Homogeneous Cell-Based Signal Transduction Assays using a Novel 3-D Cell Culture Matrix

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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
- Performing assays using cells grown on the GEM matrix simplifies the experimental process and provides results that are equivalent to control data generated with cells cultured using standard 2D methods
- 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

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, mTORC1 and mTORC2, both of which contribute to tumor cell growth. Here we provide results for an AlphaScreen SureFire assay for measuring mTORC1 phosphorylation of p70 S6 kinase at its threonine(389) residue, both expressed at endogenous levels in the cancer cell line MCF-7. The assay was developed using standard 2-D cell culture techniques, as well as cells grown on a novel three-dimensional (3-D) magnetic alginate microcarrier. Each microcarrier is coated with a covalently-bound gelatin coating to promote cell adhesion. The small sub-micron magnetic particles embedded within the alginate core serve as a way to simplify culture manipulations. The cells are propagated using a simple, small, benchtop cell culture chamber. Since the cells grow on the microcarriers, trypsinization is not needed to split and maintain cultures. Magnets are used to pull the microcarriers out of suspension during media changes. When cells are needed for downstream applications, an aliquot of the culture is simply aspirated from the tube used to grow the cells. As the alginate core is optically clear and non-autofluorescent, cells can remain on the microcarrier during the assay process. The assay was validated with each culture technique by examining mTORC1 activation through insulin stimulation and inhibition with rapamycin, using the filter-based optical system and tungsten lamp of a multi-mode microplate reader.

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.

3-D Cell Propagation

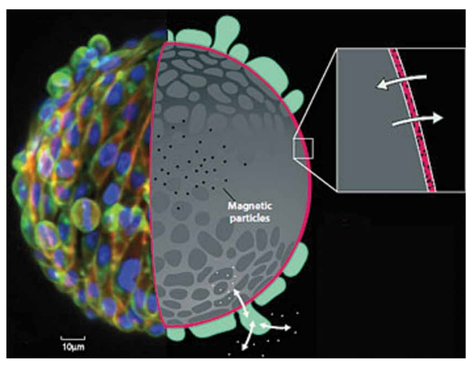


Figure 2 – GEM™ Global Eukaryotic Microcarrier.

The GEM (Global Eukaryotic Microcarrier) is a novel alginate three-dimensional cell microcarrier. The alginate, as an unbranched polysaccharide, possesses a chemical structure similar to components of the extracellular matrix and provides a unique porous surface that mimics *in vivo*-like metabolic function. The GEM is non-autofluorescent and optically clear, allowing for cell assay directly on the substrate, and also contains paramagnetic particles allowing for easy manipulation of cells on the microcarrier. The GEM is coated with a molecular layer of a protein coating allowing for attachment of unique cell types.

Figure 3 – BioLevitator™ Cell Culture Chamber

The BioLevitator is a benchtop hybrid of an incubator and bioreactor. The BioLevitator demonstrates the convenience of three-dimensional GEM culture with four independently controlled temperature and CO_2-regulated cell culture vessels. This unique device controls positioning of the GEM during manual or automated handling using an internal magnet, has a touch screen interface that allows for easy data monitoring and collection, allows for increased 'walk-away' time by minimizing the number of manual intervening steps, and finally is easily integrated into a fully automated cell system.

AlphaScreen *SureFire* p-p70 S6 Kinase (Thr389) Assay

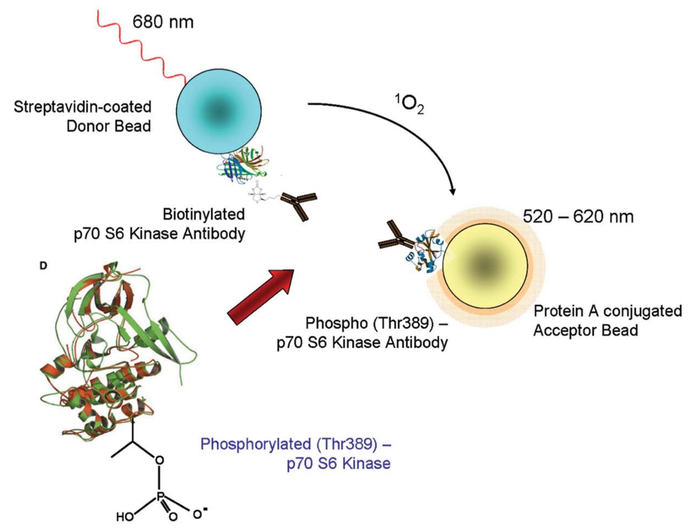
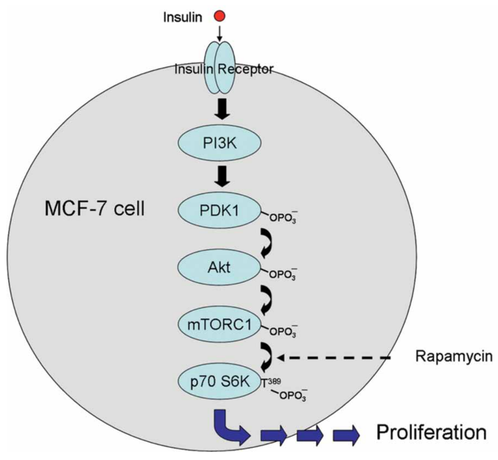


Figure 4 – Representation of AlphaScreen SureFire p-p70 S6 Kinase(Thr389) Assay.

