Chapter 8

In Vitro Genotoxicity/Mutagenicity Testing of Food Packaging

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Abstract

To ensure the safe use of packaging materials and food contact materials, genotoxicity assessment is one of the requirements of regulatory agencies around the globe. Thus, it is essential to carry out preliminary tests to clarify this possible mechanism. The *Salmonella*/mammalian-microsome mutagenicity test, widely known as the Ames test, is a rapid, relatively simple procedure for testing chemicals for mutagenicity as well as for offering provision for the metabolism of otherwise nonmutagenic chemicals to their potentially DNA-reactive forms. However, a single test is not sufficient to detect all relevant genotoxic mechanisms in tumorigenesis. Thus, in order to complement the results in the Ames test and to contribute to the elucidation of the effects, ensuring their use or not, mutagenicity at the chromosomal level must also be evaluated. In the micronucleus (MN) assay, chromosomal damages induced by chemical products are evaluated. The MN is expressed in dividing cultured cells because fragments from damaged chromosomes or whole chromosomes that lag during anaphase become enveloped by nuclear membrane, independently from the main nucleus during telophase, prior to cell division. Together, these tests detect the most relevant events for the multistep process of malignancy, that is, gene mutations, clastogenicity, and aneugenicity. Detailed descriptions of the protocols used for detection of point mutations and chromosomal damage induced by food packaging in vitro are given in this chapter.

Key words Micronucleus test, Ames test, *Salmonella*/microsome assay, Genetic toxicology

1 Introduction

The genotoxic/mutagenic potential tests reveal, at the first level of evidence, the genotoxicological effect of edible and/or food contact packaging materials, having their safe use in mind. So, these tests are always required irrespective of the extent of migration (*see* Chapter 4 on IAS and NIAS, Chapter 5 on PFAS, and Chapter 6 on migration) and the resulting human exposure to these materials [1], because even though human exposure levels may be quantitatively low, these substances are considered to be of high toxicological concern if they act as DNA reactive mutagens [2].

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Commonly, the toxicological tests of food contact materials are focused on single substances and their genotoxicity. However, most of the time, the material is not available as a pure chemical and the chemical identity is not known, causing people to be exposed to mixtures of chemicals [3], that can or cannot interact with genetic material. This interaction may result, under certain circumstances, not only in cancer, but also in degenerative conditions such as accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases (in case of accumulation of DNA damage in somatic cells) and in spontaneous abortions, infertility, or heritable damage in the offspring and/or subsequent generations resulting in genetic diseases (in case of DNA damage in germ cells) [4]. Thus, sample preparation procedures need to be optimized and standardized and approaches on the concept of safe level of food packaging materials should be discussed. Regulatory agencies around the globe have conducted research and developed both guidance and regulations for safety assessments of materials intended to contact food. Although the food packaging safety assessment structures developed by these agencies have similar principles, they differ in the application of these principles [5], which is necessary for new approaches to meet this legal obligation for authorization applications of packaging materials.

DNA damage is a complex biological process involving several modes of actions, determining the cellular fate and the severity of the hazard. Currently, a wide variety of bioassays are available to assess the genotoxic potential of chemicals and materials. These assays have been evaluated for their ability to correctly predict the adverse effects of matter and are often used as screening tools [6].

The *Salmonella/Escherichia coli* microsome assay (Ames test) is required by regulatory authorities worldwide in order to identify substances that can produce genetic damage that leads to gene mutations (base substitution type mutations—*S.* Thyphimurium strains TA1535, TA100, TA102, and TA104, and *E. coli* strains WP2 uvrA or WP2 uvrA (pKM101), frameshift mutations—*S.* Thyphimurium strains TA1537, TA1538, TA97a, and TA98) [7]. The bacterial strains and mutagenicity test procedure, developed by Bruce Ames and published in 1973 [8, 9], still retain a primary role in the testing of chemicals and materials for commercial use [7].

