

Homogeneous Cell-based Signal Transduction Assays

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Overview

- Mammalian Target of Rapamycin (mTOR) protein kinase represents a key target in today's drug discovery efforts due to its point of convergence for multiple cell signaling pathways.
- The AlphaScreen® SureFire® p-p70 S6 Kinase(Thr389) Assay provides an easy to use, and sensitive method to assay mTOR activity.
- The tungsten halogen lamp and filter-based system of the Synergy™ H4 Hybrid Multi-Mode Microplate Reader provide a flexible, easy to use method to excite and measure AlphaScreen assay signal.
- Validation data, as well as insulin receptor agonist/antagonist data, demonstrate how the combination of assay and instrumentation can provide relevant data to measure activity of this important drug target.

BioTek Instrumentation



Figure 1 – Synergy H4 Hybrid Multi-Mode Microplate Reader.

The Synergy H4 combines a filter-based and monochromator-based detection system, as well as a xenon flash lamp and tungsten halogen lamp, in one unit. The filter-based system and tungsten lamp can be used when maximum excitation and high sensitivity are a requirement. The ability to provide constant excitation, and a highly sensitive detection system incorporating filters and dichroic mirrors, makes the reader ideal for use with AlphaScreen SureFire assays.

Reader Setup

Instrument		BioTek Catalog #
Detection Component		
Excitation Filter 1	680/30 nm	7082229
Excitation Filter 2	Plug	N/A
Emission Filter 1	Plug	N/A
Emission Filter 2	570/100 nm	7082264
Dichroic	635 nm Cutoff	7139635
Optimized Instrument Settings		
Light Source	Tungsten	Delay after Plate Movement
Measurements per Data Point	15	Dynamic Range
Filter Switching per Well		Enabled

Figure 2 – Synergy H4 AlphaScreen Reader Settings.

Insulin-mTORC1 Signal Transduction

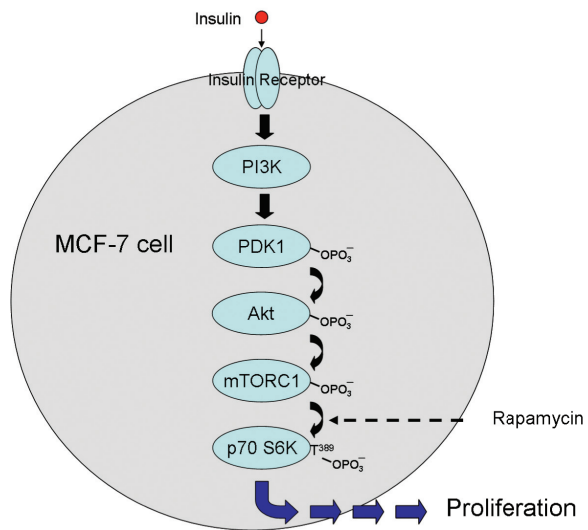


Figure 3 – Insulin-induced activation of mTORC1 serving as a model for constitutive activity common to some cancers resulting in uncontrolled cell proliferation. The small molecule rapamycin passes through the cell membrane and directly inhibits the mTORC1 phosphorylation of p70 S6 kinase at the threonine389 residue.

AlphaScreen SureFire p-p70 S6 Kinase (Thr389) Assay

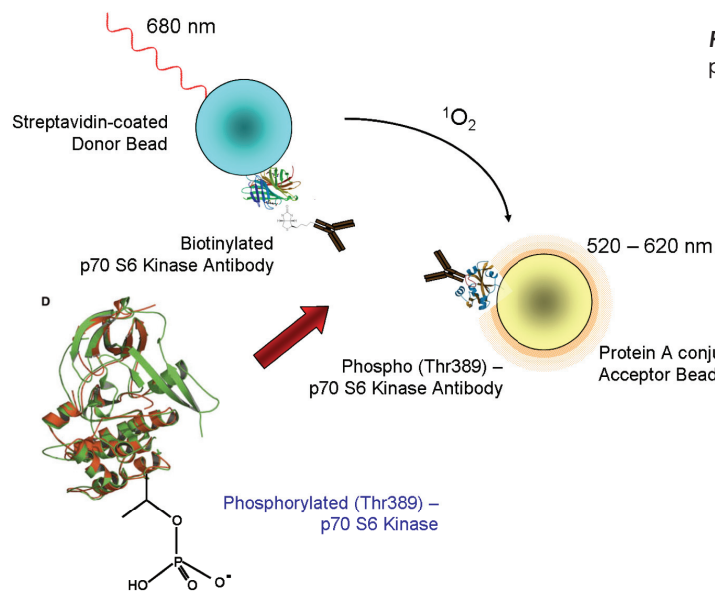


Figure 4 – Representation of AlphaScreen SureFire p-p70 S6 Kinase(Thr389) Assay.

