invitrogen™ | MOLECULAR PROBES®

PRODUCT INSERT

MONOCLONAL ANTIBODIES TO HUMAN FETAL HEMOGLOBIN

Product Code	Form	Volume	Antibody*	Excitation (nm)	Peak Emission (nm)	Matching Isotype Controls	
MHFH00†	Purified	0.5 ml	100 µg	N/A	N/A	Mouse IgG1 Purified	Code MG100
MHFH05	APC	0.5 ml	100 min.	600-650	660	Mouse IgG1 APC	Code MG105

PRODUCT DESCRIPTION

Mouse monoclonal antibody to human fetal hemoglobin (HbF)

Clone: HBF-1

Isotype: Mouse IgG1

Lot No.: See label **Expiration:** See label

Buffer: Phosphate buffered saline (PBS)

Preservative: 0.1% ***sodium azide***. Sodium azide is an extremely toxic and dangerous compound particularly when combined with acids or metals. Solutions containing sodium azide should be disposed of properly.

Stabilizer: For conjugated products only, a highly purified grade of BSA has been added as a stabilizing agent.

STORAGE & HANDLING

Store reagents at 2-8°C. For fluorochrome conjugated antibodies only, light exposure should be avoided. Use dim light during handling, incubation with cells and prior to analysis. It is recommended that investigators dilute only the quantity to be used within one week.

PRODUCT CHARACTERIZATION

Antigen Specificity: According to the literature this antibody recognizes fetal hemoglobin expressed in fetal red cells and adult F cells[4].
Leukocyte Workshop Status: N/A

PRODUCT QUALITY CONTROL

Each lot is tested by flow cytometry using FETALTROL™ fetal red cell controls. FETALTROL cells are produced by Trillium Diagnostics, LLC, Portland, ME, and distributed by Invitrogen Laboratories (Catalog # FH101). Based on this testing it is recommended that 5 µl of antibody be used per 1-2 x 10^5 red cells. Because results may vary, it is suggested that each investigator determine the optimal amount of antibody to be used for each application.

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* Antibody value assigned is based on the Optical Density at 280 nm.

† Although the antibodies indicated by the product codes MHFH00 and MHFH05 are not intended for I.V.D. use, the booklet describing the 510K clinical study for the I.V.D. product has been included for reference.

FOR RESEARCH USE ONLY ... NOT FOR THERAPEUTIC OR IN VITRO DIAGNOSTIC USE

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Anti-HbF - Flow Cytometry Protocol

Specimen:

Well mixed EDTA anticoagulated whole blood

Materials & Equipment:

1. 12x75 mm disposable polystyrene tubes (Falcon #2052), with rack
2. Pipette tips, yellow (1-200 µl) and blue (200-1000 µl)
3. Adjustable pipettes (5-40, 40-200 & 200-1000 µl)
4. Vortex mixer
5. DAC II cell washer (Baxter), optional
6. Multipurpose flow cytometer

Additional Reagents and Suggested Source:

1. PBS - 0.1% BSA

   Phosphate buffered saline (PBS, Sigma #1000-3)

   a. qs packet to 1.0 L with deionized H_20.
   b. Add 1.0 g. of Bovine Serum Albumin (BSA, Sigma #A3294).
   c. pH to 7.4.
   d. Store at 4°C.
   e. Expiration: 7 days

2. 0.05% Glutaraldehyde

   Dilute 25% Glutaraldehyde (Sigma #G5882) with PBS to .05% Glutaraldehyde.
   a. 100 µl diluted to 50 ml
   b. Store stock (25% Glutaraldehyde) at –20°C.
   c. Expiration: See container
   d. Store working solution (0.05%) at 4°C.
   e. Expiration: Make fresh each day of use
   f. Use **cold**

3. 0.1% Triton X-100

   Dilute Triton X-100 (Sigma #X100) to 0.1% with PBS-0.1% BSA 50 µl diluted to 50ml.
   a. Store stock (Triton X-100) at room temperature.
   b. Expiration: See container
   c. Store working solution (0.1% Triton X-100) at 4°C.
   d. Expiration: 30 days or visible growth
   g. Use **cold.**

4. 1% Formaldehyde

   Dilute 10% Formaldehyde - methanol free (Polysciences, Inc. #04018) with PBS-0.1% BSA to 1%, pH to 7.4.
   a. Store stock (10% Formaldehyde - methanol free) at room temperature
   b. Expiration: See container
   c. Store working solution (1% Formaldehyde) at 4°C.
   d. Expiration: 7 days
   e. Use **cold.**

Staining Procedure:

