Development, Automation and Miniaturization of High-Throughput Serine/Threonine Kinase Assays Using the LANCE® *Ultra* Platform

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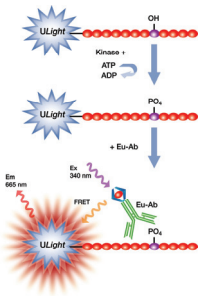
1 Introduction

Protein kinases are involved in the regulation of many aspects of the cell cycle, including proliferation, differentiation, secretion and apoptosis. Aberrant protein kinase expression or functioning is a cause or consequence of many human diseases. As a result, kinases have become extremely popular for drug discovery programs and this has prompted the development of many kinase assay technologies suitable for high-throughput screening (HTS). In the new LANCE® *Ultra* platform, the classical APC acceptor dye has been replaced by the new small molecular weight red-shifted dye U*Light*™, which allows direct labeling of peptide substrates. This enables the use of fewer assay components, simplifying assay set-up, whilst maintaining very low compound interference rates typically seen using europium chelate and red-shifted emission dye TR-FRET pairs.

In this poster, we initially present data describing the development and optimization of two LANCE *Ultra* serine/threonine kinase (Ser/Thr) assays in a 384-well format using manual pipettors. We then show the automation and miniaturization of both kinase assays to demonstrate the potential of the LANCE *Ultra* technology, combined with the JANUS® Modular Dispense Technology™ (MDT) Automated Workstation, to reduce assay costs while maintaining HTS robustness.

2 Assay Principle



In LANCE *Ultra* kinase assays, the phosphorylation of a U*Light* peptide substrate is detected with a specific anti-phospho-peptide antibody (Ab) labeled with europium chelate molecules (Eu). 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 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.

3 General Protocol for Manual Assay

Step 1: Enzymatic Reaction

2.5 µL enzyme
+ 2.5 µL inhibitor
+ 5 µL U*Light*-peptide/ATP

→ 384-well OptiPlate™ (20 µL assay)

↓ Incubate 1h at RT

+ 5 µL EDTA

Step 2: Detection

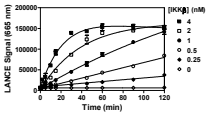
+ 5 µL Eu-Ab

↓ Incubate 1h at RT

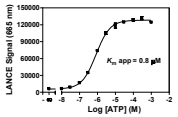
Read on EnVision™
(excitation at 320 nm, emission at 665 nm)

4 IKKβ Manual Assay Development

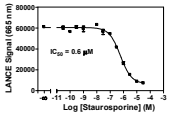
1- Enzymatic Time Course



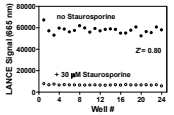
2- ATP Titration



3- Enzyme Inhibition Curve



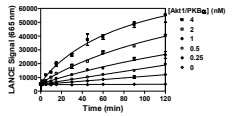
4- Z'-factor Determination



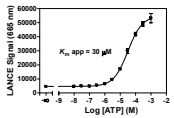
LANCE *Ultra* assay reagents: U*Light*-IkappaB-alpha (Ser32/36) peptide (TRF0113)
Europium-anti-phospho-IkappaB-alpha (Ser32/36) (TRF0206)

5 Akt1/PKBα Manual Assay Development

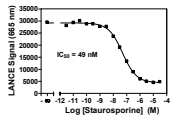
1- Enzymatic Time Course



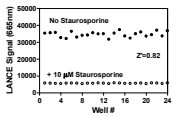
2- ATP Titration



3- Enzyme Inhibition Curve



4- Z'-factor Determination



LANCE *Ultra* assay reagents: U*Light*-Crosstide (GSK-3α, Ser21) peptide (TRF0106)
Europium-anti-phospho-Crosstide (GSK-3α, Ser21) (TRF0202)

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Assay Conditions for Automated Assays

	IKK β	*Akt1/PKBα*
Staurosporine	30 µM (1% DMSO)	10 µM (2% DMSO)
Enzyme	2 nM	2 nM
Substrate	50 nM	50 nM
ATP	2 µM	30 µM
Incubation time	90 min	60 min
EDTA	10 mM	10 mM
Eu-Ab	2 nM	2 nM
Detection time	1 h	1 h

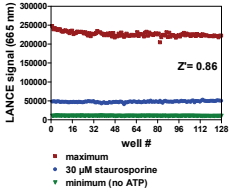
Assays were initially developed and optimized in 384-well plate (20 µL) using manual pipettors. Automation and miniaturization to the low -volume 384-well (10 µL assay) and 1536-well (5 µL assay) formats were then conducted by maintaining final concentration of reagents in both the kinase reaction and detection steps.

