



FlowCelect™
PI3K/MAPK Dual Pathway Activation
and Cancer Marker Detection kit

25 Tests

Cat. No. FCCS025100

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Application

Examination of cell signaling pathways and monitoring their activation status have been extremely important for researchers to understand the detailed mechanisms of cellular functions and the cause of various diseases. Many signal transduction pathways have been implicated to lead to multiple outcomes such as apoptosis, cell differentiation, cell growth and cell proliferation, all of which have been extensively studied for the treatment of various cancers and autoimmune diseases.

The study of cell signaling pathways has been made easier with the use of activation status-specific and phospho-specific antibodies. Measurement of protein phosphorylation with phospho-specific antibodies has given insight into kinase signaling cascades [1]. Multi-parameter phospho flow cytometry is a powerful tool for studying multiple pathways in a mixed cell population at the same time.

Much excitement in the field of signal transduction has centered on the discovery of increasing cross-talk among signaling pathways [2]. Recent evidence has suggested that communication between the PI3K and MAPK pathways exist downstream from the cell surface [2,3,4]. The ability for signaling pathways to cross-talk adds an extra dimension and complexity when evaluating pathways of interest. Since signal transduction pathways are an elaborate highway of events, the ability to monitor these key intracellular “checkpoints” simultaneously provides researchers a very powerful tool for analyzing complicated cell events such as cancer cell proliferation by measuring the activity of multiple cell signaling pathways.

Millipore’s FlowCelect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit is designed to allow the researcher to cross examine both the PI3K and MAPK signaling pathways simultaneously, as well as cell proliferation. This kit provides three directly conjugated antibodies which are optimized for multi-color flow cytometry applications for the detection of Akt phosphorylation, ERK1/2 phosphorylation, and Ki-67 cancer marker expression. Ki-67 has been indicated to be a reliable tumor proliferative marker in cancer cells [5]. The fraction of Ki-67 positive cells, often defined as the “proliferative fraction”, has prognostic value in many tumors [6].

Although the use of phospho-specific antibody staining as a biomarker may give some measure of target activation, it may not necessarily correlate with the desired biological effect (e.g. growth inhibition or apoptosis). Researchers would likely benefit from the inclusion of both phospho-specific signal transduction markers (such as pERK and pAkt) and a proliferation marker (such as Ki-67) to allow a true measure of treatment evaluation at the level of the tumor [7].

All FlowCelect™ kits are optimized on the bench-top Guava® flow cytometry systems, which saves valuable time and sample volume. All kits contain optimized fixation, permeabilization, wash and flow buffers to provide researchers with a complete solution for simultaneous detection of multiple pathway activations. With the Guava platform and FlowCelect™ kits, researchers can finally have an easy, reliable and fully validated solution to study the complex cell signaling pathways right in the comfort of their own lab.

Test Principle

Recent evidence suggests that there is cross-talk between the PI3K and the MAPK signaling pathways. Antibodies against phosphorylated Akt (pAkt) and phosphorylated ERK (pERK) can be used to examine PI3K/MAPK interactions. Additionally, to interrogate the interaction between these two pathways, the cell proliferation marker, Ki-67, can be used to validate the final biological effect.

Millipore's FlowCelect™ PI3K/MAPK Dual Activation and Cancer Detection Kit is designed to examine this cross-talk in a multi-parametric fashion by providing three fully validated and optimized antibody biomarkers to measure specific cell signaling events in flow applications. The three antibodies provided in the kit are Anti-phospho-Akt Alexa Fluor 488 conjugate, Anti-phospho-ERK R-Phycoerythrin conjugate, and Anti-Ki-67 PerCP conjugate. By utilizing all three antibody biomarkers simultaneously in flow applications, researchers now have the ability to thoroughly evaluate the "cross-talk" between PI3K and MAPK pathways, and further, by measuring their effect on Ki-67 expression, to determine the consequence of their interplay in cell proliferation and differentiation.

Anti-phospho-Akt and Anti-phospho-ERK1/2 antibodies are provided in the kit to measure cell-based PI3K and MAPK activity. It has been suggested that the phosphorylation of Akt can result in the inhibition, or dephosphorylation, of phospho-Raf on Ser 259 [2]. By inactivating Raf, this will essentially block the MAPK signaling pathway resulting in an inactivated phospho-ERK. In some situations, a surface receptor (such as IGF-1) will activate both the PI3K and MAPK pathways leading to the phosphorylation of both Akt and ERK. However, since there is cross-talk between the two pathways, it is critical to investigate their interactions in both a spatial and temporal manner.

In a recent study, we have examined the effects of IGF-1 activation by the addition of Insulin on both the PI3K and MAPK signaling pathways on HEK293 cells. An experiment was performed while implementing critical time points for signaling evaluation: activation at 3 minute and at 5 minute time intervals. The resulting responses indicate that cross-talk is observed, noted by a sharp decrease in ERK expression. This transient response is attributed to the phosphorylation of Akt, which in turn will shut off phosphorylated ERK as noted above.

In order to validate the biological effect of phospho-antibody activation by a given stimulus, cell cycle marker Ki-67 is used since it is a true measurement of the "proliferative fraction". Ki-67 is present in all phases of the cell cycle except for G₀. However, Ki-67 can only be detected in flow cytometry when cells are going through M phase as Ki-67 expression patterns are punctate in all other phases producing weaker signals. But by using a cell cycle arrest reagent, Cell Cycle Stop™, cell proliferation measured by Ki-67 expression can be accurately determined as cells are arrested at M phase and are clearly visible in flow cytometry analysis. In order to accurately measure the cell proliferative activity by Ki-67 expression, however, cells must be treated for at least 12 hours with a combination of Cell Cycle Stop™ and a given cell stimulus to fully achieve enough circulating cells to be captured in M-phase. Additionally, prior to cell treatments all cultures must be serum starved for 24 hours to essentially reset the cell cycle and bring most circulating cells back to G₀ [10].

