FlowCellect™ Src Activation Dual Detection kit

25 Tests

Cat. No. FCCS025154

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

Millipore's FlowCellect™ 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.

Src is a non-receptor tyrosine kinase involved in many key signaling cascades which has been implicated to play a major role in tumorigenesis. Elevated Src expression has been seen in multiple solid tumors including breast cancer. In fact, Src kinase activity is greatly increased in breast cancer tissue compared with normal breast tissue [1, 2]. Src plays a role in signaling and cross talk between growth-promoting pathways, such as ER and EGFR family signaling pathways, known to be active in breast cancer [3].

Because of its essential role in many intracellular signaling pathways, interrupting Src signaling may disrupt oncogenic pathways. Src physically interacts with activated receptor tyrosine kinases (RTKs) and creates a positive-regulatory loop that contributes to the robustness and persistence of RTK signaling [4]. Over-expression of RTKs due to their amplification or mutation is a very common occurrence in solid tumors, suggesting that a dysregulated interplay between RTKs and Src may effect an abnormally elevated Src activity. Src can associate with the over-expressed EGFR to cause synergistic mitogenicity, which can represent one mechanism by which Src contributes to the growth of tumor cells [5, 6].

In a high percentage of human neoplasms such as colorectal, breast, prostate, pancreas, head and neck, and lung carcinoma, to name a few, Src is over-expressed or hyperactivated, thus, making its dysregulation a major oncogenic signature found in cancer [7].

In all, a comprehensive understanding of Src activity and its association with both upstream (e.g. RTKs) and downstream signaling cascades can provide the researcher with useful information which will be important in understanding the intrinsic nature of tumor formation and assist in the development of anti-neoplastic agents. Millipore's FlowCellect™ Src Activation Dual Detection Kit is designed to allow the researcher to monitor and accurately measure phospho-specific Src activation in a population of cells.

All FlowCellect kits are optimized on guava® bench top flow cytometers. FlowCellect kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study the Src 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 FlowCellect™ Src Activation Dual Detection kit includes two directly conjugated antibodies, a phospho-specific Anti-phospho-Src (Tyr416)-Alexa Fluor® 488 and an Anti-Src-Alexa Fluor® 647 conjugated antibody to measure total levels of Src. This two color flow cytometry kit is designed to detect the extent of Src pathway activation by measuring the Src phosphorylation relative to the total Src 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 Src activation after stimulation. Moreover, simultaneous measurement of both total and phospho-Src 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.

Src pathway activation in response to receptor tyrosine kinase stimuli (e.g. EGFR stimulated by EGF) plays a significant role in the regulation of essential cellular processes. Dysregulation of Src signaling makes Src a key molecule in evaluation of tumor progression as it can provide oncogenic signals for cell survival, mitogenesis, angiogenesis, and metastasis [1]. As a result, newer generations of Src inhibitors are being actively investigated as cancer therapeutics. When stimulating A431 cells with EGF for 5 minutes the total protein level remains constant compared to unstimulated cells, while phospho-Src 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-Src 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.

For Research Use Only; Not for use in diagnostic procedures

Kit Components

- 20X Anti-phospho-Src (Tyr416) Alexa Fluor® 488: (Part No. CS206379) One vial containing 150 µL.
- 20X Anti-Src-Alexa Fluor® 647: (Part No. CS206380) One vial containing 150 µL.
- 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 Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of exceeding 400 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water (for Buffer dilutions)
8. A431 cells or cells of interest
9. EGF, human recombinant (Part No. 01-107)
10. Isotype control: mouse IgG1- Alexa Fluor 647 (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 - 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:** The fluorochrome conjugated antibody should always be stored at 2 - 8ºC 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 - 8ºC.

   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 - 8ºC.

Assay Instructions

Note: This assay protocol has been optimized for human A431 cells. However, this kit is suitable for measuring the extent of Src 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% CO_2. 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 µL 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 µL 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 µL 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-Src-Alexa Fluor 488 **or** Anti-Src-Alexa Fluor 647 to each sample well.

