

Standard Operating Procedure

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HEALTH EFFECTS LABORATORY

Standard Operating Procedure

Comet assay

Written by: MDU	Valid from: 02.06.2017	Code: HEL11T005
QA (name and sign): ESI <i>ESI</i>		Version: 5
Approved (name and sign): MDU <i>MDU</i>		Page 1 of 16

1. INTRODUCTION

The Comet assay (single cell gel electrophoresis) is a simple, widely used method for detection of DNA damage in cells with nuclei. The method is widely used for detection of strand breaks as well as specific DNA lesions, such as oxidized purines and pyrimidines, and is considered a useful method for genotoxicity testing *in vitro* as well as *in vivo*.

2. PRINCIPLE

Cells are embedded in agarose on a microscope slide and lysed in detergent solution with high concentration of salt (NaCl) to dissolve membranes, cytoplasm, and most of the soluble cell contents, including histones. DNA remains as a series of loops attached to the nuclear matrix. The loops are supercoiled, because although the histones have been removed, the winding of the DNA (formerly around the nucleosomes) remains. When DNA is subjected to an electrophoretic field, it tends to move towards the anode due to positive charge of the DNA. DNA in the gel-embedded nucleoids is very compact, thus movement of intact supercoiled DNA loops is very limited. However, if a single break is present in the loop, the supercoiling is relaxed and the loop is free to extend under the electrophoretic field and move towards the the anode. When DNA is stained and examined microscopically, images resembling comets are seen; the tail consists of loops of DNA that have moved out from the head. The amount of DNA in the tail reflects the number of relaxed loops, reflecting the number of breaks in the DNA. Over a certain range of damage (including background level), there is a near-linear relationship between the proportion of DNA in the tail and the number of breaks. For human-related genotoxicity the most suitable cells are human or mammalian cells growing in suspension or attached to the surface. One of the most commonly applied cells is TK6 cells.

The standard comet assay measures single- and double-strand breaks. Modified version of the assay with lesion-specific enzymes can detect specific DNA lesions, such as oxidized purines (with formamidopyrimidine DNA glycosylase, Fpg, or the mammalian counterpart, 8-oxoguanine DNA glycosylase, OGG1) or oxidized pyrimidines (with endonuclease III, Endo III). The enzyme incubation takes place after lysis, removing the damaged base and leaving an apurinic/aprimidinic (AP) site that is converted into a break by the AP lyase/endonuclease which is normally associated with the repair enzyme, or by the alkaline conditions of the assay.



3. MATERIAL AND REAGENTS

Material

Culturing plates
Cell culture dishes
Glass slides (S8400, Sigma)
Cover slips 22x22mm
Micro centrifuge tubes 1.5ml (Eppendorf)

Sterile tubes 10ml
Pasteur pipettes – 2, 5, 10 ml
Automatic pipettes
Multi channel pipette (optional)


Reagents, media and sera

Cells
Cell culture medium (according to SOP for cultivating of the specific cell line)
Fetal Bovine Serum, Qualified
Penicillin-Streptomycin
Other supplements of culture medium required for the specific type of cell line (e.g. antibiotics, non essential aminoacids, sodium pyruvate, etc.)
Trypsin-EDTA
1% methylene blue
Countess® Cell Counting Chamber Slides and trypan blue (0.4%) kit or Bürker chamber
Phosphate buffered saline (PBS)
CaCl₂

MgCl₂
Agarose - Electrophoresis grade
Agarose - Low melting point (LMP)
Triton X-100
DAPI (4'6-diamidine-2-phenylindol dihydrochloride or SYBr
Bovine serum albumin (BSA)
H₂O₂, 30%;
NaOH (Mw = 39.99)
EDTA (Mw = 372.24)
Tris (Mw = 121.14)
NaCl (Mw = 58.45)
KCl (Mw = 74.55)
Na₂HPO₄.12H₂O (Mw = 358.141)
HEPES (Mw = 260.3)
KOH (Mw = 56.11)

4. EQUIPMENT

Laminar Flow Hood
Light Microscope
Countess / Bürker chamber with cover glass
Pipettes
Microwave
CO₂ incubator
Centrifuge
Water bath or heat block

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Vortex mixer
 Incubator 4°C
 Electrophoresis equipment
 Fluorescent microscope (with CCD camera)
 Perceptive Instruments/Metasystem software for image analysis

5. PREPARATION PROCEDURES

5.1 Preparation of solutions, positive control, and enzymes

5.1.1 Agarose LMP 0.8 %


Dissolve 0.8 g LMP agarose in 100 ml PBS.

