



FlowCollect™ PLC- γ 1 Activation Dual Detection kit
25 Tests

Cat. No. FCCS025145

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

Application

Millipore's FlowCelect™ Activation Dual Detection kits are a series of flow cytometry products which include a pair of antibodies that bind to the same protein; one to detect total protein expression and another to detect the phosphorylated form of the same target. Using two parameter analysis, we can achieve target specific detection of phosphorylation and, by doing so, eliminate false positives while enhancing the signal to noise ratio.

Phospholipase C (PLC) is a group of enzymes that play a critical role in signal transduction during the hydrolysis of membrane phospholipids as a response to extracellular stimuli. PLC- γ 1 is known for its involvement in growth factor dependent signal transduction. PLC- γ 1 is made up of 2 SH2 domains, one SH3 domain, and 2 tyrosine phosphorylation sites. Almost all growth factors can stimulate tyrosine phosphorylation and subsequent activation of PLC- γ 1 [1].

Activation of PLC- γ 1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) which in turn produces two second messengers, diacylglycerol (DAG) and inositol 1,4,5- triphosphate (IP3). DAG serves to activate protein Kinase 3, while IP3 mediates the release of calcium. These processes have been implicated in many cellular physiological functions, such as secretion, cell proliferation, cell growth, and differentiation [2]. PLC- γ 1 tyrosine phosphorylation, IP3 formation, and Ca²⁺ mobilization are all provoked by nearly all growth factor receptors. Receptor tyrosine kinase phosphorylation at Tyrosine 783 is essential for IP3 formation [3, 4].

Unlike other PLC-isozymes (PLC- β and PLC- δ isozyme), PLC- γ 1 is involved in cellular proliferation. The expression level of PLC- γ 1 is increased in human colorectal cancer, human breast carcinomas, familial adenomatous polyposis, and human skins under hyperproliferative conditions [5, 6, 7, 8]. The rise of PLC- γ 1 level in cancer tissues was accompanied with increase of the PIP2-hydrolyzing activities. In a case study using specific antibodies, the expression levels of all three distinct PLC isozymes (PLC- β 1, - γ 1, and - δ 1) were investigated in adenoma and carcinoma tissues from familial adenomatous polypis patients. Even though there were no apparent increases in levels of PLC- β 1 and PLC- δ 1, a significant increase in the PLC- γ 1 level was detected in adenoma and carcinoma as compared with normal mucosal tissue. Furthermore, the level of PLC- γ 1 increased as the normal mucosa progresses from adenoma to carcinoma sequentially [9].

PLC- γ 1 is a pivotal element in T-cell receptor signaling. In T cells, TCR-ligation activates PLC- γ 1 isozymes. The role of Ca²⁺ mobilization by PLC- γ 1 in this process is to activate phosphatase calcineurin, which in turn dephosphorylates and activates NFAT [10].

In all, a comprehensive understanding of PLC- γ 1 activity and its association with various growth factor receptors can provide the researcher with key information in their studies involving cell growth, cell proliferation, and cell differentiation. Millipore's FlowCelect™ PLC- γ 1 Activation Dual Detection Kit provides the researcher with a tool to evaluate and accurately measure phospho-specific PLC- γ 1 activation in a population of cells.

All FlowCelect kits are optimized on guava® bench top flow cytometers. FlowCelect kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study the PLC- γ 1 signaling pathway right in the comfort of their own lab. Both antibodies provided in the kit are carefully titrated and optimized together to ensure maximal

performance when run in multiplex, alleviating the need for any additional optimization. This kit contains optimized fixation, permeabilization, wash, and flow buffers to provide researchers with a complete solution for cell signaling analysis.

