Application Note

# Optimization and Validation of Millipore's HTRF® PI3 Kinase Assay for Inhibitors to Class IA Phosphionositide 3-Kinase Isoform Activity

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*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 ($PIP_3$). 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 $IC_{50}$ of different PI 3-kinase isoforms with numerous known enzyme inhibitors.*

## Introduction

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. 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 screening and pharmacology data for this important target class.

## HTRF PI3 Kinase Assay

This HTRF® PI3 Kinase Assay makes use of the specific, high affinity binding of the GRP1 pleckstrin homology (PH) domain to $PIP_3$, the product of a Class 1A or 1B PI3-Kinase acting on its physiological substrate $PIP_2$. During the detection phase of the assay, a complex is generated between the GST-tagged PH domain and biotinylated short chain $PIP_3$. The biotinylated $PIP_3$ and the GST-tagged PH domain recruit fluorophores (Streptavidin-Allophycocyanin and Europium-labeled anti-GST respectively) to form the time-resolved fluorescence resonance energy transfer (TR-FRET) architecture. Upon excitation at 330 nm, energy is transferred from Europium to the Streptavidin-Allophycocyanin, which emits at 665 nm. The TR-FRET complex can be disrupted in a competitive manner by non-biotinylated $PIP_3$, a product formed in the PI3-Kinase assay. The Europium will then emit at its characteristic 620 nm wavelength.


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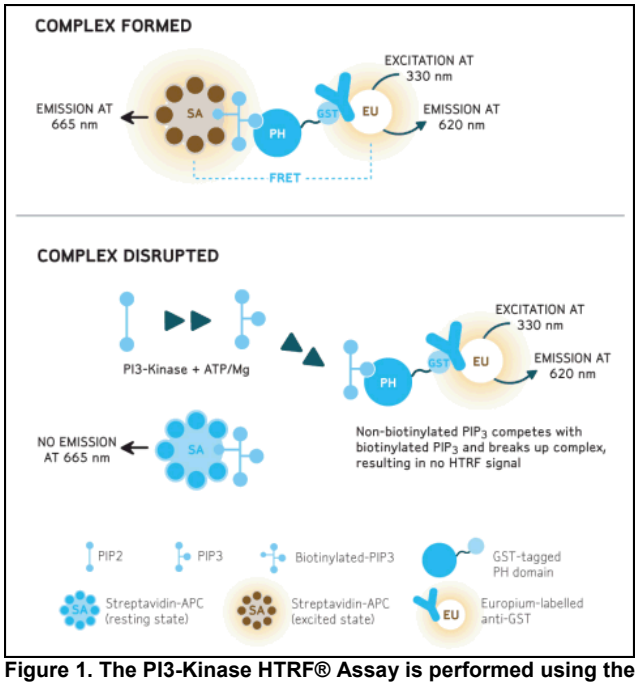



**Figure 1. The PI3-Kinase HTRF® Assay is performed using the following steps.** 1. Inhibitor, PI3-Kinase, $PIP_2$ substrate, and ATP are incubated together for 30 minutes at RT. 2. Stop solution, containing biotinylated $PIP_3$ is added to the well, preventing further substrate phosphorylation. 3. Detection mix is added, containing the GSTtagged GRP1 pleckstrin homology (PH) domain, and fluorophores.

## BioTek Instrumentation

The BioTek Synergy™ 4 Hybrid Multi-Mode Microplate Reader was used to measure the 330Ex/665Em signal, indicative of an inhibited PI3-Kinase reaction, as well as the 330Ex/620Em signal from an uninhibited reaction. Deep blocking filters and a dichroic mirror were incorporated to separate the two emission signals, and eliminate any significant interference from stray light.