Using multi-parametric flow analysis, researchers are able to cross-examine these signaling events and their biological consequence simultaneously, providing a biological correlation between pathway activation and cancer proliferation.

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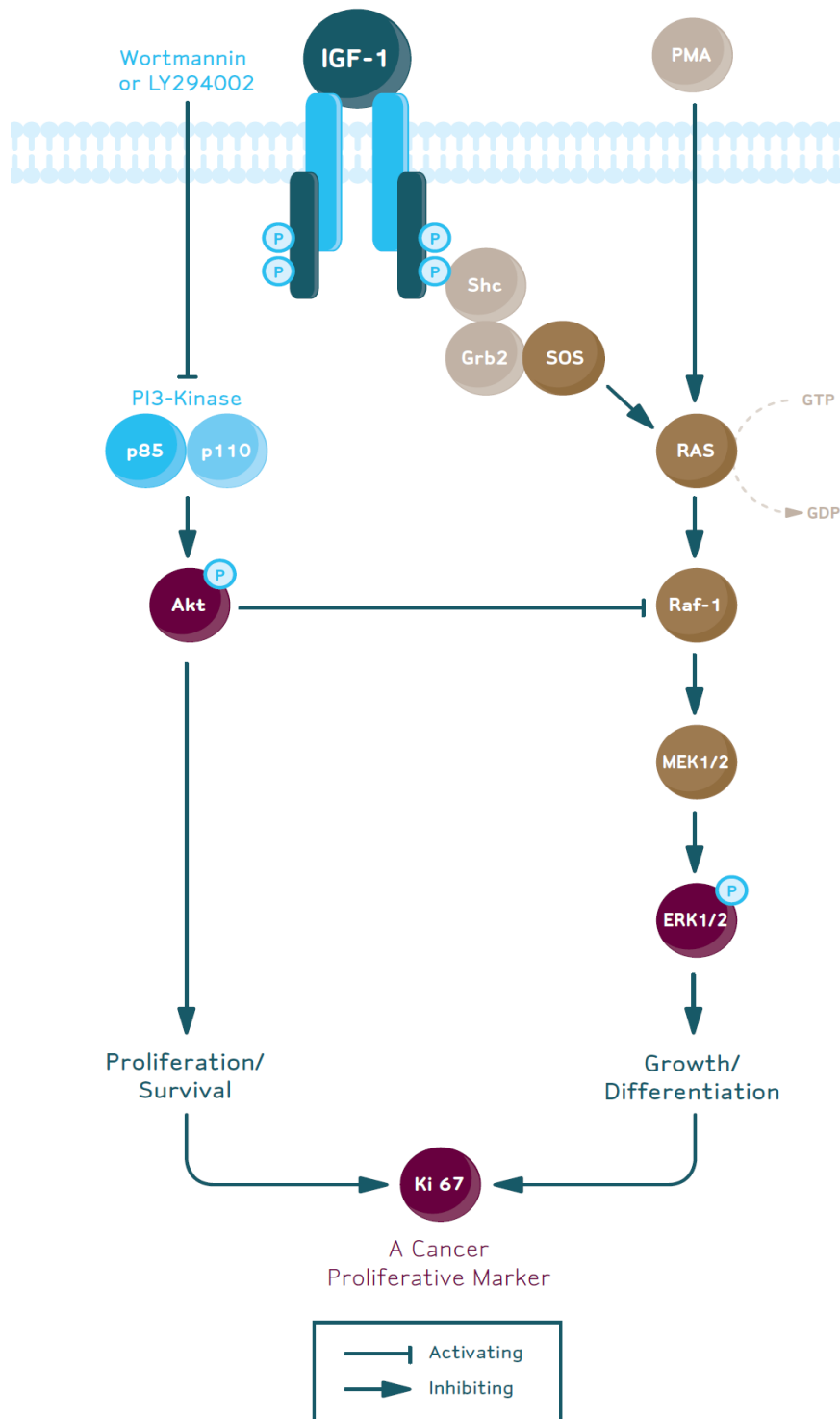
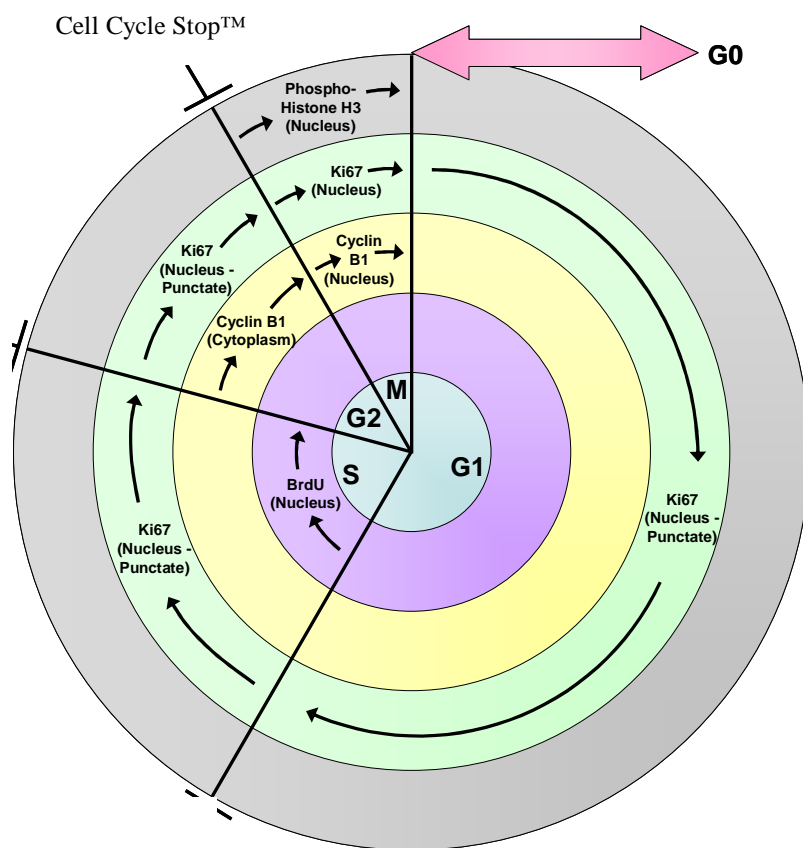


Figure 1. Cross-talk between the PI3K/MAPK signaling pathways Evidence suggests that the PI3K and Ras pathways can intersect by cross-talk among their downstream effectors. Millipore’s FlowCelect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit provides two essential phospho-specific antibodies for the researcher to fully interrogate the relationship between the PI3K and MAPK pathways, phospho-Akt and phospho-ERK1/2, respectively. Additionally, the proliferation marker Ki-67 provides the assurance of confirming the biological effect.

