Flexibility in the Development of LANCE® *Ultra* Assays for Tyrosine Kinases using General and Specific Substrates

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1 Introduction

Of the 518 known human kinases, 90 are tyrosine (Tyr) kinases. Protein Tyr kinases play a key role in signal transduction and normal cell growth. They are also involved in numerous proliferative diseases like cancer and atherosclerosis, in addition to a number of autoimmune diseases. For these reasons, Tyr kinases are often targets for new drug discovery in many laboratories involved in basic R&D, disease and therapeutics research, and HTS.

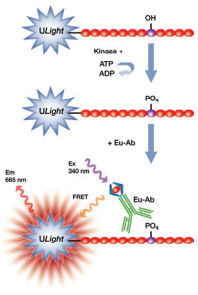
PerkinElmer now offers LANCE *Ultra*, a new ultra-HTS compatible TR-FRET technology highly suited for kinase inhibitor screening. Five new Tyr kinase substrates will soon be available in the *Ultra* format. U*Light*-poly GT (4:1) and U*Light*-poly GAT (1:1:1) are general substrates that can be phosphorylated non-specifically by Tyr kinases and can be used to quickly set up a kinase assay for an enzyme with unknown physiological substrate. Alternatively, specific U*Light*-peptide substrates corresponding to the phosphorylation site sequence from the physiological protein are available: U*Light*-IRS-1(Tyr983), U*Light*-JAK-1(Tyr1023), and U*Light*-CDK1(Tyr15). The phosphorylated substrates are detected using Eu-labeled anti-phospho-Tyr antibodies.

HTS-compatible assays have been developed for each substrate. Tyr kinase profiling of the five substrates illustrates the applicability of the substrates for a wide variety of Tyr kinases.

2 Materials

Item	Supplier	Cat.#
U*Light*™-poly GT (4:1)	PerkinElmer LAS, Inc.	TRF0100
U*Light*-poly GAT (1:1:1)	PerkinElmer LAS, Inc.	TRF0101
U*Light*-IRS-1(Tyr983)	PerkinElmer LAS, Inc.	TRF0120
U*Light*-JAK-1(Tyr1023)	PerkinElmer LAS, Inc.	TRF0121
U*Light*-CDK1(Tyr15)	PerkinElmer LAS, Inc.	TRF0122
Eu-anti-phospho-Tyr (PY20) antibody	PerkinElmer LAS, Inc.	AD0066
Eu-anti-phospho-Tyr (PT66) antibody	PerkinElmer LAS, Inc.	AD0068
Kinases	Carna Biosciences	various
LANCE Detection Buffer 10X	PerkinElmer LAS, Inc.	CR97-100
Staurosporine	Sigma-Aldrich, Inc.	S4400
ATP	Sigma-Aldrich, Inc.	A2383
0.5 M EDTA pH 8	Invitrogen Corp.	15575-020
White OptiPlate™-384	PerkinElmer LAS, Inc.	6007290
TopSeal™-A	PerkinElmer LAS, Inc.	6005250
EnVision® Multilabel Reader	PerkinElmer LAS, Inc.	2103-0010
Mirror: LANCE/DELFIA Dual	PerkinElmer LAS, Inc.	2100-4160
Excitation Filter: UV2(TRF) 320 nm	PerkinElmer LAS, Inc.	2100-5060
Emission Filter: Eu 615 nm	PerkinElmer LAS, Inc.	2100-5090
Emission Filter: LANCE 665 nm	PerkinElmer LAS, Inc.	2100-5110

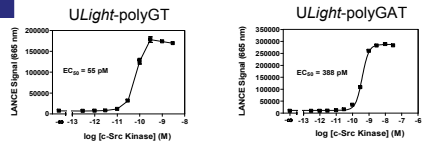
3 Assay Principle



In LANCE *Ultra* kinase assays, the phosphorylation of a U*Light*-labeled peptide substrate is detected with a specific anti-phospho-peptide antibody (Ab) labeled with europium chelate molecules (Eu). U*Light* is an innovative low-molecular weight red-shifted dye. The binding of the Eu-antibody (donor) to the phosphorylated U*Light* peptide (acceptor) substrate brings both the donor and acceptor dye molecules into close proximity. Upon irradiation at 320 or 340 nm, the excited europium chelate transfers its energy to the nearby U*Light* dye molecule that will in turn emit light at 665 nm. The intensity of light emission is proportional to the level of the U*Light* peptide phosphorylation.