Melt the agarose by careful heating in the microwave: stop microwave after about 10-15 seconds, shake the flask to ensure uniform heating and that it does not boil, start microwave, heat for seconds, stop, and check again. Repeat until the fluid is clear and completely dissolved. Make small aliquots (e.g. 10 ml pr bottle/falcon tube) and keep at 4°C.

The day of experiment you take one aliquot and melt it in the microwave until it is completely dissolved, following the same procedure as described above. Do not boil it!

5.1.2 Stock solutions to be used for making the Comet Assay solutions:

Compound	Concentration	Molecular weight (g/mol)	Quantity in 0.5L distilled H ₂ O	Quantity in 1 L distilled H ₂ O	Storage
NaOH	8 M	40.00	160 g	360 g	Room temperature
Na ₂ EDTA	0.2 M	372.24	37.2 g	74.4 g	4-8°C
Tris-HCl	4 M	121.10	242.2 g	484.4 g	Thermostat at 37°C
KOH	8 M	56.1056	224.4 g	448.8 g	Room temperature

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5.1.3 Lysis solution:


Compound	Concentration	Molecular Weight (g/mol)	Quantity for 2 L in distilled H ₂ O	Quantity for 1 L in distilled H ₂ O	Alternatively: take from stocks to make 1 L in distilled water
NaCl	2.50 M	58.44	292.20 g	146.1 g	
Na ₂ EDTA	0.10 M	372.24	74.45 g	37.23 g	
Tris-HCl	0.01 M	121.10	2.42 g	1.21 g	2.5 ml of 4M stock kept in thermostat 37C
NaOH	8 M	Start adding NaOH to ensure that EDTA dissolves and adjust to pH 10 . Be careful not to exceed the desired pH. Note: it takes several hours to dissolve all reagents and to adjust pH!!			
On the day of experiment, add 1 ml Triton X-100 per 100 ml lysis buffer before use and mix properly with magnetic blender. Keep at 4°C.					

5.1.4 Preparation of Enzyme reaction buffer for Endo III and Fpg (**Buffer F**):

Concentration	Compound	Molecular weight (g/mol)	Quantity/ L	10x (0.5 L)	10x (1 L)
0.04 M	HEPES	238.30	9.53 g	47.66 g	95.32 g
0.10 M	KCl	74.56	7.46 g	37.28 g	74.56 g
0.0005 M	EDTA	372.24	0.19 g	0.93 g	1.86 g
0.2 mg/mL	BSA		0.20 g	1 g	2 g
Adjust to pH 8.0 with KOH 8M (approx. 40 mL).					
Can be made as 10X stock, adjusted to pH 8.0 and kept in freezer at -20°C					

5.1.5 Electrophoresis buffer solution:

Concentration	Compound	Quantity for 1L	Quantity for 2 L
0.3 M	NaOH	12 g	24 g
0.001 M	Na ₂ EDTA	0.37 g	0.74 g
pH should be measured and should be > 13 after mixing the ingredients without adjustment. Keep at 4°C.			

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5.1.6 PBS solution:

8 g/l NaCl
 0.2 g/l KCl
 1.15 g/l Na₂HPO₄
 0.2 g/l KH₂PO₄

Adjust **pH to 7.2** with NaOH (8M).

You may also prepare 20X stock solution by multiplying all compounds by 20.

Alternatively: Dissolve 1 tablet of PBS as described on the box.

5.1.7 Preparation of H₂O₂ for positive control:


Stock solution in fridge: **9.8 M** (30% w/w i.e. 30g/100mg).

Dilution A: 100 mM H₂O₂: 10 µl stock solution (9,8 M) in 970 µl of PBS solution.

Recommended concentration to apply for the cells is 0.1 µM. To make 50 ml 100 µM solution for a jar: **add 50 µl from dilution A in 50 ml PBS.**

5.1.8 Preparation of stock TRIS-EDTA - TE buffer for dilution of SYBERGold:

Concentration	Compound	Molecular weight (g/mol)	Quantity for 2 L in distilled H ₂ O	Quantity for 1 L in distilled H ₂ O	Alternatively: take from stocks to make 1 L in distilled water)
2.5 mM	Tris-HCl	121.10	0.604 g	0.302 g	625 µl 4M Tris-HCl pH 7.5
4 mM	Na ₂ EDTA	372.24	2.976 g	1.488 g	400 µl 0.2M Na ₂ -EDTA (pH 8)
					Add 870 ml ddH ₂ O
Adjust to pH 7.5-7.8 using HCl					
The TE buffer stock solution can be stored at room temperature for months (working solution can be stored at 4°C for weeks).					

