echelon
an Æterna Zentaris company

Echelon Biosciences Inc.
675 Arapeen Drive, Suite 302
Salt Lake City, UT 84108 Telephone
866-588-0455
Fax 801-588-0497
echelon@echelon-inc.com
www.echelon-inc.com

Technical Data Sheet

For research use only
Not intended or approved for diagnostic or therapeutic use.

Product Name: **Luminescent Assay for PI 3-Kinase Activity**
AlphaScreen™ Assay for Detection and Quantification of PI3-Kinase Activity

Product Number: K-1300

This product is intended for use with the Amplified Luminescent Proximity Homogenous Assay (AlphaScreen™) technology and requires the researcher to obtain donor and acceptor beads from the AlphaScreen™ GST (Glutathione-S-Transferase) Detection Kit, (PerkinElmer Life Sciences #6760603,C, M or R), which must be purchased separately from PerkinElmer Life Sciences. A microplate reader with AlphaScreen™ capabilities is also requir

I. Components (500 assay points)

Kit includes:

- $PI(4,5)P_2$ Substrate Solution (K-1301)
- $PI(3,4,5)P_3$ Standard Solution (K-1302)
- $PI(3,4,5)P_3$ Detector Pellet (K-1303)
- Biotinylated $I(1,3,4,5)P_4$ Solution (K-1305)

Researcher must provide

- Buffers for reactions and dilutions
- Source of PI3-Kinase
- AlphaScreen™ GST Detection Kit (PerkinElmer Life Sciences, #6760603C, M, or R)
  384-well white Optiplates™ (#6007299, PerkinElmer Life Sciences) as needed
- Plate reader equipped for AlphaScreen™ detection, such as the AlphaQuest™, Envision™ or Fusion™ Universal Microplate readers (PerkinElmer Life Sciences).

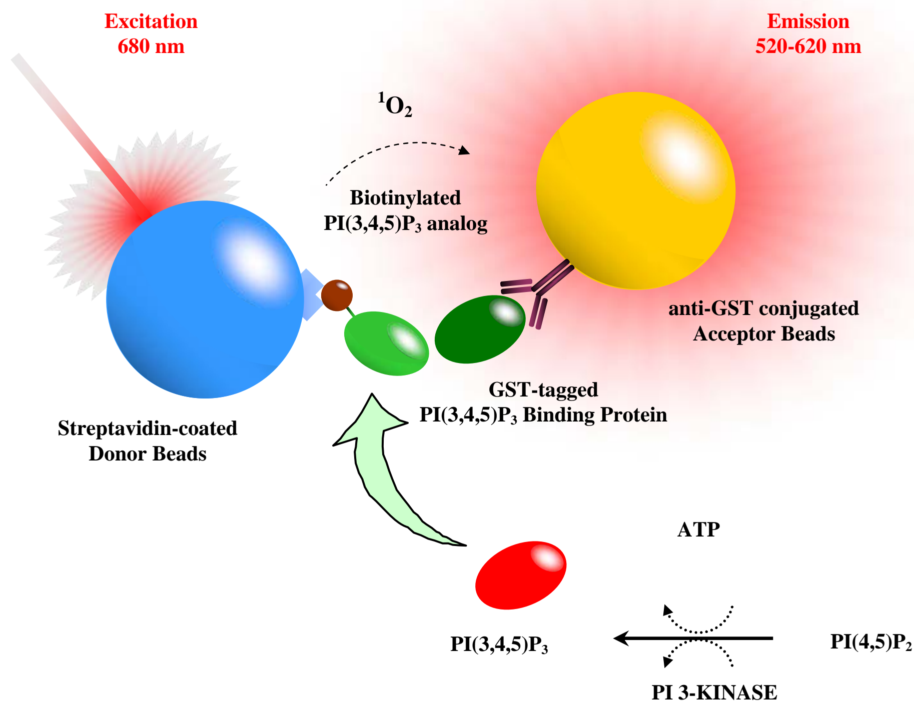


Diagram courtesy of PerkinElmer Life Sciences

II. Assay Principles

AlphaScreen™ technology is based on the emission of light (520-620nm) by Acceptor beads activated by the proximity of Donor beads. The interaction of biotinylated-PI(3,4,5)P_3 and the 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 highly amplified signal is detected upon excitation of the Donor beads at 680 nm when singlet state oxygen ($^1O_2$) molecules are generated and diffuse to excite Acceptor beads.

When PI(3,4,5)P_3 is generated via phosphorylation of PI(4,5)P_2 by PI 3-Kinase, the products of the enzymatic reaction compete with biotinylated PI(3,4,5)P3 for the interaction of the PI(3,4,5)P_3 detector protein. In the absence of this interaction, proximity of the Donor and Acceptor beads is decreased , producing a loss of luminescent signal which is inversely related to enzyme activity.

