

Brad Larson¹, Peter Banks¹, Peter Brescia¹, Paul Held¹, Andrew Plater²

¹BioTek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, VT USA, ²Millipore UK Ltd., Dundee, UK

Introduction

Here we demonstrate the capabilities of a high throughput homogeneous time-resolved fluorescence (HTRF®) PI3-Kinase assay from Millipore. The assay kit provides a universal method for assaying all Class I PI3-Kinases in a homogeneous 384 well format, and has been constructed using a Pleckstrin homology (PH) domain to bind to (a biotinylated form of) 3,4,5- phosphatidylinositol (PIP₃). Method optimization and characterization of assay performance were carried out using a BioTek Instruments Synergy™ 4 Hybrid Multi-Mode Microplate Reader. Assay validation was demonstrated by determining the IC₅₀ of different PI 3-kinase isoforms with numerous known enzyme inhibitors.

Abstract

Phosphoinositide 3-kinases (PI 3-kinases) constitute a family of related enzymes that phosphorylate the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns). PI 3-kinases have been linked to an extraordinarily diverse group of cellular functions including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. Class IA PI 3-kinases are heterodimeric molecules composed of one of five different regulatory subunits (p85α, p55α, p50α, p85β or p55γ) and one of three different catalytic subunits (p110α, p110β, or p110δ). They are attractive small molecule drug targets as aberrant activity is linked to numerous diseases including cancer, inflammation and diabetes. Yet development of assays to screen compounds against these targets has been problematic due to the difficulty of generating antibodies that bind specifically to only one of the phosphorylation states of PtdIns (e.g., 4,5-PtdIns [PIP-2], 3,4,5-PtdIns [PIP-3], etc.). To combat this problem, Millipore has developed a PI3-Kinase HTRF® Assay that makes use of the specific, high affinity binding of the GRP1 pleckstrin homology (PH) domain to PIP₃, the product of a Class IA or 1B PI3-Kinase acting on its physiological substrate PIP₂. During the detection phase of the assay, a complex is generated between the GST-tagged PH domain and biotinylated short chain PIP₃, forming the basis for the FRET architecture. By combining this assay with BioTek's Synergy™ 4 Hybrid Multi-Mode Microplate Reader, which has been previously validated to read all HTRF assays, researchers gain a way to generate high-throughput screening and pharmacology data for this important target class.

Millipore PI3-Kinase HTRF® Assay

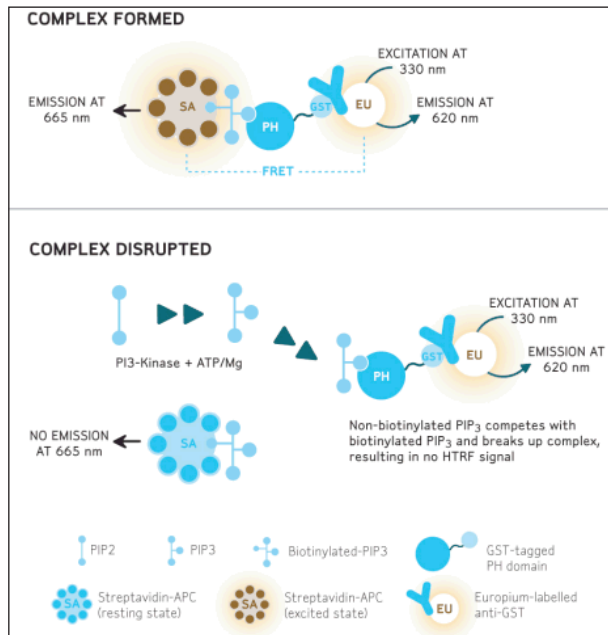


Figure 1 – The PI3-Kinase HTRF® Assay is performed using the following steps. 1. Inhibitor, PI3-Kinase, PIP2 substrate, and ATP are incubated together for 30 minutes at RT. 2. Stop solution, containing biotinylated PIP3 is added to the well, preventing further substrate phosphorylation. 3. Detection mix is added, containing the GST-tagged GRP1 pleckstrin homology (PH) domain, and fluorophores. In an inhibited reaction, a complex is generated between the PH domain and the biotinylated PIP₃. The biotinylated PIP₃ and the GST-tagged PH domain recruit fluorophores, (Streptavidin-Allophycocyanin and Europium-labeled anti-GST (respectively) to form the fluorescence resonance energy transfer (FRET) architecture. The FRET complex can be disrupted in a competitive manner by non-biotinylated PIP₃, a product formed in the PI3-Kinase assay.