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5.1.9 Preparation of SYBR®Gold (SG) for staining of slides:

- Thaw the purchased stock and make aliquots of 50 µl to be stored in freezer at -20 °C.
- **For making 10X stock solution of SYBR®Gold:**
 1. Take one aliquot of 50 µl of sybr gold from freezer and thaw it at room temperature (avoid direct light)
 2. Add 450 µl DMSO
 3. Make small aliquots of 5 µl into eppendorf tubes and freeze at -20°C in box labeled 10X SYBER Gold
- **Staining of gels using drops on slide:**
 1. Preparation of 2000x SYBERGold working solution in TE buffer working solution: Take one aliquot (5 µl) 10x SYBERGold from box in freezer and add 995µl of TE buffer.
Cover from light.
 2. Place a drop of 2000x SYBERGold on top of each gel, and put a cover slip 22 x 60 mm on top of the 12 minigels.
 3. Leave in dark for 5-10 min before visualization in the fluorescence microscope.

NOTE 1. Do not freeze the working solution

NOTE 2. TE buffer (TRIS-EDTA) working solution is stored in the fridge at 4°C.

5.2 Preparation of enzymes (Fpg and EndoIII)

The enzymes ENDO III) and Fpg) are isolated from bacteria containing over-producing plasmids. Because such a high proportion of protein is the enzyme, a crude extract is perfectly satisfactory; in our experience there is no non-specific nuclease activity at the concentrations employed. The enzyme extracts are best obtained from a laboratory producing them. On receipt, the enzyme (which should have been refrigerated in transit) should be dispensed into small aliquots (e.g 5 µl) and stored at -80°C. The final dilution depends on titration provided by supplier. If not provided, titration should be performed in our lab to find the final working concentration..

Fpg

- Enzyme is generally provided by other laboratories. Usually the enzyme received is concentrated and will need further dilutions. Depends on the instruction from each supplier about how to dilute and what is the final working concentration to use, we follow general steps as below unless it is instructed differently from the supplier:



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- Step 1: Thaw the received amount of enzyme on ice and make few aliquots as soon as possible (e.g. 50 µl/tube). Then, freeze the vials in box at -80°C. Label the box with the concentration indicated by the supplier and name it Stock 1 original batch.
- Step 2: It is recommended to make a second stock solution immediately up on receive (Stock 2) from the original stock: Take 1 enzyme aliquot from Stock 1 and dilute into buffer F with 10% glycerol (e.g dilute the enzyme 1:100). Note. The number of steps and stocks to make depend on your original concentration and the final working concentration required.
- Use final stock (e.g. stock 2 or stock 3) to make the working solution.

For use, in general we take one enzyme aliquot (e.g.10 µl) from final stock made and dilute it in buffer F (no glycerol) to achieve the final concentration needed. Keep on ice all the time until use. Prepare enough amount of enzyme working solution for all slides: e.g. 200 µl on top of each slide of 12 gel should be sufficient. Do not refreeze this working solution. The final working dilution of Fpg used to incubate with nucleoids on gels depends on titration experiments) (provided by supplier or titration experiment done in our lab).


Endo III (is more stable)

- Dispense the stock solution into 5 µl aliquots and refreeze at -80°C.
- Take one of these aliquots and dilute 100X (5 µl enzyme + 495 µl Buffer F)
- Dispense this into 10 µl aliquots, label as '100X diluted' and freeze at -80°C.
- Upon use: follow description on box. Normally, dilute one of these 10 µl aliquots 30X by adding 290 µl buffer F and keep on ice until you add it to the gels.

5.2 Pre-coating of slides for gels

We recommend to use ordinary clear glass slides pre-coated with agarose. The slides for pre-coating should be grease-free; clean with ethanol if necessary (soak the slides in alcohol for about 24 hours and then wipe dry with a clean tissue).

1. Agarose solution should be at 55°C, so after melting 0.5% standard normal melting point agarose (NMP) in H₂O (e.g. 99,5 ml water + 0,5 g NMP) in the microwave, fill a suitable vessel (a Coplin jar or a narrow beaker) with warm agarose and leave in a water bath at 55°C for approximately 15 minutes.
2. Dip one slide vertically by holding it from the frosted area. The solution should cover the slide until part of the frosted area is covered.
3. Drain off excess agarose by holding the slide vertically for some seconds, then wipe the back part of the slide with a tissue and leave the slide on the bench to dry overnight.

