MICRONUCLEUS TEST – MODIFICATION WITH CYTOCHALASIN B

S U M M A R Y:

1. Principle of the Micronucleus test

The Micronucleus test is a comprehensive, quick and sensitive method for measuring DNA damage – micronuclei (MNi). Micronuclei are chromatin-containing structures in cytoplasm surrounded by a membrane without any detectable link to the cell nucleus. They are formed by exclusion of whole chromosomes or chromatin fragments during cell division. MNi are scored specifically in once divided binucleated cells.

The micronuclei are biomarkers of chromosome breakage and/or whole chromosome loss.

2. Chemicals and Materials

Chemicals – p.a. or reagent grade is required

A549 cells incubation

Dulbecco´s modified Eagle´s medium (DMEM) with 1.0 g/L glucose, with pyruvate, without L glutamine (LONZA BE12-707F/12)
Fetal bovine serum (FBS), EU standard (LONZA DE14-801F/12)
L-glutamine (200 mM) (LONZA BE17-605E)
Gentamicin sulfate 10 mg/ml (LONZA 17-519L)
Trypsin/EDTA (1x) contains 0.5 g/L trypsin 1:250 and 0.2 g/L Versene® (EDTA) (LONZA BE17-161E)
DMSO (Sigma D2650)

cytochalasin B Stock solution	100 mg of cytochalasin B dissolve in 5 ml DMSO, store at - 20°C
cytochalasin B Work solution	1 ml of the Stock solution add to 9 ml of DMSO

Other Chemicals and Solutions

Methanol	reagent gr., Merck, USA
Acetic Acid, 99 %	p.a., Penta Chrudim
Kalium chloride	p.a., Merck
Cytochalasin B	reagent gr., Sigma – Aldrich
dH_2O	LGE, Millipore
0,55 % solution of KCl (Hypotonic solution)	0.55 g KCl dissolve in 100 ml of dH_2O keep at 37°C
methanol :acetic acid (Fixation solution)	3 : 1 don´t store
DAPI Stock solution	6 µg DAPI (4',6-Diamidino-2-Phenylindole) in 1 ml H_2O store at –20°C
DAPI Work solution	4 µl of the Stock solution 100 µl of antifade, store at 4°-10°C
mounting medium for fluorescence VECTASHIELD (H 1000)	Vector Laboratories, Inc. store at 4 °C

3. Laboratory Equipment

Equipment

- laminar box
- fridge (4°C - 10°C)
- freezer (–20°C)
- centrifugal machine (minimum 1 000 ot/min)
- thermostat (37°C ± 1°C)
- fluorescent microscope Axio Imager Z1(Zeiss) connected to Metafer (MetaSystems)
- fluorescent filter for DAPI
- optical microscope Olympus BX41
- vortex

Other Equipment

- water pump
- automatic pipettes
- Pasteur pipettes, tips, etc.
- Microscopic slides – wash before use under the stream of water, store in dH_2O in a fridge
- germicidal lamps
- analytical scales
- alarm clocks

4. Protocols

Safety Instructions

Handle hazardous chemicals (Acetic Acid, Cytochalasin B, Methanol) with adequate precaution. Always use gloves and other protective laboratory equipment. Manipulate with Acetic Acid in digestore.

4.1 Cell culture incubation

A549 cells are grown at 37°C in 72 cm^2 flasks with 15-30 ml Dulbecco´s modified Eagle´s medium to 70-80% confluency. Then we treat cells with extract samples for 72h at 37^0C. After 44. hours we add 40 µl of Cytochalasin B Work solution (final concentration in flask is 5µg/ml) and mix properly. After 72 hours we harvest the cells.

4.2 Microscopical slides preparation

1. After 72 h incubation pour off the cultivation medium to 50 ml tube. Wash twice the cells in the cultivation flask with 5 ml of PBS (phosphate buffered saline, pH=7.4) – always add PBS to 50 ml tube.
2. Pipette 2 ml of trypsin to each cultivation flask and incubate at 37°C approx. 4 minutes. Then add twice 5 ml of PBS, always resuspend the cells and add the suspension to 50 ml tube with the medium and PBS.
3. Centrifuge 10 minutes at 1 000 ot/min, 20°C.
4. Aspirate the supernatant, resuspend the cell pellet and pipette the suspension to centrifuge tube. Add 10 ml of Hypotonic solution (37°C) while shaking on a shaker and incubate 5 minutes at room temperature.
5. Centrifuge 5 min at 1 000 ot/min, aspirate the supernatant.
6. Gently add 5 ml of cold Fixation solution (drop by drop) while shaking.
7. Centrifuge 5 min at 1 000 ot/min, aspirate the supernatant.
8. Add 5 ml of cold Fixation solution while shaking.
9. Centrifuge 5 min at 1 000 ot/min, aspirate the supernatant.
10. Add 5 ml of cold methanol while shaking.

