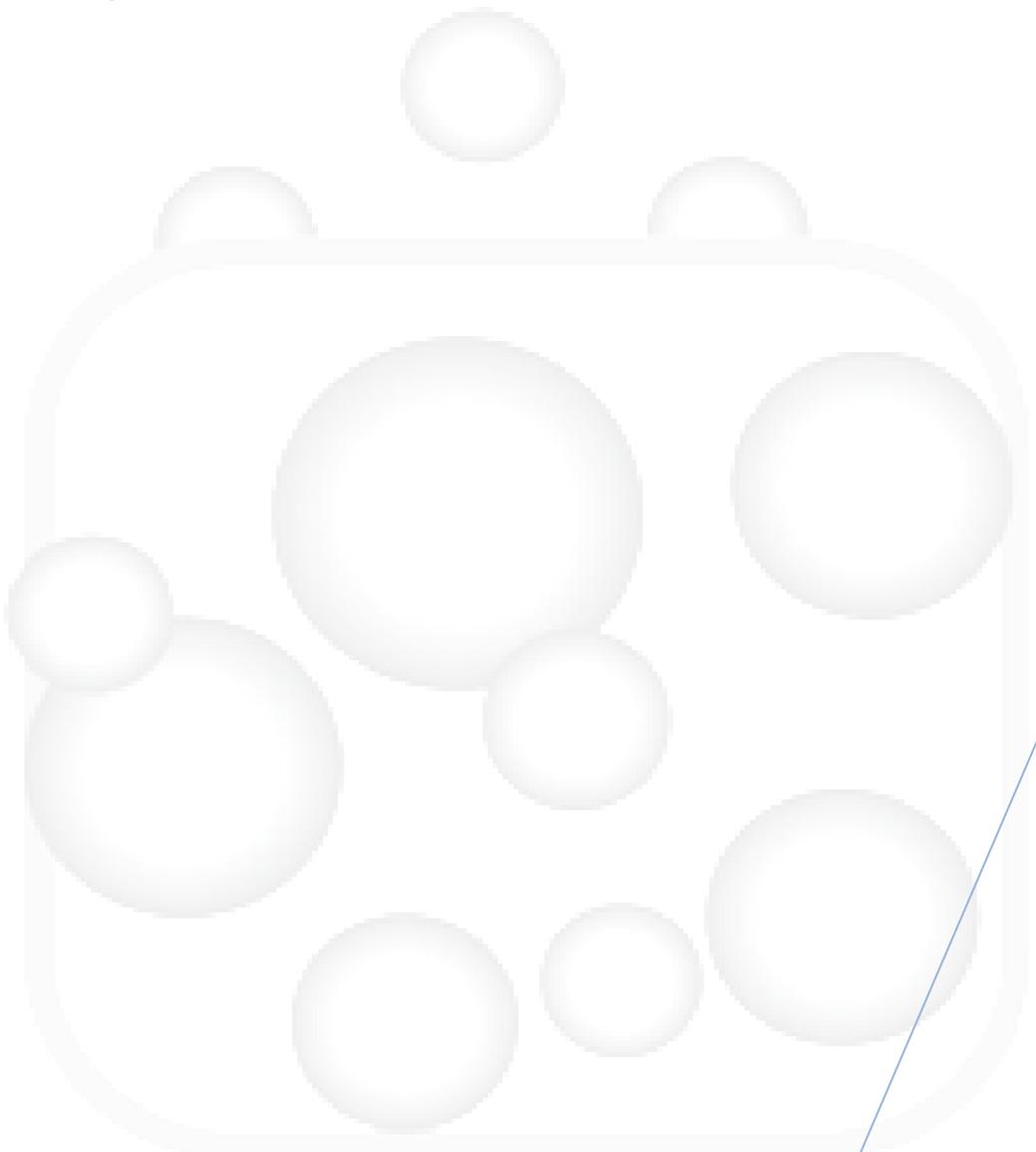




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**Pharmaceutical Nanotechnology**  
Tabriz University of Medical Sciences

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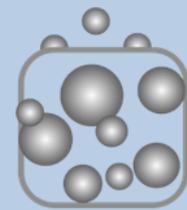


## **Flow Cytometry (FACS) Protocols**

PSR

The BD FACSCalibur™ platform allows users to perform both cell analysis and cell sorting in a single benchtop system. The system supports a wide variety of research and clinical applications and is complemented by a broad suite of intuitive software solutions to streamline analysis for a wide range of applications including enumeration of lymphocyte subsets, stem cells, residual white blood cells, and reticulocytes.

Golchin. A  
**12/30/2015**



RCPN

## Table of Contents

Cell Preparation Flow Cytometry Protocols .....	77
I. Preparation of human peripheral blood mononuclear cells (PBMC).....	77
II. Preparation of cells for Flow Cytometry .....	79
III. Preparation of peritoneal macrophages, bone marrow, thymus and spleen cells.....	81
Direct Staining Flow Cytometry Protocols .....	82
I. Direct immunofluorescence staining surface epitopes of cells and blood.....	82
II. Direct immunofluorescence staining of immunoglobulin light chains on B lymphocytes in whole blood.....	83
Indirect Staining Flow Cytometry Protocols .....	85
Indirect immunofluorescence staining of surface epitopes of cells and blood .....	85
Intracellular Staining Flow Cytometry Protocols .....	90
I. Direct immunofluorescence staining of intracellular cytokines in blood .....	90
II. Direct immunofluorescence staining of intracellular antigens: Methanol plus Leucoperm™ Accessory Reagent method.....	91
III. Direct Immunofluorescence Staining of Intracellular Antigens: Digitonin Method.....	93
Cell Cycle Staining Flow Cytometry Protocols.....	95
I. Immunofluorescence staining of cells in combination with PI staining of cells for cell cycle analysis .....	95
II. Propidium iodide staining of cells for cell cycle analysis.....	97
III. BrdU staining of cells for cell cycle analysis.....	98
References.....	100

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The BD FACSCalibur™

## Cell Preparation Flow Cytometry Protocols

### I. Preparation of human peripheral blood mononuclear cells (PBMC)

Leukocytes are the most commonly analyzed cells in flow cytometry. Leukocytes can be obtained from whole blood and a variety of tissues, such as spleen, lymph node, bone marrow and thymus. Red blood cells (RBC) present in whole blood or cell preparations are a contaminant in flow cytometry assays that must be removed or lysed in order to properly gate leukocytes.

