FlowCellect™ Chemokine Receptor CCR3 Surface Expression Identification kit

100 Tests

Cat. No. FCCR300412

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Application

The FlowCellect™ GPCR identification kits are designed for flow cytometry applications, which can be used for the identification and quantification of the surface expression level of GPCRs on any cells. The kit uses a GPCR antibody validated for flow cytometry to detect receptor expression, and also includes both positive and negative control cells with well characterized receptor expression levels for the purpose of quantification.

Chemokine receptors are very attractive targets because they are highly expressed on immune cells and regulate leukocyte activation and trafficking. Many of these targets have been indicated to play a key role in certain disease states such as HIV infection, Rheumatoid Arthritis, and Diabetes. The expression levels of these receptors often reflect the different states of infectious diseases and inflammation.

Chemokine receptors are seven transmembrane G-Protein Coupled Receptors (GPCRs). The chemokine receptor family comprises of eighteen GPCR targets, in which ten are CC chemokine receptors, seven are CXC chemokine receptors, and one is a CX_3C chemokine receptor. Millipore provides 11 GPCR surface identification kits with an initial focus on the chemokine receptor family.

Eosinophils are major effector cells implicated in a number of chronic inflammatory diseases in humans, particularly bronchial asthma and allergic rhinitis. The chemokine receptor 3 (CCR3), a GPCR activated by chemokines eotaxin 1/2, MCP-3, MCP-4, and RANTES, mediates selective recruitment of eosinophils into tissue and thus has recently become an attractive biological target for therapeutic intervention [1]. It is widely expressed on cells involved in allergic inflammation, such as basophils, mast cells, airway epithelial cells, and potentially TH2 T-lymphocytes. Allergen-induced eosinophil infiltration into airways is reduced or eliminated in CCR3 and eotaxin 1/2 knockout mice and in mice treated with antibodies directed against CCR3 [2,3]. CCR3 antagonists are currently being developed for the treatment of asthma and other allergic disorders.

The control cells provided in the kit are based on the ChemiScreen™ GPCR stable cell lines available from Millipore. Both the positive and negative control cell lines have been fully characterized using techniques such as radioligand binding assay to determine the receptor number for each cell type [4]. Using this information, the researcher can determine the number of receptors per cell on any given cell type.

Test Principle

Millipore's FlowCellect™ GPCR Surface Identification kit is designed to provide an easier and more efficient way of characterizing GPCR expression on a given cell type of interest. This kit includes optimized buffers and a high affinity, specific antibody capable for detecting the GPCR target in question. Also included in the kit is a secondary antibody conjugate to R-Phycoerythrin, which is a bright fluorochrome ideal for measuring endogenous levels of GPCR expression on native cells. By following the steps outlined in the assay procedure, researchers can easily identify which native cell lines would be suitable for performing secondary assays in support of their structure-activity drug discovery efforts.

Additional test applications include the identification of high, medium, and low expressing cell cultures during the clonal selection process. When a researcher decides to build a stable cell line in-house, it is essential that the clone of choice will provide the GPCR surface expression needed to perform drug screening. Choosing the wrong clone can adversely affect a primary screen if the chosen clone

cannot detect lower affinity compounds. This can lead to a lower hit rate, which can result in screening limitations when identifying potential blockbuster compounds.

Another added feature is the ability to quantitatively measure GPCR cell surface expression using flow cytometry as the method for detection. Provided in the kit are two vials of Millipore's ChemiScreen™ GPCR cell lines, serving as assay controls. Using patented technology, Millipore's ChemiScreen™ GPCR cell lines retain high surface expression as illustrated by the relatively high maximal number of binding sites (B_{max} value) when compared to a native system.

By using Millipore's FlowCellect™ Chemokine Receptor Surface Expression Identification kit, the researcher has the ability to identify a native GPCR expressing cell for biological assay development; the researcher can also easily select clonal stable cell lines for these GPCRs; finally, the researcher can monitor the changes of these receptor expression levels during a disease state.

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Kit Components

1. 100X Mouse anti-human CCR3 Monoclonal Antibody; Clone ID [5E8]: (Part No. CS202145) One 100 µL vial
2. 500X Secondary Antibody conjugated to R-Phycoerythrin: (Part No. CS202129) One 20 µL vial
3. Antibody Diluent: (Part No. CS202130) One 1 mL vial
4. Fixation Buffer: (Part No. CS202122) One 13 mL bottle
5. 10X Wash Buffer: (Part No. CS202123) One 13 mL bottle
6. 5X Assay Buffer: (Part No. CS202124) One 55 mL bottle
7. Positive Control Cell Culture for CCR3 : (Part No. CS202144) One vial supplied with $4x10^6$ cells
8. Negative Control Cell Culture for CCR3 : (Part No. CS202121) One vial supplied with $4x10^6$ cells

