BD Pharmingen™

BrdU *In-Situ* Detection Kit

Instruction Manual

Cat No. 550803
Cat No. 551321

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Kit Contents

Kit	Size	Cat. No.
BrdU *In-situ* Detection Kit	50 slides	550803
Biotinylated Anti-BrdU Antibody	0.5 ml	51-75512L
Diluent Buffer	15 ml	51-2373KD
BD™ Retrievagen A, Solution 1	10 ml	51-7540KD
BD™ Retrievagen A, Solution 2	50 ml	51-7541KD
Fixation Buffer	10 ml	51-2374KD
DAB Chromogen	0.75 ml	51-7549KD
DAB Buffer	10 ml	51-7548KD
Streptavidin-HRP	6 ml	51-7642KD
Control Slides	5 slides	51-7550KD
BrdU *In-situ* Detection Kit II	200 slides	551321
Biotinylated Anti-BrdU Antibody	2.0 ml	51-75512X
Diluent Buffer	40 ml	51-2373KC
BD™ Retrievagen A, Solution 1	40 ml	51-7540KE
BD™ Retrievagen A, Solution 2	200 ml	51-7541KE
Fixation Buffer	40 ml	51-2374KC
DAB Chromogen	1.0 ml	51-7549KE
DAB Buffer	20 ml	51-7548KE
Streptavidin-HRP	20 ml	51-7642KC
Control Slides	5 slides	51-7550KD

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Introduction

Cell proliferation is an important and highly studied cellular function. Different approaches to measurement of proliferation include the identification of mitotic figures and the use of autoradiography to identify [3H]-thymidine incorporation into DNA by proliferating cells.[1] In 1982, Gratzner *et al.* reported an approach to the detection of cells in S (synthetic)-phase of cell cycle, based on the use of antibodies against bromodeoxyuridine (BrdU), a thymidine analog.[2] Cellular incorporation of BrdU during S phase can be readily detected by anti-BrdU-specific antibodies following membrane permeabilization. Subsequent quantitation of cells positive for BrdU is possible using routine methods for cell analysis such as flow cytometry or immunohistochemistry.

One advantage of the immunohistochemical detection of BrdU compared to [3H]-thymidine incorporation and subsequent autoradiography is that this approach takes much less time and provides high resolution.[3-5] Immunohistochemical staining for incorporated BrdU has been used to determine proliferation rates and cell cycle kinetics by measuring the labeling indices of different cell populations in normal and cancerous tissues.[6-9]

The most common technical difficulty in obtaining effective staining for BrdU is the inability of the antibody to penetrate between the DNA strands. Typically, methods such as treatment with HCl, ethanol fixation, enzymatic digestion, or microwave pre-treatment are used to permeabilize the cell and relax the DNA to make the BrdU accessible to the antibody. Unfortunately, treatment with HCl or enzymatic digestion destroys architectural detail and more importantly reduces or destroys immunoreactivity to other antigens. To mitigate this problem and improve immunostaining, alternative fixatives such as methacarn or antigen retrieval techniques using heat and citrate buffer, can be employed to unmask the antigenic sites leading to improved immunostaining. Use of these antigen retrieval methods are also compatible with simultaneous detection of other antigens.[10-14]

Overview

BrdU *In-Situ* Detection Kit

The BD Pharmingen™ BrdU *In-Situ* Detection Kit is designed for immunohistochemical staining of BrdU in frozen sections, formalin-fixed paraffin-embedded sections, and cultured or isolated cells on slides. Our monoclonal antibody against BrdU works in all species tested (human, mouse, and rat) and provides improved specific staining with minimal background. The directly biotinylated mouse antibody eliminates the need for a species-specific secondary antibody thus allowing it to be used in mouse tissues. The BrdU *In-Situ* Detection Kit features our BD™ Retrievagen A, an antigen retrieval solution designed specifically to unmask antigenic sites, preserve tissue morphology, and enable simultaneous staining of other surface antigens in conjunction with BrdU. This important feature of the kit enables study of the proliferation state of phenotypically defined cells within the micro-environment of tissues. Consistent results are assured by providing all critical reagents in addition to a comprehensive series of protocols for staining BrdU in paraffin-embedded and frozen tissue sections and cultured cells, and in conjunction with other antigens. Control slides are provided in the kit to serve as a reference.

