BD Pharmingen™ BrdU Flow Kits

Instruction Manual

FITC BrdU Flow Kit
Catalog No. 559619 (50 tests)
Catalog No. 557891 (4 x 50 tests)

APC BrdU Flow Kit
Catalog No. 552598 (50 tests)
Catalog No. 557892 (4 x 50 tests)

BD

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Regulatory information

BD flow cytometers are Class 1 Laser Products.

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History

Revision	Date	Change made
23-12721-00 Rev. 01	11/2011	New document
Rev. 2	3/2015	Updated Warnings section

Contents

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1

Introduction

This section covers the following topics:

Purpose of the kit

BrdU staining

The immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometric analysis provide a high-resolution technique to determine the frequency and nature of individual cells that have synthesized DNA. In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle.[1-4] The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies. The levels of cell-associated BrdU are then measured by flow cytometry. Often, staining with a dye that binds to total DNA such as 7-amino-actinomycin D (7-AAD) is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (ie, G0/1, S, or G2/M phase defined by 7-AAD staining intensities).[5,6]

Prolonged exposure of cells to BrdU allows for the identification and analysis of actively cycling, as opposed to non-cycling, cell fractions. Pulse labeling of cells with BrdU at various time points, permits the determination of cell-cycle kinetics. BrdU incorporation studies have been used in a variety of experimental protocols. These include in vitro and in vivo (eg, intraperitoneal injection or administration via drinking water) labeling systems.

An important feature of the BD Pharmingen™ BrdU Flow Kit is that it provides reagents for immunofluorescent BrdU staining with a protocol that is compatible with the use of additional fluorescent antibodies specific for other cellular molecules. These molecules may include cell surface antigens or intracellular proteins (eg, cytokines, cyclins, and other proteins) the expression or activity of which may be related to the cell's activation, entry, and progression through cell cycle or cell death. This is possible because the BrdU Flow Kit staining protocol avoids DNA denaturing agents such as acid, ethanol, and high temperatures that can result in altered cellular light-scattering characteristics and limit the recognition of cellular antigens by fluorescent antibodies.[7-11]

Other uses of the kit

Fluorescent antibodies that are capable of recognizing cell surface antigens or proteins in cells that have been fixed with paraformaldehyde and permeabilized with saponin can be used with the BrdU Flow Kit. However, not all antibody clones and fluorochromes are compatible with paraformaldehyde fixation. With the combination of reagents, the expression levels of various surface or intracellular proteins can be measured by flow cytometry relative to the cell's DNA synthetic activity (BrdU incorporation level). For example, the BrdU Flow Kit can be used with fluorescent anti-cytokine antibodies in time-course analyses of cultured cells following in vitro mitogenic stimulation of quiescent lymphoid cell populations. In this way, the levels of a particular cytokine (eg, the T-cell growth and differentiation factor, IL-2) that are expressed prior to, at, and following the onset of DNA synthesis (during the first major round of cell-cycle activity) can be studied.

Many high-resolution studies of this type are possible with the use of the BD Pharmingen BrdU Flow Kit and other selected flow cytometry reagents. The kit ensures consistent results by providing the critical reagents necessary to implement the staining protocol. These individual components have been rigorously tested for their suitability to perform multiparameter analyses of incorporated BrdU levels, cell surface antigen expression, and expression of intracellular antigens by individual cells. BrdU uptake can also be analyzed in frozen or paraffin embedded tissue sections. The BD Pharmingen™ BrdU In-Situ Kits (Catalog Nos. 550803 and 551321) provide the reagents that allow you to perform two-color staining in tissue sections.

Limitations

Assay limitations

The BD Pharmingen BrdU Flow Kit staining procedure includes the fixative paraformaldehyde. Paraformaldehyde can alter epitopes on antigens and inhibit recognition by some antibodies after fixation. It is important that the antibody reagents used to stain proteins with this procedure be capable of binding to paraformaldehyde-fixed epitopes. Reagents that are compatible with other fixatives (eg, ethanol) may not work with the BD Pharmingen BrdU Flow Kit staining procedure.

Reagent limitations

Both the BD Perm/Wash™ Buffer (1X) and the BD Cytoperm™ Permeabilization Buffer Plus should be used with fixed cell samples only. Use of these buffers on unfixed cells will cause cell damage.

Kit contents

Contents

The kits (Catalog Nos. 559619 and 552598) contain the following components. Because some kit components are stored at 4°C and others are stored at –80°C, the kit components are shipped separately. See Storage and handling (page 12) for details on storage.

Reagent	Quantity
Fluorochrome-conjugated anti-BrdU Antibody	One 65-µL vial
BD Cytofix/Cytoperm™ Buffer	One 25-mL bottle
BD Perm/Wash™ Buffer (10X)	Two 25-mL bottles
BD Cytoperm™ Permeabilization Buffer Plus	One 10-mL bottle
7-AAD	One 1-mL vial

The following items are shipped separately.

Reagent	Quantity
BrdU (10 mg/mL)	Five 0.5-mL vials
DNase	Five 300-µL vials

Reagents

Some kit reagents are supplied as concentrated stock solutions and need to be diluted either with deionized water, 1X Dulbecco's PBS (DPBS), or with BD Perm/Wash Buffer. See Reagent preparation (page 18) for information. The concentrations of the kit components follow.

Fluorochrome-conjugated anti-BrdU Antibody. A single vial contains 65 µL of fluorochrome-conjugated anti-BrdU antibody stock solution and is sufficient for staining 50 samples (10^6 cells/sample). The FITC BrdU Flow Kit (Catalog No. 559619) comes with 65 µL of FITC-conjugated anti-BrdU antibody. The APC BrdU Flow Kit (Catalog No. 552598) comes with 65 µL of APC-conjugated anti-BrdU antibody.