Leukocytes in whole blood are predominately lymphocytes, macrophages/monocytes and granulocytes. If you are interested in staining granulocytes, you need only lyse the RBC. If you are interested in purifying peripheral blood mononuclear cells (lymphocytes, and macrophages/monocytes only), you must purify your PBMC using a density gradient procedure.

**Lysing Red blood Cells:** Many commercially available reagents are available that lyse RBCs. Most are simply ammonium chloride solutions. RBC lysis buffer can easily and inexpensively be made:

- 10x RBC Lysis Buffer

- 90 g. NH<sub>4</sub>Cl (0.155M)
- 10 g. KHCO<sub>3</sub> (0.01M)
- 370 mg. EDTA (0.1mM)
- Dissolve in one liter of ddH<sub>2</sub>O
- Filter sterilize through .22 micron filter

This solution can be stored at 4°C for months. Dilute 1:10 in ddH<sub>2</sub>O before use. RBC may be lysed before or after staining. Mix 200 µL of whole blood with 2 mL of lysis buffer, incubate at room temperature for 5 minutes, spin down at 300 x g in order to remove lysis buffer. Repeat if necessary. The cells are ready for analysis or staining.

**Note:**

Leukocytes should not be left in lysis buffer for extended periods of time, as cell morphology and viability will be negatively affected.

The figure on the left displays incomplete lysis of RBCs (and inadequate FSC voltage), the figure on the right displays optimal lysis of RBCs.

**PBMC isolation:**

Human PBMC are isolated using a density gradient technique. The two most commonly used density gradient solutions are Ficoll-Paque PLUS from GE Healthcare Life Sciences and Histopaque-1077 from Sigma Aldrich.

The separation can be done in either 15 or 50 mL conical tubes.

1. Aliquot 1 mL of Ficoll-Paque PLUS or Histopaque-1077 for every mL of peripheral blood to be processed. Let Ficoll-Paque PLUS or Histopaque-1077 warm to room temperature as density is temperature-dependent.
2. Mix 1 mL of blood with 1 ml of a balanced salt solution, normally PBS.
3. Overlay the mixed blood solution on top of the Ficoll-Paque PLUS or Histopaque-1077. Ratio should be 2 mL of the mixed blood + PBS solution for every 1 mL of Ficoll-Paque PLUS or Histopaque-1077.
4. Centrifuge at 400 x g for 35 minutes at room temperature. During this step, the granulocytes, platelets and RBCs pellet to the bottom of the tube and the PBMC float over the Ficoll-Paque PLUS or Histopaque-1077.
5. Carefully aspirate the PBMC from the Ficoll-plasma interface.
6. Wash the PBMC with PBS at 300 x g at 4°C twice.

If you are working with murine whole blood or other tissues, the density gradient product Lympholyte ([https://www.cedarlanelabs.com/Reagents/Listing/Lympholyte\\_Special](https://www.cedarlanelabs.com/Reagents/Listing/Lympholyte_Special)) has been manufactured at the specific density for the purification of murine lymphocytes. The isolation procedure is similar to the Ficoll procedure.

## II. Preparation of cells for Flow Cytometry

For the preparation of single cells derived from tissue culture cell lines.

Single cells must be suspended at a density of  $10^5$ - $10^7$  cells/mL to keep the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2,000-20,000 cells/second. Phosphate Buffered Saline (PBS) is a common suspension buffer.

The most straight forward samples for flow cytometry are non-adherent cells from tissue cell culture. Here we describe methods for both tissue culture cell lines and adherent tissue culture cell lines. However analysis may be required from cells derived from other sources, Bio-Rad has a protocol (FC3) for the preparations of tissues from a variety of sources including; peritoneal macrophages, thymus cells, spleen cells and bone marrow. Further to this a protocol (FC2) is also available for the sample preparation of human peripheral blood mononuclear cells.

### Reagents

- Phosphate buffered saline (BUF036A) containing 1% bovine serum albumin (PBS/BSA).
- PBS.
- 1x Accutase solution
- Trypsin 0.25%

### Methods

#### Preparation of cells stored in liquid nitrogen

1. Prepare PBS/BSA.
2. Carefully remove cells from liquid nitrogen storage.
3. Thaw cells rapidly in a 37°C water bath.
4. Resuspend cells in cold PBS/BSA buffer and transfer them to a 15 ml conical centrifuge tube.
5. Centrifuge at 300-400 g for 5 min at 4°C.

6. Discard supernatant and resuspend pellet in an appropriate amount of cold (4°C) PBS/BSA, such as  $10^7$  cells/mL.

**Note:** higher viability can be obtained by allowing the cells to recover in culture media overnight.

### **Preparation of tissue culture cell lines in suspension**

This method provides a general procedure for use with tissue culture cells in suspension.

1. Prepare PBS/BSA. Decant cells from tissue culture flask into 15 ml conical centrifuge tube(s).
2. Centrifuge at 300-400 g for 5 minutes at room temperature.
3. Discard supernatant and resuspend pellet in 10 mL of room temperature PBS/BSA.
4. Centrifuge at 300-400 g for 5 minutes at room temperature.
5. Discard supernatant and resuspend to a minimum concentration of  $1 \times 10^7$  cells/mL in cold (4°C) PBS/BSA.

### **Preparation of adherent tissue culture cell lines**

This method provides a general procedure for use with adherent tissue culture cells

1. Prepare PBS/BSA.
2. Harvest cells by enzymatic release using 1x Accutase solution or 0.25% trypsin, followed by quenching with media containing serum. (Note: epitopes may be cleaved when using the enzymatic digestion method. Cells can also be harvested by gently scraping them into culture media).
  - a) Remove the culture medium and eliminate residual serum by rinsing cell monolayers with sterile, room temperature PBS.
  - b) Slowly add 1x Accutase solution or 0.25% Trypsin to cover the cell monolayer.
  - c) Incubate at 37°C for up to 10 minutes.
  - d) After incubation gently tap the flask and the cells will detach and slide off in one sheet to the bottom of the flask.
  - e) Add growth medium and re-suspend the cells by gently pipetting.