The Ames test uses amino acid-dependent strains of *S.* Typhimurium and *E. coli*, each carrying different mutations in various genes of either histidine operon, in *S.* Typhimurium bacteria, or tryptophan operon in *E. coli* making them auxotrophic for the corresponding amino acids. These mutations act as hot spots for mutagens that cause DNA damage by different mechanisms. In addition, some strains may have (i) rfa mutations, which cause changes in the lipopolysaccharide barrier of the bacterial cell wall, thus facilitating the entry of large molecules (all *Salmonella* strains);

(ii) deficiency of the nucleotide excision repair system, preventing the bacterium from repairing the damage that has been done to its genetic material (uvrB detection in *Salmonella* strains, except TA102, or uvrA mutation in *E. coli* strains); and (iii) the plasmid R factor (plasmid pKM101) that confers resistance to ampicillin and induces an error-prone DNA repair pathway (strains TA97, TA97a, TA98, TA100, TA102, and WP2 uvrA (pKM 101)). Together, these mutations give the strains greater sensitivity in detecting several mutagens [10, 11].

Due to the inability to synthesize histidine, these strains cannot grow and form colonies in the absence of this essential amino acid [12]. However, when they are exposed to agents that induce new mutations in the gene, this function is restored and the auxotrophic character is reversed, allowing bacteria to grow and form colonies [11]. Thus, the assay detects the mutational reversion of his-dependent bacteria to his-independent colonies (*Salmonella*) or trp-dependent bacteria to trp-independent colonies (*E. coli*). The mutagenic potential of a compound can then be calculated from the number of colonies that are formed on the plate by the concentration of the compound used [12].

Another consideration about the test is the addition of the so-called S9 fraction, obtained from rat liver, which contains xenobiotic metabolizing enzymes for the identification of mutagenic agents of indirect action, which must be metabolized in order to become active [13]. Therefore, in the absence (−S9) or presence (+S9) of metabolic activation, the monitoring of the direct and indirect actions of a compound, respectively, is possible, guaranteeing the faithful identification of agents that cause gene mutations.

For the detection of chromosomal damage, the micronucleus (MN) test is a widely used method that detects chromosomal loss and breakage, being used as biomarker for the identification of clastogenic and aneugenic agents [14]. The chromosomal changes identified in the MN test are verified by counting circular structures surrounded by nuclear membrane, called MN, which are formed by chromosomal fragments or whole chromosomes that were delayed during anaphase and were not incorporated into the nuclei of daughter cells [15].

This test can be performed in vitro, using cultured primary human or other mammalian peripheral blood lymphocytes and several rodent cell lines such as CHO (Chinese hamster ovary cells), V79 (Chinese hamster lung fibroblasts, male), CHL/IU (*Cricetulus griseus*, Chinese hamster lung fibroblasts, female), and L5178Y (mouse lymphoma) cells, or human cells, such as TK6 (human spleen lymphoblasts). Other cell lines such as HT29 (human colorectal adenocarcinoma), Caco-2 (Caucasian colon adenocarcinoma), HepaRG (hepatic stem cell line), HepG2 cells (liver hepatocellular carcinoma), A549 (adenocarcinomic human alveolar basal epithelial cells), and primary Syrian Hamster Embryo cells

have been used for MN testing, but have not been extensively validated to date. Therefore, the choice of these cells should be justified [16]. The MN test can still be performed in vivo using hematopoietic rodent cells [17].

The OECD 487 Test Guideline [16] allows the use of protocols with and without cytochalasin B (cytoB). CytoB inhibits actin polymerization and blocks cytokinesis, and cells that have completed one cell cycle after treatment can be distinguished from undivided cells by their binucleate appearance. The advantage of using cytoB is that it allows the clear identification that treated and control cells have divided in vitro, and also provides a simple assessment of cell proliferation, allowing for the identification and analysis of MN only in cells that have completed one mitosis. The use of protocols without cytokinesis block can be accomplished, provided there is evidence that the cell population analyzed has undergone mitosis.

