In Situ Cell Proliferation Kit, FLUOS

Kit for the detection of 5-bromo-2’-deoxyuridine (BrdU) incorporated into cellular DNA by flow cytometry, immunocyto- and immunohistochemistry using an fluorescein conjugated monoclonal antibody.

Cat. No. 11 810 740 001
For 100 tests

1. Introduction

A broad range of biological and biomedical research depends on the ability to distinguish DNA synthesizing cells from resting cells. Assays to measure DNA synthesis usually involve the use of radiolabeled nucleosides, particularly the [3H] or [14C] isotopes of thymidine. These DNA precursors are incorporated into the genomic DNA during the S phase (DNA replication) of the cell cycle. Therefore, short incubation periods (15–60 min) of cells with e.g., [3H]-thymidine will label only cells going through the S phase of the cell cycle (= proliferating cells). The labeled cells are subsequently detected by autoradiography.

Detection of S phase cells is a potentially unique means for determining the kinetics of cycling cells within heterogeneous cell populations. This is particularly relevant for the study of tumors to determine the frequency of cycling cells (= growth fraction). Since it has been shown that 5-bromo-2’-deoxyuridine (BrdU), a thymidine analogue, shares S phase labeling characteristics with [3H]-thymidine (1, 2), immunohistochemical detection of BrdU incorporation into DNA has become a powerful tool for identifying cells in which DNA synthesis has occurred (3, 4). Individual BrdU-labeled cells are detected by immunochemical analysis using this In Situ Cell Proliferation Kit, FLUOS.

Advantages of the In Situ Cell Proliferation Kit, FLUOS
• Offers a non-radioactive alternative to tissue autoradiography
• No cross reactivity with endogenous Immunoglobulins
• No radioactive waste is produced
• Results are obtained within 3–4 hours
• Reagents are provided in a stable form, optimized and quality controlled to give reproducible performance.

2. Product description

2.1 Kit contents

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Content</th>
<th>Label</th>
<th>Cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 mM BrdU in PBS, pH 7.4, 1,000× conc., 1 ml, filtered through 0.2 μm pore size membrane</td>
<td>BrdU labeling reagent</td>
<td>Red, flip up</td>
</tr>
<tr>
<td>2</td>
<td>Monoclonal anti-BrdU-antibody (clone BMG 6H8), F(ab’)2, fragments, conjugated with fluorescein 5 × conc., lyophilized</td>
<td>Anti-BrdU-FLUOS</td>
<td>White</td>
</tr>
<tr>
<td>3</td>
<td>PBS based buffer, containing 0.5% BSA and 0.1% Tween 20, 100 ml</td>
<td>Incubation buffer</td>
<td>Blue</td>
</tr>
</tbody>
</table>

Specificity
Anti-BrdU antibody specifically binds to BrdU. It shows no crossreactivity with any endogenous cellular components such as thymidine or uridine. The antibody binds only to BrdU incorporated into DNA after denaturation/partial degradation of double stranded DNA.

Stability
The kit is stable until expire date (see lot-specific imprint) at +2 to +8°C. For stability and recommended storage conditions of working solutions see section 4.1.

3. Application

Assay principle
The assay (figure 1) is an immunocyto-/immunohistochemical technique which uses a mouse monoclonal antibody against BrdU. The procedure involves:

A: Growing animal tissue or cells in vitro and labeling them with BrdU. Alternatively, injecting the BrdU labeling reagent into an animal, to label the DNA in vivo, then sacrificing the animal and preparing tissue sections. Only proliferating cells incorporate BrdU into their DNA.

B: Fixing BrdU-labeled tissue or cells and denaturing the cellular DNA by acid (HCl).

C: Detecting incorporated BrdU with a fluorescein-conjugated anti-BrdU monoclonal antibody, F(ab’)2 fragments (anti-BrdU-FLUOS).

D: Analyzing the samples under a fluorescence microscope or on a flow cytometer.

3.2 Sample material

• Cell lines, freshly isolated cells, tissue explants labeled with BrdU in vitro.

• If cells in the S phase only are to be labeled, the sample should be incubated with BrdU only for a short period of time (e.g., 30–60 min with 10 μM BrdU).

Note: Under these conditions the addition of 5-fluoro-2’-deoxyuridine does not enhance the incorporation of BrdU (5).

• Cells, tissue sections labeled with BrdU in vivo.

4. Preparation of the solutions

4.1 Preparation of the working solutions

1. BrdU labeling solution

For in vitro labeling (see section 5.1), predilute 0.1 ml BrdU labeling reagent (bottle 1) with 9.9 ml sterile culture medium (resulting concentration: 100 μM BrdU). Stability of this solution: stable for 1 month at +2 to +8°C; for long term storage in aliquots at −15 to −25°C.