Kit Components

All the components of the BrdU kit must be stored at 4°C. Some solutions require diluting or mixing prior to use. Instructions for handling, preparation, and storage of kit components follow:

Anti-BrdU Antibody

One vial of biotinylated anti-BrdU antibody, for the detection of incorporated BrdU. Prior to use, dilute the stock solution 1:10 with the Diluent Buffer. Store at 4°C. Do not freeze.

> *Note*: The biotinylated mouse anti-BrdU antibody can be used on mouse tissues.

BD™ Retrievagen A (pH 6.0) Solution 1 and Solution 2

The BD™ Retrievagen A solutions form an antigen retrieval system that mediates the unwinding of the DNA to expose the incorporated BrdU for antibody staining. To make a working solution, take 9 ml of Solution 1 and 41 ml of Solution 2 and bring up the volume to 500 ml with distilled water. This working solution can be stored at 4°C for up to one month. Store the stock solutions at 4°C.

Fixation Buffer

The Fixation Buffer is provided to fix frozen sections and isolated or cultured cells on slides. Alternatively, frozen sections may be fixed for 2 minutes in acetone. Store at 4°C.

> *Note*: Additional fixation is not required for paraffin sections as they have been previously fixed in formalin.

Diluent Buffer

Ready-to-use Diluent Buffer is provided to dilute the anti-BrdU antibody to a working concentration and to permeabilize the cell membrane. This solution contains both blocking proteins and detergents to reduce background and permeabilize the cells. Store at 4°C.

> *Note*: When staining cultured or isolated cells it is necessary to incubate the slides in the Diluent Buffer to permeabilize the cell membrane prior to staining.

Streptavidin-HRP

The Streptavidin-HRP (horseradish peroxidase) solution is provided for the detection system. The Streptavidin binds to the biotinylated anti-BrdU antibody and the HRP enzyme is used with the DAB substrate system to visualize the results. Store at 4°C.

> *Note*: Do not use sodium azide as a reagent preservative. Sodium azide inactivates the horseradish peroxidase enzyme over time.

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DAB Buffer and Chromogen

The DAB (Diaminobenzidine) substrate system is provided to visualize the BrdU staining pattern via the HRP enzyme. DAB Buffer and Chromogen are provided separately to prevent complex formation. To prepare a working solution, add one drop of DAB chromogen for every 1 ml of DAB Buffer. DAB Buffer and Chromogen must be stored separately at 4°C.

Note: Working DAB substrate solution must be mixed immediately prior to use, and cannot be stored after initial use.

Control slides

Five control slides containing formalin-fixed, paraffin-embedded sections of mouse small intestine are provided. Each slide has a section of small intestine from a mouse that has been intra-peritoneally injected with BrdU (positive control) and a section of the same tissue from a control mouse injected with phosphate buffered saline (negative control). One of the five slides is stained for BrdU using the BrdU *In-Situ* Detection Kit and can be used as a reference. Control slides can be stored at room temperature, however storing at 4°C is recommended for optimal preservation of antigens.

Additional Reagents Required

BrdU

BrdU is required for *in vitro* or *in vivo* labeling and must be purchased separately (Cat. No. 550891).

H_2O_2 Solution

A stock solution of H_2O_2 (30%) is required for blocking endogenous peroxidase.

Phosphate Buffer Saline (PBS)

Recipe for 1 × PBS (1 liter)

KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8.0 g
Na_2HPO_4 $7H_2O$	2.16 g
pH	7.2 – 7.4

Warnings and Precautions

1. Biotinylated anti-BrdU antibody contains 0.09% Sodium Azide. Sodium Azide yields a highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
2. DAB Buffer contains <5% hydrogen peroxide. Avoid contact with skin and eyes.
3. DAB chromogen contains <2% 3 – 3-diaminobenzidine which is a suspected carcinogen. Handle with care and dispose according to regulations.
4. Fixation Buffer contains 5% v/v phosphate buffered formalin, a suspected carcinogen. Avoid contact with skin, eyes, and mucous membranes.

BrdU labeling

Prior to BrdU immunostaining for detection of proliferating cells, it is necessary to label the cells or tissue with BrdU. Labeling can be done either *in vitro* or *in vivo*. Both protocols are provided.