Thus, the bacterial reverse gene mutation test and the in vitro MN assay detect two main genetic endpoints, that is, gene and chromosome mutations, respectively. Therefore, these tests are currently considered equally appropriate in a standard genetic toxicology battery for predicting potential human risks [18].

2 Materials

1. General laboratory glassware (flasks, bottles, graduated cylinders, etc.).
2. Petri dishes (100 × 15 mm^2).
3. Sterile glass tubes (50 × 16 mm^2) with caps.
4. Test tube racks.
5. Pipets (1, 2, 5, and 10 mL).
6. Pipettors (adjustable volumes).
7. Sterile pipette tips.
8. Cryogenic tubes for freezing down permanent and working cultures.
9. Colony counter (manual or electronic).
10. 6-Well Microtiter Plate (flat-bottom).
11. Conical tubes (15 mL).
12. Microscope slides.
13. Cell culture flasks.

General Apparatuses

1. Autoclave.
2. Shaking incubator set at 120 rpm and 37 °C.

3. Incubator for the GM agar plates.
4. Oven, heating, or water bath set at 43–48 °C to maintain temperature of top agar.
5. Boiling water bath or microwave oven for melting top agar.
6. Magnetic stirrers.
7. Analytical balances (up to 0.001 g).
8. Water purification system to generate distilled water.
9. Ultrafreezer or liquid nitrogen tank.
10. Refrigerator/freezer.
11. Biological safety cabinet.
12. Inverted light microscope.
13. Incubator with humidified atmosphere of 5% carbon dioxide (CO_2) and temperature of 37 °C for cell growth.
14. Cytocentrifuge.
15. Microscope with excellent optics for bright-field and fluorescence examination of stained slides at ×1000 magnification.

Chemicals

2.1 Salmonella/*Microsome assay (*Salmonella *Test; Ames Test)*

1. Agar.
2. Glucose.
3. D-biotin.
4. L-Histidine·HCl.
5. Sodium chloride.
6. Oxoid nutrient broth No. 2.
7. Monobasic sodium phosphate.
8. Dibasic sodium phosphate.
9. Magnesium chloride.
10. Potassium chloride.
11. D-glucose-6-phosphate disodium.
12. Nicotinamide adenine dinucleotide phosphate sodium salt.
13. Mammalian tissue homogenate (S9 fraction).
14. Dimethyl sulfoxide (DMSO) or other solvents that maximize extraction of the substances present in the food packaging materials.
15. Glycerol.
16. Positive control chemicals (*see* **Note 1**).

2.2 Micronucleus Test

1. Cell growth media: Eagle's Minimal Essential Medium (EMEM), Dulbecco's Modified Eagle's medium (DMEM), RPMI, Ham's F10, Ham's F12 (check the most suitable one for each test cell).
2. Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.
3. Fetal bovine serum (FBS).
4. Cytochalasin B.
5. Dyes: Giemsa, acridine orange or panoptic (check the most suitable one for each test cell).
6. DMSO or other solvents that maximize extraction of the substances present in the food packaging materials.
7. Positive control chemicals (*see* **Note 1**).

3 Methods

Extraction

Water and culture medium are the most commonly used solvents. In case of water, it is advised not to exceed a maximum concentration of 10 vol% because of molarity changes on the medium and dilution of nutrients.

If other than well-established solvents/vehicles are used, their inclusion should be supported by data indicating their compatibility with the test system and their ability to maximize extraction of the substances present in the food packaging materials. They must not interfere with cell proliferation, metabolic activation, and must not induce DNA changes.

The International Standard ISO 10993-12 [19] suggests that the extraction of substances from films (thickness <0.5 mm) should be carried out at 37 ± 1 °C for 72 ± 2 h. Other conditions for extraction also are described, but care should be taken that this does not alter the chemical characteristics of food packaging. The surface area of the films must be of 6 cm^2 to the volume of 1 mL of extraction vehicle. When surface area cannot be determined, a mass/volume of extracting fluid shall be used [19].

Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

Food packaging extract is added directly to the test systems and/or diluted prior to treatment if it interferes with bacterial or cellular growth and survival.

3.1 Salmonella/Microsome Assay (Salmonella Test; Ames Test)

According to the OECD Guidelines [20], at least five strains of bacteria should be used. The recommended combination of strains is TA1535; TA1537, TA97a or TA97; TA98; TA100; and TA102 of *S.* Typhimurium, *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101).

Preparation of Permanent Cultures

Receive strains on a small sterile filter disk embedded in nutrient agar; first wipe the disk across the surface of a nutrient agar plate, and then transfer the disk to 5 mL of nutrient broth. For lyophilized culture, aseptically add 1 mL of nutrient broth to rehydrate the culture (a process which should take up to 2 min), then transfer the rehydrated culture to 4 mL of nutrient broth. Then, transfer a drop of the culture to a nutrient agar plate and streak the inoculum for individual colonies across the surface of the plate [11].

If single colonies are observed after overnight incubation at 37 °C, pick one healthy looking colony and restreak it for individual colonies on minimal agar medium (GM agar) plate supplemented with biotin and histidine for *S.* Typhimurium strains, and tryptophan for *E. coli* strains for purification and verification of genotypic characteristics [11, 20].

Other genotypic characteristics that should be similarly checked: ampicillin resistance in strains TA98, TA100 and TA97a or TA97, and WP2 uvrA (pKM101); ampicillin + tetracycline resistance in strain TA102 to assess the presence or absence of R-factor plasmids; rfa mutation in *S.* Typhimurium through sensitivity to crystal violet, and uvrA mutation in *E. coli* or uvrB mutation in *S.* Typhimurium, through sensitivity to ultraviolet light [20]. The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature [11, 13].

The strains grown in nutrient broth at a density of 1 to 2×10^9 colony-forming unit (CFU) mL^{-1} (Optical $Density_{540\ nm}$ between 0.1 and 0.2) are frozen with 0.5 mL of a sterile cryopreserver, such as glycerol or DMSO for the culture (final concentration, 10 vol%); mix well and dispense 1-mL aliquots in pre-marked sterile cryogenic tubes.

The tester strains should be kept frozen in ultrafreezer (−80 °C) or liquid nitrogen.

Preparation of Solutions and Plates

1. *Minimal agar medium (GM agar) plates*: Add 15 g of agar to 900 mL distilled water. Autoclave it for 15 min at 121 °C (1.5 atm; relative pressure). Add 20 mL of sterile Vogel-Bonner E medium (VB salts), and mix thoroughly, then add 50 mL of a sterile glucose (40 or 8 vol%) solution; again, swirl thoroughly. Dispense the agar medium in 100 × 15 mm^2 Petri dishes (approximately 25 mL per plate). After the solidification, the plates must be stored in an oven at 37 °C for 48 h (*see* **Note 2**).
2. *Histidine/biotin solution (0.5 mM)*: Add 124 mg of D-biotin and 96 mg of L-Histidine·HCl to 1000 mL distilled water. Autoclave it for 30 min at 121 °C (1.5 atm; relative pressure).

