AlphaScreen™ PI 3-kinase Assay: A Homogeneous, High-Throughput Assay for Screening Modulators of PI 3-Kinase Activity.

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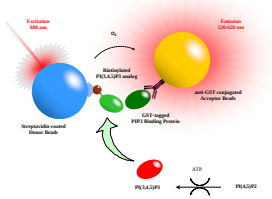
1 Abstract

Phosphoinositide 3-kinases (PI 3-kinases) are lipid kinases that have become important drug targets for cancer, inflammation and diabetes because of their implication in cell proliferation, differentiation and endocytosis. To simplify and to increase the throughput of screening at PI 3-kinase in drug development, we have combined PerkinElmer's AlphaScreen™ homogeneous screening technology with Echelon BioSciences specialized phosphoinositide and detection reagents.

This simple screening method is based on the binding reaction of a biotinylated $PI(3,4,5)P_3$ probe to a $PI(3,4,5)P_3$ detector-GST fusion protein (Echelon Biosciences Incorporated #K-1300). This interaction is detected using the AlphaScreen™ GST (Glutathione-S-Transferase) detection kit (PerkinElmer Life Sciences #6760603,C, M or R).

Data will be presented showing how the two isoforms of PI 3-kinase (α and γ) assay were optimized for use in a HTS environment. Enzyme assays further demonstrated the effectiveness of the platform for traditional enzymatic studies and quantitative pharmacological studies of PI 3-kinases show how the platform can be used to characterize drug hits in a secondary screening laboratory.

2 PI 3-kinase Assay Principle



3 Methodology

The AlphaScreen™ technology is based on the emission of light (520-620nm) by Acceptor beads activated by the proximity of Donor beads. Biological interactions between the biotinylated-$PI(3,4,5)P_3$ and $PI(3,4,5)P_3$ binding protein brings both Acceptor and Donor beads together producing a cascade of chemical reactions and leading to the amplified AlphaScreen signal. This high amplified signal is detected upon excitation of the Donor beads at 680nm when singlet state oxygen (O_2) molecules are generated and diffuse to excite Acceptor beads.

Conversely, in the absence of specific biological interactions and thus, no proximity between the Donor and Acceptor beads, the singlet oxygen molecules are undetected by the Acceptor beads and there is a resulting low signal.

- **The AlphaScreen GST (Glutathione-S-Transferase) detection kit (PerkinElmer Life Sciences #6760603,C, M or R) is composed of Donor beads conjugated with streptavidin and Acceptor beads conjugated with anti-GST.**
- **The PI 3-kinase kit (Echelon Biosciences (#K-1300) is composed of biotinylated $PI(3,4,5)P_3$ probe, $PI(3,4,5)P_3$ detector-GST binding protein, substrate and standard.**

4 Assay Procedure

Assays were performed in quadruplicate on 3 separate occasions (unless stated otherwise). Assays were performed in 384-well white plates in a final volume of 25 µl as follows:

1. Add 2.5µl of kinase buffer or test compounds*.
2. Add 5µl of enzyme prepared in kinase buffer.
3. Add 2.5µl of substrate prepared in kinase buffer.

Enzymatic reaction - Incubate at room temperature#

4. Add 5µl of biotinylated-PIP3 prepared in detection buffer. (10nM final).
5. Add 5µl of PIP3 binding protein prepared in detection buffer (10nM final).
6. Add 5µl of a mixture of Donor and Acceptor beads (20µg/ml final for each) prepared in detection buffer (Tris 10mM, NaCl 150mM, Tween-20 0.1%, EDTA 7.5mM, DTT 1mM at pH 7.4).

AlphaScreen detection- Incubate at 23°C for 120 minutes in darkness.

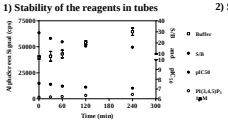
7. Read on the Fusion-alpha or the AlphaQuest-HTS analyzers (PerkinElmer)

* Pre-incubate the test compounds (inhibitor) with the enzyme before to add the substrate.
# For standard curves, steps 1, 2 and 3 were substituted by the addition of 5µl of $PI(3,4,5)P_3$ standard at various concentrations and 5µl of kinase buffer.

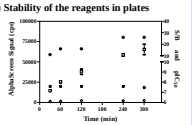
5 Standard Curve Assays

The performance of the PI 3-kinase AlphaScreen kit was tested in standard curve assays in various paradigms 1) stability of the reagents in tubes before loading into the plate, 2) stability of the assay in the plate over time, 3) selectivity of $PI(3,4,5)P_3$ detection over other phosphoinositides and 4) tolerance of the assay to the organic solvent DMSO. *Data are representative of 2 similar experiments.*

1) Stability of the reagents in tubes



2) Stability of the reagents in plates



- **IC50 values are slightly shifted to the right due to pre-coupling of the reagents in the tube. However, the window values (S/B ratio) are slightly decreasing over time due to higher background.**
- **Based on IC50 and S/B ratio values, the equilibrium of the assay is reached after 60 min and maintained over time up to 5 hours.**