Note: If you run out of anti-BrdU antibody, an additional BrdU flow kit must be purchased. We do not recommended using any other anti-BrdU antibody clone or formulation from the BD Biosciences catalog in conjunction with this kit.

BD Cytofix/Cytoperm™ Buffer. A 25-mL bottle of BD Cytofix/Cytoperm Buffer is provided in a ready-to-use formulation. BD Cytofix/Cytoperm Buffer constitutes a single-step fixation and permeabilization reagent, designed for use in intracellular staining. It contains a mixture of the fixative paraformaldehyde and the detergent saponin. This reagent serves to preserve cell morphology, fix cellular proteins, and permeabilize cells for the subsequent immunofluorescent staining of intracellular proteins.

BD Perm/Wash Buffer. Two 25-mL bottles contain a concentrated (10X) stock solution of BD Perm/Wash Buffer. The BD Perm/Wash Buffer mixture contains fetal bovine serum and the reversible permeabilization detergent reagent saponin.

BD Cytoperm Permeabilization Buffer Plus. One 10-mL bottle of buffer is provided. It is specially formulated for the BrdU Flow Kit and is used as a staining enhancer and secondary permeabilization reagent. It contains fetal bovine serum. One hundred microliters of this buffer is used for each sample. BD Cytoperm Permeabilization Buffer Plus can be purchased separately from BD (Catalog No. 561651, 10 mL).

7-AAD. One 1-mL vial of 7-AAD is provided. 7-amino-actinomycin D (7-AAD) is a fluorescent dye for labeling DNA for flow cytometric analysis. It contains fetal bovine serum. Twenty microliters of 7-AAD is used for staining each sample (10^6 cells/sample).

Note: Source of all serum proteins is from USDA-inspected abattoirs located in the United States.

BrdU and DNase

BrdU. Five vials of BrdU Solution are provided. Each vial contains 0.5 mL of a 10-mg/mL BrdU (32.5-mM) solution diluted in 1X DPBS. The BrdU solution provided is prepared aseptically (0.22-µm filtered), and contains no preservative; therefore we recommend that the solution be handled under aseptic conditions. This stock solution can be injected intraperitoneally (IP) into animals or diluted to a 1-mM solution for in vitro labeling. For in vivo labeling by IP injection, see In vivo labeling of mouse cells with BrdU (page 21).

DNase. Five vials of DNase are provided. Each vial contains 300 µL of a 1-mg/mL solution of DNase in 1X DPBS. DNase can be purchased separately from Sigma (Catalog No. D-4513).

Storage and handling

Storage

Antibody, buffers, and 7-AAD. Store the fluorochrome-conjugated anti-BrdU antibody, BD Cytofix/Cytoperm Buffer, BD Perm/Wash Buffer, BD Cytoperm Permeabilization Buffer Plus, and 7-AAD at 2 to 8°C. Keep the fluorochrome-conjugated anti-BrdU antibody and 7-AAD protected from light.

Unused portions of 1X BD Cytofix/Cytoperm Buffer can be stored at 2 to 8°C.

BrdU and DNase. Store the BrdU and DNase at –80°C.

The BrdU solution has been shown to be stable for up to 4 months at 2 to 8°C, or it can be refrozen. Avoid multiple freeze-thaw cycles.

DNase stock solution (1 mg/mL) may be refrozen once before it loses activity.

Warnings and precautions

Danger. BD Cytofix/Cytoperm™ Buffer (Fixation and Permeabilization Solution; component 51-2090KE) contains 4.2% formaldehyde (w/w).

Hazard statements

Harmful if inhaled.

Causes skin irritation.

Causes serious eye damage.

May cause an allergic skin reaction.

Suspected of causing genetic defects.

May cause cancer. Route of exposure: Inhalative.

May cause respiratory irritation.

Precautionary statements

Wear protective clothing / eye protection.

Wear protective gloves.

Do not breathe mist/vapours/spray.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

If skin irritation or rash occurs: Get medical advice/ attention.

The anti-BrdU antibody and 7-AAD contain sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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2

Before you begin

This section covers the following topics:

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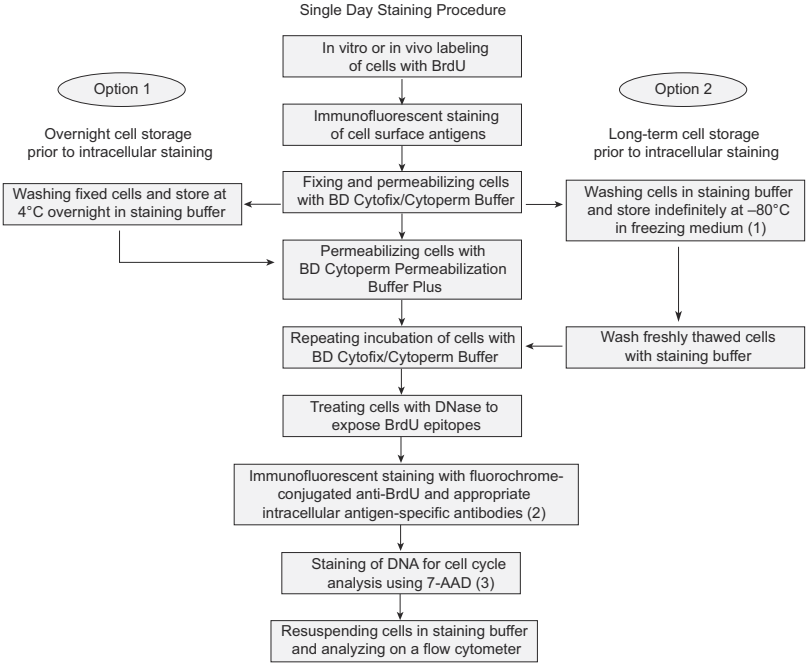
BrdU Flow Kit protocol overview

Options for storing cells prior to staining

The BD Pharmingen BrdU Flow Kit staining procedure offers several time-saving options for sample handling. With this staining protocol, it is possible to stain and analyze samples in a single day. The entire staining procedure requires approximately 3 hours.