3. *Top agar supplemented with histidine/biotin*: Add 6 g of agar and 6 g of sodium chloride to 900 mL of distilled water. Autoclave it for 30 min at 121 °C (1.5 atm; relative pressure). For the test, melt the top agar in a microwave oven or in boiling water, and then add 100 mL of limited histidine and biotin solution (0.5 mM). The top agar is used to deliver the bacteria, chemical, and buffer or S9 mix to the bottom agar and it is one of the most critical medium components in the Ames test because it contains the trace amount of histidine (0.05 mM) for limited growth and biotin at a concentration of 0.05 mM, which is in excess of what is needed for the growth of the *Salmonella* strains.
4. *Nutrient broth*: Add 0.75 g of nutrient broth (Oxoid nutrient broth No. 2) to 30 mL water. Autoclave it for 15 min at 121 °C (1.5 atm; relative pressure).
5. *Sodium phosphate buffer, 0.1 mM, pH 7.4*: After mixing 120 mL monobasic sodium phosphate (0.1 M) and 880 mL of dibasic sodium phosphate (0.1 M), adjust pH to 7.4 using 0.1 M dibasic sodium phosphate solution. Autoclave it for 30 min at 121 °C (1.5 atm; relative pressure). The buffer is used for testing chemicals in the absence of metabolic activation (*see* **Note 3**).
6. *Co-factors for S9 mix*: A number of commercial vendors provide S9 preparations, as Molecular Toxicology, in the United States. Once the S9 mix is prepared, it should be kept on ice for the duration of the experiment. The metabolic activation system consisted of 4% S9 fraction, 1% of magnesium chloride at 0.4 mol L^{-1}, 1% of potassium chloride at 1.65 mol L^{-1}, 0.5% of D-glucose-6-phosphate disodium at 1 mol L^{-1}, 4% of nicotinamide adenine dinucleotide phosphate sodium salt (NADP) at 0.1 mol L^{-1} in 50% of phosphate buffer at 0.2 mol L^{-1}, and 39.5% of sterile distilled water.

Inoculum

For each experiment, individual culture flasks are inoculated with each strain. Inoculate 0.1 mL of the tester strain cultures in 30 mL of Oxoid nutrient broth No. 2 and place on a shaker in the dark and gently shake (100 rpm) for 11–14 h (overnight) at 37 °C. On the next morning, remove the cultures from the incubator and keep at room temperature away from direct fluorescent light. It is essential that the cultures used in the experiment contain a high titer of viable bacteria. The titer may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

Experimental Procedure

The procedure described here pertains to the preincubation method.

1. To the sterile glass tubes maintained at room temperature, add the following components in this order, with mild mixing after each addition:
   - 0.5 mL of metabolic activation (S9) mix or sodium phosphate buffer.
   - Different volumes of the food packaging extract. Include untreated, solvent/vehicle and strain-specific positive controls, both with and without metabolic activation, in each assay (*see* **Notes 1** and **4**).
   - 0.1 mL overnight culture of the bacterial strain.
2. Incubate the mixture at 37 °C for 20 min.
3. To each tube, add 2 mL of molten top agar supplemented with histidine/biotin maintained at 43–48 °C. The content of the test tubes is then mixed and poured onto the surface of GM agar plates (*see* **Note 5**).
4. When the top agar has solidified (2–3 min), the plates are inverted and incubated at 37 °C for 48 h.
5. The colonies are then counted, and the results are expressed as the number of revertant colonies per plate. If colonies cannot be counted immediately after the 48 h incubation, the plates can be stored in a refrigerator for up to 2 days. All plates must be removed from the incubator and counted at the same time (Fig. 1).

Preliminary experiments are useful to determine toxicity and insolubility of the food packaging samples. Cytotoxicity may be detected in the final population on the GM agar plate after the 48-h incubation by a thinning of the background lawn, which may be accompanied by a decrease in the number of revertant colonies,

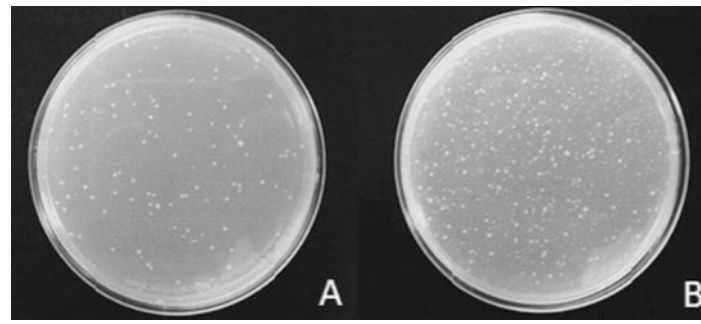

Fig. 1 Petri dishes with revertant colonies of *Salmonella* Typhimurium (TA102 strain). (**a**) Untreated control; (**b**) Positive control (Mitomycin C)

absence of background lawn (i.e., complete absence of growth), or by presence of pinpoint non-revertant colonies (generally in conjunction with an absence of background lawn).