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of exceeding 1100 x g
5. Mechanical vortex
6. Flow Cytometry instrument capable of performing single cell analysis

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.
- The R-Phycoerythin conjugated Secondary antibody is light sensitive and must be stored in the dark.
- 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 is shipped at -20ºC. The anti-human CCR3 Monoclonal Antibody (Part # CS202145), Secondary Antibody conjugated to R-Phycoerythrin (Part # CS202129), 5X Assay Buffer (Part # CS202124), and Antibody Diluent (Part # CS202130) should be stored at 2 - 8ºC immediately upon receipt. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature. Both positive and negative control frozen cell cultures must be stored at -80 ºC immediately upon receipt.

All kit components are stable up to six (6) months if stored and handled correctly. **For the control cell cultures, please avoid repeated freeze/thaw cycles as this will affect the integrity of the product.**

Discard any remaining reagents after the expiration date

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 $1x10^6$ cells: Add 500 µL of the Fixation Buffer to 500 µL of 1X Wash Buffer to make a final volume of 1 mL. 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.

4. **Mouse anti-human CCR3 Monoclonal Antibody (Clone ID [5E8])**

   The primary antibody is provided at a 100X stock concentration. Prior to use, dilute stock antibody to 10X by adding 100 µL of anti-human CCR3 monoclonal antibody to 900 µL of antibody diluent. Mix thoroughly by gently pipetting up and down avoiding bubbles. Prepared 10X primary antibody is stable with no detectable loss of activity up to one year when stored at 2 - 8ºC.

   Note: Depending on the needs of the end user (dependent on cell type) a broad or narrow range of antibody dilution may be preferred.

5. **Secondary Antibody conjugated to R-Phycoerythrin**

   The secondary antibody is provided at a 500X stock concentration. Prior to use, dilute stock antibody to 10X by adding 20 µL of secondary antibody to 980 µL of deionized water. Mix thoroughly by gently pipetting up and down avoiding bubbles. Prepared 10X secondary antibody is stable with no detectable loss of activity up to one year when stored at 2 - 8ºC.

   Note: Depending on the needs of the end user (dependent on cell type) a broad or narrow range of antibody dilution may be preferred.

Preparation of Control Frozen Cell Cultures

Two vials of frozen cell cultures are provided in each assay kit. One vial contains the recombinant CCR3 ChemiScreen™ GPCR cell line (Part No. CS202144) fixed and frozen at 4 million cells ($4x10^6$ cells) per vial, which will serve as a positive control. The second vial contains the Chem-1 ChemiScreen™ parental cell line (Part No. CS202121), fixed and frozen at 4 million cells ($4x10^6$ cells) per vial, which will serve as a negative control.

1. Remove frozen cell vials (positive and negative controls) from -80ºC. Allow to thaw on ice.
2. Prepare 1X Wash Buffer (as indicated above) and chill on ice or by refrigeration.
3. Remove desired amount of cells and place in a centrifuge tube.
4. For every $1x10^6$ cells, add 5 mL of 1X Wash Buffer to cells and pipette up and down gently to ensure proper cell dispersion.
5. Place centrifuge tube(s) into a tabletop centrifuge and spin sample(s) down for 3 minutes at 1100 x g.
6. Remove sample(s) from centrifuge and discard supernatant, saving the pellet.
7. Repeat steps #4 - #6 twice more to remove any excess DMSO from cell suspension.
8. Save the cell pellet(s) in preparation for cellular staining and analysis.

Preparation of Test Cell Samples

This kit is designed to evaluate CCR3 expression on any cell line chosen by the end user. Test samples can be evaluated using live cell analysis or by cell fixation. Below are the guidelines for preparing cells for fixation.

a. 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 Wash Buffer to collect the cells. Place cell suspension into a centrifuge collection tube. Using a hemacytometer, remove a small sample and count to determine cell numbers. Also note cell viability.
4. Place centrifuge tube into a tabletop centrifuge and spin down samples for 3 minutes at 1100 x g.
5. Aspirate supernatant and discard, saving only the cell pellet.
6. Gently resuspend the cell pellet by adding 1X Fixation Buffer at 1 mL per $1x10^6$ cells. Allow to incubate on ice for 20 minutes.
7. Place centrifuge tube into a tabletop centrifuge and spin down the sample for 3 minutes at 1100 x g.
8. Remove the sample from centrifuge and discard supernatant, saving the pellet.
9. For every $1x10^6$ cells, add 5 mL of 1X Wash Buffer to cells and pipette up and down gently to ensure proper cell dispersion.
10. Repeat steps #7 - #9 twice more to remove any excess fixation buffer from cell suspension.
11. Save the cell pellet 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 - #11 as noted above (In the "For adherent cells" section)

Assay Instructions

Note: During all steps in the assay procedure, keep all reagents on ice. Test samples can be examined using live cell analysis or after cell fixation. 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: test sample, positive and negative controls as described above. Place on ice.

