

# Cellomics<sup>®</sup> Phospho-mTOR Activation Kit

## High-Content Screening Reagents

2232.0

Number	Description
8408302	<b>Phospho-mTOR Activation Kit</b> , sufficient materials for 5 × 96 wells
8408303	<b>Phospho-mTOR Activation Kit</b> , sufficient materials for 50 × 96 wells

Kit Contents:	8408302	8408303
Phospho-mTOR Primary Antibody	120 µl	1.2 ml
DyLight™ 549 Conjugated Goat Anti-Rabbit IgG	72 µl	1 ml
Hoechst Dye	30 µl	165 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml	-
Wash Buffer II (10X Dulbecco's PBS with Tween <sup>®</sup> -20)	100 ml	-
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton <sup>®</sup> X-100)	100 ml	-
Thin Plate Seal Assembly	7/pack	-

**Storage:** Upon receipt immediately store the Phospho-mTOR Primary Antibody at -20°C. Store all other components at 4°C. Keep vials containing the fluorescent antibody and Hoechst Dye solutions protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

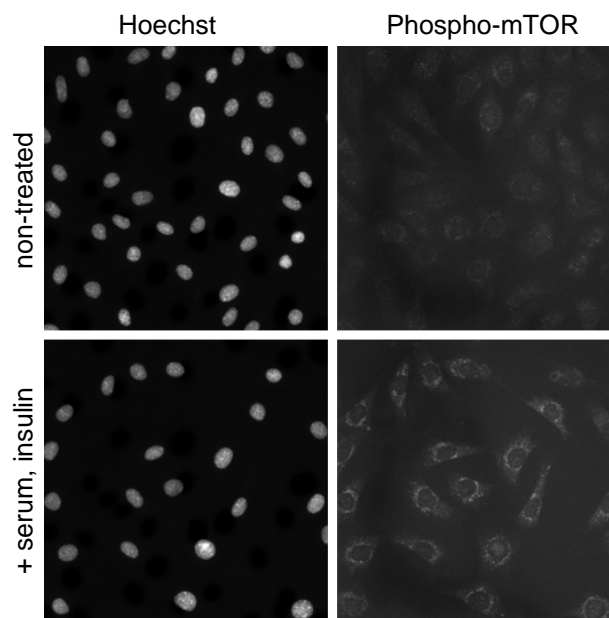
**Warning:** Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

## Introduction

The Thermo Scientific Cellomics Phospho-mTOR Kit measures phosphorylation of mTOR, a kinase involved in the initiation of ribosome biogenesis and translation. The kit contains a monoclonal rabbit anti-phospho-mTOR antibody, a goat anti-rabbit secondary antibody conjugated to Thermo Scientific DyLight 549 Fluorophore and various other reagents and buffers required for immunofluorescence detection of mTOR for high-content screening (HCS) assays.

The mammalian target of rapamycin (mTOR) is a protein kinase that regulates the initiation of protein translation, significantly influencing cell growth. Consequently, mTOR is responsive to a diversity of cellular signals including growth factors, nutrients and the cellular metabolic state. Two of the major signaling pathways that regulate mTOR activation are the PI-3 kinase through RTK signal transduction and AMPK, an energy sensing pathway. Under favorable growth conditions, the negative regulators of mTOR, TSC1 and 2 are phosphorylated by AKT, allowing mTOR to dissociate from the TSC complex for activation. mTOR is then phosphorylated, and through phosphorylation activates S6 kinase and dissociates 4E-BP-1 from the cap complex to promote protein translation. Cancer cells are often mutated in the PI-3 kinase and mTOR pathways to promote constitutive cell growth, and members of this pathway are attractive targets for anti-cancer drugs.

Phospho-mTOR was activated after a brief stimulation with serum and insulin in serum-starved or rapamycin-treated C2C12 muscle cells resulting in an increase in cytosolic intensity, visualized as spots in the perinuclear region of the cell (Figure 1). The phospho-mTOR assay was optimized with the Thermo Scientific ArrayScan Reader using the Compartmental Analysis BioApplication Software Module. Phospho-mTOR can be quantitated using the cytosolic intensity or the cytosolic spot intensity. Cells stained using this kit also can be imaged using fluorescence or confocal microscopy.



**Figure 1. Activation of phospho-mTOR after serum stimulation in C2C12 muscle cells.** Cells were serum-starved for 24 hours, followed by 15 minute stimulation with 10% serum and 50 ng/ml insulin. Cells were then fixed and stained according to the kit protocol. Cells were labeled with Hoechst 33342 dye for nuclear staining and imaged using the ArrayScan V<sup>TI</sup> HCS Reader.