Alternatively, samples may be fixed and stored for various lengths of time prior to staining. Due to the time intervals required for cell activation, BrdU incubation, and other factors that are necessary to prepare cells prior to staining, you may wish to store samples and complete the staining protocol at a later time.

- If short-term sample storage prior to staining is desired, Option 1 in the following flow chart allows you to store cells overnight after the initial fixation step.
- If longer sample storage is desired, Option 2 allows you to store frozen samples indefinitely, following the initial fixation step.



The following notes pertain to the numbers in parentheses found in the flow chart.

1. Recipe for Freezing Medium: 10% dimethyl sulfoxide (DMSO) + 90% heat-inactivated fetal bovine serum (FBS).
2. The immunofluorescent staining of cell surface antigens can be done at the same time as staining intracellular antigens provided the antibodies recognize paraformaldehyde-fixed epitopes.
3. If you do not wish to stain for total DNA content, then the 7-AAD staining step can be omitted and fluorescent data for another parameter can be measured in that channel.

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Required materials

Materials required but not provided

In addition to the reagents provided in the BD Pharmingen BrdU Flow Kit, the following items are also required.

- For the FITC kit, you will need a flow cytometer equipped with a 488-nm laser capable of detecting FITC and 7-AAD. For the APC kit, you will need a flow cytometer with a 488-nm laser capable of detecting 7-AAD and a 633- to 640-nm laser capable of detecting APC.
- Falcon® 12 x 75-mm sample acquisition tubes for a flow cytometer (Catalog No. 352008)
- Staining buffer, for example, BD Pharmingen™ Stain Buffer (FBS) [Catalog No. 554656] or 1X DPBS + 3% heat-inactivated FBS + 0.09% sodium azide

Reagent preparation

Introduction

Dilute the following reagents prior to use.

Procedures

Fluorochrome-conjugated anti-BrdU Antibody. Dilute an appropriate amount of the antibody stock solution 1:50 with 1X BD Perm/Wash Buffer. Fifty microliters of the diluted antibody is used to stain each sample.

BD Perm/Wash Buffer. Dilute the concentrated stock buffer 1:10 with deionized water. Store unused portions of 1X BD Perm/Wash Buffer at 4°C.

3

Staining protocol

This section covers the following topics:

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In vitro labeling of cells with BrdU

Introduction

Many different protocols for in vitro BrdU labeling of cells have been reported.[12-15] We have found that incubating cells with BrdU at a final concentration of 10 µM in cell culture medium (ie, 10 µL of 1-mM BrdU per mL of culture medium) was effective for labeling a wide variety of human and mouse cell lines and normal cell populations.[15,16] Prolonged exposure of cells to BrdU allows for the identification of actively cycling cell populations. Pulse labeling of cells by brief BrdU exposures at various time points permits the determination of cell-cycle kinetics.

Use cells from the same population that are not BrdU-labeled as a negative staining control for this assay. This allows you to determine background staining levels for the anti-BrdU monoclonal antibody.

Before you begin

For pulse-labeling experiments, the choice of time points and lengths of time for pulsing depends on the test cell population's rate of cell cycle entry and progression. For example, an effective length of time for pulsing an actively proliferating cell line (eg, CTLL-2 cells) is 30 to 45 minutes (ie, when the cells are in the logarithmic phase of cell proliferation).

Determine time points and pulse-labeling time intervals that are optimal for each different cell line or cell population within a particular experimental system.

Dilute the BrdU stock (10-mg/mL BrdU solution) to a 1-mM solution by adding 31 µL to 1 mL of either 1X DPBS or culture medium (this is a dilution of 32X). Add 10 µL of the 1-mM solution to each mL of culture medium to obtain a final concentration of 10 µM. The molecular weight of BrdU is 307.1.

Procedure

To label cells in vitro:

1. Carefully add 10 µL of BrdU solution (1 mM BrdU in 1X DPBS) directly to each mL of tissue culture medium.

   Avoid disturbing the cells in any way (eg, centrifugation steps or temperature changes) that may disrupt the normal cell cycling patterns. The cell culture density should not exceed 2 x 10^6 cells/mL.

2. Incubate the treated cells for the desired length of time.

In vivo labeling of mouse cells with BrdU

Introduction

Two common methods reported for in vivo BrdU labeling of cells include the intraperitoneal (IP) injection of a BrdU-containing solution into mice and the feeding of mice with BrdU that is added to their drinking water.[16-22] However, these methods have not been routinely tested at BD Biosciences.

Injecting BrdU via the intraperitoneal route

A 10 mg/mL solution of BrdU in sterile 1X DPBS is provided for in vivo use. Inject mice IP with 100 to 200 µL (1–2 mg) of BrdU solution.[17,19,21] Incorporation of BrdU can be readily detected in thymus and bone marrow in as little as 1 hour post injection.