In vitro Labeling of Cultured Cells and Cell Lines with BrdU

Many different protocols for *in vitro* BrdU labeling of cells have been reported.[15-21] We have found that incubating cells with BrdU at a final concentration of 10 µM in cell culture medium is effective for labeling a wide variety of human and mouse cell lines and normal cell populations.[16]

To label cells *in vitro*, carefully add 10 µl of BrdU solution (1 mM BrdU in 1× Dulbecco's PBS) for each ml of tissue culture medium. 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. The incubation time with BrdU is dependent 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). Investigators should determine the incubation times 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 negative cell staining control.

In vivo Labeling of Mouse Cells with BrdU

Two common methods used for *in vivo* BrdU labeling of tissues and cells include intraperitoneal injection of a BrdU-containing solution into mice and addition of BrdU to the animal's drinking water.

1. Intraperitoneal Method:

   Make a working solution of BrdU in PBS at 1 mg/ml. Inject mice via the intraperitoneal route with 1 ml (1 mg) of BrdU solution.[17,19,21] Incorporation of BrdU can be detected in thymus and bone marrow in as little as 1 hr post injection. At 24 hr post injection BrdU can be detected in most tissues.

2. Drinking water method:

   Dilute BrdU to 0.8 mg/ml in the drinking water.[18,20] The BrdU mixture should be made fresh and changed daily. Prolonged feeding of BrdU can have toxic effects. Some investigators have reported lethal effects associated with 14 days of continuous BrdU feeding. For long-term studies, some investigators have reported that feeding mice with BrdU for 9 consecutive days, followed by a change over to normal water, has worked effectively. BrdU incorporation by cells from these animals has been detected past 70 days.

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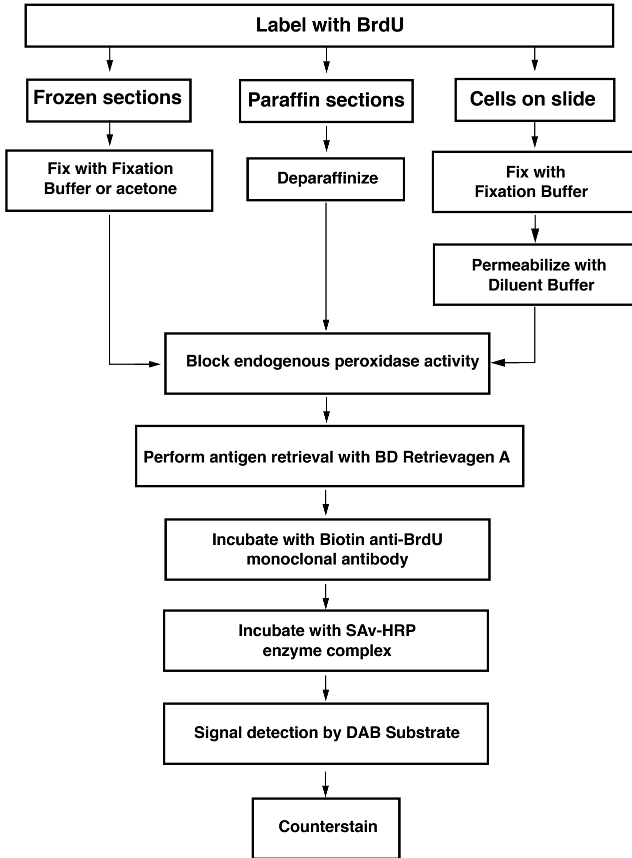
Protocol for Immunostaining for BrdU

The following section describes the immunohistochemical technique to stain BrdU in formalin-fixed, paraffin-embedded sections, frozen sections, and cultured cells/cytospins. Tissue is harvested and processed for paraffin or frozen sections by standard tissue processing procedures.



Formalin-fixed, Paraffin-embedded Sections

1. Deparaffinize slides in 2 changes of xylene (or xylene substitute) for 5 minutes each.
2. Transfer slides to 100% alcohol. Perform 2 changes for 3 minutes each time. Transfer once through 95% alcohol for 3 minutes.
3. Block endogenous peroxidase activity by incubating slides for 10 minutes in **3% H_2O_2** in PBS.
4. Rinse slides in PBS, 3× for 5 minutes each time.
5. Continue with antigen retrieval as described below.

