Technical Data Sheet

Apoptosis, DNA Damage and Cell Proliferation Kit

Product Information

Material Number: **562253**
Size: 50 Tests

Component: **51-9007685AK**
Description: Apoptosis, DNA Damage and Cell Proliferation Kit - Part A

Component: **51-9007685BK**
Description: Apoptosis, DNA Damage and Cell Proliferation Kit - Part B

Description

Kit Contents

Description of Components	Material No.	Size	Storage	Vials
Kit Part A				
PerCP-Cy™5.5 Mouse Anti-BrdU	51-9007682	50 test	4ºC	1
Alexa Fluor® 647 Mouse Anti-H2AX (pS139)	51-9007683	50 test	4ºC	1
PE Mouse Anti-Cleaved PARP (Asp214) Antibody	51-9007684	50 test	4°C	1
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution	51-2090KE	25 ml	4°C	1
BD Perm/Wash™ Buffer (10X)	51-2091KE	25 ml	4°C	2
BD Cytofix/Cytoperm™ Plus Permeabilization Buffer	51-2356KC	10 ml	4°C	1
DAPI	51-9007681	100 µl	4°C	1
Kit Part B				
BrdU (10 mg/ml)	51-2420KC	5 mg	-80°C	5
DNase	51-2358KC	300 µl	-80°C	5

Apoptosis, DNA Damage and Cell Proliferation Kit

Multiparameter flow cytometry provides a powerful tool for resolving mechanisms by which individual cells in homogenous or mixed cell populations maintain viability, enter and progress through cell cycle or undergo cell death. For this purpose, the Apoptosis, DNA Damage, and Cell Proliferation Kit was designed with the inclusion of fluorescent antibodies specific for incorporated BrdU, phosphorylated H2AX (γH2AX) and cleaved PARP. These probes along with optimized protocols enable multicolor flow cytometric analysis of proliferation, DNA damage and apoptosis, respectively, by individual cells within samples.

Immunofluorescent staining of cells that have incorporated Bromodeoxyuridine (BrdU, an analog of the DNA precursor thymidine) and flow cytometric analysis provides a high resolution technique to determine the frequency and nature of individual cells that have synthesized DNA. Exposure of cells to BrdU in either *in vitro* or *in vivo* experimental model systems allows for BrdU incorporation by actively cycling cell fractions. Pulse labeling of cells with BrdU at various time points, permits the determination of cell-cycle kinetics. Flow cytometric analysis of BrdU incorporation can readily be combined with the simultaneous analysis of cellular phosphorylated H2AX and cleaved PARP levels. Phosphorylated H2AX functions to recruit and localize DNA repair proteins or cell cycle checkpoint factors to DNA-damaged sites. In this way, phosphorylated H2AX promotes DNA repair and maintains genomic stability. Double-stranded DNA breaks caused by replication errors, apoptosis, or other physiological processes (including, immunoglobulin and TCR gene recombinations) and DNA damage caused by ionizing radiation, UV light, or cytotoxic agents lead to H2AX phosphorylation on serine 139, H2AX (pS139), to induce its function. PARP (Poly [ADP-Ribose] Polymerase) is a nuclear chromatin-associated enzyme that is involved in DNA repair. During apoptosis, Caspase-3 cleaves PARP resulting in its inactivation and the inability of cells to repair DNA damage. For this reason, the 89 kDa-cleaved fragment of PARP serves as a marker of cellular apoptosis.