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Introduction of BrdU through drinking water

Dilute BrdU to 0.8 mg/mL in the drinking water. The BrdU mixture should be made up fresh and changed daily.[18,23] Prolonged feeding of BrdU can have toxic effects for the animal. Some researchers have reported lethal effects associated with 14 days of continuous BrdU feeding.[21] For longer term studies, some researchers have reported that feeding mice with BrdU for 9 consecutive days followed by a changeover to normal water has worked effectively.[18] BrdU incorporation by cells from these animals has been detected past 70 days.[18]

BrdU Flow Kit staining protocol

Before you begin

When treating 10 or more samples, thaw the entire vial of DNase solution and add 700 µL of 1X DPBS to make a working stock solution of 300 µg/mL. If treating fewer than 10 samples, take a 30-µL aliquot (1 mg/mL) of DNase solution per sample and refreeze the remaining 1 mg/mL DNase at –80°C.

Procedure

In addition to the labeled cells, stain an aliquot of unlabeled cells for use as a negative control.

To stain the cells:

1. (Optional) Stain cell surface antigens.
   a. Add BrdU-pulsed cells (10^6 cells in 50 µL of staining buffer) to 12 x 75-mm tubes.
   b. Add fluorescent antibodies specific for cell-surface markers in 50 µL of staining buffer (eg, BD Pharmingen Stain Buffer [FBS] Catalog No. 554656) per tube and mix well.

c. Incubate cells with antibodies for 15 minutes on ice.

d. Wash cells by adding 1 mL of staining buffer per tube, centrifuge for 5 minutes at 200 to 300*g*, and discard the supernatant.

2. Fix and permeabilize the cells with BD Cytofix/ Cytoperm Buffer.

   a. Resuspend the cells in 100 µL of BD Cytofix/ Cytoperm Buffer per tube.

   b. Incubate the cells for 15 to 30 minutes at room temperature or on ice.

   c. Wash the cells with 1 mL of 1X BD Perm/Wash Buffer. Centrifuge for 5 minutes at 200 to 300*g*, and discard the supernatant.

      Note: The presence of some precipitate in the 10X BD Perm/Wash stock buffer is common. The precipitate will not affect the performance of the buffer. If desired, you can remove the precipitate prior to use by filtering the diluted 1X BD Perm/ Wash Buffer through a 0.45-µm–pore filter.

3. Incubate the cells with BD Cytoperm Permeabilization Buffer Plus.

   a. Resuspend the cells in 100 µL of BD Cytoperm Permeabilization Buffer Plus per tube.

   b. Incubate the cells for 10 minutes on ice.

   c. Wash the cells in 1 mL of 1X BD Perm/Wash Buffer (as in step 2c).

4. Re-fix cells.
    a. Resuspend the cells in 100 µL of BD Cytofix/ Cytoperm Buffer per tube.
    b. Incubate the cells for 5 minutes at room temperature or on ice.
    c. Wash the in 1 mL of 1X BD Perm/Wash Buffer (as in step 2c).
5. Treat cells with DNase to expose incorporated BrdU.[24,25]
    a. Resuspend the cells in 100 µL of diluted DNase (diluted to 300 µg/mL in DPBS) per tube, (ie, 30 µg of DNase/10^6 cells).
    b. Incubate cells for 1 hour at 37°C.
    c. Wash the cells in 1 mL of 1X BD Perm/Wash Buffer (as in step 2c).
6. Stain BrdU and intracellular antigens with fluorescent antibodies.
    a. Resuspend the cells in 50 µL of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU and/or antibodies specific for intracellular antigens.
    b. Incubate the cells for 20 minutes at room temperature.
    c. Wash the cells in 1 mL of 1X BD Perm/Wash Buffer (as in step 2c).

Note: Proceed to step 8 if you do not wish to stain for total DNA levels.

7. Stain total DNA for cell cycle analysis. Resuspend the cells in 20 µL of the 7-AAD solution.
8. Resuspend the cells in 1 mL of staining buffer.

9. Acquire stained cells on a flow cytometer. For optimal resolution, acquire using a low flow rate. Run at a rate no greater than 400 events per second.

Samples may be stored overnight at 4°C, protected from light, prior to analysis by flow cytometry.

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4

Instrument setup

This section covers the following topics:

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Instrument setup guidelines

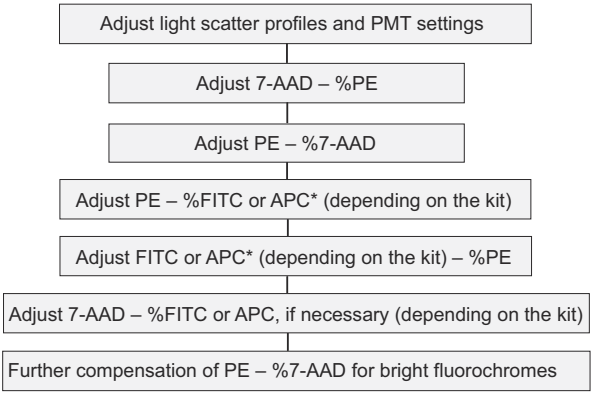
Introduction

The information in the following instrument setup sections is intended as an example of the type of setup necessary for samples stained using the BrdU staining procedure. FITC BrdU instrument setup example (page 29) is specific to the BD Pharmingen FITC BrdU Flow Kit (Catalog No. 559619) and APC BrdU instrument setup example (page 32) applies to the BD Pharmingen APC BrdU Flow Kit (Catalog No. 552598).

The instrument adjustments required might vary between instruments and between individual samples in a given experiment. It is often necessary to make further adjustments for different combinations of fluorescent-conjugated antibodies. We recommend that you refer to a textbook on flow cytometry or on cell-cycle analysis by flow cytometry for more information.[26,27]

Cytometer setup flow chart

The following flow chart shows you the general steps involved in instrument setup.



*Typically little or no compensation is necessary between PE and APC.