Frozen Sections

1. Fix frozen sections in Fixation Buffer for 15 minutes.
2. Alternatively, frozen sections can be fixed in cold acetone for 2 minutes at –20°C.
3. Wash slides in PBS 2× for 5 minutes each.
4. Block endogenous peroxidase activity by incubating slides for 10 minutes in **0.3% H_2O_2** in PBS.
5. Rinse slides in PBS, 3× for 5 minutes each time.
6. Continue with antigen retrieval as described below.

Cultured Cells or Cytospins

1. Fix cultured cells on chamber slides, or isolated cells in cytospins, in Fixation Buffer for 15 minutes.
2. Wash slides 2× in PBS for 5 minutes each time.
3. Incubate slides with Diluent Buffer for 30 minutes to permeabilize the cells.
4. Wash slides 2× in PBS for 5 minutes each time.
5. Block endogenous peroxidase activity by incubating slides for 10 minutes in **0.3% H_2O_2** in PBS.
6. Rinse slides 3× in PBS for 5 minutes each time.
7. Continue with antigen retrieval as described below.

Antigen Retrieval and Immunostaining Staining of BrdU

1. Prepare a working solution of BD™ Retrievagen A by mixing 9 ml of Solution 1 with 41 ml of Solution 2 and bringing up the volume to 500 ml with distilled water. Place the slides in a coplin jar containing the working solution of BD Retrievagen A and heat in a microwave oven to 193°F (89°C). Other sources of heat such as pressure cooker or hot water bath may be used. Hold the slides at that temperature for 10 minutes. Remove the coplin jar with the slides, cover the jar tightly and allow the solution to slowly cool down to room temperature for 20 minutes.

   Note: It is important to allow the temperature to slowly ramp down.
2. Rinse slides 3× in PBS, 5 minutes each time.
3. Dilute the biotinylated anti-BrdU antibody 1:10 in the Diluent Buffer. Apply the antibody to the sections on the slide (100 µl/slide) and incubate for 1 hour in a humidified chamber.
4. Rinse slides 3× in PBS, 2 minutes each time.
5. Apply the ready-to-use Streptavidin-HRP to each slide and allow to incubate for 30 minutes, at room temperature.

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6. Rinse slides 3× in PBS, 2 minutes each time.
7. Prepare DAB substrate solution by adding 1 drop of DAB chromogen to 1 ml of DAB Buffer.
8. Apply DAB substrate solution to cover the tissue sections and incubate for 5 minutes or less until the desired color intensity is developed.

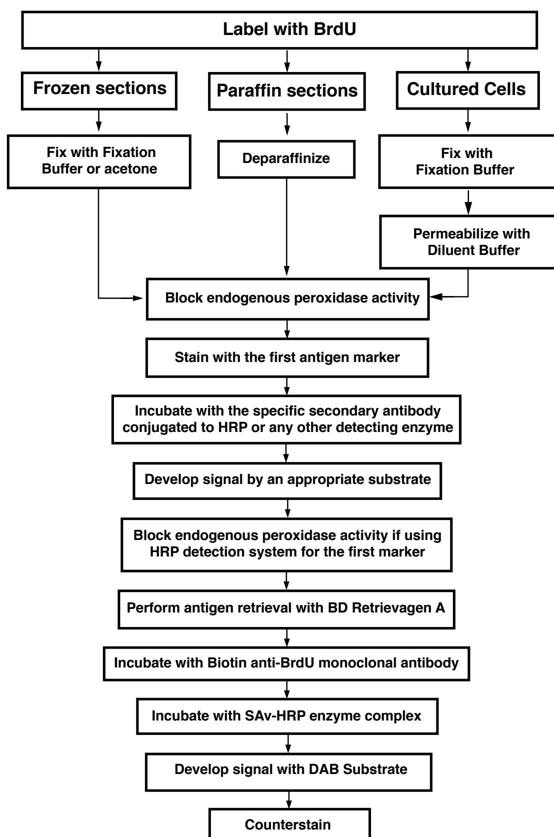
   Note: The mixed DAB substrate solution cannot be stored.
9. Rinse the slides 3× in water, 2 minutes each time.
10. Counterstain slides:
    a. Stain in hematoxylin for 30 – 60 seconds.
    b. Rinse thoroughly in water.
11. Dehydrate through 4 changes of alcohol (95%, 95%, 100%, 100%) for 5 minutes each.
12. Clear in 3 changes of xylene (or xylene substitute) and coverslip.

