Fluorokine®
Biotinylated Human Tpo

Catalog Number: NFTP0
100 Tests

Reagents Provided

Biotinylated rhTpo (100 reactions): Lyophilized protein.

2 mL Avidin-Fluorescein: Avidin conjugated with fluorescein (10 µg/mL) at an f:p ratio of 5:1.

600 µL Negative Control Reagent (60 reactions): A protein (soybean trypsin inhibitor) biotinylated to the same degree as the cytokine (5 µg/mL).

300 µL Blocking Antibody (15 reactions): Polyclonal goat IgG anti-human Tpo antibody.

60 mL RDF1 10X Cell Wash Buffer: A buffered saline-protein solution specifically designed to minimize background staining and stabilize specific binding.

Reagents Not Provided

- PBS (Dulbecco's PBS)
- human or mouse IgG

Storage

All Reagents: 2° - 8° C

Intended Use

Designed to quantitatively determine the percentage of cells bearing cytokine receptors within a population and to estimate the receptor density for human cytokine stem Thrombopoietin (Tpo) on cell surfaces by flow cytometry.

Principle of the Test

Washed cells are incubated with the biotinylated cytokine that in turn binds to the cells via specific cell surface receptors. The cells are then directly incubated with avidin-fluorescein, which attaches to the receptor-bound biotinylated cytokine. Unbound biotinylated cytokine participates in an amplification reaction with the bound cytokine that results in an enhanced signal without compromising specificity. Cells expressing the specific cytokine receptors are fluorescently stained, with the intensity of staining proportional to the density of the receptors. Relative receptor density is then determined by flow cytometric analysis using 488 nm wavelength laser excitation.

Reagent Preparation

Biotinylated rhTpo: Add 1 mL of sterile distilled water to the lyophilized product. Mix gently until the product is completely dissolved. **Reagent yields optimal activity when used within 60 days of reconstitution.**

1X RDF1 Wash Buffer: Add 1 mL of 10X concentrate to 9 mL of sterile distilled water. Reagent should be maintained on ice during use and stored at 2° - 8° C for no more than 2 months.

Sample Preparation

Peripheral Blood Cells: Whole blood collected in heparinized tubes should be processed by standard Ficoll-Hypaque gradient separation techniques to isolate mononuclear cells. Ficoll and contaminating serum components should be removed by washing the cells twice with PBS and then resuspending the cells in PBS or 1X RDF1 buffer. Cells should be resuspended to a final concentration of 4×10^6 cells/mL.

Cultured Cells: Cells from continuous or activated cultures should be centrifuged at 500 x g for 5 minutes and then washed twice with PBS to remove any residual growth factors that may be present in the culture medium. Cells should then be resuspended in PBS to a final concentration of 4×10^6 cells/mL.

Note: Adherent cell lines may require pretreatment with 0.5 mM EDTA to facilitate removal from substrate. Cells that require trypsinization to enable removal from substrate should be further incubated in medium for 6 - 10 hours on a rocker platform to enable regeneration of the receptors. The use of the rocker platform will prevent reattachment to the substrate.

Sample Staining

Add 10 µL of biotinylated cytokine reagent to 25 µL of the washed cell suspension in a 12 x 75 mm tube for a total reaction volume of 35 µL. As a negative staining control, an identical sample of cells should be stained with 10 µL of biotinylated negative control reagent.

1. Incubate the cells for 30 - 60 minutes at 2° - 8° C.
2. Add 10 µL of avidin-FITC reagent to each tube. **(DO NOT WASH CELLS PRIOR TO ADDING AVIDIN-FITC)**.
3. Incubate the reaction mixture for a further 30 minutes at 2° - 8° C **in the dark**.
4. Wash the cells twice with 2 mL of 1X RDF1 buffer to remove unreacted avidin-fluorescein and resuspend the cells in approximately 0.2 mL of 1X RDF1 for final flow cytometric analysis.

Specificity Testing

1. (Optional) Cells that are to be stained could be pretreated with purified mouse or human IgG (10 µL of 1 mg/mL/10^6 cells) for 15 minutes at room temperature in order to block Fc-mediated interactions. (*Cells should not be washed of excess IgG for this assay*).
2. **In a separate tube**, 20 µL of anti-human Tpo blocking antibody is mixed with 10 µL of Tpo-biotin and allowed to incubate for 15 minutes at room temperature.
3. To the tube containing the anti-Tpo blocking antibody and Fluorokine mixture, add 1×10^5 Fc-blocked cells in a volume of 25 µL.
4. The reaction is then allowed to proceed as described in steps 1 - 4 above.

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1-800-343-7475

The human Tpo biotin kit has been tested for its ability to stain the human TF-1 and MO7e cell lines. Staining specificity has been determined through reduction in signal intensity observed when the staining reaction is carried out in the presence of 100 molar excess unconjugated Tpo or in the presence of the blocking antibody. These inhibition reactions were carried out under limiting concentrations of Tpo biotin (see technical note).

Background Information

Thrombopoietin (Tpo) is a growth factor known to stimulate megakaryocyte precursors to increase the circulating levels of platelets (1). Mature Tpo is a 332 amino acid protein that results from the proteolytic cleavage of a 21 amino acid signal peptide from the precursor protein. The predicted Tpo sequence yields a protein with a molecular weight of 35 kDa, however naturally-derived Tpo has been documented to have a large variation in molecular weight due to glycosylation differences as well further proteolytic cleavages (2, 3). There is a high degree of sequence homology (> 70%) between Tpo from different species, including human, rat , canine, mouse and porcine.

Further understanding of the action of Tpo was facilitated by the identification of its receptor c-Mpl. C-Mpl is a proto-oncogene derived from the myeloproliferative leukemia virus (4, 5). Human and mouse c-Mpl exhibit 81% sequence homology at the amino acid level (6). Alternative mRNA splicing events give rise to multiple isoforms of the receptor, including a soluble form. The Tpo receptor has been reported by various criteria to be present on hematopoietic stem cells and in cells of megakaryocytic lineage (7, 8). However controversy remains on the extent of c-mpl expression on pluripotent stem cells. Whether Tpo exerts its effects throughout the maturation process of stem cells to megakaryocytes to platelets is not clear, however evidence appears to support the notion that Tpo may have a more pronounced effect on the later stages of megakaryocytes differentiation (8 - 11). Tpo may also act in concert with a variety of other cytokines including SCF, IL-3, IL-6 and IL-11. Of interest is the finding that the ability of Tpo to induce cellular proliferation in a variety of leukemic and non-leukemic cell lines did not always correlate the level of c-mpl expression (12).

References

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Technical Notes

Some cell lines can exhibit high non-specific staining with labeled cytokines. This effect can be compensated for by reducing the amount of labeled cytokine used in the reaction. We suggest that each user determine the optimal concentration of labeled cytokine by performing a dilution curve staining analysis on known receptor positive and negative cells. Normally, dilutions ranging from 1:2 to 1:10 are sufficient. Dilution of labeled cytokine should be made in 1X RDF1 Wash Buffer.

Warning: Contains sodium azide as a preservative - sodium azide may react with lead and copper plumbing to form explosive metal azides. Flush with large volumes of water during disposal.