MEDETOX

Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic

(LIFE10 ENV/CZ/651)

COMET ASSAY
(SINGLE CELL GEL ELECTROPHORESIS)

SUMMARY

1. PRINCIPLE OF THE METHOD

In the alkaline version of Single Cell Gel Electrophoresis (SCGE = Comet assay) the DNA is denaturated and next during the electrophoresis DNA migrates (as a polyanion) to anode. For visualization of DNA damage, observations are made of Ethidium Bromide stained DNA using a fluorescent microscope. A Comet assay analyser Lucia G software linked to a CCD camera is used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the lenght of DNA migration and the percentage of migrated DNA.

2. LABORATORY EQUIPMENT

a. Electrophoresis

Source of voltage: model OSP-300, Owl, USA

Horizontal gel electrophoresis apparatus: model A-5, Owl, USA

b. Image analysis

Fluorescent microscope VANOX – BHS, Olympus, Japan

CCD camera UDS CCD – 1300B

Software - Comet assay analyser Lucia G , LIM, Prague

3. SAMPLE PREPARATION

3.1 A549 cells incubation and cell harvesting

A549 cells are grown at 37°C in 25 cm^2 flasks with 10 ml Dulbecco´s modified Eagle´s medium (with 10% FBS) to 70-80% confluency. Then we treat cells with tested samples for 4h and 24h at 37^0C (in medium with 1% FBS). After 4/24 hours we harvest the cells.

1) Pour off the medium from the cultivation flask and wash twice with 1 ml of trypsin (add 1 ml of trypsin, pour off, add 1 ml of trypsin and pour off).
2) Incubate the cells at 37°C for 5-10 minutes.
3) Add 1 ml of cold PBS and replace the cell suspension with Pasteur pipette to marked microtubes. Protect from light and store at fridge until the analysis (Comet assay - standard alkaline version and modified protocol for oxidative damage detection).

A549 cells incubation

Dulbecco´s modified Eagle´s medium (DMEM) with 1.0 g/L glucose, with pyruvate, without L glutamine (LONZA BE12-707F/12)
Fetal bovine serum (FBS), EU standard (LONZA DE14-801F/12)
L-glutamine (200 mM) (LONZA BE17-605E)
Gentamicin sulfate 10 mg/ml (LONZA 17-519L)
Trypsin/EDTA (1x) contains 0.5 g/L trypsin 1:250 and 0.2 g/L Versene® (EDTA) (LONZA BE17-161E)
DMSO (Sigma D2650)

3.2. Cell concentration calculation

1) Drop 50 µl of the suspension on a slide with a Bürker counting chamber, cover with a coverslip and wait 2 – 5 minutes
2) Count cells under the microscope in **16 big squares** (= 0.064 mm3 = **0.064 µl**)
3) Calculate the cell concentration in 1 ml (**X**):

$$\mathbf{X} = \frac{\mathbf{N\ (number\ of\ cells)}}{\mathbf{0.064}} \mathbf{\ x\ 1000\ (x\ 2)}$$

x 2 if the suspension is diluted 1 : 1 for the Trypan blue viability test

3.3. Trypan blue viability test

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Trypan blue
0.2% solution in PBS

Procedure

1) Mix 50 µl of the sample with 50 µl 0.2% solution of trypan blue in PBS.
2) Drop 50 µl of the suspension on a slide with a Bürker counting chamber.
3) Cover the slide with a coverslip and count cells under the microscope.
4) Determine number of viable cells (cells with a clear cytoplasm) and nonviable cells (cells with a blue cytoplasm).

4. CHEMICALS AND SOLUTIONS

(all from Sigma Aldrich, except for NaCl – Lachema)

Na_2EDTA (ethylenediamine-tetraacetic acid, disodium salt, dihydrate)
Triton X-100
TRIS (trisbuffer hydrochloride, trizma hydrochloride)
NaOH
NaCl
DMSO
Ethidium Bromide

STOCK SOLUTIONS

Phosphate Buffered Saline – PBS (Ca^{++} and Mg^{++} free)

pH 7.4, store at 4°C

NaCl	8.00 g
KCl	1.44 g
KH_2PO_4	0.24 g
Na_2HPO_4	1.44 g

Add 800 ml dH_2O, adjust pH to 7.4 and adjust volume to 1 l.

Lysing stock solution

pH 10, store at room temperature

2.5 M NaCl	146.40 g
100 mM EDTA	37.20 g
10 mM Tris	1.20 g

adjust pH to 10 approx. 8.00 g NaOH

add 800 ml dH_2O, adjust pH, bring to 1 l, autoclave.

