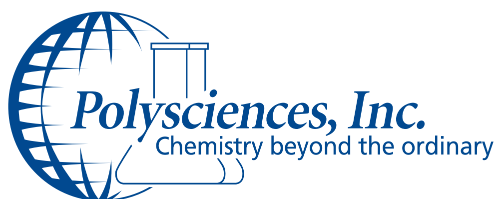

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Flow-Fix

2% Flow Fix Paraformaldehyde Fixative Kit

Directions:

1. Find Fixative solution in 500ml bottle
2. Filter 250ml 0.2 M phosphate buffer into same container as fixative solution, to make 500ml of the 2% Flow Fix
3. Final solution is 2% paraformaldehyde in 0.1 M phosphate buffer

25037A	0.2M phosphate buffer in 250ml bottle
25085B	Fixative solution in 500ml bottle
25085-1	combined MSDS info on label

Flow Fix, 2% Paraformaldehyde Fixation Buffer is a sodium azide free buffer comprised of a neutral pH-buffered saline *(i.e., Dulbecco's Phosphate-Buffered Saline)* that contains 2% w/v paraformaldehyde. Sodium Azide –Free. Do not add sodium azide to buffers if you are concerned with recovering cell function.

e.g. Sodium azide inhibits metabolic activity and is kept separate to increase shelf life. After mixing and aliquoted, you may freeze. Thawed aliquots are stable at 40°C for up to two weeks. You may store frozen aliquots for up to five years. You may aliquot solution and sterilize them by autoclaving at 20 minutes, 121°C, liquid cycle, in order to store at room temperature..

Paraformaldehyde Fixation of Hematopoietic Cells Stained with Fluorochromes

This fixation buffer is intended to preserve human and rodent lymphoid cells for the subsequent immunofluorescent staining. Flow Fix can also be used to preserve the light-scattering characteristics and fluorescence intensities of human and rodent hematopoietic cells that have been stained by immunofluorescence for subsequent flow cytometric analysis.

PREPARATION AND STORAGE

Store at 4°C and protect from prolonged exposure to light. *(See notes above)*

RECOMMENDED ASSAY PROCEDURE

Flow Fix can be used to fix unstained cells for subsequent immunofluorescent staining of intracellular cytokines. The suitability of fixing cells for immunofluorescent staining depends on whether the fluorescent antibodies can specifically detect their cognate antigens in a fixed form. With respect to intracellular cytokines. Many companies offer these types of cytokines. For the staining of antigens expressed on the surface of fixed cells, several fluorescent antibodies directed against mouse cell surface antigens have been identified to be useful. Flow Fix can also be used to fix cells after immunofluorescent staining in order to preserve the light-scattering signals and fluorescent intensities of cells for analysis at a later time. Flow Fix may be useful to avoid the capping or shedding of fluorescent antibodies and/or surface antigens during the period before flow cytometric analysis.

PROCEDURE FOR FIXING CELLS WITH FLOW-FIX

1. Pellet 10e6 suspended cells (by centrifugation (250 - 300 xg) and carefully remove supernatants to avoid cell loss.
2. Add either 200µl (for microwell plates) or 500 µl (for tubes) aliquots of cold DPBS containing protein and NaN3, gently resuspend cells, pellet, and remove supernatants.
3. Repeat step 2.
4. Add either 100 µl (for microwell plates) or 250 µl (for tubes) aliquots of fixation buffer to each cell pellet and resuspend the cells by either pipetting or vortexing. Incubate cells with fixation buffer for 15-30 minutes at 4°C. (Cell aggregation can be avoided by vortexing prior to the addition of the fixation buffer.
5. Fixed cells should be washed and suspended in a buffer that contains protein and NaN_3. Store the fixed cells at 4°C (protected from light) for subsequent immunofluorescent staining of intracellular cytokines. It is recommended that fixed cell samples be read as soon as possible, i.e., within one week.

For the immunofluorescent staining of intracellular cytokines, cells that have been previously fixed with Flow Fix can be washed two times in a buffer that contains protein and NaN_3 followed by incubating the cells for at least 10 minutes (4°C) in a buffer containing the cell-permeabilizing agent, saponin. The fixed and permeabilized cells can then be stained.

PROCEDURE FOR FIXING IMMUNOFLUORESCENTLY-STAINED CELLS WITH FLOW-FIX

Cells stained by immunofluorescence for cell surface antigens can be fixed as described above and stored (4°C, protected from light) for subsequent analysis by flow cytometry (or fluorescence microscopy).