Pipetting Protocols for the Kinase Assays

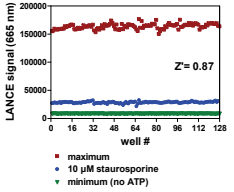
Volume (µl)	*384-well OptiPlate Manual assay*	*384-well ProxiPlate Automated assay*	*1536-well OptiPlate Automated assay*
Total Assay volume	20	10	5
Compound / Vehicle control	2.5	2	1
Enzyme	2.5	2	1
U*Light*-substrate/ATP mix	5	2	1
EDTA	5	-	-
Eu-Ab	5	-	-
EDTA / Eu-Ab Detection mix	-	4	2

7 Intra-Plate Variability in Low-Volume 384-well Format (1h Detection Time)

IKKβ

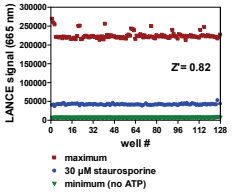


Akt1/PKBα

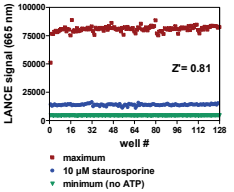


8 Intra-Plate Variability in Low-Volume 384-Well Format (O/N Detection Time)

IKKβ

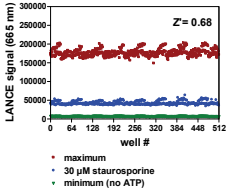


Akt1/PKBα

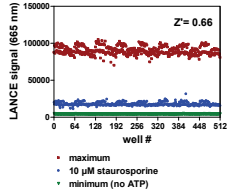


9 Intra-Plate Variability in 1536-Well Format (1h Detection Time)

IKKβ



Akt1/PKBα



10 Inter-Plate Variability Assessment

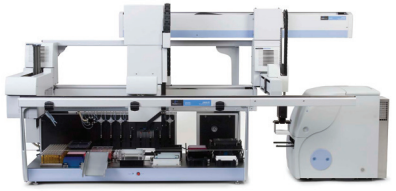
IKKβ

		384-well ProxiPlate (10 µL)			1536-well OptiPlate (5 µL)		
		S/B	CV (%)	Z'-factor	S/B	CV (%)	Z'-factor
Day 1	Detection at 1h	4.5	3.6	0.82	4.0	6.0	0.65
	Detection O/N	5.4	4.2	0.82	4.7	8.6	0.55
Day 2	Detection at 1h	4.8	2.8	0.86	4.2	5.5	0.68
	Detection O/N	5.6	5.9	0.76	4.8	9.3	0.53

Akt1/PKBα

		384-well ProxiPlate (10 µL)			1536-well OptiPlate (5 µL)		
		S/B	CV (%)	Z'-factor	S/B	CV (%)	Z'-factor
Day 1	Detection at 1h	5.8	2.8	0.87	5.3	9.0	0.56
	Detection O/N	5.8	4.3	0.81	5.3	11.4	0.45
Day 2	Detection at 1h	5.7	4.8	0.78	5.1	6.5	0.66
	Detection O/N	5.7	5.5	0.76	5.2	9.0	0.56

11 Automated Workstation



The JANUS MDT™ Automated Workstation with a 384-tip modular arm was used to dispense reagents to the low-volume 384 and 1536-well plates. The Envision MultiLabel Reader was used to read all assay plates.

12 Summary and Conclusions

- Two Ser/Thr LANCE *Ultra* kinase assays were initially developed and optimized in 384-well plates (20 µL assay) using manual pipettors. Both assays were found to be suitable for HTS purposes as illustrated by Z'-factors of 0.80 (IKKβ) and 0.82 (Akt1).
- Automation and miniaturization to the low-volume 384-well (10 µL assay) and 1536-well (5 µL assay) formats were then conducted by maintaining final concentration of reagents in both the kinase reaction and detection steps.
- Automated and miniaturized assays showed a satisfactory assay quality for both the low-volume 384 and the 1536-well formats (Z' above 0.7 and 0.6, respectively).
- The current preliminary data demonstrate that the LANCE *Ultra* platform, combined with the JANUS MDT Automated Workstation, is capable of automation and substantial miniaturization of Ser/Thr kinase assays with the potential for reduction of reagents without compromising assay quality.
- Further work will include testing of the JANUS MDT NanoHead™ dispense head combined with new 1536-well plates in order to reduce assay volume to ≤ 2 µL.