22. For multiplexing, resuspend the cells in 90 µL of assay buffer and add 5 µL of Anti-phospho-Src-Alexa Fluor 488 **and** add 5 µL of Anti-Src-Alexa Fluor 647 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

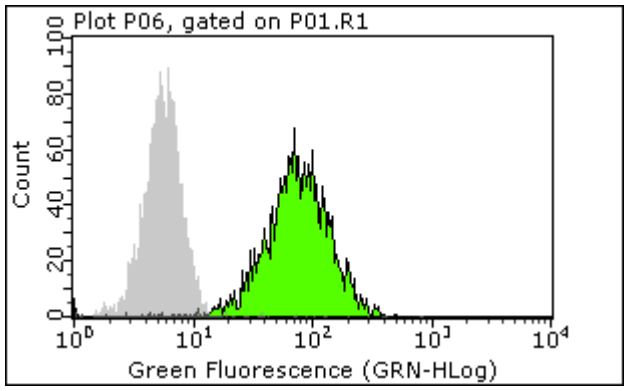

Figure 2. Analyzed Single Parameter Data for Anti-phospho-Src (Tyr416) A431 cells were treated with 100 ng/mL of EGF and then stained with phospho-Src-Alexa Fluor 488 (green). Untreated A431 cells (grey) were also stained and results are shown overlaid in each plot.

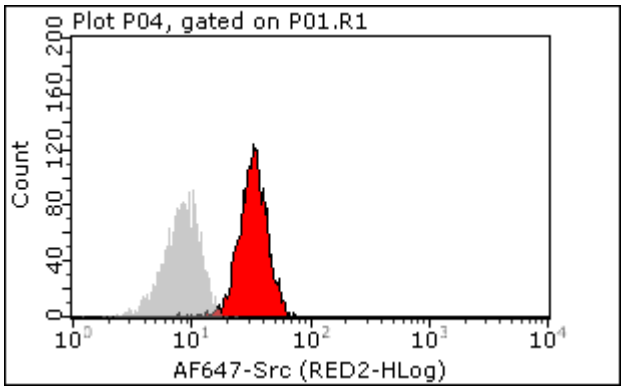

Figure 3. Analyzed Single Parameter Data for Anti-Src (total Src) on A431 cells Untreated A431 cells were stained with Anti-Src-Alexa Fluor 647 (red) versus an isotype control (grey).