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4. Mark the coated side with a pencil mark in one corner on the frosted end (e.g. top left) as when dry it is difficult to tell which side is coated.
5. Dried pre-coated slides can be stacked together, packed in slide boxes and stored indefinitely (years).

5.4 Media, culture conditions & stocks

Cells are cultured in complete culture medium and incubated in culture dishes or flasks in humidified atmosphere at 37° C, 5% CO₂ as described in independent SOPs for cell culturing.

5.5 Cell lines and preparation of cultures

Cells used for testing chemicals/nanoparticles etc. for genotoxicity should be used ideally after passaging them 3-4 times after establishing the culture from a frozen stock. Upon use, the cells should be recently sub-cultured or given fresh medium, to ensure that they are in a proliferating state. Monolayer cells should be used when 50-80% confluent.

5.6 Exposure


Negative and positive controls should always be included in the experiment.

The concentration range should be established with regard to expected genotoxicity, cytotoxicity, solubility in the test system and changes in pH or osmolarity. At least 3 concentrations of the test compound should be used in addition to controls. For relatively non-cytotoxic compounds the maximum concentration will be 1 mg/ml, 1 ml/ml, or 0.001M, whichever is the lowest. *Non-cytotoxic concentration should preferably be selected and applied.*

Proliferating cells are exposed to the test substance for defined period of time. It is recommended to use both short (0.5-4 hours) and long term (24-72 hours) exposure. The experiment should be repeated 1-2 times (2-3 independent repeats), and it is recommended to include replica gels in each experiment to increase the robustness of the results.

Positive controls

Positive control (s) should be selected based upon the criteria of the specific study. As reference standard for the comet assay these positive controls should be used: *H₂O₂* for detection of strand breaks and *RO 19-8022 (photosensitiser)* for detection of oxidised purines.

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Treatment of cells with H₂O₂

1. Put the slide with the embedded cells into a jar with 100 µM H₂O₂ in PBS for 5 min
2. Wash 2x2 minutes in PBS
3. Put the slides into lysis buffer in a separate jar - not together with the other treated slides!

Ro 19-8022 (photosensitiser)

Stock: Obtained from Hoffmann La Roche (contact angela.perrin@roche.com)


Dissolve in 70% ethanol at 1 mM and store in small aliquots in microtubes at -20°C. Avoid excessive light during preparation and wrap tubes in aluminum foil.

Working solution: 1-2 µM in PBS

To make 1 µM Ro 19-8022: 1 µl of 1mM solution into 999 µl of PBS.

Treatment of cells with RO 19-8022 (positive control for Fpg enzyme)

1. Any cells can be used for treatment with RO 19-8022
2. Cells are treated in suspension.
3. Spin cells following SOP for cells and pour off medium.
4. Wash cells with PBS and spin again.
5. Resuspend the cells in pellet.
6. Add 2-5 ml of cold PBS (depending on size of the Petri dish) containing Ro 19-8022 working solution to a Petri dish. Cells should be in suspension in ordinary density, so adjust volume and/or size of petri dish according to that.
Note: Avoid excessive light during preparation.
7. Place Petri dish with cells (in suspension) and Ro on ice 30 cm from a 250 W halogen lamp and irradiate for 4 min.
8. Spin the cells, remove Ro solution,
9. Wash with PBS.
10. Spin and resuspend in medium with serum. Count the cells and adjust cell density to about 1x10⁶ cells/ml..
11. Prepare slides for the comet assay and/or freeze the cells. Cells can be frozen as small amount of cells per aliquot at -80°C (e.g. 5 x 10⁵ in 0.5 ml) and thawed the day of experiment. About 5-10x10⁴ cells should be appropriate to be embed in 0.8% LMP agarose (1:3-4x) (see section 6.4.1).

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6. PROCEDURE

6.1 Before treatment:

- Ensure there are coated slides available
- Ensure that all solutions needed are available and with right temperature, including cold H₂O
- Seed cells 1-2 days before exposure
- Day before treatment, thaw 50 ml Buffer F (Fpg enzyme reaction buffer with bovine serum albumin) in fridge overnight

6.2 The morning of treatment

- Add triton-X to the lysis solution and keep it in fridge until use (at least 30 min before use).

6.3 Measurement of cell survival

AlamarBlue assay is the preferred method for measuring cytotoxicity (see HEL16T008 AlamarBlue assay). Other method can be applied, like Relative Growth Activity (see HEL11T002).