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 four 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.

Figure 5 – 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.



Cell Culture Procedure

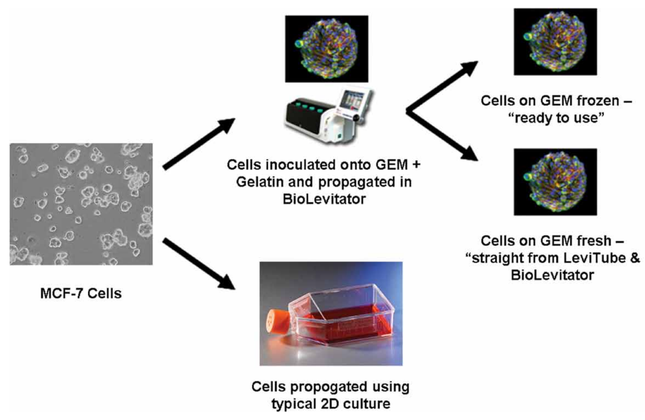


Figure 6 – MCF-7 Cell Propagation Process.

Assay Procedure

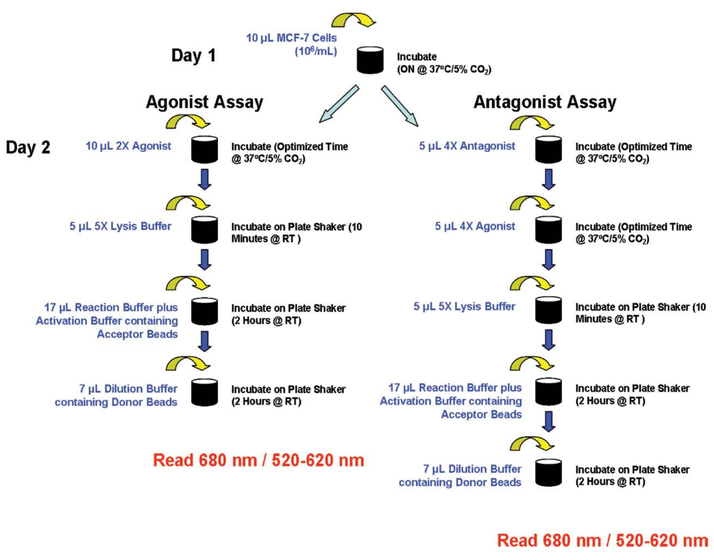


Figure 7 – AlphaScreen SureFire p-p70 S6 kinase assay protocol.

Assay Optimization

Insulin was added to 2D cultured MCF-7 cells, as well as freshly propagating and frozen MCF-7 cells on the GEM substrate, at a 1X concentration of 0 µM and 10 µM. The assay plate was then incubated at 37°C/5% CO_2 for 30 minutes. The AlphaScreen assay was then carried out as explained previously. A fold difference ranging from 10-15 was seen between no cell background subtracted values for each cell condition tested.

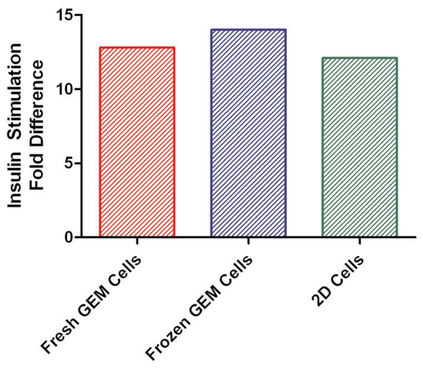


Figure 8 – Assay window between 0 µM and 10 µM insulin.

An insulin titration was performed in order to validate proper detection of the activation of the insulin receptor with each cell condition. The EC_{50} values determined were then used to determine the EC_{80} concentrations of insulin to use with Rapamycin inhibition studies.