Analysis of the Results

After the plates are removed from the incubator, the colonies are counted, and the results are expressed as mean revertant colonies per plate ± standard deviation for each dose of the test sample and the controls. A sample is considered a mutagen (it induces point mutations by base substitutions or frameshifts in the genome of either *S.* Typhimurium and/or *E. coli*) if it produces a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. The determination of a positive vs. a negative result is made through evaluation procedures for comparing dosed plates with the concurrent solvent/vehicle control plates, including a requirement for a specific fold increase (2- or 3-fold, specific to the bacterial strain).

3.2 Micronucleus Test

Experimental Procedure

1. In 6-well plates, seed 100,000 cell per well in 2 mL of cell growth media (*see* **Note 6**) with 10% of fetal bovine serum (complete culture medium). Prepare an appropriate number of wells for the experiment from a single pool of cells. These cells should be in exponential growth phase at the time of treatment.
2. Incubate the plates at 37 °C in a 5% CO_2 atmosphere for 24 h. This time is necessary for the adhesion of cells to the plate, for the formation of a semiconfluent cell monolayer, and for the progression of cells to the exponential growth phase.
3. After incubation, the solutions for the treatment groups must first be prepared in a culture medium in an amount sufficient for the treatment of the test. For treatment, it is optional to change the culture medium or not.
4. Three non-cytotoxic concentrations of the food packaging extract should be evaluated. Include untreated, solvent/vehicle, and positive controls in each treatment series (*see* **Notes 1** and **7**). Prepare duplicate cultures/wells at each experimental test point.
5. Smoothly homogenize the cultures with cross movements, avoiding bubble formation.
6. For the treatment, cells should be exposed to the food packaging extract without metabolic activation for 3–6 h, and sampled at a time equivalent to about 1.5–2.0 normal cell cycle lengths after the beginning of treatment. To conclude a negative

outcome, cells should be continuously exposed without metabolic activation until sampling at a time equivalent to about 1.5–2.0 normal cell cycle lengths [19]. After treatment, all plates are placed in a CO_2 incubator at 37 °C and 5% CO_2.

7. After the end of the exposure time, analyze the appearance of the cultures under an inverted microscope, mainly regarding the presence of precipitation, morphology, and cell death.
8. After the incubation period, remove the culture medium containing the test sample and wash the wells twice with 2 mL of PBS suitable for cell cultures.
9. Add fresh medium (2 mL per well).
10. Then add 20 μL per well of the 3 μg mL^{-1} pre-prepared cytochalasin B solution.
11. The plates must be placed in the CO_2 incubator for 1.5–2 normal cell cycle lengths.

Harvest

It is important to cast a water film on the slides, so that they can be ready and cold (2–8 °C) for use. To do this, wash the slides with neutral detergent, rinse them under running water, and then in distilled water. Then, the slides should be immersed, one by one, in distilled water and raised to check if a water film has formed on them. If the film does not form, wash again. The vial containing the slides must be kept and refrigerated until the moment of use. There is also another possibility of cleaning the slides, this being cleaning them in 70% ethanol and distilled water.

12. Collect the medium from each well into appropriately labelled 15-mL centrifuge tubes to avoid loss of detached mitotic cells.
13. The cells must be washed twice with PBS (2 mL), the first washing being reserved in the centrifuge tube and the second washing must be discarded. Remove excess PBS from each well with a pipette.
14. Add 0.3 mL of 0.1% trypsin to each well to bring cell monolayers into suspension. Time lapse (approximately 5 min) and temperature of incubation (37 °C) are indicative and should be standardized in each laboratory based on visual observations of cell detachment, as trypsin activity may vary among different lots.
15. When monolayer cells are completely detached, inactivate trypsin with 0.7 mL of complete culture medium (the medium reserved in the centrifuge tubes can be used) to block enzymatic digestion.

