# GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

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# National Food Safety Standard -

# Test of Mammalian Cell Micronucleus in Vitro

食品安全国家标准 体外哺乳类细胞微核试验

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# National Food Safety Standard - Test of Mammalian Cell Micronucleus in Vitro

## 1 Scope

This Standard specifies the basic test methods and technical requirements for test of mammalian cell micronucleus in vitro.

This Standard is applicable to evaluate the genotoxic effects of test substances.

## 2 Terms and Definitions

### 2.1 Micronucleus

When the chromosomes regularly enter the daughter cells to form the nucleus at the late stage of mitosis, the entire chromatids or acentric fragments or rings of chromosomes that remain in the cytoplasm. In the final stage, one or several regular subnuclei are formed separately, which are contained in the cytoplasm of the cell.

### 2.2 Centromere

During cell division, the area where the chromosomes connect with the spindle fibers allows the daughter chromosomes to move to the two poles of the daughter cells in an orderly manner.

### 2.3 Aneuploidy

A variation type in which one or more complete chromosomes are missed or added to the diploid chromosome complement.

### 2.4 Aneuploidy mutagen

A substance that acts on the mitosis or meiotic cycle of cells to cause abnormal cell division and induce aneuploidy.

### 2.5 Chromosome break

A chromosome arm has a gap whose length is greater than the width of the chromosome arm.

### 2.6 Chromosome clastogen

min, sterilize or filter.

#### 4.3.1.3 Coenzyme-II (oxidized) solution

Weigh Coenzyme-II under aseptic conditions and dissolve it with sterile distilled water to prepare a 0.025 mol/L solution, which is prepared immediately before use.

#### 4.3.1.4 Glucose-6-phosphate sodium salt solution

Weigh glucose-6-phosphate sodium salt, dissolve it with distilled water to prepare a 0.05 mol/L solution, and filter and sterilize it. It shall be prepared immediately before use.

### 4.3.2 Induction and preparation of S9 component of rat liver

Choose healthy male adult SD or Wistar rats, with a body weight of 150g~200g, about 5 weeks old ~ 6 weeks old. Dissolve the polychlorinated biphenyl (Aroclor1254) in corn oil at a concentration of 200g/L; and perform an intraperitoneal injection aseptically at a weight of 500mg/kg. Then the animals are sacrificed 5d later; and the animals are fasted for 12h before sacrifice.

It may also be prepared by the combined induction method of phenobarbital sodium and β-naphthoflavone. Rats are given phenobarbital sodium and β-naphthoflavone by oral gavage. The doses are both 80 mg/kg body weight for 3d. After fasting for 16h, the animals are decapitated and sacrificed. Other operations are the same as polychlorinated biphenyl induction.

After the animals are sacrificed, the liver is taken out. After weighing, the liver is washed with fresh ice-cold 0.15 mol/L potassium chloride solution for several times in order to remove hemoglobin, which can inhibit the activity of microsomal enzymes. Add 3 mL of 0.1mol/L potassium chloride solution per gram of liver (wet weight); and move it into an ice bath together with the beaker. Cut the liver with sterile scissors and place it in a glass homogenizer (less than 4000 r/min, 1 min~2 min) or tissue homogenizer (less than 20000 r/min, 1 min) to make liver homogenate. The above operations should pay attention to aseptic and local cold environment.

Centrifuge the prepared liver homogenate in a low-temperature (0°C~4°C) high-speed centrifuge at 9000g for 10 min. Aspirate the supernatant as the S9 component and distribute it in sterile cryotubes, about 2mL per tube. It is best to use liquid nitrogen or dry ice to quickly freeze and store at a low temperature of -80°C.

After the S9 component is prepared, the protein content (Lowry method) shall be determined by sterility inspection; and the protein content per mL should not exceed 40mg; and the biological activity of the indirect mutagenic agent shall be qualified and stored at -80°C low temperature or frozen and dried, and the shelf life is no more than 1 year.

c) Take 50mL of the first solution and add it to 50mL of the second solution; and mix well, which is 1/15 mol/L phosphate buffer solution with pH 6.8.

# 5 Test Methods

### 5.1 Test substance

The solid test substance shall be dissolved in a suitable dissolvent and diluted to an appropriate concentration. The liquid test substance may be used directly or diluted to an appropriate concentration for use. The test substance shall be prepared aseptically and immediately before use; otherwise, it must be confirmed that storage shall not affect its stability.

### 5.2 Cell

Chinese hamster lung cell lines (V79, CHL) or ovarian cell lines (CHO), mouse lymphoma cell lines (L5178Y), human peripheral blood lymphocyte lines (such as TK6) and primary cultured cells may be selected. It is recommended to use the CHL or L5178Y cell line. Cells shall be checked for stability of the chromosome number and mycoplasma contamination before use.