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Single Day Staining Procedure

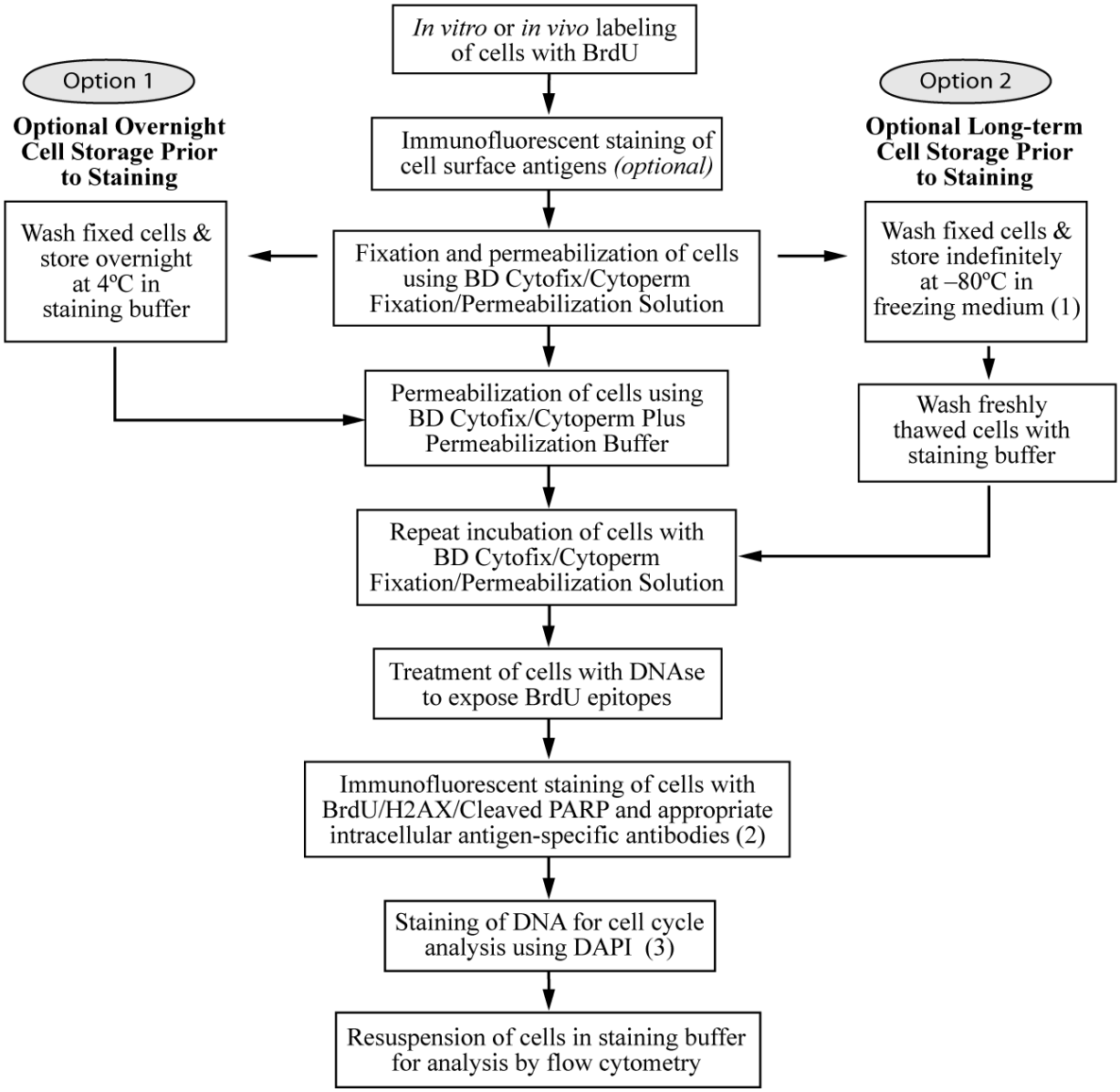

Figure 1. Overview: The BD Pharmingen™ Apoptosis, DNA Damage, and Cell Proliferation Kit Staining Protocol. *(1) Recipe for Freezing Media: 10% dimethyl sulfoxide (DMSO) + 90% heat-inactivated Fetal Bovine Serum (FBS). (2) The immunofluorescent staining of cell surface antigens can be done at the same time as staining intracellular antigens provided that the antibodies recognize paraformaldehyde-fixed epitopes. (3) If staining for total DNA content is not desired, then the DAPI staining step can be omitted and fluorescent data for another parameter can then be measured in the UV or Violet channel.*

Preparation and Storage

The monoclonal antibody was purified from tissue culture supernatant or ascites by affinity chromatography.