Lanier LL, Warner NL. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J Immunol Methods. 1981; 47(1):25-30. (Methodology)

Sander B, Andersson J, Andersson U. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol Rev. 1991;119:65-93.(Methodology)

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General Procedure:

1. Harvest, wash cells and adjust cell suspension to a concentration of 1-5x10^6 cells/ml in ice cold PBS, 10% FCS, 1% sodium azide. Cells are usually stained in polystyrene round-bottom 12 x 75mm tubes. However, they can be stained in any container for which you have an appropriate centrifuge e.g. test tubes, eppendorf tubes, and 96-well round bottomed microtiter plates. In general, cells should be spun down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.

If you are using reagents with sodium azide as a preservative it is recommended to stain with ice cold reagents and at 4ºC, since low temperature and presence of sodium azide prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity.

2. Add 0.1-10 µg/ml of the primary labeled antibody. Dilutions, if necessary, should be made in 3% BSA/PBS (Propidium iodide can also be added at this point for dead cell exclusion).
3. Incubate for at least 30 minutes at room temperature or 4ºC. This step will require optimization.
4. Wash the cells 3X by centrifugation at 400g for 5 minutes and resuspend them in 500µl to 1ml of ice cold PBS, 10% FCS, 1% sodium azide. Keep the cells in the dark on ice or at 4ºC in a refridgerator until your scheduled time for analysis.
5. Analysis. For best results, analyze the cells on the flow cytometer as soon as possible. Same day analysis. You may resuspend cells in Flow Fix to prevent deterioration, which also prepares you for step three below, if you wait longer than an hour or up to several days.
6.

FIXATION FOR DIRECT/INDERECT LABELING OF CELLS

(This will stabilize the light scatter and inactivate most bio hazardous agents). Controls will require fixation using the same procedure. Cells should not be fixed if they need to remain viable. There are several methods available, please refer to fixation in the. The fixation for different antigens will require optimization by the user.

Indirect labelling requires two incubation steps; the first with a primary antibody followed by a compatible secondary antibody. The secondary (and not the primary) antibodies have the fluorescent dye (FITC, PE, Cy5, SR.Fluor, CellVue, etc.) conjugated. Please note that this is a general protocol and you may need to adapt it for your applications.

General Procedure:

1. Harvest, wash the cells and determine the total cell number.

Cells are usually stained in polystyrene round bottom 12x75 mm falcon tubes. However, they can be stained in any container for which you have an appropriate centrifuge e.g. test tubes, eppendorf tubes, and 96-well round bottom microtiter plates. In general, cells should be spun down hard enough that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.

It is always useful to check the viability of the cells it should be around 95% not less than 90%. Resuspend cells to approximately 1-5 x 10^7 cells/ml in ice cold PBS, 10% FCS, 1% sodium azide. Use cold solutions in this case 4°C.

1. Add 100µl of cell suspension to each tube.
2. Add 0.1-10µg/ml of the primary antibody. Dilutions, if needed should be made in 3% BSA/PBS.
3. Incubate for at least 30 minutes at 4°C or room temperature in the dark.
4. Wash cells 3X by centrifugation at 400g for 5 minutes and resuspend them in ice cold PBS. You may need to adjust the conditions of the centrifugation (the force and the time) for the cell types used.
5. Dilute the fluorochrome-labeled secondary antibody in 3% BSA/PBS at the optimal dilution (according to the manufacturer's instructions) and then resuspend the cells in this solution.
6. Incubate for at least 20-30 minutes at 4°C or room temperature. This incubation must be done in the dark.
7. Wash cells 3X by centrifugation at 400g for 5 minutes and resuspend them in ice cold PBS, 3% BSA, 1% sodium azide.
8. Store the cell suspension immediately at 4°C in the dark.
9. Analysis: For best results, analyze the cells on the flow cytometer as soon as possible.

Controls will require fixation using the same procedure. Cells should not be fixed if they need to remain viable. There are several methods available. The fixation for different antigens will require optimization by the user.

1. Flow Fix for 10-15 minutes only, 100µl per sample.
2. Acetone or methanol

NB polystyrene/plastic tubes are not suitable for use with acetone.

Add 1ml ice-cold acetone to each sample. Mix gently. Place at -20°C for 5-10 minutes. Centrifuge, wash twice in PBS 1% BSA.