3. Centrifuge at 300-400 g for 5 min.
4. Discard supernatant and resuspend pellet in fresh, room temperature PBS/BSA to wash off any remaining cell debris and proteins.
5. Centrifuge at 300-400 g for 5 minutes at room temperature.
6. Discard supernatant and resuspend pellet in an appropriate amount of room temperature PBS/BSA.
7. Count cells using a hemocytometer or an automated cell counter such as the TC20™ Automated Cell counter.
8. Once counted dilute the cells with cold (4°C) PBS/BSA to a minimum concentration of  $1 \times 10^7$  cells/mL.

### III. Preparation of peritoneal macrophages, bone marrow, thymus and spleen cells

This method provides a general procedure for use with cell suspension cells acquired from the peritoneum, bone marrow, thymus and spleen. A certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications.

#### Reagents

1. Phosphate buffered saline (BUF036A) containing 1% bovine serum albumin (PBS/BSA).
2. Ammonium chloride lysis buffer: 0.16M ammonium chloride, 0.17M Tris, pH7.20.
3. optional PBS/BSA with 25 µg/mL DNase I or 5 mM EDTA to reduce cell aggregates

#### Methods

1. Prepare a single cell suspension from relevant tissue. Keep cells on ice to minimize cell death, which can lead to cell aggregation. Addition of DNase I or EDTA can also reduce aggregation. Large aggregates can be removed by passing through a 40 µm cell strainer.
2. Centrifuge at 300-400 g for 5 minutes at 4°C.
3. Discard supernatant and re-suspend pellet in 10 mL ammonium chloride lysis buffer.
4. Mix and incubate for 2 minutes at 4°C. **Do not exceed this time.**
5. Centrifuge at 300-400 g for 5 minutes at 4°C.

6. Discard supernatant and resuspend pellet in 10mL cold (4°C) PBS/BSA
7. Centrifuge at 300-400 g for 5 minutes at 4°C.
8. Discard supernatant and re-suspend pellet to a final volume of 10ml with cold (4°C) PBS/BSA.
9. Count cells using a hemocytometer or an automated cell counter such as the TC20™ Automated Cell Counter.
10. Adjust suspension if necessary to give a final count of  $0.7 - 1.2 \times 10^7/\text{mL}$ .

### Notes

- The following should be considered when designing your Flow Cytometry experiments:
  - To avoid unspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents e.g. Bio-Rad's Mouse Seroblock reagent.
  - Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion;
    - a) Isotype controls used to determine if the staining is specific.
    - b) Unstained cells should always be included in the experimental set-up to monitor autofluorescence.
- For all multi-color Flow Cytometry experiments it is advisable to include compensation controls and Fluorescence minus One (FMO) controls, which assist with identifying gating boundaries.

## Direct Staining Flow Cytometry Protocols

### I. Direct immunofluorescence staining surface epitopes of cells and blood

Applicable when the fluorophore is directly linked to the primary antibody, for example, RPE, FITC, and Alexa Fluor conjugates. RPE conjugates should always be handled in the dark.

#### Note:

Specific methodology for blood appears in [ ] brackets.

### Reagents

- Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- Erythrolyse red blood cell lysing buffer (AbD Serotec product code BUF04)

- Anticoagulant (Note: for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (Note: dissolve on heated stirrer and cool before use)

## Method

1. Prepare cells appropriately; refer to the Preparation of Cells for Flow Cytometry section for further information. Adjust the cell suspension to a concentration of  $1 \times 10^7$  cells/mL with cold (4°C) PBS/BSA.

[Whole blood samples may be used undiluted unless the cell count is high, as in leukemia samples. Use appropriate anticoagulant.]

2. Aliquot 100  $\mu$ L of the cell suspension [or whole blood] into as many test tubes as required.
3. Add antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 min, avoiding direct light.
4. Wash cells with 2 mL cold (4°C) PBS/BSA, centrifuge at 300–400 g for 5 min at 4°C, and discard the resulting supernatant.

[To the blood suspension add 2 mL freshly prepared erythrolyse red blood cell lysing buffer and mix well. Incubate for 10 min at room temperature. Centrifuge at 300–400 g for 5 min at room temperature and discard the supernatant. Wash with 2 mL room temperature PBS/BSA, centrifuge at 300–400 g for 5 min at room temperature, and discard the supernatant. Proceed to step 5.]

5. Resuspend cells in 200  $\mu$ L cold (4°C) PBS or with 200  $\mu$ L 0.5% paraformaldehyde in PBS if required.
6. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

## II. Direct immunofluorescence staining of immunoglobulin light chains on B lymphocytes in whole blood

The immunofluorescent staining of immunoglobulin expression by B lymphocytes in whole blood requires a procedure to remove serum immunoglobulin (which otherwise interfere and block staining with immunoglobulin-specific antibodies). This method provides a general procedure for use with the majority of Bio-Rad reagents. In some cases specific

Recommendations are provided on product datasheets, and these methods should always be used in conjunction with the product and batch specific information provided with each vial. A certain level of technical skill and immunological knowledge is required for the successful design and

implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications.

