5-bromo-2'-deoxyuridine detection using low concentration of hydrochloric acid and Exonuclease III

Protocol for fluorescence microscopy including Image cytometry

Protocol

1. Incubate the cells with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for the required time.

**If necessary another halogenated nucleoside analogue can be used including 5-trifluoromethyl-2'-deoxyuridine (TFdU).*

2. Wash with 1× PBS
3. Fixation

A. Formaldehyde fixation: Fix the samples with 1 - 2% formaldehyde in 1× PBS (10 min, room temperature), wash with 1× PBS and permeabilise with 0.2% Triton X-100 (10 min, room temperature). Then, wash the samples with 1× PBS (3x, 1 hr in total, room temperature).

**Generally, a longer wash step is not critical, but its shortening leads to a decrease of the signal.*

B. Ethanol fixation: Fix the samples with 70% ethanol (30 min-several weeks, -20°C)

4. BrdU detection

A. Wash with 150 mM NaCl and 3 mM KCl (3×).

**This step serves for washing out 1× PBS or ethanol. The residual 1× PBS neutralises the acid environment in the next step leading to a decrease of the signal.*

B. Incubate in a solution of 5 – 20 mM HCl in 150 mM NaCl and 3 mM KCl (20 min, 25°C).

**The concentration of HCl depends on the fixation protocol and antibody used. Test it first. According to our data, 20 mM HCl usually works well.*

**Although a higher temperature leads to an increase in the number of DNA breaks, it simultaneously leads to an increase in the cell structure damage. On the other hand, the lower temperature requires the prolongation of the incubation time and/or higher concentration of acid for sufficient BrdU revelation.*

**In the case of cells grown on coverslips this and all additional steps can be performed on drops of solutions.*

**The circular coverslips with a diameter of 12 mm provide enough cells for the analysis of the cell cycle using image cytometry.*

C. Wash with 1× buffer for exonuclease III (3x).

**This step serves for the neutralisation of the acidic environment. According to our observations, one wash step is enough.*

D. Incubate the samples in a mixture of primary anti-BrdU antibody, exonuclease III (0.4 U/µl) and 1× buffer for exonuclease III (30 min, 37°C).

**If RNA has to be removed (e.g. if propidium iodide is used for DNA labelling), add RNase A (100 µg/ml) to the mixture.*

**For the antibody clone B44 the optimal concentration is 0.25 – 0.5 µg/ml, for Bu20a 2.5 – 5 µg/ml (for other anti-BrdU antibodies, test the optimal concentration).*

**Use 30 µl of the mixture per circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.*

**If it is necessary to incubate the samples at room temperature, increase the exonuclease III concentration twice.*

**In the case of the detection of additional cellular components using specific antibodies, add the required primary antibody to the mixture described in step D and secondary antibody to the mixture described in step G.*

E. Shortly wash with 1× PBS (2×, 1 min or less in total). Incubate with 0.2% formaldehyde in 1× PBS (10 min, room temperature).

**This step serves for the stabilization of an antibody with incorporated BrdU, otherwise, the antibody-BrdU complex can dissociate quickly (Ligasová et al. 2017, PLoS One 12(3): e0174893).*

**It is possible to use a methanol-stabilised stock solution of formaldehyde or a solution prepared from paraformaldehyde in 1× PBS.*

**The quick wash followed by the stabilisation step is important in the ethanol-fixed cells only in the case of some antibodies. This step is necessary e.g. for antibody clone Bu20a, however not for clone B44. The data about some other clones are available in the above-mentioned paper On the contrary, for the formaldehyde-fixed cells, this step is important for the majority of the antibodies tested.*

**If the stabilisation step is not used, the signal can be improved by a quick reaction with the secondary antibody although the stabilisation step usually allows faster and more effective stabilisation of the antibody.*

F. Wash with 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (3× 5 min).

**It is possible to perform only one wash without a significant effect on the secondary antibody reaction.*

G. Incubate the samples with the secondary antibody (dilution according to the manufacturer or testing) and DAPI (10 µM) diluted in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (30 min, room temperature).

**Use 30 µl of the mixture per circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.*

H. Wash in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (3× 5 min).

I. In the case of coverslips, remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on the filter paper. Apply drops of the mounting medium on the glass slide (3 – 5 µl) and carefully lay the coverslips onto the mounting medium. Avoid the creation of any air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 µl of the mounting media per well.

J. Use nail polish to seal the coverslips and to protect the medium from drying. The well plates and coverslips can be stored at −20°C. In the case of well plates, seal them with parafilm to protect the medium from drying.

Please, if you use this protocol, cite this source: **Ligasová et al. 2017, PLoS One 12(4): e0175880**