Test Principle

Millipore's FlowCelect™ PLC- γ 1 Activation Dual Detection kit includes two directly conjugated antibodies, a phospho-specific Anti-phospho-PLC- γ 1 (Tyr837)-Alexa Fluor® 488 and an Anti-PLC- γ 1-PE conjugated antibody to measure total levels of PLC- γ 1. This two color flow cytometry kit is designed to detect the extent of PLC- γ 1 pathway activation by measuring PLC- γ 1 phosphorylation relative to the total PLC- γ 1 expression in any given cell population. By doing such, the levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of PLC- γ 1 activation after stimulation. Moreover, simultaneous measurement of both total and phospho-PLC- γ 1 confirms target specificity of the phosphorylation event. Together, a total and phospho antibody duo performed in multiplex provides an enhanced and more reliable detection of the phospho: total ratio within a mixed population.

PLC- γ 1 is devoted to tyrosine kinase-dependent signaling for mitogenic responses to diverse agents such as growth factor activation of receptor tyrosine kinases and stimulation of immune cell receptors. Signaling pathways that depend on tyrosine kinases communicate with the PLC- γ family [11]. Because of its involvement in many key signaling cascades resulting from receptor tyrosine kinase activation, PLC- γ 1 plays a critical role in tumor progression and cancer-related illnesses. When stimulating A431 cells with EGF for 2 minutes the total protein level remains constant compared to unstimulated cells, while phospho-PLC- γ 1 levels are increased in all cells indicating that there is no competition between the two antibodies for their target epitopes.

The antibody pair provided in the kit have been carefully titrated to ensure the ability to measure Total and phospho-PLC- γ 1 simultaneously on the same protein for accurate determination of protein level and activation. Sufficient reagents are provided to perform 25 two-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

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Kit Components

- 20X Anti-phospho-PLC- γ 1 (Tyr783) Alexa Fluor[®] 488: (Part No. CS206377) One vial containing 150 μ L.
- 20X Anti-PLC- γ 1-PE: (Part No. CS206378) One vial containing 150 μ L.
- Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL.
- 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL.
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL.
- 1X Permeabilization Buffer: (Part No. CS203284) Two bottles containing 14 mL.

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. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 μ L
4. Tabletop centrifuge capable of exceeding 400 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water (for Buffer dilutions)
8. ViaCount Reagent (Catalog No. 4000-0040)
9. A431 cells or cells of interest
10. EGF, human recombinant (Part No. 01-107)
11. Isotype control: mouse IgG- PE (based on user preference)

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 fixation solution containing paraformaldehyde. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found by contacting Millipore technical services).
- The conjugated antibody is 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.
- Do not use reagents beyond the expiration date of the kit.

Storage

This kit 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:** The fluorochrome conjugated antibody should always be stored at 2 - 8°C and stored in the dark.

All kit components are stable up to six (6) 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 with deionized water prior to use. 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

Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8°C.

Assay Instructions

Note: This assay protocol has been optimized for human A431 cells. However, this kit is suitable for measuring the extent of PLC- γ 1 target-specific detection of activation via phosphorylation on a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

Flow Kit Staining Protocol

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.

I. Cell Culture and Stimulation

1. Seed 6 million A431 cells per T-175 flask overnight in a 37°C incubator with 5% CO₂. Cells should be at about 70% confluent the next day.
2. Aspirate growth media from T-175 flask and gently wash with 10 mL of PBS to remove any residual growth media. **NOTE:** From this point on, media must be serum-free.
3. Add 40 mL of serum-free media to the flask for 24 hours.
4. Aspirate to remove the serum-free media from the T-175 flask and gently wash cell monolayer with 10 mL of PBS. Aspirate off the PBS.
5. Add 2 mL Accutase to detach cells. Incubate for 5 minutes.

6. Gently tap the sides of the flask to dislodge cell monolayer and then immediately add 10 mL of serum-free media to collect cells. Transfer cell suspension into a 50 mL conical tube. Pipet the cells several times to achieve a single cell suspension.
7. Determine cell numbers by using ViaCount or a hemacytometer and note cell viability. Healthy cells should be above 90% viable.
8. After cell numbers have been determined, centrifuge cells at 670 x g for 5 minutes to pellet cells.
9. Aspirate to remove the supernatant and resuspend cell pellet at 2 million cells per mL in fresh serum-free media.
10. Divide cells into two separate 50 mL conical tubes. Note volume of cell solution --One tube will serve as the "treated" or experimental sample while the other will serve as the "untreated" or control.
11. Place both conical tubes in a 37°C water bath for 20 to 30 minutes to bring cells back to 37°C.
12. Add 100 ng/mL of EGF into the tube labeled "treated" and the same volume of serum-free media to the tube labeled "untreated" for 2-5 minutes.