    Note: When using AEC Substrate do not dehydrate in alcohol or use xylene. Perform an aquamount after counterstaining.

Two-color Immunohistochemical Staining of Other Antigens with BrdU

Simultaneous staining of other antigens with BrdU is possible, but is dependent on the conditions the other antigens can endure. Some modifications to the general protocol include:

1. If the antibody works only in frozen sections, perform staining in frozen sections using acetone for fixation followed by antigen retrieval and anti-BrdU staining.
2. If the antibody works in paraffin sections and requires no pre-treatment, then perform the first antigen staining followed by antigen retrieval and anti-BrdU staining.
3. If the antibody works in paraffin sections and requires antigen retrieval then perform antigen retrieval in the beginning followed by the first antigen staining and then anti-BrdU staining.
4. Purified or enzyme conjugated formats of the antibody for the first antigen with the appropriate secondary antibody (if necessary) and detection system can be used.



Two–color Immunostaining Protocol

Described below is a representative protocol for staining another antigen together with anti-BrdU.

Fix sections in acetone or fixation buffer, if required, and block for endogenous peroxidase activity with H_2O_2 solution as mentioned in ***Protocol for Immunostaining for BrdU***.

1. Dilute the purified primary antibody appropriately in Diluent Buffer and apply to the tissue sections. Incubate for 1 hour in a humidified chamber.
2. Rinse slides 3× in PBS, 2 minutes each time.
3. Dilute the HRP-conjugated secondary antibody to the appropriate concentration and apply to tissue sections. Incubate for 30 minutes.
4. Rinse slides 3× in PBS, 2 minutes each time.
5. Use AEC substrate to develop color.
6. Wash 3× in PBS, 3 minutes each time.

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7. Block endogenous peroxidase again with a **3% solution of H_2O_2** in PBS for the paraffin sections or a **0.3% solution of H_2O_2** in PBS for the frozen sections and cultured cells. This step will also inactivate any of the remaining HRP.
8. Wash 3× in PBS, 5 minutes each time.
9. Stain for incorporated BrdU by following the protocol for ***Antigen Retrieval and Immunostaining for BrdU*** on page 10.

Representative Examples of BrdU Immunostaining

Shown here are a few examples of BrdU staining in paraffin-embedded sections, frozen sections, and cultured cells. Immunohistochemical pictures of double–color staining are also included.