3. Prepare 1X Primary Antibody by adding 10 μL of 10X antibody to 90 μL of 1X Assay Buffer, for each sample of 250,000 cells. For example if running 3 tests, add 30 μL of 10X primary antibody to 270 μL Assay Buffer.
4. Add 100 μL of 1X Mouse anti-human CCR3 Monoclonal Antibody to each sample pellet consisting of 250,000 cells. If testing larger samples, add the appropriate amount of antibody to adjust for the increase of cell numbers. For example, if testing a sample of 2 million cells, add 800 μL of 1X Mouse anti-human CCR3 Monoclonal Antibody.
5. Allow primary antibody to incubate with the cell population for one hour on ice.

*(**Caution: Avoid long term cell incubations with the primary antibody. Incubations lasting longer than 90 minutes can result in non-specific staining of the wild type control cells.)*

6. After one hour incubation with the primary antibody, add 5 mL of 1X Assay Buffer and place test tube containing cell culture into a tabletop centrifuge and spin sample for 3 minutes at 1100 x g.
7. Aspirate supernatant and discard, saving only the cell pellet.
8. Gently add 5 mL of 1X Assay Buffer to disrupt the cell pellet. Pipette sample up and down to ensure complete homogeneity.
9. Replace centrifuge tube back into tabletop centrifuge and spin sample for 3 minutes at 1100 x g.
10. Repeat washing steps #7 to #9 one more time to completely remove any residual primary antibody from the cell suspension.
11. After the third washing step, place the cell pellet(s) on ice and prepare the secondary (2^{o}) antibody in the same manner as the primary antibody, adding 10 μL of 10X Secondary Antibody to 90 μL 1X Assay Buffer for each 250,000 cell sample.
12. Allow secondary antibody to incubate with the cell population for 45 minutes to one hour on ice.
13. Repeat the washing steps as described in detail in steps #6 to #10.
14. At the completion of the final washing step, resuspend the cell pellet at $0.5x10^{6}$ cells per milliliter of 1X Assay Buffer.
15. Begin cellular analysis

Calculation of Results

Determining number of molecules per cell:

As described by Schertler *et al.* (1992), a rough guide for converting expression levels has been created for GPCRs relative to maximal number of binding sites (B_{max}) determined by radioligand binding assay. For a given cell line, approximately 10^6 molecules per cell correspond to about 10 pmol/mg of membrane protein.

Example Results:

1. Determined in a radioligand binding assay, ChemiScreen™ positive control cells yield a B_{max} value of 5 pmol/mg. This corresponds to approximately 500,000 receptors per cell.
2. In flow analysis using the Guava EasyCyte™ Plus, a Mean Fluorescent Intensity (MFI) of 588 is determined for the positive control cells with 98% of the cells positively identified.
3. A native cell line (unknown) produces a MFI of 273 when evaluated on the same system, with 96% of the cells positively identified.
4. By dividing the MFI value determined by the unknown against the MFI value determined for the positive control cells, the total number of receptors can be calculated for the unknown cell line.

Total # of cell surface receptors **= Unknown cell line MFI / ChemiScreen™ "positive" Control Cells MFI**

Calculation:
273 / 588 = 0.464 or 46.4% receptors relative to the positive control

Where 46.4% of 500,000 receptors per cell equal 232,000 receptors per cell (calculated for the unknown).

Values can be further extrapolated to 2.32 pmol/mg protein for the unknown cell line