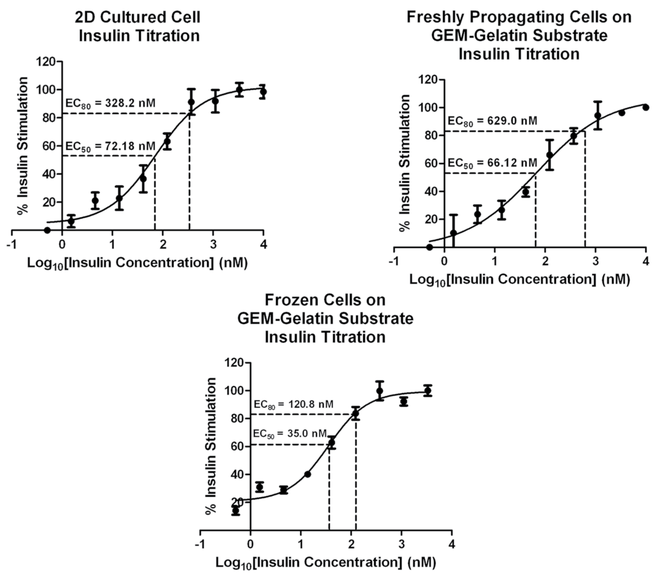


Figure 9 – Insulin titration curve. Serial 1:3 titrations tested between 10,000 – 0 nM insulin.

Insulin Agonist/Antagonist Verification

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 IC_{50} value.

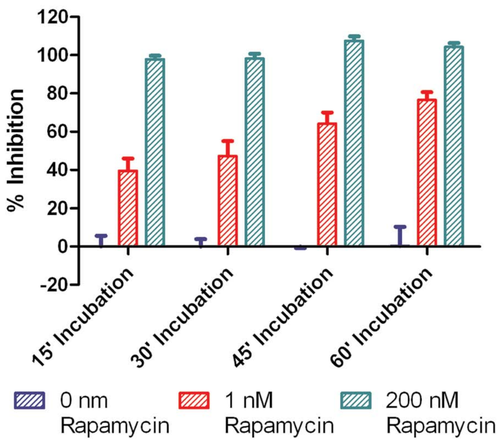


Figure 10 – Rapamycin incubation test results.

2D cultured MCF-7 cells, as well as freshly propagating and frozen MCF-7 cells on the GEM substrate, were dispensed to the assay plates (10 µL) at a concentration of 10^6 cells/mL and incubated overnight at 37°C/5% CO_2.

Rapamycin was serially titrated 1:3 to create a 12 point dilution series. 5 µL from each point on the titration was then added to the plate in quadruplicate. Final 1X concentrations ranged from 200-0 nM. Following the 30 minute incubation period, 5 µL of the appropriate 4X EC_{80} insulin concentration was added to each well. The AlphaScreen assay was then carried out as explained previously.

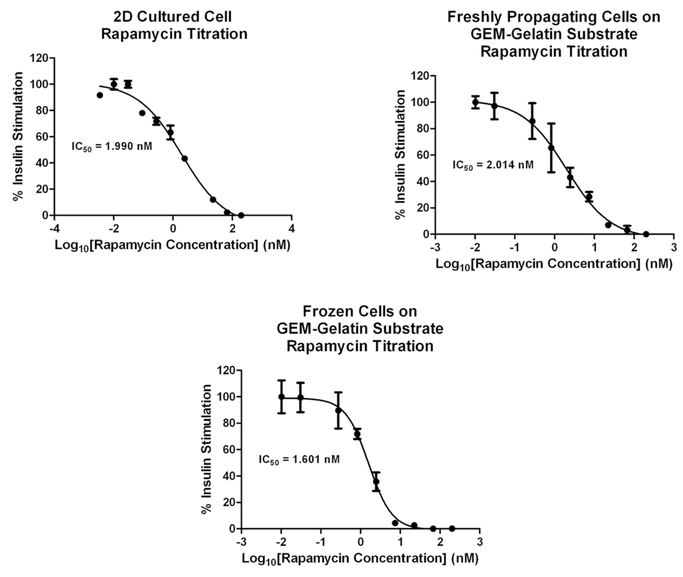


Figure 11 – Rapamycin titration using 1X reagents.

Conclusions

1. The AlphaScreen *SureFire* p-p70 S6 Kinase (Thr389) Assay provides the ability to monitor activity of the insulin receptor cell signaling pathway.
2. Cells grown and assayed on the GEM substrate provide a simplified process for cell propagation, as well as equivalent pharmacology data, when compared to traditional 2D cultured cells.
3. The tungsten halogen lamp on the Synergy H4 provides a flexible, yet robust way to provide the proper excitation required for AlphaScreen assays.
4. 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.
5. The combination of assay, GEM substrate, and instrument provide a unique capability to measure the activity of the mTOR protein kinase.

[1]Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer*. 2004;4:335-348. [2]Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006;124:471-484. [3]Pouysségur J, Dayan F, Mazure N. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature*. 2006;441:437-443. [4]Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 2006;441:424-430. [5]Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Disc*. 2006;5:671-688.