Standard Operating Procedure (SOP)

Aim of SOP (tick box)

☐ Munition detection or identification	☒ Toxicity
☐ Sampling	☐ In situ exposure studies
☐ Chemical analysis	☒ Bioassays
☐ Bioindicators/biomarkers	

22. Comet assay (applied to zebrafish embryos)

version 1.1

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Scope

This Standard Operating Procedure describes the alkaline comet assay using zebrafish embryos (*Danio rerio*) as test species. This method is used to determine the genotoxicity of chemicals in single cells isolated from whole zebrafish embryos and can be applied to individual substances or mixtures of chemicals. The procedure described here is carried out according to the alkaline comet assay method of Singh et al. (1988)[1] with modifications for zebrafish embryos according to Kosmehl et al. (2008)[2]. In the field of ecotoxicology, this method can be used for biological effect monitoring to quantify the genotoxic effects of pollutants in addition to the analysis of pollutants.

Summary of the method

The comet assay is a method for the quantification of DNA damage induced by chemicals. Single and double strand breaks in the DNA of different cells can be detected under alkaline conditions. These strand breaks can be observed *in vivo* or *in vitro*, which can either have a temporary effect, be lethal to the cell or lead to a mutation that represents a permanent viable change. This SOP specifically describes the comet assay with zebrafish embryos (*Danio rerio*), meaning the *in vivo* exposure of the embryos for 48 hours (see SOP on zebrafish embryo acute toxicity test, ZFET), followed by isolation of the single cells and the comet assay.

For a robust statement on the genotoxicity of the tested substance in zebrafish cells, concentrations should be chosen that are lower than the acute toxicity of the substance in zebrafish. Three independent replicates per test concentration are preferred. A proper analytical method for the tested chemical is recommended to measure the tested nominal concentrations in relation to their actual concentrations.

Safety aspects

Depending on the chemicals to be tested, the corresponding risk assessments have to be considered before starting work. If further instructions are required, contact the local safety officer or the laboratory manager.

Documentation

The test report should include the following information, please also refer to the SOP for the ZFET and OECD guideline No. 236[3].

Information on test chemical:

- Water solubility, IUPAC or CAS name, CAS number, purity and additional relevant physico-chemical properties.
- For mixtures: Chemical identity where possible, relevant physico-chemical properties of the constituents.

Test procedure:

- Information on the test conditions of ZFET.

Results:

- List of effects in each well/embryo at each time of study or at the end of exposure, including controls.
- Information on incidents in the course of the test that may have influenced the results.
- The photos of the microscope slides/fluorescent comets, the evaluation files and if available boxplots of the genotoxic effects.

Methods

Equipment:

- For ZFET equipment see respective SOP.
- Fluorescence microscope (200 x magnification) with a suitable camera.
- Electrophoresis apparatus with power supply.
- Stainless steel or glass troughs with inserts for horizontal and vertical storage.
- Heating block.
- Refrigerated centrifuge.
- Vortex mixer.
- pH meter.
- Glass/glass homogenizer, e.g. Potter-Elvehjem type.
- Pipettes, volume adjustable including tips.
- Ice tub.
- Microwave oven.
- Reaction tubes, Eppendorf.
- Haemocytometer/Neubauer counting chamber.
- DURAN bottles of suitable volumes.
- Frosted microscope slides including cover glasses.
- Disposable plastic Pasteur pipettes (3 mL).

Chemicals:

- Benzocaine, CAS 94-09-7.
- Agarose, low melting point, CAS 9012-36-6.
- Agarose normal melting point, CAS 9012-36-6.
- Sodium chloride, NaCl, CAS 7647-14-5.
- Ethylenediaminetetraacetic acid, EDTA, CAS 60-00-4 60-00-4.
- Tris(hydroxymethyl)aminomethane, Tris, CAS 77-86-1.
- Triton X-100, CAS 9002-93-1.

- Sodium hydroxide, NaOH, CAS 1310-73-2.
- Hydrochloric acid (37%), HCl, CAS 7647-01-0.
- Phosphate-buffered saline tablets, PBS.
- Hydrogen peroxide, H_2O_2, CAS 7722-84-1.
- Fluorescent dye for DNA, e.g. Midori Green (Biozym, Germany).
- Trypan Blue, CAS 72-57-1.
- Ethanol

Exposure of zebrafish embryos:

Zebrafish embryos are exposed to the test chemical according to the procedure described in the ZFET SOP. The exposure time can be chosen between 48 h and 96 h. The test concentrations should be lower than the acute toxicity of the test chemical in order to be able to detect the genotoxic effects which are not superimposed by acute toxicity.

