5-bromo-2'-deoxyuridine detection using DNase I and Exonuclease III

Protocol for flow cytometry

Stock solutions to be prepared:

10× Buffer for exonuclease III

660 mM Tris, pH 8, 30°C
6.6 mM $MgCl_2$
Dissolve in deionised water, aliquot and store at -20°C.

Protocol

1. Incubate the cells with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for the required time. 30 minutes is commonly used for cell cycle analysis.

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

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2. Centrifuge the samples (500× *g*, 5 min).
3. Discard the supernatant and add 1× PBS (10 ml if 15 ml tubes are used).
4. Centrifuge the samples (500× *g*, 5 min).
5. Discard the supernatant and add 3 ml of 1× PBS. Mix by pipetting.
6. Shake the tube and slowly drop 7 ml of 100% ethanol cooled to -20°C during the shaking. Put the samples into the freezer and incubate at least 60 min.
7. Centrifuge the samples (500× *g*, 5 min).
8. Discard the supernatant and add 2 ml of 1× buffer for exonuclease III, mix by pipetting.
9. Repeat step 7 and step 8 once again.
10. Centrifuge the samples (500× *g*, 5 min).
11. Discard the supernatant and add 150 - 200 µl of the mixture composed of exonuclease III (0.4 U/µl), DNase I (2 U/µl), the primary antibody in 1× buffer for exonuclease III and 0.1 mM $CaCl_2$ and incubate 30 min at 37°C. During incubation, gently shake the tube, do not vortex.

**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).*

**Too long incubation in this mixture and/or high concentration of DNase I can result in the lowering of DNA-derived signal after its staining with the specific dyes. Higher concentration of exonuclease III can be used without the risk of lowering of DNA- or BrdU-derived signals.*

**In the case of the detection of additional cellular components using specific antibodies, add the required primary antibody to the mixture described in step 11 and secondary antibody to the mixture described in step 14.*

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12. Add 3 ml of 1× PBS, mix by pipetting and immediately add 15 µl of 35% stabilised formaldehyde (incubate 5 min at room temperature).

**This step serves for the stabilization of 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.*

13. Centrifuge the samples (500× *g*, 10 min).
14. Discard the supernatant and add 150 µl of the mixture composed of the secondary antibody (dilution according to the manufacturer or testing), propidium iodide (10 µg/ml) or DAPI (10 µM) diluted in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl. Mix by pipetting (10×) and incubate for 30 min at room temperature.
15. Centrifuge the samples (500× *g*, 10 min).
16. Wash with 1× PBS

Please, if you use this protocol, cite this source: **Ligasová et al. 2017, PLoS One 12(3): e0174893**