## Reagents

- Phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA).
- PBS
- Erythrolyse red blood cell lysing buffer
- Anticoagulant (Note: for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (Note: dissolve on heated stirrer and cool before use)

## Method

1. Collect blood into appropriate anticoagulant such as EDTA, heparin or acid citrate dextrose.
2. Aliquot 2-3 mL of whole blood into a 50 mL centrifuge tube. Add 20-25 mL of PBS/BSA, pre-warmed to 37°C and mix well.
3. Centrifuge at 300-400 g for 5 minutes at 37°C. Carefully aspirate the supernatant, taking care not to disturb the cell pellet, and resuspend the pellet in the residual supernatant.
4. Repeat steps 3 and 4 twice more for a total of three washes.
5. Resuspend in 2-3 mL cold (4°C) PBS/BSA.
6. Aliquot 100 µL of the washed blood into the required number of test tubes.
7. Add appropriate volume of antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 minutes, avoiding direct light.
8. Add 2 mL of freshly prepared erythrolyse red cell lysing buffer and mix well.
9. Incubate for 10 minutes at room temperature.
10. Centrifuge at 300 - 400 g for 5 minutes, discard supernatant.
11. Wash with 2 mL of room temperature PBS/BSA, centrifuge at 300-400 g for 5 minutes at room temperature and discard the supernatant.

12. Resuspend cells in 200  $\mu\text{L}$  cold ( $4^{\circ}\text{C}$ ) PBS or with 200  $\mu\text{L}$  of 0.5% paraformaldehyde in PBS if required.
13. Acquire data by Flow Cytometry. Analyze fixed cells within 24 hours.

### Notes

- The cell washing procedure described above has no effect upon other cell surface antigens, and has been shown not to affect the recovery of individual cell subsets.
- To avoid unspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents e.g. Bio-Rad's Mouse Seroblock reagent.
- Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion;
  - Isotype controls used to determine if the staining is specific.
  - Unstained cells should always be included in the experimental set-up to monitor autofluorescence.
- For all multi-color Flow Cytometry experiments it is advisable to include compensation controls and Fluorescence minus One (FMO) controls, which assist with identifying gating boundaries.

## Indirect Staining Flow Cytometry Protocols

### Indirect immunofluorescence staining of surface epitopes of cells and blood

#### What to bring

- Your filtered cells
- Extra media/buffer
- Tubes to sort into with your media in the tubes (or whatever buffer you want your sorted cells to land in)
- If using the cloning sort option, your 96 well plates with media in each well
- A sample of unstained cells, pos &/or neg controls, and compensation (single color) controls for each stain

#### Important! Biohazard precaution!

Due to the fact that cell sorting generates aerosols and the cells are generally not submitted in any fixative, the biohazard risks are much greater than in analytic flow cytometry done on a machine without a jet-in-air flow nozzle. It is therefore imperative to discuss any biohazard issues with Leslie before setting up your experiment. At CSU, we do have an aerosol evacuation

system which removes micro droplets and particulates greater than 0.12 micron. They are removed under vacuum, and trapped in an Ultra Low Penetration Air (ULPA) filter. Please inform Leslie of the Biological Safety Level (BSL) of your sample.

## Sample Preparation

The single most important issue for a successful sort would have to be proper sample preparation. This can be broken down into four separate components:

1. Single Cell Suspensions
2. Optimized Sample Concentration
3. Proper Sort Buffer Recipe
4. Expedient Sample Processing

### Single Cell Suspensions

In order for the sorter to function properly and to be able to deliver the proper results, the cells must be in a single cell suspension, and remain that way for the duration of the sort. This becomes a more important factor when working with adherent cell lines or tissues. Achieving a single cell suspension is the goal for a perfect sample preparation. One of the easiest tricks is to remove any large aggregates is by filtration (typically through fine 30-50um nylon mesh). There are two options. The first option, and the easiest, is to use a 12x75 mm Falcon test tube with a cell strainer cap. Simply pipet the cell suspension through the top of the cap. The second option is a little more involved. To minimize sample loss, use the techniques described below. The following tools are required (sterilize as necessary):

- 1 syringe sufficient to hold sample and one additional mL (retain cap)
- 1 Luer-Lock needle (typically 18 gauge)
- 1 *15mm x 15mm* piece of nylon mesh (with a 30-50 um mesh)
- 1 razor blade

The next step is to aspirate the sample up through the syringe needle such that your sample is loaded along with at least 1.0mL of air as a void volume.

Once the sample has been aspirated along with the 1.0mL of void volume, carefully dispose of the needle. Maintain the syringe in an upright position so that the void bubble remains at the tip of the syringe.

The next step is to sandwich the piece of nylon mesh over the tip of the syringe using the original syringe tip. The idea is to use the tip to hold the filter mesh in place. Once the tip is in place, carefully cut off the first few mm of the tip. This will allow a hole for the filtered sample to pass through. Additionally, take care to maintain the void bubble towards the tip so no sample is accidentally lost during this step.

At this point you need to invert the needle so the void bubble rises to the plunger. Now the sample is ready to be passed through the filter mesh and the void bubble should ensure no sample remains in the syringe.

## **Optimized Sample Concentration**

Cells must be at the proper concentration in order for the sorter to function optimally. Simply put, cells that are too concentrated will have a lower recovery due to coincidence aborts (two cell that are too close together will be rejected by the machine in order to ensure purity) and cells that are too dilute will have a longer processing time (or if they are processed faster, an increased signal CV).

## **Ideal Cell Suspension Concentrations**

Having the sample too concentrated, or too diluted can be problematic. There is no ideal concentration that works for all cell types and sort set-ups. It is a matter of understanding some of the issues and deciding what factors are most relevant to a given cell type and experimental design.

- **Coincidence Aborts**

While the sorter is evaluating which cell to sort, it must also determine whether it can do so in a manner that ensures the sorted material remains pure. The sorter makes this decision based on the proximity of events in time. If the desired event is too close to a potential undesired event, the machine will abort (not sort) the desired event to ensure purity. There are other modes of sorting that favor recovery over purity, so if total cell numbers are more important than purity, we can accommodate this. This should explain why we would not want the sample so concentrated that it becomes difficult to space the cells far enough apart while they are going through the sorter. If recovery is a prime concern, aborts tend to be the issue.

- **Signal CVs and Sensitivity**

If the sample is too dilute, to get them to run at a reasonable rate can cause another set of problems. To get them to run at the appropriate rate, the sample differential must be increased. If the differential is too high, the CV's (coefficients of variation) start to become higher. This ultimately leads to less resolution and lower sensitivity. If you are trying to separate two very close populations, the CV becomes more important.

