# FlowCellect™ PI3K-mTOR Signaling Cascade Mapping kit

25 Tests

## Cat. No. FCCS025210

**FOR RESEARCH USE ONLY**
**Not for use in diagnostic procedures**

USA & Canada Phone: +1 (800) 437-7500 Fax: +1 (951) 676-9209
www.millipore.com

## 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. Multi-parameter phospho flow cytometry is a powerful tool for studying multiple pathways in a mixed cell population at the same time.

Signaling through the PI3K/Akt/mTOR pathway is constitutively active in many cancer cells due to the loss of PTEN (tumor suppressor), exposure to carcinogens, activation of growth receptors, as well as mutations or amplifications of PI3K or Akt. The relevance of this pathway in difficult to treat cancers makes theses signaling nodes prime targets in advancing anticancer therapies.

Millipore's FlowCellect™ PI3K-mTOR Signaling Cascade Mapping kit includes two directly conjugated phospho-specific signaling antibodies which are optimized for multi-color flow cytometry applications to analyze the mTOR pathway in great detail. The phosphorylation of Akt is indicative of the upstream PI3K signaling, marking the cells initiation into proliferation or cell survival. Phosphorylated-Ribosomal S6 protein is indicative of downstream mTOR and p70S6K signaling leading to protein translation.

By supplying both of these antibodies in one kit, Millipore provides the researcher with a complete solution for examining the mTOR pathway, which can be useful for the elucidation of drug effectiveness in cancer research.

All FlowCellect™ 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 FlowCellect™ 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

The phosphoinositide 3-kinase (PI3K) pathway plays an important role in tumor growth and angiogenesis. PI3K signaling regulates tumor growth and angiogenesis by activating Akt, which will in turn activate its downstream effectors [5]. One of the major downstream targets from Akt signaling is the mammalian target of rapamycin (mTOR). mTOR is an important therapeutic target as it has been indicated to play an active role in many cancers. The mTOR pathway plays a central role in regulating protein synthesis and ribosomal protein translation. Deregulations in mTOR signaling are frequently associated with tumorigenesis, angiogenesis, tumor growth, and metastasis [6]. Antibodies against phosphorylated Akt (pAkt) and phosphorylated Ribosomal Protein S6 (pRiboS6) can be used to fully interrogate the mTOR pathway in greater detail.

Millipore's FlowCellect™ PI3K-mTOR Signaling Cascade Mapping kit is designed to measure the PI3K and mTOR activities of the PI3K/mTOR signaling cascade simultaneously. The kit provides two fully validated and directly conjugated antibodies against pAkt and pRiboS6 biomarkers. By utilizing both phospho-specific antibodies simultaneously in flow cytometry applications, researchers now have the ability to thoroughly evaluate and dissect two key stages of the PI3K/mTOR signaling cascade for their cancer research.

Each kit will provide enough material for 25 two-color sample tests. The kit includes all optimized fluorescently labeled antibodies and buffers necessary for cell preparation and analysis. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

*For Research Use Only; Not for use in diagnostic procedures*

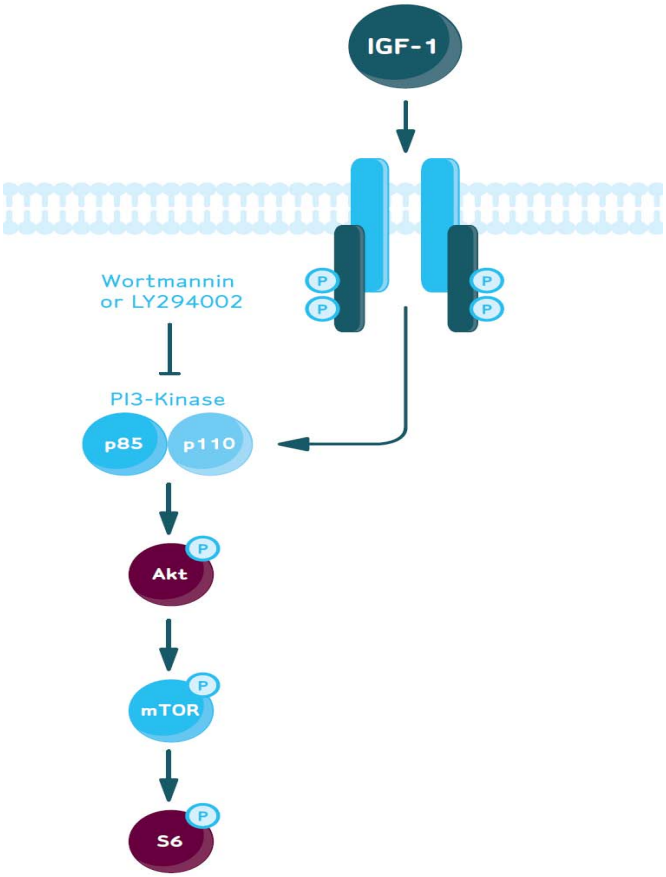


**Figure 1. Diagram of the PI3K/mTOR Signaling Pathway.** The mammalian target of rapamycin (mTOR) has proven to be an important new therapeutic target in cancer research. Being a central regulator of cell proliferation, angiogenesis, and cell survival, the mTOR pathway has huge implications in cancer regulation.
By understanding this critical pathway by close examination of both upstream and downstream markers (p-Akt and p-Ribosomal Protein S6, respectively) researchers can now fully understand and address this target in greater detail.