FITC BrdU instrument setup example

Experiment details

Mouse S-phase T cells were labeled with 10 µM of BrdU for 1 hour. The cells were processed according to the BrdU Flow Kit protocol. The samples were stained with FITC anti-BrdU, 7-AAD, and a PE-labeled surface marker.

Note: PE was included to illustrate the amount of compensation necessary when including PE in the experiment. If you do not use PE, proceed directly to Adjusting the BrdU/7-AAD profile (page 32).

Adjusting scatter and PMTs

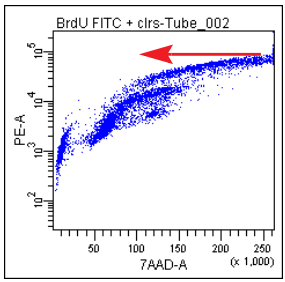
To adjust scatter and PMTs:

1. Adjust the FSC vs SSC parameters so that the cell populations are on scale.
2. Adjust the PMTs so that the negative populations fall between channels 10^2 and 10^3.

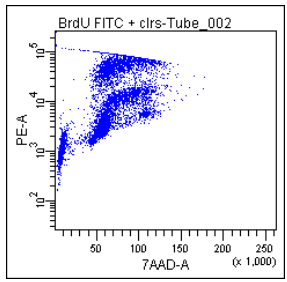
Adjusting compensation for 7-AAD and PE

To adjust compensation for 7-AAD and PE:

1. Adjust 7-AAD–%PE to bring the PE signal to the left (setting is ~209).



uncompensated 7-AAD–%PE



compensated 7-AAD–%PE

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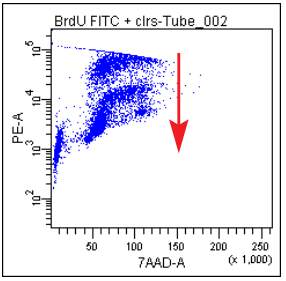
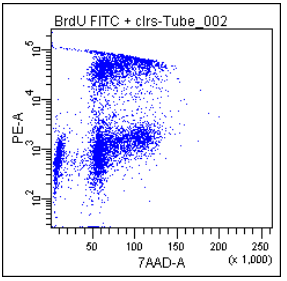
2. Adjust PE–%7-AAD to bring the PE signal down (setting is ~3.0).

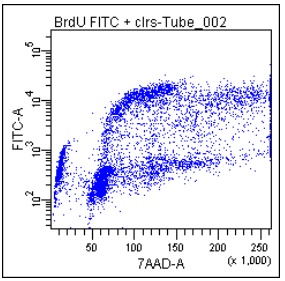


uncompensated PE–%7-AAD

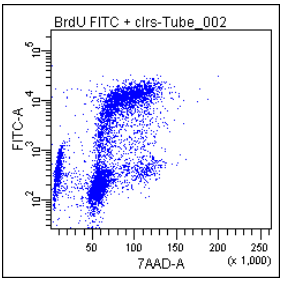


compensated PE–%7-AAD

The BrdU plot gets adjusted from the 7-AAD–%PE and PE–%7-AAD compensation adjustments. The following uncompensated plot is an example of how the data appears when PE is included.



uncompensated 7-AAD vs FITC

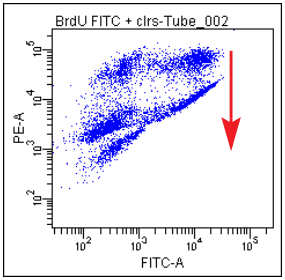


compensated 7-AAD vs FITC

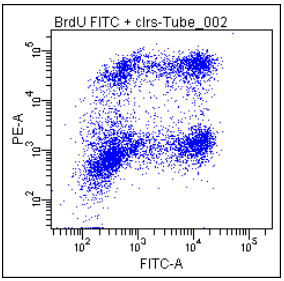
Adjusting compensation for PE and FITC

To adjust compensation for PE and FITC:

1. Adjust PE–%FITC to bring the FITC signal down (setting is ~65.4).

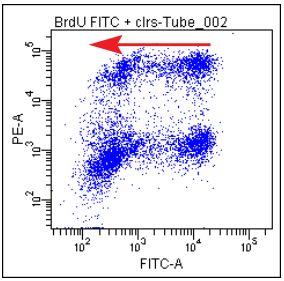


uncompensated PE–%FITC

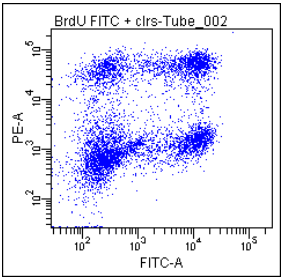


compensated PE–%FITC

2. Adjust FITC–%PE to bring the PE signal slightly to the left (setting is ~0.4).



uncompensated FITC–%PE



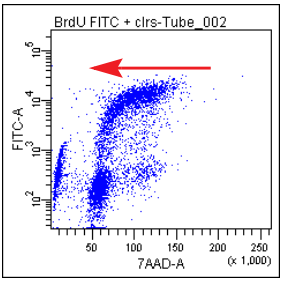
compensated FITC–%PE

Adjusting the BrdU/7-AAD profile

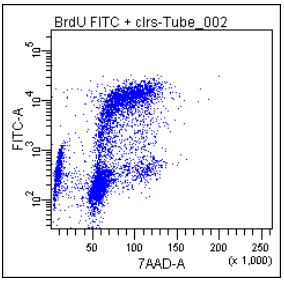
Start here if you stained using only the contents of the FITC BrdU Flow Kit with no additional drop-in antibodies.