For in vivo labeling (see section 5.2), use the BrdU labeling reagent (bottle 1) undiluted. The BrdU labeling reagent is stable at +2 to +8°C until control date (see lot-specific imprint).

For life sciences research only. Not for use in diagnostic procedures.
Add prewarmed (37°C) culture medium to the working solution: cannot be stored. Stability of the undiluted anti-BrdU-FLUOS stock solution: stable at +2 to +6°C in the dark for 12 months.

Tissue slices
- Add prewarmed (37°C) culture medium to the freshly isolated tissue.
- Cut tissue sample with a sharp blade to obtain slices approx. 1 mm thick and 2 mm² in area. The cutting should also be performed in prewarmed culture medium.
- Transfer the tissue slices into a cell culture tube or petri dish containing a suitable amount of prewarmed culture medium and add 1/10 volume BrdU labeling solution (I) to the culture medium in which the slices are being incubated (final concentration: 10 µM BrdU).
- Incubate the tissue slices for 65–90 min at 37°C in a humidified atmosphere (5% CO₂). The incubation time in the presence of BrdU (labeling period) depends on the type of tissue used and the individual experimental requirements.
- Remove the labeling medium carefully, add fresh prewarmed culture medium to the tissue slices and incubate for 15–25 min at 37°C in a humidified atmosphere (5% CO₂).
- Process tissue slices for frozen sectioning or paraffin embedding (see section 5.2).

Negative control
For in vitro samples, negative controls should be performed by leaving the BrdU-labeling which should result in a totally unainted preparation.
- Also short labeling intervals result in preparations containing unlabeled cells (G0, G1 and G2/M) which could serve as controls.

5.2 In vivo labeling with BrdU and sample processing
- Inject the animal with the undiluted BrdU labeling reagent (bottle 1). 1 ml of the BrdU labeling reagent per 100 g body weight is suitable for most applications. It is recommended to inject the BrdU labeling reagent intraperitoneally.
- Sacrifice the animal approx. 2–4 h later and remove the tissue or organ under study.
- Process tissue samples for frozen sectioning or paraffin embedding.

Frozen sections
- Freeze the tissue immediately after removal to avoid damage caused by proteolytic enzymes and freeze rapidly to avoid damage of the tissue by ice crystal formation.
- Plunge the tissue into freezing isopentane and store the sample material frozen until required for sectioning.
- Cut the frozen tissue in a cryostat as thin as possible, preferably 3–5 µm.
- Transfer the sections directly to a clean, poly L-lysine- or chromalaun-gelatine-coated glass slide. Most tissues should be air-dried at +15 to +25°C prior to fixation.
- For fixation of the tissue sections and immunostaining, proceed further as outlined below starting from point 6.2.1.

Paraffin embedded sections
Immerse the tissue immediately after removal in 10% neutral buffered formalin for 8–10 hours.
- Use standard dehydration and paraffin wax-embedding procedures to process the fixed tissue. **Note:** The paraffin wax temperature should not exceed 58°C to avoid loss of tissue integrity.
- Cut sections in a microtome as thin as possible, preferably 3–5 µm at +15 to +25°C.
- Use standard procedures to dewax and rehydrate the tissue sections.
- For immunostaining of the dewaxed and rehydrated tissue sections proceed as outlined below starting from point 6.2.3.
6. Immunostaining

6.1 Procedure for flow cytometry

6.1.1 Fixation
Resuspend the cell pellet in 0.5 ml PBS and inject the cell suspension into 5 ml fixative (IV), which should result in a monodispersed cell suspension. Incubate for 30 min at 4°C. Do not resuspend the cell pellet with fixative, because cells will aggregate and a single cell analysis will be impossible.

6.1.2 Washing
Wash the cells with PBS and centrifuge cells at 200 × g for 5 min.

6.1.3 Denaturation
Resuspend the pellet in 500 μl HCl-denaturation solution (V) and incubate for 10–20 min at +4 to +25°C. After denaturation add 2 ml PBS and centrifuge at 300 × g for 10min (sedimentation of denatured cells requires elevated speed). Check pH value, which should be above pH 6.5 (if pH is lower repeat incubation with PBS). To block unspecific binding incubate the cells with 500 μl incubation buffer (bottle 3) for 10 min at +15 to +25°C.

6.1.4 Immunodetection
Sediment cells (300 × g, 10 min) and resuspend pellet in 50 μl anti-BrdU-FLUOS antibody working solution (II). Incubate for 45 min at 37°C in a humid chamber.

6.1.5 Washing
Wash cell suspension in PBS twice.

6.1.6 Analysis
Resuspend cells in 0.5–1 ml PBS analyze on a flow cytometer (use 488 nm excitation and a 515 nm longpassfilter for detection). For bivariate analysis, e.g., cell cycle analysis, add 1μg/ml propidium jodid (figure 2) or counterstain with a specific rhodamin- or phycoerythrin-conjugated antibody for the detection of any other antigen.