## Additional Materials Required

- Insulin (e.g., Sigma, Product No. I-1882)
- Formaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Collagen-I coated 96-well microplates (e.g., BD Biocoat<sup>®</sup> Plates, Product No. 354407)
- Fetal bovine serum (FBS)
- Hank's Balanced Salt Solution (-Ca, -Mg) (e.g., Thermo Scientific HyClone, Product No. SH30031.02)

## Cell Preparation Information

- This protocol is optimized for C2C12 muscle cells (American Type Culture Collection, Product No. CRL-1772). IMR-90, NIH 3T3 and HeLa cells have also been used successfully. Using other cell lines might require optimization.
- For routine culture of C2C12 cells use DMEM complete medium (HyClone, Product No. SH30088) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin.
- Dilute cells when they reach 70-80% confluence (2-3 times per week) at a ratio of 1:8-1:10. Use cells at a passage number ≤ 12. Cells will differentiate if allowed to become over-confluent and may be more variable in response if later than passage 12.
- For phospho-mTOR activation, cells must be shifted into medium without serum or growth factors.
- Forty-eight hours before performing the assay, harvest cells by trypsinization, dilute into DMEM Complete Medium and determine cell density. Dilute cells to  $2.5-4 \times 10^4$  cells/ml with DMEM Complete Medium and add 100 µl of the cell suspension per well of a 96-well microplate (recommended plating density = 2,500-4,000 cells/well).
- Grow cells for 16-20 hours at 37°C in 5% CO<sub>2</sub>. Gently aspirate media to dryness, and wash two times with HBSS. Aspirate and replace solution with 100 µl/well of DMEM medium without serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Incubate cells for 20-24 hours at 37°C in 5% CO<sub>2</sub>.

**Note:** Some cell lines may require 48 hours of serum-starvation before stimulation. Prepare cells as indicated above except serum-starve for 48 hours before adding serum and insulin to the media. For C2C12 cells, 48 hours of serum-starvation will also induce autophagy.

## Alternate Cell Preparation

**Note:** As a replacement for serum starvation, cells may be treated with rapamycin before stimulation with serum and insulin.

- Twenty-four hours before performing the assay, harvest cells by trypsinization, dilute into DMEM Complete Medium and determine cell density. Dilute cells to  $2.5-4 \times 10^4$  cells/ml with DMEM Complete Medium and add 100  $\mu$ l of the cell suspension per well of a 96-well microplate (recommended plating density = 2,500-4,000 cells/well).
- Grow cells for 16-20 hours at 37°C in 5% CO<sub>2</sub>. Gently aspirate media to dryness, and wash two times with HBSS. Aspirate and replace solution with 100  $\mu$ l/well of DMEM medium without serum containing 250 nM rapamycin, 100 units/ml of penicillin and 100  $\mu$ g/ml of streptomycin. Incubate cells for 60-90 minutes at 37°C in 5% CO<sub>2</sub>.
- Continue with stimulation as described in the Procedure Section.

## Phospho-mTOR Activation Kit Protocol

### A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 20 ml 10X Wash Buffer II to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 9 ml of 16% formaldehyde to 3 ml of 1X Wash Buffer just before use.
Blocking Buffer	Add 2.5 ml of FBS to 47.5 ml of 1X Wash Buffer to make 50 ml of Blocking Buffer (5% FBS Blocking Buffer). Prepare just before each assay.
Primary and Secondary Antibody Dilution Buffer	Add 60 $\mu$ l of FBS to 12 ml 1X Wash Buffer (0.5% FBS dilution buffer). Prepare just before each assay.
1X Permeabilization Buffer	Add 10 ml of 10X Permeabilization Buffer to 90 ml of ultrapure water for a final volume of 100 ml. Store buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 24 $\mu$ l of Phospho-mTOR Primary Antibody to 6 ml of Primary and Secondary Antibody Dilution Buffer. Prepare just before each assay.
Secondary Antibody Staining Solution	Add 0.6 $\mu$ l of Hoechst Dye and 12 $\mu$ l of the DyLight 549 Goat Anti-Rabbit to 6 ml Primary and Secondary Antibody Dilution Buffer. Prepare solution just before each assay.