- **Cell Adhesion and Clumping**

Adherent cells are trickier to sort than suspension cell types. Adherent cells typically like to stick to each other given the opportunity. Careful cell preparation and media can help avert this problem, but so can cell concentration. These sticky cell types typically like to be slightly more dilute to mediate clump formation. There is no absolute formula for this, it is determined by trial and error for a given cell type. The take home message is to consider running very sticky samples slightly more dilute than the below suggestions.

The following is a list of concentration ranges based on machine set-up which typically correlates with cell type:

Nozzle Size	Cell Types	Concentration (per mL)
70 um	lymphocytes, thymocytes	8-15 x 10 <sup>6</sup>
80 um	activated subsets, smaller cell lines	7-10 x 10 <sup>6</sup>
100 um	larger adherent cells	5-9 x 10 <sup>6</sup>

These guidelines are in place to suggest where to start with, but it might be easier to tend towards a little too concentrated (towards the high end of the recommended range), but bring some additional sample buffer with you so things can be diluted as necessary.

### Proper Sort Buffer Recipe

This is probably one of the most important factors to achieve an ideal sort. A properly designed buffer recipe will help maintain a single cell suspension as well as keep the cells in a good physiological state. Culture media is typically a poor sort buffer (although it can be modified).

The proper design of sort buffer for both your pre-sort sample and your collected sample is crucial for a successful sort. The following will be a basic recipe and some suggestions for modifications that might be relevant to your particular experiment. Culture media is not an ideal sort buffer for two reasons: the pH regulation fails under normal atmosphere causing the media to become basic and the calcium chloride in most culture medias is not compatible with the phosphate component of the instrument sheath buffer (the Basic Sorting Buffer without additional protein) leading to precipitation of calcium phosphate crystals. Following the suggested recipes below will help maximize the recovery and viability of your sorted cells.

#### Basic Sorting Buffer:

- 1x Phosphate Buffered Saline (Ca/Mg<sup>++</sup> free)
- 1mM EDTA
- 25mM HEPES pH 7.0
- 1% Fetal Bovine Serum (Heat-Inactivated)
- 0.2um filter sterilize, store a 40C
- antibiotics (pen and strep)

#### Optioal Collection Media:

- Fetal Bovine Serum only  
or
- Your own culture media with antibiotics  
or

- PBS if collecting cells for RNA or DNA

### **For Clean Lymphoid Cells**

The buffer can be simplified to HBSS with 1% FBS. The additional cations in the recipe promote better viability. Since these cells are not prone to clump, the lack of EDTA is not a problem.

### **For Sticky Cells**

Raise the concentration of the EDTA to 5mM and use FBS that has been dialyzed against Ca/Mg<sup>++</sup> free PBS. Some activated cells become clumpy and the chelators (EDTA) help reduce cation-dependent cell to cell adhesion.

### **For Adherent Cells**

In order to achieve good single cell preparations; one must start at the moment of detaching your cells from the plate. Typically, the trypsin (or other detachment buffer) is quenched with culture media or a PBS/FBS buffer. This is problematic because it reintroduces the cations that facilitate the cells reattaching to the plate (or each other). One must use a cation-free FBS buffer in order to stop the detachment. Additionally, the level of EDTA can be increased if necessary (but too much EDTA can be deleterious).

### **For Samples with High Percentage of Dead Cells**

If there are a large number of dead cells in the prep, it is likely that there is soluble DNA from the dead cells that will come out of solution. This DNA will start to coat the cells and lead to severe clumping. The addition of 10U/mL DNAase II to the buffer recipe will help reduce DNA associated clumpiness.

**Extremely Important: After cells are sorted into collection tubes, centrifuge cells to remove diluted buffer and replenish with fresh culture media.**

These suggestions should help to optimize sample preparation for both enhanced viability and enhanced recovery. It may require some more comprehensive modifications evolving from these simplistic guidelines.

### **Expedient Sample Processing**

The sample must be prepared in as short a time as feasible to minimize stress on the cells as sorting is a relatively harsh process. Much of this can be achieved by simplifying the staining process and staggering the sample preps if more than one sample is being sorted.

### **Controls**

Appropriate control samples must be included in all experiments. The control could include:

- A sample of unstained cells
- Positive and negative controls

- Compensation (single color) controls for each stain as cells or compensation beads

For surface staining of cells; usually three controls are needed for each experiment. The controls must undergo the same treatment (i.e., preparation) as all the tubes in an experiment. An unstained control is used to detect auto-fluorescence or background staining innate to the cells of interest. Auto-fluorescence can be a significant problem, particularly in systems that contain monocytes/macrophages, cultured cells, or activated cells. An isotype control (i.e., where an antibody is used that has the same immunoglobulin isotype as the test antibody, but a different specificity which is known to be irrelevant to the sample being analyzed) is needed to determine whether fluorescence emitted is due to non-specific binding of the fluorescent antibody. A positive control is highly desirable (although not always available) to prove that the test antibody and/or procedure is working properly. The positive control should include cells known to be positive for the marker of interest.

If more than one color (fluorescent conjugate) will be used within the same experiment, a single-color control must be prepared for each of the dyes used. This will allow for compensation from spectral overlap between the stains. An unstained control is also important for multi-color compensation. In any experiment where two or more fluorochromes (multi-color analysis) are used to characterize the cells of interest, the need for fluorescence compensation must be checked. Fluorescence compensation is the mathematical subtraction of the fluorescence of one fluorochrome from the fluorescence of another fluorochrome. This is necessary because emission spectra from multiple fluorochromes can overlap. For this reason, in a sample that contains 2 colors such as fluorescein isothiocyanate and phycoerythrin, fluorescence due to the FITC will be detected by the electronics that are set up to detect fluorescence produced by the phycoerythrin. If this were not subtracted from the phycoerythrin fluorescence, the phycoerythrin signal would be falsely elevated. To determine the correct amount of fluorescence compensation, cells stained with only one fluorochrome-conjugated antibody are analyzed individually. This must be done for each of the fluorochromes used in the experiment, with an unstained control as the initial region.