II. Fix and Permeabilize Cells

13. Immediately after stimulation, fix cells by adding an equal volume of ice-cold Fixation Buffer into both tubes for 20 minutes on ice.
14. Centrifuge fixed cells at 670 x g for 5 minutes in a 4°C centrifuge and aspirate off supernatant.
15. Wash cells once with 5 mL of 1x Wash Buffer and repeat step #14 above.
16. Resuspend each cell pellet to 5 million cells per mL and add 100 μ L of cells into a "V" bottomed 96-well plate. (see guava manual for instrument compatible plates) NOTE: Plate must be placed on ice before adding cells.
17. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.
18. Permeabilize cells by adding 100 μ L of ice-cold Permeabilization Buffer and incubate on ice for 20 minutes. Gently resuspend each sample during this 20 minute incubation.
19. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.
20. Wash cells with 200 μ L of a 1x Wash Buffer and repeat step #19 above.

III. Cell Staining and Flow Analysis

21. For single color staining, resuspend the cells in 95 μL of assay buffer and add 5 μL of either Anti-phospho-PLC- γ 1-Alexa Fluor 488 **or** Anti-PLC- γ 1-PE to each sample well.
22. For multiplexing, resuspend the cells in 90 μL of assay buffer and add 5 μL of Anti-phospho-PLC- γ 1-Alexa Fluor 488 **and** add 5 μL of Anti-PLC- γ 1-PE to each sample well.
23. Incubate cells for one hour on ice in the dark.
24. Add 100 μL of 1x Wash Buffer to the 100 μL of diluted antibodies already in the wells and centrifuge at 670 x g for 5 minutes at 4°C. Discard supernatant.
25. Wash with 200 μL of 1x Wash Buffer and centrifuge cells at 670 x g for 5 minutes at 4°C. Discard supernatant. Repeat once more.
26. Resuspend cells in each well with 200 μL of 1x ice-cold Assay Buffer.
27. Perform flow cytometry analysis

Sample Data

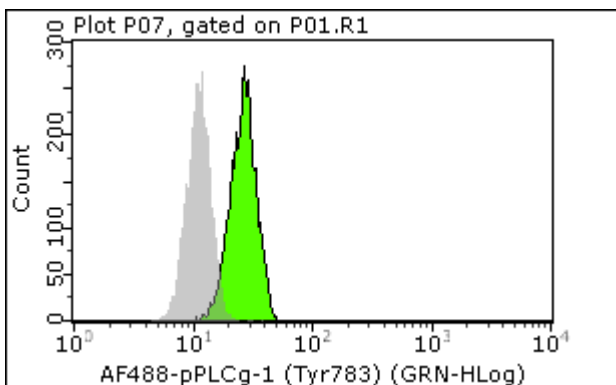


Figure 2. Analyzed Single Parameter Data for Anti-phospho-PLC- γ 1 (Tyr783) A431 cells were treated with 100 ng/mL of EGF and then stained with phospho-PLC- γ 1-Alexa Fluor 488 (green). Untreated A431 cells (grey) were also stained and results are shown overlaid in each plot.

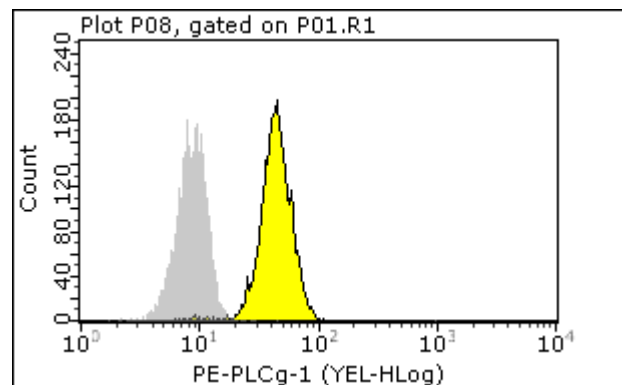


Figure 3. Analyzed Single Parameter Data for Anti-PLC- γ 1 (total PLC- γ 1) on A431 cells Untreated A431 cells were stained with Anti-PLC- γ 1-PE (yellow) versus an isotype control (grey).