## Kit Components

1. 20X Anti-phospho-Ribosomal Protein S6 (Ser235)-PerCP conjugated Monoclonal Antibody: (Part No. CS203308) One vial containing 150 μL antibody.
2. 20X Anti-phospho-Akt1/PKBα (Ser473)-Alexa Fluor 488 conjugated Monoclonal Antibody: (Part No. CS203310) One vial containing 150 μL antibody.
3. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer.
4. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer.
5. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer.
6. 1X Permeabilization Buffer: (Part No. CS203284) Two bottles, each containing 14 mL buffer.

## Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS, cell dislodging buffers, etc.
3. Wortmannin 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)

## 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 - 8ºC. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature upon receipt. **Caution:** Fluorochrome conjugated antibodies should always be stored at 2 - 8ºC 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 - 8ºC.

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

2. **Fixation Buffer**

   For every $1x10^6$ cells: Add 250 µL of the Fixation Buffer to 250 µL of 1X Wash Buffer to make a final volume of 500 µL. Stir to homogeneity. Prepared 1X Fixation Buffer is stable up to one month. Store at 2 - 8ºC.

3. **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 - 8ºC.

## Preparation of Test Cell Samples

<u>For adherent cells:</u>

1. Aspirate growth media from tissue culture flask and wash cell monolayer using PBS or HBSS to wash away any residual growth media.
2. Gently harvest adherent cells using a mild enzyme such as trypsin or an EDTA-based cell dissociation buffer. Gently tap the side of the flask to dislodge cell monolayer and add 1X PBS to collect the cells.
3. Transfer suspension into a centrifuge collection tube. Determine cell numbers using a hemacytometer. Also note cell viability.
4. Divide desired amount of cells into to 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 tabletop centrifuge and spin down 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, adding 500 µL of 1X Wash Buffer for every 1 million cells is recommended.

8. Cell treatment: This will be performed at the end user's own discretion. If you are investigating the activation or inhibition of the PI3K/mTOR 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 compound inhibition on Jurkat cells, treat one centrifuge tube containing a cell suspension with 1 μM Wortmannin for one hour at 37°C. The other centrifuge tube will serve as the untreated negative control)*.
9. Following cell treatment, place centrifuge tubes into a tabletop centrifuge and spin down samples for 5 minutes at 400 x g. Aspirate supernatant and discard, saving only the cell pellets.
10. Gently resuspend the cell pellets by adding 1X Fixation Buffer at 500 μL per $1x10^6$ cells. Allow to incubate on ice for 20 minutes.
11. Following cell fixation, place centrifuge tubes into a tabletop centrifuge and spin down the samples for 5 minutes at 400 x g.
12. Remove the samples from centrifuge and discard fixation reagent, saving only the pellets. ***Caution:*** *Depending on cell type, cells may appear glassy and may be difficult to visualize after the fixation step. 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.*
13. For every $1x10^6$ cells fixed, add 500 μL of 1X Wash Buffer to cells and pipette up and down gently to ensure proper cell dispersion.
14. Again, place centrifuge tubes into a tabletop centrifuge and spin down the samples for 5 minutes at 400 x g (washing step). Carefully aspirate supernatant and save the cell pellet.
15. For every 1 million cells, add 500 μL 1X Permeabilization Buffer to each tube and resuspend cells to ensure complete homogeneity. Allow cells to incubate on ice for 20 minutes (***Note:*** *Samples may be stored in Permeabilization buffer overnight at 4°C if necessary).*
16. Repeat steps 13 - 14 once more (washing steps) to ensure that there is no residual Permeabilization Buffer remaining.
17. Save the cell pellets in preparation for cellular staining and analysis.

**b. 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 - #17 as noted above (In the "For adherent cells" section)

## 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 below 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. All samples will be resuspended in a final volume of 100 µL 1X Assay Buffer per test.
3. Cell Staining: For every cell sample (treated and untreated), add 5 µL 20X Anti-phospho-Ribosomal Protein S6 (Ser235) PerCP conjugate Monoclonal Antibody and 5 µL 20X Anti-phospho-Akt1/PKBα (Ser473) Alexa Fluor 488 conjugate Monoclonal Antibody to 90 µL of 1X Assay Buffer for a final volume of 100 µL. Allow to incubate in the dark on ice for one hour.
4. Along with the test samples, prepare single antibody staining for both Anti-phospho-Ribosomal Protein S6 (Ser235) and Anti-phospho-Akt1/PKBα (Ser473) by preparing 5 µL 20X antibody into 95 µL 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 µL of 1X Assay Buffer and place test tube(s) containing cells into a 4˚C tabletop centrifuge and spin sample 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 µL 1X Assay Buffer to disrupt the cell pellet. Pipette sample up and down to ensure complete homogeneity.
8. Replace centrifuge tube into 4˚C tabletop centrifuge and spin sample for 5 minutes at 400 x g.
9. At the completion of the washing step, resuspend the cell pellet at $0.5x10^6$ cells per milliliter of 1X Assay Buffer.
10. Begin cellular analysis

## Sample Results

The FlowCellect™ PI3K-mTOR Analysis Kit is designed to analyze the PI3K-mTOR signaling pathway by providing the biomarkers suitable for the complete interrogation of both the upstream and downstream signaling nodes for mTOR activity.