## **Intracellular Staining Flow Cytometry Protocols**

### **I. Direct immunofluorescence staining of intracellular cytokines in blood**

Whole blood protocol for analysis of intracellular cytokines this is a rapid and simple approach to the analysis of intracellular cytokines by flow cytometry. It permits the analysis of small samples, and avoids any possibility of generating artefactual results during the separation of peripheral blood cells by density gradient centrifugation.

The stimulation conditions described are suitable for IFN gamma, IL-2 and TNF alpha. Different conditions may be needed for other cytokines.

The procedure requires a reagent kit to fix and permeabilize cells. There are several available but we recommend Leucoperm™.

**Note:** All blood samples must be collected into heparin anti-coagulant. EDTA interferes with the cell stimulation process and, therefore, must be avoided.

1. Aliquot 0.5mL of blood separately into 2 tubes, then add 0.5mL of cell culture medium (without any additives) to each sample.
2. To one tube (the resting population), add monensin to a final concentration of 3mM.
3. To the other tube (activated cells), add PMA, ionomycin and monensin to a final concentration of 10 ng/mL, 2mM and 3mM, respectively.
4. Incubate for 2–4 hours at 37°C in a 5% CO<sub>2</sub> atmosphere.
5. At the end of the incubation period aliquot 100µL samples into the appropriate number of tubes.
6. Add cell surface antibodies at this stage (if needed for your experiment) and incubate for 15 minutes.
7. Add 100µL of Leucoperm™ Reagent A per tube and incubate for 15 minutes. This reagent fixes cells in suspension.
8. Wash twice with PBS containing 0.1% sodium azide and 1% BSA.
9. Add 100µL of Leucoperm™ Reagent B (permeabilizes cells) and the required anti-cytokine antibodies.
10. Incubate for 20 minutes.
11. Wash twice using the PBS buffer, and analyze by flow cytometry.

## **II. Direct immunofluorescence staining of intracellular antigens: Methanol plus Leucoperm™ Accessory Reagent method**

The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC7. “Direct Staining of intracellular Antigens by Flow Cytometry using Leucoperm™ does not provide the desired results. This method is particularly suitable for the detection of nuclear antigens, such as PCNA and Ki67. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with the product and batch specific information provided with each vial. A certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications. Specific methodology for blood appears in [ ] brackets.

## Reagents

- Leucoperm Accessory Reagent (Product code BUF09). Includes Reagent A (cell fixation agent) and Reagent B (cell permeabilization agent).
- Phosphate buffered saline (Product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS.
- Erythrolyse red blood cell lysing buffer
- Anticoagulant (Note: for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required.)
- Optional: 0.5% (w/v) paraformaldehyde in PBS (**Note:** dissolve on heated stirrer and cool before use)

## Method

1. Harvest cells and determine the total number present.
2. Adjust cell suspension to a concentration of  $1 \times 10^7$  cells/ml in PBS/BSA. [Whole blood samples may also be used. Bio-Rad recommends the use of EDTA anti-coagulant in these circumstances, although satisfactory results may be obtained using heparin or acid-citrate dextrose.]
3. Add 100  $\mu$ L of cell suspension [or whole blood] to the appropriate number of test tubes. If required, perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash cells once in 2ml cold (4°C) PBS/BSA and discard the supernatant.
5. Re-suspend cells in 100  $\mu$ L cold (2-8°C) Leucoperm™ Reagent A (cell fixation agent) per  $1 \times 10^6$  cells.
6. Incubate for 10 minutes at 2-8°C.
7. Add 500  $\mu$ L of ice cold absolute methanol, vortex and incubate for 10 minutes at 2-8°C.
8. Add 3 ml cold (4°C) PBS and centrifuge for 5 minutes at 300-400 g at 4°C.
9. Remove supernatant and add 100  $\mu$ l Leucoperm™ Reagent B (cell permeabilization agent) per  $1 \times 10^6$  cells.

10. Add the directly conjugated antibody at the vendor-recommended dilution and incubate at 4°C for at least 30 minutes, avoiding direct light. [To the blood suspension add 2mL freshly prepared erythrolyse red cell lysing buffer and mix well.
11. Incubate for 10 min at room temperature.
12. Centrifuge at 300-400 g for 5 min and discard the supernatant.
13. Wash with 2ml room temperature PBS/BSA, centrifuge at 300-400 g for 5 min at room temperature and discard the supernatant. Continue to step 9.]
14. Resuspend cells in 2 mL cold (4°C) PBS and centrifuge at 300-400 g at 4°C.
15. Discard supernatant.
16. Resuspend cells in 200 µL cold (4°C) PBS for immediate analysis or with 200 µL 0.5% formaldehyde in PBS if required.
17. Acquire data by flow cytometry. Analyze fixed cells within 24 hours.

### Notes

- Phycoerythrin conjugates are not suitable for the detection of cell surface antigens using this method due to damage of the RPE at low temperatures.
- To avoid unspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents e.g. Bio-Rad's Mouse Seroblock reagent.
- Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion; Isotype controls used to determine if the staining is specific.
- Unstained cells should always be included in the experimental set-up to monitor autofluorescence.
- For all multi-color Flow Cytometry experiments it is advisable to include compensation controls and Fluorescence minus One (FMO) controls, which assist with identifying gating boundaries.

### III. Direct Immunofluorescence Staining of Intracellular Antigens: Digitonin Method

Alternative method for cell permeabilization required prior to intracellular staining that does not require the use of Leucoperm™ Accessory Reagent. The detection of intracellular antigens requires a cell permeabilization step prior to staining. This method provides an alternative procedure for use when protocol FC7. "Direct staining of intracellular antigens: Leucoperm Accessory Reagent method" does not provide the desired results. This method is particularly

suitable for the detection of the T cell antigen receptor zeta chain using MCA1297. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with the product and batch specific information provided with each vial. A certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications. Note: This procedure causes a reduction in Forward Scatter (FSC) signal, so the flow cytometer set up may need to be adjusted to compensate.