Two parameter analysis using total and phospho antibodies

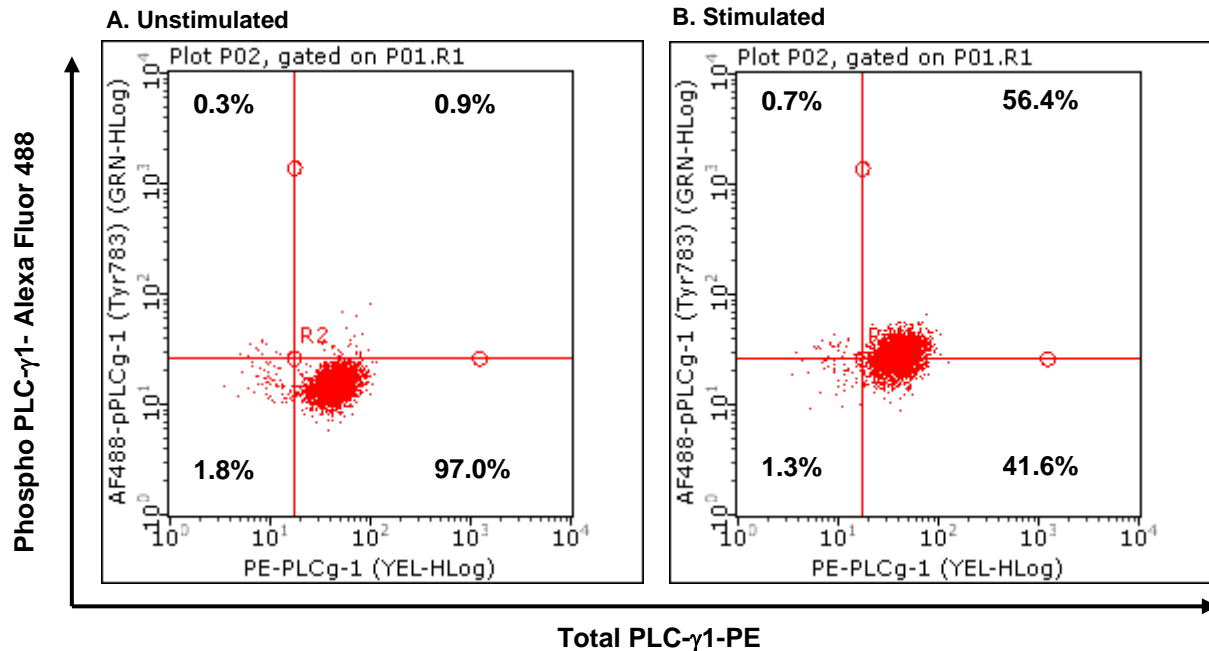


Figure 4. Dual Parameter Analysis of Total and Phospho PLC- γ 1 on A431Cells Unstimulated A431 cells are stained with both phospho-PLC- γ 1-Alexa Fluor 488 and Anti-PLC- γ 1-PE (A), where there is no indication of PLC- γ 1 activation via phosphorylation, but only on total PLC- γ 1 as noted by 97.0% of cells. However, once A431 cells were stimulated with 100 ng/mL EGF, simultaneous measurement of both total and phospho PLC- γ 1 confirms target specificity of the phosphorylation event as indicated by the double positive cell population (B) as indicated by the 0.9% to 56.4% increase of double positive staining.

Technical Hints

- 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 that all steps be performed 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. Guava flow cytometers have 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 (Catalog No. SCNY00060; 60 µM) After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow stream takes place.
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*

References

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Related Products

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2. FlowCollect™ EGFR/STAT3 Pathway Activation Kit (Catalog No. FCCH025111)
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