The antibody was conjugated with PerCP-Cy5.5 under optimum conditions, and unconjugated antibody and free PerCP-Cy5.5 were removed. Storage of PerCP-Cy5.5 conjugates in unoptimized diluent is not recommended and may result in loss of signal intensity.

The antibody was conjugated to Alexa Fluor® 647 under optimum conditions, and unreacted Alexa Fluor® 647 was removed.

The antibody was conjugated with R-PE under optimum conditions, and unconjugated antibody and free PE were removed.

Application Notes

Recommended Assay Procedure:

Kit 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. Instructions for handling, preparation, and storage of kit components is as follows:

PerCP-Cy™5.5 Mouse Anti-BrdU Antibody. This vial contains 250 µl of the stock solution of PerCP-Cy™5.5 Anti-BrdU antibody that is sufficient for staining 50 samples (10^6 cells/sample). This fluorescent antibody should be stored in the dark at 4°C. Add 5 µl of the stock antibody solution to the sample.

Alexa Fluor® 647 Mouse Anti-H2AX (pS139) Antibody. This vial contains 250 µl stock solution of Alexa Fluor® 647 Anti-H2AX (pS139) antibody that is sufficient for staining 50 samples (10^6 cells/sample). This fluorescent antibody should be stored in the dark at 4°C. Add 5 µl of the stock antibody solution to the sample.

PE Mouse Anti-Cleaved PARP (Asp214) Antibody. This vial contains 250 µl of stock solution of PE Anti-Cleaved PARP (Asp214) conjugated antibody that is sufficient for staining 50 samples (10^6 cells/sample). This fluorescent antibody should be stored in the dark at 4°C. Add 5 µl of the stock antibody solution to the sample.

DAPI (4',6-diamidino-2-phenylindole dihydrochloride) is a fluorescent dye for labeling DNA for flow cytometric analysis. Stock solution is at 1 mg/ml in water. Add 1 µl of the DAPI stock solution per ml of staining buffer. One vial of DAPI is provided and should be stored in the dark at 4ºC.

BD Cytofix/Cytoperm™ Plus Permeabilization Buffer. BD Cytofix/Cytoperm Plus Permeabilization Buffer is specially formulated for the Apoptosis, DNA Damage, and Cell Proliferation Kit and is used as a staining enhancer and secondary permeabilization reagent (100 µl/sample). One 10 ml bottle of BD Cytoperm Plus Buffer is provided and should be stored at 4°C.

N*ote*: The BD Cytoperm Plus Buffer should be used with fixed cell samples only. Use of this buffer with unfixed cells will cause cell damage.

BrdU. Each vial contains 0.5 ml of a 10 mg of BrdU/ml (32.5 mM) solution diluted in 1X DPBS. The BrdU solution is prepared aseptically (0.22 µm filtered) and contains no preservative; therefore it is recommended that the solution be handled under aseptic conditions. This stock solution can be injected intraperitoneally (i.p.) into animals or diluted to a 1 mM solution for *in vitro* labeling of cells. For *in vivo* labeling of S-phase cycling cells by i.p. injection, proceed to the *in vivo* labeling section of the Technical Data Sheet (page 4). To label cells *in vitro*, first dilute the stock (10 mg of BrdU /ml solution) to a 1 mM solution by adding 31 µl of the BrdU stock solution to either 1 ml of 1X DPBS or culture medium (this is a dilution of 32X). Add 10 µl of the 1 mM BrdU solution to each ml of culture medium to obtain a final concentration of 10 µM. The molecular weight of BrdU is 307.1. Five vials of BrdU Solution are provided and should be stored at -80°C.

