A Novel PI 3-Kinase Assay Utilising HTRF® Technology on the RUBYstar

Christine J. Rossant, Jason P. Brown and Marie L. Marshall.
Upstate Ltd., Cambridge, UK.

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- Homogeneous assay for PI 3-Kinase α, β, γ and δ
- The assay is able to detect as little as 0.5 pmoles of PIP3
- Ratiometric HTRF® readout can reduce interference by coloured compounds
- Robust assay with Z'>0.6

Introduction

Inositol phospholipids provide a wide range of cellular signals that play crucial roles in normal cell function. These lipids are interconverted by families of kinases and phosphatases, generating at least seven different lipid species that differ with respect to the number and distribution of phosphate groups around the inositol ring.
Type I PI 3-Kinases (p110 α, β, γ and δ) convert phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3) upon activation by growth factors, insulin and G-protein coupled receptors. PIP3 is a ubiquitous second messenger that binds to protein effector molecules such as protein Ser/Thr kinases (PKB, PDK1), Tec family protein tyrosine kinases, and regulators of monomeric G-proteins (e.g. GRP-1). These binding interactions modulate cellular processes such as growth, proliferation, motility, apoptosis and mediation of the metabolic actions of insulin.
PI 3-Kinases have traditionally been assayed using labour intensive radiometric methods. In this application note we describe a novel assay format for measuring PI 3-Kinase activity (data is presented for human PI 3-Kinase γ) that exploits the specific protein/lipid interactions described above. This assay has been configured into a robust homogeneous format utilising Homogeneous Time Resolved Fluorescence (HTRF®) technology.

Materials and Methods

The Upstate PIProfiler™ assay utilises the specific high affinity binding of the GRP-1 pleckstrin homology (PH) domain to PIP3 (the product of PI 3-Kinase γ acting on its physiological substrate PIP2). The principle of the Upstate PIProfiler™ PI 3-Kinase assay is shown in figure 1.

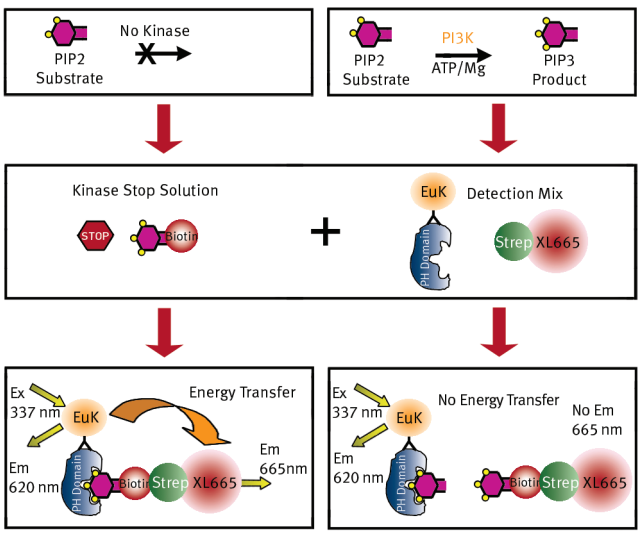

Fig. 1: Principle of the Upstate PIProfiler™ PI 3-Kinase assay. More information on this assay can be found at: www.upstate.com/features/piprofiler.asp

In the absence of PIP3 a sensor complex is formed consisting of Europium cryptate (EuK) labelled anti-GST monoclonal antibody, a GST tagged GRP1 PH domain, biotinylated short chain PIP3 and Streptavidin-Allophycocyanin (Strep-XL665). Excitation of the Europium cryptate in the complex results in the transfer of energy to the XL665 and a stable fluorescent emission at 665nm. The PIP3 product formed by PI 3-kinase γ activity displaces biotin-PIP3 from the complex resulting in a loss of energy transfer and thus a decrease in signal. The HTRF ratio of Em665nm (XL665)/Em620nm (EuK) *10000 is determined to correct for quenching effects of compounds on the signal and the time-resolved component minimises the interference of compound autofluorescence and background fluorescence.

This method is a modification of that described in Gray, A., Olsson, H., Batty, I.H., Priganica, L., and Downes, C.P. (2003) Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signalling lipids in cell and tissue extracts. Analytical Biochemistry **313**, 234-245.

PI 3-Kinase γ (h) was incubated with PIP2 and Mg^{2+}/ATP for 30 minutes at room temperature in a reaction volume of 20 µL. 5 µL stop buffer containing EDTA and biotin-PIP3 was added followed by 5ul of detection buffer comprising Europium cryptate labelled anti-GST monoclonal antibody, GST tagged GRP1 PH domain, and Strep-XL665. The stop and detection buffers were added to both assay wells and control wells (plus/minus kinase).Time resolved fluorescence was measured at 620nm and 665nm after excitation at 337nm using the BMG LABTECH RUBYstar and HTRF counts determined according to the following formula HTRF=10000*(Em665nm/Em620nm).

Results and Discussion

The sensitivity of the assay was demonstrated by titration of a PIP3 standard with the sensor complex. The PIP3 competed with the biotin-PIP3 in a dose-dependent manner (EC_{50} 92nM/1.85pmoles), with the assay able to detect as little as 0.5 pmoles of PIP3. A PIP2 standard was included to demonstrate the binding specificity of the GRP1 PH domain for PIP3 over the PIP2 substrate. Figure 2 demonstrates that although the GRP1 PH domain is able to bind PIP2, the EC_{50} (›100 µM/2000pmoles) for this interaction shows at least 500-fold lower affinity than that observed for PIP3.

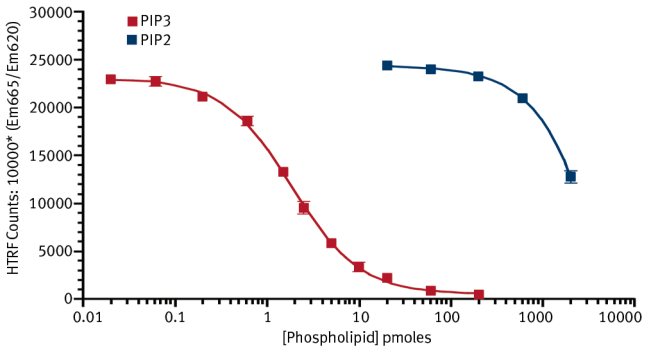

Fig. 2: PIP3 and PIP2 displacement of Biotin-PIP3 from the sensor complex

A titration of PI 3-Kinase γ (h) was then carried out at 10 and 100 µM ATP to determine enzyme concentrations that would be suitable for compound screening applications. The HTRF counts obtained at each enzyme concentration were converted to pmoles PIP3 formed using a PIP3 standard curve. Linear product formation was observed up to 10ng/well PI 3-Kinase γ (h) at 100 µM ATP and up to 20ng/well PI 3-Kinase γ (h) at 10 µM ATP, with less than 5% conversion of substrate to product at all concentrations tested.

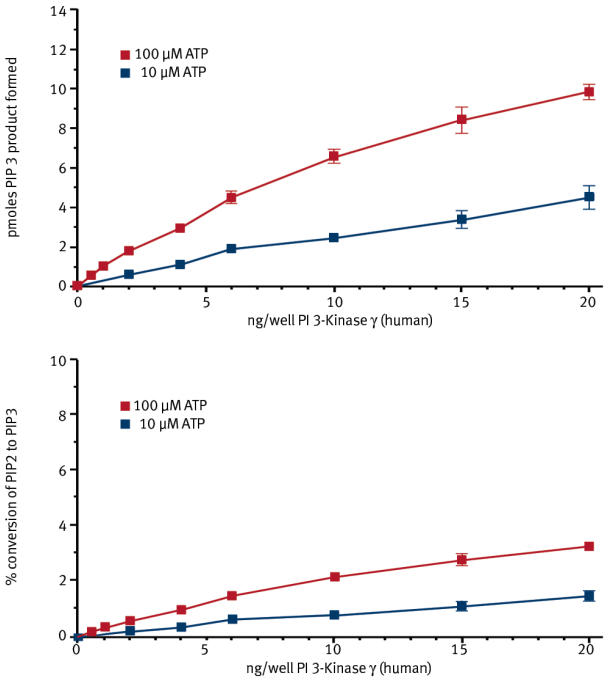

Fig. 3: Titration of PI 3-Kinase γ (h) and % substrate conversion

The utility of the assay for screening applications was demonstrated by titration of two known PI 3-Kinase inhibitors. Wortmannin is a fungal metabolite that irreversibly inhibits PI 3-Kinase activity by alkylating an active site lysyl residue thus preventing substrate binding. LY-294002 ('Lilly compound') is a reversible inhibitor that competes for the ATP-binding site of the enzyme. Wortmannin and LY294002 gave IC_{50} values in agreement with those found in the literature (see figure 4).

