INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® Micronucleus Kit

High-Content Screening Reagents

1826.1

Number	Description
K11-0001-1	**Micronucleus Kit,** sufficient materials for 5 × 96 wells

Kit Contents:	K1100011
Cellular Dye	35 µl
Permeability Dye	25 µl
Cytokinesis Block	5 x 100 µg
Hoechst Dye	2 x 28 µl
Wash Buffer (10X)	100 ml

Storage: Upon receipt store Cellular and Permeability Dye at -20°C. Store remaining kit components at 4°C. Keep vials containing Cellular, Permeability and Hoechst dyes protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. Cytokinesis blocking agent (cytochalasin B) is a toxin. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The micronucleus assay is an important component of genetic toxicology screening programs.[1-3] Micronuclei arise from acentric chromosomes and lagging whole chromosomes.[4,5] Chromosome mutations of both structure and number are implicated in many human diseases. There is substantial evidence that chromosome mutations and related events in oncogenes and tumor suppressor genes of somatic cells are involved in induction and/or progression of some cancers in humans and experimental animals. Thus, the purpose of the *in vitro* micronucleus assay is to detect those agents that modify chromosome structure and segregation in such a way as to lead to induction of micronuclei in interphase cells. Traditional quantitative assessment of micronucleus induction in 1,000 or more cells per slide is a labor-intensive, manual task. The *in vitro* micronucleus assay uses cultures of established cell lines, cell strains or primary cell cultures. The cells used are selected based on their ability to grow in culture and their spontaneous micronuclei frequency. Analysis of micronuclei induction in human lymphocyte cultures indicates that the most convenient stage to score micronuclei is during the binucleate, interphase stage.[6, 7] Such cells have completed one cell division after chemical treatment and are therefore capable of expressing micronuclei. Treating cultures with an inhibitor of actin polymerization (cytochalasin B) results in the trapping of cells at the binucleate (or multinucleate) stage where they can be easily identified (Figure 1).[6, 7] Measuring the relative frequencies of binucleate to mononucleate cells within a culture also provides a simple method of measuring treatment toxicity.[8] Spontaneous micronuclei formation may occur – baseline frequency of spontaneous micronuclei is approximately 1%; micronucleus frequency of cells treated with a genotoxic agent is approximately 3-fold higher or better (Figure 2).

The Micronucleus HCS Reagent Kit and protocol have been optimized for quantification of micronuclei in multinucleate cells. The kit provides reagents for fluorescence detection of micronuclei and nuclei, their cellular domains, and any membrane permeability changes upon treatment of cells grown on collagen-I coated microplates. The kit reagents, in combination with the Thermo Scientific ArrayScan® Readers and the Micronucleus BioApplication software, enable automated plate handling, focusing, image acquisition, analysis and quantification of micronuclei.

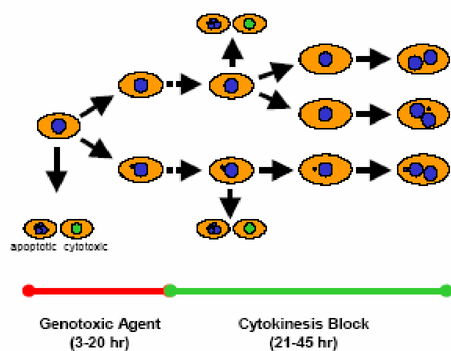

Figure 1. Principle of micronucleus induction using the cytokinesis-block method and the Micronucleus HCS Reagent Kit. Although rare, spontaneous micronuclei may occur; however, upon adding cytochalasin B (after adding a genotoxic agent), cells either undergo apoptosis, permeability changes, blocking in cytokinesis (binucleate cells) or blocking with the induction of micronuclei.

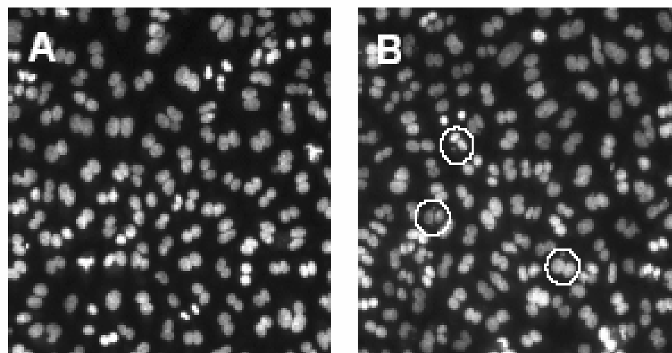

Figure 2. CHO-K1 cells with and without genotoxic treatment. Panel A: Control cells treated only with cytochalasin B. **Panel B:** Cells were exposed to mitomycin C (50 ng/ml) for 20 hours and blocked with cytochalasin B for 28 hours. Examples of cells containing micronuclei are circled.