Note: The BrdU solution has been shown to be stable for up to 4 months at 4˚C or can be refrozen. Avoid multiple freeze-thaw cycles.

DNase. Each vial contains 300 µl of a 1 mg of DNase/ml solution in 1X DPBS. When staining 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.

Note: If fewer than 10 samples are being treated with DNase, take a 30 µl aliquot of (1 mg/ml) DNase solution/sample and refreeze the remaining 1 mg/ml DNase at -80°C.

Note: DNase stock solution (ie, at 1 mg DNase/ml) may be refrozen once before it loses activity. A total of 100 µl of the working stock is used to treat each cell sample (ie, 30 µg of DNase/10^6 cells) with incubations performed at 37°C. Five vials of DNase are provided and should be stored at -80°C.

BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution. BD Cytofix/Cytoperm Fixation/Permeabilization Solution constitutes a single step fixation and permeabilization reagent that is 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. A 25 ml bottle of BD Cytofix/Cytoperm Fixation/Permeabilization Solution is provided in a ready to use formulation. BD Cytofix/Cytoperm Fixation/Permeabilization Solution should be stored at 4°C.

BD Perm/Wash™ Buffer (10X). Each 25 ml bottle contains 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. The concentrated stock buffer should be diluted 1:10 with deionized water; unused portions of 1X BD Perm/Wash Buffer may be stored at 4°C. The two bottles of 10X BD Perm/Wash Buffer that are provided should be stored at 4°C.

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, the precipitate may be removed prior to use by filtration of the diluted 1X BD Perm/Wash Buffer through a 0.45 µm-pore filter.

Note: The BD Perm/Wash Buffer (1X) should be used with fixed cell samples only. Use of this buffer on unfixed cells will cause cell damage.

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

Protocols for Labeling Cells with BrdU

***In vitro*-labeling of cultured cells and cell lines with BrdU**

Many different protocols for *in vitro* BrdU labeling of cells have been reported. 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. 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.

To label cells *in vitro*, carefully add 10 µl of BrdU solution (1 mM BrdU in 1X DPBS) directly to each ml of tissue culture media. For this step, it is important to avoid disturbing the cells in any way (eg, by centrifugation steps or temperature changes) that may disrupt their normal cell cycling patterns. The cell culture density should not exceed 2 × 10^6 cells/ml. The treated cells are then incubated for the desired length of time. For pulse-labeling experiments, the choice of time points and lengths of time for pulsing depend 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 30-45 minutes (ie, when the cells are in the logarithmic phase of cell proliferation). Researchers should determine time points and pulse-labeling time intervals that are optimal for each different cell line or cell population within a particular experimental system. Cells from the same population that are not BrdU-labeled are the recommended negative staining control for this assay. This will allow determination of background staining levels for the anti-BrdU monoclonal antibody.

Methods for *in vivo*-labeling of mouse cells with BrdU

Two common methods reported for *in vivo* BrdU labeling of cells include the intraperitoneal (i.p.) injection of a BrdU-containing solution into mice and the feeding of mice with BrdU that is added to their drinking water. Please note however that these methods are not routinely tested at BD Biosciences Pharmingen.

Method No. 1: Method for 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 i.p. with 100-200 µl (1-2 mg) of BrdU solution. Incorporation of BrdU can be readily detected in thymus and bone marrow derived cells in as little as 1 hr post injection.

Method No. 2: 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. 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. 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. BrdU incorporation by cells from these animals has been detected past 70 days.

Apoptosis, DNA Damage and Cell Proliferation Kit Staining Protocol

1. Immunofluorescent staining of cell surface antigens.
   a. Add BrdU-pulsed cells (10^6 cells in 50 µl of staining buffer) to flow cytometry tubes.
   b. Add fluorescent antibodies specific for cell-surface markers in 50 µl of staining buffer (eg, BD Pharmingen Stain Buffer (FBS) Cat. No. 554656) per tube and mix well.
   c. Incubate cells with antibodies for 15 minutes on ice.
   d. Wash cells 1X by adding 1 ml of staining buffer per tube, centrifuge (5 min) at 200-300*g*, and discard supernatant.