6 Standard Curve Assays

3) Specificity of $PI(3,4,5)P_3$ binding

Competitors	Standard Curve S/B	Standard Curve IC50 (nM)
PI(3,4,5)P3	15 ± 2	24 ± 4
PI(3,4)P2	~1	>1000
PI(4,5)P2	~1	>1000
PI(3)P	~1	>1000
PI	~1	>1000

- **The PI 3-Kinase AlphaScreen assay appears to have selectivity for PI(3,4,5)P3 over the other phosphoinositides PI, PI(3)P, PI(3,4)P2 and PI(4,5)P2.**

4) Resistance to DMSO

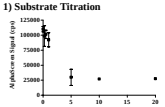
DMSO	Standard Curve S/B	Standard Curve IC50 (nM)
0%	17 ± 3	29 ± 6
0.5%	20 ± 1	29 ± 2
1%	24 ± 4	33 ± 8
2%	27 ± 3	35 ± 4
5%	40 ± 6	31 ± 1

- **The assay is resistant to 2 % DMSO without affecting S/B ratio and IC50 values.**

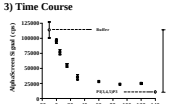
• S/B ratio value represents the window size of the assay. In standard curves it is calculated from the counts at the top of the curve divided by that of the bottom. In enzymatic assay it is calculated from the maximum counts (obtained in absence of enzyme) divided by the minimum count (in presence of enzyme where PIP3 is generated).

7 Enzymatic Assays PI 3-Kinase Alpha

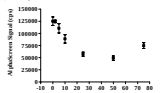
1) Substrate Titration



3) Time Course



2) Enzyme Titration



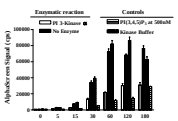
- **Optimal substrate concentration: 5µM/well**
- **Optimal enzyme concentration: 25ng/well**
- **Optimal reaction time: 60 minutes**

Km= 3µM

Data are representative of 2 similar experiments

8 Enzymatic Assays PI 3-Kinase Alpha

1) Stability of the assay in plates



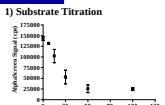
2) Resistance to DMSO

DMSO	S/B ratio calculated from PI 3-Kinase Alpha vs No Enzyme
0%	3.1 ± 0.7
0.5%	3.2 ± 0.3
1.0%	2.9 ± 0.3
2.0%	2.3 ± 0.3
5.0%	2.6 ± 0.5

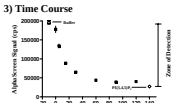
- **The equilibrium of the assay in the plate is reached after 60 min and maintained up to 3 hours**
- **The enzymatic assay appears to tolerate to up to 1% of DMSO without detrimental impact.**

9 Enzymatic Assays PI 3-Kinase Gamma

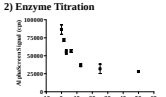
1) Substrate Titration



3) Time Course



2) Enzyme Titration



- **Optimal substrate concentration: 20µM/well**
- **Optimal enzyme concentration: 10ng/well**
- **Optimal reaction time: 60 minutes**

Km= 45µM

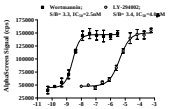
Data are representative of 2 similar experiments

10 Pharmacology

1) PI 3-Kinase Alpha

ALPHA buffer composition: Hepes 5mM, $MgCl_2$ 2.5mM, ATP 25µM at pH 7.4.

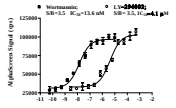
- **IC50 values of wortmannin (3nM) and LY-294002 (4µM) correlate with those reported in the literature.**



2) PI 3-Kinase Gamma

GAMMA buffer composition: Tris 20mM, $MgCl_2$ 4mM, NaCl 10mM, ATP 25µM at pH 7.4.

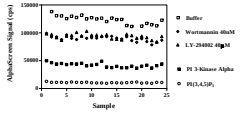
- **IC50 values of wortmannin (14nM) and LY-294002 (4µM) correlate with those reported in the literature.**



11 Precision Assays (Z' Determination)

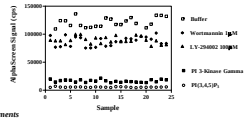
1) PI 3-Kinase Alpha

- **PI 3-Kinase vs Wortmannin, Z=0.48**
- **PI 3-Kinase vs LY-294002, Z=0.51**
- **Buffer vs $PI(3,4,5)P_3$ standard, Z=0.78**



2) PI 3-Kinase Gamma

- **PI 3-Kinase vs Wortmannin, Z=0.55**
- **PI 3-Kinase vs LY-294002, Z=0.59**
- **Buffer vs $PI(3,4,5)P_3$ standard, Z=0.76**



Data are representative of 2 similar experiments

12 Conclusion

- The data presented here demonstrates the robustness of the assay in for stability in the plate, selective recognition to $PI(3,4,5)P_3$ phosphoinositide, resistance to DMSO and precision (Z'>0.5 in presence of inhibitors). Further, expected pharmacological profiles were obtained with common PI 3-kinase inhibitors, wortmannin and LY-294002.
- In summary, the PI 3-Kinase AlphaScreen assay kit offers a high performance and precise means to characterize and screen both PI 3-Kinase alpha and gamma enzyme isoforms.