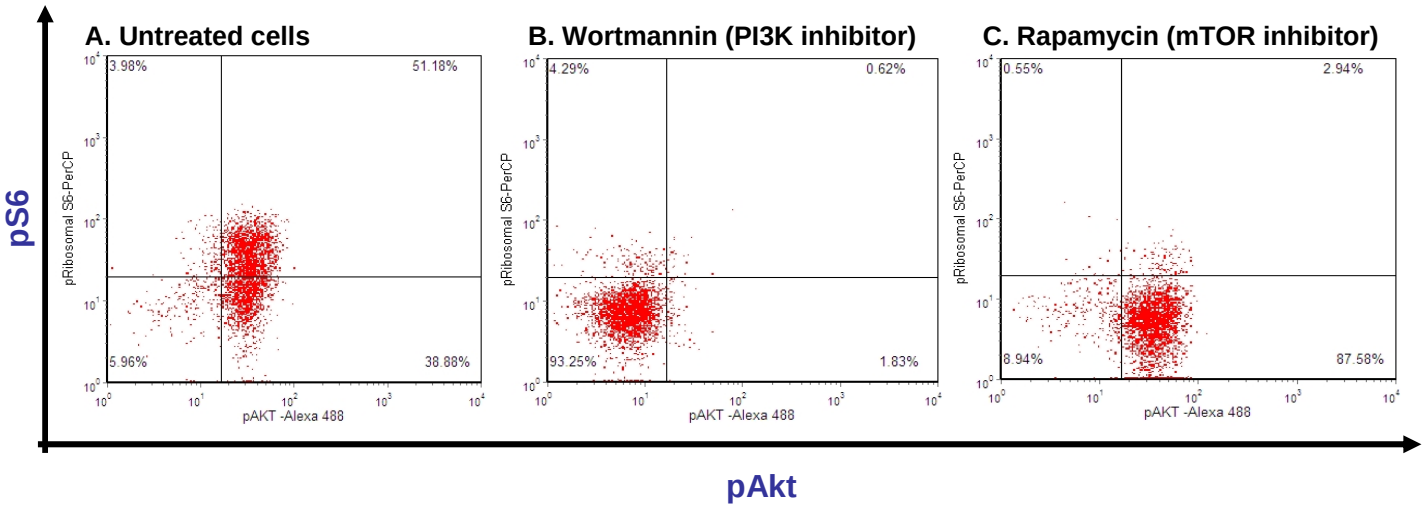


**Figure 2. PI3K and mTOR inhibition on Jurkat cells** The scatter plots above represent the phospho-specific activities of both phospho-Akt (Ser473)-Alexa Fluor 488 conjugate and phospho-Ribosomal Protein S6 (Ser235) -PerCP conjugate in Jurkat cells, in which the PI3K and its

downstream mTOR activities are constitutively activated **(A: untreated cells)** as noted. But after the addition of a specific PI3K inhibitor, Wortmannin, all PI3K signaling biomarkers are down-regulated as shown in **(B)**. However, when a specific mTOR inhibitor Rapamycin is used alone, only phospho-Ribosomal Protein S6 activity is affected without any effect on the phosphorylation of Akt **(C)**.

## Technical Hints

- All kit antibodies, 5X Assay Buffer and 1X Permeabilization Buffer should always remain at 2 - 8ºC, 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 | • If storing at -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC 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 | This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following:<br>• 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.<br>• 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)<br>• 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 | • 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.<br>• 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 | • 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 | • 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.<br>• 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***

## References


1. Krutzik, P.O. *et al.* (2003) Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A.* Oct;55(2):61-70
2. LoPiccolo, J. *et al.,* (2008) Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat.* 11(1-2):32-50.
3. Iwenofu, O.H. *et al.* (2008) Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. *Mod Pathol.* 21(3):231-7.
4. Heinonen, H. *et al.* (2008) Deciphering downstream gene targets of PI3K/mTOR/p70S6K pathway in breast cancer. *BMC Genomics.* 2008 Jul 24; 9:348.
5. Jiang, B.H. *et al.* (2009) Chapter 2: PI3K/PTEN Signaling in Angiogenesis and Tumorigenesis. *Adv Cancer Res.* 102C:19-65.
6. Jiang, B.H. *et al.* (2008) Role of mTOR in anticancer drug resistance: perspectives for improved drug treatment. *Drug Resist Updat.* 11(3): 63-76.


## Related Products

1. FlowCellect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog # : FCCS025100)
2. FlowCellect™ Multi-STAT Activation Profiling Kit (Catalog # : FCCS025550)

## Warranty

**Millipore Corporation** (“Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**



Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals

**Cat No. FCCS025210**

June 2010
Revision C: FCCS025210MAN