IMPORTANT: The luminescent signal produced by the AlphaScreen™ assay is dependent on the release of singlet oxygen by the Donor beads and transfer to the Acceptor beads. Thus, chemicals which act as singlet oxygen scavengers can affect the assay by quenching the production of luminescence. These include, but are not necessarily limited to: transition metals (Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{2+}), azide and ascorbic acid) . The presence of these compounds in enzyme preparations or other buffers can interfere with the determination of enzyme activity using the AlphaScreen™ assay.

III. Buffer Preparation

Kinase Buffer for PI 3-Kα	**Kinase Buffer (for PI 3-Kα and γ)**	**Detection Buffer**
2.5 mM $MgCl_2$	4 mM $MgCl_2$	10mM Tris, pH 7.4
5 mM Hepes pH 7.4	20 mM Tris pH 7.4	150 mM NaCl
25 µM ATP	10 mM NaCl	7.5 mM EDTA
	25 µM ATP	1 mM DTT
		0.1% Tween-20

Kinase Reaction Buffers and Detection Buffer must be made fresh prior to each experiment. If it is desired that buffers be made and stored prior to use, ATP and dithiothreitol (DTT) must be omitted and fresh ATP and DTT added prior to the start of the experiment.
Note: Two different recipes for kinase buffer are provided. One is suitable for determining the activity of PI 3-Kα isoforms and the other is suitable for determining activity of both PI 3-Kα and PI 3-Kγ isoforms.

IV. Reagents:

PI(4,5)P_2 substrate. Each tube contains 110 µL of 1 mM diC_8 PI(4,5)P_2. Store frozen at -20 °C, stable for at least 6 months. Prior to each experiment, prepare the required amount of working substrate solution for that day only by diluting into the appropriate Kinase Buffer. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after dilution into Kinase Buffer.

PI(3,4,5)P_3 for standards. Each tube contains 20 µL of 25 µM diC8 PI(3,4,5)P_3 in water. Store frozen at -20 °C, stable for at least 6 months. Prior to each experiment, prepare the required amount of standard solutions for that day only by diluting into the appropriate Kinase Buffer. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after dilution into Kinase Buffer.

Biotinylated I(1,3,4,5)P_4. Each tube contains 140 µL of 1 µM biotin- I(1,3,4,5)P_4 in water, enough for 500 assay points. Store frozen at -20 °C, stable for at least 6 months. Prior to each experiment, prepare the required amount of biotin- I(1,3,4,5)P_4 solution for that day only by diluting 1/20 in Detection Buffer for a

50 nM working solution. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after dilution into Detection Buffer.

PI(3,4,5)P_3 Detector. Each pellet contains 0.140 nanomoles of PI(3,4,5)P_3 detector. Reconstitute in 140 µLof milliQ H_20 for a 1µM stock. The reagent should be used fresh after reconstitution. Prior to each experiment, prepare the required amount of PI(3,4,5)P_3 detector solution for that day only by diluting 1/20 in Detection buffer for a 50 nM working solution. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after dilution into Detection Buffer.

AlphaScreen™ GST detection kit (must be purchased separately from PerkinElmer). Each vial provided by PerkinElmer contains Donor Beads and Acceptor Beads already reconstituted in liquid. Both stock solutions are at 5 mg/mL. Store at 4 °C in the dark, stable for at least 6 months. Prior to each experiment, prepare the required amount of Beads mixture solution for that day only by diluting 1/50 in Detection Buffer for a 100 µg/mL working solution. Keep working solution protected against light. Do not reuse solution on subsequent days after dilution into Detection Buffer.

V. Setting up a Standard Curve

You may want to run a sample standard curve before running any enzyme reactions. It may also be helpful to set up a standard curve of diC8 PI(3,4,5)P_3 alongside enzyme reactions to allow determination of the amount of PI(3,4,5)P_3 produced.

From a 25 µM stock of PI(3,4,5)P_3, dilute 1:5 in kinase buffer for a 5 µM working solution. From the 5 µM solution, make seven 3-fold serial dilutions into Kinase Buffer to generate a standard curve of PI(3,4,5)P_3 ranging from 1 µM to 460 pM final concentration, as outlined in the Table below. Also set up a standard containing 5 µL Kinase Buffer without PI(3,4,5)P_3 competitor. The Table below outlines the concentrations of standard solutions, and the final PI(3,4,5)P_3 concentrations and amounts per well.

The Standard Curve is set up directly in the wells of a 384-well white Optiplate. Final volume per point is 25 µL.

1) Add 5 µL of each standard solution into wells.

2) Add 5 µL of the appropriate Kinase buffer into wells.