BioTek Instrumentation

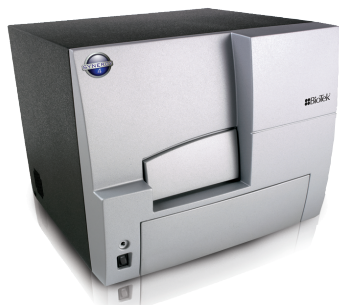


Figure 2 – The optical hybrid design of the Synergy™ 4 combines a fluorescence filter-based detection system and a monochromator-based detection system in one compact unit. The instrument was used to quantify the 330/620 and 330/665 signal from all assay plates.

Instrument		BioTek	Optimized
Detection Component		Catalog Number	Instrument Settings
Excitation Filter	330/80 nm	7082263	Delay after Plate Movement 100 msec
Emission Filter 1	620/10 nm	7082265	Measurements per Data Point 20
Emission Filter 2	665/8 nm	7082266	Lamp Energy High
Dichroic	365 nm	7138365	Top Probe Vertical Offset 7.00 mm

Table 1 – Synergy™ 4 HTRF Instrument Settings

Assay Optimization – Enzyme Titration

Assay Setup

1. Add 5 µL of 10% DMSO or compound in 10% DMSO.
2. Add 10 µL of 2X enzyme/PIP2 substrate.
3. Add 5 µL of 4X ATP, shake for 60 seconds, and incubate at RT for 30 minutes.
4. Add 5 µL of Stop Solution, and 5 µL of Detection Mix, shake for 60 seconds, and incubate at RT overnight (15 – 18 hours).
5. Read microplate on Synergy™ 4.

Class 1A PI3-Kinase	Project Reference
PI3 Kinase (p110α/p85α)	PI3 Kinase α
PI3-Kinase (p110α/p65α)	PI3 Kinase α Mutant
PI3 Kinase (p110β/p85α)	PI3 Kinase β
PI3 Kinase (p110δ/p85α)	PI3 Kinase δ

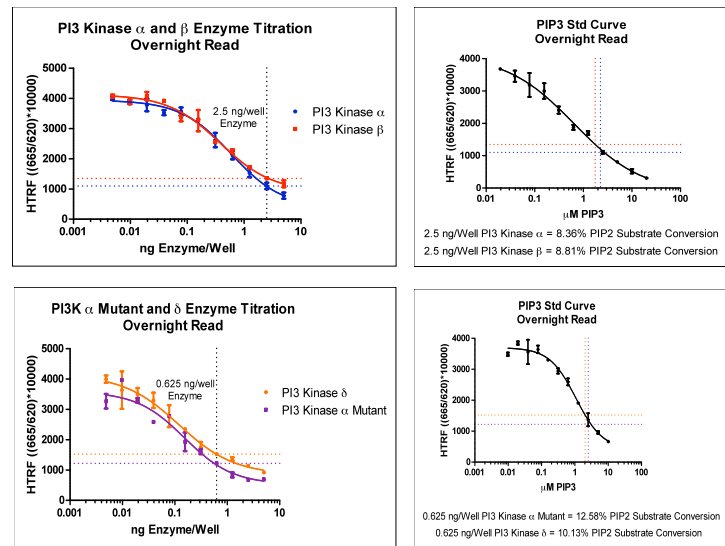


Figure 3 – PI3-Kinase Titrations. Enzymes titrated from 5 – 0.005 ng/well. ATP concentration equaled 200 µM and PIP2 substrate equaled 10 µM. PIP3 standard curve run with concentrations ranging from 10 – 0.01 µM.