Additional Materials Required

- Genotoxic Agents: mitomycin C (EMD Biosciences, Product No. 47589), bleomycin sulfate (EMD Biosciences, Product No. 203401)
- Formaldehyde (37%) (Fisher, Product No. F79-500)
- Microplates, clear, collagen-I coated (BD, Product No. 354407)

Cell Preparation Information

- Cellomics recommends that you perform a density study for your cell type of interest in order to determine the Day 0 seeding density that results in 70-80% confluency at the time of fixation.
- Protocol optimized for CHO-K1 cells (ATCC, Product No. CCL-61)
- Culture CHO-K1 cells in F-12K Nutrient Mixture medium containing the following supplements (F12-K Complete Medium): F12-K Nutrient Mixture (Kaighn's Modification), 10% fetal bovine serum (heat-inactivated), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.
- Split cells when they reach 70-80% confluency (every 2-3 days) at a dilution of 1:10 to 1:20.
- For plating cells, harvest cells with trypsin-versene mixture (BioWhittaker, Product No. 17-161F), dilute into F12-K Complete Medium and determine cell density.
- Adjust cell density to approximately 3×10^4 cells/ml in F12-K Complete Medium and add 100 µl of the cell suspension to each well of a 96-well collagen-I coated microplate (=3,000 cells/well).
- Incubate 18-24 hours at 37°C in 5% CO_2.

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Micronucleus Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 10 ml 10X Wash Buffer to 90 ml of ultrapure water. Store diluted solution at 4°C for up to 7 days.
Cellular Dye Solution	Add 5.5 µl Cellular Dye to 11 ml F12-K medium. Prepare just before each assay. Store remaining stock solution at -20°C.
Genotoxic Agent	**Mitomycin C (MMC):** Reconstitute MMC to 500 µg/ml with hot water. (Refer to the manufacturers' instructions for appropriate storage conditions.) Dilute stock 1:100 in F12-K medium. Add 110 µl of dilution to 11 ml of F12-K medium for a final concentration of ~50 ng/ml. Prepare just before each assay. **Bleomycin Sulfate:** Reconstitute bleomycin (15 U stock) with 750 µl ultrapure water to make a stock concentration of 20 U/ml. (Refer to the manufacturers' instructions for appropriate storage conditions.) Dilute stock 1:100 with F12-K medium. Add 137.5 µl of dilution to 11 ml F12-K medium for an approximate final concentration of 0.0025 U/ml. Prepare just before each assay.
Cytokinesis Blocking Agent (cytochalasin B)	Add 10 µl ethanol to 1 vial of Cytokinesis Block to create a 10 mg/ml stock concentration. Store stock solution at 4°C for up to 6 months. Add 6.6 µl to 11 ml F12-K medium. Prepare just before each assay.
Permeability Dye Solution	Add 3.6 µl Permeability Dye to 6 ml F12-K medium. Prepare just before each assay. Store remaining stock solution at -20°C.
Fix/Hoechst Dye Solution	Add 1.2 ml 37% formaldehyde and 6 µl Hoechst Dye to 10.8 ml 1X Wash Buffer. Warm to 37°C. Prepare just before each assay.

B. Procedure

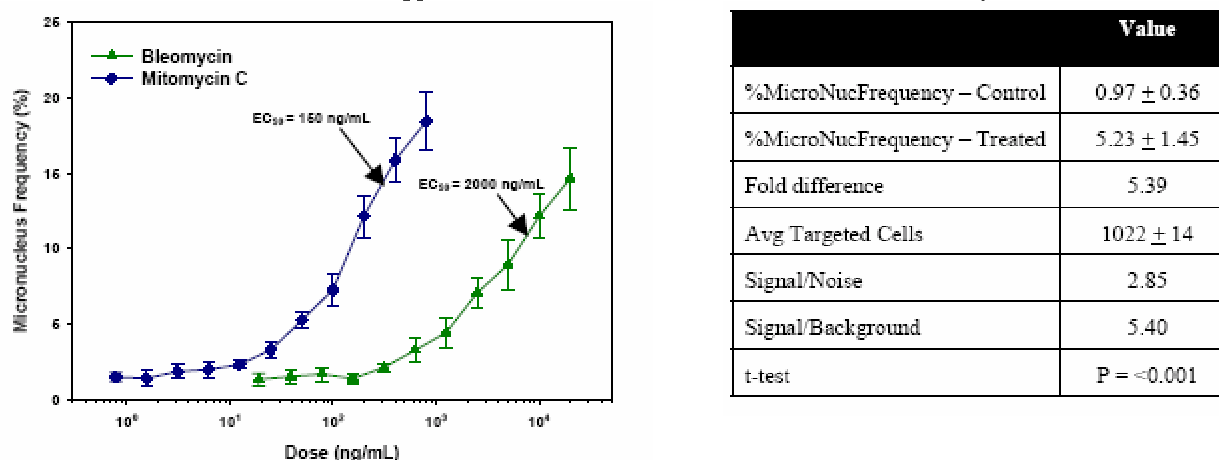
Note: Use 100 µl per well unless indicated otherwise. Protocol requires approximately 5 hours of hands-on time to perform over a 4-day period (including initial plating).

