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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

Version 07
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Store at +2 to +8°C

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 [^{3}H] or [^{14}C] 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.,* [^{3}H]-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 [^{3}H]-thymidine (1, 2), immunochemical 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

2.1Kit contents

Bottle	Content	Label	Cap
1	10 mM BrdU in PBS, pH 7.4, 1,000 × conc., 1 ml, filtered through 0.2 µm pore size membrane	BrdU labeling reagent	Red, flip up
2	Monoclonal anti-BrdU-antibody (clone BMG 6H8), F(ab')$_2$ fragments, conjugated with fluorescein 5 × conc., lyophilized	Anti-BrdU-FLUOS	White
3	PBS based buffer, containing 0.5% BSA and 0.1%Tween 20, 100 ml	Incubation buffer	Blue

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

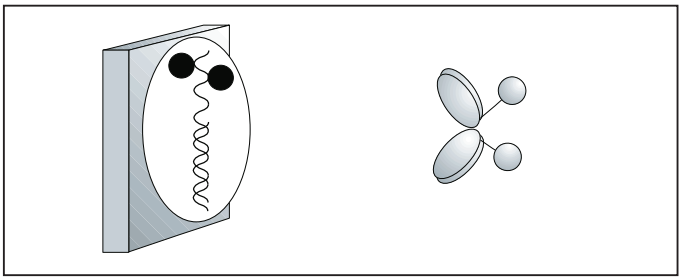
The assay (figure 1) is an immunocyto-/immunohistochemical technique which uses a mouse monoclonal antibody against BrdU. The procedure involves:

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 denaturating 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.



cells with partially degraded DNA, labeled with BrdU

Anti-BrdU-FLUOS F(ab')$_2$-fragment

Fig.1: Test principle

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

I.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).

II.Anti-BrdU-FLUOS antibody working solution:	Dissolve lyophilizate in 1 ml bidest. water. Immediately before use, dilute anti-BrdU-FLUOS stock solution 1:5 with incubation buffer (bottle 3). Stability of the working solution: cannot be stored. Stability of the undiluted anti-BrdU-FLUOS stock solution: stable at +2 to +8°C in the dark for 12 months.

4.2 Additionally required solutions

III.Washing solution	PBS
IV.Fixative solution	Ethanol-fixative (prepare fixative by mixing 3 volumes of glycine solution (50 mM, pH 2.0) with 7 volumes of absolute ethanol)
V.Denaturation solution	4 M HCl.
VI.Trypsin solution	(optional)**:** Trypsin (0.05% in PBS and 0.05% $CaCl_2$). Enzymatic digestion with trypsin is recommended for best results before or after acid denaturation of DNA.
VII.Mounting Media	Citifluor. Best results are received without mounting: put a drop of PBS onto the specimen and cover with cover slip (not suited for long term storage).

5. Labeling of cells

5.1 *In vitro* labeling with BrdU and sample processing

Adherent cells

- Grow cells on cover slips or on chamber slides to a confluency of about 50%. For flow cytometry, grow cells in tissue culture flasks.
- Add $^1/_{10}$ volume BrdU labeling solution (I) to the culture medium in which the cells are growing. For example, add 10µl of the BrdU labeling solution (I) to the cells if they were incubated in 100 µl culture medium (final concentration: 10µM BrdU).
- Incubate the cells for 30–60 min at 37°C in a humidified atmosphere (5% CO_2). The incubation time in the presence of BrdU (labeling period) depends on the cell type used (generation time) and the individual experimental requirements.
- Remove the labeling culture medium by aspiration or tapping and wash the cover slips or chamber slides 3 times in PBS.
- For flow cytometric analysis, prepare a monodispersed cell suspension by trypsin treatment or any other method, established in your laboratory.
- For fixation of the cells and immunostaining, see section 6. Immunostaining.