Figure 2. Measuring Ki-67 Expression in the Cell Cycle by using Cell Cycle Stop™ Ki-67 expression is evident throughout the cell cycle. However, in G1/S/G2 phases of the cycle Ki-67 is punctate, making it difficult to visualize in flow applications due to the weaker intensity. By treating cells overnight with cell cycle arrest reagent Cell Cycle Stop™, all proliferating cells can be properly measured in M-phase as a result of intense Ki-67 expression.



Kit Components

1. 20X Anti-phospho-Erk1/2 (Thr202/Tyr204, Thr185/Tyr187)- R-Phycoerythrin conjugated Monoclonal Antibody: (Part No. CS203329) One vial containing 150 μ L antibody.
2. 20X Anti-phospho-Akt1/PKB α (Ser473)- Alexa Fluor 488 conjugated Monoclonal Antibody: (Part No. CS203310) One vial containing 150 μ L antibody.
3. 20X Anti-KI-67- PerCP conjugated Monoclonal Antibody: (Part No. CS203324) One vial containing 150 μ L antibody.
4. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer.
5. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer.
6. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer.
7. 1X Permeabilization Buffer: (Part No. CS202125) One bottle containing 13 mL buffer.
8. Cell Cycle Stop™: (Part No. CS203335) One vial containing 1 mg lyophilized material.

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS w/o Ca²⁺ or Mg²⁺, cell dislodging buffers, etc.
3. PMA or other cell treatment compounds
4. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
5. Tabletop centrifuge capable of exceeding 400 x g
6. Mechanical vortex
7. Flow Cytometry instrument capable of performing single cell analysis
8. Deionized water (for Buffer dilutions)
9. Dimethyl sulfoxide (DMSO)

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Kit contains a paraformaldehyde solution (fixation solution). Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, the product supplied in vials may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.

Storage

This kit is shipped and must be stored at 2 - 8°C. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8°C or at room temperature upon receipt. **Caution:** Fluorochrome conjugated antibodies should always be stored at 2 - 8°C and stored in the dark.

All kit components are stable up to four (4) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. Wash Buffer

Wash buffer is supplied at 10X concentration and should be diluted to 1X prior to use. Add 1 mL of the 10X wash buffer to an appropriate container, and adjust volume to 10 mL with deionized water. Mix thoroughly. Prepared 1X wash buffer is stable up to one year. Store at 2 - 8°C.

Note: 10X Wash Buffer can be stored at RT to avoid any potential precipitate formation

2. Assay Buffer

Add 50 mL of 5X Assay Buffer to an appropriate container, and adjust volume to 250 mL with deionized water. Stir to homogeneity. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8°C.

3. Cell Cycle Stop™

Reconstitute each vial with 133 µL DMSO. Mix thoroughly to ensure that lyophilized material is completely dissolved. Store at -20°C. To avoid multiple freeze/thaw cycles, it is recommended to aliquot material into single use vials immediately upon reconstitution.

Preparation of Test Cell Samples

This kit is designed to cross examine both the PI3K and MAPK signaling pathways simultaneously, as well as cell proliferation on any cell line chosen by the end user. Below are the guidelines for preparing cells for antibody staining.

I. Measuring the activation or inhibition of Akt and ERK signaling pathways

Prior to performing any cell treatments, serum starvation for 24 hours is recommended as it may enhance the activity of the provided biomarkers on most cell types. Although this step may be cell line dependent, it has been demonstrated that cell cycle marker Ki-67 expression can be more accurately measured since starvation will reset the cell cycle back to G₀ phase.

For adherent cells:

1. Aspirate growth media from tissue culture flask and wash cell monolayer using PBS or HBSS to wash away any residual growth media.
2. Obtain cell culture by gentle harvesting using a mild enzyme such as trypsin or an EDTA-based cell dissociation buffer.
3. Gently tap the side of the flask to dislodge cell monolayer and add 1X PBS to collect the cells. Place cell suspension into a centrifuge collection tube. Determine cell numbers using a hemacytometer. Also note cell viability.
4. Divide desired amount of cells into two separate centrifuge tubes: One tube will serve as the “treated” sample (experimental) and the second tube will function as the “untreated” sample (control). Typical sample sizes range from 500,000 to 1 million cells.