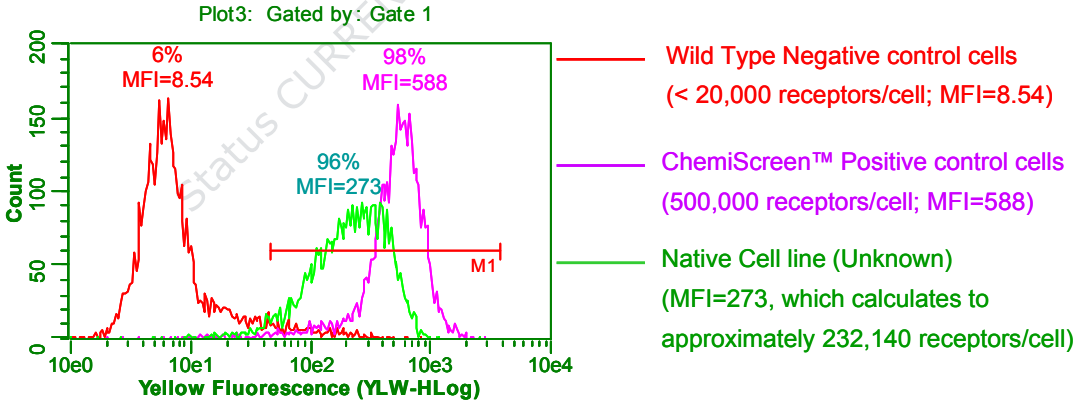
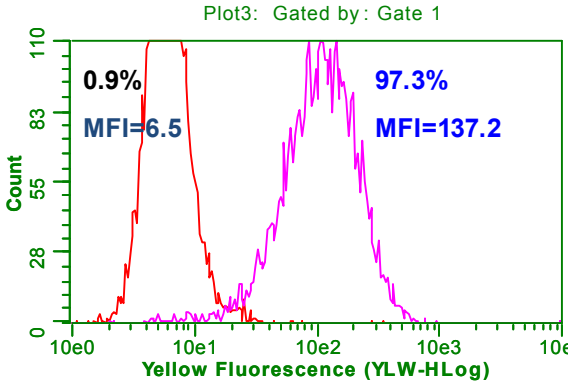


Figure 1. The histograms above illustrate how using Millipore's FlowCellect™ GPCR Surface Identification Kit can be used to quantitatively measure cell surface expression for any given cell type. The histogram on the far right (pink) represents the positive control cells, the histogram in the middle (green) represents the unknown, and the histogram on the far left (red) is the negative control cells. Because Millipore's ChemiScreen™ GPCR products have been fully characterized using Pharmacology techniques such as radioligand binding, now the researcher can use this information to calculate cell surface receptors based on a given B_{max} value and flow detected mean fluorescence intensity (MFI).

Sample Results

Using Flow Cytometry as the method for detection, Millipore's GPCR Cell Surface Identification kit will assist the researcher identify their GPCR of interest on any cell type. Moreover, the researcher will also be able to quantitatively measure the cell surface expression of a given cell by using the frozen control cells provided, making this kit a suitable alternative to traditional radioligand binding assays.



- Recombinant CCR3 ChemiScreen™ GPCR cell line (Part No. CS202144) yields an approximate B_{max} value = 2.0 pmol/mg protein,
- Chem-1 ChemiScreen™ host cell line (Part No. CS202121) yields a B_{max} value < 0.2 pmol/mg protein,
- Value conversion:

  a) ~200,000 CCR3 receptors per cell (positive control cells)

  b) < 20,000 CCR3 receptors per cell (negative control cells)

Figure 2. The histogram on the left (red) represents the negative, null control cells and the histogram on the right (pink) represent the positive control cells. As seen above, the mouse anti-human CCR3 antibody is highly selective and ideal for detecting CCR3 expression on the cell surface.

Technical Hints

- All kit buffers and antibodies should always remain at 2 - 8ºC, both prior and during use.
- Frozen control cells should be properly stored at -20ºC when not in use (cell cultures can also be stored at -80ºC for long term storage).
- 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.
Instrument Clogging	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. 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	• 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. Fujisawa T *et al.* (2000). Chemokines induce eosinophil degranulation through CCR-3. *J. Allergy Clin. Immunol.* 106: 507–513.
2. Fulkerson PC *et al.* (2006) A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc. Natl. Acad. Sci. USA* 103: 16418-16423.
3. Grimaldi JC *et al.* (1999). Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). *J. Leukoc. Biol.* 65: 846–853.
4. Schertler GFX (1992). Overproduction of membrane proteins. *Curr Opin Struct Biol.* 2: 534-544.

Related Products

1. FlowCellect™ CCR1 Surface Expression Identification Kit (Catalog # : FCCR100410)
2. FlowCellect™ CCR2b Surface Expression Identification Kit (Catalog # : FCCR200411)
3. FlowCellect™ CCR4 Surface Expression Identification Kit (Catalog # : FCCR400413)
4. FlowCellect™ CCR6 Surface Expression Identification Kit (Catalog # : FCCR600414)
5. FlowCellect™ CCR7 Surface Expression Identification Kit (Catalog # : FCCR700415)
6. FlowCellect™ CXCR1 Surface Expression Identification Kit (Catalog # : FCXR100410)
7. FlowCellect™ CXCR2 Surface Expression Identification Kit (Catalog # : FCXR200411)
8. FlowCellect™ CXCR3 Surface Expression Identification Kit (Catalog # : FCXR300412)
9. FlowCellect™ CXCR4 Surface Expression Identification Kit (Catalog # : FCXR400413)
10. FlowCellect™ CXCR6 Surface Expression Identification Kit (Catalog # : FCXR600414)

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