To adjust compensation for 7-AAD and FITC:

1. Adjust 7-AAD–%FITC to bring the FITC signal to the left (setting is ~88).



uncompensated 7-AAD–%FITC



compensated 7-AAD–%FITC

APC BrdU instrument setup example

Initial instrument settings

When staining cells with anti-BrdU APC, the initial setup is similar to the FITC anti-BrdU conjugate shown in the previous section. Shown here are cells stained with APC anti-BrdU, 7-AAD, and a PE-labeled surface marker. The important difference here is that APC anti-BrdU is detected with a separate laser.

Note: PE was included to illustrate the amount of compensation necessary when including PE in the experiment. If you do not use PE, proceed directly to Adjusting the BrdU/7-AAD profile (page 34).

Adjusting scatter and PMTs

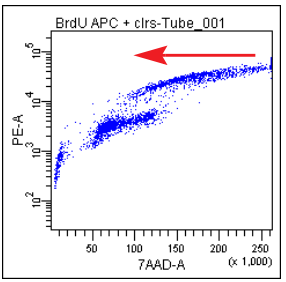
To adjust scatter and PMTs:

1. Adjust the FSC vs SSC parameters so that the cell populations are on scale.
2. Adjust the PMTs so that the negative populations fall between channels 10^2 and 10^3.

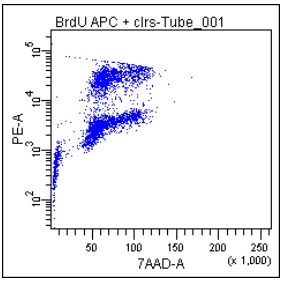
Adjusting compensation for PE and 7-AAD

To adjust compensation for PE and 7-AAD:

1. Adjust 7-AAD–%PE to bring the PE signal to the left (setting is ~308).

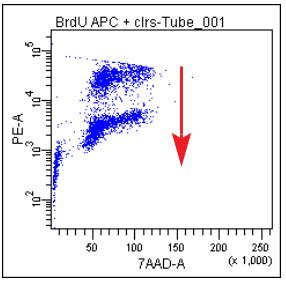


uncompensated 7-AAD–%PE

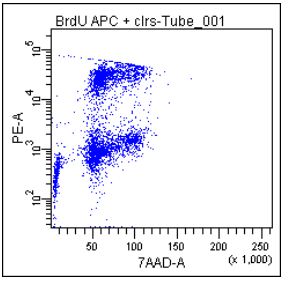


compensated 7-AAD–%PE

2. Adjust PE–%7-AAD to bring the PE signal down (setting is ~0.5).



uncompensated PE–%7-AAD



compensated PE–%7-AAD

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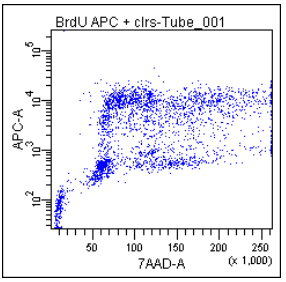
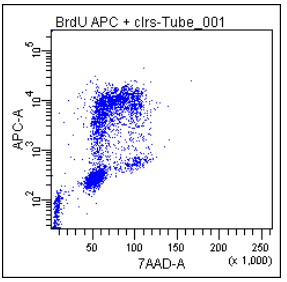
The BrdU plot gets adjusted from the 7-AAD–%PE and PE–%7-AAD compensation adjustments. The following uncompensated plot is an example of how the data appears when PE is included.



uncompensated 7-AAD vs APC



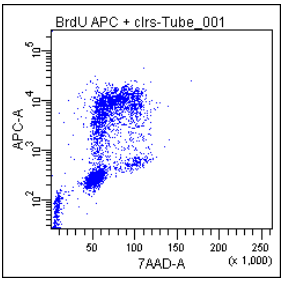
compensated 7-AAD vs APC

Adjusting the BrdU/7-AAD profile

Start here if you stained using only the contents of the APC BrdU Flow Kit with no additional drop-ins.

To adjust compensation for 7-AAD and APC:

1. If the APC is very bright, adjust compensation between 7-AAD and APC. The following plot looks correct without compensation. The compensation settings for 7-AAD–%APC and APC–%7-AAD are both 0.



uncompensated 7-AAD vs APC

5

Analysis

This section covers the following topics:

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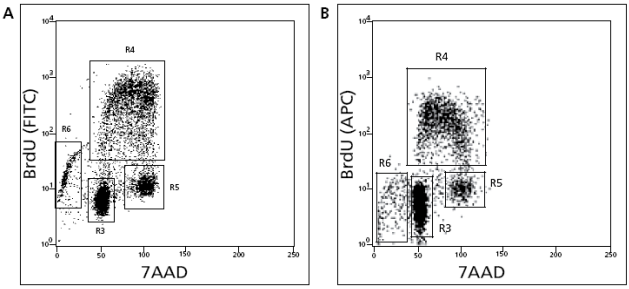
Analysis of stained cell samples

Introduction

Some of the data presented in the following examples was acquired using a flow cytometer equipped with a 488-nm laser, which excites FITC, PE, and 7-AAD. When staining with fluorochromes such as APC, flow cytometers with an additional laser light source were used. With the addition of each different fluorochrome used for multicolor staining, it becomes more critical to properly compensate overlaps in detection of emitted fluorescence signals. Fluorescence signals from 7-AAD are typically acquired in the linear signal amplification mode, whereas fluorescence signals generated by other fluorochromes are acquired in a logarithmic mode.

BrdU and total DNA staining

The cell cycle positions and DNA synthetic activities of cells can be determined by analyzing the correlated expression of total DNA and incorporated BrdU levels.



Regions for the quantitative cell cycle analysis of populations that have been stained for incorporated BrdU and total DNA levels.