**Figure 2. Synergy™ 4 Multi-Mode Microplate Reader.** The instrument was used to quantify the 330/620 and 330/665 signal from all assay plates.

| Instrument Detection Component | | BioTek Catalog Number | Optimized Instrument Settings | |
|---|---|---|---|---|
| Excitation Filter | 330/80 nm | 7082263 | Delay After PI. 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.**

## Materials and Methods

| Component | Vendor | Part Number |
|---|---|---|
| Black 384-Well Assay Plate | Corning | 3573 |
| HTRF PI3 Kinase Assay | Millipore | 33-017 |
| PI3 Kinase (p110α/p85 α) | Millipore | 14-602 |
| PI3 Kinase (p110α/p65 α) | Millipore | 14-790M |
| PI3 Kinase (p110β/p85 α) | Millipore | 14-603-K |
| PI3 Kinase (p110δ/p85 α) | Millipore | 14-604 |
| ATP | Sigma Aldrich | A7699 |
| Wortmannin | EMD Biosciences | 681675 |
| Quercetin | EMD Biosciences | 551600 |
| LY294002 | EMD Biosciences | 440204 |
| PI3KB-Inh VI | EMD Biosciences | 528113 |

**Table 2. Project component list.**

### Assay Setup

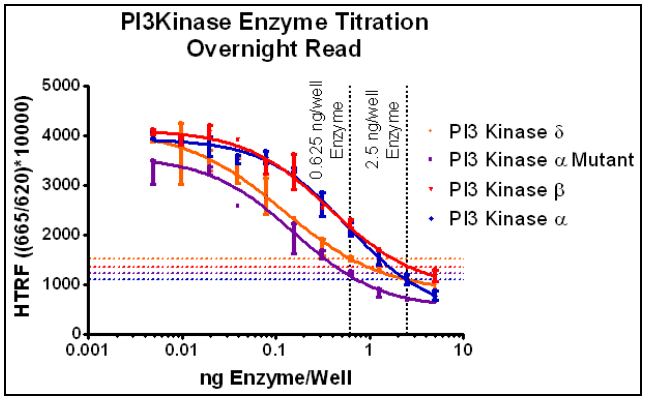
1. Add 5 µL of 10% DMSO or compound in 10% DMSO.
2. Add 10 µL of 2X enzyme/$PIP_2$ substrate.
3. Add 5 µL of 4X ATP, shake plate for 60 seconds, and incubate at RT for 30 minutes.
4. Add 5 µL of Stop Solution, and 5 µL of Detection Mix, shake plate for 60 seconds, and incubate at RT overnight (15-18 hours).
5. Read plate on Synergy™ 4.

## Results and Discussion

Our intent in this application note is to optimize assays for a series of Class IA PI3-kinases. The optimization requires determining optimal ATP and enzyme concentrations, and assay incubation conditions. Validation studies include the demonstration of assay performance, Z'-Factor at 10% ATP conversion levels, and pharmacology.

### Enzyme Titration

Enzyme titration curves were generated to determine the $EC_{85}$ concentration of enzyme. This concentration is sufficient to create an acceptable assay window. $PIP_3$ standard curves were also run in order to determine the percent $PIP_2$ substrate that was converted to $PIP_3$. A conversion level around 10% was targeted in order to ensure that initial rate velocity was being preserved. All enzyme reactions were allowed to proceed for 30 minutes.



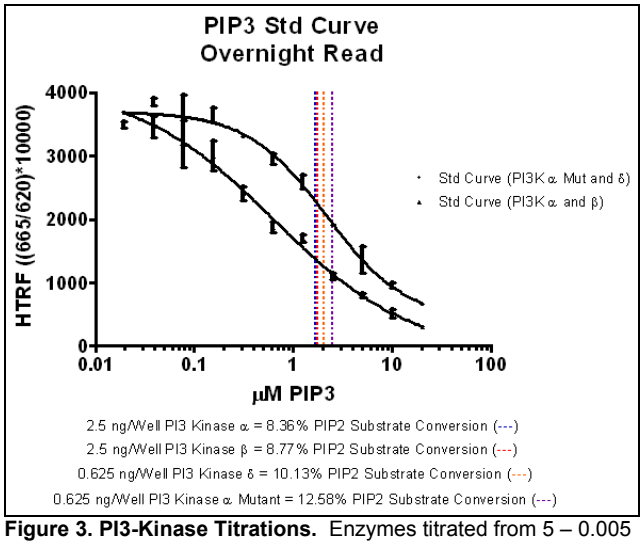




**Figure 3. PI3-Kinase Titrations.** Enzymes titrated from 5 – 0.005 ng/well. ATP concentration equaled 200 μM and $PIP_2$ substrate equaled 10 μM. $PIP_3$ standard curves were run with concentrations ranging from 20 – 0.02 μM.