5. Place centrifuge tubes into a 4°C tabletop centrifuge and spin samples for 5 minutes at 400 x g.
6. Aspirate supernatant and discard, saving only the cell pellets.
7. Using a pipette, gently disrupt and mix the cell pellets in 1X Wash Buffer to ensure complete homogeneity. Buffer volumes will depend on the end user's preference, however, typically 250 µL of buffer will be sufficient for 1 million cells.
8. **Cell treatment:** To be performed at the end user's own discretion. If you are investigating the activation or inhibition of a given signaling pathway by administering a small molecule or drug, this can be performed at this step. *(For example, if you are investigating the effects of PMA stimulation on Jurkat cells, treat one centrifuge tube containing a cell suspension with 100ng/mL of PMA for 5 minutes at 37°C. The other centrifuge tube will serve as the untreated negative control).*
9. Immediately following cell treatment, add an equal volume of Fixation Buffer to cell samples. For example, if the sample contains 3 million cells in 750 µL of 1X Wash Buffer, add 750 µL of Fixation Buffer to the centrifuge tube. Mix well to ensure complete homogeneity. Allow sample(s) to incubate on ice for 20 minutes.
10. Following cell fixation, place centrifuge tubes into a 4°C tabletop centrifuge and spin down the samples for 5 minutes at 400 x g.
11. Remove the samples from centrifuge and discard supernatant, saving only the pellets.
12. For every 1 million cells, add 200 µL 1X Permeabilization Buffer to each tube and gently resuspend cells to ensure complete homogeneity. Allow to incubate on ice for 5 minutes.
13. Immediately after cell permeabilization, perform a washing step by placing each centrifuge tube into a 4°C tabletop centrifuge and spin down samples for 5 minutes at 400 x g.
14. Aspirate supernatant and discard, saving only the cell pellets. Resuspend in 1X Assay Buffer (500 µL per million cells should be sufficient for proper cell washing). Repeat steps 5 and 6 to completely remove any residual 1X Permeabilization Buffer from the cell pellets.
Caution: *Depending on cell type, cells may appear glassy and may be difficult to visualize after fixation/permeabilization. Be extra cautious when aspirating supernatant, making sure to keep the cell pellet intact. Additionally, cell washing can lead to some cell loss. Be careful when performing all washing steps to ensure proper cell numbers prior to assay.*
15. Save the cell pellets in preparation for cellular staining and analysis.

For suspension cells:

1. Obtain cell culture by gently pipetting cell suspension up and down to ensure complete homogeneity.
2. Remove a small sample from the tissue culture flask, and using a hemacytometer count and determine cell numbers. Also note cell viability.
3. Repeat steps 4 - 15 as noted above (In the "For adherent cells" section)

II. Monitoring cell proliferation by measuring Ki-67 expression

As indicated previously, accurately measuring Ki-67 expression will require a minimum of 24 hours serum starvation prior to cell treatment. Additionally, during serum starvation cells will need to be treated with the provided Cell Cycle Stop™ reagent overnight to ensure that proliferating cells can be captured post stimulation.

For adherent and suspension cells:

1. Sixteen to 20 hours prior to cell staining, treat cell culture with Cell Cycle Stop™ reagent. Overnight treatment with Cell Cycle Stop™ is sufficient for a next morning assay. It is recommended to have the cell density at 1 million cells per milliliter (suspension cells), or have the tissue culture flask approximately 50% confluent (for adherent cells).

Note: *It is critical that cell cultures are **not** over-confluent during the time of assay; otherwise, accurate measurements of cell cycle activity cannot be properly achieved.*

2. For every 1 million cells add 4 µL of Cell Cycle Stop™ reagent to growth media.
3. Next, treat cells with desired stimulant. This step will be performed at the end user's own discretion. For example, if you are investigating the proliferative effects of adding a compound stimulant such as Insulin on HEK293 cells, treat the cells with 100 nM Insulin and allow incubation at 37°C overnight.

4. If testing suspension cells where cell harvesting is not required, skip to step #7.

For adherent cells, the following morning (approximately 16 to 20 hours from cell treatment), aspirate growth media from tissue culture flask and wash cell monolayer using PBS or HBSS to wash away any residual growth media (adherent cells).

5. Obtain cell culture by gentle harvesting using a mild enzyme such as trypsin or an EDTA-based cell dissociation buffer.
 6. Gently tap the side of the flask to dislodge cell monolayer and add 1X PBS to collect the cells.
 7. Place centrifuge tube into a 4°C tabletop centrifuge and spin sample for 5 minutes at 400 x g.
 8. Aspirate supernatant and discard, saving only the cell pellet(s). Resuspend the pellet(s) in 1X Wash Buffer to ensure complete homogeneity. Buffer volumes will depend on the end user's preference; however, typically 250 µL of buffer will be sufficient for 1 million cells.
 9. Add equal amounts of Fixation Buffer to cell mixture. Mix gently to ensure complete homogeneity. For example, if 4 million cells are in 1 mL of Wash Buffer, add 1 mL of Fixation Buffer for a final of 2 mLs. Allow to incubate on ice for 20 minutes.
 10. Following cell fixation, place centrifuge tubes into a 4°C tabletop centrifuge and spin down the samples for 5 minutes at 400 x g.
 11. Remove the sample from centrifuge and discard supernatant, saving the pellets.
 12. For every 1 million cells, add 200 µL 1X Permeabilization Buffer to each tube and gently resuspend cells to ensure complete homogeneity. Allow to incubate on ice for 5 minutes.
 13. Place centrifuge tube into a 4°C tabletop centrifuge and spin sample for 5 minutes at 400 x g.
 14. Remove the sample from centrifuge, discard supernatant, and resuspend the cell pellets in 1X Assay Buffer. Again, 500 µL per million cells will be sufficient for proper cell washing and removal of any residual Permeabilization Buffer. Place centrifuge tubes back into a 4°C tabletop centrifuge and spin down the samples for 5 minutes at 400 x g. Repeat cell washing step twice 2X.
- Caution:** *Depending on cell type, cells may appear glassy and may be difficult to visualize after fixation/permeabilization. Be extra cautious when aspirating supernatant, making sure to keep the cell pellet intact. Additionally, cell washing can lead to some cell loss. Be careful when performing all washing steps to ensure proper cell numbers prior to assay.*
15. Save the cell pellets in preparation for cellular staining and analysis.

Assay Instructions

Note: During all steps in the assay procedure, keep all reagents on ice. After preparation of the cell cultures, follow the guidelines listed to ensure proper cell staining for optimal analysis.