The same controls are required as for the ZFET (see ZFET SOP), whereby 60 embryos should be used for the negative control in order to have enough embryos left that can be used for the positive control of the Comet Test (H_2O_2).

Preparation of solutions needed for comet assay:

The solutions can be prepared in advance and stored in the refrigerator, the day before starting the comet assay.

Lysis buffer

146.11 g NaCl (2.5 mol/L), 37.23 g EDTA (100 mmol/L), 1.21 g Tris (10 mmol/L), 7.9 g NaOH and fill up to 1 L with water. Adjust the pH to 10 with NaOH. Before using the lysis buffer, add 1% of Triton X-100 to the lysis buffer.

Electrophoresis buffer

12 g NaOH (300 mmol/L), 0.35 g EDTA (1 mmol/L) and fill up to 1 L with water.

Neutralization buffer

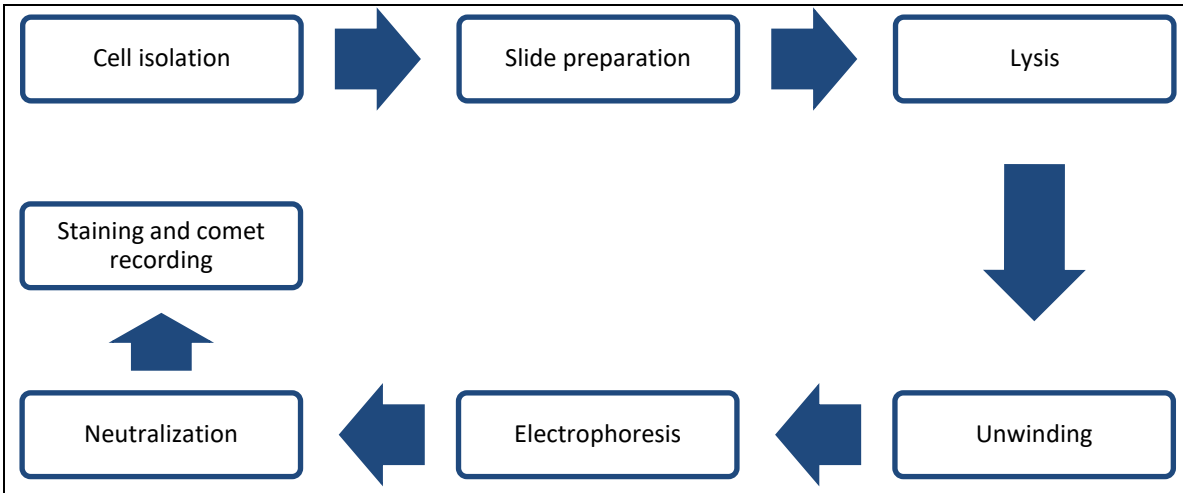
Tris-stock solution: 48.46 g Tris (0.4 mol/L) and fill up to 1 L with water.

HCl stock solution: 16.5 mL HCl (37%) and fill up to 1 L with water.

The neutralization buffer (around 200 mL per trough) is prepared by mixing 50 mL of tris-stock solution, 80 mL of HCl stock solution and 70 mL of water.

Phosphate buffer saline, PBS

PBS can be prepared by dissolving one PBS tablet in the specified amount of water.



Cell isolation from whole zebrafish embryos:

Before starting cell isolation, 20 embryos of the negative control are exposed to 0.35% H_2O_2 solution, which is known to cause DNA damage[4], for 20 min and are used subsequently for cell isolation.

To obtain single cell suspensions from zebrafish embryos, 20 surviving embryos of each treatment, including controls, are pooled and immersed in a saturated benzocaine solution for anesthesia. Afterwards, a glass/glass homogenizer is used for homogenization of the embryos in 1.3 mL PBS. The solution is filtered through 70 µm filter mesh, transferred in a 1.5 mL reaction tube and centrifuged at 2000 g for 10 min at 4 °C. From now on the reaction tubes should be kept on ice whenever possible. After resuspension in 500 µL PBS, the solution is centrifuged again at 1800 g for 7 min at 4 °C, the supernatant is discarded and the pellets are resuspended in a final volume of 150 µL PBS, which is used for the comet assay and trypan blue assay.