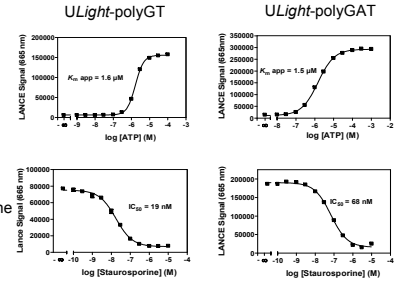
General substrates

4 Enzyme titration

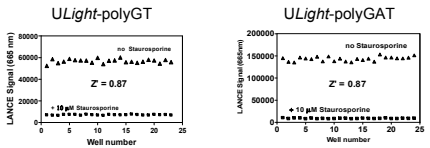


General Protocol:
5 µL of 2X Src kinase in kinase buffer (50 mM HEPES pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT and 0.01% Tween-20)
5 µL of 2X U*Light* -Substrate/ATP mix in kinase buffer (final 50 nM substrate)
Incubate 60 min at RT
5 µL of 4X EDTA in detection buffer (final 10 mM EDTA)
Incubate 5 min at RT
5 µL of 4X Eu-Antibody (AD0066) in detection buffer (final 2 nM)
Incubate 60 min at RT and read on EnVision instrument

5 ATP titration and staurosporine curve



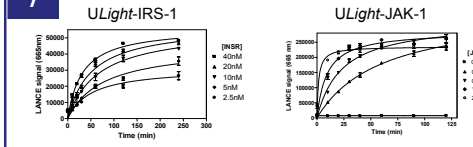
6 Z'-factor determination



Summary:
Src kinase assays using U*Light*-polyGT and U*Light*-polyGAT as substrates have been developed. The enzyme is more efficient in phosphorylation of U*Light*-polyGT, but the apparent Km for ATP in the presence of the different substrates is similar. Excellent precision is obtained in a Z` experiment for both substrates.

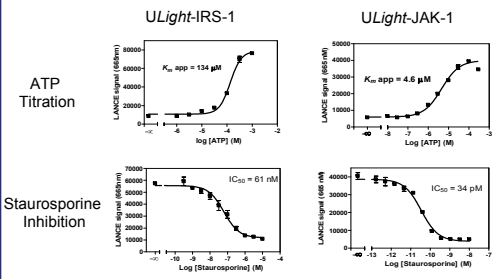
Specific substrates

7 Enzyme time course

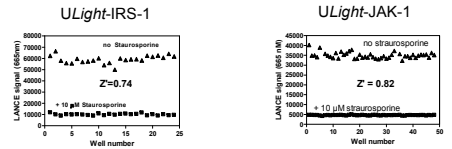


Peptide sequences: IRS-1 (CKKSRGDYMTMQIG), JAK-1 (CAGAGAIETDKEYYTVKD)
General Protocol:
5 µL of 2X INSR or JAK3 kinase in kinase buffer
5 µL of 2X U*Light* -Substrate/ATP mix (final 50 nM U*Light*-JAK-1 or 200 nM U*Light*-IRS-1)
Incubate up to 240 min at RT
5 µL of 4X EDTA in detection buffer (final 10 mM EDTA)
Incubate 5 min at RT
5 µL of 4X Eu-Antibody (AD0068) in detection buffer (final 2 nM)
Incubate 60 min at RT and read on EnVision instrument

8 ATP titration and staurosporine curve



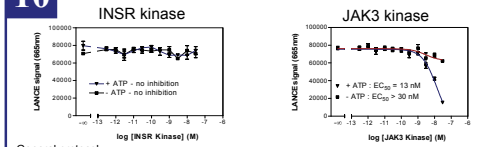
9 Z'-factor determination



Summary:
Kinase assays using U*Light*-IRS-1 and U*Light*-JAK-1 as substrates have been developed using INSR and JAK3 kinases, respectively. Excellent precision is obtained in a Z` experiment for both substrates.