### B. Procedure

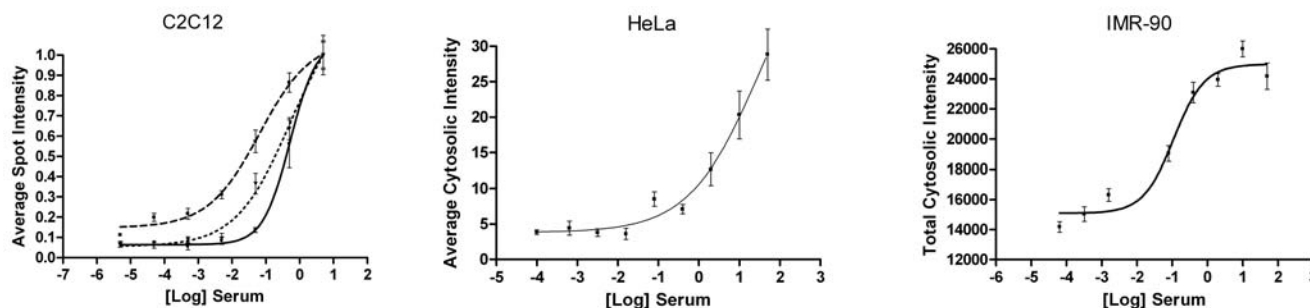
1. Prepare a 2X stimulation solution by adding 2 ml of FBS and 100 ng/ml of insulin to 8 ml of DMEM no-serum media. Add 100  $\mu$ l/well to the treated side of the plate and 100  $\mu$ l/well of DMEM-no serum media to the control side of the plate.
2. Incubate plate for 15 minutes at 37°C.
3. Gently overlay 100  $\mu$ l of Fixation Solution to each well. Incubate plate in a fume hood at room temperature (RT) for 25 minutes.
4. Aspirate Fixation Solution completely and wash plate twice with 100  $\mu$ l/well of 1X Wash Buffer.
5. Aspirate Wash Buffer completely, add 100  $\mu$ l/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.
6. Aspirate Permeabilization Buffer and wash plate twice with 100  $\mu$ l/well of 1X Wash Buffer.
7. Aspirate Wash Buffer, add 100  $\mu$ l/well of Blocking Buffer and incubate at RT for 15 minutes.
8. Aspirate Blocking Buffer, add 50  $\mu$ l/well of Primary Antibody Solution and incubate for 1 hour at RT.
9. Aspirate Primary Antibody Solution and wash with 100  $\mu$ l/well of 1X Wash Buffer II.
10. Aspirate Wash Buffer II and wash twice with 100  $\mu$ l/well of 1X Wash Buffer.
11. Aspirate Wash Buffer, add 100  $\mu$ l/well of Blocking Buffer and incubate for 15 minutes at RT.
12. Aspirate Blocking Buffer, add 50  $\mu$ l/well of staining solution containing the secondary antibody and incubate at RT for 45 minutes.
13. Aspirate Staining Solution and wash plate twice with 100  $\mu$ l/well of 1X Wash Buffer II.

14. Aspirate Wash Buffer II and wash plate twice with 100  $\mu$ l/well of Wash Buffer.
15. Aspirate Wash Buffer and replace with 150  $\mu$ l/well of Wash Buffer.
16. Seal plate and evaluate on the ArrayScan Reader. Store plates at 4°C.

## Additional Information

### A. Dose-response Curves

Activation of C2C12, HeLa and IMR-90 cells was measured in response to treatment with serum  $\pm$  insulin for 15 minutes after 24 (or 48 for HeLa and IMR-90) hour serum-starvation (Figure 2). The average cytosolic spot, average cytosolic or total cytosolic intensities were used for quantitation.



**Figure 2. Dose-response curves of phospho-mTOR in cells treated with serum after serum-starvation.** C2C12 cells were treated for 15 minutes with serum and insulin.  $EC_{50} = 0.37 \pm 0.27\%$  serum and  $0.037 \pm 0.027 \mu\text{g/ml}$  insulin. HeLa cells were treated for 15 minutes with serum. IMR-90 cells were treated for 15 minutes with serum.  $EC_{50} = 0.1\%$  serum. Each data point represents eight wells. C2C12 curves represent separate data from three replicate plates. Single curves represent data from a single plate. Serum concentrations were derived from serial dilutions of five- or ten-fold, where 2 = 100%, and 1 = 10%.

### B. Performance Robustness

Assay robustness was ascertained by determining the  $Z'$  for the difference in average cytosolic spot intensities in non-treated cells (min, minus serum) and treated cells (max, plus serum, insulin) for 15 minutes. The mean  $\pm$  SD of the  $Z'$  factor was determined from three plates of C2C12 cells that were treated identically and was  $0.26 \pm 0.12$ , with a coefficient of variation of  $13.3\% \pm 5.6$ .

DMSO tolerance: The assay performance using these kits was robust when compounds were added in DMSO up to a maximum concentration of 1% DMSO.

### C. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

DyLight 549 Conjugates = 550/568 nm

Hoechst Dye = 350/461 nm

### D. Recommendations for Automation

- **Plating Cells:** To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop Combi or WellMate Dispensers.
- **Dead Volumes:** Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- **Nonspecific Binding:** Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- **Mixing:** Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.

- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time that plates with live cells are out of a controlled CO<sub>2</sub> environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.
- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

**Table 1.** Suggested volumes to use for different cell culture plates.

<u>Kit Component</u>	<u>96-Well Plates</u>	<u>384-Well Plates</u>	<u>24-Well Plates</u>
	<u>(<math>\mu</math>l/well)</u>	<u>(<math>\mu</math>l/well)</u>	<u>(<math>\mu</math>l/well)</u>
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
Wash Buffer II	100	25	400
1X Permeabilization Buffer	100	25	400
Blocking Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

## Compatible BioApplication Software Modules

S50-5019-1 or S50-2019-1      **Molecular Translocation BioApplication**

S50-5011-1 or S50-2011-1      **Target Detection BioApplication**

S50-5017-1 or S50-2017-1      **Compartmental Analysis BioApplication**

## References

- Petroulakis, E., *et al.* (2006). mTOR signaling: implications for cancer and anticancer therapy. *Br. J. Cancer* **94**: 195-9.
- Sarbassov, D.D., *et al.* (2005). Growing roles for the mTOR pathway. *Current Opin Cell Biol.* **17**: 596-603.
- Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.
- Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4**:67-73.

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