Buffer for the alkaline electrophoresis (300 mM NaOH/1 mM EDTA)

Always prepare fresh:

1) 10 N NaOH
2) 200 mM EDTA (7.4448 g EDTA in 100 ml dH_2O)
   Store max. 2 weeks!!

Neutralization Buffer (TRIS)

pH 7.5, store at room temperature – pH is not stable!

0.4 M Tris 48.5 g

adjust to 1000 ml with dH_2O

adjust pH to 7.5 with concentrated HCl

Staining stock solution

0.1% solution of Ethidium Bromide (0.1 g in 100 ml dH_2O), store at room temperature

LMP (low melting point) agarose (red)

(Amresco, USA: Agarose II for low –gel applications)

Store at 4°C, max. 2 months.

0.75% agarose: 0.15 g in 20 ml PBS

1) heat on the magnetic stirrer until near boiling and the agarose dissolves
2) put aside for a minute, heat again until near boiling
3) repeat the step 2)
4) **aliquot 160 µl** to red microtubes

NMP (normal melting point) agarose (blue)

(Sigma: Agarose Type I, low EEO)

Store at 4°C, max. 2 months.

0.75% agarose: 0.15 g in 20 ml PBS

1) heat on the magnetic stirrer until near boiling and the agarose dissolves
2) put aside for a minute, heat again until near boiling
3) repeat the step 2)
4) **aliquot 250 µl** to green microtubes

Slides coating with agarose

- pour 1% water solution of agarose into the high and narrow beaker
- dip the slide, wipe underside of slide to remove the agarose and lay the slide in a tray on a flat surface to dry, then air dry at 60°C in a stove
- mark the slide side without agarose layer with a diamond

For the experiment prepare:

A) LYSING SOLUTION:

1) fresh Triton X-100 dilute 100x in lysing stock solution (mix on the magnetic stirrer)
2) add 17 ml of 10% DMSO
3) mix and cool in a fridge at 4°C!

Calculation:

1 photographical tray = 10 slides = 168.5 ml of the solution

- 1.5 ml of Triton X-100
- 150 ml of lysing stock solution
- 17 ml of 10% DMSO (2 ml DMSO + 18 ml dH_2O)

2 photographical trays = 20 slides = 337 ml of the solution

- 3 ml of Triton X-100
- 300 ml of lysing stock solution
- 34 ml of 10% DMSO (4 ml DMSO + 36 ml dH_2O)

2 photographical trays + 1 small photographical tray (24 slides)= 421.25 ml

- 3.75 ml of Triton X-100
- 375 ml of lysing stock solution
- 42.5 ml of 10% DMSO (5 ml DMSO + 45 ml dH_2O)

B) ELECTROPHORESIS BUFFER

NaOH Stock solution	60 ml
EDTA Stock solution (200 mM)	10 ml
dH_2O	1930 ml

Total volume is 2 L.

C) ETHIDIUM BROMIDE

Stock solution of Ethidium Bromide	50 µL
dH_2O	900 µL

D) OTHER EQUIPMENT

- microscope slides with agarose + mechanical pencil
- coverslips 22 x 22 mm
- LMP agarose
- NMP agarose
- micropipettes with sterile tips
- pack-ice, polystyrene box
- alarm clock

5. WORKING PROTOCOL

5.1. Standard alkaline version

(detection of alkali-labile sites, single and double strand breaks in DNA)

1) Melt NMP and LMP agarose at 90°C and then put LMP into water bath (37°C).

2) Drop 110 µl of NMP agarose (min. 60°C warm) on the base slide (pre-heated at 50°C) and immediately place the coverslip on it. Put the slides on ice packs until agarose layer harden (5 – 10 minutes). Prepare 2 slides from each microtube with agarose.

Next steps perform under the yellow light !

3) Add 20 µl of the cell suspension (conc. 10^6/ml) to the microtube with 150 µl LMP agarose and mix properly.

4) Gently slide off the coverslip and add another agarose layer on the base slide = 75 µl LMP agarose with cells, cover with the coverslip and put on ice packs until this layer harden (5 minutes, prepare 2 slides from 1 microtube).

5) Carefully remove the coverslip and slowly lower slide into cold freshly made Lysing solution. Protect from light and refrigeratefor a min. 1 hour (max. 24 hours).