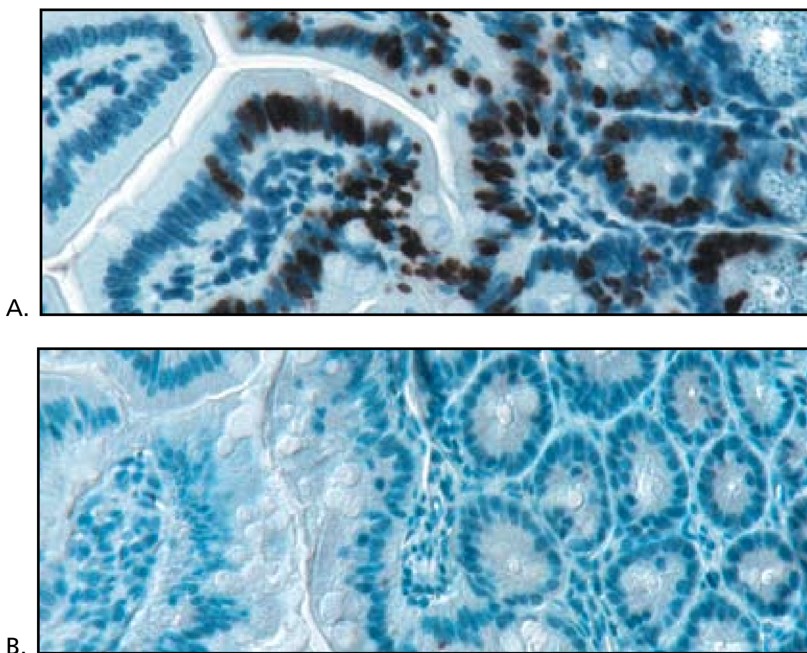

Figure 1. Immunohistochemical staining of BrdU in paraffin sections. BALB/c mice were injected with 1 mg of BrdU via the intra-peritoneal route. After 24 hrs the spleen, thymus, and gastro-intestinal tract were harvested and processed for paraffin sections. Mice injected with PBS served as the negative control. Immunohistochemical staining of BrdU was performed using the BrdU *In-Situ* Detection Kit on paraffin sections of the mouse gastro-intestinal tract. Proliferating cells in the crypts that incorporated BrdU can be identified by the dark brown color in their cell nuclei (A) in contrast to the control (B). Magnification 400×.

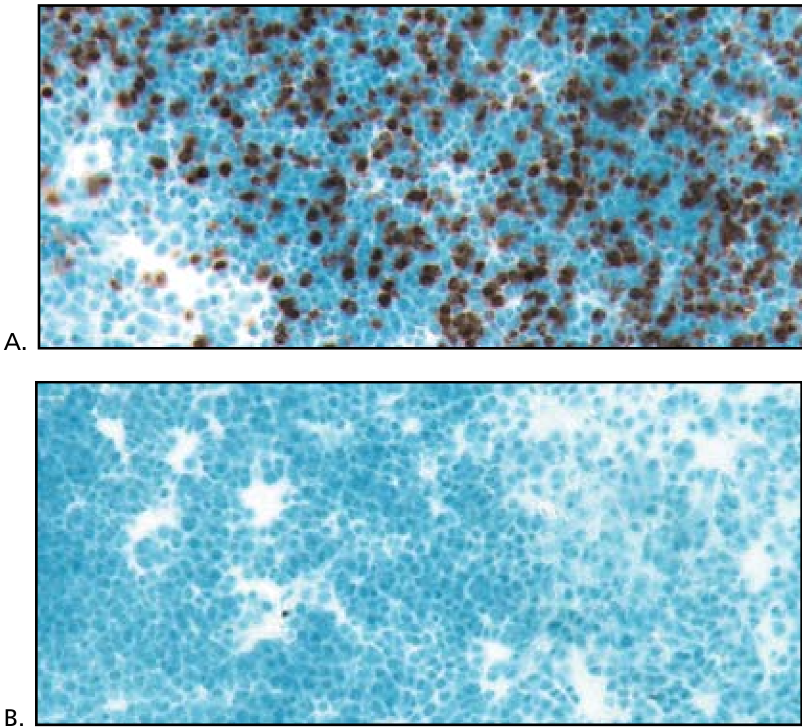

Figure 2. Immunohistochemical staining for BrdU in frozen sections. BALB/c mice were injected with 1 mg of BrdU via the intra-peritoneal route. After 24 hrs the spleen, thymus, and gastro-intestinal tract were harvested and processed for frozen sectioning. Mice injected with PBS served as the negative control. Immunohistochemical staining of BrdU was performed using the BrdU *In-Situ* detection Kit on acetone-fixed frozen sections of the mouse thymus. Cells that incorporated BrdU can be identified by the dark brown color in their cell nuclei (A) in contrast to the control (B). Magnification 400×.