### Enzyme Reaction Progression

Enzyme titration time course experiments were also conducted to ensure the linearity of the reaction at the 30 minute time point. Reactions were stopped at 5, 15, 30, and 60 minutes. All $R^2$ values for each enzyme tested were above 0.9 at 30 minutes. Figure 4 shows representative data for this set of experiments.

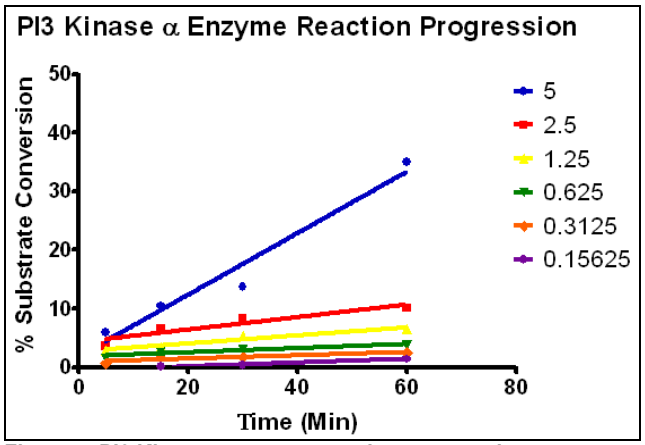



**Figure 4. PI3 Kinase α enzyme reaction progression.**

### ATP Titration

An ATP titration was then conducted with each PI3-Kinase using the $EC_{85}$ enzyme levels previously determined. The $EC_{50}$ 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.

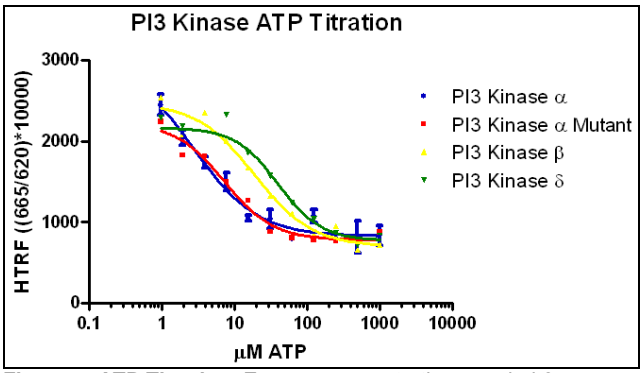
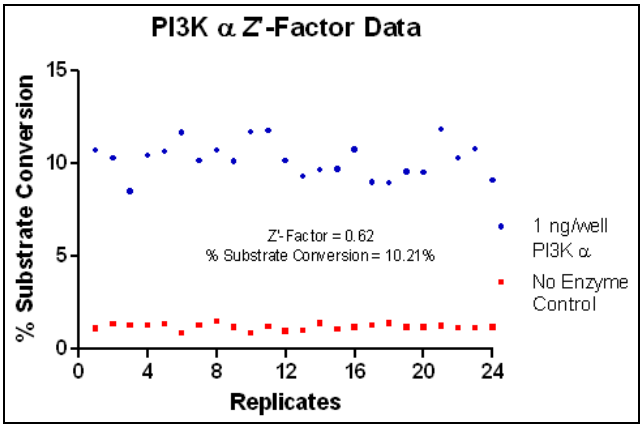
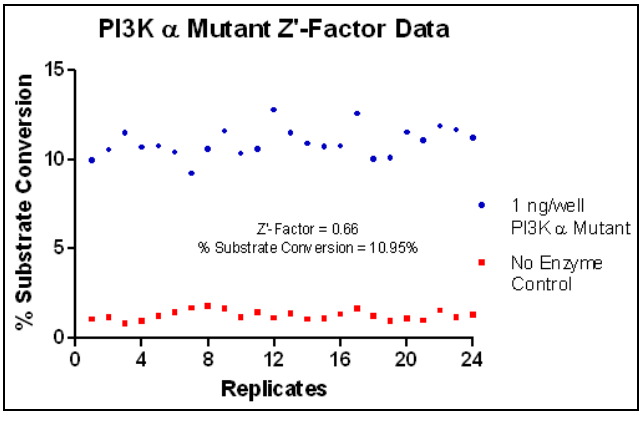