2. Fix and permeabilize cells with BD Cytofix/Cytoperm Fixation/Permeabilization Solution.
   a. Resuspend cells with 100 µl of BD Cytofix/Cytoperm Fixation/Permeabilization Solution per tube.
   b. Incubate cells for 15-30 minutes at room temperature or on ice.
   c. Wash cells 1X with 1 ml of 1X BD Perm/Wash Buffer, centrifuge as in step 1d and discard supernatant.

3. Incubate cells with BD Cytofix/Cytoperm Plus Permeabilization Buffer.
   a. Resuspend cells with 100 µl of BD Cytofix/Cytoperm Plus Permeabilization Buffer per tube.
   b. Incubate cells for 10 minutes on ice.
   c. Wash cells 1X by adding 1 ml of 1X BD Perm/Wash Buffer (as in Step 2c).

4. Re-Fixation of cells
   a. Resuspend cells with 100 µl of BD Cytofix/Cytoperm Fixation/Permeabilization Solution per tube.
   b. Incubate cells for 5 minutes at room temperature or on ice.
   c. Wash cells 1X by adding 1 ml of 1X BD Perm/Wash Buffer (as in Step 2c).

5. Treatment of cells with DNase to expose incorporated BrdU.
   a. Resuspend cells with 100 µl of diluted DNase (diluted to 300 µg/ml in DPBS) per tube, (ie, 30 µg of DNase to each tube).
   b. Incubate cells for 1 hour at 37°C.
   c. Wash cells 1X by adding 1 ml of 1X BD Perm/Wash Buffer (as in Step 2c).

6. Stain BrdU, H2AX (P139), Cleaved PARP (Asp214) and intracellular /surface antigens with fluorescent antibodies.
   a. Resuspend cells with 20 µl BD Perm/Wash Buffer + PerCP-Cy™5.5 Anti-BrdU (5 µl/test), Alexa Fluor® 647 Mouse Anti-H2AX (pS139) (5 µl/test), PE Anti-Cleaved PARP (Asp214) (5 µl/test) antibodies with or without other antibodies specific for intracellular or surface antigens.
   b. Incubate cells for 20 minutes at room temperature.
   c. Wash cells 1X by adding 1 ml of 1X BD Perm/Wash Buffer (as in Step 2c).

7. Optional - Staining of total DNA for cell cycle analysis.

a. Resuspend cells with 1 ml of DAPI solution (1 µg/ml).

b. Analyze stained cells with a flow cytometer (run at a rate no greater than 400 events/sec.) and acquire multiparameter data files.

Note: If you perform the optional staining in Step 7, you are done and can proceed to analyze your cell samples. Otherwise, proceed to Step 8 if the staining of total DNA levels is not desired.

Note: Samples may be stored overnight at 4°C, protected from

8. Resuspension of cells for Flow Cytometric Analysis.

a. Add 1 ml of staining buffer to each tube to resuspend cells.

b. Analyze stained cells with a flow cytometer (run at a rate no greater than 400 events/sec.) and acquire multiparameter data files.

Note: Samples may be stored overnight at 4°C, protected from light.

Recommended controls for instrument setup

Each individual antibody is recommended as the control for instrument set up.

1. PerCP-Cy™5.5 Anti-BrdU (Display in Log Mode)
2. PE Anti-Cleaved PARP (Asp214) (Display in Log Mode)
3. Alexa Fluor® 647 Mouse Anti-H2AX (pS139) (Display in Log Mode)
4. DAPI (Display in Linear Mode)

Use the autocompensation in BD FACSDiva™ software to compensate for the experiment. For the DAPI signal, select the entire DNA histogram population. DAPI is displayed in the linear mode and the G0/G1 peak is set to the 50,000 channel on the histogram.