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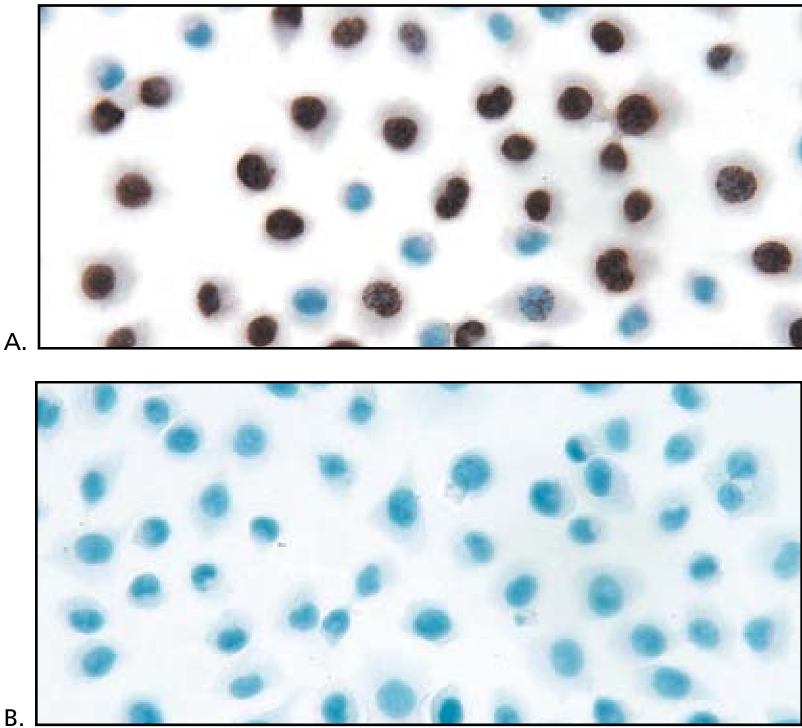

Figure 3. Immunohistochemical staining for BrdU in cultured L929 cells. L929 cells, a mouse fibroblast cell line, were grown on chamber slides at a cell density of 3×10^5 cells/well. During their logarithmic phase of cell division they were pulsed with a 10 µM solution of BrdU for 1 hour. L929 cells in culture media served as the control. Immunohistochemical staining for BrdU was performed using the BrdU *In-Situ* Detection Kit on the cultured cells. Cells that incorporated BrdU can be identified by the dark brown color in their cell nuclei (A) in contrast to the control (B). Magnification 400×.

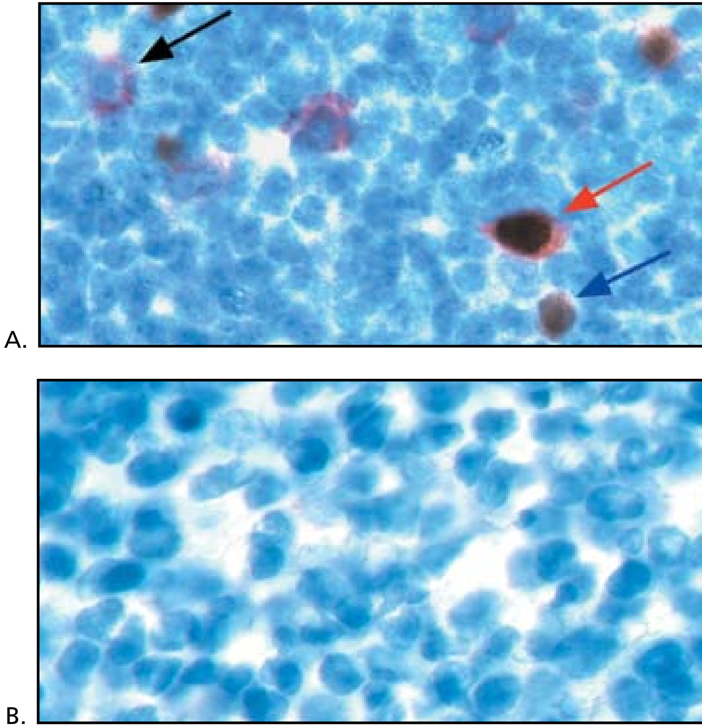

Figure 4. Double–color immunostaining for BrdU and CD45/B220. BALB/c mice were injected with 1 mg of BrdU via the intra-peritoneal route and after 24 hrs the spleen, thymus, and gastro-intestinal tract were harvested and processed for paraffin sectioning. Double-color immunostaining was performed on the paraffin sections of the mouse spleen using the BrdU *In-Situ* Detection Kit with the DAB substrate to label the BrdU and anti-CD45/B220 antibody with AEC substrate to label the B lymphocytes. Cells positive for BrdU alone can be identified by the dark brown staining of the nucleus (blue arrow), B lymphocytes can be identified by the reddish color on the cell membranes (black arrow), and B lymphocytes that have incorporated BrdU can be identified by the double color labeling of dark brown in their nucleus and red color on the cell surface (red arrow) (A). Figure 4B is an isotype control of the same experiment. Magnification 1000×.

