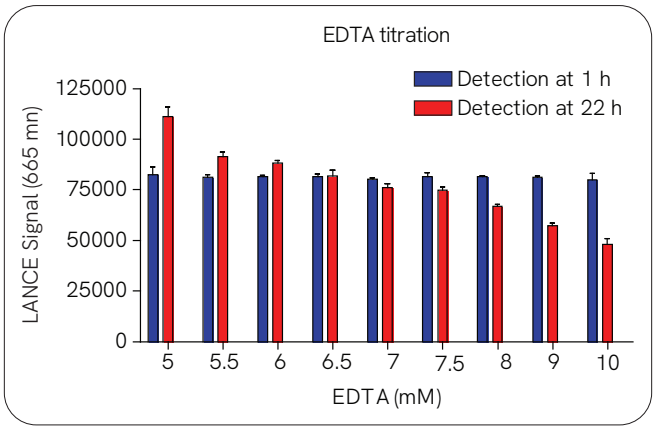


Figure 4. AMPKα1 enzyme (2 nM) was incubated with U*Light*-SAMS peptide (50 nM) in kinase assay buffer in the presence of 30 µM ATP. Reactions were stopped after 30 min by the addition of EDTA at concentrations ranging from 5 to 10 mM (final concentrations in 20 µL total assay volume). U*Light*-SAMS peptide phosphorylation was detected by the addition of 2 nM Eu-labeled anti-phospho-Acetyl-CoA Carboxylase antibody. The LANCE signal was measured after 1 and 22 h on the EnVision multilabel plate reader.

EDTA Titration

A titration was performed to determine the concentration of EDTA, that could stop effectively the kinase reaction without affecting the LANCE signal over time.

Final concentrations of EDTA ranging from 5 to 10 mM were used to stop the AMPKα1 kinase reaction. As shown in Figure 4, at 5 mM EDTA, the kinase reaction was not stopped effectively, since a 30% increase in signal was observed after 22 h incubation. At EDTA concentrations of 7.5 mM or higher, a dose-dependent decrease in signal was observed after 22 h. At concentrations ranging from 5.5 to 7 mM EDTA, no significant effect on signal was observed. In light of these results, a concentration of 6 mM EDTA was selected to stop LANCE *Ultra* kinase reactions.

Staurosporine IC_{50} stability at 6 mM EDTA

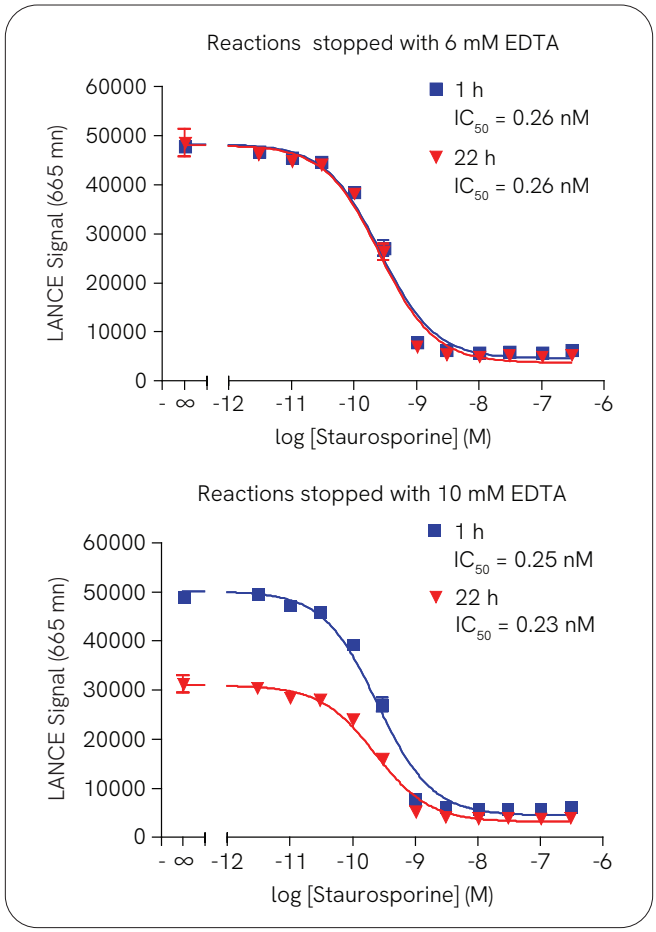


Figure 5. AMPK 1 enzyme (2 nM) was incubated with ULight-SAMS peptide (50 nM) in the presence of serial dilutions of staurosporine (3 pM to 0.3 µM) in kinase assay buffer supplemented with 30 µM ATP and 2% DMSO (final concentrations in 10 µL of the kinase reaction). Reactions were stopped after 30 min by the addition of 5A) 6 mM EDTA or 5B) 10 mM EDTA. ULight-Acetyl-CoA Carboxylase peptide phosphorylation was detected by the addition of 2 nM Eu-labeled anti-phospho-Acetyl-CoA Carboxylase and measured after 1 and 22 h on the EnVision multilabel plate reader.