### **Reagents:**

- Phosphate buffered saline (BUF036A) containing 1% bovine serum albumin (PBS/BSA)
- PBS
- 0.5% (w/v) paraformaldehyde in PBS (Note: dissolve on heated stirrer and cool before use)
- 0.05% (v/v) Tween 20 in PBS
- 10 µg/ml digitonin in PBS

### **Method:**

1. Prepare cells appropriately; refer to protocol FC1. 'Preparation of cells for flow cytometry' for further information. Adjust the cell suspension to a concentration of  $1 \times 10^7$  cells/mL with cold PBS/BSA.
2. Aliquot 100 µL of the cell suspension into the required number of tubes.
3. If required perform staining of cell surface antigens using appropriate directly conjugated monoclonal antibodies at 4°C, avoiding direct light.
4. Wash twice in cold (4°C) PBS/BSA by pelleting cells at 300-400 g and 4°C for 5 minutes. Discard supernatant.
5. Add 50 µL PBS/BSA to each tube, followed by 100 µL 0.5% paraformaldehyde.
6. Incubate for 20 minutes at room temperature.
7. Wash twice with 3 mL 0.05% Tween 20. Pellet cells at 300-400 g and 4°C for 5 min.
8. Decant supernatant. Add 100 µL of cold 10 µg/mL digitonin and the directly conjugated antibody at the vendorrecommended dilution and incubate for at least 30 minutes at 4°C, avoiding direct light.

9. Wash twice with 3 mL 0.05% Tween 20. After each wash step pellet cells at 300-400 g and 4°C for 5 minutes.
10. Resuspend in 200 µL PBS.
11. Acquire data by Flow Cytometry. Analyze fixed cells within 24 hours.

### **Notes:**

- The following should be considered when designing your Flow Cytometry experiments:
- To avoid unspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents e.g. Bio-Rad's Mouse Seroblock reagent.
- Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion; - Isotype controls used to determine if the staining is specific - Unstained cells should always be included in the experimental set-up to monitor autofluorescence.
- For all multi-color flow cytometry experiments it is advisable to include compensation controls and Fluorescence minus One (FMO) controls, which assist with identifying gating boundaries.

## **Cell Cycle Staining Flow Cytometry Protocols**

### **I. Immunofluorescence staining of cells in combination with PI staining of cells for cell cycle analysis**

This method provides a general procedure for DNA staining for cell cycle analysis using propidium iodide when you need to stain for other intracellular antigens. 70% ethanol is optimal for DNA so if you do not need to stain for other antigens we recommend Protocol FC12 "Propidium iodide staining of cells for cell cycle analysis". This method will allow you to stain for DNA and cellular antigens, however these are guidelines only and the incubation times may need to be adjusted for different cell types and different antibodies.

### **Reagents**

- Phosphate buffered saline (PBS)
- 70% Ethanol in DI water
- 2% (w/v) paraformaldehyde in PBS
- 0.1% saponin (w/v), 1% (w/v) bovine serum albumin in PBS

- Nucleic acid staining solution (1x PBS, 100 ug/mL RNase A)

## Methods

1. Prepare cells appropriately; refer to protocol 'Preparation of cells for flow cytometry' for further information.
2. Fix in 2 mL 2% paraformaldehyde for 30 min on ice.
3. Centrifuge at 500 x g for 5 minutes, decant supernatant.
4. Resuspend in 2-5 ml cold (4°C) 70% ethanol. Add drop wise to cell pellet while vortexing. Fix for at least 30 min on ice. **Note:** specimens can be left at this stage for several weeks.
5. Centrifuge at 500 x g for 10 minutes, decant supernatant.
6. Wash twice with 0.1% saponin at 300-400 g at 4°C for 5 min, discard the supernatant.
7. Resuspend in 100 mL of 0.1% saponin and add the directly conjugated antibody at the vendor-recommended dilution and incubate for at least 30 min at 4°C, avoiding direct light.
8. Wash twice in PBS. Pellet cells at 300-400 g and 4°C for 5 min. Discard supernatant.
9. Resuspend in 500 mL nucleic acid staining solution and incubate for 30 min at room temperature avoiding direct light.
10. Add propidium iodide e.g. 1-2 drops of ReadIDrop™ propidium iodide.
11. Analyze cells by flow cytometry. The propidium iodide should be read on the appropriate channel in the linear scale. Doublets should be gated out using the Area vs. Height or Width depending on your instrument.

## Notes

Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion;

- A known positive sample
- Isotype controls used to determine if the staining is specific
- Unstained cells should always be included in the experimental set-up to monitor autofluorescence
- For all multi-color flow cytometry experiments it is advisable to include compensation controls and Fluorescence minus One (FMO) controls, which assist with identifying gating boundaries.

## II. Propidium iodide staining of cells for cell cycle analysis

Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry.

The DNA of mammalian, yeast, plant or bacterial cells can be stained by a variety of DNA binding dyes. The premise of these dyes is that they are stoichiometric, i.e. they bind in proportion to the amount of DNA present in the cell.

In this way cells that are in S phase will have more DNA than cells in G1. They will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content. The cells in G2 will be approximately twice as bright as cells in G1.

DNA-binding dyes include propidium iodide (PI), 7-aminoactinomycin-D (7-AAD), Hoechst 33342, 33258 and S769121, TO-PRO-3, 4'6'-diamidino-2-phenylindole (DAPI), DRAQ5™ and DRAQ7™.

In most cases, cells must be fixed or permeabilized to allow entry of the dye which is otherwise actively pumped out by living cells. For fixation, alcohol or aldehyde is commonly used. Alcohol is a dehydrating fixative which also permeabilizes. This will allow easy access of the dye to the DNA and gives good profiles (low coefficient of variation, CV). The disadvantage is that it is often incompatible with fluorescent proteins and some surface markers.

If fluorescent proteins or surface markers need to be examined simultaneously, use of an aldehyde (cross-linking) fixative, usually paraformaldehyde is more appropriate. This may lead to poorer quality profiles (higher CVs) but will allow simultaneous detection of other fluorochromes and membrane-bound proteins. However, paraformaldehyde will usually not permeabilize the cell membrane, and so further sample processing is required.

