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Standard Operating Procedure

Title	***HTS Comet Assay with and without FPG - 20 wells***
Subtitle	Comet assay with and without FPG using Trevigen 20-well slides
NANoREG Work package/task:	WP5 / Task 5.06
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Date finalised	January 2016
Document name	NANoREG D5.07 SOP 11 HTS Comet Assay with and without FPG - 20 wells
Key words:	

Version	***Date***	***Reason of change***
1	2017/07/10	Harmonisation according NANoREG template for SOPs
2		
3		
4		

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Comet assay with and without FPG using Trevigen 20-well slides SOP - Institute of Experimental Medicine - NANoREG Task 5.6

The protocol is based on the SOP for the comet assay provided to the NANoREG project partners by NILU (HEALTH EFFECTS LABORATORY SOP code HEL11T005, v.2, Comet assay with and without repair enzymes) and manufacturer's instructions for the Trevigen 20-well slides (CometSlide™ 20 Well, Trevigen, Cat No 4252-02K-01)

- HEALTH EFFECTS LABORATORY (2013). Standard Operating Procedure Comet assay with and without repair enzymes. Code HEL11T005, v2.
- Trevigen Instruction (2016). CometAssay® HT, Reagent Kit for Higher Throughput Single Cell Gel Electrophoresis Assay Catalog # 4252-040-K.

Valid from: January 2016

Level of throughput: middle throughput

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Chemicals, Reagents, and Media

Bovine Serum Albumin (Sigma, A5418)

EDTA (Sigma, E4884)

Fetal Bovine Serum (FBS) (Sigma, F6178)

FPG (provided by Dr. Andrew Collins) – aliquots of 30 x dilutions in FPG buffer with BSA and glycerol

Glycerol (Sigma, G5516)

HCl (Sigma, 435570)

HEPES (Sigma, H3375)

Hydrogen peroxide solution (Sigma, H1009)

KCl (Sigma, P4504)

KOH (Sigma, P5958)

Low Melting Point (LMP) Agarose (Trevigen, 4250-050-02)

Lysis Solution (Trevigen, 4250-050-01)

Minimum Essential Medium (MEM) with HEPES and Glutamax™-I (Gibco, 42360-024)

NaOH (Sigma, S5881)

Phosphate Buffered Saline (PBS) (Ca++, Mg++ free) (Gibco, 10010-015)

SYBR ® Safe DNA gel stain 10,000X concentrate in DMSO (Invitrogen, S33102)

Trizma® base (Sigma, T1503)

Trypan Blue Solution (Gibco, 15250061)

Trypsin-EDTA solution: (Sigma, T4049)

Cell culturing

A549 cells are cultured in MEM with HEPES and Glutamax supplemented with 10% FBS in humified atmosphere at 37°C, 5% CO_2. Cell are passaged every 2-3 days and kept under 90% confluency.

Cell treatment

A549 cells are exposed to the tested substances or media only (negative control) for a defined period of time (24h).

Comet Assay Procedure

Solutions

Alkaline Unwinding Solution	50 mL
NaOH pellets	0.4 g
200mM EDTA, pH 8	250 µL
ddH_2O	49.75 mL
Prepare fresh (before each electrophoresis run); keep at room temperature.	

Alkaline Electrophoresis Solution	1 L
NaOH pellets	8 g
500mM EDTA, pH 8	2 mL
ddH_2O	998 mL
Prepare fresh (before each electrophoresis run); cool down to 4°C.	

TE Buffer, pH 7.5	30 mL
500mM EDTA	60 µL
50mM Tris-HCl, pH 7.5	6 mL
ddH_2O	23.94 mL
Store at room temperature.	

SYBR Green Staining solution	30 mL
SYBR Safe DNA Gel Stain	3 µL
TE Buffer, pH 7.5	30 mL
Store at 4°C, use the solution within 14 days.	

FPG Buffer (10X)	100 mL
HEPES	9.53 g
KCl	7.45 g
EDTA	1.46 g
BSA	0.22 g
KOH	up to pH 8
ddH_2O	100 mL
Aliquots can be frozen at -20°C (before freezing, add glycerol to the final concentration of 10 %).	