6.2 Procedure for slides and coverslips

6.2.1 Rehydration
Rehydrate sample material (frozen sections, cells grown on slides or cover slips, cytospin preparations, cell smear preparations, cell suspensions) in PBS.

6.2.2 Fixation
Fix the sample material with fixative solution (IV) for 45 min at RT.

6.2.3 Washing
Wash the slides or cover slips 2 times in PBS.

6.2.4 Enzymatic digestion (required for tissue sections)
Cover the preparation with trypsin solution (VI) and incubate for 5–15 min at 37°C to obtain best results.

6.2.5 Denaturation
Incubate preparation in 4 M HCl for 10–20 min at +15 to +25°C. After denaturation, incubate the specimen with PBS (3 × 5 min) to neutralize the pH. Check pH value, which should be above pH 6.5 (if pH is lower repeat incubation with PBS). Incubate 10 min with 50–100 μl incubation buffer (bottle 3) to block unspecific binding.

Alternatively
Denaturation could also be achieved by incubation with 5 μl/ml DNase I recombinant Grade 1, simultaneously with the anti-BrdU-FLUOS incubation. Prepare a suitable volume (50 μl) of anti-BrdU-FLUOS antibody working solution (II) containing DNase I recombinant and incubate for 60 min at 37°C in a humid chamber.

6.2.6 Immunodetection
Cover the preparation according to its size with a suitable volume (50 μl) of anti-BrdU-FLUOS antibody working solution (II) and incubate for 45 min at 37°C in a humid chamber.

Note for application on tissue sections
Use lintfree tissue (e.g., Kimwipe) to remove excess liquid from exposed glass areas. Avoid touching of the preparation. Dry areas before adding antibody solution. For reduction of unspecific fluorescence in tissue sections, incubate specimen in sulphorhodamin 101 (20 μg/ml in PBS) for 5min and wash with PBS once before anti-BrdU-FLUOS incubation.

6.2.7 Washing
Wash the slides or cover slips 3 times in PBS.

6.2.8 Embedding
If cover slips were used: Wipe the furthest edge of the cover slips as dry as possible. Put one small drop of an appropriate mounting medium (e.g., Citifluor) onto a glass slide and press the cover slide carefully onto the glass slide.

If glass slides were used: Cover the preparation with an appropriate mounting medium (e.g., Citifluor) and overlay a cover slide.

Note: Any embedding medium reduces fluorescence and results in an appearance of more unspecific fluorescence of the specimen. If preparations are not needed for long term storage, use PBS and cover with glass for microscopic analysis.

6.2.9 Analysis
Evaluate by fluorescence microscopy (use 488 nm excitation and a 515 nm longpassfilter for detection).

7. References


8. Ordering Information

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<th>Detection by</th>
<th>Roche Products</th>
<th>Cat. No.</th>
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<td>BrdU labeling and Detection Kit I</td>
<td>11 298 736 001</td>
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<td></td>
<td>BrdU labeling and Detection Kit II</td>
<td>11 299 964 001</td>
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<td></td>
<td></td>
<td>BrdU labeling and Detection Kit III</td>
<td>11 444 011 001</td>
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<td>Cell Proliferation Kit, FLUOS</td>
<td>11 810 740 001</td>
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<td></td>
<td></td>
<td>ELISA</td>
<td>11 647 229 001</td>
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<td>Cell Proliferation ELISA, BrdU (colorimetric)</td>
<td>11 689 915 001</td>
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<td>Cell Proliferation ELISA, BrdU (chemiluminescent)</td>
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<td>Anti-BrdU-Fluorescein</td>
<td>11 202 693 001</td>
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<td>Anti-BrdU - Peroxidase, Fab fragments</td>
<td>11 585 800 001</td>
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<td>Measurement of metabolic activity</td>
<td>Quantification in microtiter plate</td>
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<td>Cell Proliferation Kit (XTT)</td>
<td>11 465 015 001</td>
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<td>Cell Proliferation Reagent WST-1</td>
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9. Quick reference protocols