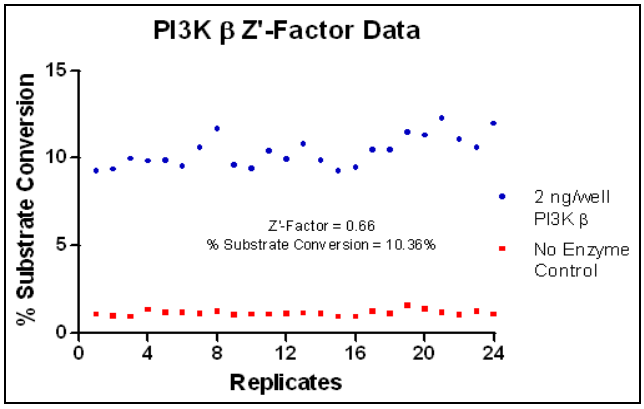

**Figure 5. ATP Titration.** Enzyme concentration equaled 2.5 ng/well for PI3-Kinase α and β, and 0.625 ng/well for PI3-Kinase α Mutant and δ. $PIP_2$ substrate equaled 10 μM. ATP titrated from 1000 – 1.0 μM.

### 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.





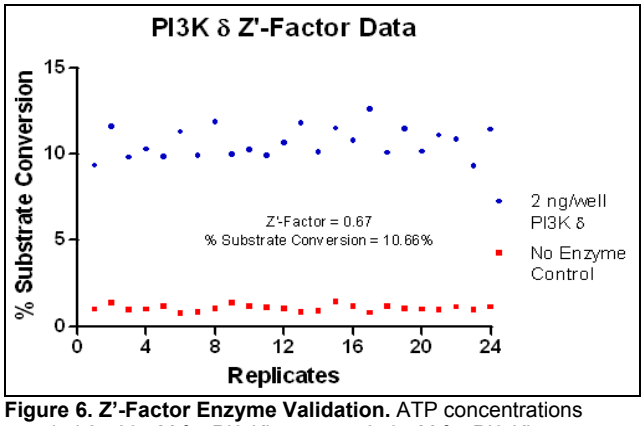




**Figure 6. Z'-Factor Enzyme Validation.** ATP concentrations equaled 2.789 µM for PI3-Kinase α, 7.379 µM for PI3-Kinase α Mutant, 19.19 µM for PI3-Kinase β, and 40.73 µM for PI3-Kinase δ. $PIP_2$ substrate equaled 10 µM.

### PI3-Kinase Inhibitor Profiling

Final validation 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. $IC_{50}$ values were generated for each compound with each PI3-Kinase isoform.

| PI3 Kinase Isoform | Assay Component Concentrations | | |
|---|---|---|---|
| | Enzyme (ng/well) | ATP (µM) | $PIP_2$ Subst (µM) |
| PI3K α | 1 | 2.789 | 10 |
| PI3K α Mutant | | 7.379 | |
| PI3K β | 2 | 19.19 | |
| PI3K δ | | 40.73 | |

