



**"Genetic Toxicology: DNA Damage and Repair/
Unscheduled DNA Synthesis in Mammalian Cells
in vitro"**

1. INTRODUCTORY INFORMATION

• Prerequisites

- Solid, liquid, vapour or gaseous test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- Melting point/boiling point
- pH (where appropriate)
- Vapour pressure data (if available)

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION

The guideline for Unscheduled DNA Synthesis (UDS) in mammalian cells *in vitro* describes procedures utilizing primary mammalian cells or continuous cell lines to detect DNA repair synthesis. The endpoint of unscheduled DNA synthesis is measured by the uptake of radioactive labeled nucleotide e.g. ^3H -TdR using autoradiographic or liquid scintillation counting (LSC) procedures. UDS may also be measured using *in vivo* systems.

• Principle of the method

The UDS test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical or physical agents. The test is based commonly on the incorporation of tritium-labelled thymidine (^3H -TdR) into the DNA of mammalian cells which are not in the S-phase of the cell cycle. The uptake of ^3H -TdR may be determined by autoradiography or by LSC of DNA from the treated cells. Mammalian cells in culture, unless primary rat hepatocytes are used, are treated with the test substance with and without exogenous mammalian metabolic activation.

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B. DESCRIPTION OF THE TEST METHOD

• Preparations

Test substance

The test substance and control substances should be prepared in growth medium, or dissolved in an appropriate vehicle and then further diluted in growth medium, for use in the assay. The final concentration of the vehicle should not affect cell viability.

Cells and culture conditions

Primary cultures (e.g. rat hepatocytes), human lymphocytes or established cell lines (e.g. human diploid fibroblasts) may be used in the assay. Appropriate growth media, CO₂ concentration, temperature and humidity should be used in maintaining cultures. Established cell lines should be periodically checked for mycoplasma contamination.

• Test conditions

Number of cultures

At least two cell cultures for autoradiography are necessary for each experimental point. For UDS determination using LSC, six cell cultures, or less if scientifically justified, are needed for each experimental point.

Controls

Concurrent positive and negative (vehicle) controls with and without metabolic activation should be included in each experiment.

Examples of positive controls for the rat hepatocyte assay include 7, 12-dimethylbenzanthracene (7, 12-DMBA) and 2-acetylaminofluorene (2-AAF). In the case of established cell lines, 4-nitroquinoline-N-oxide (4-NQO) is an example of a positive control for both the autoradiographic and LSC assays performed without metabolic activation. N-dimethylnitrosamine is an example of a positive control compound when metabolic activation systems are used.

Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response, should be used. The highest concentration should elicit some cytotoxic effects. Rela-

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tively water-insoluble compounds should be tested up to their limit of solubility. For freely water-soluble non-toxic chemicals, the highest concentration should be determined on a case-by-case basis.

Metabolic Activation

Except for primary cells with intrinsic metabolic activity, cells should be exposed to the test substance both in the presence and absence of an appropriate mammalian metabolic activation system.

• Performance of the test

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37° C.

Short term cultures of mammalian hepatocytes are established by allowing freshly dissociated hepatocytes to attach themselves to the growing surface.

Human lymphocyte cultures are set up using appropriate techniques.

Treatment of the cultures with the test substance

– *Primary mammalian hepatocytes*

Freshly isolated mammalian hepatocytes are treated with the test substance in a medium containing ³H-TdR for an appropriate length of time. At the end of the treatment period, medium should be removed from the cells, which are then rinsed, fixed and dried. Slides should be dipped in autoradiographic emulsion (alternatively, stripping film may be used), exposed, developed, stained and counted.

Another technique uses parallel BrdU-incorporation and subsequent density gradient centrifugation, in addition to radiolabelling, by which replicating DNA and UDS-DNA can be separated before the liquid scintillation counting.

– *Established cell lines and lymphocytes, using an autoradiographic technique*

Cell cultures are exposed to the test substance for appropriate durations. These times will be governed by the nature of the substance, the activity of the metabolic activation system and

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the type of cells. To detect the peak of UDS, $^3\text{H-TdR}$ should be added either simultaneously with the test substance or within a few minutes after exposure to the test substance. The choice between these two procedures will be influenced by possible interactions between test substance and $^3\text{H-TdR}$.

In order to discriminate between UDS and normal semi-conservative DNA replication, the latter may be reduced or inhibited, for example, by the use of an arginine-deficient medium, low serum content, or by hydroxyurea in the culture medium.

– *Established cell lines and lymphocytes, using LSC measurement of UDS*

Prior to treatment with test substance, entry of cells into S-phase should be blocked as described above; cells should then be exposed to test chemical as described for autoradiography. At the end of the incubation period, DNA should be extracted from the cells and the total DNA content, and the extent of incorporated radiolabel, determined.

It should be noted that, where human lymphocytes are used in both the above techniques, the suppression of normal semi-conservative DNA replication is unnecessary in unstimulated cultures.

Analysis

– *Autoradiographic determinations*

In determining UDS in cells in culture, S-phase nuclei are not counted. Slides should be coded before counting. At least 50 cells per culture should be counted. Several widely separated random fields should be counted on each slide. The amount of $^3\text{H-TdR}$ incorporation in the cytoplasm should be determined by suitable methods.

The result should be confirmed in an independent experiment.

– *LSC determinations*

Six cultures (or fewer, if scientifically justified) should be used at each concentration and in the controls.

The result should be confirmed in an independent experiment.

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3. DATA AND REPORTINGS

- **Treatment of results**

Data should be presented in tabular form.

- *Autoradiographic determinations*

The extent of ³H-TdR incorporation in the cytoplasm and the number of grains found over the cell nucleus should be recorded separately.

Mean, median and mode may be used to describe the distribution of the extent of ³H-TdR incorporation in the cytoplasm and the number of grains per nucleus. The percentage of cells showing UDS may provide useful information.

Data should be evaluated using appropriate statistical methods.

- *LSC determinations*

For LSC determinations, incorporation of radiolabel should be reported as dpm/μg DNA. The mean dpm/μg DNA with standard deviation may be used to describe the distribution of incorporation.

Data should be evaluated using appropriate statistical methods.

- **Evaluation of results**

There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in radiolabel incorporation (expressed either as grains per nucleus or as dpm/μg DNA). Another criterion may be the detection of a reproducible and statistically significant positive response for at least one of the test points.

A test substance producing neither a statistically significant dose-related increase in radiolabel incorporation (expressed either in grains per nucleus or as dpm/μg DNA) nor a statistically significant and reproducible positive response at any one of the test points is considered not active in this system.

- **Test report**

The test report should include the following information, if applicable:

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- cells used, density and passage number at time of treatment, number of cell cultures;
- methods used for maintenance of cell cultures including medium, temperature and CO₂ concentration;
- test substance, vehicle, concentrations used in the assay and rationale for their selection;
- details of metabolic activation systems;
- treatment schedule;
- positive and negative controls;
- procedures used to block entry of cells into S-phase;
- autoradiographic technique used;
- procedures used for DNA extraction and determination of total DNA content in LSC;
- dose-response relationship;
- statistical evaluation;
- discussion of results;
- interpretation of results.

4. L I T E R A T U R E

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