Cell-based Assay for the Quantification of mTORC1 Activation and Inhibition in a Breast Cancer Cell Line

Endogenous Cell-based Kinase Assay using the AlphaScreen® SureFire® p70 S6 Kinase Kit and Synergy™ 2 Alpha

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Targeting dysregulated cellular phosphorylation is a general drug discovery strategy for a variety of indications, particularly cancer. Here we demonstrate an assay using the cancer cell line MCF-7 to monitor endogenous phosphorylation of p70 S6 kinase by mTORC1 in a homogeneous format suitable for high throughput screening.

Introduction

Mammalian Target of Rapamycin (mTOR) is a protein kinase predominantly found in the cytoplasm of the cell. It acts as a central regulator of many biological processes that are essential for cell proliferation, angiogenesis, and cell metabolism [1-3]. mTOR exerts its effects primarily by turning on and off the cell's translational machinery, resulting in protein synthesis.

mTOR is a key downstream intracellular point of convergence for a number of cellular signaling pathways. These diverse signaling pathways are activated by a variety of growth factors (including vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), hormones (insulin, estrogen, progesterone), and the presence or absence of nutrients (glucose, amino acids) or oxygen [4,5]). One or more of these signaling pathways may be abnormally activated in patients with many different types of cancer, resulting in deregulated cell proliferation, tumor angiogenesis, and abnormal cell metabolism [1,4,5].

mTOR exists in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), both of which contribute to tumor cell growth. Here we provide results for an AlphaScreen assay for measuring mTORC1 phosphorylation of p70 S6 kinase at its threonine(389) residue, expressed at endogenous levels in the cancer cell line MCF-7. mTORC1 activation is produced through insulin stimulation and inhibition with rapamycin. Figure 1 displays the signal transduction pathway induced by insulin stimulation in MCF-7 cells that leads to p70 S6 kinase phosphorylation at its threonine residue at position 389 of its primary structure.

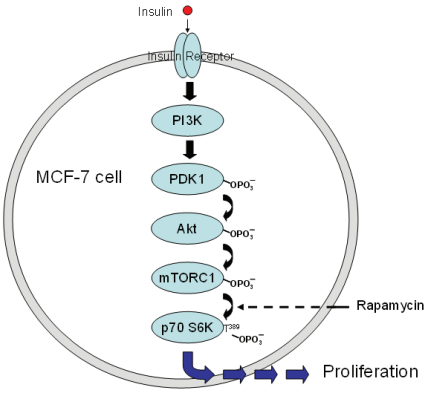

Figure 1. Insulin-induced activation of mTORC1 serving as a model for constitutive activity common to some cancers resulting in uncontrolled cell proliferation. The small molecule rapamycin passes through the cell membrane and directly inhibits the mTORC1 phosphorylation of p70 S6 kinase at the threonine 389 residue.

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AlphaScreen® SureFire® p70 S6 Kinase Assay Principle

AlphaScreen® SureFire® cellular assays utilize sandwich immunoassay techniques to quantify endogenous protein phosphorylation. It is an end point assay requiring cell lysis and release of the phosphorylated protein into the assay medium for quantification using acceptor and donor beads conjugated with the antibodies necessary for the assay. In this 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 (Figure 2).

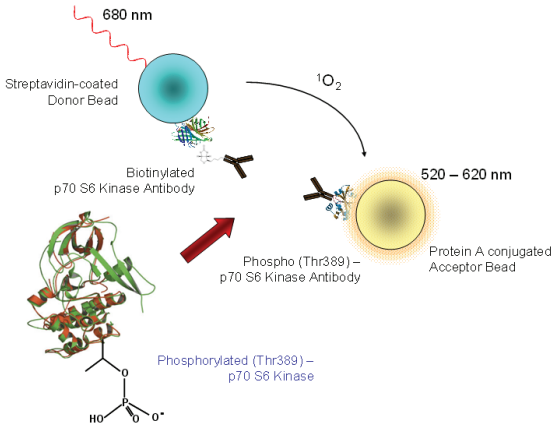


Figure 2. Representation of AlphaScreen® SureFire® p-p70 S6 Kinase(Thr389) Assay.

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 µsec 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.

Materials and Methods

Materials

The SureFire p70 S6 kinase (T389) Phosphorylation Kit was obtained from TGR Biosciences (Catalog # TGR70S10K) and the AlphaScreen IgG Detection Kit (Protein A) from PerkinElmer (Catalog # 6760617M). Insulin, Human Recombinant, was obtained from Sigma-Aldrich (Catalog # 91077C) and Rapamycin from Tocris Bioscience (Catalog # 1292). MCF-7 cell line was obtained from ATCC (ATCC Number: HTB-22). Standard sized assay microplates used in this study where white, 384-well, tissue culture-treated, from Corning Life Sciences (Catalog # 3570).