11. Centrifuge 5 min at 1 000 ot/min, aspirate the supernatant except for approx. 0.5 ml.
12. Resuspend the cell pellet in the approx. 0.5 ml of methanol and drop on the wet cold slides.
13. Mark the slides with the sample number and let dry overnight at room temperature.

4.3 Fluorescent staining for automatic microscopical evaluation

1. Mix 100 µl of VECTASHIELD mounting medium with 4 µl of DAPI Stock solution (conc. 6 µg/ml).
2. Mix properly, store at fridge protected from light.
3. Drop two drops (2 x 10 µl) of the mixture on the slides with fixed cells and cover with the coverslip (24 x 50 mm).

   Score the slides. During the evaluation store slides at fridge protected from light.

4.4 Non-fluorescent staining for manual microscopical evaluation

Stain the dry slides with 5% Giemsa solution for 3 – 4 minutes, wash properly with water and let dry at room temperature.

5% Giemsa solution

Giemsa	5 ml
Sörens buffer	15 ml
dH_2O	80 ml

5. Microscopical slides evaluation

5.1 Automated microscopical analysis

Objects scored during automatic analysis:

1.Binucleated cells without micronuclei – scored as intact (**obr. 1a**)

2.Binucleated cells with 1 micronucleus (**obr. 1b, c**)

3.Binucleated cells with 2 micronuclei (**obr. 1d**)

Automated image analysis procedure

1. Metasystems Metafer MNScore device - Metafer MNScore separately identifies mononucleated cells or BNC and MN in the vicinity of these cells. The parameters for identification of both is stored in a set of parameters (called a classifier). We evaluate 1000 -3000 binucleated cells (BNC). The evaluation of higher numbers of BNC allows improvement of statistical power. (For more information see - D. Varga, T. Johannes, S. Jainta, S. Schuster, U. Schwarz-Boeger, M. Kiechle, B. Patino Garcia and W. Vogel, An automated scoring procedure for the micronucleus test by image analysis, Mutagenesis 19 (2004) 391-397.)
2. Adjustment of the classifier:
   For the nuclei: sharpen (3,4); object threshold: 20%; minimum area: 4000 in 1/100 μm^2; maximum area: 20 000 in 1/100 μm^2; maximum relative concavity depth: 120 in 1/1000; maximum aspect ratio: 1500 in 1/1000; maximum distance: 180 in 1/10 µm; maximum area asymmetry: 90%; region of interest radius: 300 in 1/10 µm; maximum object area in the region of interest (ROI): 2000 in 1/100 μm^2.
   For the micronuclei: medianv (3), medianh (3), average (3,1), sharpen (5,5); object threshold: 15%; minimum area: 100 in 1/100 μm^2; maximum area: 2100 in 1/100 μm^2; maximum relative concavity depth: 1000 in 1/1000; maximum aspect ratio: 1720 in 1/1000; maximum distance: 290 in 1/10 µm.
3. In the next step visually check the BNC gallery.

Analysis criteria
Criteria for selecting the cells:

1. The cells should be binucleated.

2. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
3. The two main nuclei in a binucleated cell should not overlap.

Criteria for scoring micrunuclei (MNi):

1. MNi are morphologically identical to but smaller than nuclei.
2. The diameter of MNi usually varies between 1/16th and 1/3rd of the mean diameter, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a binucleated cell.
3. MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