1. Make sure that all reagents have been prepared properly prior to use. Concentrated assay components must be diluted to a working 1X solution.
2. Obtain cell pellets: Treated test sample and untreated negative control as described above. Place on ice. Completely resuspend cell pellets by gently adding 100 μ L of 1X Assay Buffer. Arrange sample tubes as follows: **(for a detailed example of the kit application, please refer to the Appendix section)**
3. **Cell Staining:** For every cell sample (treated and untreated), add 5 μ L 20X Anti-phospho-Erk1/2 (Thr202/Tyr204, Thr185/Tyr187) R-Phycoerythrin conjugate Monoclonal Antibody, 5 μ L 20X Anti-phospho-Akt1/PKB α (Ser473) Alexa Fluor 488 conjugate Monoclonal Antibody, and 5 μ L 20X Anti-KI-67 APC conjugate Monoclonal Antibody to 85 μ L of 1X Assay Buffer for a final volume of 100 μ L. Allow to incubate in the dark on ice for one hour.
4. Along with the test samples performed in multiplex, prepare single antibody staining for all three markers: Anti-phospho-Erk1/2 (Thr202/Tyr204, Thr185/Tyr187), Anti-phospho-Akt1/PKB α (Ser473) and Anti-KI-67 by preparing 5 μ L 20X antibody into 95 μ L 1X Assay Buffer for every 250,000 cells. These samples will assist in setting voltages and compensation for the multiplex stained samples. Allow to incubate in the dark on ice for one hour.
*(**Caution: Avoid long term cell incubations with the antibody. Incubations lasting longer than 2 hours can result in non-specific staining and high background for non-treated cells)*
5. After one hour incubation, for every 1 million cells add 500 μ L of 1X Assay Buffer and place test tube(s) containing cell culture into a 4°C tabletop centrifuge and spin for 5 minutes at 400 x g.
6. Aspirate supernatant and discard, saving only the cell pellet.
7. For every 1 million cells, gently add 500 μ L 1X Assay Buffer to disrupt the cell pellet. Pipette sample up and down to ensure complete homogeneity.
8. Replace centrifuge tube back into 4°C tabletop centrifuge and spin for 5 minutes at 400 x g.
9. At the completion of the washing step, resuspend the cell pellet at 0.5×10^6 cells per milliliter of 1X Assay Buffer.
10. Begin cellular analysis

Sample Results

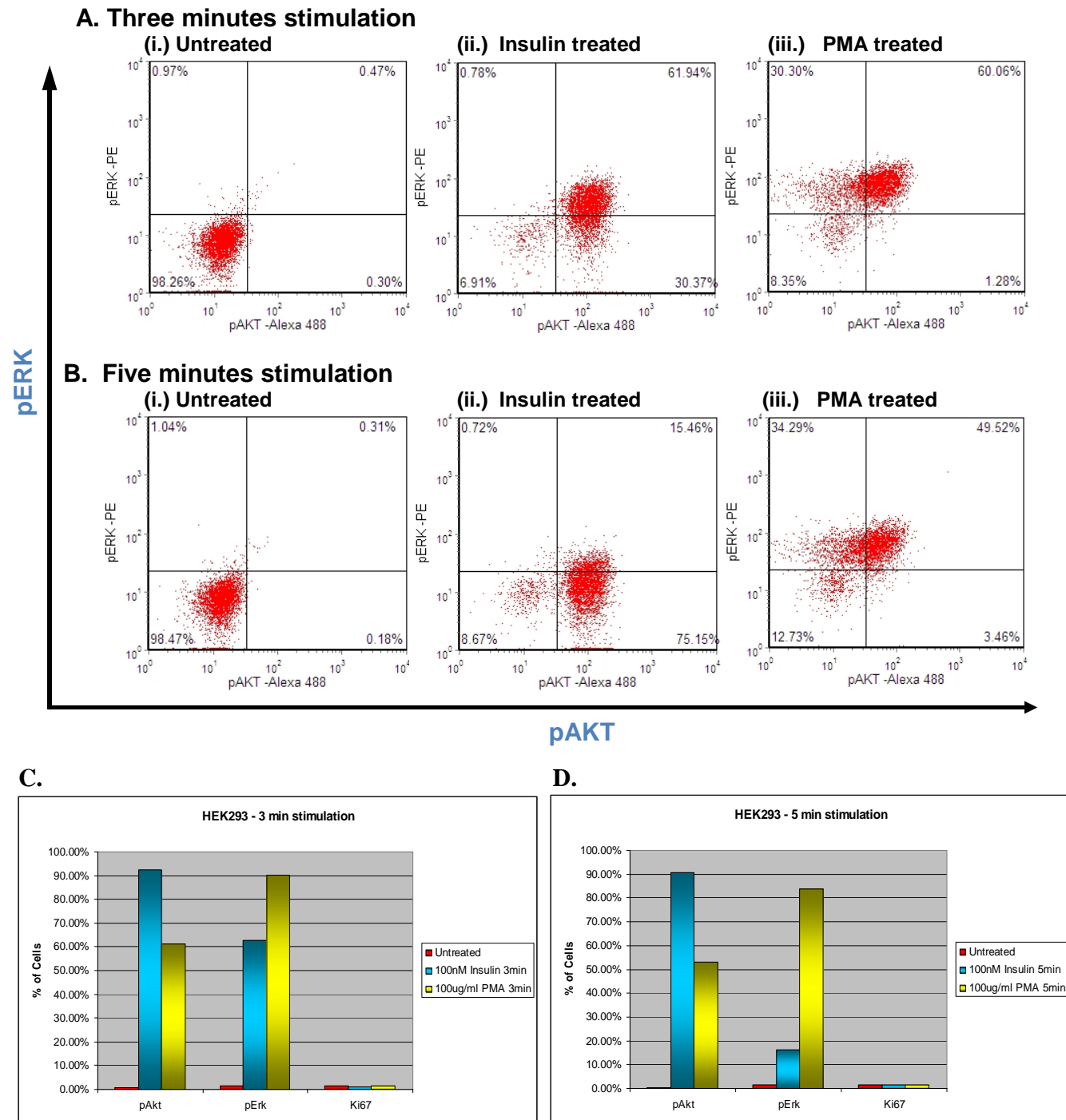


Figure 3. As described in Guan et al. [8], it was shown that cross-talk between the PI3K/Akt and MAPK signaling pathways is evident in HEK293 cells. Above are sample results of HEK293 cells stimulated at 3 minutes and 5 minutes by Insulin and PMA as shown in A and B, respectively. As previously indicated, although Insulin will activate both pathways independently, the activation of the PI3K pathway noted by the phosphorylation of Akt inhibited the activation of ERK [9]. The dot plots illustrate the cross-talk that exists between these two signaling pathways. This is demonstrated by the sharp decrease in ERK phosphorylation between 3 minutes and 5 minutes (see bar graphs; C, D).