Biotinylated p70 S6 Kinase Antibody binds to streptavidin-coated donor beads, while Phospho (Thr389)-p70 S6 Kinase Antibody binds to Protein A coated acceptor beads. Upon activation of the insulin signal transduction pathway, phosphorylated p70 S6 kinase is created. Following addition of the donor and acceptor bead mixes, the donor bead-antibody conjugate will bind to the p70 S6 kinase, while the acceptor bead-antibody conjugate will bind to the phosphorylated Thr389 residue.

During the detection step, acceptor beads are excited at 680 nm. The photosensitizer in the bead, phthalocyanine, produces singlet oxygen. This form of oxygen has a limited lifetime prior to falling back to ground state. Within its 4 microsecond half-life, singlet oxygen can diffuse approximately 200 nm in solution. In the presence of phosphorylated p70 S6 kinase, the beads are in close proximity to one another. Energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead, subsequently culminating in light production at 520-620 nm. In the absence of the phosphorylated kinase, the beads are not brought into close proximity to one another. The excited singlet oxygen then falls back to ground state and no signal is produced.

MCF-7 Cell Propagation

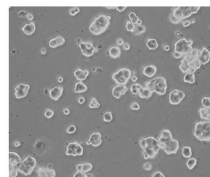
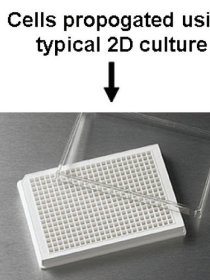


Figure 5 – MCF-7 Cell Propagation and Plating Process.



Cells plated in Corning® 384-well white TC treated plates and serum starved in 0.1% serum containing media overnight

Assay Procedure

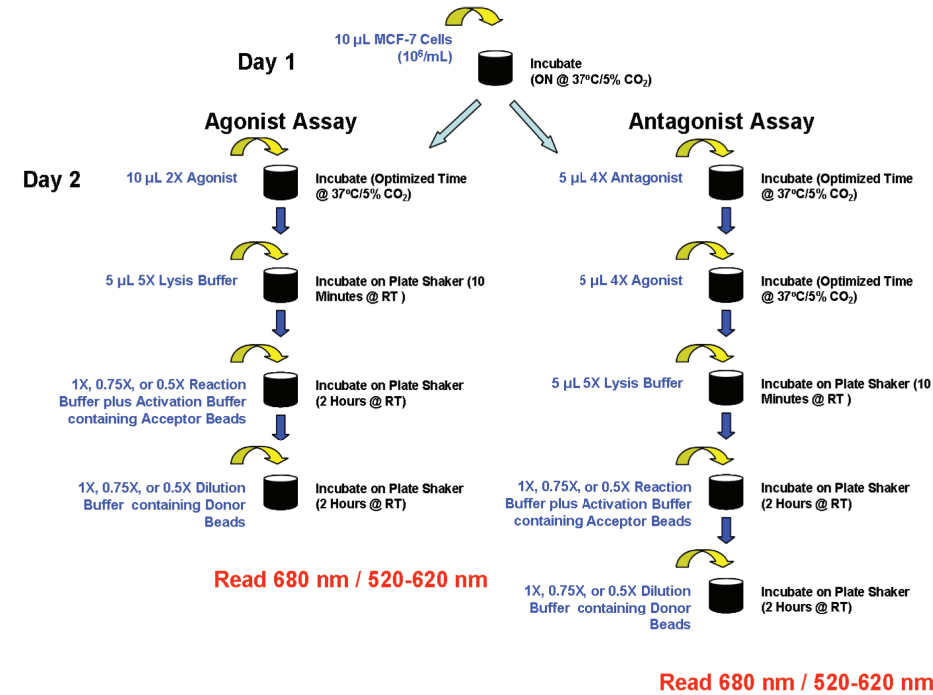


Figure 6 – AlphaScreen SureFire p-p70 S6 Kinase Assay Protocol.

Assay Optimization

Insulin was added to the cells at a 1X concentration of 0 µM and 10 µM. The assay plate was then incubated at 37°C/5% CO₂ for 30 minutes. The AlphaScreen assay was then carried out as explained previously. A 12.1 fold difference was seen between no cell background subtracted values for each concentration of insulin.

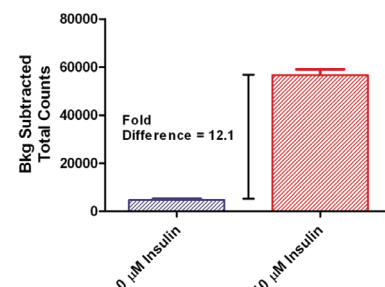


Figure 7 – Assay window between 0 µM and 10 µM Insulin.