PROTOCOL FOR IMMUNOCYTOCHEMISTRY

Solutions

1. FlowFix, 4% paraformaldehyde
2. 100mL 0.05% (v/v) Tween-20 in PBS (PBT)
3. 10mL 1% (w/v) BSA in PBT
4. 20mL 0.1% BSA in PBT (diluted from the 1% solution)

Protocol

1. Plate cells in a 4 chamber glass slide.
2. Pour off the media from the wells, wash each chamber twice quickly with 0.5mL PBS, then fix the cells by adding 0.5mL FlowFix, 4% paraformaldehyde to each chamber and incubating for 15 minutes.
3. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each, and then incubate the cells for 30 minutes. in 0.5mL of 1% BSA.
4. Wash cells in PBT 3X as previously described, then incubate cells in 0.2mL of the working concentration of the primary antibody (about 1:200), diluted in 0.1% BSA, for 60 minutes at room temperature.

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5. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each.
6. Incubate cells in 0.2mL of the working concentration of the second antibody (about 1:500), diluted in 0.1% BSA, for 40 minutes at room temperature.
7. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each.
8. Incubate cells in the Streptavindin-HRP (three drops) for 40 minutes at room temperature.
9. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each.
10. Incubate the cells in 0.2mL of ABS for 1 minute at room temperature.
11. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each.
12. Add three drops of Hematoxylin for 5 minutes at room temperature.
13. Wash cells 3X in 0.5mL PBT (per chamber), 5 minutes each. Once again fix cells for 15 minutes in 0.5mL of 4% PFA.
14. Remove the plastic chamber piece and sealer holding it in place completely, then place 1 drop of mounting solution Aqua Polymount on each sheet of cells and add coverslips to each sheet.

IMMUNOHISTOCHEMISTRY PROCEDURES

Day 1

1. Deparaffination - Heat slides on warmer that has been preheated at 65°C-70°C until wax melts (~15 minutes) (this is heat induced epitope retrieval-HIER or use L.A.B solution). Prepare 40mL (plastic bottle) or 45mL (glass bottle for xylene) of each of the following solutions and place slide in bottle for specified amount of time.

Xylene 1	5 min	70% EtoH	5 min
Xylene 2	5 min	30% EtoH	5 min
100% EtoH	5 min	H_2O	2 min
95% EtoH	5 min	H_2O	2 min

2. Put slides in beaker of 0.01M citrate buffer (pH=6.0), and place this beaker in a separate larger beaker of boiling water. Citrate buffer should be 92°C-98°C when slide placed in it. Use L.A.B. here or catalog numbers of citrate buffer. To prepare citrate buffer, use 18mL buffer A, 82mL buffer B, 900mL water.

   Buffer A: 0.1M sodium citrate (29.41g sodium citrate/1000mL H_2O).
   Buffer B: 0.1M citric acid (9.56 g citric acid/500mL H_2O).

   Keep slides in citrate buffer (over boiling water) at this temperature for 20 minutes. Allow slides to cool in citrate buffer to room temperature.

3. Wash slide with 3% H2O2 (1.5 mL H2O2 in 48.5 mL MeOH) for 10 minutes.
4. Obtain container to hold slides and place wet paper towel in bottom to keep tissue environment moist at all times. Draw around the tissue pieces on the slide with pap pen to prevent leaking. Wash with PBS (rinsing buffer) for 2 minutes, twice. To prepare rinsing buffer, follow kit instructions for specific volume. In between and after washes dab back of slide, and front of slide around tissue to dry.
5. Incubate in block serum (is the same as the secondary antibody) for 30 minutes. Use 2 drops per tissue section.
6. Incubate with the primary antibody at 4°C overnight. Dilute primary antibody at 1:50. Keep primary antibody on ice at all times. Use 100µL per slide (so 2µL primary antibody diluted with 98µL PBS). Put 50µL of this solution on each tissue section (or enough to cover).

Day 2

7. Wash with PBS for 5 minutes, twice.
8. Incubate with the secondary antibody for 30 minutes at room temperature. Use 2 drops for each tissue section.
9. Wash with PBS for 5 minutes, twice.
10. Incubate with streptavidin-HRP for 30 minutes at room temperature.
11. Wash with PBS for 5 minutes, twice.
12. Add 50µL of DAB (chromagen reagent) to each slide. For preparation of DAB solution, see kit instructions. Keep container of DAB solution covered in foil because it is light sensitive. Look at slide under microscope. When you see the backgroud turn brown, wash in H_2O for 10 seconds.
13. Counterstain with Heamatoxylin for 5 minutes. Use 50µL for each tissue section (or enough to cover). Observe slide under microscope.
14. Wash with H_2O for 5 seconds, twice.
15. Place in 95% EtoH for 15 seconds.
16. Place in 100% EtoH for 15 seconds.
17. Place in Xylene for15 seconds.
18. Add 2 drops mounting solution Aqua Polymount to slide and add coverslips to each tissue section to save slides.