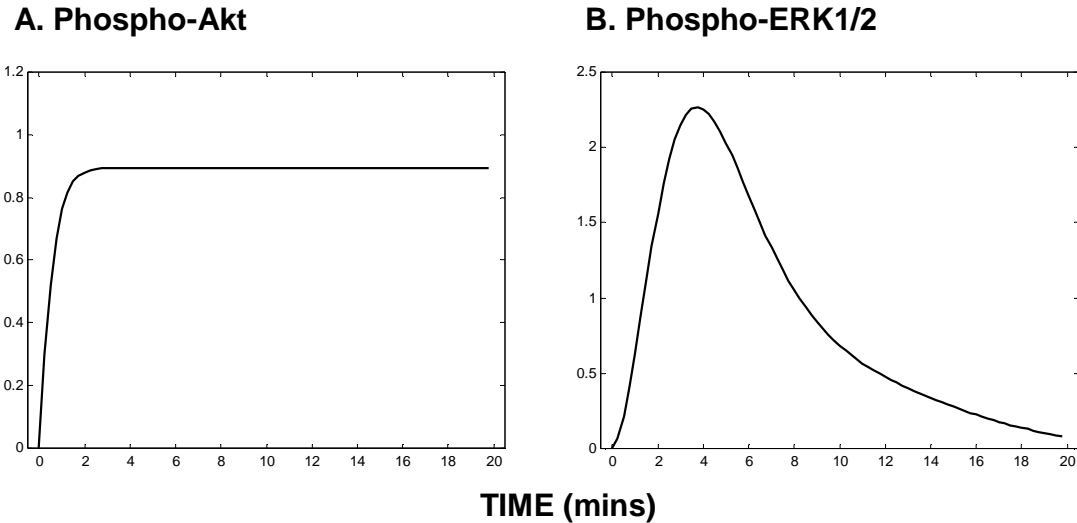
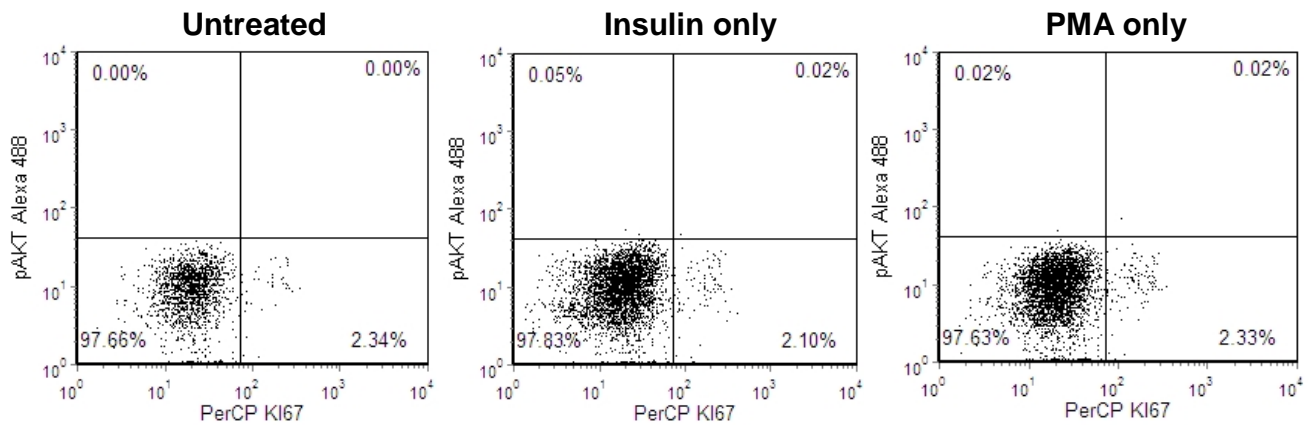


Figure 4. Kinetic profiles of phospho-Akt and phospho-ERK1/2 antibodies in response to insulin treatment on HEK293 cells As illustrated by Kim and Ryu from the Combinatorial and Computational Mathematics Center at Postech [11], both phospho-Akt **(A)** and phospho-ERK1/2 **(B)** are immediately activated by 30nM insulin on HEK293 cells. However, after 5 minutes the activation of phospho-ERK1/2 decreases substantially as indicated in the kinetic profiles above. Phosphorylated Akt, however, sustains the response. This transient signal clearly demonstrates the cross-talk between the PI3K and MAPK signaling pathways. This validates the results and we have seen in flow cytometry applications.