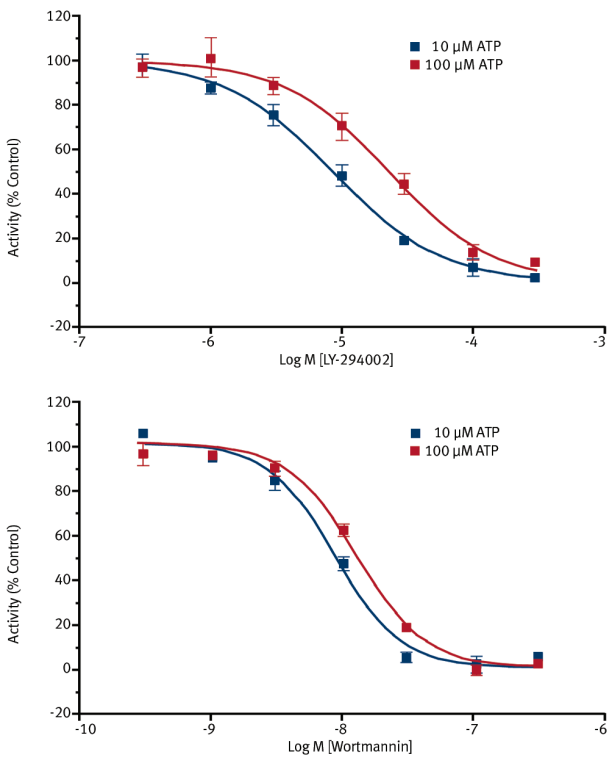

Fig. 4: Inhibition of PI 3-Kinase γ (h) by LY-294002 and wortmannin

Assay interference by coloured compounds was tested. Coloured compounds are a considerable issue in HTS as they can result in the generation of false positive/negative data. In the PI 3-Kinase HTRF assay although the XL665 and Europium cryptate counts may be quenched, the ratiometric HTRF readout can reduce this effect (see figure 5).

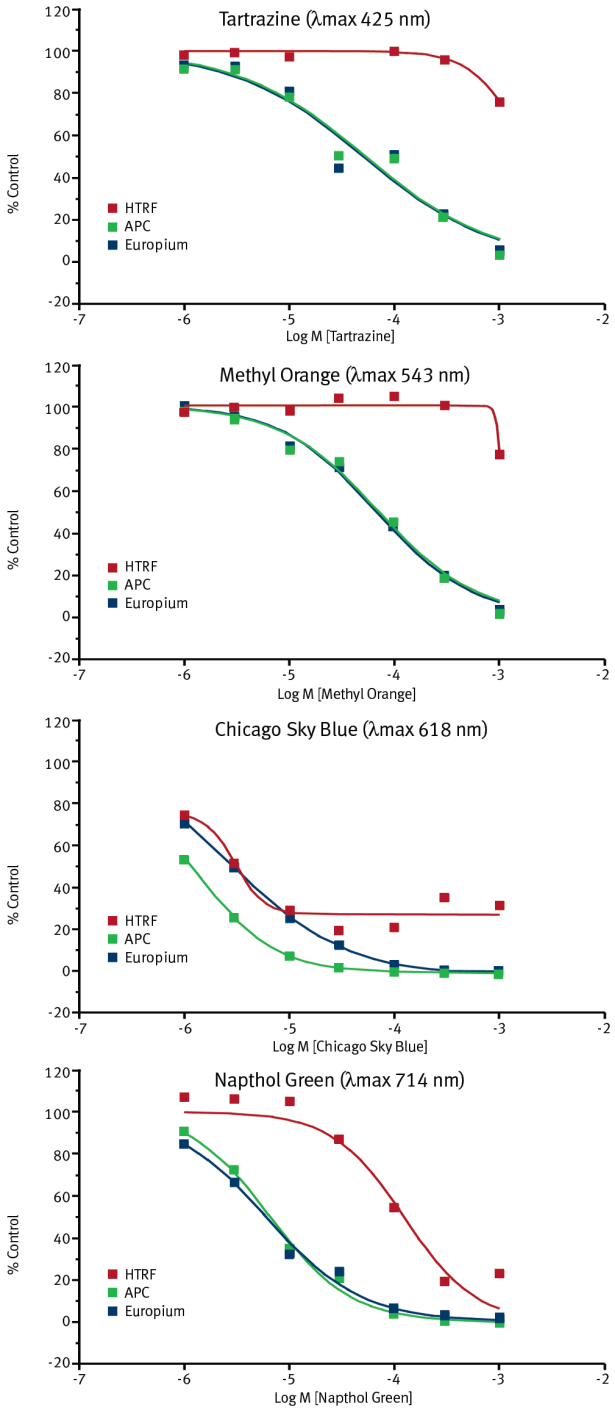

Fig. 5: Effect of coloured compounds on the PI 3-Kinase assay

Conclusion

Utilising Homogeneous Time Resolved Fluorescence (HTRF®) technology from Cisbio international and the BMG LABTECH RUBYstar, this assay yields high quality data that enables screening of compounds for PI 3-Kinase inhibitory activity. The assay is robust with a Z prime value of ›0.6 and standard inhibitors yield literature validated IC_{50} values.
More information on this assay can be found at:
www.upstate.com/features/piprofiler.asp

Headquaters:

Germany:	BMG LABTECH GmbH	Tel: +49 781 96968-0
Australia:	BMG LABTECH Pty. Ltd.	Tel: +61 3 59734744
China:	BMG LABTECH Co. Ltd.	Tel: +86 10 6424063
France:	BMG LABTECH SARL	Tel: +33 1 48862020
UK:	BMG LABTECH Ltd.	Tel: +44 1296 336650
USA:	BMG LABTECH Inc.	Tel: +1 919 806 1735
	www.bmglabtech.com	info@bmglabtech.com

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