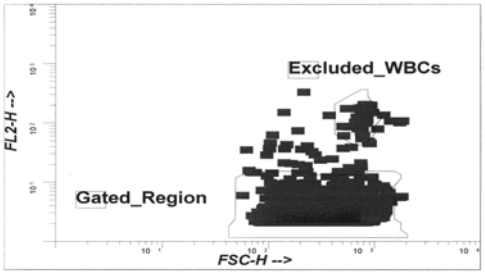
1. Measure the red blood cell (RBC) count of the specimen.
2. Fix 2.5 x 10^7 RBCs (or 10 µl Whole Blood) in 1 ml of cold 0.05% Glutaraldehyde for 10 minutes at room temperature. Vortex after the addition of cells.
3. Wash times **three (3)** with 2 ml of PBS-0.1% BSA.
4. Resuspend the cell pellet by vortex in 0.5 ml 0.1% Triton X-100. Incubate for 3-5 minutes at room temperature.
5. Wash times **one (1)** with 2 ml of PBS-0.1% BSA.
6. Resuspend the cell pellet in 0.5 ml PBS-BSA by vortexing or gentle pipetting
7. Add 10 µl of this suspension (about 1-2 x 10^5 cells) to 5 µl of the antibody and 70 µl of PBS 0.1% BSA. Incubate at room temperature (maximum) for 15 minutes.
8. Wash times **two (2)** with 2 ml of PBS-0.1%BSA. For MHFH00 follow steps 9 through 11. For MHFH05 proceed directly to step 11.
9. Resuspend cell pellet by vortexing and add the appropriate amount of fluorochrome-conjugated secondary antibody. The amount of secondary antibody added should be determined empirically by each investigator. Incubate at room temperature in the dark for 15 minutes. Invitrogen offers a number of products for this purpose (Catalog #'s M35001, M35004, M35005, M35006 and M35017).
10. Wash times **two (2)** with 2 ml of PBS-0.1%BSA.
11. Resuspend cell pellet by vortex in 0.5 ml of 1% Formaldehyde. Store tubes in the dark in the refrigerator until ready for flow cytometric acquisition.

Flow Cytometric Acquisition:

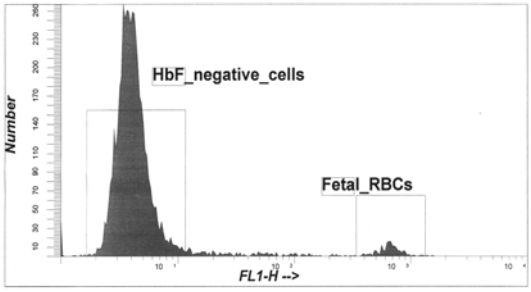
1. It is assumed that the instruments have been standardized and properly quality controlled according to laboratory procedure.
2. List mode files of at least 50,000 events should be collected for log FSC, Log SSC, and log fluorescence signals for both the fluorochrome conjugated to the anti-HbF antibody (eg. FL1 for FITC - conjugated antibody) and another signal to detect autofluorescence of leukocytes in the specimen (eg. FL2 for FITC-conjugated antibody). Compensation settings between the two fluorescence signals should be optimized to separate the autofluorescence of leukocytes and the antibody-stained HbF containing cells. This can be done using fresh blood samples with 1-5% ABO-matched newborn or cord blood added.
3. Sample handling and flow rates should be selected to minimize doublets and coincidence signals.
4. Voltage settings for the detectors are used consistently for standardizing results. A change in the laser, the FSC detector, the SSC PMT or the FL1 PMT will require adjustment of these settings. When selecting a new voltage setting, place the negative population fully within the first decade.

Data Analysis:

1. Data is analyzed using instrument software or third party offline programs. The red cell population can be gated using light scatter parameters. However, this may not be sufficient to ensure elimination of all leukocytes from analysis. With this staining procedure leukocytes show significant autofluorescence. This can be used to exclude leukocytes from analysis of HbF-containing red cells. For example:



2. Using negative and positive controls, region markers can be set to identify subpopulation positions and proportion. For example:



Quality Control:

1. Invitrogen recommends the use of FDA-cleared FETALTROL™ fetal red cell controls for this purpose. These cells are most appropriate for establishing this assay and setting markers as their values are close to expected clinical ranges. FETALTROL cells are produced by Trillium Diagnostics, LLC, Portland, ME, and distributed by Invitrogen Laboratories. (Catalog Code # FH101).
2. Cord blood or newborn peripheral blood are the best alternative sources of highly fluorescent fetal cells. See the illustration above.
3. Peripheral blood from a normal male is the best source of a negative control.
4. Mixtures of ABO matched newborn blood and normal blood, stabilized in Alsevers's Solution, provide additional controls.

5. Alsevers's Solution

   a. 0.33 g. Chloramphenicol
      0.50 g. Citric Acid $C_6H_8O_7$
      20.5 g. Dextrose $C_6H_{12}O_6$
      2.0 g. Inosine
      0.5 g. Neomycin sulphate
      4.2 g. Sodium chloride NaCl
      8.0 g. Trisodium citrate $C_6H_5Na_3O_7{\cdot}2H_2O$
   b. Dissolve citric acid, detrose, sodium chloride and trisodium citrate in approximately 600 ml of distilled water.
   c. Add chloramphenicol, inosine and neomycin sulphate, mix well.
   d. Dilute to 1.0 L with deionized water.
   e. Store at 4°C.

   Expiration: 90 days or with visible microbial growth

Limitations of Procedure:

1. Run all positive controls **after** running the unknown specimens. As this assay deals with rare event detection, positive controls may contaminate patient specimens due to instrument carry over.
2. This procedure requires a number of washes that are essential and should not be eliminated. Use of a Blood Bank type cell washer will significantly reduce the tedium of this procedure.
3. Fixed nucleated cells demonstrate high autofluorescence and should not to be