Without Cell Cycle Stop™



Co-treated with Cell Cycle Stop™

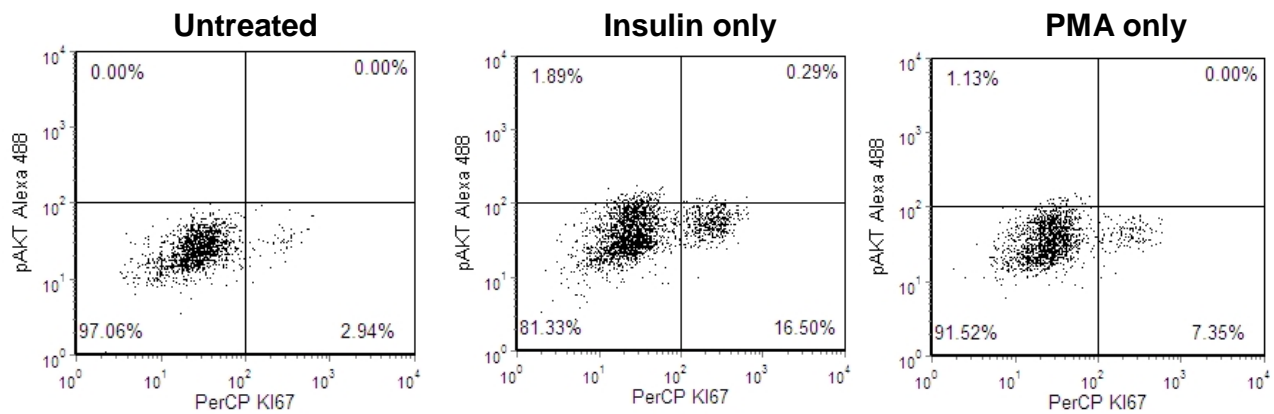


Figure 5. The effects of Cell Cycle Stop™ on Ki-67 staining After 24 hours starvation to cause cell cycle arrest at G₀ phase of the cell cycle [10], HEK293 cells were then stimulated with either Insulin or PMA overnight and measured for cell proliferation the next day (approximately 16-20 hours). As indicated by the dot plots above, using Cell Cycle Stop™ greatly enhances the ability to measure circulating cells which normally are difficult to visualize since Ki-67 expression in G₁/S/G₂ phases are punctate and produce weaker signals in flow applications.

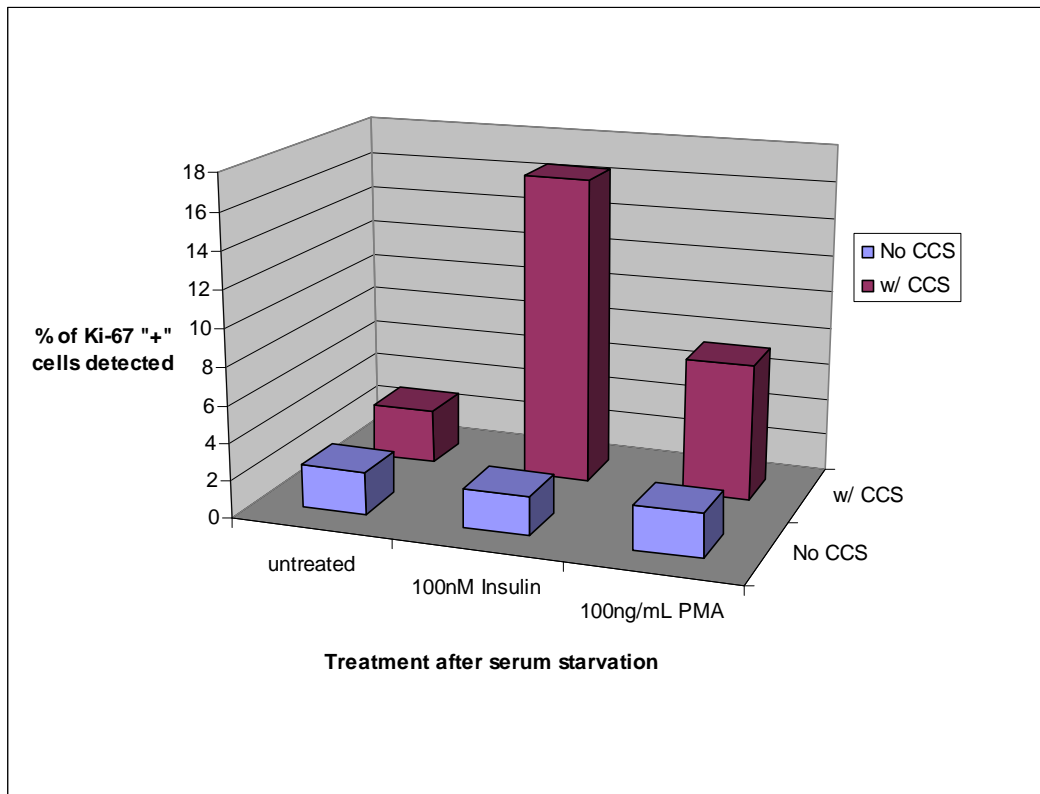


Figure 6. Bar graph representing the effects of Cell Cycle Stop™ As seen by the bar graph, the proliferative effect by Insulin and PMA stimulation was captured by the increase in Ki-67 expression after using Cell Cycle Stop™ versus without treatment.

Technical Hints

- All kit antibodies, 5X Assay Buffer and 1X Permeabilization Buffer should always remain at 2 - 8°C, both prior and during use.
- Mix samples thoroughly before use; avoid excessive bubbling.
- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended to perform all steps in a smaller collection tube (e.g. centrifuge tube)
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash buffer	<ul style="list-style-type: none"> If storing at -20°C, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37°C water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	<p>This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following:</p> <ul style="list-style-type: none"> Decreasing number of cells for analysis. The Guava EasyCyte™ Plus has the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Any cell densities in excess can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter. Adherent or sticky cells can result in cellular clumping. Use a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed (Cat#: SCNY00060; 60 µM) After many uses, it is possible that the fluid system on the Guava EasyCyte™ Plus requires cleaning. Run a Quick Clean procedure to clean the fluid system during or after an assay. This will prevent any material from forming within the glass capillary walls.
Cellular Analysis	A loss or lack of signal	<ul style="list-style-type: none"> Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.
Cellular Analysis	Background and/or non-specific staining of cells	<ul style="list-style-type: none"> Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.
Cellular Analysis	Variability in day to day experiments	<ul style="list-style-type: none"> Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. When using the Guava EasyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (*See Analytical Sensitivity and Detection Limits Section for Guava Check standards)

*For further support, please contact Millipore's Technical services at +1(800) 437-7500

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Related Products

1. FlowCollect™ PI3K-mTOR Signaling Cascade Mapping Kit (Catalog # : FCCS025210)
2. FlowCollect™ Multi-STAT Activation Profiling Kit (Catalog # : FCCS025550)

Appendix

In order to fully validate the kit components and the utility of the three antibody biomarkers in determining cell signaling activation and resulting cell proliferation, we conducted an experiment using HEK293 cells in response to both Insulin and PMA stimulation. Anti-phospho-Akt and Anti-phospho-ERK1/2 signaling antibodies were used in measuring the cross-talk in a temporal manner. And to further validate phospho-antibody activity upon Insulin activation, biological cell cycle marker Anti-Ki-67 is used to measure cells captured in the mitotic phase, an accurate determination of cell proliferation.