16. Add these cell suspensions to their respective centrifuge tubes and centrifuge them for 5 min at 1000 rpm.
17. Aspirate the supernatant carefully using a glass pipette, leaving approximately 0.5–1.0 mL as the pellet protection margin.
18. With the aid of the pipette, resuspend the cells, gently homogenizing the pellet (20×) and keep the solution inside the pipette so that all samples come into contact with the potassium chloride (KCl; 0.075 M) at the same time.
19. Add 3 mL of KCl previously cooled to 2–8 °C and incubate for approximately 7–10 min at room temperature. During this period, gently resuspend the cells, mixing 40× with the pipette.
20. After incubation with KCl, add 0.5 mL of the fixative (3:1 v/v methanol-glacial acetic acid), prepared at the time of use and kept at room temperature, and mix gently.
21. Centrifuge the cultures for 5 min at 1000 rpm. Remove the supernatant, leaving approximately 0.5–1.0 mL.
22. Homogenize the pellet (20×) and keep the solution inside the pipette.
23. Add 5 mL of fixative and mix immediately.
24. Centrifuge the cultures for 5 min. Remove the supernatant.
25. Add 5 mL fixative again and mix.
26. In this step, cultures should be kept in a refrigerator (2–8 °C) for at least 1 h and/or until the slides are prepared.
27. At the time of making the slides, centrifuge the tubes, remove the supernatant, and finally produce an appropriately concentrated cell suspension, maintaining approximately 0.5 mL of fixative.
28. Approximately five drops cellular solution should be dripped under the identified slide. Prepare at least three slides for each experimental point, labeled with the identity of the culture. Leave the slides to dry at room temperature prior to staining for at least 1 day. After this period, stain the slides or store in slide boxes.
29. The slides can be stained with 3 vol% Giemsa in tap water, 0.0125% (w/v) acridine orange in PBS, panoptic, among others, depending on the most suitable one for each test cell (Fig. 2).

Analysis of the Results (*See* Note 8)

The frequencies of cells with MN (with one, two, and more than two MN) are recorded. A total of 6000 binucleated cells are scored per treatment, corresponding to 2000 cells per treatment per repetition. Attention should be given to ensure that MN are scored only

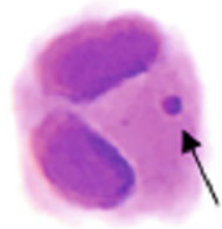

Fig. 2 Microphotograph (1000× magnification) of a micronucleated binucleated HepG2 cell. Staining with Giemsa (3%)

in binucleated cells and not in multinucleated cells, because multinucleated cells are not once-divided cells and tend to have greatly elevated MN frequencies relative to binucleated cells, which would result in inaccurate genome damage estimates [17].

A sample is considered a mutagen in this assay if statistically significant increases in the proportion of micronucleated cells over the negative/solvent controls (reference point for comparison in the statistical evaluation of the results) are observed at one or more concentrations.

Furthermore, the determination of the Cytokinesis-Block Proliferation Index (CBPI) may be used to calculate cell proliferation. CBPI indicates the average number of nuclei per cell. Cells with well-preserved cytoplasm containing 1–4 nuclei are scored. Analyze 1500 cells per treatment for a total of 500 cells per repetition. CBPI is calculated using the following formula [16]:

$$\mathrm{CBPI} = \frac{((\text{No.mononucleate cells}) + (2 \times \text{No.binucleate cells}) + (3 \times \text{No.multinucleate cells}))}{(\text{Total number of cells})}$$

4 Notes

1. Examples of suitable positive controls for *Salmonella*/microsome assay (to confirm the reversion properties and specificity of each tester strain, and the efficacy of the metabolic activation system): 4-nitro-*o*-phenylenediamine (TA98 and TA97a, 10 μg per plate); sodium azide (TA100, 1.25 μg per plate); mitomycin C (TA102, 0.5 μg per plate), in the absence of S9 and 2-anthramine (TA98, TA100, TA 97a, 1.25 μg per plate), 2-aminofluorene (TA102, 10 μg per plate), in the presence of S9.