Enzyme titration curves were generated to determine the EC₈₅ concentration of enzyme. PIP3 standard curves were also run in order to determine the percent PIP2 substrate that was converted to PIP3. A conversion level around 10% was targeted in order to ensure that initial rate velocity was being preserved. Component concentrations for this experiment are listed in Figure 3. The enzyme reactions were allowed to proceed for 30 minutes.

Figure 4 also shows the reaction progression at various enzyme levels from 5 through 60 minutes. This was done to once again ensure the linearity of the reaction at the enzyme concentration and reaction time chosen.

Conclusions

1. The instrument settings, and sensitivity of the Synergy™ 4 are able to deliver dependable time-resolved FRET readings in 384-well format.
2. Millipore's PI3-Kinase HTRF® assay is easily optimized, and yields results that are consistent with established literature values.
3. The combination of BioTek's Synergy™ 4, and Millipore's PI3-Kinase HTRF® assay create an ideal solution for high-throughput screening of compounds for this important target class.

Enzyme Reaction Progression

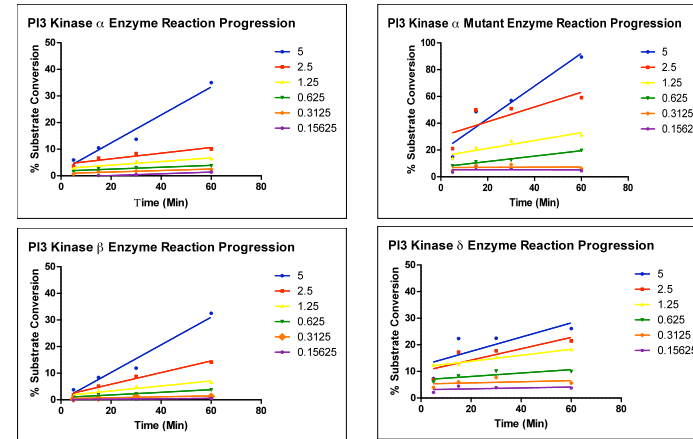


Figure 4 – Enzyme Reaction Progression. Reactions containing 5 – 0.15625 ng/well PI3-Kinase tracked from 5 – 60 minutes.

Assay Optimization – ATP Titration

An ATP titration was then conducted with each PI3-Kinase using the EC₈₅ enzyme levels previously determined. The EC₅₀ concentration of ATP for the individual kinases was chosen. This was the concentration required to show a 50% change between the maximum and minimum HTRF levels.

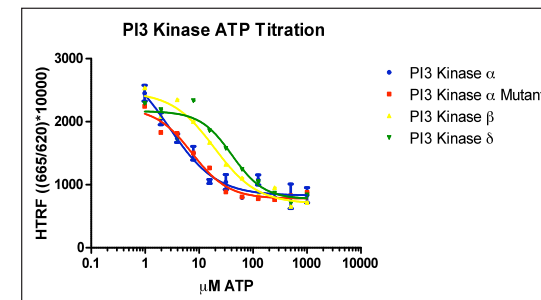


Figure 5 – ATP Titration. Enzyme concentration equaled 2.5 ng/well for PI3-Kinase α and β, and 0.625 ng/well for PI3-Kinase α Mutant and δ. PIP2 substrate equaled 10 µM. ATP titrated from 1000 – 1.0 µM.

Z'-Factor Enzyme Confirmation

Z'-Factor experiments were performed in order to confirm the enzyme concentration required to generate a satisfactory Z' score. This was necessary before proceeding with pharmacology studies. Enzyme levels yielding a Z' score above 0.6 were chosen for the proceeding experiments.