Suspension cells

- Adjust cell concentration to about $2 \times 10^5 - 1 \times 10^6$ cells/ml and add $^1/_{10}$ volume BrdU labeling solution (I) to the culture medium in which the cells are being incubated (final concentration: 10 µM BrdU).
- Incubate the cells for 30–60 min at 37°C in a humidified atmosphere (5% CO_2). The incubation time in the presence of BrdU (labeling period) depends on the cell type used (generation time) and the individual experimental requirements.
- Centrifuge the cells for 5 min at 200 × *g* and remove the supernatant carefully.
- Resuspend the cell pellet in fresh culture medium and centrifuge the cell suspension for 5 min at 200 × *g*.
- Preparation for flow cytometry: Resuspend the cells in 500 µl PBS and inject the cells into 5 ml of fixative (see 6.1).
- Preparation of cytospins: Resuspend the cells in culture medium to obtain a concentration of approx. 3×10^5 cells/ml and centrifuge 100 µl of this cell suspension onto a clean poly L-lysine-coated glass slide with a cytocentrifuge. Air-dry the samples at +15 to +25°C. For fixation of the cells and immunostaining, proceed as outlined below starting from point 6.2.2.
- Preparation of cell smears: Resuspend the cells in culture medium to obtain a concentration of approx. 5×10^7 cells/ml and place 1 drop of this cell suspension on one end of a clean, poly L-lysine-coated glass slide. To obtain a cell smear, draw the liquid over the glass slide by using a second clean slide. Air-dry the samples at +15 to +25°C. For fixation of the cells and immunostaining, proceed as outlined below starting from point 6.2.2.

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 mm^2 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 45–90 min at 37°C in a humidified atmosphere (5% CO_2). 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 15–25min at 37°C in a humidified atmosphere (5% CO_2).
- 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 unstained 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 an ultramicrotome 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

Negative control The preparation of a non-labeled animal for negative control purposes is not necessary, because BrdU-negative cells (G0, G1 and G2/M) appear in any tissue section. Specific nuclear staining discriminates between labeled and BrdU-negative cells.

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 fixativ, 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 +15 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 for excitation and a 515 nm bandpassfilter 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 U/ml DNase I recombinant Grade I*, simultaneously with the anti-BrdU-FLUOS incubation (6.2.6). 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 lindfree 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 glas for microscopic analysis.

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

* available from Roche Diagnostics

7. References

1 Chwalinski S. et al. (1988) *Cell Tissue Kinet.* **21,** 317.
2 Kellett M. et al. (1992) *Epithelial Cell Biol.* **1,** 147.
3 Gratzner, H.G. (1982) *Science* **218,** 474.
4 Vanderlaan, M. & Thomas, C.B. (1985) *Cytometry* **6,** 501.
5 Ellwart, J. & Dörmer P. (1985) *Cytometry* **6,** 513.

8. Ordering Information

Parameter	Detection by	Roche Products	Cat. No.
BrdU labeling of proliferating cells	▪ *In Situ* Assay	▪ BrdU labeling and Detection Kit I ▪ BrdU labeling and Detection Kit II ▪ BrdU labeling and Detection Kit III ▪ *In Situ* Cell Proliferation Kit, FLUOS	11 296 736 001 11 299 964 001 11 444 611 001 11 810 740 001
	▪ ELISA	▪ Cell Proliferation ELISA, BrdU (colorimetric) ▪ Cell Proliferation ELISA, BrdU (chemiluminescent)	11 647 229 001 11 669 915 001
	▪ Single reagents for *in situ* assays and ELISA specifications	▪ Anti-BrdU ▪ Anti-BrdU-Fluorescein ▪ Anti-BrdU -Peroxidase, Fab fragments	11 170 376 001 11 202 693 001 11 585 860 001
Measurement of metabolic activity	▪ Quantification in microtiter plate	▪ Cell Proliferation Kit I (MTT) ▪ Cell Proliferation Kit II (XTT) ▪ Cell Proliferation Reagent WST-1	11 465 007 001 11 465 015 001 11 644 807 001

9. Quick reference protocols

9.1 Required solutions (for 10 tests)

Solution	Content
I	**BrdU labeling solution** ▪ 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). ▪ For *in vivo* labeling: Use the undiluted BrdU labeling reagent (bottle 1).
II	**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 400 µl incubation buffer (bottle3).
III	**Washing solution:** Prepare 1 l PBS
IV	**Fixative solution:** Prepare 50 ml ethanol (70%) in 50mM glycine buffer, pH 2.0.
V	**Denaturation solution:** Prepare 50 ml HCl solution (4M).
VI	**Trypsin solution (optionally, required for tissue sections):** Prepare 2 ml PBS based solution containing 0.05% trypsin* and 0.05% $CaCl_2$.