### 5.3 Selection of test scheme

The test is divided into two schemes: using and not using cytoB. Scheme-1: After the cells are treated with the test substance, use cytoB before mitosis; and then observe and analyze the micronucleus rate of the cells (binuclear cells) that have completed one mitosis. When using human lymphocytes, it is recommended to use Scheme-1; because different sources of cells, their cycle are different; and not all cells respond to Phytohemagglutinin (PHA). Scheme-2: Do not use cytoB; and then observe and analyze the micronucleus rate of the cells after the cells are treated with the test substance. If there is evidence that proves the test substance interferes with the activity of cytoB, or cytoB may affect the growth of cells (such as mouse lymphoma cell lines), then it is recommended to adopt the Scheme-2.

### 5.4 Dose

#### 5.4.1 Dose setting

At least 3 detection doses shall be set. When the test substance is not cytotoxic, set at least 2 doses from the highest dose downward. Generally, the interval coefficient may be 2~3. When there is cytotoxicity, the dose range shall cover from 55%±5% cytotoxicity to almost no cytotoxicity.

#### 5.4.2 Selection of the highest dose

If there is no literature or historical data to prove that the used dissolvent is not mutagenic, a blank control shall be set.

**5.5 Test procedures**

**5.5.1 Preparation of cell**

Inoculate a certain number of cells in a culture dish (bottle). When harvesting the cells, if the cells in the culture dish (bottle) is not overgrown, it shall be regarded as the standard. Adherent cells are generally preferred to grow to about 85%.

**5.5.2 Treatment of test substance**

**5.5.2.1** Application scheme-1: Aspirate the culture solution; wash the cells with phosphate buffer solution. Add serum-free culture solution and a certain concentration of the test substance (if metabolic activation is required, add S9 mix at the same time); and place it in the incubator for 3h~6h. After the end, aspirate the culture solution containing the test substance; wash the cells with PBS. Add fresh culture solution containing 10% serum and cytoB; continue to culture for 1.5~2.0 normal cell cycles and then collect the cells.

For lymphocytes, the most effective method is to start the test substance treatment 44h~48h after mitogen (e.g.: PHA) stimulation, at which time the cells begin to enter the division cycle.

If the test result of the short-term treatment for 3h~6h is negative or unclear, a long-term treatment test without S9 is required. The cells are treated with cytoB and the test substance for 1.5~2.0 normal cell cycles; and the cells are collected after the treatment is completed.

If it is known or suspected that the test substance (e.g.: nucleosides) may affect the cell cycle (especially P53 active cells), then the cell harvest time shall be extended by 1.5~2.0 normal cell cycles.

**5.5.2.2** Application scheme-2: it is the same as 5.5.2.1, except that cytoB is not added.

**5.5.3 Cell harvesting and slicing**

Cells shall be harvested and sliced separately for each culture. If the cell mixed liquor is well dispersed, hypotonic treatment is not required.

**5.5.3.1 Digestion**

Adherent cells are digested with 0.25% trypsin solution. After the cells fall off, add a culture solution containing 10% fetal bovine or calf serum to stop the trypsin effect; mix well; and put it in a centrifuge tube to centrifugate at 800 r/min~ 1000 r/min for 5min; and discard the supernatant. Suspended cells do not need to be digested and

used.

# 6 Data Processing and Result Judgment

### 6.1 Data processing

The data is listed by different doses; and the indicators include cytotoxicity, the number of observed cells, the number of micronucleus cells and micronucleus cell rate. The micronucleus cell rate of each dose group of the test substance, the blank control group, the negative control group (dissolvent control group), and the positive control group are processed by appropriate statistical methods (such as $X^2$ test).

### 6.2 Judgment of results

The following two situations may determine that the test substance is a positive result in this test system:

a) The increase in the rate of micronucleus cells caused by the test substance is statistically significant and is related to the dose;

b) The increase in the rate of micronucleus cells caused by the test substance under any dosage condition is statistically significant and repeatable.

# 7 Test Report

**7.1** Test name, test organization name, contact information, and report number.

**7.2** The name and contact information of the test entrusting organization, and the date of sample acceptance.

**7.3** The start and end date of the test, the person in charge of the test project, the technical person in charge of the test organization, and the date of issuance.

**7.4** Test summary.

**7.5** Test substance: name, identification information, CAS number (if known), purity, physical and chemical properties and stability of the test substance related to this test.

**7.6** Solvent.

**7.7** Cell line: the source and name of the cell line.

**7.8** Test conditions: dose, metabolic activation system, standard mutagens, operation procedures, etc.

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