Additional experiments were performed to determine if a reduction in EDTA concentration used to stop the reaction would affect the IC_{50} value for the inhibitor staurosporine over time. Staurosporine inhibition curves were performed using 6 mM and 10 mM EDTA (Figure 5).

Figure 5A shows that staurosporine inhibition curves obtained after incubation times of 1 and 22 h superimpose perfectly when kinase reactions are stopped with 6 mM EDTA. There was no signal reduction or shift in IC_{50} values. At 10 mM EDTA however, a decrease of approximately 40% of the maximal signal was observed (Figure 5B). As expected, signal reduction in the presence of 10 mM EDTA does not lead to a shift of the IC_{50} value for staurosporine.

Staurosporine inhibition

To evaluate the effect of reducing EDTA concentrations from 10 mM to 6 mM on other kinases assays, staurosporine inhibition curves were performed for 13 additional enzymes in combination with a selection of LANCE *Ultra* U*Light* peptides and Eu-anti-phosphosubstrate antibodies (Table 1). All kinase reactions included ATP at a concentration near the apparent K_m of the enzyme for ATP, except for CAMK1α, where ATP was deliberately added at a concentration significantly higher (500 µM).

Table 1 lists IC_{50} values obtained for staurosporine inhibition. Signal was measured after incubations of 1 and 22 h. This table also shows the percent reduction of maximal signal over time. At the two EDTA concentrations, IC_{50} values for staurosporine are almost identical and remain constant over time. As expected, major improvements in signal stability were observed when reactions were stopped with 6 mM instead of 10 mM EDTA. A notable exception is the CaMK1α assay, where ATP was added at 500 µM. For this enzyme, signal decreased by 71% in the presence of 10 mM EDTA while 6 mM EDTA still caused a major decrease of 55% of the maximal signal. This observation indicates that in addition to EDTA, ATP concentrations above 100 µM may contribute to signal decrease over time.

Table 1: IC_{50} values for staurosporine inhibition determined for 14 different kinases.

			6 mM EDTA*			10 mM EDTA*		
Kinase	Substrate	ATP in kinase reaction (µM)	IC_{50} (nM) after 1 h	IC_{50} (nM) after 22 h	Signal Reduction (%) after 22 h	IC_{50} (nM) after 1 h	IC_{50} (nM) after 22 h	Signal reduction (%) after 22 h
AMPKα1	U*Light*-SAMS	30	0.26	0.26	0	0.25	0.23	36
Aurora A	U*Light*-PLK	5	1.81	1.74	0	1.99	1.74	32
CaMK1α	U*Light*-SAMS	500	2.80	2.43	55	2.73	3.00	71
ERK1	U*Light*-MBP	4	657	674	0	595	658	31
IKKβ	U*Light*-IkappaB-α	1	300	340	0	260	250	27
JAK2	U*Light*-IRS-1	10	0.16	0.16	0	0.22	0.20	40
JAK3	U*Light*-JAK1	10	0.32	0.32	0	0.32	0.33	34
MAPKAPK2	U*Light*-MBP	10	500	500	19	500	0.39	49
MSK1	U*Light*-Crosstide	3	2.60	2.60	0	2.70	388	38
PKA	U*Light*-CREBtide	1.5	1.00	1.10	0	1.00	1.10	22
PKC	U*Light*-PKC	10	0.49	0.48	2	0.46	0.44	46

*Assays were performed as optimized previously. Kinase reactions were stopped with either 6 or 10 mM EDTA and signal was read with the EnVision multilabel plate reader after 1 and 22 h of incubation.

Conclusions

- Signal stability of LANCE *Ultra* kinase assays was improved remarkably by titrating carefully the EDTA concentration required to stop the reactions.
- A ratio of EDTA/Mg^{2+} of 1.2 (final concentrations of 6 mM EDTA and 5 mM Mg^{2+}) effectively stopped kinase reactions, while preserving the LANCE signal intensity overnight.
- Of the 14 kinases assayed, IC_{50} values for staurosporine were identical regardless of the EDTA concentration used to stop the assay or the incubation time after the addition of EDTA and antibodies (1 h or 22 h).
- ATP at high concentrations creates a synergistic effect with EDTA resulting in signal decrease over time. This effect can be minimized by keeping ATP concentrations below or near 100 µM while maintaining the EDTA concentration at 6 mM.

Recommendations

In kinase assays including 10 mM $MgCl_2$, a final concentration of 10 mM EDTA in the total reaction volume can be used to stop the reaction when the Eu-anti-phosphopeptide antibodies are incubated in the reaction for 4 h or less. However, if plates are to be read after more than 4 h, it is recommended to limit the EDTA concentration to 6 mM, or to the minimum concentration that will effectively stop the enzymatic reaction. The EDTA concentration required to stop a kinase assay will vary depending on the concentration of divalent cations present in the assay.