IMMUNOFLUORESCENT STAINING PROCEDURES

Day 1

1. Deparaffination - Heat slides on warmer that has been preheated at 65°C-70°C until wax melts (~15 minutes). Prepare 40mL (plastic bottle) or 45mL (glass bottle for xylene) of each of the following solutions and place slide in bottle for specified amount of time (keep solutions fresh weekly), using the following sequence.

Xylene 1	5 min	70% MeoH	5 min
Xylene 2	5 min	30% MeoH	5 min
100% MeoH	5 min	H_2O 1	2 min
95% MeoH	5 min	H_2O 2	2 min

2. Put slides in beaker of 0.01M citrate buffer (pH=6.0), and place this beaker in a separate, larger beaker of boiling water. Citrate buffer should be 92°C-98°C when slide placed in it.
3. Keep slides in citrate buffer (over boiling water) at this temperature for 20 minutes. Allow slides to cool in citrate buffer to room temperature.
4. Obtain container to hold slides and place wet paper towel in bottom to keep tissue environment moist at all times. Draw around the tissue pieces on the slide with pap pen to prevent leaking. Wash with PBST (PBS with 0.1% Tween 20) for 10 minutes, twice. In between and after washes dab back of slide and front of slide around tissue to dry.
5. Incubate in block serum (is the same as the secondary antibody) for 30 minutes. Dilute to 10% with 1% milk.
6. Incubate with the primary antibody at 4ºC or room temperature overnight. Dilute primary antibody at 1:50 with 1.5% serum and 0.5% milk. Keep primary antibody on ice at all times. Put 50µL of this solution on each tissue section.

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Day 2

7. Wash with PBST for 10 minutes, 3X.
8. Incubate with the FITC-conjugated secondary antibody for 30 minutes at room temperature. Dilute 1:200 in PBST. Put 50µL of this solution on each tissue section.
9. Wash with PBST for 10 minutes, 3X.
10. Place the slides on the towel and use the cotton tip dipping with xylene cleaning the PAP pen trace carefully. Don't touch any tissue section edge.
11. Cover with Vecta shield for delay fluorescence quenching.

NEGATIVE CONTROL (OF THE PRIMARY ANTIBODY)

Incubate with the block serum (the same as the secondary antibody).

PARAFORMALDEHYDE FIXATION OF HEMATOPOIETIC CELLS STAINED WITH FLUOROCHROMES

This procedure is used to preserve hematopoietic cells stained with fluorochrome conjugated monoclonal antibodies for subsequent analysis by fluorescence microscopy or flow cytometry. The fixed cells may be stored for at least one week at 4°C in the dark. This method of fixation does not significantly alter the light scatter or fluorescence properties of human lymphoid cells or transformed cell lines.

CELLS

Human cells stained by a direct or indirect immunofluorescence technique *(See Direct Immunofluorescence Staining of Cell Surfaces and Indirect Immunofluorescence Staining of Cell Surfaces)*.

REAGENT Flow-Fix

EQUIPMENT

Refrigerated centrifuge fitted with swinging bucket rotor (4°C)

Ice bucket

PROCEDURE

1. Following the last incubation with antibody or fluorescent second-step reagent, centrifuge the cells at 250 x g for 10 minutes. Remove the supernatant.
2. Add 1mL of cold 1X PBS per 106 stained cells. Gently vortex the cells and centrifuge at 250 x g for 10 minutes. Remove the supernatant.
3. Repeat Step 2. Loosen pellet gently.
4. Add 0.3 to 1.0mL of cold Flow Fix solution to the pellet. Vortex the cell suspension immediately.
5. Store the fixed cells at 4°C in the dark.

NOTE

Cells may also be fixed following staining in microtiter plates using these modifications:

i) Following the last staining step, centrifuge the cells (~106/well) in microtiter plates at 250 x g for 5 minutes. Carefully remove the supernatant.

ii) Add 100µL PBS to each well. Centrifuge at 250 x g for 5 minutes. Carefully remove the supernatant.

iii) Repeat Step (ii).

iv) Add 100µL of cold Flow Fix solution to each well and mix thoroughly at once.

v) Store covered in the dark as above. Transfer into tubes for analysis. Dilute to proper volume with Flow Fix, 1% . Cells fixed at least 4 hours may be diluted in PBS.

REFERENCE

Lanier LL, Warner NL. Paraformaldehyde Fixation of Hematopoietic Cells for Quantitative Flow Cytometry (FACS) Analysis. J Immunol Meth. 1981;47:25

ORDERING INFORMATION

Cat. #	Description	Sizes
25037	Flow-Fix, 1% Paraformaldehyde Fixative Kit	500 ml - 1L

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In The U.S. FAX: 1-800-343-3291 • 215-343-0214

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