   For MN test, the most commonly used positive control agents are: methyl methanesulfonate, mitomycin C, 4-nitroquinoline-N-oxide, cytosine arabinoside, benzo(a)pyrene,

Table 1 Recipe for Vogel-Bonner medium E (50X)

Ingredients	Quantity per liter
1. Warm distilled water (about 50 °C)	670 mL
2. Magnesium sulfate ($MgSO_4.H_2O$)	10 g
3. Citric acid monohydrate	100 g
4. Potassium phosphate, dibasic, anhydrous (K_2HPO_4)	500 g
5. Sodium ammonium phosphate ($Na_2NH_2PO_4.4H_2O$)	175 g

cyclophosphamide, colchicine, or vinblastine. Concentrations should be defined in preliminary tests.

2. Vogel–Bonner (VB salts) medium E.

   The ingredients must be added in the order indicated below. Make sure that each salt is dissolved thoroughly by stirring it on a magnetic stirrer before adding the next salt (Table 1).

   - The agar should never be autoclaved together with the VB salts and glucose.
   - The plates can be stored at 4 °C for several weeks when placed in sealed plastic bags to prevent dehydration. Before use, the plates should be warmed up to room temperature and examined for excess moisture.

3. Sodium phosphate, monobasic (0.1 M): To 1 L of water, add 13.8 g $NaH_2PO_4.H_2O$.

   Sodium phosphate, dibasic (0.1 M): To 1 L of water, add 14.2 g $Na_2HPO_4.H_2O$.

4. At least five different concentrations of the food packaging extract should be selected for the test, and the interval between each concentration should be approximately half log ($\sqrt{10}$). At least three plates for each dose level and for the controls is recommended.

   Extracts obtained from aqueous solvents can be used at levels up to approximately 1 mL per plate before they interfere with the gelling of the top agar, while organic solvents are often used at a maximal dose of 0.1 mL per plate [21].

   Untreated control: also called spontaneous control, as it aims to demonstrate the rate of spontaneous reversion of each strain and that no deleterious or mutagenic effects are induced by the chosen solvent.

   Solvent/vehicle control: solvent or vehicle alone, without test substance, and treated in the used maximum volume in the

treatment group. In the Ames test, the solvent/vehicle control is also called negative control [20].

Positive control (*see* **Note 1**).

5. It is important to quickly swirl the plates after the addition of the top agar to the surface of the GM agar plates to ensure an even distribution of the top agar that contains the bacteria, test sample, and S9 mix or buffer.
6. The most common culture media for the cell lines mentioned in the Introduction section are Eagle's Minimal Essential Medium (EMEM), Dulbecco's Modified Eagle's medium (DMEM), RPMI, Ham's F10, Ham's F12.
7. When cytochalasin B is used, the most appropriate method to assess cytotoxicity is to calculate the cytokinesis-block proliferation index (CBPI). CBPI: the proportion of second-division cells in the treated population relative to the untreated control.

   Untreated control: also called negative control, only 2 mL of complete culture medium.

   Solvent/vehicle control: solvent or vehicle alone, without test substance, and treated in the used maximum volume in the treatment group.

   Positive control (*see* **Note 1**).
8. MN are morphologically identical, but smaller than nuclei. The diameter of MN usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which correspond to 1/256th and 1/9th of the area of one of the main nuclei in a binucleated cell, respectively. MN are non-refractile and they are not linked or connected to the main nuclei. MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. Moreover, MN usually have the same staining intensity as the main nuclei, but occasionally staining may be more intense.

   For analysis of CBPI, mono-, bi, and multinucleated cells are viable, with an intact cytoplasm and normal nucleus morphology containing one, two, and three or more nuclei, respectively.

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