With fixed cells, samples may be accumulated, stained and analyzed at the conclusion of an experiment. Alcohol-fixed cells are stable for several weeks at 4°C. Aldehyde fixed cells are stable for 2 to 3 days.

An alternative method to allow the DNA dye into the cells is to permeabilize them with a detergent. This can be Triton X-100 (0.1%) or NP40 (0.1%). Saponin is not a recommended permeabilizing reagent for DNA analysis as it does not permeabilize the nuclear membrane well. Permeabilized cells cannot be stored for as long as fixed ones and should be processed within hours.

It is also usually necessary to combine a fixation (paraformaldehyde) and permeabilization (Triton X-100) for the intracellular staining. Other methods are also available, e.g. use of citrate buffers (in combination with detergent), although these are not so widespread. There are also some dyes that will enter live cells and quantitatively bind to DNA, these include Hoechst 33342, DRAQ5™ (ab108410) and the DyeCycle dyes.

The method used will depend on the experiment and the information required. In this protocol, PI is used to label DNA content.

### Reagents

- 70% Ethanol
- Propidium iodide (stock solution 50 µg/mL)

- Ribonuclease I (stock 100 mg/mL)

## Method

1. Harvest the cells in the appropriate manner and wash in PBS.
2. Fix in cold 70% ethanol. Add drop wise to the pellet while vortexing. This should ensure fixation of all cells and minimize clumping.
3. Fix for 30 min at 4°C.
4. Wash 2 X in PBS. Spin at 850 g in a centrifuge and be careful to avoid cell loss when discarding the supernatant especially after spinning out of ethanol.
5. Treat the cells with ribonuclease. Add 50  $\mu$ L of a 100  $\mu$ g/mL stock of RNase. This will ensure only DNA, not RNA, is stained.
6. Add 200  $\mu$ L PI (from 50  $\mu$ g/mL stock solution).

## Analysis of results

1. Measure the forward scatter (FS) and side scatter (SS) to identify single cells.
2. Pulse processing is used to exclude cell doublets from the analysis. This can be achieved either by using pulse area vs. pulse width or pulse area vs. pulse height depending on the type of cytometer.
3. PI has a maximum emission of 605 nm so can be measured with a suitable band pass filter.

## III. BrdU staining of cells for cell cycle analysis

Cell cycle and apoptosis are very important functional parameters to assess the cellular metabolism, physiology and pathology. Several techniques have been developed to quantitate these parameters utilizing the differential staining of fluorescent dyes. We are describing four different flow cytometric methods, two for the discrimination of cell cycle phases (A and B) and two for the simultaneous assessment of cell cycle and apoptosis (C and D).

### Bromodeoxyuridine/Propidium Iodide

The classical method for the analysis of cell cycle distribution is the flow cytometric measurement of DNA content which can simultaneously determine the incorporation of Bromodeoxyuridine (BrdU). The procedure requires that DNA is partially denatured to expose incorporated BrdU to a specific antibody. Denaturation is necessary because antibodies developed so far bind only to BrdU in single-strand DNA. The remaining undenatured DNA is then stained with Propidium Iodide (PI). Green fluorescence from the fluorescein-conjugated

antibody is a measure of BrdU incorporation. Red fluorescence from the PI is a measure of DNA. The protocol described here uses high-molarity HCl for the denaturation of DNA. Furthermore, this method may be utilized either for unfixed or for fixed cells in suspension.

## Materials

- BrdU
- Washing buffer
- HCl 4 M, Borax buffer
- Anti-BrdU antibody
- Goat-anti-mouse-FITC antibody, PI buffer

## Method

1. Cells ( $1 \times 10^6$ /mL) are incubated with BrdU 10  $\mu$ M at final concentration, for 30 min at 37°C in controlled atmosphere.
2. Wash twice at 500 g for 1 min using the washing buffer.
3. Resuspend in 0.5 mL of washing buffer and 0.5 mL of HCl 4 M.
4. Mix accurately and incubate for 30 min at room temperature.
5. Wash once as in step 2.
6. Resuspend in 1 mL of Borax buffer.
7. As in step 5.
8. Resuspend in 200  $\mu$ L of washing buffer and label with 5  $\mu$ L of mAb ant-BrdU.
9. Incubate for 1 hour at 4 °C in the dark.
10. As in step 5.
11. Resuspend in 200  $\mu$ L of washing buffer and label with 4  $\mu$ L of goat-anti-mouse FITC-conjugated antibody.
12. Incubate for 30 min at 4 °C in the dark.
13. As in step 5.

14. Resuspend in 200  $\mu$ L of washing buffer and 200  $\mu$ L of PI buffer.
15. Incubate for 15-30 min at 4°C in the dark.
16. Analyse with flow cytometer equipped with a 488 nm argon laser.

## **Background information**

In this procedure fixed cells by 4% PFA in Phosphate Buffer Saline (PBS) can be utilized. In this case to wash cells once in PBS before to start at step 1 is necessary.

Moreover, both direct and indirect immunofluorescence can be used. The BrdU incorporation is more evident using the indirect method.

## **Anticipated results**

It is recommended to perform each experiment using a negative and a positive control sample. The negative sample, in order to have a correct setting of instrument, is assessed following all the steps except step 8. The positive sample, in order to make sure that the method works, is assessed by using a proliferating cell line (such as U937, K562, MOLT4, etc.) following all steps.

## **Time considerations**

The protocol is simply but it require a quite long time. Indeed, for few samples, more or less 4 hours are required. The duration of the method is obviously depending on the number of samples.

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Good luck!



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RCPN

Document ID: RCPN9	
Document name	Flow Cytometry (FACS) Protocols
Document arrangement	Mohanna Osali, Rahimeh Mousavi
Date	12/30/2015
Full file name	Flow Cytometry (FACS) Protocols
Document Description	The BD FACSCalibur™ platform allows users to perform both cell analysis and cell sorting in a single benchtop system. The system supports a wide variety of research and clinical applications and is complemented by a broad suite of intuitive software solutions to streamline analysis for a wide range of applications including enumeration of lymphocyte subsets, stem cells, residual white blood cells, and reticulocytes
Author / translator	Golchin. Asal