Danger: *BD Cytofix/Cytoperm™ Fixation/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.

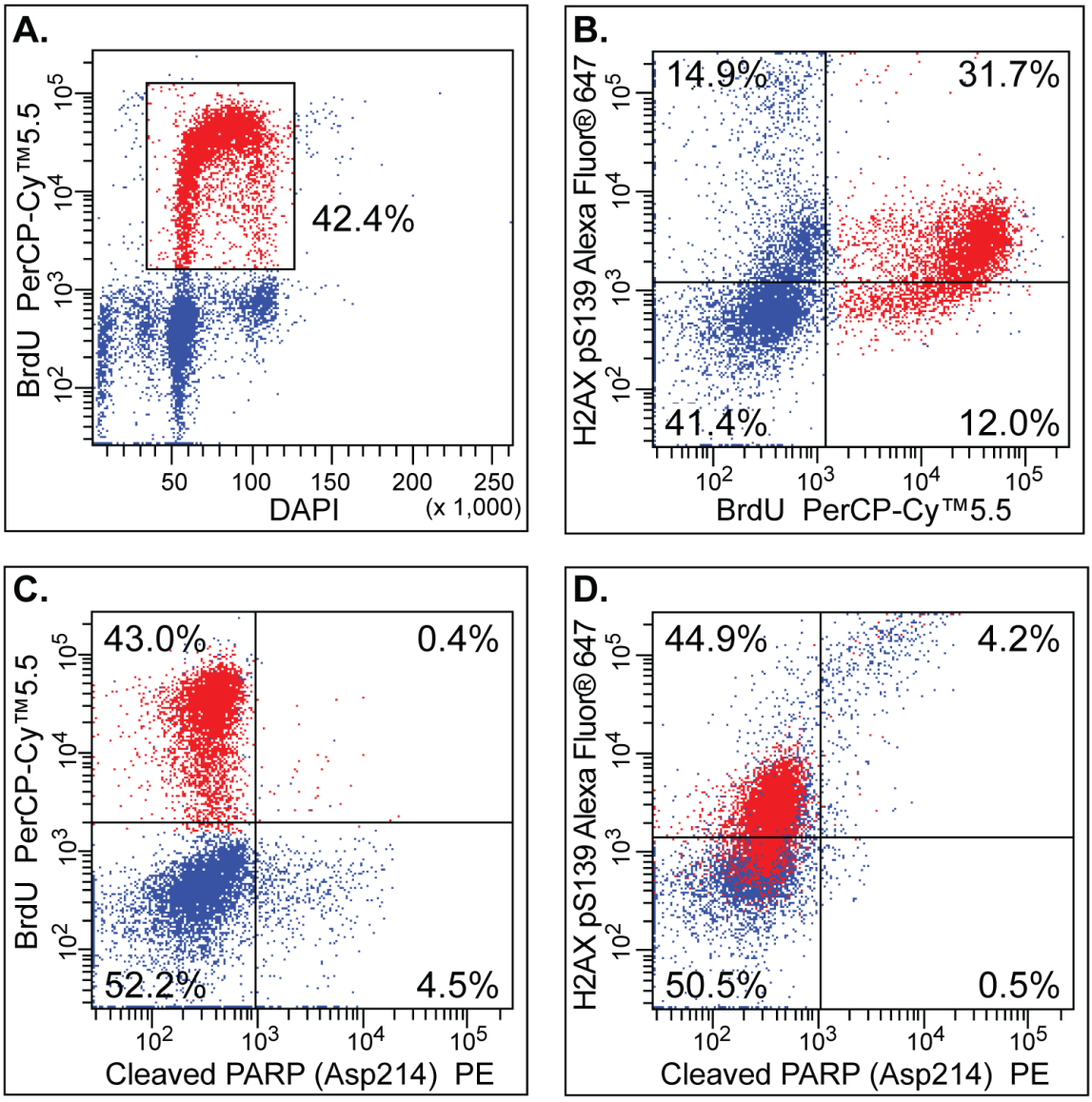

Figure 2. Multiparameter cell cycle analysis of stimulated human peripheral blood mononuclear cells (PBMC). *PBMC were stimulated with Purified NA/LE Mouse Anti-Human CD3 (Cat. No. 555329) and Purified NA/LE Mouse Anti-Human CD28 (Cat. No. 555725) antibodies for 3 days. The activated cells were harvested and re-plated in complete medium and labeled with 50 μM BrdU for 1 hr. The cells were then harvested and analyzed by immunofluorescent staining and multicolor flow cytometric analysis using a BD™ LSR II Flow Cytometer System. Panel A: DAPI versus BrdU PerCP-Cy™5.5 staining profile for activated PBMC. Panel B: BrdU PerCP-Cy™5.5 versus H2AX (pS139) Alexa Fluor® 647 profile. Panel C: Cleaved PARP (Asp214) PE versus BrdU PerCP-Cy™5.5 profile. Panel D: Cleaved PARP (Asp214) PE versus H2AX (pS139) Alexa Fluor® 647 profile. BrdU-positive cells are color-gated red whereas BrdU-negative cells are colored blue.*

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Suggested Companion Products

Catalog Number	Name	Size	Clone
554656	Stain Buffer (FBS)	500 mL	(none)

Product Notices

1. This reagent has been pre-diluted for use at the recommended Volume per Test. We typically use 1 × 10^6 cells in a 100-µl experimental sample (a test).
2. Alexa Fluor® is a registered trademark of Molecular Probes, Inc., Eugene, OR.
3. Alexa Fluor® 647 fluorochrome emission is collected at the same instrument settings as for allophycocyanin (APC).
4. The Alexa Fluor®, Pacific Blue™, and Cascade Blue® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc. for research use only, excluding use in combination with microarrays, or as analyte specific reagents. The Alexa Fluor® dyes (except for Alexa Fluor® 430), Pacific Blue™ dye, and Cascade Blue® dye are covered by pending and issued patents.