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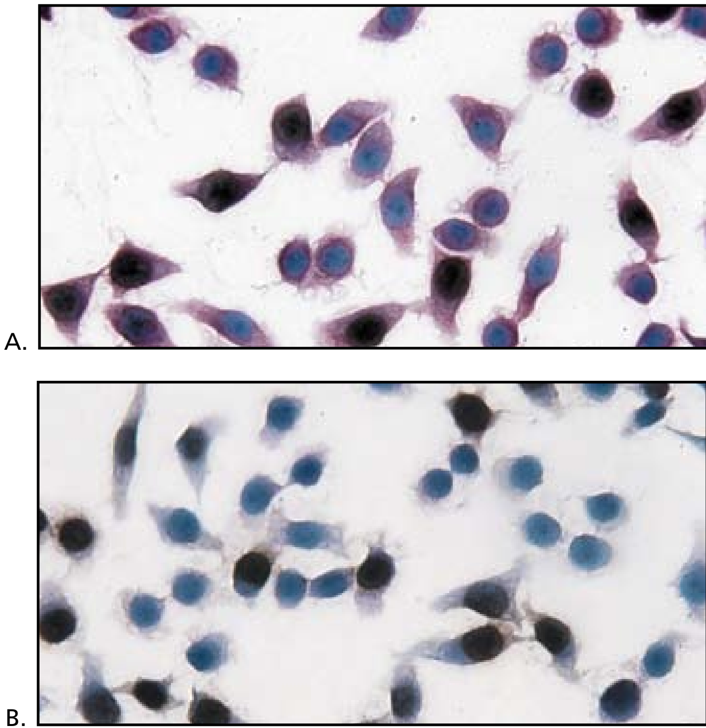

Figure 5. Double-color immunostaining for BrdU and Cytokeratin. HeLa cells were plated at a concentration of 3×10^4 cells/ml and pulsed with a 10 µM solution of BrdU for 2 hours. Immunostaining was performed on the cells with our BrdU *In-Situ* Detection Kit with DAB substrate to label the BrdU and anti-cytokeratin antibody with AEC substrate to label the cytoskeletal filaments in the cytoplasm. Cells positive for BrdU and cytokeratin can be identified by the dark brown staining of the nucleus and red staining of the cytoplasm (A). Cells stained with BrdU alone can be identified by the dark brown labeling of their nuclei (B) Magnification 1000×.

Troubleshooting Guide

This section addresses some of the common problems encountered in immunohistochemical staining.

Overstaining or Weak Staining

The intensity of BrdU staining is dependent on the amount of BrdU incorporation. Our recommended dilutions in this manual work well with our protocol for BrdU incorporation. However, overstaining or weak staining can be resolved by using the optimal dilution of the biotinylated anti-BrdU antibody.

The length of incubation times can also affect the staining intensity. It is important to follow the protocol for the incubation times during the various steps. However, incubation times with anti-BrdU antibody or the streptavidin-HRP can be adjusted to give the desired staining intensity.

Weak staining may be due to a variety of other factors such as:

a. The primary antibody does not recognize the antigen due to incorrect or over-fixation.

b. Incomplete embedding or antigen retrieval procedures.

c. Incompatibility of counterstain or mounting media which may have dissolved the chromogen reaction product.

d. Reagents are past their expiration date.

Non-specific Background Staining

Excessive staining in structures of the tissue that should be negative is termed non-specific background staining and may be due to any of the following reasons:

a. Inadequate rinsing between steps.

b. Tissue was allowed to dry out with the reagents during the immunostaining protocol.

c. Tissue contains endogenous biotin or endogenous enzyme.

To avoid non-specific background staining.

a. Ensure adequate washing of slides between steps.

b. Do not allow slides to dry out at any time.

c. If tissue has endogenous biotin it may be blocked by an avidin-biotin block or other suitable protein block.

Two-color Immunostaining

Double-color immunostaining is more complicated. It is important to choose antibodies that are compatible with the fixation, embedding, and antigen retrieval treatments. Performing single-color staining of the BrdU and the other marker prior to and in parallel with the double-color staining can be very helpful. Complete quenching of the enzyme activity after the first marker is developed is critical to prevent crossover of the first antigen into the next.

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Notes

Notes

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