**Table 3. Assay component concentrations.**

| PI3 Kinase Isoform | Compound $IC_{50}$ Values (µM) | | | |
|---|---|---|---|---|
| | Wortmannin | Quercetin | LY294002 | PI3Kβ-Inh VI |
| PI3K α | 0.03/0.0089[1] | 1.02/0.87[1] | 0.98/1.1[1] | 3.77/3.3[1] |
| PI3K α Mutant | 0.10 | 1.49 | 2.86 | 9.75 |
| PI3K β | 0.06 | 1.52 | 0.98 | 0.47 |
| PI3K δ | 0.38 | 0.93 | 4.77 | 1.84 |

**Table 4. $IC_{50}$ 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.

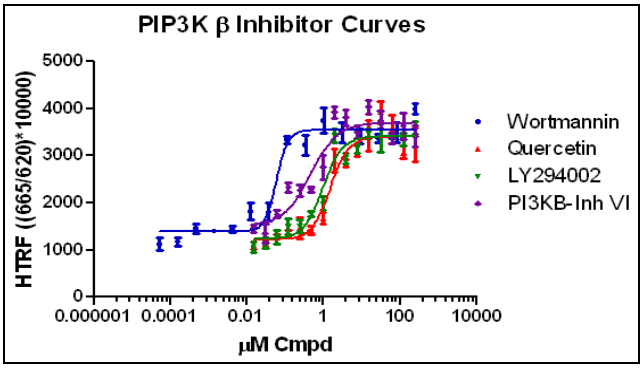



**Figure 7.** Representative inhibition curves for PI3-Kinases with test compounds. PI3-Kinase β curves shown.

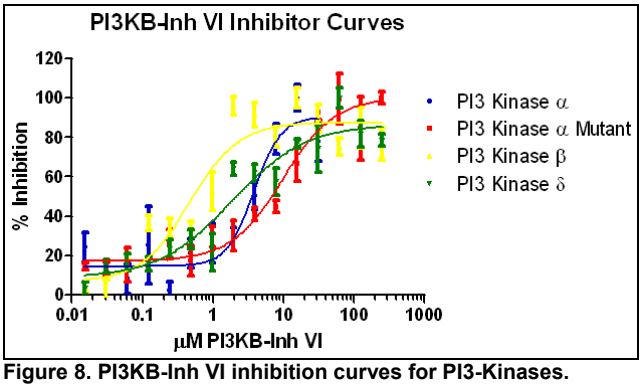



**Figure 8. PI3KB-Inh VI inhibition curves for PI3-Kinases.**

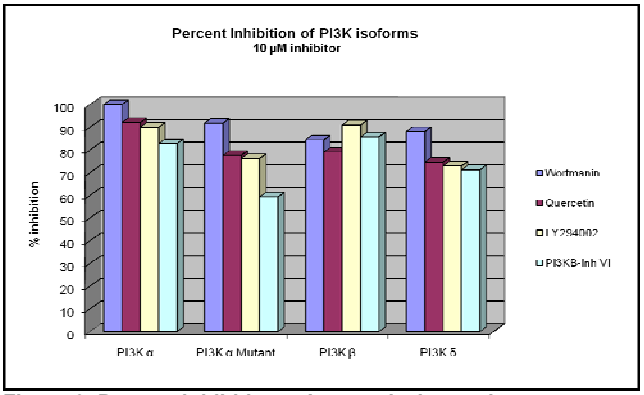



**Figure 9. Percent inhibition values at designated enzyme concentrations.**

## Conclusions

Millipore's PI3-Kinase HTRF® assay is easily optimized, uses low concentrations of enzyme and yields results that are consistent with established literature values. In addition, the instrument settings, and sensitivity of BioTek's Synergy™ 4 are able to deliver dependable time-resolved FRET readings in 384-well format. With the increased demand for assays that deliver reliable pharmacology results, and instrumentation that fits into tightening capital budgets, the combination of Synergy™ 4, and the PI3-Kinase HTRF® assay create an ideal solution for screening of compounds for this important target class.

## References

Caliper Life Sciences. LabChip Mobility-Shift Assay: Phosphotidylinositol-3 Kinase PI3Ka. LC3000-AP-212 June 08