6.4 Comet assay procedure for 12 gels system:

6.4.1 Cells embedding

- 1) Exposure of the cells
- 2) Before the end of exposure, melt the agarose carefully in the microwave oven and leave on a heating block at about 37-40°C. Make sure the agarose is at the right temperature before adding it to the cells.
- 3) At the end of exposure, remove the test compound and wash with PBS (except for nanomaterials)
- 4) Take appropriate number of cells (adherent cells needs to be trypsinized) into a 96 well plate. About 5-10 x10⁴ cells should be appropriate, which will give approx 200-300 cells/5µl LMP agarose gel).
- 5) Embedding the cells in agarose (1:3-4x agarose).
Add agarose into the wells with cells, and mix properly.

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- 6) Add 5-10 μ l drops of gels on the slide placed on top of cooled metal blocks. The maximum number of gels pr slide is 12. You can use the multichannel pipettes to make the 12 gels per slide.

Note: Work quickly as the agarose polymerize fast

6.4.2 Lysis

Place the slides in lysis solution in a (vertical) staining jar.

1. Leave at 4°C for at least 1 h. Can also stay overnight (24 hours) or longer on lysis solution.


6.4.3 Enzyme treatment

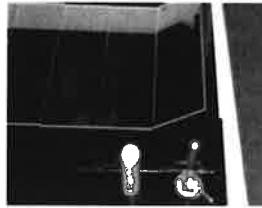
Fpg (formamidopyrimidine DNA glycosylase), EndoIII (endonuclease III),

1. Get enzyme from freezer (keep on ice)
2. Wash slides 2x10 minutes in Buffer F
3. Meanwhile, dilute enzyme in Buffer F according to procedure described above. Keep on ice until use.
4. Add about 200 μ l of enzyme solution (or buffer alone, as control) on top of each slide, and cover with thin plastic foil
5. Put slides into moist box (prevents desiccation) and incubate at 37°C for 30 min with Fpg enzyme or for 45 min with Endo III enzyme
6. It is recommended to place slides into fridge for 5-10 minutes for the gels to stabilize just to avoid losing gel drops after the incubation with enzymes.

6.4.4 Alkaline treatment (20 min)

1. Place slides on platform in the tank, forming complete rows (gaps should be filled with blank slides). It is also recommended to place one slide at the end of the tank (see picture below) to avoid moving of slides when you fill the tank with electrophoresis solution
2. Put the tank into the cooling incubator.
3. Gently add 520 ml cold electrophoresis buffer into the tank until slides are immersed just enough to be covered. Make sure that tank is leveled. Leave for 20 min to equilibrate.

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6.4.5 Electrophoresis (20 min)

1. Put lid with cables onto the tank – red on red and black on black electrode. The tank should still be in the cooling incubator.
2. Start the power supply and run the electrophoresis at 25 V for 20 min.

If there is too much electrolyte covering the slides, the current may be so high that it exceeds the maximum - so set this at a higher level than you expect to need. If 25 V (1.2 V/cm) is not reached, remove some solution by a pipette
Normally the current is around 300 mA but this is not crucial.

6.4.6 Neutralisation

1. After electrophoresis, gently move the slides into jars with cold PBS and wash for 5 minutes
2. Thereafter wash the slides in jar with cold ddH₂O for 5 minutes
3. Dry the slides horizontally laying at room temperature (normally overnight)

6.4.7 Staining for scoring

The slides must be dried before staining. Preferably we use SYBr Gold (0.1 µl/ml in TE buffer) as described above, but DAPI (1 µg/ml DAPI solution in distilled H₂O) may also be used. Both staining solutions are stored in the -20 freezer.

Use 50 µl and spread it evenly in small droplets to cover the whole 12 gels and cover with preferably large cover slip. Do scoring in the fluorescence microscope.