5. This product is subject to proprietary rights of Amersham Biosciences Corp. and Carnegie Mellon University and made and sold under license from Amersham Biosciences Corp. This product is licensed for sale only for research. It is not licensed for any other use. If you require a commercial license to use this product and do not have one return this material, unopened to BD Biosciences, 10975 Torreyana Rd, San Diego, CA 92121 and any money paid for the material will be refunded.
6. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
7. Caution: 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.
8. Cy is a trademark of Amersham Biosciences Limited. This conjugated product is sold under license to the following patents: US Patent Nos. 5,486,616; 5,569,587; 5,569,766; 5,627,027.
9. Please observe the following precautions: Absorption of visible light can significantly alter the energy transfer occurring in any tandem fluorochrome conjugate; therefore, we recommend that special precautions be taken (such as wrapping vials, tubes, or racks in aluminum foil) to prevent exposure of conjugated reagents, including cells stained with those reagents, to room illumination.
10. PerCP-Cy5.5–labelled antibodies can be used with FITC- and R-PE–labelled reagents in single-laser flow cytometers with no significant spectral overlap of PerCP-Cy5.5, FITC, and R-PE fluorescence.
11. PerCP-Cy5.5 is optimized for use with a single argon ion laser emitting 488-nm light. Because of the broad absorption spectrum of the tandem fluorochrome, extra care must be taken when using dual-laser cytometers, which may directly excite both PerCP and Cy5.5™. We recommend the use of cross-beam compensation during data acquisition or software compensation during data analysis.
12. For fluorochrome spectra and suitable instrument settings, please refer to our Multicolor Flow Cytometry web page at www.bdbiosciences.com/colors.
13. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

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