Synergy™ 2 Alpha Settings

The Synergy™ 2 Alpha is a filter-based system and uses a spectrally-filtered Tungsten lamp for excitation of donor beads. 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. Table 1 below depicts the Synergy 2 Alpha settings required for high performance AlphaScreen detection.

Instrument Detection Component		BioTek Catalog #
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 Cut-off	7139635

Optimized Instrument Settings			
Light Source	Tungsten	Delay After Plate Movement	250 msec
Filter Switching / Well		Enabled	

Table 1. Synergy™ Alpha Reader Settings used in this work.

mTORC1 Activation and Inhibition Protocols

All experiments began with plating 10 µL aliquots of MCF-7 cells (10,000 cells/well) in 0.1% serum-containing media and incubating overnight at 37 °C, 5% CO2. Slightly different protocols were used whether mTORC1 activation or inhibition was pursued according to Figure 3.

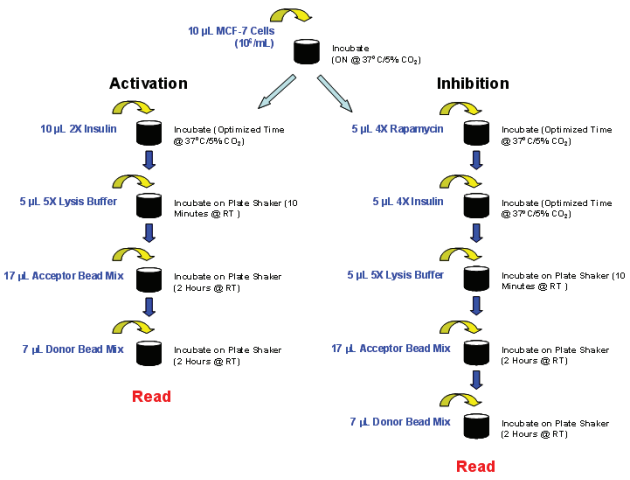
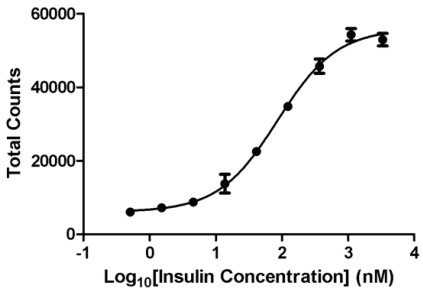


Figure 3. AlphaScreen® SureFire® p-p70 S6 Kinase Assay Protocol.

Results and Discussion

Insulin Activation of mTORC1

An insulin dose response curve is shown in Figure 4. A signal to background ratio (S/B) of 9.1 is evident from the range on insulin concentrations used. The EC50 value was determined to be 86.2 nM. From the graph, an EC80 concentration of insulin (360 nM) was used for Rapamycin inhibition studies.



EC50	86.16

Figure 4. Insulin dose response curve. Serial 1:3 titrations tested between 3000 – 0 nM insulin.

We also investigated the reduction in use of acceptor and donor beads and its impact on S/B in the activation protocol. This will serve to minimize the costs associated with the assay. Table 2 demonstrates the effect of using a 1.33x and 2x reduction in acceptor and donor beads.

AlphaScreen Bead Reduction	Total Assay Volume (µL)	Acceptor Bead Volume (µL)	Donor Bead Volume (µL)	S/B
1x	49	17	7	9.1
1.33x	42.8	12.5	5.3	8.4
2x	37	8.5	3.5	6.6

Table 2. Insulin fold stimulation values using 3000 and 0 nM insulin and 1x, 1.33x, and 2x reduction in AlphaScreen® bead mix volumes.

While there is an expected reduction in S/B as acceptor and donor bead concentrations are reduced, the assay still retains an acceptable S/B ratio suitable for screening using half as many beads.

Rapamycin Inhibition of mTORC1 Activity

Figure 5 demonstrates a Rapamycin dose response curve inhibiting insulin activation using its EC80 concentration established in Figure 4. An IC50 of 6.7 nM was found indicative of potent inhibition. A S/B of 5.3 was evident indicating good assay performance.

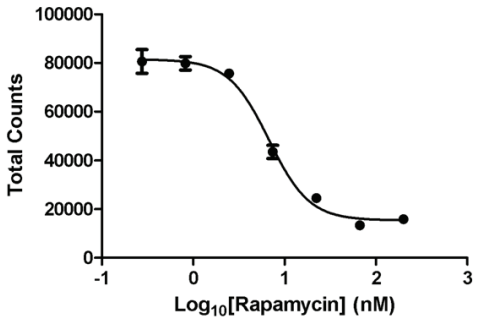


Figure 5. Rapamycin dose response curve using 1x reagents.

Conclusions

We have demonstrated the ability to monitor endogenous mTORC1 activation and inhibition in the cancer cell line MCF-7 using the AlphaScreen SureFire p70 S6 kinase kit and Synergy Alpha. S/B ratios are acceptable for screening applications. We have also developed a simple "mix and read" protocol that can utilize standard 384 well microplates.

References

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