7. CHRONOLOGICAL PLANNING

Day 0 cell inoculation for experiment for adherent cells (for suspension cells start at Day 1)

Day 1 or 2 Treatment with tested compound

Day 1, 2, or 3 (depending on length of exposure) – the day of end of treatment: test performance

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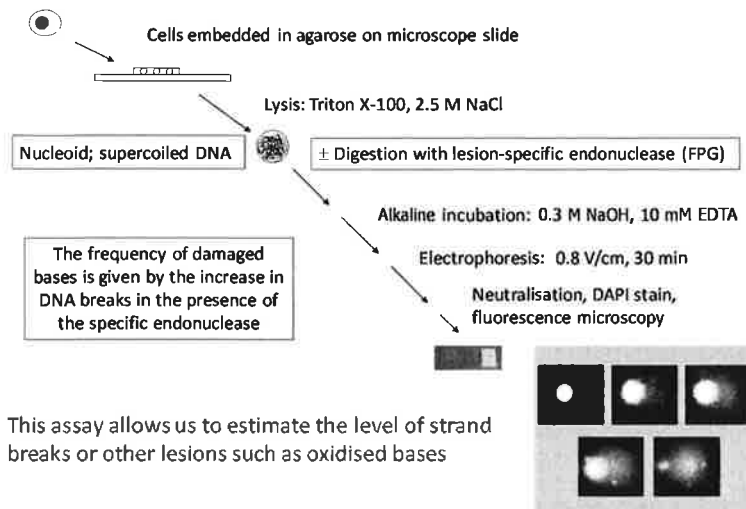
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Any day after gels are dried: Scoring and analysis

The comet assay (with lesion-specific enzymes) - principle



Comet Assay – a quick overview:

1. Expose the cells for a certain period of time
2. Embedding of the cells into LMP agarose and drop gels on slides
3. Positive controls (e.g. H₂O₂, RO+/- cells)
4. Lysis
5. Enzyme treatment
6. Alkaline treatment
7. Electrophoresis
8. Neutralization
9. Drying
10. Staining
11. Scoring
12. Data analysis

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8. EVALUATION/DATA ANALYSIS, DATA SHEETS AND DATA PRESENTATION

Quantification

Computer image analysis

Slides are analyzed by fluorescence microscope using image analysis system Comet assay IV (Perceptive instruments) or Metasystem by scoring of 50 cells per gel (2 gels per concentration). These are software which, linked to a closed circuit digital camera mounted on the microscope, automatically analyses individual comet images. The programs are designed to differentiate comet head from tail, and to measure a variety of parameters including tail length; % of total fluorescence in head and tail; and 'tail moment'. We use % **DNA in tail** as the most informative parameter.

Alternatively comets could be evaluated by visual scoring (100 cells per gel). During one study, the same method of analysis should be used.

Calculate the median of each gel. Then the **mean of medians** (for each concentration) is used to represent the result. To evaluate significance of the results, statistical methods such as Mann-Whitney and Kruskal Wallis or alternatively ANOVA can be used. Use a statistical software, and level of significance is set to 0.05.


9. INTERPRETATION OF RESULTS

A result is classified as cytotoxic if the viability was reduced at least 20% from the viability of control culture. Results should always be compared with survival measurements to distinguish true DNA damage from apoptosis or necrosis.

Criteria for determining a genotoxic effect are:

- a concentration-related induced DNA damage or at least genotoxic response in one concentration with cell viability more than 60% compared to control
- reproducible response

Biological relevance of the results should be considered first. A test substance, for which the results do not meet the above criteria, is considered non-genotoxic.

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Positive results in an *in vitro* DNA damage test on human or mammalian cells indicate that the test substance is potentially genotoxic to humans. A positive concentration response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce DNA damage in the cultured mammalian cells used.

10. RECORDING OF TEST PERFORMANCE AND REPORT

Performance of the test is recorded on Test performance sheet template where all procedure is documented from start to the end. The sheet must be dated and signed by person performing the test.


After all results are collected from at least one experiment and one repeat, data are analyzed and report needs to be written. The report should contain details of the test performance including test conditions, information on tested substance and controls, concentrations used, critical points and deviation, if any, data evaluation and the interpretation of results. Report on Quality control should be included.

11. CRITICAL PHASES

- Exposure of cells with compound – be sure that right concentration is included into correctly marked vial/well with cells
- Cells placed at correct spot on the slide
- Enzyme treatment - placing enzyme and Buffer F on the correct gel position

12. IMPORTANT ASPECTS

- Work with cells must be performed in sterile conditions in laminar box.
- The temperature of the incubator must be set to 37°C, and the temperature must be monitored and recorded.
- Density (confluence) of cells during cultivation
- Treatment of cells is critical – concentration preparation, treatment time and washing
- Cell processing before lysis must be on ice and/or rapidly performed
- Enzyme treatment (temperature, moisture, length of incubation)
- Electrophoresis conditions (Voltage, temperature, length of electrophoresis)

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Corrections from version 4 to version 5: Minor changes in H₂O₂ dilutions and Evaluation/data analysis