Region	Cell population
R3	G0/G1
R4	S phase
R5	G2 + M
R6	apoptotic

Plot A: The measurement of cell-incorporated BrdU (with anti-BrdU FITC) and total DNA content (with 7-AAD) in D10.G4.1 cells. The D10.G4.1 cells were cultured with 10 µM of BrdU for 30 minutes. As shown by the regions applied to the 7-AAD vs BrdU dot plot, flow cytometric analysis of cells stained with the reagents provided in the BrdU Flow Kit allowed for the discrimination of cell subsets that were apoptotic—defined as sub-G0/G1 (R6, 5.6% of cells) or resided in G0/G1 (R3, 38.6%), S (R4, 38.6%), or G2 + M (R5, 14.4%) phases of the cell cycle and had recently synthesized DNA.[5,6] The 7-AAD signal data is displayed on a linear scale, as shown on the x-axis.

Plot B: The measurement of cell-incorporated BrdU (with anti-BrdU APC) and total DNA content (with 7-AAD). Human PBMCs were stimulated with immobilized anti-human CD3 antibody, clone HIT3a, 10 µg/mL for plate coating (Catalog No. 555336), soluble anti-human CD28 antibody, clone CD28.2 at 2 µg/mL (Catalog No. 555725), recombinant human IL-2 at 10 ng/mL (Catalog No. 554603), and recombinant human IL-4 at 20 ng/mL (Catalog No. 554605) for 2 days. The cells were then washed and subsequently expanded for 3 days in culture with medium containing recombinant IL-2 and IL-4. Finally the cells were harvested and restimulated for 4 hours with PMA (Sigma, Catalog No. P-8139, 5 ng/mL) and

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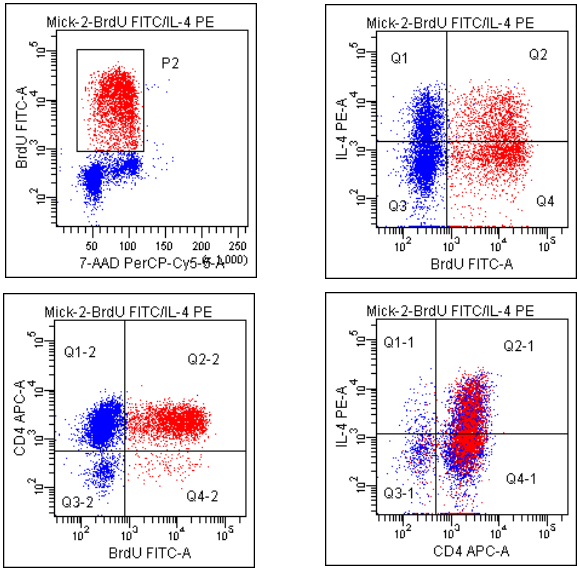
ionomycin (Sigma, Catalog No. I-0634, 500 ng/mL). Twenty micromoles of BrdU was added for the final hour. Regions are as in figure A with R6 being apoptotic (3.31%), R4 S phase (23.5%), R3 G0/G1 (64.3%), and R5 G2 + M (6.1%).

Sample data using the FITC BrdU Flow Kit protocol

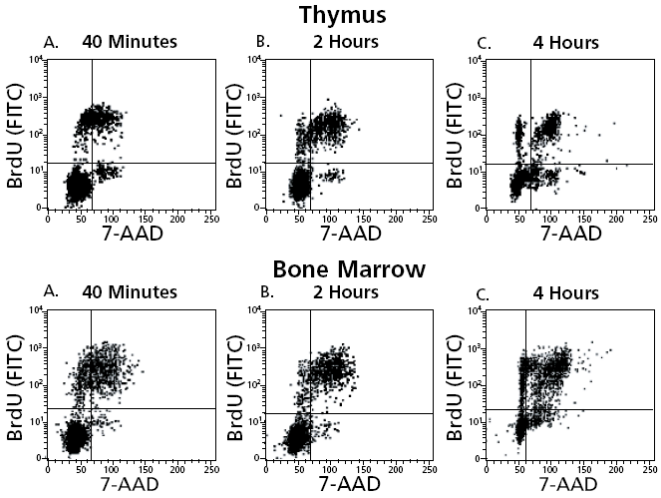
Spleen cells from a BALB/c mouse were primed in vitro and restimulated with PMA, ionomycin, and Brefeldin A (a protein transport inhibitor to promote intracellular cytokine accumulation). During the final 1 hour of culture, the cells were pulsed with 50 µM of BrdU. The cells were then harvested and stained with anti-BrdU FITC, 7-AAD, anti-mouse IL-4 PE, and anti-mouse CD4 APC. The plots depict two-color staining patterns generated from the reanalyzed flow cytometric data for these cells.



Multicolor flow cytometric analysis of stimulated mouse spleen cells that synthesized DNA and/or produced IL-4.

BrdU time-course study

C57BL/6 mice were intraperitoneally injected with 1 mg of BrdU for various time intervals. The animals were sacrificed at 40 minutes, 2 hours, or 4 hours post injection. Thymus and bone marrow cells were removed and stained for BrdU and 7-AAD.



Time-course study of in vivo BrdU pulsing in mice.

Plots A show bone marrow and thymus cells obtained from mice that were pulsed with BrdU for 40 minutes. Notice the characteristic BrdU/7-AAD "horseshoe" fluorescence staining profile. Plots B show staining patterns for bone marrow and thymus cells from mice pulsed for 2 hours. The characteristic horseshoe pattern is present. In addition, a population of cells that have incorporated BrdU but reside in the G0/G1 compartment is discernible (ie, BrdU-positive cells without increased 7-AAD content). Plots C show bone marrow and thymus cells from mice pulsed for 4 hours. The profile now shows a large population of BrdU-positive cells that are

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in G0/G1. The characteristic BrdU/7-AAD horseshoe pattern is much less discernible.