6) Remove slides from the Lysing Solution a lower them into alkaline Electrophoresis Buffer. Let slides sit in this buffer for 20 minutes to allow for unwinding of the DNA (**unwinding** = differnt period according to the cell type used in the experiment).

7) Fill the buffer reservoir with freshly made Electrophoresis buffer until the liquid level completely covers the slides. Turn on power supply to U= 36 V (voltage value depends on the box format) and adjust the current to 300 mA by raising or lowering the buffer level (amperage value is regulated in relation to the buffer volume).

8) Place slides on the horizontal gel box near anoda end.

9) Electrophorese the slides for 30 minutes at 4°C (16°C).

10) Turn off the power. Gently lift the slides from the buffer and place on a drain tray. Coat the slides with Neutralization Buffer (1 ml), let sit for at least 5 minutes. Drain slides and repeat two more times.

11) Drain slides, drop 100 µl Ethidium Bromide solution on the slide and cover with a coverslip. Incubate for 8 minutes at room temperature.

12) Slide off the coverslip and dip in chilled distilled water (3 x 5 minutes) to remove excess stain. Then drop 100 µl of dH_2O and place the coverslip over it. The slides are scored immediately or dried before staining.

Store slides for 1 – 24 hours until scoring in a fridge with humid atmosphere.

Slide draining – slides for later evaluation

A) Fixation and draining

- after the electrophoresis wash twice with Neutralization Buffer (á 1 ml, 5 minutes)
- methanol fixation – keep the slides for 15 minutes in cold 100% methanol (100 µl) for dehydration.
- wash twice with dH_2O (1 ml, 5 min.)
- air dry the slides overnight

B) Rehydration and Staining

- Rehydrate the slides with chilled distilled water for 1 hour and stain with Ethidium Bromide as in step 11, cover with a fresh coverslip.

5.2. Modified protocol for oxidative damage detection

1) Prepare slides according to standard protocol.
2) Lower slides into the Lysing solution for 60 minutes (slides for Endo III and ½ of controls) and for 75 minutes (slides for FPG and ½ of controls).
3) After 60 minutes – wash slides for Endo III and ½ of controls with Endobuffer (4°C) – 3x5 min.
4) Add 40 µl of Endo III solution (40 µl endobuffer to control samples), cover with a coverslip and incubate in thermostat for 45 minutes at 37°C.
5) After 75 minutes – wash slides for FPG and ½ of controls with Endobuffer (4°C) – 3x5 min.
6) Add 40 µl of FPG solution (40 µl endobuffer to control samples), cover with a coverslip and incubate in thermostat for 30 minutes at 37°C.
7) Lower all slides into alkaline Electrophoresis Buffer. Let slides sit in this buffer for 20 minutes to allow for unwinding of the DNA.
8) Electrophoresis 30 min, 36 V, 300 mA.
9) Next steps perform according to standard protocol.

Positive control: 100 µM H_2O_2

Endobuffer preparation:

KCl (g)	7,45	14,90	29,80	37,25	44,70
Hepes (g)	9,53	19,06	38,12	47,65	57,18
EDTA (g)	1,46	2,92	5,84	7,30	8,76
BSA (g)	0,20	0,40	0,80	1,00	1,20
Volume (ml)	1000	2000	4000	5000	6000

Enzyme preparation procedure:

Endo III (z r.1998, A. Collins, crued extract)
Mix 2 µl of extract and 1000 µl of Endobuffer with BSA, aliquot 250 µl to microtubes and store in a freezer. Dilute 1:1 (250 µl + 250 µl of endobuffer with BSA) before each application.
40 µl is dropped on each slide = the solution is for 12 slides, the whole volume for 48 slides. Keep the residual of the solution in a freezer.

FPG (z r.1998, A. Collins, crued extract)
Mix 2 µl of extract and 600 µl of Endobuffer with BSA, aliquot 50 µl to microtubes and store in a freezer.. Dilute 1 : 10 (50 µl + 450 µl endobuffer with BSA) before each application.
40 µl is dropped on each slide = the solution is for 12 slides, the whole volume is for 144 slides. Discard the residual of the solution.

6. EVALUATION OF DNA DAMAGE

Prepare 2 slides of each sample. Evaluate 50 cells per one slide = 100 cells per sample. The DNA damage is expressed in %„tail DNA“.

7. HAZARDOUS CHEMICALS

Handle Ethidium Bromide with adequate precaution as it is known carcinogen. Always use gloves and avoid aspiration during weighting.
Manipulate with NaOH and HCl only in digestore.