5-BROMO-2'-DEOXYURIDINE DETECTION USING LOW CONCENTRATED HYDROCHLORIC ACID AND EXONUCLEASE III

PROTOCOL FOR FLOW CYTOMETRY

Protocol

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

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

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 150 mM NaCl and 3 mM KCl. Mix by pipetting.

**This step serves for washing out 1× PBS. The residual 1× PBS neutralises the acid environment that is used in step 8. The neutralization leads to a decrease of the signal.*

6. Slowly drop 7 ml of 100% ethanol pre-cooled to -20°C. Put the samples into the freezer and incubate at least for 60 min.

**It is possible to leave the samples in the freezer for several weeks without the loss of the signal. A shorter time did not result in a decrease of the signal, however it can result in the gradual loss of cells.*

7. Centrifuge the samples (500× *g*, 5 min).
8. Discard the supernatant and add 3 ml of 10 – 20 mM hydrochloric acid (HCl) in 150 mM NaCl and 3 mM KCl. Mix by pipetting (10×) and incubate for 20 min at 25°C. During incubation gently shake the tube, do not vortex.

**Although the higher temperature leads to an increase in the number of DNA breaks, it simultaneously leads to an increase in 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. The concentration of HCl depends on the antibody used. Test it first. According to our data, 20 mM HCl will usually work well.*

9. Add 0.3 ml of 10× buffer for exonuclease III), mix by pipetting.

**This step serves for the neutralisation of the acidic environment.*

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) and primary anti-BrdU antibody in 1× buffer for exonuclease III (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).*

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

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

**This step is important when low stability of the particular anti-BrdU antibody-BrdU complex is expected. In other cases, this step can be skipped. However, the time of centrifugation and washing should be minimised.*

This step is necessary e.g. for anti-BrdU antibody Bu20a, however not for clone B44. The data about some other clones are available in 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 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 (final concentration: 1 - 10 µM) 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. Discard the supernatant and add 1× PBS (10 ml if 15 ml tubes are used). Mix by pipetting.
17. Centrifuge the samples (500× *g*, 10 min).
18. Discard the supernatant and add 1× PBS (10 ml if 15 ml tubes are used). Mix by pipetting.

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