The assay design comprises of eight (8) samples in total. Three of the samples are used for single colored staining, with the intent to assist in setting the voltages and compensation prior to performing

multi-parametric staining analysis. The other five (5) samples are experimental samples for testing. All eight samples are laid out as shown:

SINGLE COLOR STAINING (+ / -)			MULTI-COLOR STAINING (Akt-AF488/ERK-PE/Ki67-PerCP)--POOLED				
Tube #1	Tube #2	Tube #3	Tube #4	Tube #5	Tube #6	Tube #7	Tube #8
100nM Insulin (3 mins) + untreated (3 mins); Akt-AF488	100ng/mL PMA (3 mins) + untreated (3 mins); ERK-PE	100nM Insulin + CCS + untreated (16hr); Ki-67-PerCP	untreated	100nM Insulin (3 mins)	100nM Insulin (5 mins)	Control (CCS only)	100nM Insulin + CCS
3 and 5 minutes			16 hours		3 and 5 minutes		16 hours

Sample tubes #1- #3 are used to set instrument voltages and compensation. Each sample is “spiked” with an untreated sample to ensure that compensation is done properly.

Tube #1: 187,500 cells of 100nM Insulin (3 mins) + 62,500 cells of untreated = 250,000; Addition of 5 μ L 20X Akt-AF488 + 95 μ L 1X Assay Buffer

Tube #2: 187,500 cells of 100ng/mL PMA (3 mins) + 62,500 cells of untreated = 250,000 cells; Addition of 5 μ L 20X ERK-PE + 95 μ L 1X Assay Buffer

Tube #3: 187,500 cells of Insulin + CCS + 62,500 cells of untreated (16hr) = 250,000 cells; Addition of 5 μ L 20X Ki-67-PerCP + 95 μ L 1X Assay Buffer

Sample tubes #4- #6 are experimental samples to determine the cross-talk between the PI3K and MAPK pathways. It has been indicated that cross-talk exist, and this theory is confirmed by measuring the activation of both signaling pathways via phosphorylation of Akt and phosphorylation of ERK in a temporal manner.

Cells are serum starved for 24 hours prior to stimulation. After stimulation of the insulin receptor for three minutes, cells are immediately fixed in fixation buffer to essentially capture and freeze the phosphorylation event, resulting in both the activation of Akt and ERK1/2 signaling pathways (noted by the phosphorylation of both biomarkers--see Figure #3). Another group of cells are stimulated for five minutes and immediately fixed to capture and freeze the phosphorylation event at this time point. Comparing 3 minutes versus 5 minutes of stimulation, there is a sharp decline in the phosphorylation of ERK. This clearly displays the cross-talk as the phosphorylation of Akt has dephosphorylated Raf, which effectively abolished the activity of ERK1/2 signaling.

Tube #4: 250,000 cells of HEK untreated negative control (3 mins); 5 μ L 20X Akt-AF488 / 5 μ L 20X ERK-PE / 5 μ L 20X Ki-67-PerCP + 85 μ L 1X Assay Buffer

Tube #5: 250,000 cells of 100nM Insulin (3 mins); 5 μ L 20X Akt-AF488 / 5 μ L 20X ERK-PE / 5 μ L 20X Ki-67-PerCP + 85 μ L 1X Assay Buffer

Tube #6: 250,000 cells of 100nM Insulin (5 mins); 5 μ L 20X Akt-AF488 / 5 μ L 20X ERK-PE / 5 μ L 20X Ki-67-PerCP + 85 μ L 1X Assay Buffer

Sample tubes #7 and #8 are designed to measure cell proliferation induced by insulin stimulation by using proliferation biomarker Ki-67. Cells have been starved for 24 hours prior to any cell treatment to reset the cell cycle back to G₀. After 24 hours, sample #7 was treated with Cell Cycle Stop™ only (negative control) and sample #8 was treated with Cell Cycle Stop™ and 100nM Insulin (positive control). Both were incubated at 37°C overnight (approximately 16 hours of treatment).

The next day, samples #7 and #8 are fixed, permeabilized, and stained. The results indicate that cells have proliferated in response to insulin treatment when compared to the negative control. Moreover, Cell Cycle Stop™ enhanced the visualization of Ki-67 expression in M-phase of the cell cycle. Since Ki-67 expression is present in all phases of the cell cycle but is punctate in G1/S/G2 phases, it cannot be detected legitimately in flow cytometry. By using Cell Cycle Stop™, all cells undergoing the mitotic phase as a result of insulin stimulation are accumulated in this phase to make studying Ki-67 expression possible in flow applications.

Tube #7: 250,000 cells of HEK control (16hr); 5 µL 20X Akt-AF488 / 5 µL 20X ERK-PE / 5 µL 20X Ki-67-PerCP + 85 µL 1X Assay Buffer

Tube #8: 250,000 cells of Insulin + CCS; 5 µL 20X Akt-AF488 / 5 µL 20X ERK-PE / 5 µL 20X Ki-67-PerCP + 85 µL 1X Assay Buffer

Each tube is incubated on ice for one hour in the dark. After staining is completed, all eight cell samples are washed to remove any residual antibody and resuspended in Assay Buffer for sample and data acquisition.

All assays and data acquisition is achieved using a bench-top Guava® flow cytometry system.

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