Two parameter analysis using total and phospho antibodies

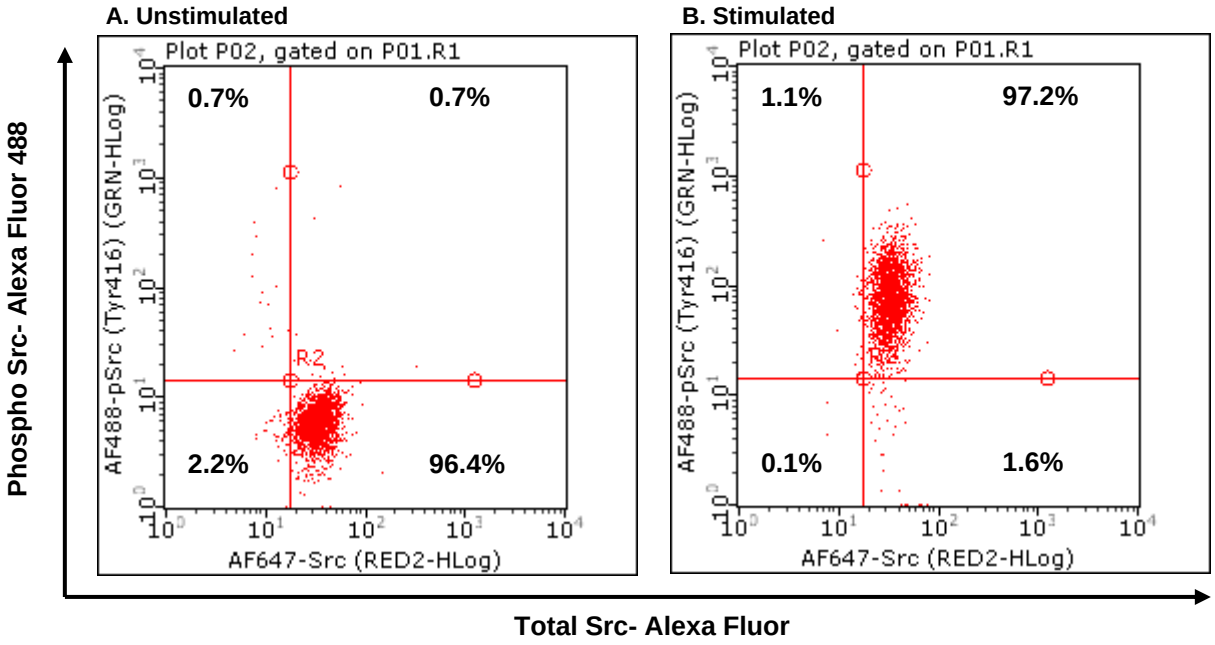

Figure 4. Dual Parameter Analysis of Total and Phospho Src on A431Cells Unstimulated A431 cells are stained with both phospho-Src-Alexa Fluor 488 and Anti-Src-Alexa Fluor 647 (**A**), where there is no indication of Src activation via phosphorylation, but only on total Src as noted by 96.4% of cells. However, once A431 cells were stimulated with 100 ng/mL EGF, simultaneous measurement of both total and phospho Src confirms target specificity of the phosphorylation event as indicated by the double positive cell population (**B**) as indicated by the 0.7% to 97.2% 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	• 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: • 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 steam takes place.
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. • 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. • 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. Summy, J.M., *et al.* (2003). Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev.* 22:337-358.
2. Yeatman, T.J., *et al.* (2004). A Renaissance for Src. *Nat Rev Cancer.* 4:470-480.
3. Slamon, D.J., *et al.* (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med.* 344:783-792.
4. Bromann, P.A., *et al.* (2004). The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene*. 23:7957-7968.
5. Maa, M.C., *et al.* (1995). Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: Implications for the etiology of multiple human cancers. *Proc Natl Acad Sci.* 92:6981-6985.
6. Biscardi, J.S., *et al.* (2000). Tyrosine kinase signalling in breast cancer: Epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res*. 2:203-210.
7. Guarino, M. (2010). Src Signaling in Cancer Invasion. *J. Cell Physiol.* 223:14-26.

Related Products

1. FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis (Catalog No. FCCH025102)
2. FlowCellect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
3. FlowCellect ™ Multi-Color DNA Damage Response Kit (Catalog No. FCCH025104)
4. FlowCellect™ DNA Damage (ATM) and Cell Cycle Analysis Kit (Catalog No. FCCH025143)
5. FlowCellect™ p38 Stress Pathway Activation Detection Kit (Catalog No. FCCS025132)
6. FlowCellect™ EGFR/MAPK Pathway Activation Kit (Catalog No. FCCS025101)
7. FlowCellect™ EGFR/STAT3 Pathway Activation Kit (Catalog No. FCCH025111)
8. FlowCellect™ PI3K-mTOR Signaling Cascade Mapping Kit (Catalog No. FCCS025210)
9. FlowCellect™ Multi-STAT Activation Profiling Kit (Catalog No. FCCS025550)
10. FlowCellect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog No. FCCS025100)
11. FlowCellect™ PI3K Activation Dual Detection Kit (Catalog No. FCCS025105)
12. FlowCellect™ MAPK Activation Dual Detection Kit (Catalog No. FCCS025106)
13. FlowCellect™ EGFR RTK Activation Dual Detection Kit (Catalog No. FCCS025107)
14. FlowCellect™ PLC-γ1 Activation Dual Detection Kit (Catalog No. FCCS025145)
15. FlowCellect™ STAT1 Activation Dual Detection Kit (Catalog No. FCCS025142)
16. FlowCellect™ STAT3 Activation Dual Detection Kit (Catalog No. FCCS025143)

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.

Alexa Fluor® is a registered trademark of Molecular Probes, Inc. Alexa Fluor® dye antibody conjugates are sold under license from Molecular Probes, Inc. for research use only.

Cat. No. FCCS025154

FCCS025154MAN
November 2010
Revision A