Kinase profiling

10 Interference by Tyr kinase in detection



General protocol:
10 µL 50 nM biotin-phospho-Tyr-peptide plus 0-30 nM kinase ± 100 µM ATP
5 µL of 4X Eu-Antibody diluted in detection buffer (final 2 nM)
5 µL of 4X U*Light* -SA diluted in detection buffer (final 50 nM)
Incubate 60 min at RT and read on EnVision instrument

Summary:
JAK3 Tyr kinase interferes in phospho-Tyr detection by binding to the anti-phospho-Tyr antibody, whereas INSR kinase does not interfere.

11 Kinase assays with U*Light* substrates

Type	Kinase	[Kinase] (nM)	Kinase assay signal/background (+ATP / -ATP) U*Light*-poly GT	U*Light*-poly GAT	U*Light*-CDK1	U*Light*-IRS-1	U*Light*-JAK-1
Receptor	ALK	10	3	3	1	2	3
	AXL	10	5	13	1	2	2
	EGFR	0.5	4	13	2	2	17
	EPHB4	0.5	7	11	1	3	24
	FGFR1	1	2	13	1	7	25
	FLT1	1	15	18	1	3	15
	HER2	20	7	8	1	1	1
	IGF1R	0.5	8	7	1	4	2
	INSR	20	6	12	2	8	3
	KIT	5	10	14	1	1	4
	MET	1	9	15	1	2	8
	PDGFRA	1	12	15	1	4	19
	RET	0.5	3	10	1	6	20
	TIE2	1	8	15	2	3	19
Cytoplasmic	BRK	5	14	10	1	1	3
	BTK	0.5	11	15	3	1	2
	FAK	20	8	18	1	1	2
	ITK	0.5	8	10	2	3	7
	JAK1	20	11	15	1	4	24
	JAK2	5	5	9	2	8	20
	JAK3	10	3	9	1	1	23
	LCK	0.5	16	19	5	1	15
	LYNa	1	9	11	8	2	18
	SRC	20	6	12	3	1	9
	TYK2	20	9	5	1	7	1
	ZAP70	20	4	9	1	1	13

S/B > 4

General protocol: same as other kinase assays. For kinase reaction the concentration of kinase was chosen to avoid interference. Used 200 µM ATP, 100 nM U*Light*-substrate, incubation 4 hours.

Summary: U*Light*-polyGAT gives acceptable S/B values for all kinases tested (26/26) and U*Light*-polyGT works for 21/26 kinases. The other substrates provide acceptable S/B values with certain kinases only.

12 Summary and Conclusions

- Two types of U*Light*-labeled substrates for Tyr kinases were developed: general and specific.
- The applicability of the substrates to the development of HTS assays is illustrated by demonstrating enzyme kinetic data, inhibition curves, and Z` experiments. The assays developed provide ideal parameters for HTS – only 3-4 additions per well, low quantities of substrate and antibody, and Z` values > 0.7.
- Tyr kinases were assayed in detection assays to determine the interference of the enzyme on antibody binding. The kinase interferes in antibody-based kinase assays due to the presence of phospho-Tyr residues on the kinases. The concentration of the kinase in the reaction must be adjusted accordingly.
- The substrates were tested with a variety of receptor and cytoplasmic Tyr kinases. All the kinases were active with U*Light*-polyGAT as substrate and 21/26 kinases were active with U*Light*-polyGT. The applicability of the general substrates to a wide variety of kinases illustrates their advantage in setting up Tyr kinase assays in a short period of time. The specific substrates showed activity only towards certain substrates. U*Light*-CDK1 prefers kinases form the Src family: LCK, LYNa, and SRC. U*Light*-IRS-1 contains the YMxM motif and select kinases of both receptor and non-receptor type were active with this peptide. Interestingly, U*Light*-JAK-1 is active with a variety of kinases.

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