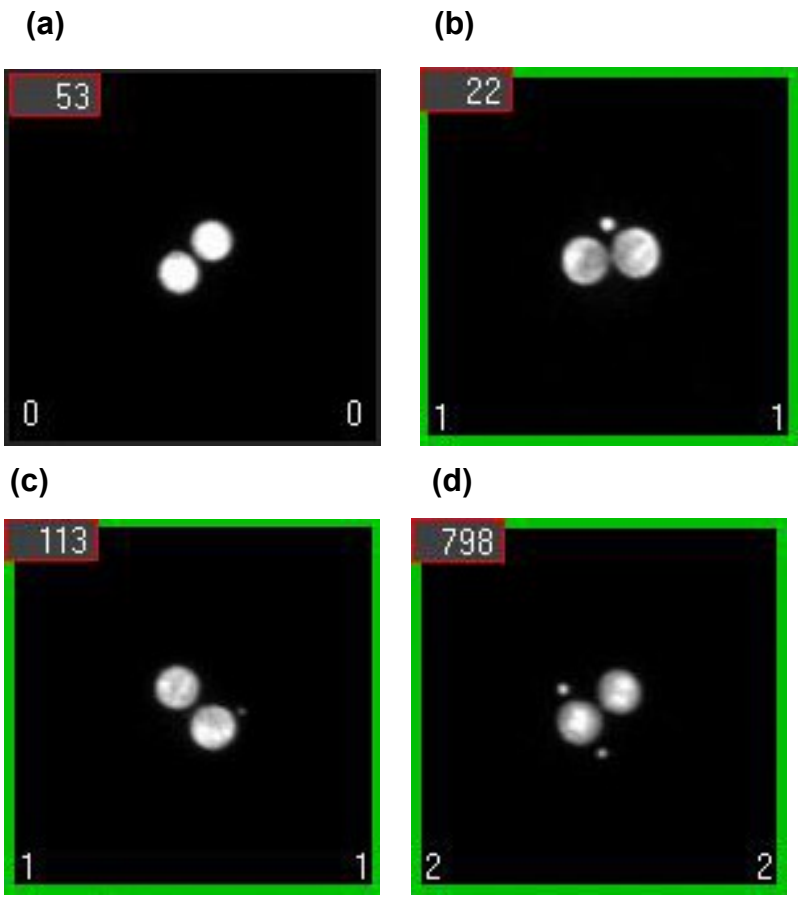


Fig.1 – Scoring of binucleated cells - Automatic analysis: **(a)** binucleated cell without the micronucleus; **(b,c)** binucleated cells with the micronucleus of different size; **(d)** binucleated cell with two micronuclei.

5.2 Manual microscopical analysis

Objects scored during manual analysis:

1.Binucleated cells without micronuclei – scored as intact (**obr. 2a**)

2.Binucleated cells with 1 micronucleus (**obr. 2b, c**)

3.Binucleated cells with 2 micronuclei (**obr. 2d**)

In manual microscopic analysis we can also score nucleoplasmic bridges (NPB - a biomarker of DNA misrepair and/or telomere end-fusion) and nuclear buds (NBUD – a biomarker of elimination of amplified DNA and/or DNA repair complexes).

Manual analysis procedure

1. Calculation of CBPI (Cytokinesis-Block Proliferation Index). The CBPI indicates the number of cell cycles per cell during the period of exposure to cytochalasin B.
   The cells (min. 500) are scored using an optical microscope Olympus BX41 at 400-fold magnification.

   Calculation of Cytokinesis-Block Proliferation Index (CBPI)

   CBPI = M1 + 2(M2) +3(M3 +M4)/N

   M1 – number of mononucleated cells
   M2 – number of binucleated cells
   M3, M4 – number of tri- or more- nucleated cells
   N – number of counted objects (recommended 500)

2. The micronuclei are scored in at least 2,000 binucleated cells using a microscope Olympus BX41 at 1000-fold magnification.
   Then the number of micronuclei in 1,000 binucleated cells is calculated (**MN/1000 BNC** = micronuclei / 1000 binucleated cells).

Analysis criteria

Criteria for selecting the cells:

1. The cells should be binucleated with an intact cytoplasm and normal nucleus morphology.

2. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
3. The two main nuclei in a binucleated cell should not overlap.
4. The two main nuclei may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.

Criteria for scoring micrunuclei (MNi):

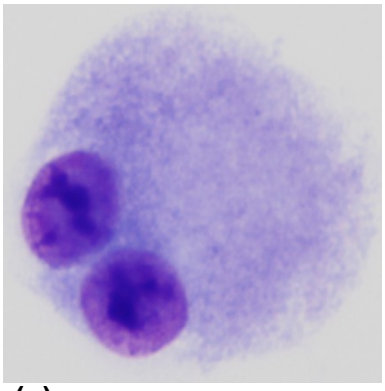
1.MNi are morphologically identical to but smaller than nuclei.

2.The diameter of MNi usually varies between 1/16th and 1/3rd of the mean diameter, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a binucleated cell.

3.MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

4.MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

(a) **(b)**



(c) **(d)**

Fig.2 – Scoring of binucleated cell - Manual analysis: **(a)** binucleated cell without the micronucleus; **(b,c)** binucleated cells with the micronucleus of different size; **(d)** binucleated cell with two micronuclei.

Literature:

M. Fenech, A. A. Morley: Measurement of micronuclei in lymphocytes. Mutation Res.147 (1985) 29-36.

M. Fenech: Cytokinesis-block micronucleus cytome assay. Nature Protocols, Vol.2 No.5 (2007).