A. Preparations

1. Pre-cool PBS (Ca^{2+}, Mg^{2+} free) to 4°C.
2. Pre-cool lysis solution to 4°C.
3. Pre-warm Trevigen slides to 37°C on lab hot plate.
4. Pre-cool ddH_2O (1L) for electrophoresis solution, electrophoresis cooling pads and electrophoresis tank to 4°C.
5. Thaw FPG buffer (10 X concentrated), keep at 4°C.
6. Melt aliquoted LMP agarose, keep at 37°C.

B. Cell harvesting

1. Remove media.
2. Wash cells twice with PBS (Ca^{2+}, Mg^{2+} free).
3. Trypsinize cells for 3 min at 37°C.
4. Add complete cold cell culture medium, count cells and determine cell viability using trypan blue.
5. Centrifuge cells at 1000 RPM, 4°C, for 5 min.
6. Remove supernatant, wash cells in cold PBS (Ca^{2+}, Mg^{2+} free).
7. Resuspend cells in cold PBS (Ca^{2+}, Mg^{2+} free) at a concentration of 1 x 10^5 cells/mL in cold PBS. Keep cells on ice until embedding into agarose.

C. Embedding cells in agarose

1. Mix cell suspension (flick with fingers).
2. Quickly add cells (1 x 10^5/mL in PBS) into melted LMP agarose (1:10) at 37°C, mix with a pipet.
3. Transfer 30µL of cells/LMP agarose mixture to appropriate wells of a 20-well slide (at 37°c on a lab hot plate) and spread the mixture all over the well with a pipet tip.
4. Move the slide row with gels away from the hot plate (to prevent drying and shrinkage of the gels).
5. After filling all wells, place the slide on pre-cooled pads (to fix the LMP agarose) - ensure an even surface.
6. Place all slides into the refrigerator (cool, dark) for 10 min.

C. Lysis

1. Immerse slides in pre-chilled lysis solution and lyse them for 1 h at 4°C.

D. Enzyme treatment

1. Thaw 50ml FPG buffer with BSA. Put aside 5 ml.
2. Wash slides twice in FPG buffer without BSA and once with FPG buffer with BSA, at 4°C, 8-10 min each.
3. Defreeze and dilute FPG: mix aliquots of 10µL with 990µL FPG buffer with BSA.
4. Dab off excess liquid with tissue, and pipet 40µL of diluted FPG or FPG buffer (as control) onto the the gels. Cover the whole slide with parafilm.

5. Incubate slides for 30 min at 37°C.

E. Unwinding

1. Place slides at 4°C for 5 min, then remove parafilm.
2. Drain excess of FPG/FPG buffer.
3. Immerse slides in freshly prepared Alkaline Unwinding solution for 20 min at room temperature in dark.

F. Electrophoresis

1. Insert slides into a tray filled with freshly prepared pre-cooled electrophoresis solution. Fill all empty positions in the tray (using blank slides). Cover slides with glass slide platform.

2. Run electrophoresis at 21V (= 1 V/cm) for 30 min (current at approximately 250mA).

G. Neutralization and Drying

1. Gently drain the excess of electrophoresis solution.
2. Wash slides twice in TE buffer and once in ddH_2O, each for 5 min.
3. Immerse the slides for 15 min in 70% ethanol, then for 15 min in 100% ethanol.
4. After drying, store samples and at a dry place (room temperature) until scoring.

H. Staining

1. Pipet 50µL of SYBR green solution on each well (20-well slides) in dark and incubate for 30 min.
2. Remove the SYBR Green solution and wash slides in ddH_2O for 5 min

I. Image analysis

1. Automated slide-scanning system (Metafer MetaCyte with CometScan) is used for unattended scanning of the slides. The scan is performed under fluorescent conditions using the 20× objective. Once a comet is accepted by the system, its intensity profile is automatically analyzed and head and tail of the comet are determined based on the intensity levels. The image gallery is checked visually and inappropriate objects (false positive, false negative, background objects) are discarded. Then 50 comets per sample are randomly selected for calculation of the median of the percentage of tail DNA.