9.1 Required solutions (for 10 tests)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>BrdU labeling solution</td>
</tr>
<tr>
<td>• For in vitro labeling: Predilute 0.1 ml BrdU labeling reagent (bottle 1) with 9.9 ml sterile culture medium (resulting concentration: 100 μM BrdU).</td>
<td></td>
</tr>
<tr>
<td>• For in vivo labeling: Use the undiluted BrdU labeling reagent (bottle 1).</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Anti-BrdU-FLUOS antibody working solution: Dissolve lyophilizate (bottle 2) in 1 ml redist. water. To prepare the working solution, dilute 100 μl anti-BrdU-FLUOS stock solution in 450 μl incubation buffer (bottle3).</td>
</tr>
<tr>
<td>III</td>
<td>Washing solution: Prepare 1 l PBS</td>
</tr>
<tr>
<td>IV</td>
<td>Fixative solution: Prepare 50 ml ethanol (70%) in 50ml glycine buffer, pH 2.0</td>
</tr>
<tr>
<td>V</td>
<td>Denaturation solution: Prepare 50 ml HCl solution (4M).</td>
</tr>
<tr>
<td>VI</td>
<td>Trypsin solution (optionally, required for tissue sections): Prepare 2 ml trypsin based solution containing 0.05% trypsin* and 0.05% CaCl₂</td>
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</tbody>
</table>

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9.2.1 Immunostaining procedure for flow cytometry

<table>
<thead>
<tr>
<th>Steps</th>
<th>Procedure</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Washing:  Wash BrdU labeled cells in PBS</td>
<td>30 min, 4°C</td>
</tr>
<tr>
<td>2</td>
<td>Fixation: Fix cells with fixative solution (IV)</td>
<td>45 min, +15 to +25°C</td>
</tr>
<tr>
<td>3</td>
<td>Washing: Wash in PBS 2 times</td>
<td>2 × 2 min, +15 to +25°C</td>
</tr>
<tr>
<td>4</td>
<td>Denaturation: Incubate cells in denaturation solution (4 M HCl)</td>
<td>10-20 min, +15 to +25°C</td>
</tr>
<tr>
<td></td>
<td>Neutralization: Wash preparation with PBS. Check pH.</td>
<td>1–3 × 5 min, +15 to +25°C</td>
</tr>
<tr>
<td></td>
<td>Blocking: Incubate cells in incubation buffer (bottle 3).</td>
<td>10 min, +15 to +25°C</td>
</tr>
<tr>
<td>5</td>
<td>Immunodetection: Resuspend cells in 50 μl anti-BrdU-FLUOS antibody working solution (II) and incubate in a humid chamber.</td>
<td>45 min, +37°C</td>
</tr>
<tr>
<td>6</td>
<td>Washing: Wash cells twice in PBS</td>
<td>2 × 2 min, +15 to +25°C</td>
</tr>
<tr>
<td>7</td>
<td>Analysis: Analyze on a flow cytometer (488 nm excitation using a 515 nm longpass filter for detection)</td>
<td></td>
</tr>
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</table>

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9.2.2 Immunostaining procedure for slides and cover slips

<table>
<thead>
<tr>
<th>Steps</th>
<th>Procedure</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rehydration: Wash BrdU labeled cells in PBS</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fixation: Fix the sample with fixative solution (IV). For formalin-fixed paraffin embedded sections: Dewax sections.</td>
<td>45 min, +15 to +25°C</td>
</tr>
<tr>
<td>3</td>
<td>Washing: Wash the slides or cover slips 2 times in PBS.</td>
<td>2 × 2 min, +15 to +25°C</td>
</tr>
<tr>
<td>4</td>
<td>Enzymatic digestion (optional): Incubate preparation in excess incubation buffer (bottle 3).</td>
<td>5 – 15 min, +37°C</td>
</tr>
<tr>
<td>5</td>
<td>Denaturation: Incubate preparation in denaturation solution (4 M HCl)</td>
<td>10 – 20 min, RT</td>
</tr>
<tr>
<td></td>
<td>Alternatively: Denature with DNase I recombinant included in the antibody solution. Then, HCl treatment and neutralization/blocking could be skipped. Proceed with step 7.</td>
<td>60 min, +37°C</td>
</tr>
<tr>
<td>6</td>
<td>Immunodetection: Cover preparation with a suitable volume of anti-BrdU-FLUOS antibody working solution (II) and incubate in a humid chamber.</td>
<td>30 min, +37°C</td>
</tr>
<tr>
<td>7</td>
<td>Washing: Wash the slides or cover slips 3 times in PBS</td>
<td>3 × 2 min, +15 to +25°C</td>
</tr>
<tr>
<td>8</td>
<td>Embedding if needed for long term storage: Cover slips: Wipe the rear of the cover slips as dry as possible. Put one small drop of an appropriate mounting medium (e.g., Citifluor) onto a glass slide and press the cover slide carefully onto the glass slide as possible. Glass slides: Cover the preparation with an appropriate mounting medium (e.g., Citifluor) and overlay a cover slip. If no long term storage is needed, put a drop of PBS onto the slide and cover with cover slip.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Analysis: Evaluate by fluorescence microscopy (use 488 nm excitation and a 515 nm longpass filter for detection)</td>
<td></td>
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</tbody>
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* available from Roche Applied Science

Changes to Previous Version

Editorial changes

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