6

Reference

This section covers the following topic:

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References

Cited publications

1. Sasaki K, Murakami T, Takahashi M. Flow cytometric analysis of cell proliferation kinetics using the anti-BrdUrd antibody. *Gan To Kagaku Ryoho*. 1989;16:2338-2344.
2. Miltenburger HG, Sachse G, Schliermann M. S-phase cell detection with a monoclonal antibody. *Dev Biol Stand*. 1987;66:91-99.
3. Vanderlaan M, Thomas CB. Characterization of monoclonal antibodies to bromodeoxyuridine. *Cytometry*. 1985;6:501-505.
4. Gratzner HG, Leif RC. An immunofluorescence method for monitoring DNA synthesis by flow cytometry. *Cytometry*. 1981;1:385-393.
5. Lacombe F, Belloc F, Bernard P, Boisseau MR. Evaluation of four methods of DNA distribution data analysis based on bromodeoxyuridine/DNA bivariate data. *Cytometry*. 1988;9:245-253.
6. Dean PN, Dolbeare F, Gratzner H, Rice GC, Gray JW. Cell-cycle analysis using a monoclonal antibody to BrdUrd. *Cell Tissue Kinet*. 1984;17:427-436.
7. Toba K, Winton EF, Bray RA. Improved staining method for the simultaneous flow cytofluorometric analysis of DNA content, S-phase fraction, and surface phenotype using single laser instrumentation. *Cytometry*. 1992;13:60-67.
8. Sasaki K, Adachi S, Yamamoto T, Murakami T, Tanaka K, Takahashi M. Effects of denaturation with HCl on the immunological staining of bromodeoxyuridine incorporated into DNA. *Cytometry*. 1988;9:93-96.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

9. Lakhanpal S, Gonchoroff NJ, Katzmann JA, Handwerger BS. A flow cytofluorometric double staining technique for simultaneous determination of human mononuclear cell surface phenotype and cell cycle phase. *J Immunol Meth*. 1987;96:35-40.

10. Houck DW, Loken MR. Simultaneous analysis of cell surface antigens, bromodeoxyuridine incorporation and DNA content. *Cytometry*. 1985;6:531-538.

11. Moran R, Darzynkiewicz Z, Staiano-Coico L, Melamed MR. Detection of 5-bromodeoxyuridine (BrdUrd) incorporation by monoclonal antibodies: role of the DNA denaturation step. *J Histochem Cytochem*. 1985;33:821-827.

12. Holm M, Thomsen M, Høyer M, Hokland P. Optimization of a flow cytometric method for the simultaneous measurement of cell surface antigen, DNA content, and in vitro BrdUrd incorporation into normal and malignant hematopoietic cells. *Cytometry*. 1998;32:28-36.

13. Mehta BA, Maino VC. Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. *J Immunol Meth*. 1997;208:49-59.

14. Endl E, Steinbach P, Knüchel R, Hofstädter F. Analysis of cell cycle-related Ki-67 and p120 expression by flow cytometric BrdUrd-Hoechst/7AAD and immunolabeling technique. *Cytometry*. 1997;29:233-241.

15. Dolbeare F, Gratzner H, Pallavicini MG, Gray JW. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc Natl Acad Sci USA*. 1983;80:5573-5577.

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16. Penit C. In vivo thymocyte maturation. BrdU labeling of cycling thymocytes and phenotypic analysis of their progeny support the single lineage model. *J Immunol*. 1986;137:2115-2121.

17. Thoman ML. Early steps in T cell development are affected by aging. *Cell Immunol*. 1997;178:117-123.

18. Tough DF, Sprent J. Turnover of naive- and memory-phenotype T cells. *J Exp Med*. 1994;179:1127-1135.

19. von Boehmer H, Hafen K. The life span of naive alpha/beta T cells in secondary lymphoid organs. *J Exp Med*. 1993;177:891-896.

20. Schittek B, Rajewsky K, Forster I. Dividing cells in bone marrow and spleen incorporate bromodeoxyuridine with high efficiency. *Eur J Immunol*. 1991;21:235-238.

21. Rocha B, Penit C, Baron C, Vasseur F, Dautigny N, Freitas AA. Accumulation of bromodeoxyuridine-labeled cells in central and peripheral lymphoid organs: minimal estimates of production and turnover rates of mature lymphocytes. *Eur J Immunol*. 1990;20:1697-1708.

22. Westermann J, Ronneberg S, Fritz FJ, Pabst R. Proliferation of lymphocyte subsets in the adult rat: a comparison of different lymphoid organs. *Eur J Immunol*. 1989;19:1087-1093.

23. Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D, Weinmaster G, Salmon P. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell*. 1996;87:483-492.

24. Carayon P, Bord A. Identification of DNA-replicating lymphocyte subsets using a new method to label the bromo-deoxyuridine incorporated into the DNA. *J Immunol Meth*. 1992;147:225-230.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

25. Gonchoroff NJ, Katzmann JA, Currie RM, Evans EL, Houck DW, Kline BC, Greipp PR, Loken MR. S-phase detection with an antibody to bromodeoxyuridine. Role of DNase pretreatment. *J Immunol Meth*. 1986;93:97-101.

26. Shapiro H M, Practical Flow Cytometry, 3rd Edition, Wiley-Liss, New York.

27. Gray JW, Darzynkiewicz Z, Eds, Techniques in cell cycle analysis, Humana Press, Clifton, New Jersey.

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