An insulin titration was performed in order to validate proper detection of the activation of the insulin receptor. The EC₅₀ value determined, 86.2 nM, was then used to determine the EC₈₀ concentration of insulin to use with Rapamycin inhibition studies.

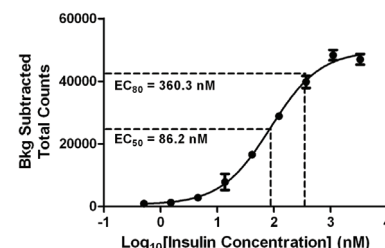


Figure 8 – Insulin titration curve. Serial 1:3 titrations tested between 3000 – 0 nM insulin.

A rapamycin incubation test was performed to determine the proper time to incubate the antagonist with the cells. Final 1X concentrations of 0, 1, and 200 nM were tested using 15, 30, 45, and 60 minute incubations. A 30 minute incubation time was decided upon due to the fact that complete inhibition was seen at 200 nM rapamycin, and close to 50% inhibition was seen at 1 nM rapamycin, which approximates the compounds IC₅₀ value.

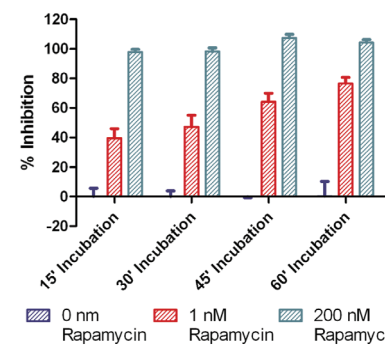


Figure 9 – Rapamycin incubation test results.

Insulin Agonist/Antagonist Verification

MCF-7 cells were dispensed to the assay plates (10 µL) at a concentration of 10⁶ cells/mL and incubated overnight at 37°C/5% CO₂. Insulin titrations were added to the cells at a final 1X concentration range of 3000-0 nM. The titration was run in agonist mode with and without 200 nM rapamycin, in order to illustrate the effect of rapamycin on insulin stimulation.

A rapamycin titration was also performed with final 1X concentrations ranging from 200-0 nM. The titration was run in antagonist mode, with the previously determined EC₈₀ concentration of insulin being added to the wells following the antagonist incubation.

Following the lysis step, 1X, 0.75X, or 0.5X volumes of acceptor and donor bead mixes were added to the assay plate wells. Acceptor bead mix volumes were 17, 12.8, or 8.5 µL, while donor bead mixes were 7, 5.3 or 3.5 µL, respectively. This was done in order to illustrate the ability of the Synergy reader to accurately detect the assay signal, no matter what volumes are decided upon by the end user.

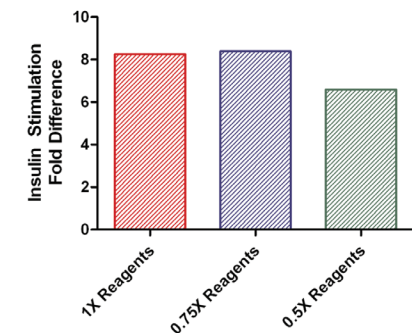


Figure 10 – Insulin fold stimulation values using 3000 and 0 nM insulin and 1X, 0.75X, and 0.5X AlphaScreen bead mixes.

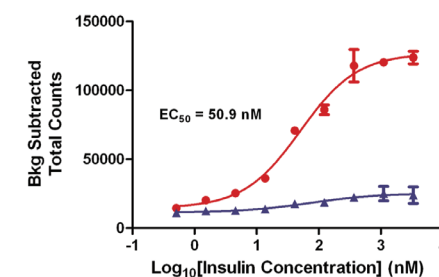


Figure 11 – Insulin titration curve plus and minus 200 nM rapamycin using 1X reagents.

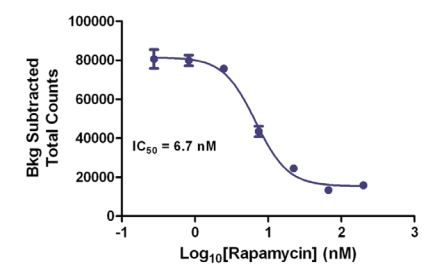


Figure 12 – Rapamycin titration using 1X reagents.

Conclusions

- The AlphaScreen SureFire p-p70 S6 Kinase(Thr389) Assay provides the ability to monitor activity of the insulin receptor cell signaling pathway.
- The tungsten halogen lamp on the Synergy H4 provides a flexible, yet robust way to provide the proper excitation required for AlphaScreen assays.
- The excitation energy provided by the tungsten lamp, combined with the sensitivity of the filter-based detection system on the Synergy H4 provide the ability to conserve reagent volumes and maximize the number of data points from the assay.
- The combination of assay and instrument provide a unique capability to measure the activity of the mTOR protein kinase.