Concentration of PI(3,4,5)P_3 Standard, nM	Final Concentration PI(3,4,5)P_3, nM	picomoles PI(3,4,5)P_3
5000 (5.0 µM)	1000 (1 µM)	25
1666	333.2	8.3
555	111	2.8
185	37	0.9
61.7	12.3.	0.3
20.5	4.1	0.1
6.8	1.37	0.033
2.3	0.46 (460 pM)	0.011

2) Add 5 µL of 50 nM biotinylated I(1,3,4,5)P_4 prepared in Detection Buffer to each well.

3) Add 5 µL of 50 nM PI(3,4,5)P_3 binding protein prepared in Detection Buffer to each well.

4) Add 5 µL of a mixture of 100 µg/ml of Donor and 100 µg/mL of Acceptor beads prepared in Detection Buffer. Final concentration of both Donor and Acceptor beads is 20 µg/mL.
IMPORTANT: The beads are light-sensitive, and should only be handled in a darkened room under subdued light (less than 100 lux). Exposure to direct light will cause a decrease in the luminescent signal produced.

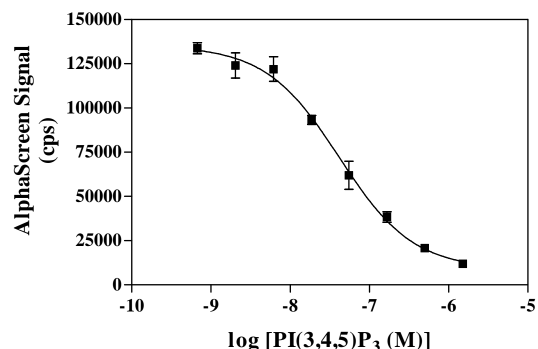
5) Seal the plate, and incubate in a dark location at room temperature (22-24 °C) for 2 hours.

6) Read plate using appropriate instrument (Fusion-alpha or AlphaQuest-HTS analyzers from PerkinElmer). The instruments manufacturer's suggested count times are 1 second/well with 300 msec excitation and 700 msec emission times. The competition of PI(3,4,5)P_3 standards for the interaction of

the PI(3,4,5)P_3 Detector and biotin-I(1,3,4,5)P_4 coated Acceptor and Donor beads will cause a decrease in the luminescent signal which is inversely proportional to the amount of PI(3,4,5)P_3 standard.
A representative standard curve is shown below.

VI. Determination of PI 3-Kinase Activity

The entire procedure, including enzymatic reaction and detection, is performed directly in the wells of a 384-well white Optiplate. The final volume per assay point is 25 µl.



1. Add 2.5µl of kinase buffer or test compound(s)* to each well.

2. Add 5µl of PI 3-Kinase Enzyme prepared in the appropriate kinase buffer to each well.
Note: It is recommended that test compound(s) be preincubated with the enzyme for 15-30 minutes prior to addition of substrate.

3. Add 2.5µl of PI(4,5)P_2 substrate prepared in kinase buffer to each well.

For PI 3-Kinase α isoforms, recommended substrate concentration in the final reaction is 5 µM by loading 2.5 µl of a 20 µM working solution , which can be obtained by diluting the 1 mM stock solution of PI(4,5)P_2 1/50 in the appropriate Kinase Buffer.

For PI 3-Kinase γ isoforms, recommended substrate concentration in the final reaction is 20 µM, by loading 2.5 µl of a 80 µM working solution , which can be obtained by diluting the 1 mM stock solution of PI(4,5)P_2 1/12.5 in the appropriate Kinase Buffer.

4. Seal plate and incubate at room temperature (22-24°C) to allow enzymatic reaction to proceed.

5. When reaction is complete, add 5µl of 50 nM biotinylated I(1,3,4,5)P_4 prepared in Detection Buffer. Addition of this reagent quenches the kinase reaction.

6. Add 5µl of 50 nM PI(3,4,5)P_3 binding protein prepared in Detection Buffer.

7. Add 5µl of mixture of 100µg/ml of Donor and 100µg/ml of Acceptor beads prepared in Detection Buffer. Final concentration of both Donor and Acceptor beads is 20 µg/ml.

IMPORTANT: The beads are light-sensitive, and should only be handled in a darkened room under subdued light (less than 100 lux). Exposure to direct light will cause a decrease in the luminescent signal produced.

8. Seal the plate, and incubate in a dark location at room temperature (22-24°C) for 2 hours.

The following Additional Controls are helpful to include:

1. No enzyme control: Substitute 5 µl of Kinase Buffer for PI 3-Kinase enzyme solution in step 2.
2. No substrate control: Substitute 2.5 µl of Kinase Buffer for PI(4,5)P_2 substrate solution in step 3.

Notice to Purchaser
Echelon Biosciences products are sold for research and development purposes only and are not to be incorporated into products for resale without written permission from Echelon Biosciences. **This kit and all non-radioactive, competitive assays for determining phosphoinositide-3-kinase (PI3-K) activity are protected by Echelon Biosciences Inc. U.S. Patent 7,067,269**. The purchase of

this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. For inquiries email busdev@echelon-inc.com.