1. Plate approximately 3,000 CHO-K1 cells/100 µl F12-K complete medium in a 96-well collagen-I coated microplate and incubate for 18-24 hours at 37°C and 5% CO_2.
2. Aspirate medium and add 75 µl of pre-warmed Cellular Dye Solution to all wells. Incubate for 1 hour at 37°C with 5% CO_2.
3. Aspirate Cellular Dye Solution and wash once with medium.
4. Remove medium and add 100 µl of pre-warmed Genotoxic Agent or media to desired wells. Incubate for 20 hours at 37°C with 5% CO_2.
5. Aspirate Genotoxic Agent and wash once with medium.
6. Remove medium and add 100 µl of pre-warmed Cytokinesis Blocking Agent to all wells. Incubate for 27.5 hours at 37°C with 5% CO_2.
7. Add 50 µl of pre-warmed Permeability Dye Solution to all wells. Return to incubator for 30 minutes.
8. Aspirate solution and wash once with medium.
9. Remove medium and add 100 µl pre-warmed Fix/Hoechst Dye Solution to each well. Incubate in fume hood at room temperature for 20 minutes. Pre-warming fixative is critical to maintaining cell integrity.
10. Aspirate Fix/Hoechst Dye solution and wash plate twice with 100 µl of 1X Wash Buffer.
11. Remove wash and add 200 µl of Wash Buffer. Seal plate. Evaluate plate immediately on the ArrayScan HCS Reader.
12. Store sealed plates in the dark at 4°C. Note that Permeability Dye might not be measurable after 48 hours post-fixation. Cellular Dye should last approximately 72 hours post-fixation. The Cellular Dye is required for the micronucleus assay. The Permeability Dye is required only for the Channel 3 cell permeability (cytotoxicity) results.

Additional Information

A. Dose Response Curves and Performance Data

CHO-K1 cells were stained with the Cellular Dye and treated with increasing concentrations of either mitomycin C (MMC) or bleomycin sulfate for 20 hours. Cells were then washed and treated with Cytokinesis Blocking Agent for 28 hours, stained with the Permeability Dye, and fixed/stained with formaldehyde and Hoechst Dye. Plates were analyzed using the ArrayScan HCS Reader with the Micronucleus BioApplication software, XF93 filter, and 20X (NA .40) objective.



	Value
%MicroNucFrequency – Control	0.97 ± 0.36
%MicroNucFrequency – Treated	5.23 ± 1.45
Fold difference	5.39
Avg Targeted Cells	1022 ± 14
Signal/Noise	2.85
Signal/Background	5.40
t-test	P = <0.001

Figure 3. Left panel: A representative dose response curve, where average EC_{50} values of 150 ng/ml (MMC) and 2 µg/ml (bleomycin) were determined. **Right panel:** Data were collected from a full 96-well plate of CHO-K1 cells run on the ArrayScan HCS Reader with at least 1,000 targeted cells/well. Columns 1-6 were control (media only) and columns 7-12 were treated with MMC (50 ng/ml).

B. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

Cellular Dye = 540/566 nm

Permeability Dye = 491/509 nm

Hoechst Dye = 350/461 nm

C. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop® Combi or WellMate® Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.

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- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

Kit Component	96-Well Plates (µl/well)	384-Well Plates (µl/well)	24-Well Plates (µl/well)
Fixation Solution	100	25	400
Wash Buffer	100	25	400
Wash Buffer II	100	25	400
Permeabilization Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

Compatible BioApplication Software Modules

S50-0018-1 **Micronucleus BioApplication**

References

1. Matter, B., and W. Schmid. (1971). *Mutation Res* **12:**417-25.
2. Heddle, J. (1973). *Mutation Res* **18:**187-90.
3. MacGregor, J., *et al*. (1983). In: Hayes AW, Schnell RC, Miya TS (eds.). *Developments in the Science and Practice of Toxicology,* pp. 555-58. Elsevier, Amsterdam.
4. Maier, P., and Schmid, W. (1976). *Mutation Res* **40:**325-38.
5. Hayashi, M., *et al.* (1984). *Mutation Res* **141:**165-69.
6. Fenech, M., and Morley, A. (1985). *Cytobios* **43:**233-46.
7. Fenech, M., and Morley, A. (1985). *Mutation Res* **147:**29-36.
8. Fenech, M. (1997). *Mutation Res* 392:11-18.
9. Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.
10. Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4:**67-73.

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