Detection of mitochondrial DNA replication using 5-bromo-2'-deoxyuridine, copper ions and exonuclease III

Stock solutions to be prepared:

Solution A *- 20 mM CuSO4 and 500 mM NaCl.*

Dissolve $CuSO_4$ in deionized water, add an appropriate volume of 1 M NaCl (stock solution) and fill with deionized water to the final volume. Store at 4°C.

Solution B **- 50 mM sodium ascorbate and 200 mM Hepes pH 7.0.**

Dissolve sodium ascorbate in deionized water, add an appropriate volume of 1 M Hepes (stock solution, pH 7.0) and fill with deionized water to the final volume. Store at −20 °C.

**As an aqueous solution of sodium ascorbate is the subject of quick air oxidation, prepare the aliquots in Eppendorf tubes and seal them with parafilm.*

Protocol

1. Incubate the cells with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for the required time.
2. Wash with 1× PBS
3. Fix the samples with 2% formaldehyde in 1× PBS (10 min, room temperature).
4. Wash in 1× PBS (3×).
5. Permeabilise the samples with 0.2% Triton X-100 in 1× PBS (10 min, room temperature).
6. Wash in 1× PBS (3×).
7. Prepare fresh working cleavage mixture from stock solutions: Add one volume of the solution A and one volume of the solution B to three volumes of deionized water just prior to DNA cleavage. Mix on vortex for 5 s.

** Pre-warm solution A, solution B and water to 20°C before use.*

8. Remove the buffer from the samples and immediately add the prepared working cleavage mixture.

**In the case of cells grown on coverslips, perform this step in a Petri dish. Do not perform it on drops of prepared mixture as it protects the sample from oxygen.*

**1 ml of mixture is enough for Petri dish with the diameter of 3.5 cm.*

**Use one coverslip per one Petri dish, otherwise, it is necessary to check the position of the coverslips during the next step to protect their mutual sliding resulting in stacking.*

9. Put the samples on the shaker for 60 s, 300 rpm, 20°C.
10. Remove the working cleavage mixture.
11. Wash the samples with 100 mM Tris–HCl, pH 7.5 (3×, 20 minutes in total).
12. Wash with 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl (3×).
13. Incubate the samples with the solution containing primary anti-BrdU antibody, exonuclease III (final concentration: 1U/µl) and 1× buffer for exonuclease III (incubate at least 30 min at room temperature).

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

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

**We tested several anti-BrdU antibodies. The highest signal provided anti-BrdU antibody clone B44 and chicken polyclonal antibody. If there is need for other antibody clone that provides low signal, you can test protocol based on the stabilization of BrdU-antibody complex by formaldehyde (Ligasová et al., 2017, PLoS ONE 12(3): e0174893). In that case, wash samples immediately after incubation with antibody with 1× PBS (2x, 1 min or less in total) and incubate with 0.2% formaldehyde in 1× PBS (10 min, room temperature).*

**In the case of the detection of additional cellular components by specific antibodies, add the required primary antibody to the mixture described in step 13 and secondary antibody to the mixture described in step 15.*

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14. Wash the samples four times in 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl.
15. Incubate the samples with the prepared secondary antibody (dilution according to the manufacturer or testing) with DAPI (1 - 10 μM) in 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl for at least 30 min at room temperature.

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

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16. Wash the samples four times in 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl.
17. 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.
18. 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., 2012, PLoS ONE 7(12): e52584**