Trypan blue assay:

In order to check the viability of the cells, the trypan blue exclusion test is applied and only cell suspensions with more than 75% viability are used for the comet assay. For this purpose 1 part of 0.4% trypan blue is mixed with 1 part of the cell suspension and incubated for 3 min[5]. Viable (unstained) and nonviable (stained) cells are counted in a drop of the suspension in haemocytometer (200 x magnification) to obtain the cell viability per treatment.

Comet assay:

Preparation of slides

The slides for the Comet Assay can be prepared one day in advance. Before the agarose gel is placed on the slide, they have to be cleaned with ethanol (99%). A first layer of 0.5% normal melting agarose gel (100 µL) is placed on the slide and dried overnight. In this form the slides can be stored for several weeks.

It is recommended to prepare both low melting agarose (0.7%, 70 mg low melting agarose in

10 mL PBS) and normal melting agarose (0.5%, 50 mg normal melting agarose in 10 mL PBS) in larger quantities and then to store them in aliquots of 1 mL in the refrigerator. The aliquots can then be heated and liquefied again in the heating block. After heating at 85 °C, the low melting agarose should be cooled down to 37 °C before mixing with the cell suspension.

Two microscope slides per treatment should be prepared with the same cell suspension in order to obtain two technical replicates and ensure a sufficient number of cells for evaluation.

After preparing the cell suspensions, the suspensions are diluted 1:2 (volume) with 0.7% low melting agarose gel, mixed carefully on the Vortex and placed on top of the first layer on the slide. Subsequently, a cover slide is placed on top in such a way that no air bubbles form under the glass. To harden the second layer the slides must be kept on ice for 3 minutes.

After 3 minutes, the cover glass is carefully removed laterally and 100 µL of 0.7% low melting agarose are applied as a third layer. Again a cover glass is placed on top and the slide is placed on ice for 3 minutes for hardening.

Lysis of the cells

The slides are incubated for 1.5 hours in the lysis buffer (200 mL in a trough). The lysis solution must be kept at a temperature of about 4 °C in the dark. For this purpose, the lysis solution is best pre-tempered in the refrigerator, then the slides in the holder are immersed in the solution and stored in the refrigerator for 1.5 hours.

Unwinding time and electrophoresis

For unwinding of the DNA, the slides are placed in the alkaline electrophoresis buffer for 20 min with the slides horizontally in the filled electrophoresis chamber. All slides are placed with the labelling field in the same direction in the electrophoresis chamber filled with electrophoresis buffer. The electrophoresis chamber is filled with buffer in such a way that the slides are covered with the buffer for about 2mm. (For unwinding, the voltage is not switched on!)

Afterwards, electrophoresis is performed at a voltage of 22 V and 300 mA for 17 min. After each electrophoresis the chamber must be filled with fresh buffer.

Neutralization

After electrophoresis, the slides (mounted in a tray) are placed in a trough filled with neutralization buffer for 20 minutes. Afterwards, the slides are stored at 4 °C in PBS until they are stained and analyzed.

Staining and recording of the comets

The slides are stained with 40 µL of the fluorescent dye solution and covered with a cover glass. Microscopy is performed under fluorescent light at 200x magnification. The wavelength has to be chosen according to the fluorescent dye used, e.g. Midori Green: 490 nm excitation and 530 nm emission wavelength. The DNA appears as green fluorescence on a black background. The damaged DNA migrates through electrophoresis towards the anode and appears under the microscope like a small comet. A sufficient number of photos (at least 100 cells per treatment) must be taken of the slides.

Assessment:

Different software can be used to evaluate the comets on the photos, e.g. CASP[6] or OpenComet (http://www.cometbio.org). For each concentration, 80 randomly selected nucleoids (40 from each slide) are evaluated with the chosen image analysis program. The DNA damage is quantified as % tail DNA and tail moment

Data evaluation:

For comparison of the DNA damage, the tail moment of the treatments are plotted against the corresponding controls. Appropriate statistical methods such as generalized linear models with gamma probability distribution can be applied to test significant differences in genotoxicity between the treatments and the controls[7].

References

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