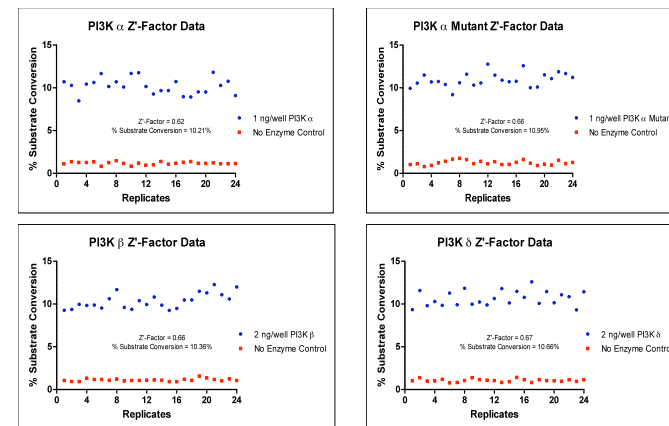


Figure 6 – Z'-Factor Enzyme Validation. ATP concentrations equaled 2.789 µM for PI3-Kinase α, 7.379 µM for PI3-Kinase α Mutant, 19.19 µM for PI3-Kinase β, and 40.73 µM for PI3-Kinase δ. PIP2 substrate equaled 10 µM.

PI3-Kinase Inhibitor Profiling

Final testing of the optimized assays involved running known inhibitors for the four PI3-Kinase isoforms. Serial 1:2 titrations were performed of Wortmannin, Quercetin, LY294002, and PI3KB-Inh VI. Final assay concentration ranges for each compound were 250 – 0.015 µM. IC₅₀ values were generated for each compound with each PI3-Kinase isoform.

Inhibitor Study Assay Components

PI3 Kinase Isoform	Assay Component Concentrations
	Enzyme (ng/well) ATP (µM) PIP2 Substrate (µM)
PI3K α	1 2.789 10
PI3K α Mutant	1 7.379 10
PI3K β	2 19.19 10
PI3K δ	2 40.73 10

Table 3 – Assay component concentrations

Results

PI3 Kinase Isoform	Compound IC ₅₀ Values (µM)
	Wortmannin Quercetin LY294002 PI3KB-Inh VI
PI3K α	0.028/0.0089 ¹ 1.023/0.87 ¹ 0.976/1.1 ¹ 3.77/3.3 ¹
PI3K α Mutant	0.097 1.491 2.857 9.751
PI3K β	0.059 1.517 0.979 0.468
PI3K δ	0.378 0.93 4.765 1.843

Table 4 – IC₅₀ values for known inhibitors. Numbers in black represent compounds tested with PI3-Kinase HTRF assays. Numbers in red represent values from literature source listed below.

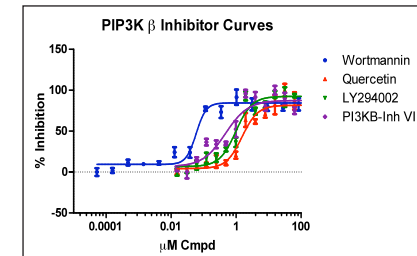


Figure 7 – Representative inhibition curves for PI3-Kinases with test compounds. PI3-Kinase β curves shown.

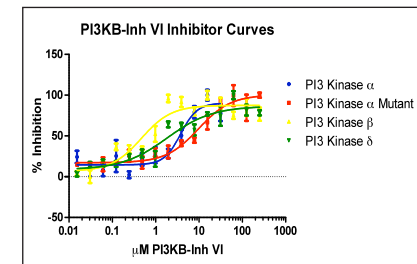


Figure 8 – PI3KB-Inh VI inhibition curves for PI3-Kinases.

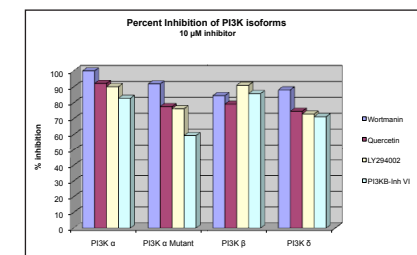


Table 5 – Percent inhibition values at designated enzyme concentrations.