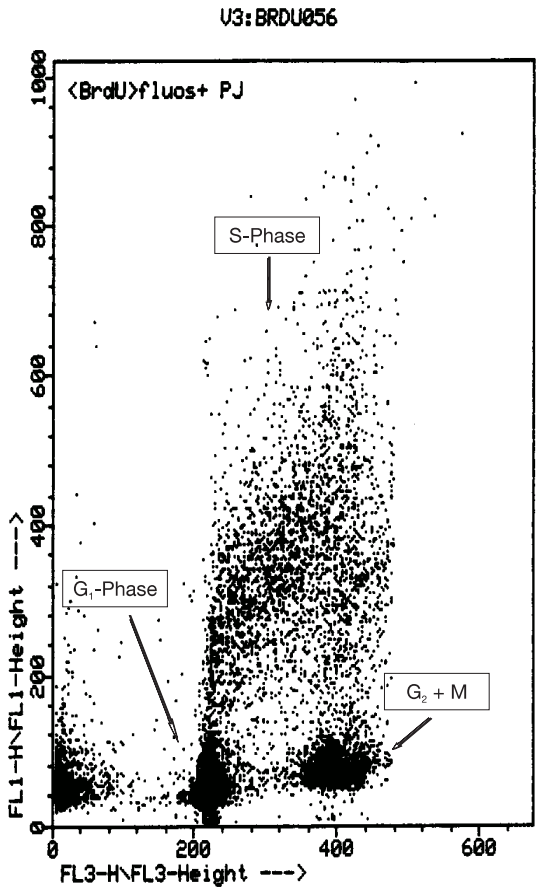


Fig. 2: U937-cells were incubated with BrdU for 30 min and subsequently stained for BrdU-incorporation according to the protocol given in this package insert. Cells were counterstained with 1 µg/ml propidium jodid. FL1-H = Fluorescein intensity, FL3-H = propidium jodid intensity of the cells.

9.2.1 Immunostaining procedure for flow cytometry

Steps	Procedure	Incubation
1	**Washing:** Wash BrdU labeled cells in PBS	
2	**Fixation:** Fix cells with fixative solution (IV)	30 min, 4°C
3	**Washing:** Wash 2 times in PBS	2 × 2 min, +15 to +25°C
4	**Denaturation:** Incubate cells in denaturation solution (4 M HCl)	10–20 min, +15 to +25°C
	Neutralization: Wash preparation with PBS. Check pH.	1–3 × 5 min, +15 to +25°C
	Blocking: Incubate cells in incubation buffer (bottle 3).	10 min, +15 to +25°C
5	**Immunodetection:** Resuspend cells in 50 µl anti-BrdU-FLUOS antibody working solution (II) and incubate in a humid chamber.	45 min, +37°C
6	**Washing:** Wash cells twice in PBS	2× 2 min, +15 to +25°C
7	**Analysis:** Analyze on a flow cytometer (488nm excitation using a 515 nm bandpassfilter for detection) or counterstain with a second specific antibody or DNA-specific dyes like propidium jodid.	

9.2.2 Immunostaining procedure for slides and cover slips

Steps	Procedure	Incubation
1	**Rehydration:** Wash BrdU labeled cells in PBS	
2	**Fixation:** Fix the sample with fixative solution (IV). For formalin-fixed paraffin embedded sections: Dewax sections.	45 min, +15 to +25°C
3	**Washing:** Wash the slides or cover slips 2 times in PBS.	2 × 2 min, +15 to +25°C
4	**Enzymatic digestion (optional):** Incubate preparation in trypsin solution (VI)	5 – 15 min, +37°C
5	**Denaturation:** Incubate preparation in denaturation solution (4 M HCl)	10 – 20 min, RT
	Alternatively: Denaturate with DNase I recombinant included in the antibody solution. Then, HCl treatment and neutralization/blocking could be skipped. Proceed with step 7.	60 min, +37°C
	Neutralization/blocking: Incubate preparation in excess incubation buffer (bottle 3). Check pH.	3 × 5 min, +15 to +25°C
6	**Immunodetection:** Cover preparation with a suitable volume of anti-BrdU-FLUOS antibody working solution (II) and incubate in a humid chamber.	30 min, +37°C
7	**Washing:** Wash the slides or cover slips 3 times in PBS	3 × 2 min +15 to +25°C
8	**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.	
9	**Analysis:** Evaluate by fluorescence microscopy (use 488 nm excitation and a 515 nm longpassfilter for detection)	

* available from Roche Diagnostics

Changes to Previous Version

Editorial changes.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany