SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Report on the final harmonised SOPs used to propose amendments to the existing OECD TGs

DELIVERABLE 5.1

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Abstract

The aim of task 5.1 was to assess selected OECD test guidelines and other well developed test methods for their applicability to hazard assessment of engineered nanomaterials (ENMs) and identify if any adaptations were necessary to reduce uncertainty in human risk assessment approaches. In this task, round robin (RR) interlaboratory comparison exercises were employed to evaluate the reproducibility and transferability of the test systems, to support the activities of the OECD working Party on Manufactured Nanomaterials (WPMN) and other standardization bodies.

Available standard operating procedures (SOPs) were critically evaluated to determine their suitability for ENMs hazard assessment and the need for potential assay adaptations. Two RRs were performed each with 4 SOPs: i) colony forming efficiency (CFE) assay, ii) comet assay (CA) and mammalian gene mutation HPRT assay for iii) adherent and iv) suspension cells), while one RR was performed using the micronucleus (MN) assay. A total of 5 ENMs were tested, TiO_2 and ZnO in RR1 and CuO, multiwalled carbon-nanotubes (MWCNTs) and Tungsten in RR2. Results were compiled and compared after RR1 and the SOPs amended accordingly before performing RR2. Data templates for data and metadata entry into the RiskGONE instance of the eNanomapper database were developed and the resulting data was uploaded.

Amended Test Guidelines (TGs) will be prepared for the test methods CFE, MN and CA, to be submitted as SPSFs to the OECD WPMN for onward development and validation, while results showed only minor adjustments are needed for the HPRT assay and thus that there is no need to amend this TG. Based on the accompanying literature search, several manuscripts on the interference of ENMs with test methods are in progress and an approach to test for interference has been implemented in the CA SOP, as well as for the colorimetric assays.

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List of Abbreviations

AB – Alamar Blue

BSA - Bovine serum albumin

CA – Comet assay

CFE – Colony Forming Efficiency

CBMN - Cytokinesis-block Micronucleus

ECACC - European Collection of Cell Cultures

ENM – Engineered Nanomaterial

Fpg - Formamidopyrimidine DNA Glycosylase

HPRT – HPRT (hypoxanthine phosphoribosyl transferase) gene mutation

INT - Interference (control)

MF - Mutant frequency

MMC - Mitomycin-C (positive control)

MN – Micronucleus

NC – Negative Control

OECD - Organisation for Economic Co-operation and Development

PC – Positive Control

RPD - Relative population doubling

RR – Round Robin (interlaboratory testing)

SB – Strand Breaks

SD - Standard Deviation

SOP – Standard Operating Procedure

SPSF- Standard project submission form

TGs - OECD Test guidelines

WMN - the Working Group of National Co-ordinators of the TGs programme

WPMN – Working Party on Manufactured Nanomaterials

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1. Introduction

This deliverable is the outcome of the RiskGONE task 5.1 *Evaluation and adaptation of in vitro safety testing OECD test guidelines (TGs) to enable ENM risk governance*. It aims to critically evaluate the nano-specific applicability of a sub-set of existing *in vitro* test methods for hazard assessment, and to identify any required adaptations to existing TGs. To identify the nano-specific adaptations, round robin (RR) pre-validation exercises (interlaboratory comparisons) were conducted to ensure that the TGs are appropriate for engineered nanomaterial (ENM) safety assessment.

Following the two rounds of RR testing using 2 different sets of ENMs, draft guidelines for harmonised test methods are being prepared to ensure that end-users can easily apply the ENM-adapted tests in their own laboratories. These draft guidelines will undergo final approval by the RG pre-council under WP2 to ensure they are readily accepted by the OECD WPMN as SPSFs for further validation and eventual acceptance by regulatory bodies. The final harmonised standard operating procedures (SOPs) will also be used to propose amendments to the existing OECD TGs, both in support of the "Malta project" (phase 2 efforts) and NANOHARMONY, extending the reach of these projects by contributing to ongoing proposed revisions of ECHA's annexes for nano-substances under REACH.

The deliverable 5.1 generated through this task includes annexes with the harmonized test guidelines as SOPs and will facilitate formal standardisation of the test systems, resulting in the provision of sustainable solutions for integrating the scientifically validated nano-specific applicability of the test methods into regulatory use, beyond the lifetime of the project. The outcome of this work will be published in several peer-reviewed journals and disseminated at conferences and scientific meetings.

Interference of ENMs with the testing methods is of major concern and has been discussed by all partners specifically within the context of assays being utilised in task 5.1. A systemic literature review has been launched to create more knowledge on reported and potential interference and corresponding mitigations. A big effort was also deployed to develop a common template for data collection which has been developed and updated several times to suit the specific needs of each method. Challenges have been faced during the RRs as a result of Covid-19 related delays in deliveries of e.g., reagents, cells, culture medium and disposables as well as lockdowns and thus closure of laboratories and staff absences as a result of self-isolation periods.

2. Test methods and experimental design of the round robins

2.1 Test methods for the round robins

Endpoints selected to focus upon in this work addressed cytotoxicity, genotoxicity, and mutagenicity. The most representative test methods for each endpoint were identified. Table 1 below highlights the endpoints and corresponding *in vitro* test methods planned for critical evaluation and from those the four that already have the most advanced progress towards evaluation of nano-specific adaptations were selected for optimization of SOPs and to perform RRs in order to identify any additional tweaks or adjustments needed to the SOPs.

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Table 1: *Test guidelines (TGs) planned to be verified, optimized and pre-validated for engineered nanomaterials (ENMs).*

Endpoint	OECD TG	Description
Cytotoxicity	TG487 TG432	Relative population doubling (TG487) Colony forming efficiency (CFE) *In vitro* 3T3 NRU Phototoxicity Test
Genotoxicity	TG487	Micronucleus assay New *in vitro* guideline for comet assay to detect strand breaks and specific deoxyribonucleic acid (DNA) lesions
Mutagenicity	TG476	Mammalian cell gene mutation test
Cell Transformation	Guidance documents 214 & 231	Cell transformation assay

Table 2 below shows the selected test methods for the RRs (RR1 and RR2) as well as the contributing partners for each test method.

Table 2: *Test guidelines (TGs) selected to be verified, optimized and pre-validated for ENMs during the RRs, and contributing partners. NILU: Norwegian institute for air research; KU: KU Leuven; IMI: Institute for medical research and occupational health; ANSES: French Agency for Food, Environmental and Occupational Health & Safety; SU: Swansea University; LIST: Luxembourg Institute of Science and Technology; INIC: Iran Nanotechnology Initiative Council; UiB: University of Bergen.*

Endpoint	OECD TG	Description	Partners RR1	Partners RR2
Cytotoxicity	TG432	Colony forming efficiency (CFE)	NILU, KU Leuven, UiB, IMI	NILU, KU Leuven, UiB, IMI
		Colorimetric and fluorometric assays: AlamarBlue, MTT, TBA and WST-8	NILU, KU Leuven, ANSES	NILU, KU Leuven
Genotoxicity		New *in vitro* guideline for comet assay to detect strand breaks and specific deoxyribonucleic acid (DNA) lesions	NILU, KU Leuven, ANSES, INIC	NILU, KU Leuven, LIST, SU
	TG487	Micronucleus assay	SU, ANSES, NILU	
Mutagenicity	TG476	HPRT Mammalian cell gene mutation test	SU, NILU	SU, NILU, KU Leuven

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2.2 Experimental design of the round robins

The TGs identified in Table 2 above were assessed for hazard characterization of ENMs by interlaboratory comparisons. The available SOPs (e.g., from previous projects such as PATROLS, NanoReg etc.) were critically evaluated to determine their suitability for ENMs hazard assessment or if any protocol adaptations were needed. The aim of the RRs was to provide pre-validation data to support future amendments/annexes to the current OECD TGs for the selected test methods or upon which to develop new TGs (CFE, CA).

Two series of RRs were performed for three of the *in vitro* assays for adaptation purposes, to ensure that the assays are fit for ENMs hazard assessment and that they can report appropriately the outcomes, while for the MN assay, only one RR was planned and performed (in progress). The tests' reproducibility, transferability, and inter-laboratory variability[1], which are essential requisite steps to facilitate standardisation and integration into test guidance, were also evaluated. For consistency, all partners purchased the selected cell lines utilised from the same provider (A549 ATCC CCL-185, V79-4 ATCC CCL-93, TK6 ECACC 13051501).

Stakeholders from different sectors, including academia (SU, UiB, KU Leuven) and government and non-government research laboratories and research institutes (NILU, LIST, IMI, ANSES, INIC) undertook the RRs exercises on the CFE assay for cytotoxicity testing, the enzyme-modified version of the CA for genotoxicity (both DNA strand breaks and oxidized base lesions), on the mammalian HPRT gene mutation test for mutagenicity and on the MN assay for chromosomal damage. The SOPs included negative and positive controls specific for each endpoint and several concentrations for each of the selected ENMs (see Tables 3 and 4 for details). Results were compiled after RR1 and required changes in the SOPs identified and implemented into the updated version of the SOPs before RR2. Regular teleconferences were undertaken to ensure effective communication and progress. For all test methods, data and metadata collection templates were tailored for data entry and upload to the RiskGONE instance of the eNanoMapper database which is available here:

(https://search.data.enanomapper.net/projects/riskgone/login/).

2.3 Selected ENMs for the round robins

For the RRs, OECD reference ENMs (defined in WP4) falling into specific groups according to their physico-chemical characteristics were selected. The ENMs agreed for use in the *in vitro* experiments are summarized in Table 3 below. Note that the ERM numbers correspond to the unique RiskGONE ENM identifiers, as defined by the NanoCommons research infrastructure project which established the European Registry of Materials (van Rijn et al., 2021; preprint of submitted article available) as a means to increase the interoperability of datasets related to individual ENMs.

For RR1, ZnO from Sigma (ERM00000063) and TiO_2 from JRC (ERM00000064) were used. The corresponding ZnO from JRC and TiO_2 from Sigma could not be included due to challenges with the dispersion protocol caused by rapid sedimentation of these materials. For RR2, CuO (ERM00000088), Nano Tungsten (ERM00000089) and MWCNTs (ERM00000325) were included. At least four concentrations of each ENM (1- 100 µg/mL) were tested in each method. The hazard testing of ENMs

[1] Reproducibility: the extent to which consistent results are obtained when an experiment is repeated.
Transferability: transfer of knowledge and skills to perform test to other laboratories.
Inter-laboratory variability: consistency of results between laboratories joining the RR, analyzed by statistical analysis by QSAR lab. More details in Annex 3 and 4.

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has been described to face issues attributed to various interferences due to their specific physico-chemical characteristics, and potential interference of the ENM with the test assays has been carefully checked and controlled, except for the interference free CFE and HPRT assays. A systematic literature review has been conducted in parallel to gather information of the type of interference reported and the mitigation actions. Several review manuscripts addressing this topic are now under preparation. The overview of the assays, ENMs and testing conditions are given in Table 4.

Particle characterization of the ENMs in the relevant test medium was performed in WP4 and is reported in the deliverable D4.1, Report on the rounds of the RRs for characterisation of ENMs. The data produced under the task will be included in the publications on the *in vitro* methods.

Table 3. *Overview of the initially selected ENM and their identifications and specifications.* Those indicated with * were finally used in the RRs.

ERM identifiers	ID	Name	CAS	type	Supplier	Supplier code	Batch	Core
ERM00000062	ERM 00000062	Titanium dioxide	1317-70-0	NPO_1486	Sigma Aldrich	637254	MKCK-4358	TiO_2
ERM00000063*	ERM 00000063	Zinc oxide	1314-13-2	NPO_1542	Sigma Aldrich	721077	MKCJ-4155	ZnO
ERM00000064*	JRCNM01005a	Titanium dioxide	13463-67-7	NPO_1486	JRC	JRCNM 01005a		TiO_2
ERM00000065	JRCNM01101a	Zinc oxide	1314-13-2	NPO_1542	JRC	JRCNM 01101a		ZnO
ERM00000088*	ERM 00000088	CuO 40nm	1317-38-0	NPO_1544	PlasmaChem			[Cu]=O
ERM00000089*	ERM 00000089	Nano Tungsten Carbide/ Cobalt Powder	12718-69-3	ENM_9000257	NanoAmor	5561HW		[Wc]-[Co]
ERM00000325*	ERM 00000325	MWCNT 3wt% AQUACYL 0303-NC7000		NPO_354	Nanocyl	AQUACYLTM AQ0303		Carbon

2.3.1 Dispersion protocol for the preparation of the test substance

The stock dispersions for each ENM tested were prepared following a harmonised SOP for the resuspension of ENMs in biological media and *in vitro* dosimetry (see deliverable D4.9 for details). The protocol was provided by WP4 (LIST). The DeLoid protocol was to be followed during RR1 for TiO_2 and ZnO ENMs, and for CuO and MWCNTs in RR2.

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During RR1, 5 mL of stock solution of TiO_2 and ZnO ENM at 5 mg/mL were prepared in MilliQ water and vortexed. Samples were sonicated using a probe sonicator which was previously calibrated by all partners as part of WP4 SOP harmonisation for ENMs dispersion, to be able to define the sonication energy that is really delivered to the solution. In the case of the TiO_2 and ZnO ENMs, the total energy to be used by all partners to disperse the particles was 322.32 J/mL.

During RR2 and specifically for Nano Tungsten Carbide/Cobalt (NanoAmor) particles, partners followed the NANOGENOTOX protocol. The NANOGENOTOX protocol was chosen here in order to allow the comparison of the results with those from previous EU projects where the same type of particles was employed. Briefly, the stock was first diluted in filtered MilliQ water containing 0.05% Bovine serum albumin (BSA) to achieve a stock solution of 2.56 mg/mL. The sample was sonicated for a specific time to be able to deliver a total energy of 7056 +/- 103 Joule in total volume of 6ml. The sample was kept on ice during the process of sonication to avoid overheating. The CuO and MWCNT ENMs were prepared following the LIST protocol (see deliverable D4.9 for details).

The obtained dispersions of each ENM were further diluted in the cell culture medium to achieve the desired concentrations to be used in each test.

Table 4. *Overview of the selected test methods, cell lines, exposure times, concentrations and controls used in the RRs. CFE, colony forming efficiency; AB, alamarBlue, TBA, tryphan blue exclusion assay; CA, comet assay; MN, micronucleus assay; NC, negative control; PC, positive control; CHL; chlorpromazine; MWCNT, multiwalled carbon-nanotubes.*

Assays	NMs-RR1	NMs-RR2		Cell line	Exposure time	Concentrations	Controls
CFE	TiO_2 ZnO	CuO MWCNT	Tungsten	A549	10 days	1-100 µg/mL or 1- 25 µg/mL	NC, PC (CHL)
AB/TBA/WST	TiO_2 ZnO	CuO MWCNT	Tungsten	A549 TK6	3, 24h	1-100 µg/mL or 1- 25 µg/mL	NC PC1 MMS PC2 H_2O_2 PC3 $KBrO_3$
CA	TiO_2 ZnO	CuO MWCNT	Tungsten	A549 TK6	3, 24h	1-100 µg/mL or 1- 25 µg/mL	NC PC1 MMS PC2 H_2O_2 PC3 $KBrO_3$
HPRT	TiO_2 ZnO	CuO MWCNT	Tungsten	TK6 V79-4	24h	1-100 µg/mL or 1- 25 µg/mL	NC PC1 MMS
MN		TiO_2 ZnO		TK6	24h	1-100 µg/mL or 1- 25 µg/mL	

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3. Results

3.1 Cytotoxicity by the colony forming efficiency CFE

3.1.1 Summary

The ultimate index of cytotoxicity is loss of cell viability, measured by a cell´s ability to survive and form colonies, which is the endpoint in the CFE assay (also called clonogenic or plating efficiency assay). The CFE assay was applied for testing of the five selected ENMs in two rounds of interlaboratory comparisons (RR1 and RR2), to evaluate its suitability for ENM hazard assessment or if any assay adaptation was needed. The SOP, a modified version from the JRC report by Ponti *et al* from 2014, was provided by NILU (**ANNEX 1**), and was applied by all participating partners: UiB, KU Leuven, IMI and NILU. Several concentrations of the ENMs were tested on A549 human lung epithelial cells, and cytotoxicity was measured as colony formation by viable cells.

Under the experimental conditions in RR1, TiO_2 was found not to be cytotoxic, while ZnO induced toxicity in a concentration-dependent manner. The results were rather consistent between the different laboratories involved. No amendments to the SOP were necessary and the same SOP was followed during RR2 to test the other 3 ENMs. In RR2, CuO and MWCNTs were found to be cytotoxic in a concentration-dependent manner, while data for Tungsten was more divergent across partners.

Being non-colorimetric and non-fluorescent, the CFE assay is specifically suitable for assessment of ENMs to avoid interference issues and was found to be a promising test method for cytotoxicity assessment of ENMs.

3.1.2 Presentation of data, summary of data, Data analysis and evaluation

For RR1 and RR2, human lung epithelial cells, A549, were exposed to the ENMs continuously for 9-12 days. Three independent experiments were performed for each ENM, with 6 replicate wells for each treatment within each experiment. CFE was measured and calculated relative to negative control (set to 100 %), exposed to cell culture medium only (rCFE). The results provided by each of the 4 partners were compiled, and the data are presented in **Figure 1**. TiO_2 ENM did not affect cell viability, while CuO, ZnO and MWCNTs reduced cell viability in a concentration-dependent manner. Tungsten showed divergent results and was found to be slightly cytotoxic by KU Leuven and IMI.

Overall, there were some differences in the data obtained by partners. The results from UiB tended to differ from the other partners for ZnO at lower concentrations and CuO at higher concentrations. For RR1, NILU and KU Leuven had very similar results, while in RR2 IMI and KU Leuven showed the most similar results for CuO and Tungsten, while the curves fitted very well for MWCNTs in the data from NILU and IMI, and lowest cytotoxicity was measured by KU Leuven, followed by UiB. The biggest discrepancy was found for CuO at 10 and 25 µg/mL, where no cytotoxicity was reported from UiB, whereas the other partners´ data showed cytotoxicity. The solvent control for the MWCNTs (the ENM dispersant) was also provided by the particles manufacturer and was shown to be highly toxic at the highest concentration (only concentration tested) (data not shown). No colonies were detected after treatment with chlorpromazine (50 µM) as the positive control (PC, data not shown) reflecting its high cytotoxicity.

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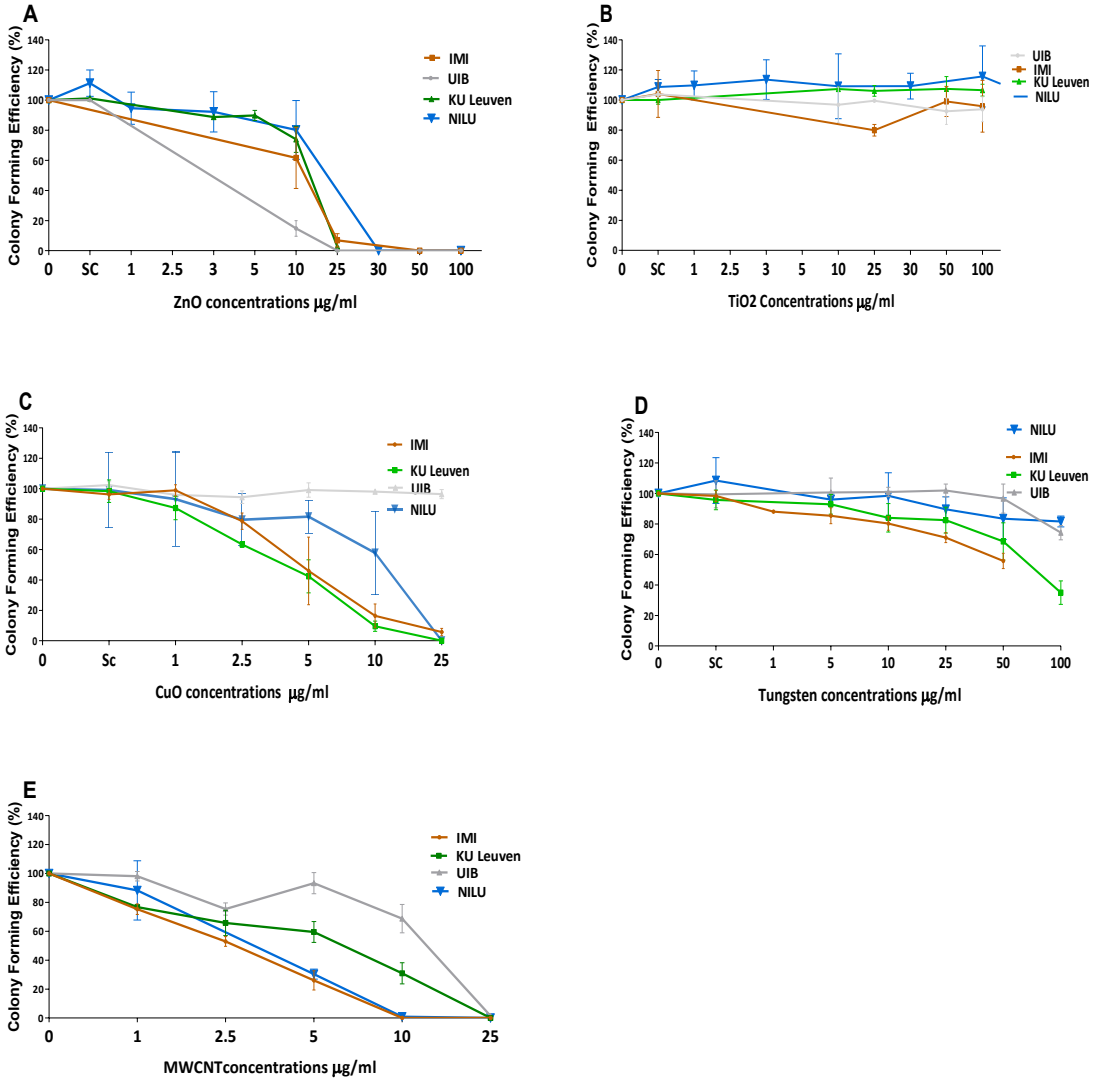

Figure 1. Relative colony forming efficiency (rCFE). Single A549 cells were treated with TiO_2 (A), ZnO (B), CuO (C), Tungsten (D), or MWCNT (E) and cell viability measured as CFE. Cell viability is shown relative to negative control (set to 100 %) (rCFE) as mean +/-SD from 3 independent experiments (mean of 6 replicate wells in each experiment) for each partner, IMI (orange), KU Leuven (green), UiB (grey), NILU (blue). SD, standard deviation.

3.1.3 Conclusion

For TiO_2 ENM no significant toxicity was detected. The results were rather consistent between the different partners involved in the interlaboratory comparison experiments. ZnO was found to be cytotoxic in a concentration-dependent manner and the data from the partners is rather consistent, although higher toxicity was detected by UiB below 25 µg/mL. Positive control chlorpromazine worked fine, and no toxicity was detected in the negative control, nor the solvent control treated cells. Surviving cells in negative control varied a bit between the partners and between the different experiments. This is probably a

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reflection of inaccuracy in cell seeding and counting of cells. Therefore, during the RR2, partners agreed to use serial cell density dilutions for more precise seeding of 30 cells per well and to be consistent on serum concentration for cell cultivation (9%). However, the results from RR2 did not show higher consistency than the RR1 data, rather the opposite for CuO and MWCNTs, e.g., UiB detected very low toxicity in RR2 for CuO, while the results for the other concentrations were close to those from NILU. Overall, the results for ZnO and tungsten were very close for all partners, whereas a bigger spread between partners was noted for ZnO and MWCNT, which may be due to difficulties in obtaining the same levels of dispersions for the ENMs.

This exercise taught us that training in the SOP is important, even for rather easy methods. Also, accuracy in number of cells seeding and thus cell counting, is of importance to avoid too large variation in the data set. It is important to have proper negative controls (NC), and preferably to include two NCs in the same experiment, since all data are calculated relative to the NC, and thus having a precise cell number seeded is critical. Another critical phase is proper handling of the ENM dispersion before exposure, with sufficient vortexing and application to the cells within a short time after preparation. Further, an appropriate dispersion protocol is critical, as well as measuring size and size distribution of the dispersion before exposure, as differences in size of the ENMs and agglomeration state will clearly influence on the results of the CFE assay, as well as any other test method for ENM exposure. Passage number of the cells could also be a confounding factor. Overall, the CFE assay seems to be a reliable and robust test method for hazard assessment of ENMs, and the modified SOP for higher throughput as developed in NanoREG (performed in 12-well plates) was applied in these RRs.

3.2 Cytotoxicity by AlamarBlue assay, TBA and WST-1 (partners NILU, ANSES, KU Leuven)

3.2.1 Summary

Cytotoxicity can also be measured by colouring of dead cells, such as the TBA assay (ANSES) which uses conjugation of (4-thiobutylamidine), or metabolic activity and conversion from non-fluorescent to fluorescent dye in metabolic active and viable cells, such as AlamarBlue (AB) assay (NILU), WST-1 assay (KU Leuven) and MTT (ANSES). These assays for cytotoxicity were performed as part of the genotoxicity testing to distinguish between primary and secondary genotoxicity detected by measuring of DNA strand breaks and oxidised DNA lesions. The cells were exposed to each ENM for 3 and 24 hours, and 2-3 independent experiments including at least 2 replicate wells for each treatment in each experiment were performed.

3.2.2 Presentation of data, summary of data, Data analysis and evaluation

Data from RR1: TiO_2 and ZnO ENMs (NILU, KU Leuven and ANSES)

Results from RR1 showed no cytotoxic effect of TiO_2 ENM after exposure of TK6 and A549 cells using the AB assay for both cells (NILU) or MTT assay (ANSES) for A549 cells or TBA assay (ANSES) for TK6 (**Figure 2**). The ZnO ENM was found to be highly toxic at both 3 and 24 h exposure (NILU) by the AB assay in both cell lines, while with the TBA assay cytotoxicity was only detected after 24h exposure of A549 cells (ANSES) (**Figure 3**).

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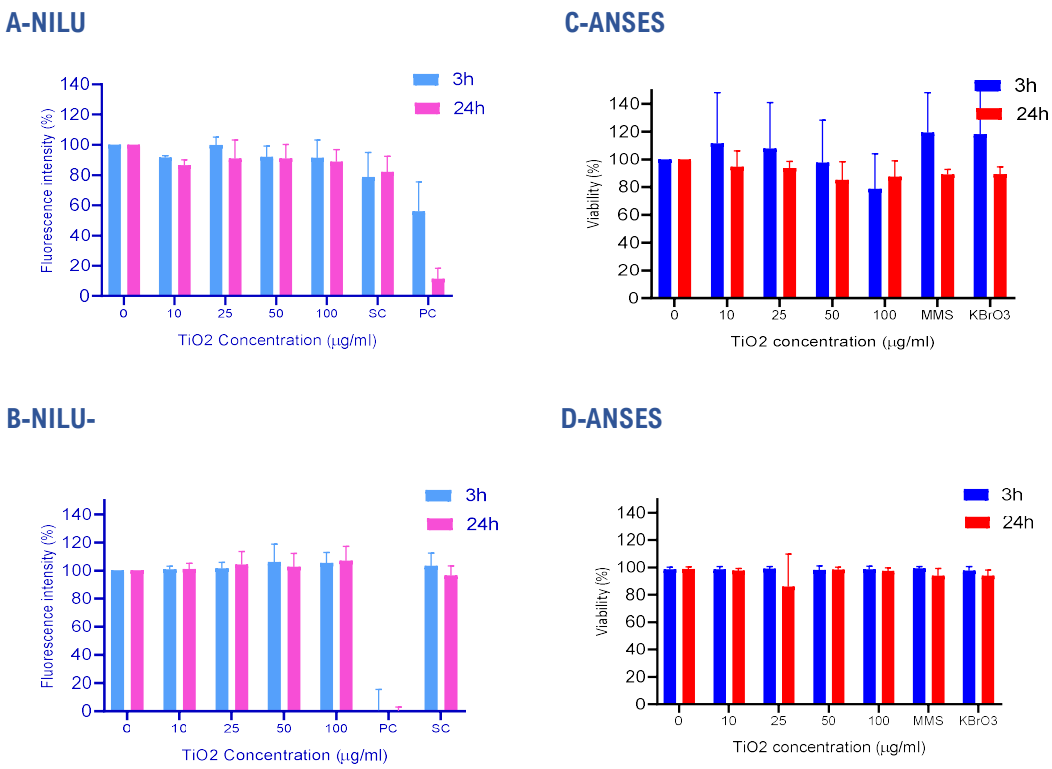

Figure 2. Cell viability after exposure of **A549** (A & C) and TK6 (B & D) cells for 3 or 24 hours to **TiO_2 ENMs** measured by the AB assay (**NILU**) and MTT assay (**ANSES**) shown as fluorescence intensity or number of coloured cells reflecting % cell viability, respectively, relative to negative control (NC) treated with growth medium only. PCs were chlorpromazine 50 µM (AB assay) or MMS and $KBrO_3$ (MTT assay). The data are presented as the mean of 3 independent experiments +/- SD. SC, solvent control; h, hours. Possible interference of ENMs with the AB test was assessed by mixing ENMs suspensions with medium + 10 % AB (no cells). No interference was seen at the highest ENM concentration (100 µg/mL) (data not shown, NILU).

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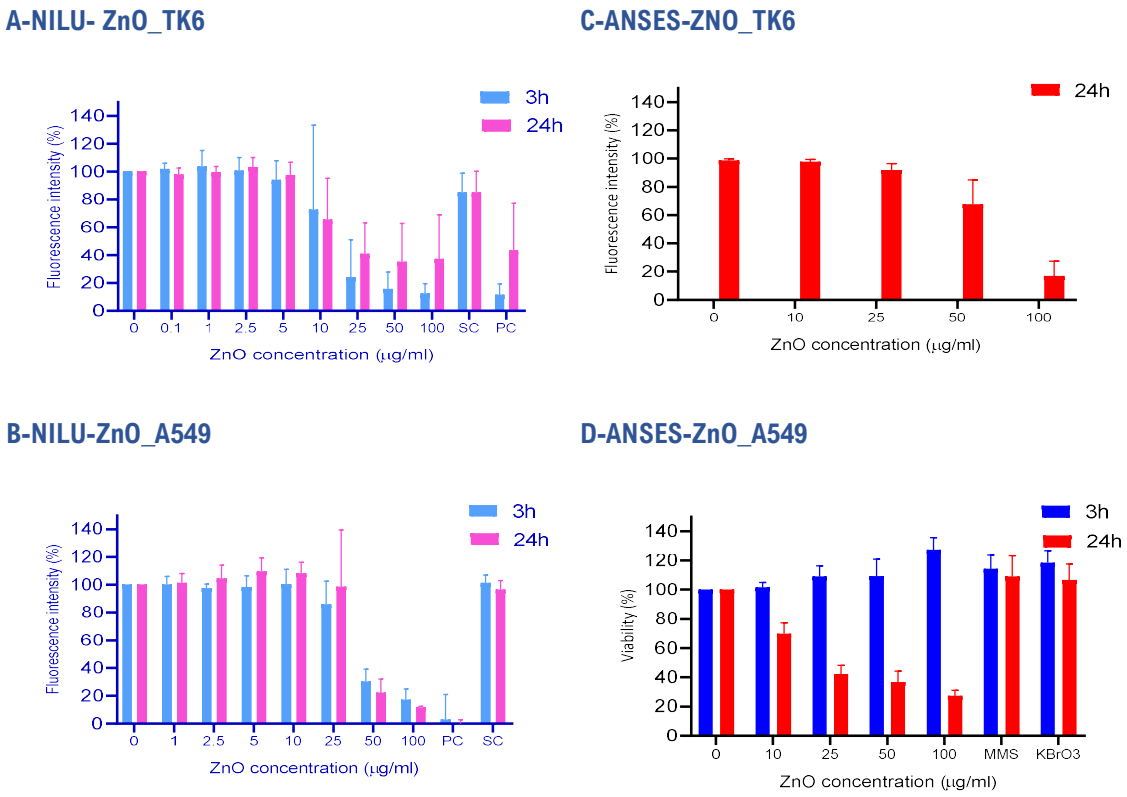

Figure 3. Cell viability after exposure of A549 and TK6 cells for 3 or 24 hours to **ZnO ENMs** measured by the AB assay in both cell lines both time points **(NILU, A & B**), by the TBA assay on TK6 after only 24h **(ANSES, C)** and by the MTT assay on A549 cells at both time points **(ANSES, D)**. Cell viability is expressed as the fluorescence intensity (AB and MTT) or number of coloured cells (TBA), relative to NC treated with growth medium only. PC was chlorpromazine 50 µM (AB assay) or MMS and $KBrO_3$ (MTT assay and TBA). The data are presented as mean of 3 independent experiments +/- SD. SC, solvent control; h, hours. Possible interference of ENMs with the AB test was assessed by mixing ENMs suspensions with medium + 10 % AB (no cells). No interference was seen at the highest concentration (100 µg/mL) (data not shown, NILU).

Data from RR2: CuO, Tungsten and MWCNT ENMs (NILU and KU Leuven)

In RR2, human lung epithelial cells A549 and human lymphoblastoid TK6 cells were exposed to Tungsten, CuO or MWCNTs for 3 or 24 hours and cell viability measured by metabolic reduction and fluorescent signal of the dyes in living cells. Two different colorimetric assays were used – AB assay (NILU) and WST-1 assay (KU Leuven). Both assays were performed for cytotoxicity testing as part of the CA. In A549 cells, Tungsten was found not to be cytotoxic, while CuO and MWCNTs reduced cell viability in a concentration-dependent manner. The two assays gave rather consistent results, except after 3 h exposure for MWCNTs, where increased fluorescence intensity was measured by AB assay, while decreased cell viability is reported by WST-1. No interference with the readouts of the assays were

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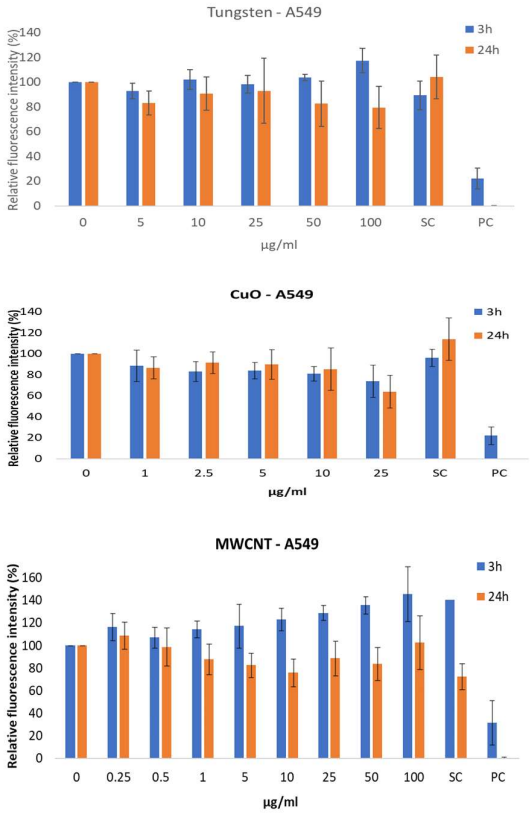
detected (INT) (**Figure 4**). In TK6 cells, Tungsten did not induce cytotoxicity, in line with the results from A549 cells. CuO reduced cell viability in a concentration-dependent manner after 24 h, and only very slight reduction was measured only with the WST-1 assay after 3h. MWCNTs reduced cell viability at the from 50 µg/mL, and the effect was most pronounced with the AB assay (**Figure 5**). Also, in TK6 cells, the results were found to be rather consistent between the two assays.

A-NILU



B-KU Leuven

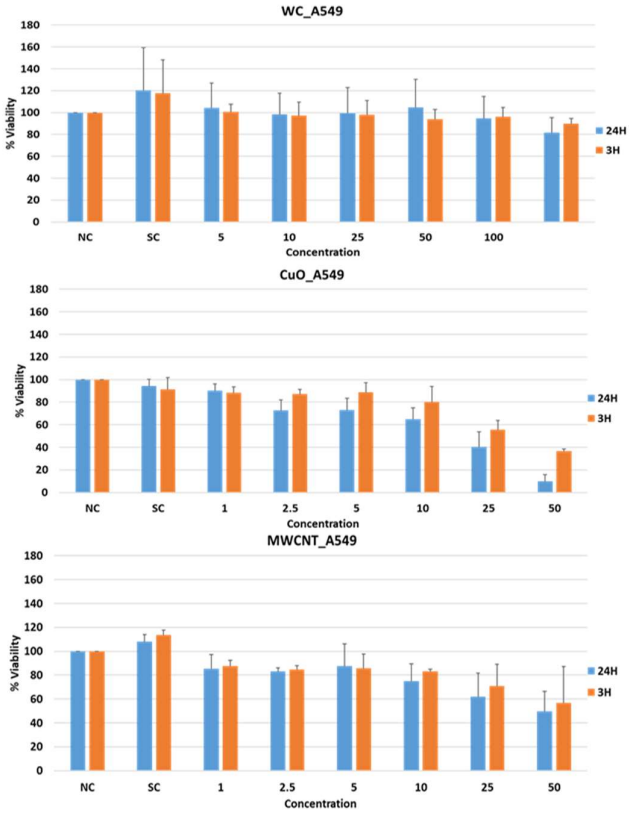


Figure 4. Cell viability after exposure of **A549** cells to **Tungsten, CuO or MWCNTs** for 3 or 24 hours measured by the AB assay in A (**NILU**) or WST-1 assay in B (**KU Leuven**), shown as fluorescence intensity relative to NC treated with growth medium only. PC, chlorpromazine 50 µM. SC, Solvent control. The data are presented as mean of 2-3 independent experiments +/- SD. Possible interference of ENMs with the AB test was assessed by mixing ENMs suspensions with medium + 10 % AB (no cells). No interference was seen at the highest concentration (100 µg/mL) (data not shown, NILU).

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A-NILU **B-KU Leuven**

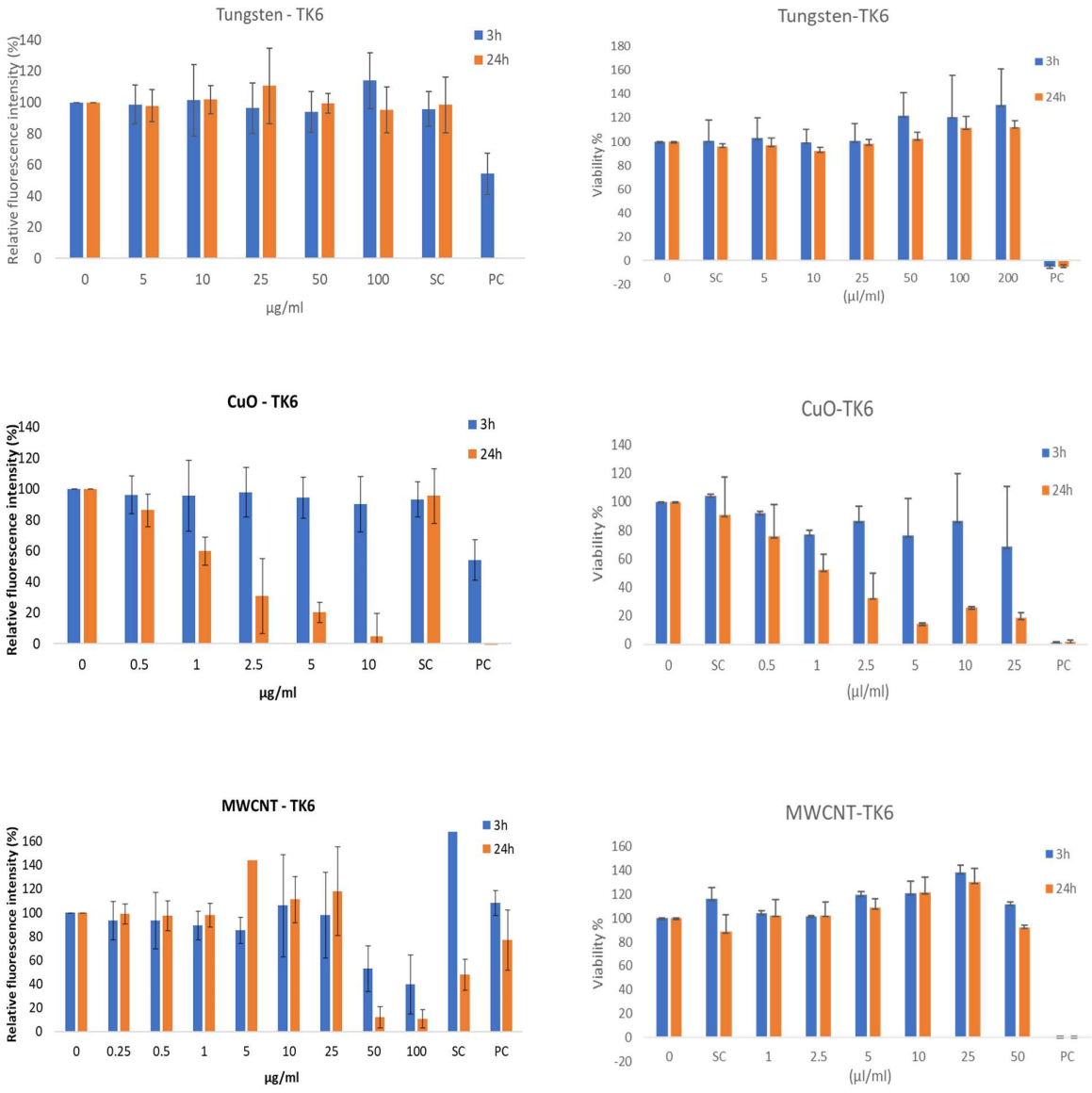

Figure 5. Cell viability after exposure of **TK6** cells to **Tungsten, CuO or MWCNTs** for 3 or 24 hours measured by the AB assay in A (**NILU**) or WST-1 assay in B (**KU Leuven**), shown as fluorescence intensity relative to NC treated with growth medium only. PC, chlorpromazine 50 µM. SC, Solvent control. The data are presented as mean of 2-3 independent experiments +/- SD. Possible interference of ENMs with the AB test was assessed by mixing ENMs suspensions with medium + 10 % AB (no cells). No interference was seen at the highest concentration (100 µg/mL) (data not shown, NILU).

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3.2.3 Conclusion

Cytotoxicity testing of the 5 ENMs in A549 and TK6 cells by three different colorimetric assays showed that there were no big differences between the AB, WST-1 and TBA assays. Effects were most pronounced after 24 h exposure, as expected. The most consistent results were seen by comparing AB and WST-1 assays, which are based on the same principle.

3.3 Genotoxicity by the Comet assay

3.3.1. Summary

The Comet assay (CA) or (single cell gel electrophoresis) is a method for detection of DNA damage in cells with a nucleus. The method is widely used for detection of strand breaks (SBs) as well as specific DNA lesions, such as oxidized purines and pyrimidines by inclusion of specific enzymes (in this case Fpg, formamido pyrimidine glycosylase protein, that detects preferably oxidized base lesions). The assay is considered a useful method for genotoxicity testing *in vitro* as well as *in vivo*. The SOP was provided by NILU to all participating partners; ANSES, KU Leuven, IMI, SU, LIST and NILU. During RR1, the main focus was the harmonization of the critical steps in the SOP between the partners, and with the COST Human Comet project. Additional PCs suitable for alkylation and oxidatively damaged DNA were included and tested by all the partners.

Possible interference of the ENMs with the CA test is a matter of concern. To investigate this aspect interference controls (INT) have been added to the experiments. In RR1 interference was assessed by mixing non exposed cells (negative control, NC) with ENMs at the highest exposure concentration used (e.g., 100 µg/mL), right before cells embedding in agarose. This aims at checking for the creation of "artificial" DNA breaks induced by ENMs after exposure and resulting in increased DNA migration during electrophoresis.

After RR1 more discussion took place among the partners about the interference possibilities and a review of the literature on this issue was performed. Based on this discussion and review work, in RR2 more conditions were to be considered to assess potential ENM interference in the CA. These possible types of interference were individuated as follows:

- Inhibition/interaction with Fpg activity
- Quenching /autoflorescence during quantification of signals
- Direct/physical interference of nanoparticles with DNA (after washing steps)
    - creating additional breaks or adducts
    - interfering by reducing or blocking the DNA migration during electrophoresis

To assess all these possible types of interference the following interference controls were prepared:

- For inhibition/interaction with Fpg activity: NC and PC for Fpg samples were trypsinized after exposure and the cells were mixed with the ENMs immediately before embedding in agarose
- Physical interference:
    - NC cells and ENMs were mixed just before embedding into agarose like in RR1
    - PC cells and ENMs were mixed just before embedding into agarose.

This last control is meant to check for possible reduced or hindered DNA migration into the agarose gel as a consequence of ENMs still being present in the gel.

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The CA SOP was applied on the human lymphoblastoid cells TK6 and the human lung epithelial cells A549. Five ENMs in total were tested in RR1 and RR2, as described in Table 4, with 3 or 24 h exposure times. Each partner provided data for 2-3 independent experiments for each ENM, cell line and time point. The inclusion of potassium bromate ($KBrO_3$) as PC gave inconsistent data. Several trial studies have been conducted in parallel to identify the best condition for $KBrO_3$. A data entry template for the RiskGONE instance of the eNanoMapper database was tailored and harmonized with partner NMBP13 project Gov4Nano.

Under the experimental conditions in RR1, TiO_2 ENMs did not induce DNA damage (SBs) after 3h or 24h exposure to A549 and TK6 cells. One partner (KU Leuven) reported DNA oxidation lesions after incubation with Fpg both at 3 and 24h in A549 cells, however the increase was not statistically significant. The ZnO ENMs seemed to induce an increase in SBs and oxidized bases (SBs+Fpg) at the higher concentrations. The interlaboratory comparisons using data from RR1 have shown that it is necessary to refine the performance of the CA in the labs and to further amend the SOP to ensure more robustness and better consistency and reproducibility of data. An amended SOP was prepared and followed during RR2 (**ANNEX 2**). One of the most important changes to the SOP based on the RR1 training, was the use the same source of Fpg (Norgenotech, Norway). During RR2, an amended SOP was distributed to all partners involved and data were completed and provided on CuO, Tungsten and MWCNT ENMs by NILU and KU Leuven. The comparison of the results showed that both partners obtained very close results during this round. The other partners LIST and SU will provide their data when the test is completely established in their lab. UoB will also contribute with A549 cells to align WP6 activity on ecotoxicity-relevant cell types with WP5 data.

3.3.2 Presentation of data, summary of data, Data analysis and evaluation

Data from RR1: TiO_2 and ZnO ENMs (NILU, KU Leuven and ANSES)

The collected data for SBs (measured as % DNA in tail), SBs+Fpg and netFpg (calculated as the difference in %DNA in tails between samples with Fpg incubation and samples without Fpg incubation) for each cell line (**A549 & TK6**) and time point (3h and 24h) were reported by each partner (**Table 5**). The SBs and SBs+Fpg data are summarized in **Figures 6, 7, 8 & 9**.

The **TiO_2** ENM did not induce a clear genotoxic effect with no SBs or oxidized base lesions in either cell line (**Figures 6 & 7).** In A549 cells, the TiO_2 ENM did induce a slight increase in oxidized base lesions in a concentration dependent manner after 3h and **24h** exposure in **A549**, however the increase was not found to be statistically significant from the negative control (**Figure 6**). Similarly, on **TK6** cells and after exposure for 3h, KU Leuven obtained a slight increase in oxidized bases which is also statistically not significant (**Figure 7**).

No significant interference with the CA results was observed to occur with the ENMs tested here and the interference control approach here used (explained in the paragraph 3.3.1.) has thus been effective in ruling out an influence from interference.

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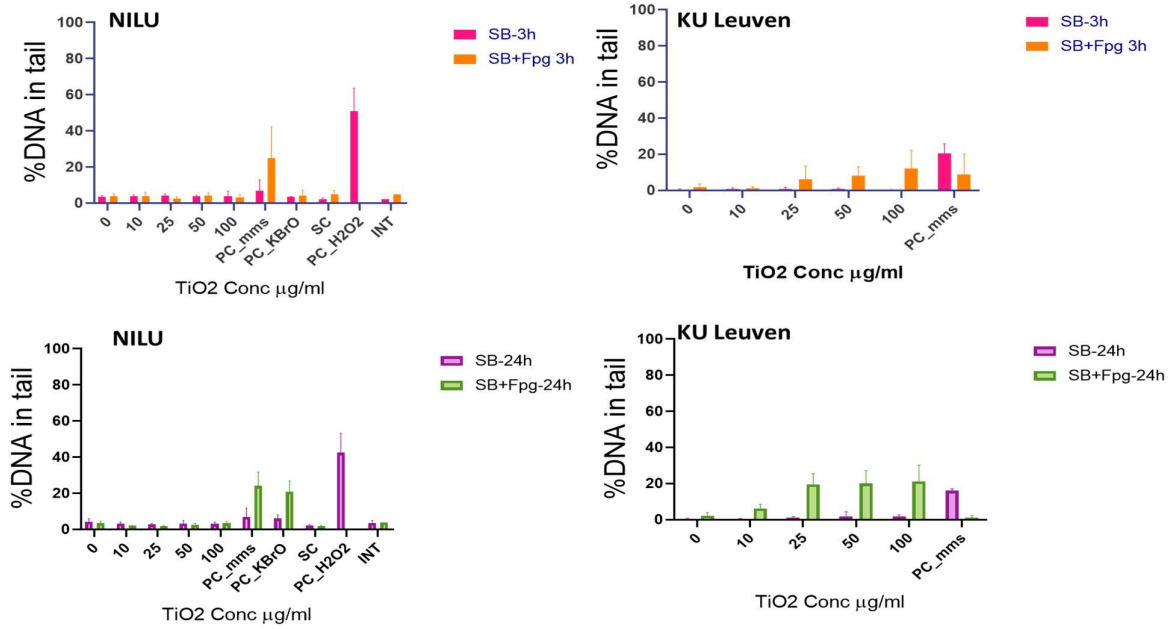

Figure 6. DNA damage measured as tail intensity induced by **TiO_2 (ERM00000064)** in **A549** cells after 3- or 24-hours exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (2% MilliQ sterile water, equivalent to the water content in the highest ENM concentration 100 µg/mL); PCs: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose). The data are presented as mean of 2-3 independent experiments +/- SD. SBs – strand breaks; SBs+Fpg – strand breaks with oxidised base lesions.

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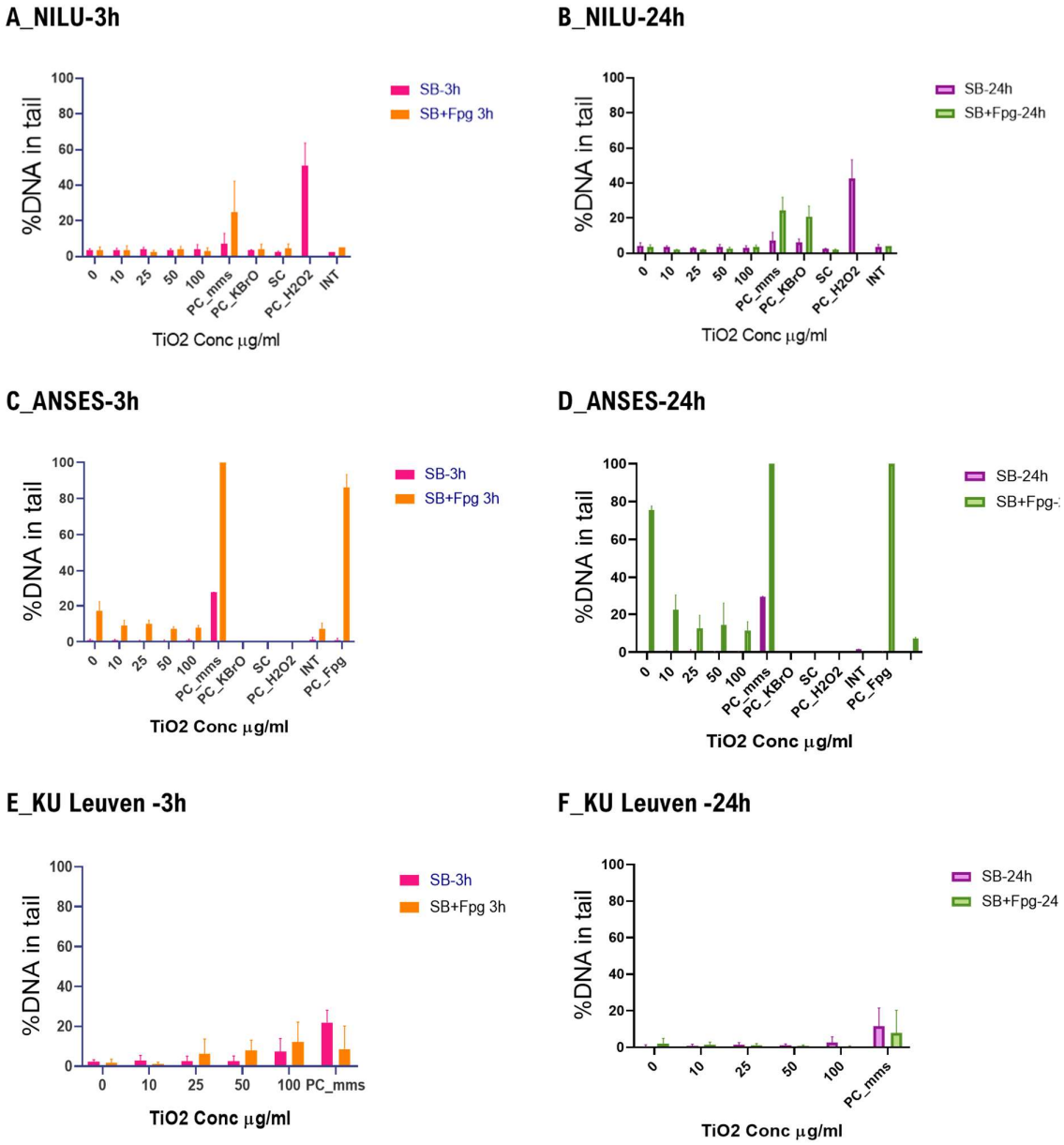

Figure 7. DNA damage measured as tail intensity induced by **TiO_2 (ERM00000064)** in **TK6** cells after 3- or 24-h exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (2% MilliQ sterile water, equivalent to the water content in the highest ENM concentration 100 µg/mL); PCs: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1 mM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose).

In the case of **ZnO** ENMs (F**igures 8 & 9**). The results reported by the three partners were slightly inconsistent. For instance, NILU didn't observe any effect of ZnO ENMs on **TK6** after 3h but only after 24h at the higher concentration 100 µg/ml (F**igure 8**). In A549 cells, and since NILU have used a wide

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range of concentrations from 1 to 100 µg/mL, the effect of ZnO was observed mainly at the higher concentrations from 25-100 µg/mL. ANSES, did show an increase in oxidized bases after 3h and KU Leuven after 24h exposure of **TK6** cells (**Figure 8**). Although, the increase is not statistically significant after 24h exposure, ANSES detected a significant increase in both SBs and oxidized bases (SB+Fpg) at 10 µg/mL of ZnO ENMs (**Figure 9).** KU Leuven had a high SBs level at all the tested concentrations including the NC after both 3 and 24h (**Figure 9)**. It is also worth mentioning that ANSES had a high background of % Tail DNA in the control cells in the Fpg condition (**Figure 7D, 8D &9D)**. This is almost certainly because ANSES used an Fpg enzyme from a different provider compared to NILU and KU Leuven. Moreover, after further tests, it was concluded that the Fpg enzyme used at ANSES was too concentrated, giving this high background signal. Indeed, control A549 cells were used in the CA with various dilutions of the Fpg enzyme. The results indicated a low % Tail DNA in control cells in absence of enzyme (0.51 +/- 0.1) while in the presence of Fpg, control cells reached a % Tail DNA of 36 +/- 3.3 strictly following the SOP but this level was decreased to 16.4 +/- 0.8 when the Fpg was further diluted (by a factor 10) (Data not shown here). Therefore, these data from ANSES indicate that the high background in % Tail DNA in control cells in the presence of Fpg could be reduced by further dilution of the enzyme in order to gain good data quality and good comparison with other partners. This is also the origin of the recommendation for RR2 to all use fpg from the same supplier.

During the RRs, several controls have been used. The $KBrO_3$ was included as PC for DNA oxidation, and H_2O_2 and MMS for SBs. $KBrO_3$ induced only a slight increase in base lesions, while H_2O_2 induced increased SBs as expected (NILU). The MMS induced an increase in SBs to a lesser extent than H_2O_2, and at varying levels between the partners.

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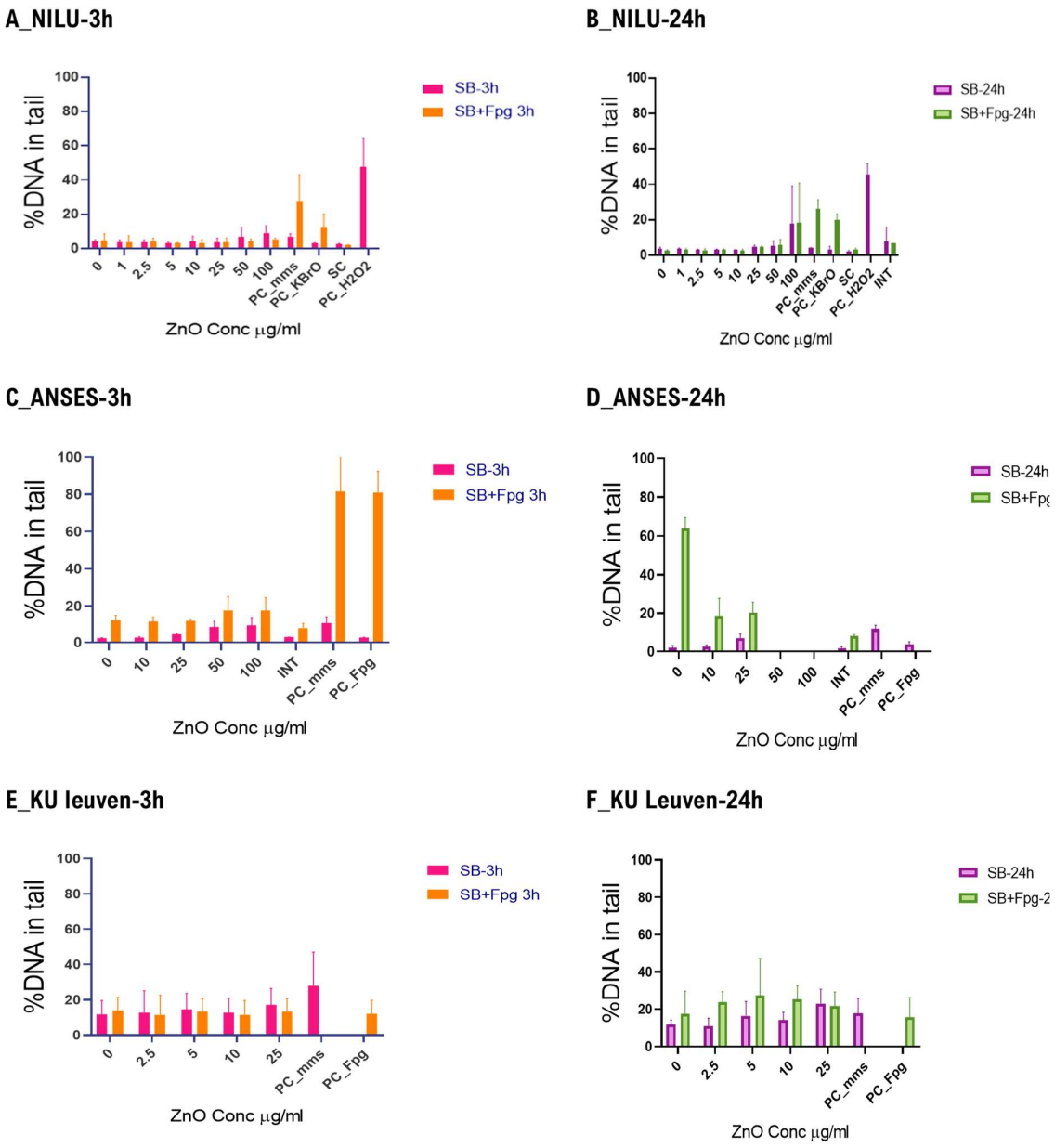

Figure 8. DNA damage measured as tail intensity induced by **ZnO (ERM00000063) in A549 cells after 3 or 24 h** exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration: 2% for 100 µg/mL of ENM, 0.5 % for 25 µg/mL of ENM); Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

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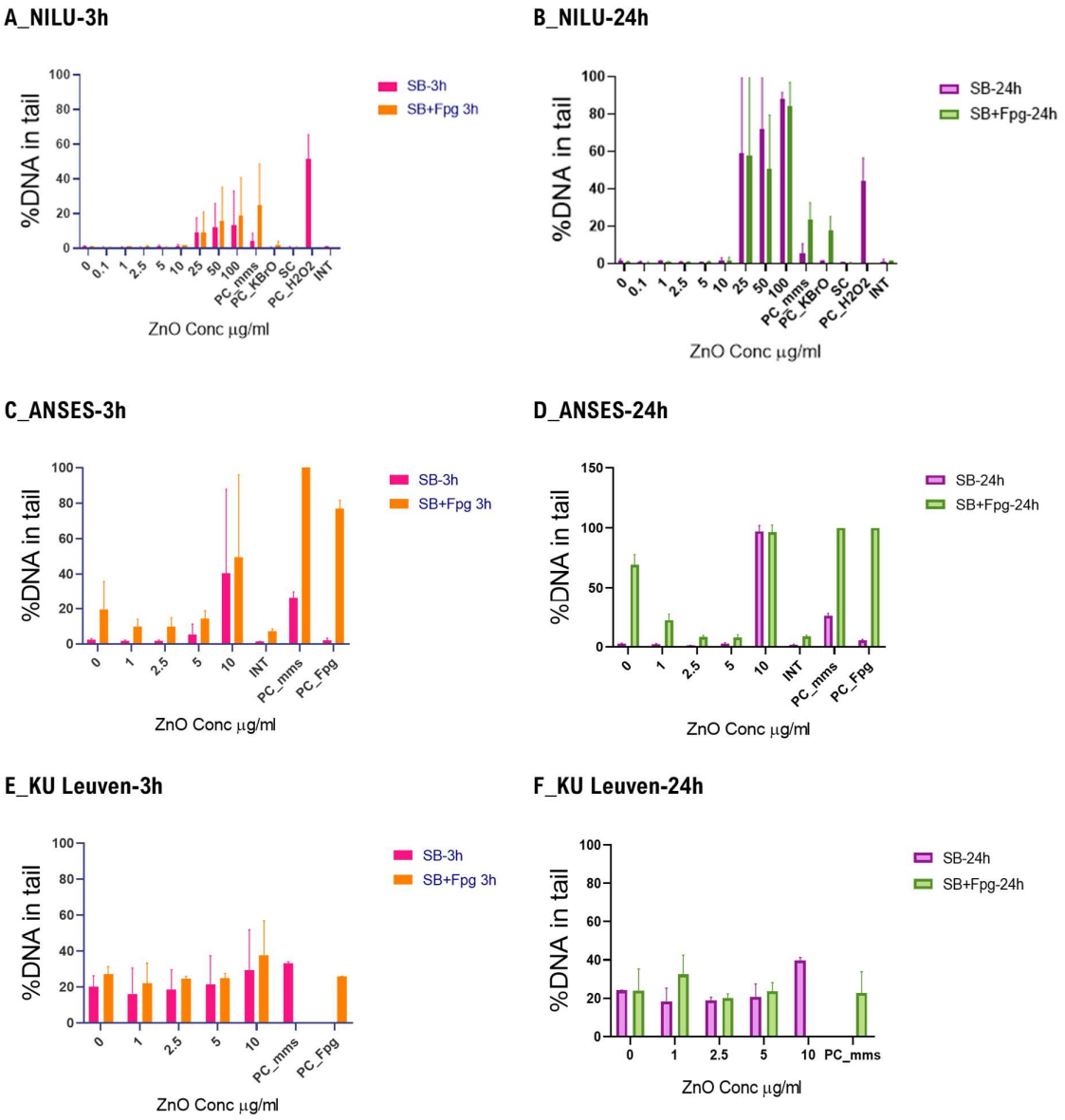

Figure 9. DNA damage measured as tail intensity induced by **ZnO (ERM00000063) in TK6 cells after 3- or 24-h** exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration, here: 0.2% for 10 µg/mL of ENM); Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min).

Detailed statistical analysis was conducted by QSAR lab (**Annex 3**). The statistical analysis indicates that the results differed in the accuracy of measurements for all measured endpoints (SBs, SBs+Fpg, netFpg) between the labs (Table 5). For **ZnO**, the results for TK6 cells were more accurate between labs, with the smallest differences between NILU and ANSES. Less accurate results were obtained for ZnO in A549

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cells by ANSES vs. KU Leuven, and for NILU vs. KU Leuven. For TiO_2 the measurements did not differ significantly in accuracy between the three labs for all endpoints (NILU, ANSES and KU Leuven) independently of cell line applied. Differences in precision have been seen with all three labs independent of ENM and cell line. The biggest differences were observed between NILU and the other two partners (ANSES and KU Leuven).

***Table 5**. Comparison of the statistical parameters from measurements from different labs.*

		NILU_ANSES						ANSES_KU						NILU_KU					
		SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24	SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24	SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24
TiO2_A549	F statistic	N.D.						N.D.											
	Test statistic																		
	p value																		
TiO2_TK6	F statistic																		
	Test statistic																		
	p value																		
ZnO_A546	F statistic																		
	Test statistic																		
	p value																		
ZnO_TK6	F statistic																		
	Test statistic																		
	p value																		

Data from RR2: CuO, Tungsten, MWCNT ENMs (NILU and KU Leuven)

Four partners have been participating in RR2, although data are ready just for NILU and KU Leuven. The data for **CuO** ENMs were consistent (note that different concentration ranges were tested in the different labs) in **A549 cells**, however not in **TK6 cells**. In TK6 cells, NILU measured after 24 h exposure an increase in both SBs and oxidized base lesions (SBs+Fpg), and only an increase in oxidized base lesions (SBs+Fpg) in **A549 cells**. However, the DNA damage was measured only at cytotoxic concentrations (**Figure 5**). **Tungsten** ENMs induced a slight concentration-dependent increase in SBs and oxidized base lesions after both 3 and 24h exposure, and the data were consistent between the labs. For **MWCNTs ENMs** no significant increase in DNA damage was detected in TK6 and a slight increase at 3h exposure in A549. Checks for interference between the tested ENMs and the CA has been performed following the SOP. The approach used to verify the occurrence of interference consisted of mixing the untreated cells with the higher concentration of each ENMs directly (no incubation) before embedding in agarose. The results obtained by both partners showed no interference occurs at this level.

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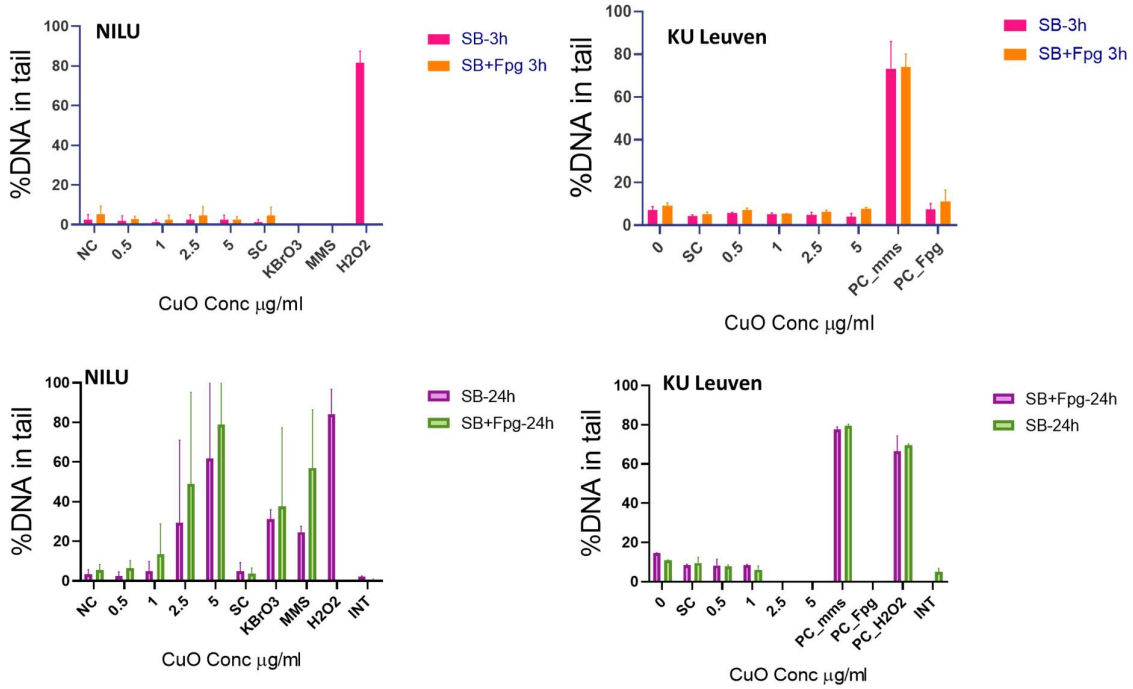

Figure 10. Summary data from NILU and KU Leuven on DNA damage measured as tail intensity induced by **CuO**, in **TK6 cells** after 3- or 24-h exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration, here: 0.1% for 5 µg/ml of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (5 µg/mL) just before embedding into agarose) and $KBrO_3$ (1mM). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

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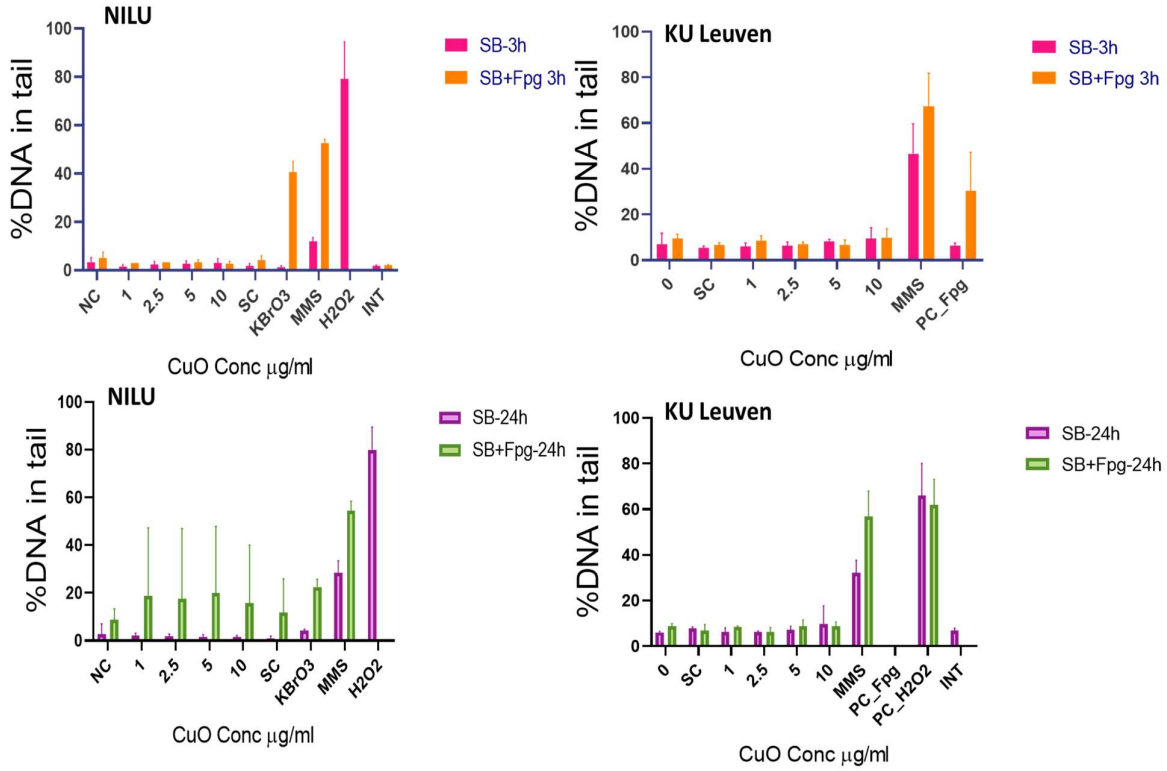

Figure 11. Summary data from NILU and KU Leuven on DNA damage measured as tail intensity induced by **CuO ENMs,** in **A549** cells after 3- or 24-hours exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD (standard deviation). SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration, here: 0.2% for 10 µg/ml of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (10 µg/ml) just before embedding into agarose). MMS, positive control (0.2 mM), H2O2, positive control (0.1 mM in jar for 5 min) and KBrO3 positive control (1mM).

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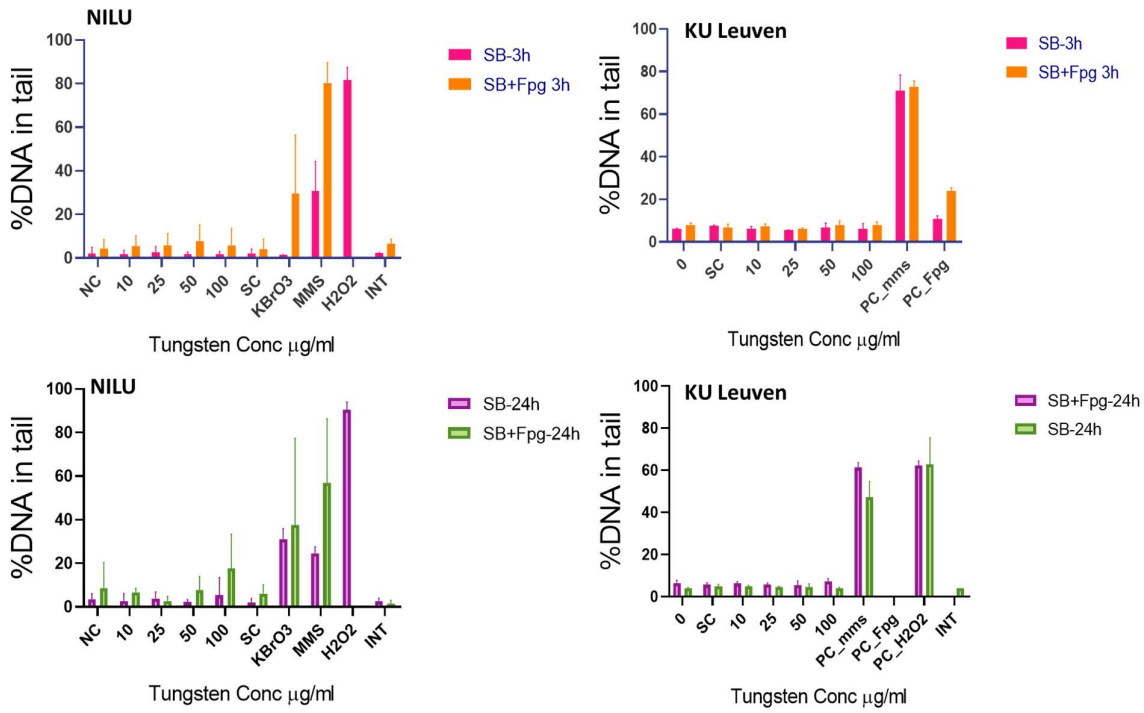

Figure 12. Summary data from NILU and KU Leuven on DNA damage measured as tail intensity induced by **Tungsten,** in **TK6** cells after 3- or 24-h exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water + 0,05%BSA equivalent to the content in the highest ENM concentration, here: 3.9 % for 100 µg/mL of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

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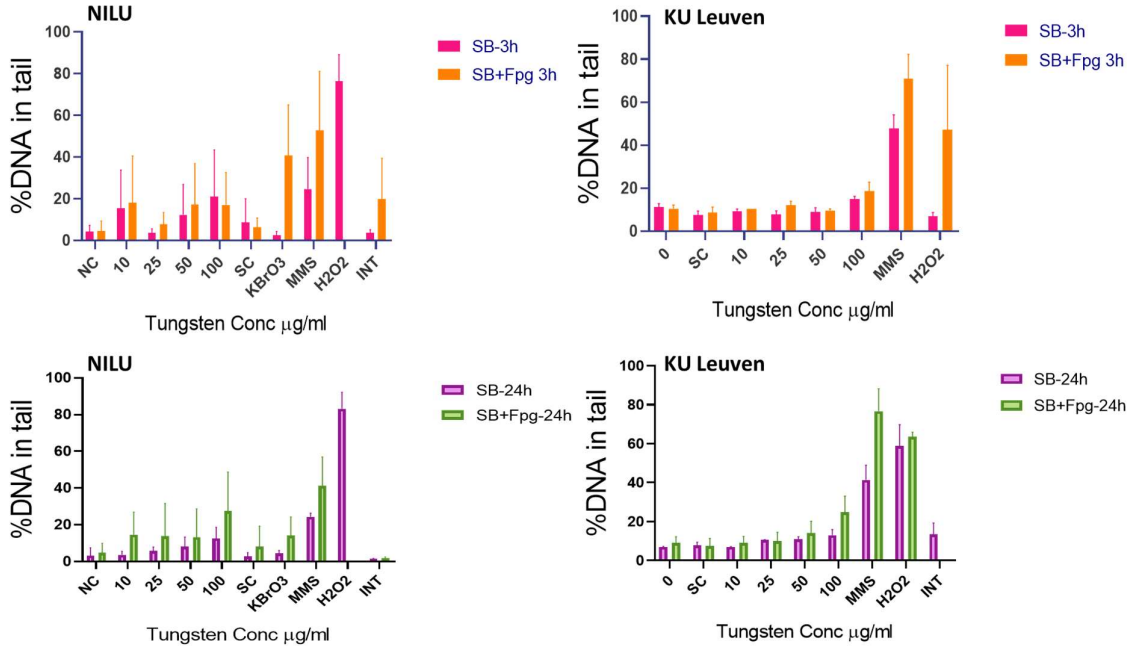

Figure 13. Summary data from **NILU and KU** Leuven on DNA damage measured as tail intensity induced by **Tungsten**, in **A549** cells after 3- or 24-hours exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water + 0,05%BSA equivalent to the content in the highest ENM concentration, here: 3.9 % for 100 µg/mL of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (100 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

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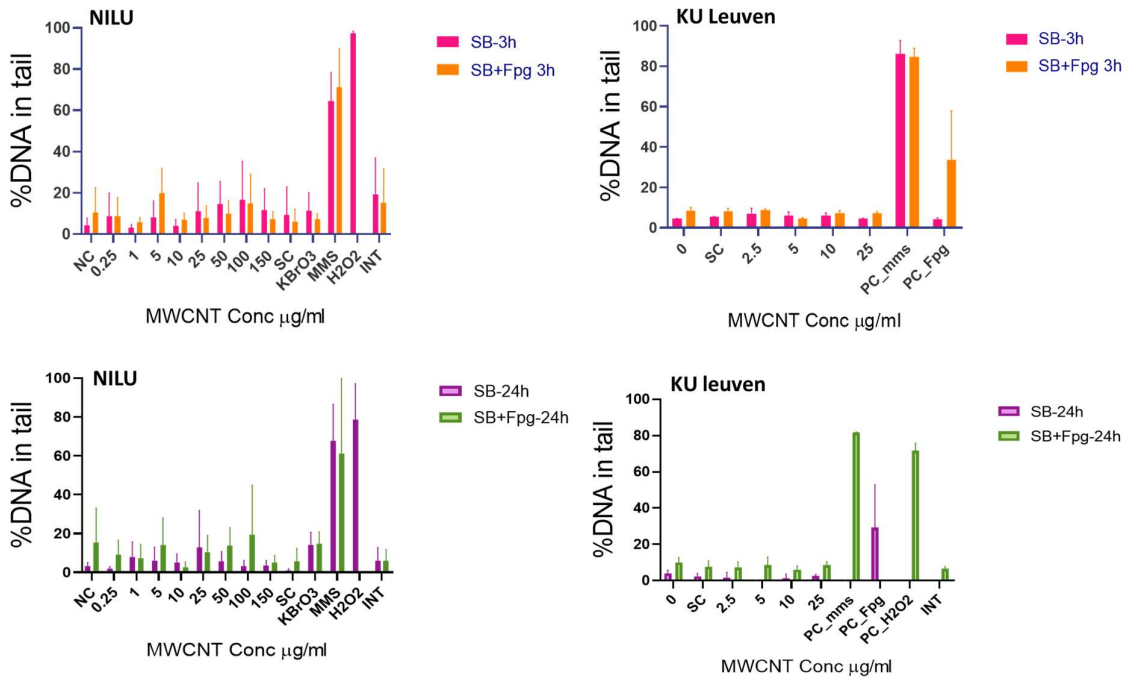

Figure 14. Summary data from **NILU and KU** Leuven on DNA damage measured as tail intensity induced by **MWCNT, in TK6 cells** after 3- or 24-hours exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration, here: 3% for 150 µg/mL and 0.5 % for 25 µg/mL of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (25 or 150 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

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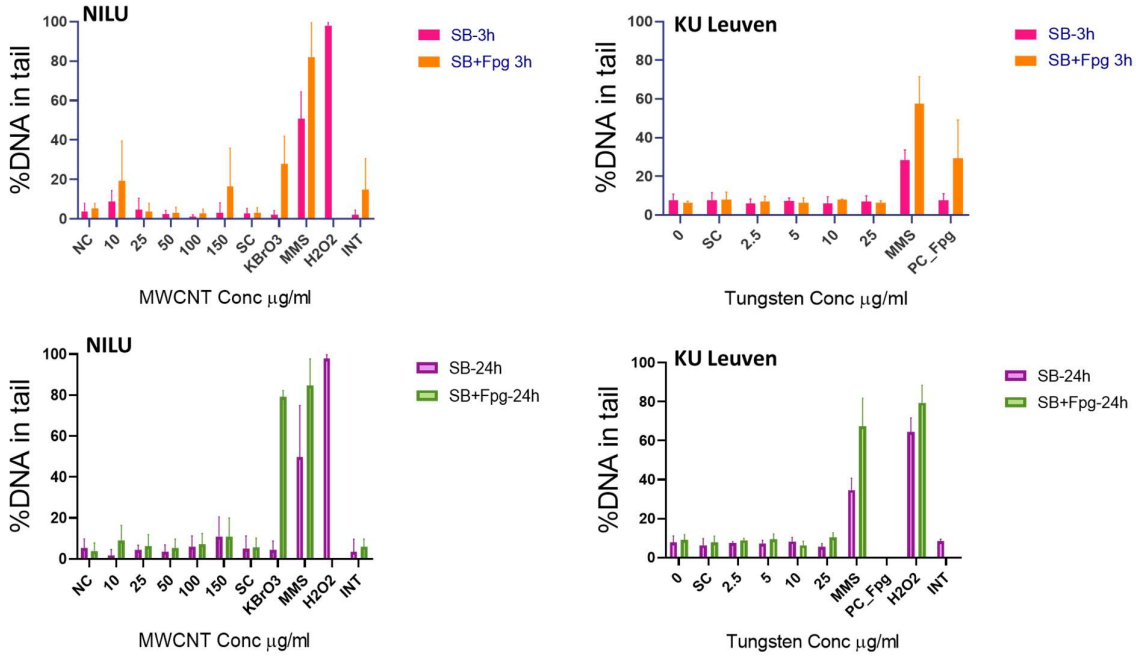

Figure 15. Summary data from NILU and KU Leuven on DNA damage measured as tail intensity induced by **MWCNT, in A549 cells** after 3- or 24-hours exposure. 2-3 independent experiments were performed with duplicate wells for each exposure within each experiment. The results are shown as mean of the median of duplicate wells, ± SD. SC, solvent control (MilliQ sterile water equivalent to the water content in the highest ENM concentration, here: 3% for 150 µg/mL and 0.5 % for 25 µg/mL of ENM). Interference control (INT, NC cells mixed with ENMs at the highest exposure concentration used (25 or 150 µg/mL) just before embedding into agarose). PC: MMS (0.2 mM), H_2O_2 (0.1 mM in jar for 5 min) and $KBrO_3$ (1mM).

3.3.3 Conclusion

The interlaboratory comparisons have shown that it is necessary to refine the performance of the CA in the labs and to further amend the SOP to ensure more robustness and better consistency and reproducibility of data. The CA is compatible with testing of genotoxicity of ENMs, but proper interference testing is needed for each ENM, as interference between the ENM and the assay can potentially occur at various stages as described in the CA SOP (Annex 2). It is further important to include cytotoxicity testing as part of the genotoxicity testing to avoid false positive results as DNA breaks occur also during apoptosis/necrosis.

A cut-off for cytotoxic response and titration of concentrations of the ENM to be tested is needed. Based on the results from RR1, the importance of the Fpg enzyme source and harmonization became clear. This was implemented in the amended SOP for RR2. The inclusion of several positive controls was also shown to be of importance, and H_2O_2 was included by all partners as PC in RR2. Conclusions from RR2 will be drawn when data are ready from all the partners. The results for NILU and KU Leuven were largely consistent.

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3.4 Gene mutation assay on *Hprt* locus

3.4.1 Summary

The hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene is located on the X chromosome of mammalian cells and is used as a model gene to investigate gene mutation and identify mutagenic materials. The HPRT assay can detect a wide array of chemicals capable of inducing DNA damage resulting in gene mutations and is significantly important for detecting point mutation induced by ENMs, as the bacterial reverse gene mutation assay (Ames test) is not appropriate for use with these materials. The HPRT methodology is such that mutations which destroy the functionality of the HPRT gene and/or protein are detected through positive selection via addition of the toxic analogue 6-thioguanine (6-TG), resulting in only HPRT-mutants being seen as live colonies when cultured in selective media. Whilst the HPRT gene mutation assay is a standardised method for investigating chemical-induced mutagenic potential, the assay has not yet been standardised for use with ENMs. An interlaboratory approach was taken whereby SU prepared an SOP for suspension cells (using, TK6 cells) and NILU prepared an SOP for the adherent cells (using the V79-4 cell line). Both groups performed their respective assays before performing each-others SOP to generate a total of 4 data sets on the agreed TiO_2 and ZnO ENMs during RR1 and CuO, Tungsten and MWCNTs during RR2. The data generated by the two labs in RR1 were uploaded into the developed data and metadata capture templates and stored within the H2020 RiskGONE – eNanoMapper database. The statistical analysis for RR1 data was completed by QSAR which reports on the precision, accuracy, and concurrence between the two laboratory data sets.

The partners agreed to conduct a second round RR2, including a new partner KU Leuven. The SOP for the HPRT assay with TK6 cells was amended and updated version was circulated. No major changes were required to the SOP for the HPRT assay with V79-4 cells. However, the SOP adopted a step of cell cleansing similar to the SOP on TK6 cells. Data from RR2 on the three ENMs were completed in V79-4 cells from both partners (NILU and SU) and only SU have completed the testing on TK6 cells as yet. Available data from RR2 are also uploaded and analysed. The RRs will help to move towards a harmonised approach for testing ENM mutagenic potential using the HPRT forward mutation assay.

3.4.2 Presentation of data, summary of data, data analysis and evaluation

a. HPRT gene mutation assay on TK6 cells

Data from RR1: TiO_2 and ZnO ENMs

At SU and NILU, MMS was used as the positive control and induced significant cytotoxicity with viability reduced to ~60% at 1.5 µg/mL (**Figures 16 & 17**). MMS also induced a highly significant mutagenic response at the HPRT locus (**Figures 16 & 17**) and was capable of producing significant mutagenicity by inducing a mutant frequency of $1.2x10^{-3}$ (**Figure 16**) and $1 x10^{-3}$ (**Figure 17**) representing an 8-fold increase over background TK6 mutant levels. Whilst neither TiO_2 nor ZnO induced any significant mutagenicity at the HPRT loci in both labs, the results from SU showed induction of significant cytotoxicity following a 24-hour exposure at 10 µg/mL and 50 µg/mL for ZnO and TiO_2 ENM respectively, whilst no cytotoxicity of any of the ENMs was detected at NILU (**Figures 16 & 17**). Background mutant frequency levels appeared much lower at NILU as opposed to SU, however this may be due to the evaluation criteria followed and the inclusion of TK6 colonies which ought to have been excluded by SU. Whilst the cytotoxic response observed in TK6 cells following 24 h ZnO exposures appears concentration-dependent, this was not the case with TiO_2. The highest concentration of TiO_2 did induce significant cytotoxicity which

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may suggest differences in agglomeration of this particle type across the concentration range. Two independent experiments including 2 duplicates each were performed at each laboratory.

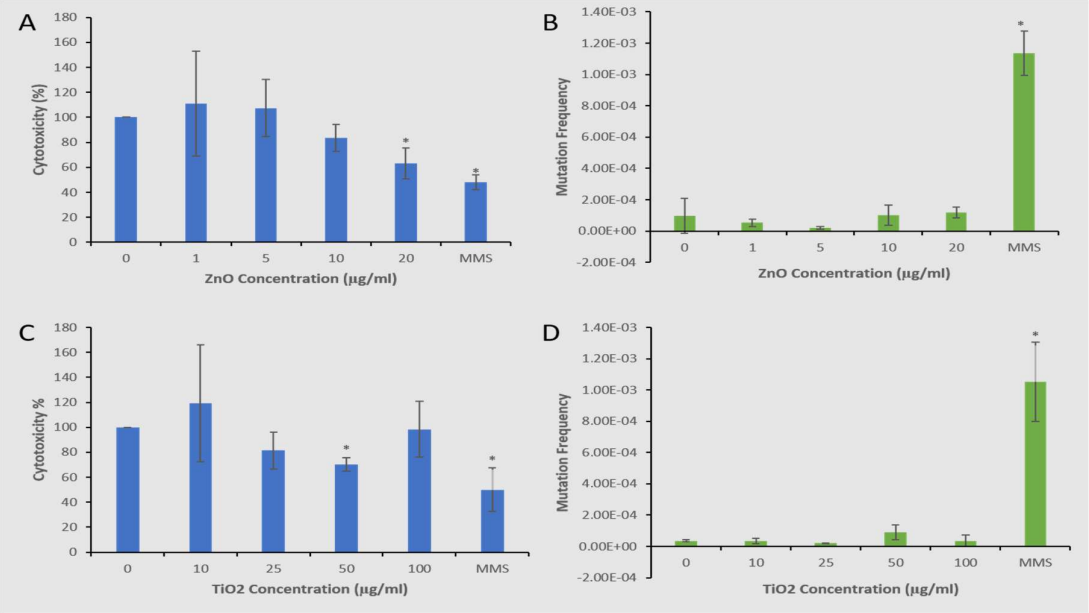

Figure 16. Data **from SU on TK6** cytotoxicity and mutant frequency following 24 -hour exposure to **ZnO** (A & B) and **TiO_2 (C & D) ENMs** from RR1. MMS was used as a positive chemical control at a concentration of 1.5µg/mL. Results were considered statistically significant (*) when p≤0.05 (n=2).

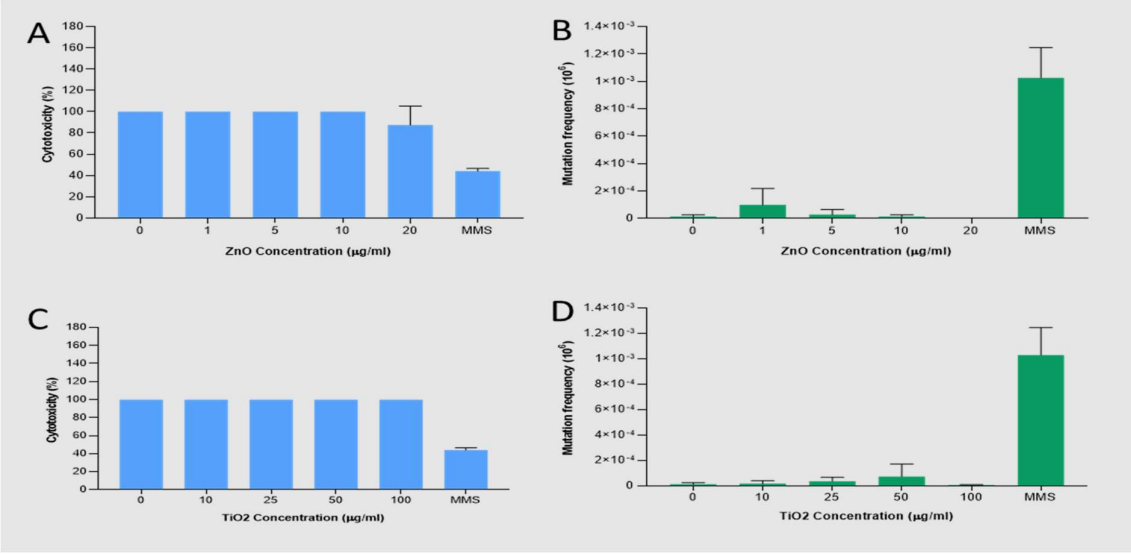

Figure 17. Data from **NILU on TK6** cytotoxicity and mutant frequency following 24 -hour exposure to **ZnO (A & B) and TiO_2** (**C & D**) ENMs from RR1 only. MMS was used as a positive chemical control at a concentration of 1.5µg/ml. Results were considered statistically significant (*) when p≤0.05 (n=2).

In summary, the ZnO and TiO_2 ENMs were cytotoxic at 20 µg/ml and 50 µg/ml respectively on TK6 cells, whilst showing no significant mutant frequency at the *Hprt* locus in both laboratories.

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ZnO and TiO_2 ENMs were not cytotoxic on V79-4 cells (SU and NILU). However, significant mutant frequency was observed (SU). The ZnO induced significant mutagenicity at concentrations of 5, 10 and 20 µg/ml, whilst TiO_2 induced significant mutagenicity at 50 and 100 µg/ml.

The statistical analysis of HPRT assay data generated on TK6 cells indicate that the obtained measurements between both partners do not differ in the accuracy of the viability or mutant frequency endpoints independently of the ENMs used. Differences in precision between both groups are evident in the cell viability endpoint however, the data from SU are closely related to the measured values by NILU (**ANNEX 4 Table 4 & Figure 3&4**). Therefore, the experimental data obtained are consistent between SU and NILU, in future it was recommended by QSAR that both groups should refine the measurement of cell viability in both SOPs.

Data from RR2: CuO, Tungsten and MWCNT ENMs (SU ONLY)

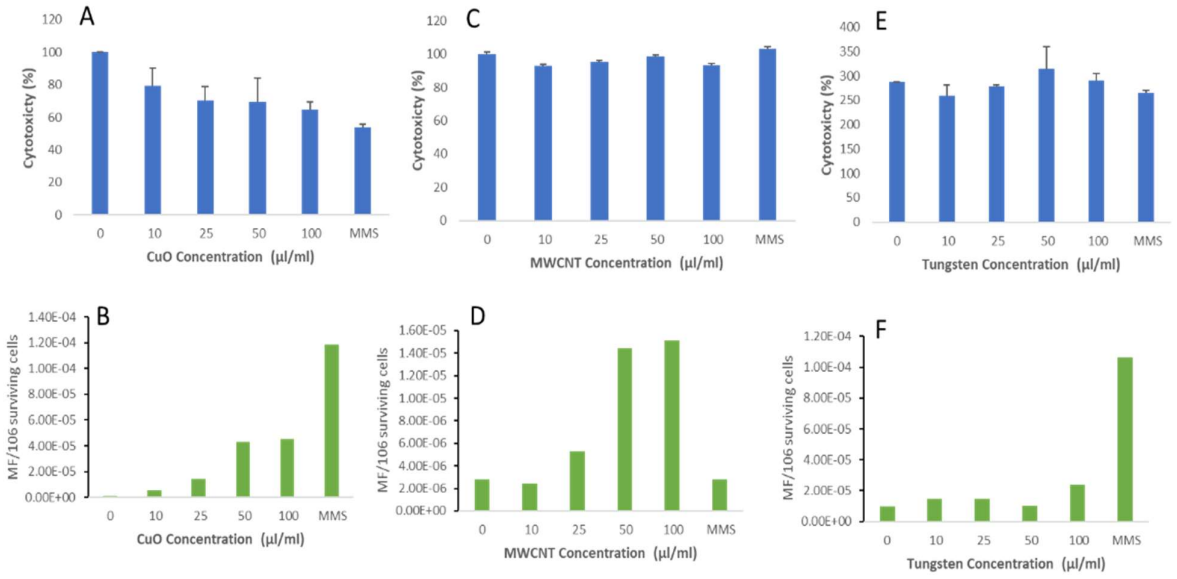

Figure 18. Data from **SU on TK6** cytotoxicity and mutant frequency following 24 -hour exposure to **CuO (A & B), MWCNT (C & D) and Tungsten (E & F)** from RR2. MMS was used as a positive chemical control at a concentration of 1.5 µg/mL (*n*=2).

During RR2, only one partner completed the testing of the three ENMs by the HPRT using TK6 cells. Tungsten ENMs didn't seem to be cytotoxic or mutagenic (**Figure 18 E&F**). The MWCNT didn't show any decrease in cell viability (**Figure 18C**). However, the MWCNT did increase the mutation frequency % at the higher concentrations tested, 25, 50 and 100 µg/mL. In the case of CuO ENMs, the particles decreased sightly the viability and increased the mutant frequency % in respect to the negative control. These results will be compared to NILU data, when all experimental work is completed.

b. HPRT gene mutation assay on V79-4 cells

For the mutagenicity evaluation by the V79-4 HPRT assay, at least 10^6 cells/mL per sample and concentration were analyzed. The frequency of spontaneous and induced mutants (the mutant frequency, MF) was measured at two time points, called 1st and 2nd harvests, was calculated relative to the results obtained on the viability measured by the plating efficiency assay which was performed in parallel to each

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harvest for mutant. Each viable cell forms a colony. Stained colonies of minimum 50 cells were counted, using a click counter (tally counter). Cell survival by plating efficiency (PE) was calculated as the number of colonies relative to the number of inoculated cells (%) following the same formula as for cytotoxicity by the CFE assay (Annex 1) and in HPRT assay in adherent cells SOP (Annex 6). The mutant frequency for the treated and control cultures was calculated as the number of mutant cells (colonies) per $1x10^5$ or $1x10^6$ surviving cells (colonies) using the following formula:

Mutant frequency (%) = (Mutant Colonies / surviving inoculated cells) x 100

Acceptance of the test was based on the following criteria (OECD TG 476):

1. The negative control was considered acceptable for addition to the laboratory historical negative control database.
2. Concurrent positive controls induced responses that were compatible with those generated in the historical positive control database and produced a statistically significant increase compared with the concurrent negative control.

Data from RR1. ZnO and TiO2 ENMs (SU and NILU)

Following the HPRT assay for V79-4 cells at SU, no cytotoxicity was observed following exposure to ZnO ENMs for 24 hours (**Figure 19**). Notably, this lack of cytotoxicity was also evident for the positive control, MMS. However, at NILU, the ZnO was slightly toxic at only the highest concentration 20 µg/mL (**Figure 19).**

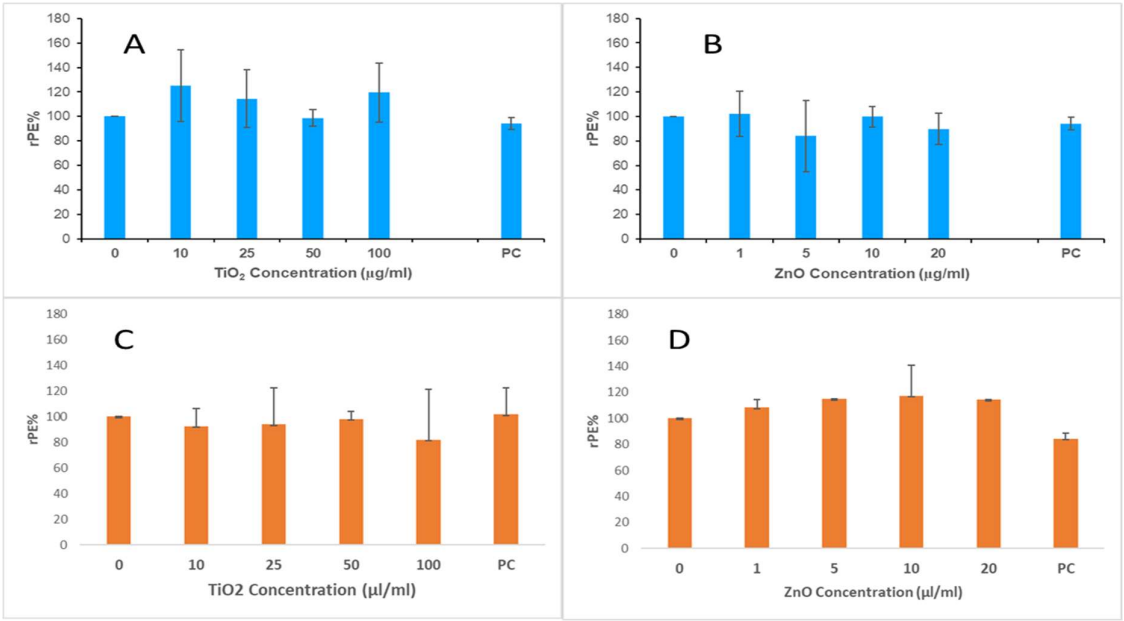

Figure 19. Data **from SU and NILU on V79-4 cells**. Relative plating efficiency **rPE** following 24-hour exposure to **ZnO and TiO_2 by SU (A&B) and NILU (C&D)**. MMS (0.1mM) was used as the positive control (PC). Results were considered statistically significant (*) when p≤0.05 (n=2).

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In **SU, V79-4** cells exposed to the **ZnO** ENMs induced significant ($p<0.05$) increase in mutant frequency (MF) at the HPRT locus. The exposure to ZnO ENMs generated significant ($p<0.05$) mutagenicity with approximate 2-fold increase over background levels in plates scored for mutation harvest 1 (**Figure 20**). Mutant harvest 2 later revealed that only the two highest concentrations tested for ZnO, 10 and 20 µg/mL were significant ($p<0.05$) (**Figure 20B**). However, at **NILU** no significant induction of mutations was observed (**Figure 20 C&D**).

In **SU, V79-4** cells exposed to **TiO_2** ENMs showed slightly more variation in cytotoxicity and plating efficiency **(Figure 20**). At SU, MF % after 1st harvest revealed significant ($p<0.05$) mutagenicity at concentrations of 50 and 100µg/mL (**Figure 20A**). This effect however was substantially different at mutant frequency after the 2nd harvest, whereby all test concentrations of TiO_2 were significant with approximate 1 to 2-fold increases over background levels (**Figure 21**). However, at **NILU** no significant induction of mutations was observed (**Figure 21 C&D**).

The positive control MMS induced a 6-fold increase over background V79-4 levels in MF for both the 1st and 2nd harvest at SU and approximate 8-fold increase over background levels at NILU. It should be noted that no cleansing of V79-4 cells was performed at the beginning of the experiment in SU, yet background levels remained low. Background MF levels for V79-4 cells appeared to show high concordance between both laboratories, indicating that the cells were handled appropriately during RR1 at Swansea University and that scoring of negative controls was performed in good accordance with NILU, where the SOP was generated.

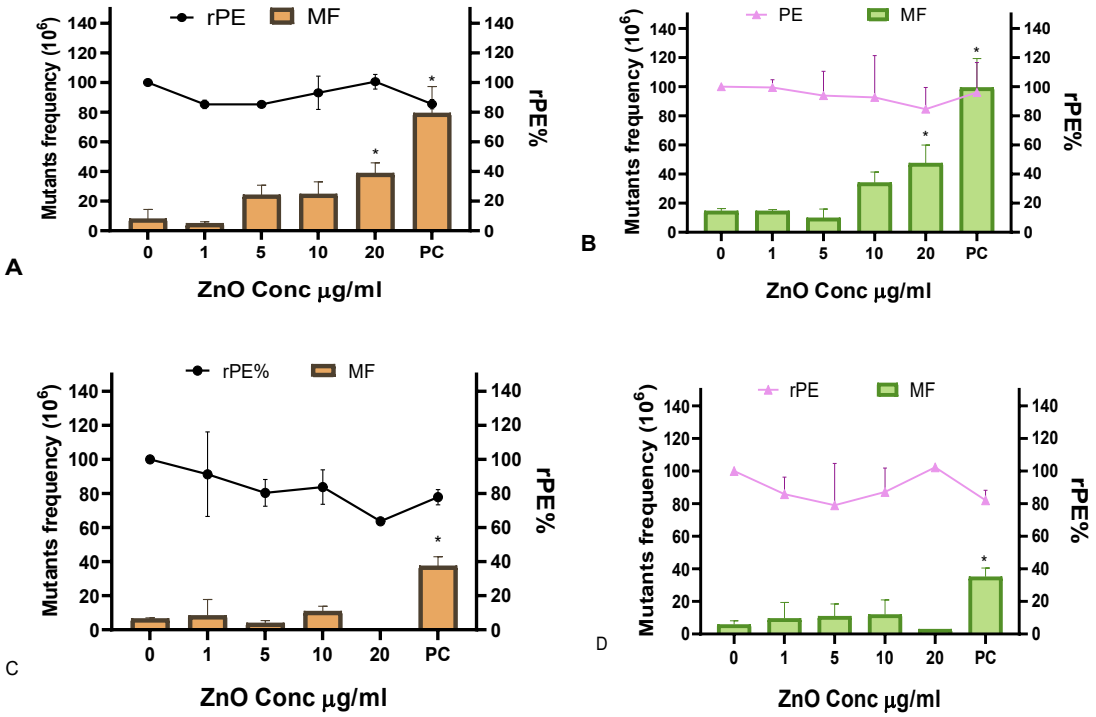

Figure 20. Data from **SU and NILU on V79-4 cell** for mutant frequency after 24-hour exposure **to ZnO ENMs.** 1st and 2nd harvest by SU (A&B) and by NILU (C&D). Lines represent the viability of cells measured by the plating efficiency. MMS (0.1 mM) was used as the PC. Results were considered statistically significant (*) when $p \leq 0.05$ ($n=2$).

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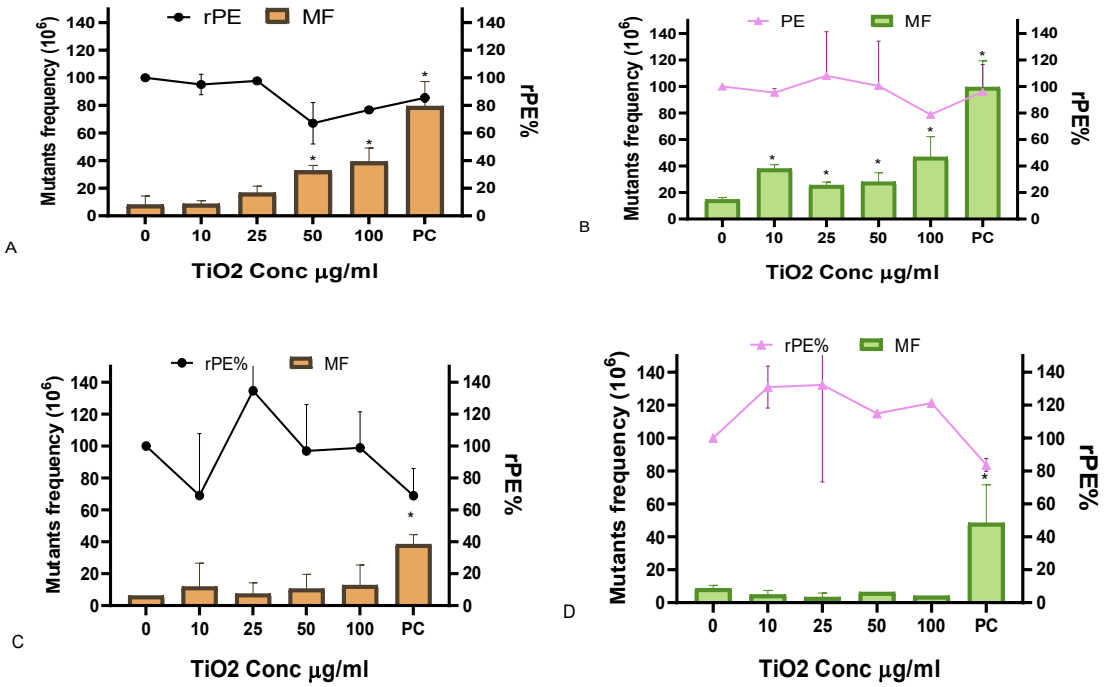

Figure 21. Data from **SU and NILU on V79-4 cell** for mutant frequency after 24-hour exposure **to TiO_2 ENMs.** 1st and 2nd harvest by SU (A&B) and by NILU (C&D). Lines represent the viability of cells measured by the plating efficiency. MMS (0.1 mM) was used as the PC. Results were considered statistically significant (*) when $p \leq 0.05$ ($n=2$).

From the data generated during RR1, both ZnO and TiO_2 were mutagenic in V79-4 exposed cells at SU following the NILU SOP. No effect was observed by NILU.

Statistical analysis conducted by QSAR on the V79-4 data revealed high concordance between both partners for plating efficiency (**ANNEX 4 Figure 1 & Table 1 and ANNEX 4 Figure 2 & Table 2**). There did however appear to be higher MF at NILU than at SU when following the SOP for V79-4 cells, which was detected in the measurement of precision by the statistical analysis. The plating efficiency proved to be highly complementary between both labs and is evident in **ANNEX 4 Figure 1**. This good level of concordance was true for both ENMs; ZnO and the TiO_2.

Briefly, ZnO ENMs data showed 40% concordance in precision and accuracy respectively when comparing the ZnO data sets, whereas the TiO_2 data between SU and NILU showed 20% precision and 40% accuracy indicating efforts could be made in future work to improve SOP robustness increasing the likelihood of comparable data.

3.4.3 Summary of Changes to SU RR1 SOP for the HPRT assay with TK6 cells

To ensure that the data generated in RR2 (which will involve 2 partners; SU, NILU) are closer in accuracy and precision a catalogue of amendments was made, and the RR2 SU HPRT assay SOP for TK6 cells is given in **ANNEX 5**, whereby the changes are included as Track Changes.

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3.4.4 Summary of Changes to NILU RR1 SOP for the HPRT assay with V79-4 cells

To ensure the data generated in RR2 are closer in accuracy and precision, the SOP for the HPRT assay with V79-4 cells has also been updated. Several detailed schemes were made to facilitate understanding of several key steps of the assay. The SOPs included also pictures of the colonies obtained in negative and positive control in both PE and MF dishes. As in the SOP for HPRT with TK6, the cleansing step of cells from spontaneous mutants have been also implemented in the SOP with V79-4. Briefly, the step consists of cultivating the V79-4 cells in selective media to remove the spontaneous mutants in locus Tk-/-. The RR2 NILU HPRT assay SOP is attached as **ANNEX 6**.

RR2- data from CuO, Tungsten and MWCNT ENMs (SU and NILU)

During RR2, both partners completed their testing of three ENMs on V79-4 cells. The three ENMs tested were found not to be cytotoxic in both labs, as no significant reduction in cell viability of V79-4 cells was detected after exposure for 24 hours up to a concentration of 100 µg/mL (**Figure 21**).

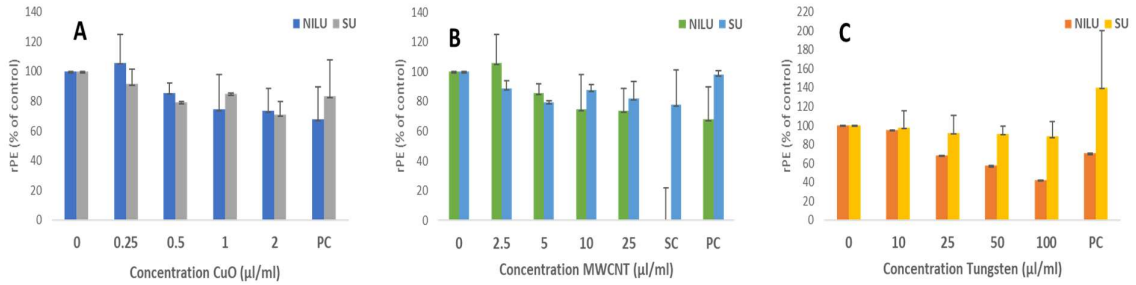

Figure 21. **Summary of cytotoxicity of CuO, (A), MWCNT (B) and Tungsten (C)** measured by the PE assay in V79-4 after exposure up to 100 µg/mL for 24 hours by two partners (**NILU) and (SU**). Bars represent relative plating efficiency, rPE, as a measure for cytotoxicity relative to untreated cells (set to 100 %). SC: Solvent control. The cells were exposed to the PC MMS (0.1 mM) for 3 h.

Two mutant harvests were performed at two different time points. Every harvest included 1-2 parallel treatments for each treatment group. The MF results from the 1st and 2nd harvests are presented (**Figures 22, 23 & 24)** for CuO, MWCNT and Tungsten ENMs respectively. Representative plates with mutant colonies of V79-4 cells are shown in **Figure 25.**

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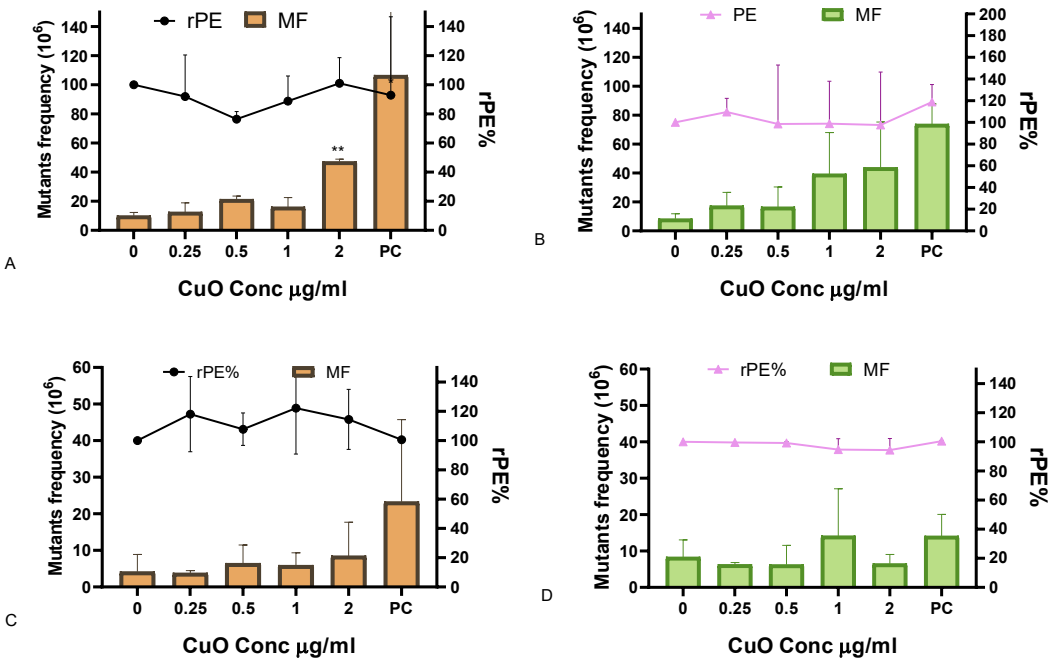

Figure 22. Summary of the effect of **CuO** on induction of HPRT gene mutations in **V79-4 cells reported by SU and NILU.** Cells were treated with four different concentrations of each ENMs including controls. Bars represents data for the mutant frequencies MF% / (1×10^6 viable cells) expressed as mean ± SEM after 1st harvests **SU (A) and NILU (C)** and after the 2nd harvest **SU (B)** and **NILU (D)**. NC: negative control (culture medium); PC: MMS; 0.1 mM, 3h. SEM: Standard error of mean. Asterisks indicate significant different effects compared to unexposed control NC analyzed by ANOVA followed by Dunnet post-hoc test (**$p < 0{,}001$).

At SU, the positive control used was MMS at 0.1mM. The MMS induced significantly higher MF (104 MF / $1x10^6$ viable cells) compared to negative control (10 MF/ $1x10^6$ viable cells). Both negative and positive controls were in accordance with our historical controls. Only the CuO ENMs induced a significative increase in MF compared to negative controls but only at 2 µg/mL. By comparison, at NILU, none of the three tested ENMs induced a significant increase in the number of MF/$1x10^6$ viable cells compared to negative control (NILU). Both MWCNT and Tungsten ENMs were not found to be cytotoxic at the tested concentrations by both partners (**Figure 23 & 24**). The MWCNT ENMs didn't induce mutagenic effect in tests by either partner (**Figure 23),** while the Tungsten ENM was found to increase the level of MF (%) compared to the negative control by SU (**Figure 24).**

We can also observe that the background MF in the negative control as well as the PC is lower in NILU compared to SU. This can be explained due to the cleansing process of cells from the spontaneous mutants.

Overall, during RR2, both partners have improved significantly their knowledge and performance of the HPRT assay. Both partners became more aware of the importance of good standardization and harmonization of protocols used in nanotoxicology. The accuracy between both partners did improve significantly from RR1 to RR2.

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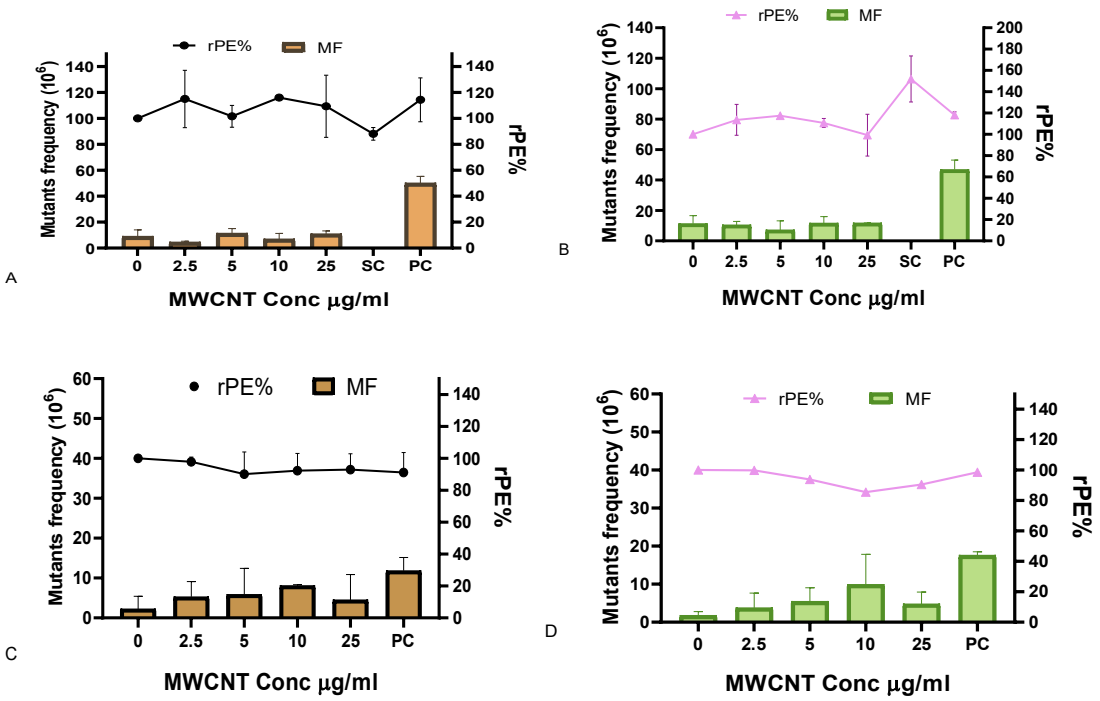

Figure 23. Summary of the effect of **MWCNT** on induction of HPRT gene mutations in **V79-4 cells reported by SU and NILU.** Cells were treated with four different concentrations of each ENMs including controls. Data represents the MF% / (1×10^6 viable cells) expressed as mean ± SEM after 1st harvests **SU (A) and NILU (C)** and after 2nd harvest **SU (B)** and **NILU (D)**. NC: negative control (culture medium); PC: positive control (MMS; 0.1 mM, 3h). SEM: Standard error of mean. Asterisks indicate significant different effects compared to unexposed control NC analyzed by ANOVA followed by Dunnet post-hoc test (**$p < 0{,}001$).

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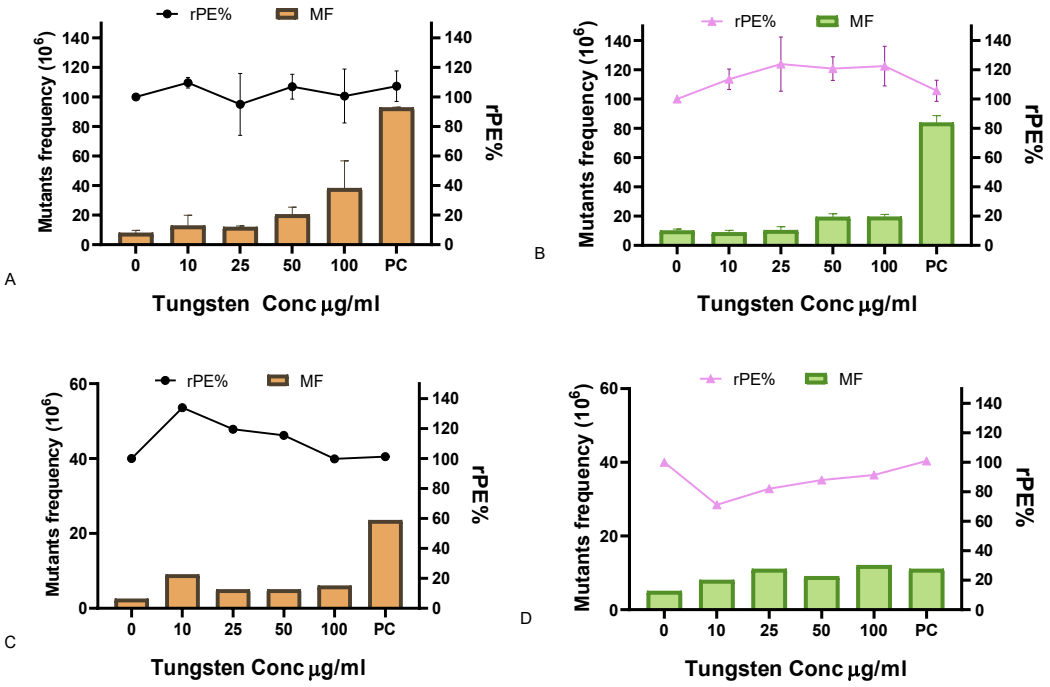

Figure 24. Summary of the effect of **Tungsten** on induction of HPRT gene mutations in **V79-4 cells reported by SU and NILU.** Cells were treated with four different concentrations of each ENMs including controls. Data represents the mutant frequencies MF% / (1×10^6 viable cells) expressed as mean ± SEM, after 1st harvests **SU (A) and NILU (C)** and after the 2nd harvest **SU (B)** and **NILU (D)**. NC: negative control (culture medium); PC: positive control (MMS; 0.1 mM, 3h). SEM: Standard error of mean. Asterisks indicate significant different effects compared to unexposed control NC analyzed by ANOVA followed by Dunnet post-hoc test (**p < 0,001).

3.4.5 Conclusion

The RR1 approach to standardize the SOP for HPRT on TK6 cells has shown good concordance in TK6 data sets pertaining to the potential toxicity of both ZnO and TiO_2. The data generated by both partners showed a high degree of **accuracy** which was confirmed with inter-laboratory statistical analysis. The **precision** of the data sets between both SU and NILU was also assessed with statistical analysis and showed 75% concordance with only the plating efficiency data for ZnO considered significantly different in NILU. The final data sets are being completed for the RR2 ENM exposures, once this is completed the final statistical analyses can be initiated to deduce the inter-laboratory concordance. As indicated by the data generated in RR2 and presented in **Figures 22, 23 & 24** it does appear that we have achieved a harmonized approach to the HPRT assay to be conducted in both TK6 and V79 cells.

From the data generated during RR1 by both SU and NILU, several key outcomes have been determined. Firstly, both SOPs have required modification to provide more clarity to untrained personnel performing the assay for the first time. This mostly pertains to the scoring of the colonies at the end of the experiments, which introduced the greatest degree of variability in both laboratories. Furthermore, extensive training materials were provided in the form of training videos which were used in conjunction with the SOPs during specially RR2. These training videos are audio-visual presentations of the key stages of the assay, including the scoring of colonies.

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At the end of both RRs, the modified SOP for the TK6 HPRT will contain a bank of exemplar images of good colony formation (inclusion criteria) and bad colony growth / dead cells (exclusion criteria). The bank of images will be supported by a training video which will examine the exemplar images and detail the reasons for inclusion and exclusion. Similarly, the SOP for V79-4 HPRT was modified including detailed schemes for each critical step which will be supported by an audio-visual training video of the key stages.

3.5 Micronucleus assay (SU, NILU and ANSES)

3.5.1 Summary

The *in vitro* cytokinesis-blocked micronucleus (CBMN) assay is the gold-standard to investigate chromosomal damage in a broad range of cell lines. The CBMN assay can be used to detect both clastogenic or aneugenic DNA damage, chromosomal breakage or the loss or gain of an entire chromosome following incubation with a test agent. The CBMN assay also provides a direct measure of cytotoxicity by performing cell counts both before and after ENM treatment or by measuring cellular proliferation at the end of the assay. The use of cytochalasin B which inhibits cell division and also internalisation of ENMs following the treatment period was opted for to reduce the total number of cells to be scored. The SOP to conduct this assay was drafted and prepared at Swansea University (SU) (**ANNEX 7**) and distributed to each partner (NILU & ANSES). The SOP has been attached as Annex 7 to this deliverable for further detailed information. Briefly, the SOP is written for human lymphoblast TK6 cells. TK6 cells would be seeded at $1x10^5$ cells/mL and exposed to a range of ENMs (ZnO, TiO_2 and Tungsten carbide-cobalt WC/Co) as well as a positive chemical control, mitomycin-C (MMC). The ENMs were handled and dispersed in accordance with the other assays mentioned above; ZnO was mixed by vortexing, followed by a weight-based calculation of the ZnO concentration in 1mL. The TiO_2 particles were sonicated in accordance with the WP4 sonication procedure. Finally, the Tungsten was dispersed by following the NanoGenoTox dispersion protocol. The concentrations and ENMs were decided on between WP5 partners to provide a comparison to other experiments such as the HPRT and CA respectively. TK6 cells were exposed to test agents for 1.5 cell cycles followed by a 1.5 cell cycle incubation with cytochalasin B. TK6 cell harvesting and fixation was then performed according to the operator preference (manual or semi-automated approach), which was detailed in the SOP. TK6 cells were then scored for the presence of micronuclei in binucleated cells (%Mn/BN) which was used as an indicator of DNA damage. The results from each institution have been summarised below in **Figures 26-27**. All data sets were analysed with one-way analysis of variance (ANOVA) with post-hoc Dunnett's applied.

3.5.2 Presentation of data, summary of data, Data analysis and evaluation

The results of the manual approach to the *in vitro* CBMN assay performed at SU revealed a concentration-dependent cytotoxic response in TK6 cells following 1.5 cell cycle exposure to ZnO ENM. The cytotoxicity induced by the ZnO at the highest test concentration of 20 µg/mL decreased the TK6 cell viability to below 50% and thus scoring of micronuclei was not performed (**Figure 25A**). The TiO_2 ENM did not induce any statistically significant cytotoxicity following a 1.5 cell cycle exposure (**Figure 25B**). The background frequency of micronuclei in binucleated TK6 cells at SU was ~0.5%Mn/BN which did change significantly following exposure to either particle type. However, the Tungsten used in this study to deduce its potential as a positive particulate control in the assay did produce a statistically significant (p<0.05) response at 100 µg/mL, an approximate 2-fold increase over background levels of micronuclei in binucleated cells. Data generated at NILU for the ZnO and TiO_2 ENMs revealed a very similar trend

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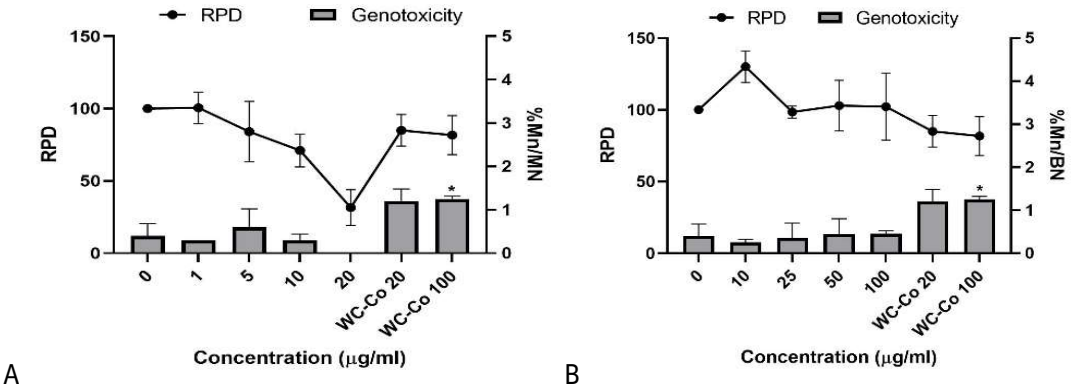
with respect to cytotoxicity of each material. In particular, the response of the TK6 cells to the ZnO ENM at the highest concentration reduced viability to 50% or less (**Figure 26A**). However, the genotoxicity of ZnO did appear to induce statistically significant levels of micronuclei at concentrations of 5- and 10 µg/mL. The data generated at NILU pertaining to the TiO_2 ENM appears to agree with the data generated at SU, showing no cytotoxicity or genotoxicity at any of the tested concentrations (**Figure 26B**).

ANSES also participated in the RR on the MNs assay. ANSES had difficulties with the fixation of the cells and the discrimination between mononucleated and binucleated cells. However, ANSES generated data in the NM assay without cytochalasin B, in a different format and using a protocol routinely used at ANSES. Data not shown and will be included as part of deliverable D5.2.



A B

Figure 25. Cytotoxicity and genotoxicity in TK6 cells exposed to ZnO (A) and TiO_2 (B) ENMs respectively measured at **SU**. Cell viability is represented by relative population doubling (RPD) and DNA damage is shown as the percentage of micronuclei in binucleated cells. MMC was used as the positive chemical control and Tungsten (WC/Co) was used as a particulate control at concentrations of 20- and 100 µg/mL (*n*=2). Data was considered statistically significant (*) when $p<0.05$.

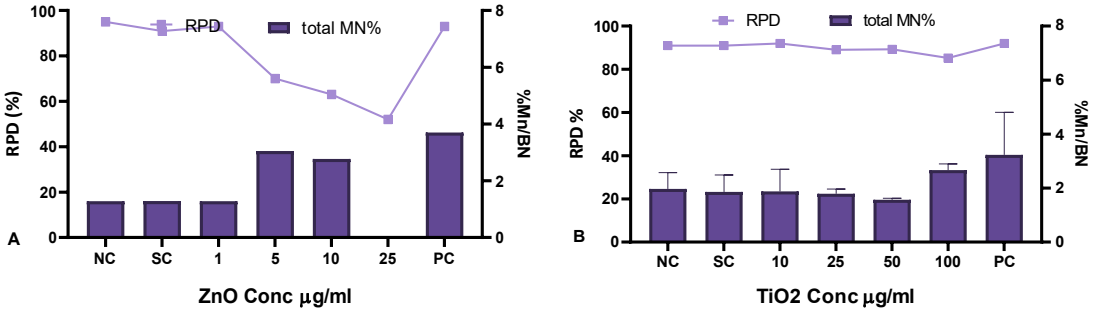


Figure 26. Cytotoxicity and genotoxicity in TK6 cells exposed to ZnO (A) and TiO_2 (B) ENMs respectively measured at **NILU**. Cell viability is represented by RPD and DNA damage is shown as the percentage of micronuclei in binucleated cells. PC, positive control MMC. Data are average of two independent experiments (TiO_2) and one experiment for ZnO ENM. Data was considered statistically significant (*) when $p<0.05$.

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3.5.3 Conclusion

The data generated by SU and NILU for the *in vitro* CBMN assay with TK6 cells appears to be in agreement with respect to the cytotoxicity of both the ZnO and TiO_2 ENMs. Furthermore, both institutions had similar data findings for the positive chemical control, MMC, which generated ~2-2.5%Mn/BN. Furthermore, a high level of agreement was observed for the genotoxicity of TiO_2, suggesting this material is not genotoxic when tested in TK6 cells at concentrations up to and including 100 µg/mL. There does appear to be differences between the data generated on the genotoxicity induced by ZnO whereby at SU there was no significant increase in the frequency of micronuclei in binucleated cells. The data generated at NILU shows concentrations of 5- and 10 µg/mL to potentially increase significantly the amount of DNA damage.

4. Conclusion

The laboratories participating to RRs included both experts as well as inexperienced labs. Knowledge and skills were transferred by training from the experts to the less experienced labs. The consistency of the results were evaluated by statistical analysis by QSAR lab. The overall exercise shows that results are in general more consistent in RR2, and a harmonized approach was achieved for the tests.

Selected SOPs for recommendation to OECD for future development

Based on the work carried out in the course of the RiskGONE project, several submissions to the OECD are planned or in progress.

- A modification of the existing test guideline for the *in vitro* micronucleus (MN) assay. This is already under development led by Swansea University. (Reference: WNT4.95_GD on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of NMs).
- The colony forming efficiency (CFE) assay, applied to cells in culture - the definitive test for cell viability; an SPSF is being prepared for submission in 2022.
- The enzyme-linked comet assay (CA) is the method of choice for detection of base alterations induced by ENMs and other chemicals. An SPSF for an *in vitro* test guideline for this method was already submitted in 2019 and is now being revised for re-submission in 2022.
- The existing test guideline on HPRT mutation assay [TG 476] does not need any modification but the particular requirements for dispersion of ENMs and the need for proof of uptake has to be taken into account. This requirement is already accepted by various regulatory agencies such as SCCS (SCCS 2019).

Relevant SOPs are planned to be published in the Special Issue of Frontiers in Toxicology, 'Methods and Protocols in Nanotoxicology' (https://www.frontiersin.org/research-topics/18580/methods-and-protocols-in-nanotoxicology). These SOPs will specifically address ENMs related issues, including possible interference with test methods.

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5. Deviations from Description of Action-impact/how you cope with them

Due to the COVID-19 pandemic all laboratories have been closed in some cases for more than a year which caused considerable delay in interlaboratory studies. Challenges have been also faced during the RRs with Covid-19 related delays in deliveries of e.g., reagents, cells, culture medium and disposables.

In spite of these challenges the work continued during the period of lock down. The partners evaluated selected protocols, conducted intensive literature searches and at regular teleconferences discussed modification of protocols taking into account specific consideration for ENMs. Two RRs have been successfully completed and results analysed.

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ANNEX 1. Colony Forming Efficiency CFE. NILU SOP

1. INTRODUCTION

Cytotoxic effects of chemicals can be determined by different methods, based on i) membrane integrity (trypan blue exclusion, neutral red uptake) ii) metabolic competence (MTT assay), or iii) relative cell growth (reflecting cell death together with inhibition of cell proliferation). However, the ultimate index of cytotoxicity is loss of cell viability, measured by ability to survive and form colonies, which is the endpoint in colony forming efficiency assay (CFE) (also called clonogenic or plating efficiency assay).

For CFE, mammalian adherent cells in culture, commonly stable cell lines, are applied. There are two options in performing plating efficiency assay, depending on how many cells are treated: a) treatment of cell population in monolayer (the assay is part of the OECD guideline 476; Plating Efficiency 1); b) treatment of individual cells in small inoculum, known as Colony Forming Efficiency (CFE), described in this SOP.

The CFE assay is a label-free method for assessment of basal cytotoxicity. Being non-colorimetric and non-fluorescent, the method is specifically suitable for assessment of nanomaterials (NMs) toxicity *in vitro* to avoid interference with the readout of the test method, which is commonly seen with optical detection methods (light absorption, fluorescence), metabolic assays (chemical reaction between the NMs and the assay components) and enzymatic assays (adsorption of assay molecules (e.g. antibodies, enzymes) on the particle surface) (Kroll et al. 2012; Guadagnini et al., 2015).

The CFE assay has been optimized and standardized for NMs testing by the JRC's Nanobiosciences Unit and validated in the interlaboratory comparison study of the Colony Forming Efficiency assay for assessing cytotoxicity of nanomaterials (Kinsner-Ovaskainen A and Ponti J, 2014).

2. PRINCIPLE

The CFE assay is performed on individual mammalian cells growing in small inoculum attached to a surface. Briefly, A549 cells are plated out in small inoculums,

30 cells per well, on 12 -well plates at 1-2 h. Then, cells are exposed to the test compound, positive and negative controls and cultured to allow for colony formation, generally for 10-12 days. Colonies are stained and counted manually.

Mandatory:

- To calculate CFE (PE), the number of colonies is expressed as % of the number of inoculated cells.

Optional:

- The relative CFE (RCFE) is the ratio of viability of treated cells and negative control cells.

- In addition to the number of colonies, reduced colony size, compared with control, indicate a delay in the cell cycle. Thus, it is possible to distinguish between cytotoxic effects (reduction of the number of colonies formed) and cytostatic effects (reduction in colony size).

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The CFE assay has already been used with different *in vitro* systems to assess cytotoxicity of a wide range of NMs, e.g., gold NMs (Coradeghini et al., 2013), silver NMs (Locatelli et al., 2012), titanium oxide NMs (De Angelis et al., 2013; Fenoglio et al., 2013; El Yamani et al., 2017), zinc oxide NMs (De Angelis et al., 2013; El Yamani et al., 2017), silica NPs (Uboldi et al., 2012), mwCNTs (Ponti et al., 2010), cerium oxide (El Yamani et al., 2017) and silver NMs (El Yamani et al., 2017).

3. MATERIAL AND REAGENTS

(Vendors and products are suggestions and not mandatory, with one exception, i.e., the cell line)

ENMs for RR1: TiO_2 – JRC NM105 (JRC), ZnO – 721077 (Sigma).

Possibly for RR2, 3 and 4 to include also TiO_2 – 637254 (Sigma), ZnO - JRC NM111 (JRC), MWCNT - JRC NM401 (JRC), depending on the results oft he RR1.

Final concentrations of ENMs in DMEM + 10% FBS: 10, 25, 50, 100 µg/ml

ENMs Dipersion: will be done as described in the "NPs dispersion protocol based on DeLoid et al 2017". **NB!** Please use the protocol delivered by WP4 (LIST) - the protocol may be ammended and the latest updated version should be used .

Cells (adherent cell) – A549 from ATCC (www.atcc.org)

Flasks 25 cm^2 or/and 75 cm^2 (Thermo Scientific™ Nunc™ Cell Culture Treated Flasks with Filter Caps)

12-well plates (Thermo Scientific Nunc cell culture plates)

Sterile plastic centrifuge tubes 15 ml + 50 ml

Microcentrifuge tubes (1.5, 5 ml)

Serological pipettes

Pipette tips

Cell culture medium: DMEM + 10% FBS

Fetal Bovine Serum, (26140-079, ThermoFisher)

Penicillin-Streptomycin (15140-122, ThermoFisher)

Other supplements of culture medium required for the specific cell line

Trypsin-EDTA (59429C, Sigma-Aldrich)

1% methylene blue (M9140-256, Sigma-Aldrich)

Phosphate buffered saline (PBS) (ThermoFisher, 10010049)

CO_2

Distilled water

Ethanol

Bürker chamber + Cover slips 22x22 mm/Countess slide

Dimethyl sulfoxide (DMSO), CAS. 67-68-5 (Sigma-Aldrich cat. number D5879)

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Trypan Blue stain 0.4% (ThermoFisher, cat number 15250 or equivalent)

Chloropromazine hydrochloride (C8138, Sigma-Aldrich) dissolved in sterile ultraclean water to a convenient stock solution concentration (e.g., 10 mM) – final concentration 30 µg/ml.

4. PREPARATION PROCEDURES

4.1 Solutions

Preparation of methylene blue (1%)

1g of methylene blue is dissolved in 100 ml of redistilled water. Filter through filtration paper. It is not necessary to sterilize it. Keep stock solution at room temperature.

4.2 Media, culture conditions & stocks

Cells are cultivated in complete culture medium and incubated in culture dishes or flasks in a cell incubator with humidified atmosphere at 37°C, 5 % of CO_2 as described in independent SOPs for cultivation of cells.

4.3 Cell lines and preparation of culture

Human A549 cells, which is an adherent cell line with high cloning efficiency is used for the assay.

Cells are thawed, put into culture medium and cultivated in a cell incubator. The cells should be sub-cultured at least 2-3 times before used in experiments. Cell cultures that reach 50-80 % of confluency in monolayer are suitable for being applied in experiments.

4.4 Exposure conditions and treatment with test substance and controls

Negative control, positive control and 4 concentrations of the test ENM (10, 25, 50, 100 µg/ml) should be applied. It is recommended to include two sets of negative controls (unexposed cells), i.e., right before and right after exposing cells to ENMs, for increased robustness of the test method. For positive control use Chloropromazine Hydrochloride 30 µg/ml.

NB! Total final volume/well is 1 ml.

Individual cells are exposed to the test substance chronically during all growing period (generally 10-12 days). When colonies are visible, they are stained and counted. The experiment should be independently repeated at least once, preferably twice.

5. PROCEDURE

Cells should be taken in the exponential growth phase (50-80 % confluence) in low passage (max P15). Briefly, seed cells in low inoculum about 1-2 h before exposure. 6 replicate exposure wells should be used. The cells are exposed chronically for the length of the experiment. At the end of the experiment the colonies are stained with methylene blue and counted.

After drying, colonies (composed of at least 50 cells) are counted manually.

1. Trypsinize and count the cells.
2. Seed 30 cells per well in 12 well plates. Use 6 replicate wells per treatment.
    a. Prepare dilution of $1x10^5$ cells/ml.

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Re-suspend well by pipetting and/or vortexing.

b. Prepare further $1x10^4$ cells/ml (10x dilution of $1x10^5$ cells/ml)
E.g., 0.1 ml of suspension of $1x10^5$ cells/ml plus 0.9 ml of medium. Vortex.

c. Prepare further $1x10^3$ cells/ml dilution
E.g., 0.1 ml of suspension $1x10^4$ cells/ml plus 0.9 ml of medium. Vortex.

d. Prepare dilution of the number of cells you want per ml. Calculate the volume needed for all wells.

3. Add 0.5 ml of cell suspension in each well to get 30 cells/well. It is important to mix the suspension prior to plating to ensure an even suspension of cells, as well as to spread the cells evenly in the wells. Remember to label both lid and the plate properly to avoid mix-up. Keep the cells in the incubator.
4. Prepare dilutions of the test substance and controls in culture medium immediately after sonication and add 0.5 ml to each well (remember to make 2x concentration since there is already half of medium in the well!)
5. Leave the plates with the cells in the incubator to form visible colonies, normally 10-12 days.
6. When colonies visible by eye are formed in negative control plates, the colonies should be stained with 1 % methylene blue. Add 20 µl of methylene blue into each well, mix and leave for minimum 30 minutes.
7. Remove staining solution into waste bottle.
8. Leave the plates to dry upside down on the bench.
9. Count the colonies.

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

Counting of the colonies

- Each viable cell forms a colony. The colonies should be counted manually, preferably with a cell counter pen. Mark each colony with a pen as you count it to avoid duplication (see figure 1).
- Only the colonies containing more than 50 cells should be counted

Calculate CFE as the number of colonies (in %) relative to the number of inoculated cells following the formula: **CFE (%) = (colonies counted / cells inoculated) x 100**

Data analysis

The numbers are normalized to negative control (cells exposed to fresh complete culture medium, set to 100%) and expressed as relative CFE:

RCFE = (average of number of colonies in treatment plate /average of number of colonies in negative control) * 100.

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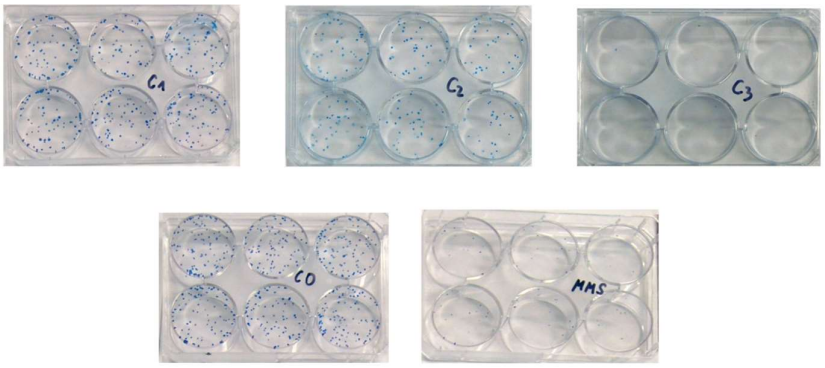

Figure 1: Example of 6 well plates with cell colonies stained with methylene blue. Each independent sample (negative control C0, positive control MMS and tested compound with concentrations C1-low, C2-middle, C3-highest) has 6 parallels.

7. INTERPRETATION OF RESULTS

Acceptance criteria for the experiment:

- Exposure to the positive control must result in significant reduction (50 %) or complete cell death (no colonies in the dish)
- High plating efficiency in negative control, should be comparable to historical control data for the cell line.

Note: Both acceptance criteria must be met for the experiment to be considered valid.

Criteria for characterizing the tested compound as cytotoxic are:

- cell viability (CFE) is reduced by at least 20 % compared to negative control
- a concentration-dependent reduction in cell viability
- reproducible effects

A test substance, for which the results do not meet the above criteria, is considered non-cytotoxic. Statistical significance will not be the only determining factor for cytotoxicity. The biological relevance of the results needs to be considered first. Statistical methods may be used as an aid in evaluating the test results.

A positive result in an *in vitro* mammalian cytotoxicity test indicates that the test substance induces a cytotoxic effect in the cultured mammalian cells used. A negative result indicates that, under the test conditions, the test substance does not induce cytotoxicity in the cultured mammalian cells used.

8. CRITICAL PHASES

- Dilution of cell suspension
- Preparation of test substance concentrations
- Exposure of cells with compound – be sure that right concentration is included into correctly marked plate with cells

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9. IMPORTANT ASPECTS

- Avoid contamination. Work must be performed in sterile conditions in laminar flow hood.
- The temperature of the incubator must be within acceptable range
- Cell counting
- Density (confluence) of cells during cultivation
- Inoculation and spreading of cells for colony formation
- Treatment of cells is critical – concentration preparation, treatment time and washing

10. HEALTH SAFETY AND ENVIRONMENT

All procedures have to be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). Gloves must be worn by operators. Only sterile equipment must be used in cell handling. Discard all the materials used following the appropriate procedure for special biological and chemical waste.

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ANNEX 2. COMET ASSAY. NILU SOP

1. INTRODUCTION

The Comet assay (single cell gel electrophoresis) is a simple method used for measuring DNA damage in eukaryotic cells. 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 as useful method for genotoxicity testing *in vitro* as well as *in vivo*.

2. PRINCIPLE OF THE ASSAY

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 to form nucleoids containing supercoiled loops of DNA linked 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 the DNA is under an electrophoretic field, it tends to migrate towards the positive electrode-anode due to its negative charge. The supercoiled DNA contained in the nucleoids is very compact and its movement is very limited. If a break (in single or double stranded DNA) is present in the DNA loop, the supercoiling is relaxed, and the loop is free to extend under the electrophoretic field and move towards the anode. When DNA is stained with specific dyes and examined microscopically, images resembling comets are seen; the tail consists of loops of DNA that have moved out from the head. 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.
The standard comet assay measures single- and double-strand breaks. Modified version of the assay by inclusion of lesion-specific enzymes can detect specific DNA lesions, such as oxidized purines (using formamidopyrimidine DNA glycosylase (Fpg), 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 bases and leaving an apurinic/apyrimidinic (AP) sites that are converted into a break by the AP lyase/endonuclease which is normally due to the repair enzyme, or to the alkaline conditions of the assay.

A positive finding with the comet assay may not be due to genotoxicity, as toxicity may also be a result of DNA migration due to cell death. Therefore, cytotoxicity testing should always be performed in parallel with the comet assay. to distinguish true DNA damage from apoptosis or necrosis.

3. QUICK OVERVIEW OF THE COMET ASSAY PROCEDURE

1. Cell inoculation
2. Treatment with test substance
3. Cell embedding in agarose
4. Lysis treatment to remove cell membranes and most of soluble cell contents
5. Enzyme treatment (for modified comet assay) (optional)

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6. Alkaline treatment or unwinding of DNA
7. Electrophoresis
8. Neutralization
9. Fixation (optional)
10. Drying of gels
11. Staining of DNA and visualization of the comets
12. Scoring of comets
13. Data analysis

4. MATERIAL AND REAGENTS

(**Vendors and products are suggestions and not mandatory**, unless otherwise requested for specific project, i.e., the cell line vendors and the NMs...)
Glass slides (S8400, Sigma)
Cover slips 22x22mm (VWR, cat number 631-0124), Cover slips 22x60mm (Thermo Fisher Sc,)
Micro centrifuge tubes 1.5ml (Eppendorf)
Sterile plastic centrifuge tubes 15 ml + 50 ml
Pasteur pipettes – 2, 5, 10 ml
Flasks 25 cm^2 or/and 75 cm^2 (Thermo Scientific™ Nunc™ Cell Culture Treated Flasks with Filter Caps)
96-well plates (Thermo Scientific Nunc cell culture plates)
Microcentrifuge tubes (1.5, 5 ml)
Serological pipettes
Pipette tips
Trypsin-EDTA (CAS. 59429C, Sigma-Aldrich)
Phosphate buffered saline (PBS) (Thermo Fisher, 10010049)
Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. number D5879- CAS. 67-68-5)
Trypan Blue stain 0.4% (Thermo Fisher, cat number 15250)
Agarose - Electrophoresis grade (Fluka, cat number 05066)
Agarose - Low melting point (LMP) (Sigma-Aldrich, cat number A9414)
Triton X-100 (Sigma-Aldrich, cat number T8787)
SYBr Gold (Thermo Fisher S11494)
Bovine serum albumin (BSA) (Sigma-Aldrich, cat number A9418)
Distilled water
Ethanol
$CaCl_2$
$MgCl_2$
H_2O_2, 30%; (Sigma-Aldrich, cat number 31642-M)
NaOH (Mw = 39.99)
EDTA (Mw = 372.24)
Tris (Mw = 121.14)
NaCl (Mw = 58.45)
KCl (Mw = 74.55)
$Na_2HPO_4.12H_2O$ (Mw = 358.141)
HEPES (Mw = 238.30)
KOH (Mw = 56.11)
MMS (CAS. M4016_ Sigma Aldrich),
Potassium Bromate KrBO3 (Cat. 34268_Fluka),

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5. EQUIPMENT

Laminar Flow Hood
Light Microscope
Countess / Bürker chamber with cover glass
Pipettes, Automatic pipettes and multi channel pipette (optional)
Microwave
CO_2 incubator
Centrifuge
Water bath or heat block
Vortex mixer
Incubator 4ºC
Electrophoresis equipment with power supplier
Fluorescent microscope (with CCD camera)
Software for image analysis (e.g Perceptive Instruments or Metasystem)

6. PREPARATION PROCEDURES

This section explains the procedure of preparation of solutions and material needed for the comet assay, including pre-coating of slides, and preparation of Fpg enzyme, cell cultures, test substance and controls.

6.1 Preparation of solutions

6.1.1 Preparation of Low melting Point Agarose (LMP) solution

The LMP solution is made in PBS and the concentration of LMP can vary between 0.6 and 1 % depending on the cell type and genome complexity. For instance, lower % of LMP can be recommended when working with plants. When working with cell cultures, we recommend to use 0.8% LMP. The LMP can be prepared in batches and stored at 4 °C in fridge.

For preparation of 0,8 % LMP: Dissolve 0.8 g LMP agarose in 100 ml PBS.

After weighing the agarose and adding the PBS, melt the solution by careful heating it 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 per bottle/falcon tube) and keep at 4 ºC.

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6.1.2 Stock solutions

Compound	Concentration	CAS	Molecular weight (g/mol)	Quantity in 0.5L distilled H_2O	Quantity in 1 L distilled H_2O	Storage
NaOH	8 M		40.00	160 g	360 g	Room temperature
Na2EDTA	0.2 M	6381-92-6 SIGMA	372.24	37.2 g	74.4 g	4-8°C
Tris base	4 M	77-86-1 CALBIOCHEM	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

6.1.3 Lysis solution

Compound	Concentration	CAS	Molecular Weight (g/mol)	Quantity for 2 L in distilled H_2O	Quantity for 1 L in distilled H_2O	**Alternatively:** take from stocks to make 1 L in distilled water
NaCl	2.5 M	7647-14-5 SIGMA	58.44	292.20 g	146.1 g	
Na2EDTA	0.1 M	6381-92-6 SIGMA	372.24	74.45 g	37.23 g	
Tris base	0.01 M	77-86-1 CALBICHEM	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 4ºC.						

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6.1.4 Enzyme reaction buffer for Fpg (BF)

Compound	Concentration	CAS	Molecular weight (g/mol)	Quantity/ L	10x (0.5 L)	10x (1 L)
HEPES	0.04 M	7365-45-9 Sigma	238.30	9.53 g	47.66 g	95.32 g
KCl	0.10 M	7447-40-7 Sigma	74.56	7.46 g	37.28 g	74.56 g
EDTA	0.0005 M	6381-92-6 Sigma	372.24	0.19 g	0.93 g	1.86 g
BSA	0.2 mg/mL	9048-46-8 Sigma		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, aliquoted e.g 50 ml per tube and kept in freezer at -20°C. The day of use: thaw one vial and dilute in cold water (50 ml BF+450 ml cold water)						

6.1.5 Electrophoresis buffer solution (Elfo)

Concentration	Compound	Quantity for 1L	Quantity for 2 L
0.3 M	NaOH	12 g	24 g
0.001 M	Na_2EDTA	0.37 g	0.74 g
pH should be measured and should be > 13 after mixing the ingredients without adjustment. Keep at 4ºC.			

6.1.6 Preparation of PBS solution

- 8 g/l NaCl
- 0.2 g/l KCl
- 1.15 g/l Na_2HPO_4
- 0.2 g/l KH_2PO_4

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.

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6.1.7 TRIS-EDTA stocks - TE buffer for dilution of SYBERGold

Concentration	Compound	CAS	Molecular weight (g/mol)	Quantity for 2 L in distilled H_2O	Quantity for 1 L in distilled H_2O	**Alternatively:** take from stocks to make 1 L in distilled water)
2.5 mM	Tris base	648310 CALBIOCHEM	121.10	0.604 g	0.302 g	625 µl 4M Tris- pH 7.5
4 mM	Na2EDTA	6381-92-6 SIGMA	372.24	2.976 g	1.488 g	400 µl 0.2M Na2-EDTA (pH 8)
						Add 870 ml ddH_2O
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).						

6.1.8 SYBR®Gold (SG) for staining

Thaw the purchased stock and make aliquots of 50 µl to be stored in freezer at -20 ºC.

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 -20ºC in box labeled 10X SYBER Gold.

6.2 Pre-coating of slides

When using microscope slides, it is recommended to use ordinary clear glass slides pre-coated with normal melting point agarose (NMP). 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**. Set a water bath at 55°C or a thermo-block heater. Prepare **0.5%** NMP solution in water (e.g., 99.5 ml water + 0.5 g NMP). Melt the agarose in the microwave. Make sure all the agarose is well dissolved and the solution is transparent. Fill a suitable vessel (Coplin jar or a narrow beaker) with the warm agarose solution and place in a water bath at 55°C for approximately 15 minutes.
2. Dip one slide vertically in the solution of agarose 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 horizontally on the bench to dry overnight.
4. Mark the coated side with a pencil mark in one corner on the frosted end (e.g., top left) to identify the coated side.
5. Dried, pre-coated slides can be stacked together, packed in slide boxes and stored (years).

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6.3 Restriction enzyme formamidopyrimidine (Fpg) preparation

For the preparation of Fpg enzyme, follow always updated instruction by supplier (Norgenotech).

The enzymes Fpg is 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.

Usually, the enzyme produced is concentrated and will need further dilutions. W**e follow general steps as below unless it is instructed differently from the producer**:

- 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.

6.4 Cell culture

Cells used for testing chemicals or nanoparticles for genotoxicity should be used ideally after passaging them 3-4 times, depending on the cell cycle of the specific cell line used. 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 60-80% confluent.

6.4.1 Cell culture composition

NOTE. Same cell culture medium (complete media) is used for cell cultivation as for the exposure. UNLESS OTHERWISE INSTRUCTED

Cells are cultured in complete culture medium and incubated in culture dishes or flasks in humidified atmosphere at 37° C, 5% CO_2. See SOP for cultivation of each specific cell line used for more details. For example for:

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A. A549 cells: cells grow in DMEM D6046 (low glucose with 4 mM L-glutamine) (Sigma), **9 % Fetal Bovine Serum (FBS)** (26140-079, ThermoFisher), 100 U/ml penicillin/100 µg/ml streptomycin solution (15140-122, ThermoFisher).

B. TK6. Cells grow in RPMI 1640 without glutamine (**31870**, GIBCO®, Life Technologies), 9 % Horse Serum (**16050122,** GIBCO®, Life Technologies or **H1138**, Invitrogen), L-Glutamine 200mM (25030-024, GIBCO®, Life Technologies), 100 U/ml penicillin/100 µg/ml streptomycin solution (15140-122, ThermoFisher).

6.4.2 Seeding of cells for exposure

The number of cells to be seeded per well, depends on the layout used, the surface area of the wells and the purpose of the study.

As an example: If you are working with 96 well format layout (Well surface area 0.32 cm^2) and using:

- A549 cell line: Seed **15.000 cells/well** in 96 well plate **(day before exposure), volume of medium per well 200 µL (YOU CAN USE ALSO 100 µL and add your treatment 2x concentrated in 100 µl)**
- TK6 cell line: **Normally we seed same day of exposure since cell don't need to be attached. Seed 15.000-20.000 cells/well in 96** well plate **(day of exposure)**. **Final volume will be 200 µL.**

NOTE. When cells are in suspension, always consider the volume of the treatment to be added to the cells to achieve your desired final volume and concentration.

Example: Seed cells in **100 µL per well and add the treatment twice concentrated in a volume of 100 µl.**

NOTE. If you start an early exposure, it is recommended also to seed your cells e.g., A549 in the morning the day before to be sure to reach 60-80% confluency before exposure. If you seed cells very late the day before exposure, you might have low confluency the day of exposure. This also depends on the cell doubling time of the cell line used.

Details about the number of wells needed for the experiment, and number of experiments to be performed, can be found in section 6.1.

6.5 Negative controls (NC) and solvents (SC)

In general cells in culture media serves as negative control (NC) and cells exposed in cell culture media to the solvent for the test substance as solvent control (SC).

6.6 Positive controls (PC)

Positive control (s) (PC) should be always included. The selected positive control is based upon the criteria of the specific study. As a reference standard for the comet assay, **Methylmethane Sulfonate MMS, hydrogen peroxide H_2O_2 and potassium bromate $KBrO_3$** are recommended. The use of cells

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exposed to Ro-photosensitizer plus light is not mandatory but advisable as PC for Fpg enzyme treatment.

1. **MMS** 0.1-0.2 mM *for 3 and 24h*
2. **$KBrO_3$** potassium Bromate (an alternative for oxidised purines control as a strong oxidizing agent) (1-4 mM for 3h and 24h).
3. ***H_2O_2*** 100 µM in PBS as an external control for detection of strand breaks.
4. ***RO 19-8022 (photosensitizer)*** for detection of oxidised purines

List of Positive controls (See examples how to make):

Compound	**Solvent**	**Diluted in**	**Recommended stock concentration**	**Final concentration to be applied**	**Exposure time**
MMS	DMSO	Cell media	1 mM	**0.1-0.2 mM**	3h and 24h
KBrO3	dH_2O	Cell media	10 mM	**1-4 mM**	3h and 24h
H_2O_2	-	PBS	100 mM	**100 µM**	5 min in Jar
RO19-8022	Ethanol	70% Ethanol +PBS	1 mM	**1-2 µM**	5-8 min

6.7 Test substances

Chemicals and Nanoparticles

When testing nanoparticles, there are steps to be followed. Depending on the state of the particles (powder or suspension) some steps such as dispersion and sonication may be required. Two dose metrics are usually followed, either µg/cm^2 or µg/ml. See Annex 3 for the list of main range of concentrations used for NMs and the conversion from µg/cm^2 to µg/ml or vis versa for each layout.

NOTE. When testing nanoparticles, potential interference should also be investigated. Remember to always keep a leftover or an amount of the tested NM to use for interference control.

6.8 Selection of test substance concentrations

Always test at least 4 concentrations of the test substance. (4 concentrations of the test substance+NC+PC+SC)

- Concentrations to apply: If the compound is cytotoxic, include at least one cytotoxic concentration (giving no more than 40 % cytotoxicity), and minimum 3 non-cytotoxic concentrations.

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NOTE. Keep on mind, when using 96 well plate format, if the compound is not cytotoxic, the highest concentration tested, considering the metrics mass/area and a final volume of 100 µl, should be 100 µg/cm^2 which is equivalent to 350 µg/ml.

If the final volume for exposure is 200 µl, then the highest will be 160 µg/ml.

7. COMET ASSAY PROCEDURE STEP BY STEP

7.1 Experimental design

A pre-designed experiment is important. NC, SC and PC and at least 4 concentrations of the test substance should be applied. It is recommended to use both short (3h) and long (24h) exposure. It is recommended to include two wells per treatment. The experiment should be repeated 2 times (3 independent experiments with duplica treatment within each experiment and also replicate slides), and it is recommended, if possible, to include replica gels per slide in each experiment to increase the robustness of the results.

NOTE. **Positive controls should be placed in a separate plate or with gaps from the other samples, to avoid any cross contamination. See template annex 2.**

7.1.1 Before exposure (Day 0)

- Ensure there are pre-coated slides available
- Ensure that all solutions needed are available including *cold* H_2O
- In case of use of adherent cells (e.g A549), cells are trypsinized and counted. The cells are seeded 15.000 cells per well (total volume of complete culture medium 200 µl) in 96-well plates the day before exposure (ideally 1 plate for 3h and 1 plate for 24h), duplicate wells pr treatment. The cells are kept in the incubator at 37°C, 5% CO_2.
- In case of suspension cells (e.g TK6), they can be seeded on the same day of exposure Day 1, at density of 10,000 cells/well.

7.1.2 The day of exposure (Day 1)

The test substance is prepared as described above. Cells seeded in duplicates are exposed to the selected concentrations including positive controls, solvent and negative control and placed back to the incubator 37°C, 5% CO_2, for the required time.

Sometime before end of the exposure, mix the lysis solution with Triton-X (1%) as described above. Keep the final lysis solution in fridge until use (at least 30 min before use). **Make sure it is cold 4°C.** Set a water bath or thermoblock heater **at 37°C.** Take one aliquot of the LMP agarose from the fridge and carefully melt it in the microwave until it is completely dissolved, following the same procedure as described above (section xxx). Do not boil it! **place the melted LMP in the pre-warmed bath or thermo-block until use.** Make sure the LMP is at the right temperature before adding it to the cells. **Make sure it is covered so it doesn't evaporate or solidify quickly.** Work quickly when making gels as the agarose polymerizes fast.

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Label the already precoated slides accordingly following your template. This can be done any time before you start your experiment. The slide label should be readable and understandable. When using blind codes for labeling, make sure the right codes are saved and accessible. Make sure you include date on each slide.

7.1.3 End of exposure and preparation of slides with gels (Day 1 or 2 depending on length of exposure)

When exposure time is finished, stop exposure by removing the treatment and washing the cells. In case of adherent cells. A step of trypsinization is needed:

- Remove the medium with the tested substances
- wash twice with PBS and trypsinise
- Add culture medium to each well to stop trypsin. E.g 100-200 µl
- After gently mixing, **take a volume of cell mixture in new empty well**.

If working with 96well plates and if seeding was 15.000 cells/well, take 50 µL. This is indicative and may vary depending on seeding number of cells and their doubling time.

In case of cells in suspension (e.g TK6): at the end of exposure, you can either collect the samples into new tubes and centrifuge, centrifuge the whole 96 well plate or directly take similarly as before the volume needed to be mixed with LMP, especially when working with nanoparticles.

7.1.4 Embedding of cells in LMP

At the end of exposure, embedd the cell into gels by mixing the cell suspension with the LMP agarose and put the gel drops on top of each precoated slide.

Depending on the size of the drops wanted, the number of cells needed can vary. In general, ca 5-10 x104 cells should be appropriate number to mix with agarose, which will give approx 200-300 cells/5µl gel.

Add LMP 0.8% agarose which is already at 37°C to every well (tube) with the cell mixture at ratio 1:4 agarose, (e.g. 50 µl cell suspension and 200 µl of LMP).

From each well, 1 gel on top of pre-coated slide is made, preferably on 2 replicas slides.

In total 2 slides are made for the standard comet assay (labeled lysis) and 2 slides for the modified comet assay with enzyme (labeled Fpg). NB. The slides should be labeled with the name of the tested material code, lysis or Fpg, number of replicate (1 or 2) and date.

NOTE 1. We are not counting cells after end of exposure when using 96 well format and specially when testing nanomaterials. Our calculation is based on the number of cells we have seeded before exposure and the number of cells we are expecting to get at the end of the treatment.

NOTE 2. Keep on mind that after 3 hours exposure the number of cells seeded is basically the same. So, the proportion 1:4 LMP should be ok. BUT after 24h, the number of cells may increase, which means we don't have the same number of cells as we started with. In this case, we will estimate differently the number of cells to take from each well. In case of A549, we can observe under the microscope how confluent the cells are and make a judgment. We

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assume that in general the cells double after 24h (except in case of toxic substance where apoptosis can happen). So, if the confluency of cells is higher than the day of seeding, we take half number of cells to mix with agarose. For example, if after 3h we are taking 50 µL, after 24h we will take 25-30 µl and mix the same way with LMP. Use the same cell mixture/LMP-ratio for the whole experiment (and also note it).

7.1.5 Test for NM interference

Testing for interference with the test method is a requirement when testing NMs due to their phys-chem properties which may cause NMs to interfere directly or indirectly with the test method. Potential interference may occur in comet assay, especially when testing relevant nanoparticles (e.g., TiO_2, nanogold). The interference may happen either by 5 hypotheses:

1. Direct/physical interference of the nanoparticles with the DNA (after lysis) creating additional breaks or adducts.
2. reducing or blocking the DNA migration during electrophoresis
3. Inhibition/interaction with Fpg activity
4. Quenching /autofluorescence during quantification of signals/scoring

5. Interference of photosensitive particles with direct light (Hanna Karlsson *et al.,* 2015) causing changes in the particles (e.g., increase reactivity/increase effect).

Therefore, it is recommended to include interference controls always when testing a nanomaterial (NM) for the first time in the cell line (not needed to include in all experiments if no interference is detected). It is not required to test same NM for interference for each cell line, as the interference is mainly to occur between the NMs and the component of the assay (reagents, fluorescence, signal).

Always include duplicate interference control exposures:

i. For the 1st hypothesis: Mix highest and middle concentration of the NM with negative control cells (trypsinize the cells and mix thereafter) and embed into gels immediately. Work fast! (the results %DNA in tail will be compared with the results for the negative control cells).

ii. For the 2nd hypothesis: Take a part of cells exposed to PC and mix with the highest and middle concentration of the NM and embed into gels. (The results %DNA in tail will be compared with the results for the positive control cells).

iii. For the 3rd hypothesis: Interference control for Fpg enzyme: Mix the NM in question with Fpg before incubating the embedded Ro-exposed cells or any embedded PC in humid box at 37°C. Compare the effect (%DNA in tail) with calibration curve for Fpg on RO-cells for example or with any positive control for oxidative damage).

iv. For the 4th hypothesis: Interference control for quenching for fluorescence (SyberGold): Mix cells from the PC directly with the highest concentration of NM and compare the PC results (%DNA in tail) with the results for the PC+NM mixed directly).

v. For the 5th hypothesis: Photosensitive materials, like TiO_2: Work under red light or switch off the light when doing exposure and embedding of gels.

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Note. There is no exposure time when performing interference.

How is this done?

After you detach cells from negative control or/and positive control, take a specific volume of cells and mix with a volume of NM tested. The idea is to achieve the highest concentration in the mixture before adding LMP 1:4. Make 2 gels per slide, 2 slides - one for lysis and one for Fpg - and you follow same steps as for the other slides.

7.1.6 Immersion of slides in lysis solution (Day 1 or 2)

Lysis for incubation should be already prepared with Triton-X and at 4°C. Place the slides in lysis solution in a (vertical) staining jar. Leave at 4°C for overnight. (You can also leave in Lysis for a specific time at least one hour **but you need to be consitant and always keep lysis duration the same within same project/set of experiments).**

7.1.7 Enzyme treatment

Fpg enzyme (formamidopyrimidine DNA glycosylase)

1. Get enzyme from the freezer (keep on ice).
2. Wash the slides labeled for buffer and Fpg 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 µl of enzyme solution (or buffer alone, as control) on top of each slide, and cover with thin plastic foil for 12 gel format and for 2 gel format place 50 µl of enzyme onto each gel and cover with coverslip.
5. Put slides into humid box (prevents desiccation) and incubate at 37°C for 30 min with Fpg enzyme.
6. At the end of incubation, gently take the humid box, remove the thin plastic foil or the cover slip.
7. 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.

As an alternative, you can incubate your slide in bath with Fpg by placing your slide in jar or box filled with the Fpg, but this can require a high amount of Fpg to be used.

NB. When not using enzyme, you skip this step and go straight to unwinding step after the lysis.

7.1.8 Unwinding in alkaline solution (20 min)

1. Place the slides in the electrophoresis tank, side by side, and add cold alkaline electrophoresis solution to cover the gels (**use always the same volume**).

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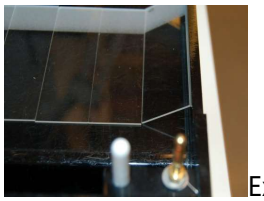
2. Fill gaps on the platform with empty slides. Make sure that tank is levelled
3. Incubate for 20 min at 4°C

 Example of how to fill in the gap.

7.1.9 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 **1 V/cm** for 20 min.
3. Electrophoresis should be run at 4°C in a cold room or a fridge.

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 1 V/cm is not reached, remove some solution by a pipette.** Normally the current is around 300 mA but this is not crucial.

Set the voltage and leave the electrophoresis running for the desired period of time by following **V/cm*min = 20**

[Optional] The best way to measure the voltage is using a voltmeter. The current (mA) should be as high as practical on the power supply. Check the link below for more information about how to measure the voltage for comet tanks. **https://www.youtube.com/watch?v=kvgZ7O25kXo&feature=youtu.be**

7.1.10 Neutralisation

1. After electrophoresis, gently move the slides into jars with cold PBS and wash for 5 minutes.
2. Wash the slides in jar with cold dH_2O for 5 minutes.
3. Dry the slides horizontally laying at room temperature (normally overnight).
4. If fixation step is to be used, don´t dry the slide but from point 2 move to fixation.

After this step, slides can be kept for months at room temperature (RT) as long as they are protected from light and dust.

7.1.11 Fixation (Optional)

A fixation step in ethanol after neutralisation can be used if desired.

1. Transfer the slides into 70% ethanol and incubate for 15 min at RT.
2. Transfer slides into absolute-ethanol and incubate for 15 min at RT.

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3. Leave the slides to dry at room temperature (RT) until next day. After this step, slides can be kept for months at RT as long as they are protected from light and dust.

7.1.12 Staining of gels

The gels must be dried before staining.

We recommend the dye SYBr Gold (0.1 µl/ml in TE buffer), but DAPI (1 µg/ml DAPI solution in distilled H_2O) or EtBromide may also be used. Staining solutions are stored in the -20°C freezer.

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 the box in the freezer and add 995 µl 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 mini-gels.
3. Leave in dark for 5-10 min before visualization in the fluorescence microscope.

NOTE. Do not freeze the working solution.

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

7.1.13 Visualisation and scoring

Slides are analyzed by fluorescence microscope (eg. Leica and objective 10x) using computer image analysis system e.g. Comet assay IV (Perceptive instruments) or any system for comet scoring e.g Metasystem, by scoring at least **50 cells per gel (2 gels per treatment group).**

Perceptive is a software which, linked to a closed-circuit digital camera mounted on the microscope, automatically analyses individual comet images. The program is 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.

To perform the scoring of the slides follow these recommendations:

* Anomalous comets at the edges or near a bubble, if present, should not be evaluated

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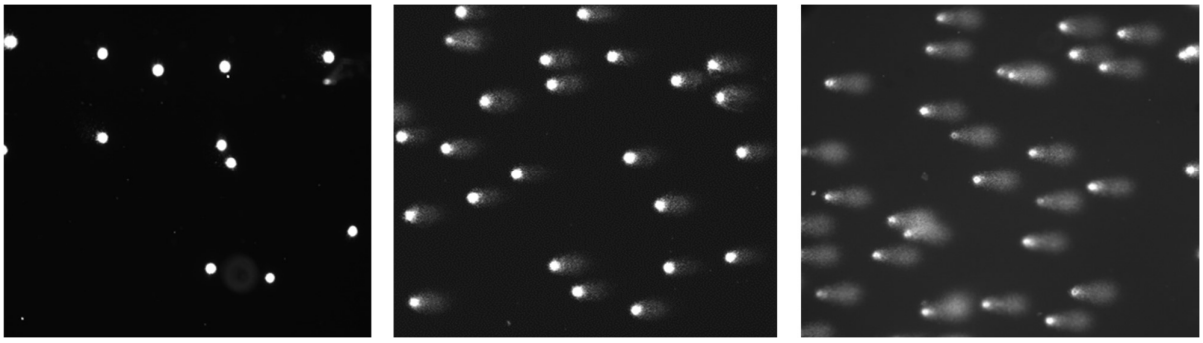

Images of comets after staining with SyberGOld.

* Highly damaged comets containing almost all the DNA in tail should be analysed

Hedgehogs (ghost cells) are cells consisting of small or non-existing head, and large diffuse tails and are considered to be heavily damaged cells, although the etiology of hedgehogs is uncertain. They may be evaluated separately if needed. |

7.1.14 Presentation of the data

At the end of image analysis, you create your data base:

- Calculate the median of the +/- 50 comets (% DNA in tail).
- Then calculate the **mean of medians** and SD (for the replica gels of the same concentration/sample within the same experiment). This value (mean and SD) is the mean DNA damage (measured by median % DNA in tail) of your replicas in the sample.

7.2 Acceptance Criteria

The acceptance criteria of the comet assay are based on the following:

- Valid negative control (section 8.1.1)
- Valid positive control (section 8.1.1)
- Adequate number of cells and concentration have been analysed.
- The criteria for selection of the highest concentration of the test substance is met.
- Quality control of test system *(mycoplasma test) is shown to be negative*

7.3 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
- Electrophoresis duration/voltage

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8. 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)
- Reduce light exposure

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*1. If we fix the conc/cm2_*

		96-well plate	6-well plate	12-plate	petri dish	unit
Maximum well volume						ml
Well area		0.320	9.600	3.800	100.000	cm2
Volume to add to each well		0.200	2.000	1.000	10.000	ml
Area-concentrations (microg/cm2)		**volume-concentrations (micrg/ml)**				
0	µg/cm2	0.00	0.00	0.00	0.00	µg/ml
0.01	µg/cm2	0.02	0.05	0.04	0.10	µg/ml
0.03	µg/cm2	0.05	0.14	0.11	0.30	µg/ml
0.05	µg/cm2	0.08	0.24	0.19	0.50	µg/ml
0.1	µg/cm2	0.16	0.48	0.38	1.00	µg/ml
0.3	µg/cm2	0.48	1.44	1.14	3.00	µg/ml
0.5	µg/cm2	0.80	2.40	1.90	5.00	µg/ml
1	µg/cm2	1.60	4.80	3.80	10.00	µg/ml
3	µg/cm2	4.80	14.40	11.40	30.00	µg/ml
5	µg/cm2	8.00	24.00	19.00	50.00	µg/ml
10	µg/cm2	16.00	48.00	38.00	100.00	µg/ml
25	µg/cm2	40.00	120.00	95.00	250.00	µg/ml
30	µg/cm2	48.00	144.00	114.00	300.00	µg/ml
50	µg/cm2	80.00	240.00	190.00	500.00	µg/ml
75	µg/cm2	120.00	360.00	285.00	750.00	µg/ml
100	µg/cm2	160.00	480.00	380.00	1000.00	µg/ml

1. If we start with the conc/ml

		96-well plate	6-well plate	12-plate		unit
Maximum well volume						ml
Well area		0.320	9.600	3.800	100.000	cm2
Volume to add to each well		0.200	2.000	1.000	10.000	ml
Area-concentrations (microg/ml)		**volume-concentrations (micrg/cm2)**				
0	µg/ml	0.00	0.00	0.00	0.00	µg/cm2
0.01	µg/ml	0.01	0.00	0.00	0.00	µg/cm2
0.03	µg/ml	0.02	0.01	0.01	0.00	µg/cm2
0.05	µg/ml	0.03	0.01	0.01	0.01	µg/cm2
0.1	µg/ml	0.06	0.02	0.03	0.01	µg/cm2
0.3	µg/ml	0.19	0.06	0.08	0.03	µg/cm2
0.5	µg/ml	0.31	0.10	0.13	0.05	µg/cm2
1	µg/ml	0.63	0.21	0.26	0.10	µg/cm2
3	µg/ml	1.88	0.63	0.79	0.30	µg/cm2
5	µg/ml	3.13	1.04	1.32	0.50	µg/cm2
10	µg/ml	6.25	2.08	2.63	1.00	µg/cm2
30	µg/ml	18.75	6.25	7.89	3.00	µg/cm2
25	µg/ml	15.63	5.21	6.58	2.50	µg/cm2
50	µg/ml	31.25	10.42	13.16	5.00	µg/cm2
75	µg/ml	46.88	15.63	19.74	7.50	µg/cm2
100	µg/ml	62.50	20.83	26.32	10.00	µg/cm2

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

ANNEX 3. QSAR Statistics summary for comet assay data RR1

WP 5. Statistical analyses for the experimental toxicological data (Comet assay)

1. Available date and purpose of the analysis

Two nanomaterials (TiO_2-JRC (064) and ZnO-Sigma (063)) were analysed using comet assay SOP for two cell lines: A549 and TK6 and in two time points: 3h and 24h. Data comes from three labs NILU, ANSES, KU Leuven. Each lab provided two or three independent experiments for each ENM, cell line and time point.

Analysed endpoints were:

Level of DNA strand breaks (SBs)

Formamidopyrimidine DNA glycosylase (Fpg)

(NetFpg)

The statistical analysis was used to determine if the obtained measurements between three labs (NILU, ANSES, KU Leuven) are consistence.

A single analysis consisted of comparing results between two labs in every possible combination: NILU-ANSES, ANSES- KU Leuven, NILU- KU Leuven.

The mean values of the conducted experiments (two or three) for each concentration and available test control were used for the statistical analysis. If data for any of the concentrations or tests were not provided for any of the laboratories, they were not taken into account in single analysis.

The statistical analysis included determining the precision and accuracy of the obtained measurements.

Precision is the degree to which future measurements or calculations yield the same or similar results — it is a measure of the spread of repeated measurement results and depends only on the distribution of random errors – it does no indication of how close those results are to the true value. To compare the precision of two independent measurements obtained during the analysis of samples with the same level of analyte content, we use the F-Snedecor test. The critical value for the cases under consideration depends on the number of tested concentrations for each particular experiment. Its values are presented in Table 1.

Accuracy refers to the degree of conformity of a measured or calculated quantity to an actual (true) value. Accuracy is closely related to precision, but it's not the same thing. A result is said to be accurate when it matches to a particular target. In the analysis, we investigated the differences between the experiments performed in two labs with a pairwise t Student's test for each ENM concentration. As noted previously, critical values depend on the number of tested concentrations and are presented in the Table below.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Table 1. Critical values of the F distribution and t distribution for different degrees of freedom (significance level = 5%).

Degrees of freedom	F distribution	t distribution
2	19.00	4.3029
3	9.28	3.1824
4	6.39	2.7764
5	5.05	2.5706
6	4.28	2.4469
7	3.79	2.3646
8	3.44	2.3060

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Comet assay analyses

a. TiO_2

- ***A549 cell line***

The average values for each analysed endpoint TiO_2 using A549 are presented in the Figure 1. The data points for different concentrations/control of TiO_2 using A549 were available only for NILU and KU lab, therefore the analysis was performed only for those labs.

The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the Table 2.

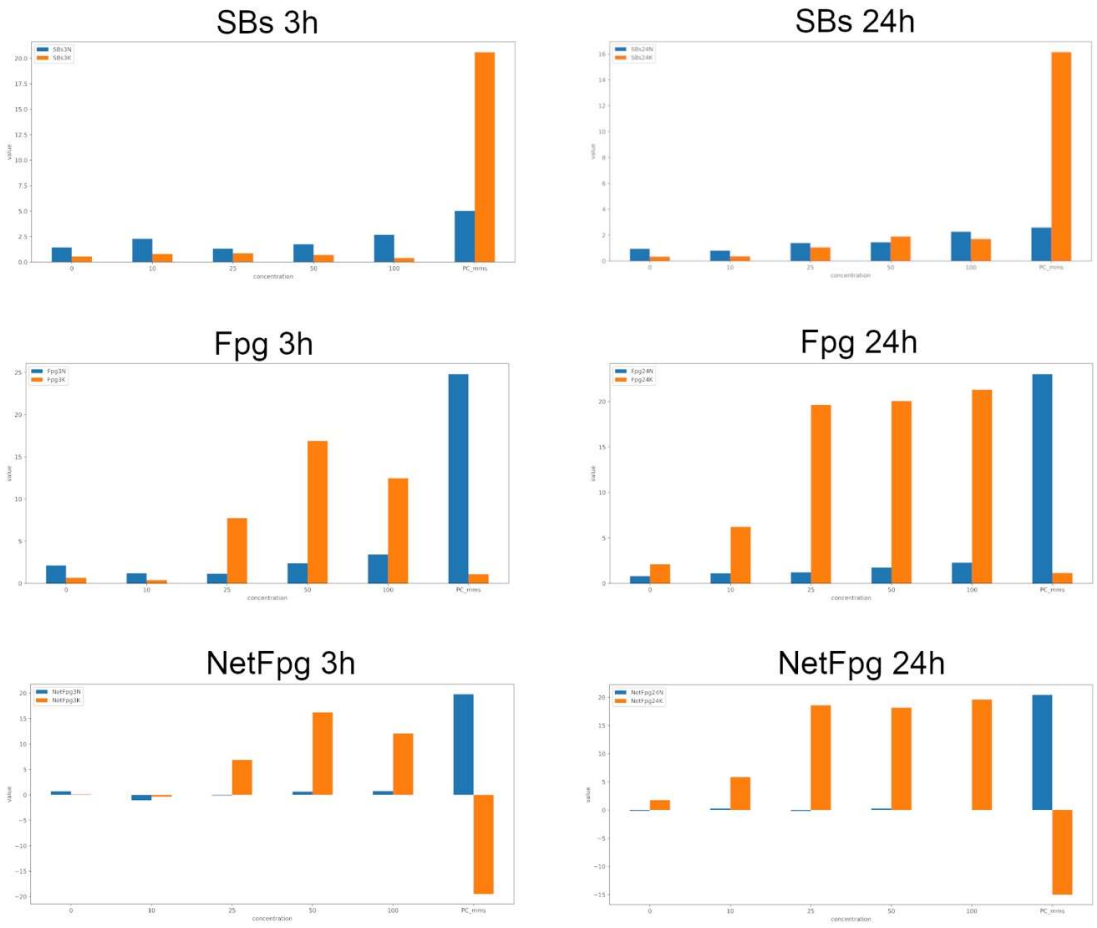

Figure 1. The average values for analysed endpoints for TiO_2 using A549. Blue colour corresponds to the values obtained in NILU lab whereases the orange line to KU Leuven.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Table 2. Comparison of the statistical parameters from measurements from different labs.

		NILU_ANSES						ANSES_KU						NILU_KU					
		SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24	SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24	SBs_3	Fpg_3	NetFpg_3	SBs_24	Fpg_24	NetFpg_24
TiO2_A549	F statistic	N.D.						N.D.											
	Test statistic																		
	p value																		
TiO2_TK6	F statistic																		
	Test statistic																		
	p value																		
ZnO_A546	F statistic																		
	Test statistic																		
	p value																		
ZnO_TK6	F statistic																		
	Test statistic																		
	p value																		

Colour red corresponds to the results of F and t values higher than the critical F and t statistics and $p<0.05$. Colour green corresponds to the results of F and t values lower than the critical F and t statistics and $p>0.05$

Precision (F-Snedecor test):

In case of Fpg_3, NetFpg_3, Fpg_24, NetFpg_24 for A549 cell line, determined F is lower than F_{crit} ($F < F_{crit}$), therefore, the conclusion follows that the obtained values of standard deviations do not differ statistically significantly between laboratories (Table 2). For those four endpoints the compared measurements between the NILU and KU labs do not differ in terms of precision.

However, in the case of Sbs_3, Sbs_24, the F>Fcrit hence the conclusion is that the measurement of these endpoints differs from each other in a statistically significant manner in terms of precision.

Accuracy (t Student test):

In case of all of analysed endpoints (SBs_3, Fpg_3, NetFpg_3, SBs_24, Fpg_24 and NetFpg_24) the determined t statistic is lower than the t_{crit} at the same time the p-value higher than 0.05 (Table 2). It means that compared measurements for TiO_2 using A549 in NILU and Ku labs do not differ in accuracy.

- ***TK6 cell line***

The average values for each analysed endpoint TiO_2 using TK6 are presented in the Figure 2. Here, the data points for different concentrations/control of TiO_2 using TK6 cell line were available for all three labs NILU, ANSES and KU.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the Table 2.

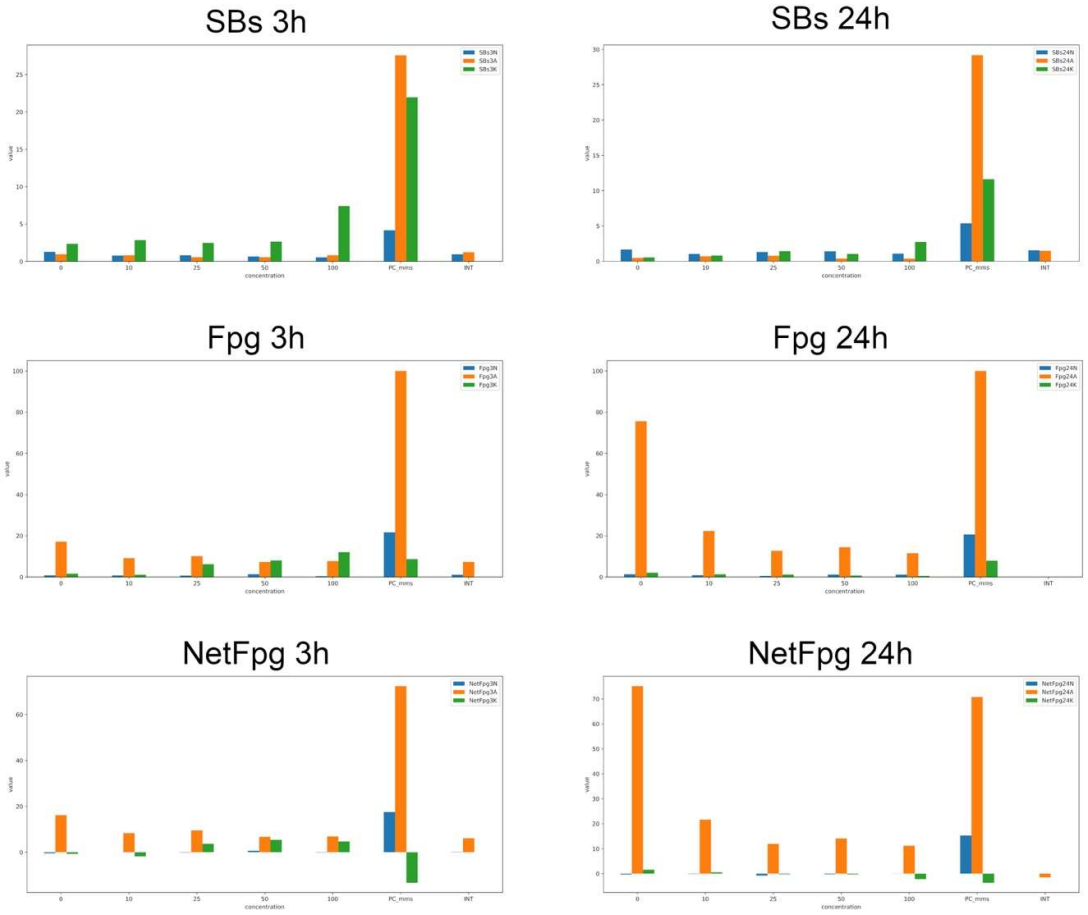

Figure 2. The average values for analysed endpoints for TiO_2 using TK6. Blue colour corresponds to the values obtained in NILU lab, the orange line to ANSES and green to KU Leuven.

Precision (F-Snedecor test):

The compared measurement between NILU and ANSES for TK6 cell line differ statistically significantly of precision in every endpoint (SBs_3, Fpg_3, NetFpg_3, SBs_24, Fpg_24 and NetFpg_24), because the determined Fs are higher than the F_{crit} ($F > F_{crit}$) (Table 2).

In case of measurements between ANSES and KU Leuven for TK6 cell line, the calculated F statistic for endpoints: SBs_3, Fpg_3 and NetFbg_3 is lower than F_{crit} (Table 2), hence the conclusion is that the measurement of these endpoints do not differ in a statistically significant of precision. However, all measurements performed after 24h of experiment indicated the differences in terms of precision ($F > F_{crit}$).

In case of measurements between NILU and KE Leuven for TK6 cell line, SBs_3, SBs_24, Fpg_24 and NetFpg_24 determined Fs are higher that F_{crit}, thus the obtained values of standard deviations differ statistically significantly (Table 2). Only two endpoints (Fpg_3, NetFpg_3) do not differ in terms of precision.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Accuracy (t Student test):

In case of following endpoints: SBs_3, Fpg_3, NetFpg_3 and SBs_24 (Table 2) the determined t statistic is lower than t_{crit} ($t<t_{crit}$) and at the same time the p values are higher than 0.05 for every comparison. It means that those endpoints do not differ in accuracy. However, in comparison between NILU-ANSES and ANSES-KU Leuven, endpoints: Fpg_24 and NetFpg_24 differ in accuracy between labs. The determined t statistic is higher than t_{crit} ($t>t_{crit}$) in every mentioned case, therefore p-value is lower than 0.05 for Fpg_24 and NetFpg_24 in comparison between NILU-ANSES and for NetFpg_24 in comparison between ANSES-KU Leuven.

The overall conclusion of the analysis TiO_2 endpoints for TK6 is that the 24-hour experiments have inferior parameters in precision and accuracy, compared to 3-hour ones. Most of analysis characterised by imprecision.

b. ZnO

- ***A549 cell line***

The average values for each analysed endpoint ZnO using A549 are presented in the Figure 3.

Here, the data points for different concentrations/control of TiO_2 using A549 were available for all three labs NILU, ANSES and KU Leuven. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the Table 2.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

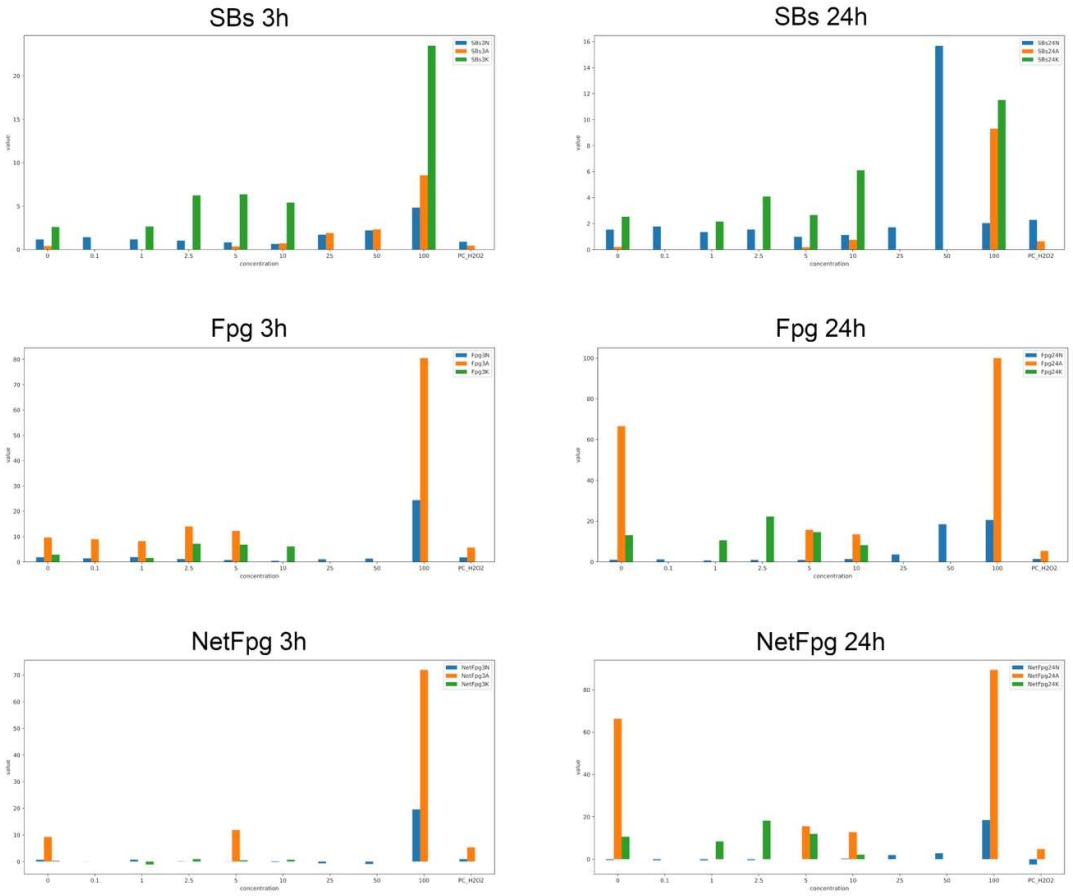

Figure 3. The average values for analysed endpoints for ZnO using A549. Blue colour corresponds to the values obtained in NILUlab, the orange line to ANSES and green to KU Leuven.

Precision (F-Snedecor test):

In the case of comparison between NILU and ANSES measurements for all endpoints (SBs_3, Fpg_3, NetFpg_3, SBs_24, Fpg_24 and NetFpg_24) the determined Fs are higher than F_{crit} ($F>F_{crit}$), therefore the conclusion follows that the obtained values of standard deviations differ statistically significantly (Table 2).

In the case of SBs_3, Fpg_3 and SBs_24 for comparison between ANSES and KU Leuven and NetFpg_3 for NILU and KU Leuven, determined Fs are lower than F_{crit} ($F<F_{crit}$) the measurements of these endpoints do not differ from each other between those labs in a statistically significant manner in terms of precision (Table 2).

Accuracy (t Student test):

In the case of all endpoints (SBs_3, Fpg_3, NetFpg_3, SBs_24, Fpg_24 and NetFpg_24) for comparison between NILU and ANSES the determined t statistic is lower than the t_{crit} and at the same time the p values are higher than 0.05. It means that compared measurement do not differ in accuracy.

The compared measurements between ANSES and KU Leuven for A549 cell line differ in accuracy for following endpoints: Fpg_3, NetFpg_3 and SBs_24, because determined t statistic is higher than t_{crit} in every mentioned case ($t>t_{crit}$) and p-values are lower than 0.05 for Fpg_3 and SBs_24 (Table 2).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

In the case of comparison between NILU and KU Leuven Fpg_3, SBs_24, Fpg_24 and NetFpg_24 the determined t statistic is higher than t_{crit} ($t>t_{crit}$) and p-values are lower than 0.05 for Fpg_24 and NetFpg_24. It means that the accuracy differs between the labs for mentioned endpoints.

The overall conclusion of analysis ZnO endpoints for A549 is that the 24-hour experiments have inferior parameters in precision and accuracy, compared to 3-hour ones. Most of analysis characterised by imprecision.

- ***TK6 cell line***

The average values for each analysed endpoint ZnO using TK6 are presented in the Figure 4.

The data points for different concentrations/control of TiO_2 using A549 were available for all three labs NILU, ANSES and KU Leuven. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the Table 2.

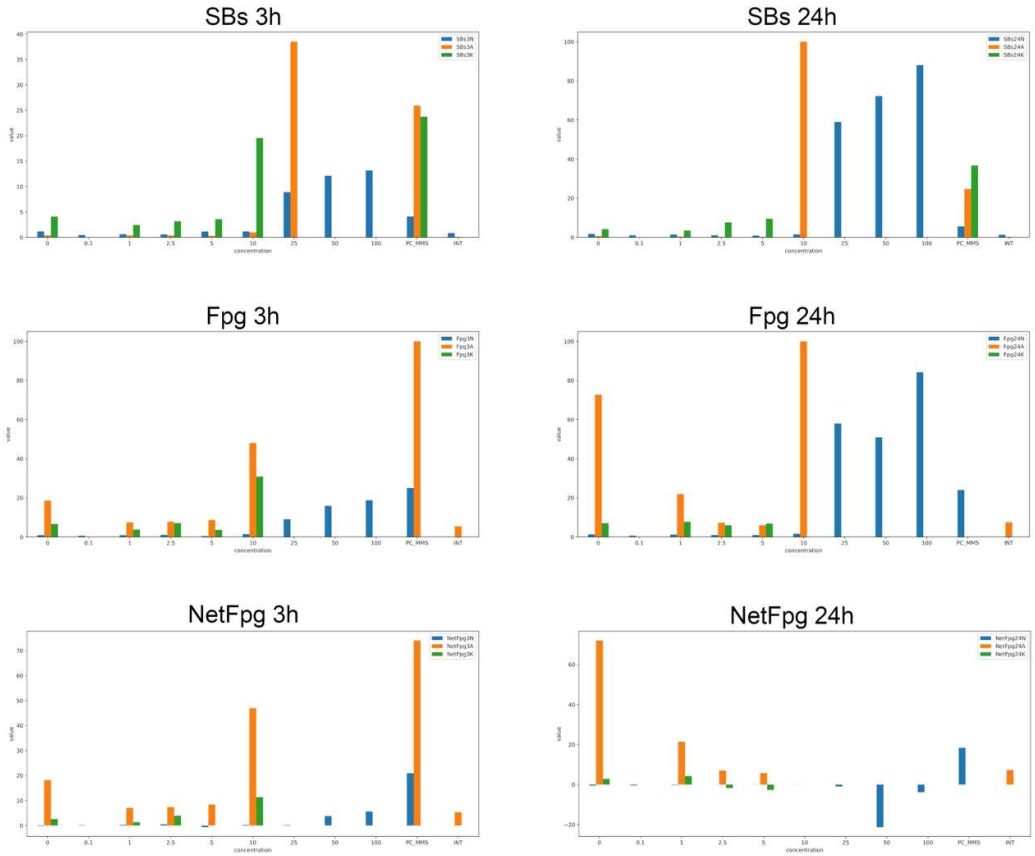

Figure 4. The average values for analysed endpoints for ZnO using TK6. Blue colour corresponds to the values obtained in NILUlab, the orange line to ANSES and green to KU Leuven.

Precision (F-Snedecor test):

In the case of all endpoints (SBs_3, Fpg_3, NetFpg_3, SBs_24, Fpg_24 and NetFpg_24) for comparison between NILU-ANSES and NILU-KU Leuven the determined Fs are higher than F_{crit} ($F>F_{crit}$), therefore the conclusion follows that the obtained values of standard deviations differ statistically significantly (Table 2).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

In the case of SBs_3, Fpg_3 and SBs_24 for comparison between ANSES and KU Leuven the determined Fs are lower than F_{crit} ($F<F_{crit}$), hence the conclusion is that the measurements of these endpoints do not differ from each other in a statistically significant manner in terms of precision (Table 2).

Accuracy (t Student test):

The compared measurements between NILU and ANSES differ in accuracy for only two endpoints: NetFpg_3 and Fpg_24. Determined t statistic is higher than t_{crit} ($t>t_{crit}$) for Fpg_24 and p-values are lower than 0.05 for NetFpg_3 (Table 2).

In the case of SBs_3 and Fpg_24 in comparison for ANSES and KU Leuven the determined t statistic is higher than t_{crit} ($t>t_{crit}$), but p-values are higher than 0.05, unlike SBs_24. It means that the accuracy differs between the labs for mentioned endpoints.

In the case of NetFpg_3 in comparison for NILU an KU Leuven the determined t statistic is higher than t_{crit} ($t>t_{crit}$) and p-value of Fpg_24 is lower than 0.05, therefore the conclusion follows that accuracy for that endpoints differs between the labs.

Summing up the statistical analysis of comet assay using A549 and TK6, indicates that obtained measurements between labs differ in the accuracy of measurements for several endpoints between the labs. Comparing the experiments performed on ZnO between two cell lines it can be concluded that the results for TK6 were more accurate, with the smallest differences between NILU and ANSES. The less accurate results for ZnO were obtained using A459 ANSES vs. KU Leuven, and NILU vs. KU Leuven.

In case of TiO_2 the measurements do not differ significantly in the accuracy for all endpoints between three labs independently of used cell line.

The differences in the precision between all three labs are seen in almost all endpoint independently on the nanomaterial and cell line. Most differences are observed in the comparisons between the NILU and the other two laboratories, and therefore the results measured in this lab have the greatest impact in low statistical significance of the precision.

Overall, in future experiments, it is necessary to refine the performance of the comet assay into three labs.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

ANNEX 4. QSAR Statistics summary for HPRT RR1

WP 5. Statistical analyses for the experimental toxicological data (HPRT assay)

1. Available date and purpose of the analysis

2 nanomaterials (ERM00000063 and ERM00000064) were analysed using HPRT SOP for two cell lines V79 and TK6. Each Lab provided two independent experiments for each cell line and the nanomaterial.

The endpoints analysed for V79 were:

Plate efficiency 0 (PE0)

Plate efficiency 1 (PE1)

Plate efficiency 2 (PE2)

Average plate efficiency 1_2 (PF1_2)

Cell viability 0 (CV0)

Cell viability 1 (CV1)

Cell viability 2 (CV2)

Mutation frequency 1 (MF1)

Mutation frequency 2 (MF2)

Average mutation frequency 1_2 (MF1_2)

The endpoints analysed for TK6 were:

Cell viability (CV)

Mutation frequency (MF)

For the statistical analysis, if for one experiment (one concentration) there were no data available, only the data for the other experiment was taking into account (not the average), otherwise the average values from two experiments within the same lab were considered.

The statistical analysis was used to determine if the obtained measurements between two labs (Swansea and Nilu) are consistence.

The statistical analysis included determining the precision and accuracy of the obtained measurements.

Precision is the degree to which future measurements or calculations yield the same or similar results — it is a measure of the spread of repeated measurement results and depends only on the distribution of random errors – it gives no indication of how close those results are to the true value. In order to compare the precision of two independent measurements obtained during the analysis of samples with the same level of analyte content, we use the F-Snedecor test. The critical value for the case under consideration was Fcrit=6.388.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Accuracy refers to the degree of conformity of a measured or calculated quantity to an actual (true) value. Accuracy is closely related to precision, but it's not the same thing. A result is said to be accurate when it matches to a particular target. In the analysis we investigated the differences between the experiments performed in two labs with pairwise t Student's test for each ENM concentration. The critical value for the case under consideration was tcrit=2.7765.

2. HPRT assay SOP V79 analyses

- **TiO_2**

The average values for each analysed endpoint TiO_2 using V79 are presented in the Figure 1. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the table below.

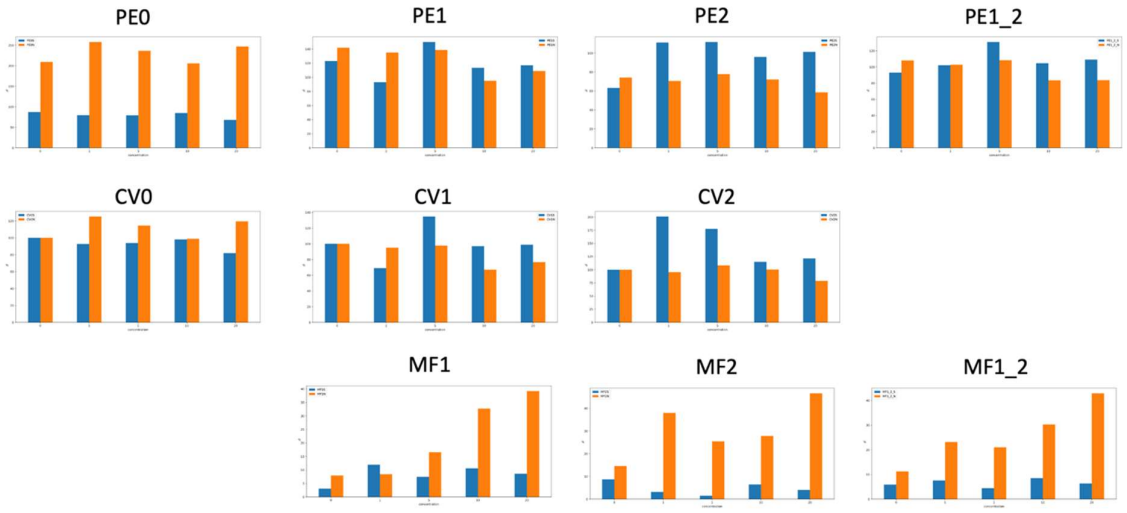

Figure 1. The average values for analysed endpoints for Ti02 using V79. Blue colour corresponds to the values obtained in Swansea whereases the orange line to Nilu lab.

Table 1. Comparison of the statistical parameters from measurements from different labs (F-Snedecor test (Fcrit=6.388), and the pairwise Student's test (tcrit=2.7765))

TiO2_V79	PE0	CV0	PE1	CV1	PE2	CV2	MF1	MF2	PE1_2	MF1_2
F statistic	9.4762	2.8351	1.0249	2.5498	7.4081	16.0832	17.6209	18.8709	1.2313	57.4209
Test statistic	11.7723	2.3340	0.4152	1.0949	2.6526	2.4428	2.0666	4.1140	1.3475	3.7580
p value	0.0003	0.0799	0.6993	0.3351	0.0568	0.0710	0.1076	0.0147	0.2491	0.0198

Colour red corresponds to the results of F and t values higher than the critical F and t statistics and $p<0.05$. Colour green corresponds to the results of F and t values lower than the critical F and t statistics and $p>0.05$.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Precision (F-Snedecor test Fcrit=6.388)

In the case of CV0, PE1, CV1 and PE1_2 determined F is lower than Fcrit (F<Fcrit), therefore, the conclusion follows that the obtained values of standard deviations do not differ statistically significantly (Table 1). The compared measurements between the Swansea and Nilu labs do not differ in terms of precision. However, in the case of PE0, PE2, CV2, MF1, MF2 and MF1_2, the F>Fcrit hence the conclusion is that the measurement of these endpoints' differs from each other in a statistically significant manner in terms of precision.

Accuracy (t Student test tcrit=2.7765)

In the case of almost all analysed endpoints (CV0, PE1, CV1, PE2, CV2, MF1, PE1_2 and MF1_2 - Table 1) the determined t statistic is lower than the tcrit (2.7765) at the same time the p value higher than 0.05. It means that compared measurement (with Swansea and Nilu labs) do not differ in accuracy. However, for three endpoints (PE0, MF2 and MF1_2) the t statistic and p value exceed the critical values therefore in this case the accuracy differs between the labs.

- ZnO

The average values for each analysed endpoint for ZnO using V79 are presented in the Figure 2. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the table below.

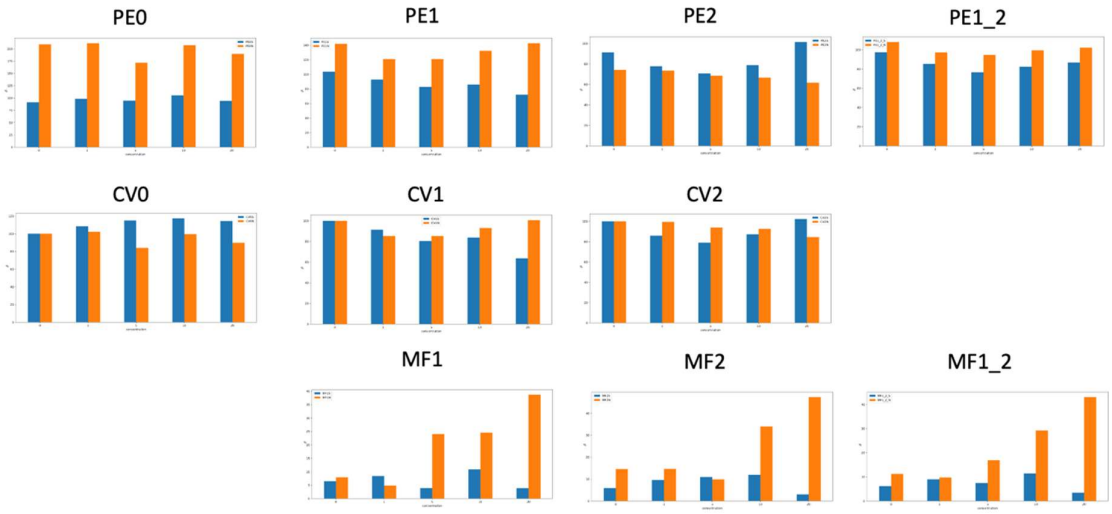

Figure 2. The average values for analysed endpoints for ZnO using V79. Blue colour corresponds to the values obtained in Swansea whereases the orange line to Nilu lab.

Table 2. Comparison of the statistical parameters from measurements from different labs (F-Snedecor test (Fcrit=6.388), and the pairwise t Student's test (tcrit=2.7765))

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

ZNO_V79	PE0	CV0	PE1	CV1	PE2	CV2	MF1	MF2	PE1_2	MF1_2
F statistic	9.3577	1.2549	1.2143	3.2680	5.6569	2.5324	21.1070	18.1830	2.2069	21.9574
Test statistic	14.0625	2.7750	6.1968	1.2136	2.2461	0.5478	1.9478	1.9552	10.1501	2.1190
p value	0.0001	0.0501	0.0034	0.2917	0.0880	0.6130	0.1233	0.1222	0.0005	0.1015

Colour red corresponds to the results of F and t values higher than the critical F and t statistics and $p<0.05$. Colour green corresponds to the results of F and t values lower than the critical F and t statistics and $p>0.05$.

Precision (F-Snedecor test Fcrit=6.388)

In the case of CV0, PE1, CV1 PE2, CV2 and PE1_2 determined F is lower than Fcrit (F<Fcrit), therefore, the conclusion follows that the obtained values of standard deviations do not differ statistically significantly (Table 2). The compared measurements between the Swansea and Nilu labs do not differ in terms of precision. However, in the case of PE0, MF1, MF2 and MF1_2 the F>Fcrit hence the conclusion that the measurement of these endpoints' methods differs from each other in a statistically significant manner in terms of precision.

Accuracy (t Student test/ C-Cochran-Cox test tcrit=2.7765)

In the case of almost all analysed endpoints (CV0, CV1, PE2, CV2, MF1, MF2, MF1_2 - Table 2) the determined t statistic is lower than the tcrit (2.7765) at the same time the p value higher than 0.05. It means that compared measurement (with Swansea and Nilu labs) do not differ in accuracy. However, for three endpoints (PE0, PE1 and PE1_2) the t statistic or p value exceed the critical values therefore in this case the accuracy differs between the labs.

Summing up the statistical analysis of HPRT assay using V79 indicates that obtained measurements between labs differ in the precision and accuracy for the PE0 endpoint independently of the used nanomaterial. The differences in the precision between two labs is also seen in the MF1 and MF2 endpoint and in the average of these values MF1_2. In overall experimental data obtained in two labs are consistent. However, in the future experiments it is necessary to refine the measurement of PE0 and the determination of the values of MF1 and MF2.

3. HPRT assay SOP TK6 analyses

- **TiO_2**

The average values for each analysed endpoint TiO_2 using V79 are presented in the Figure 1. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the table below.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

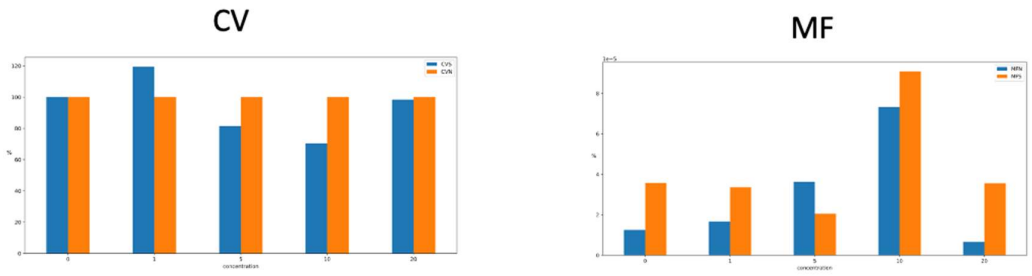

Figure 3. The average values for analysed endpoints for TiO_2 using TK6. Blue colour corresponds to the values obtained in Swansea whereases the orange line to Nilu lab.

Table 3. Comparison of the statistical parameters from measurements from different labs (F-Snedecor test (Fcrit=6.388), and the pairwise t Student's test (tcrit=2.7765))

TiO2_V79	CV	MF
F statistic	variance=0	1.0193
Test statistic	0.7191	1.8204
p value	0.5119	0.1428

Colour red corresponds to the results of F and t values higher than the critical F and t statistics and p<0.05. Colour green corresponds to the results of F and t values lower than the critical F and t statistics and p>0.05.

Precision (F-Snedecor test Fcrit=6.388)

It was impossible to determine the F value for CV endpoint. Here the variance in the cell valiability measures in NIlu lab was equal to 0. For MF determined F is lower than Fcrit (F<Fcrit), therefore, the conclusion follows that the obtained values of standard deviations do not differ statistically significantly (Table 3). The compared measurements between the Swansea and Nilu labs do not differ in terms of precision.

Accuracy (t Student test tcrit=2.7765)

For two analysed endpoints (CV and MF) the determined t statistic is lower than the tcrit (2.7765) at the same time the p value higher than 0.05. It means that compared measurement (with Swansea and Nilu labs) do not differ in accuracy.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

- ZnO

The average values for each analysed endpoint for ZnO using V79 are presented in the Figure 4. The F-Snedecor test and pairwise t Student's test were performed in order to determine the precision and accuracy of the measurements. The results are presented in the table below.

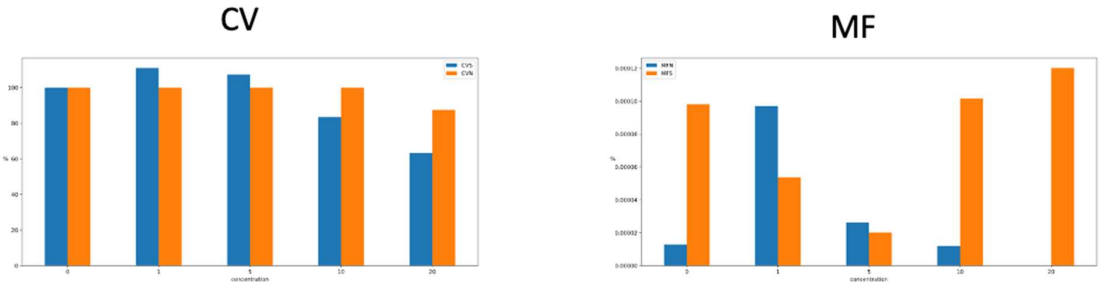

Figure 4. The average values for analysed endpoints for ZnO using TK6. Blue colour corresponds to the values obtained in Swansea whereases the orange line to Nilu lab.

Table 4. Comparison of the statistical parameters from measurements from different labs (F-Snedecor test (Fcrit=6.388), and the pairwise t Student's test (tcrit=2.7765))

ZNO_V79	CV	MF
F statistic	12.2771	1.1084
Test statistic	0.6576	1.5686
p value	0.5467	0.1918

Colour red corresponds to the results of F and t values higher than the critical F and t statistics and $p<0.05$. Colour green corresponds to the results of F and t values lower than the critical F and t statistics and $p>0.05$.

Precision (F-Snedecor test Fcrit=6.388)

In the case of CV the determined value for F is higher than the Fcrit (F>Fcrit), which means that the measurement of these endpoints differs from each other in a statistically significant manner in terms of precision. However, this result is closely related to the fact that 4 out of 5 measurements of CV in Nilu lab have the same values.

In case of MF endpoint, the determined statistic F is lower than Fcrit (F<Fcrit), therefore, the conclusion follows that the obtained values of standard deviations do not differ statistically significantly (Table 4). The compared measurements between the Swansea and Nilu labs do not differ in terms of precision.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Accuracy (t Student test tcrit=2.7765)

For two analysed endpoints (CV and MF) the determined t statistic is lower than the tcrit (2.7765) at the same time the p value higher than 0.05. It means that compared measurement (with Swansea and Nilu labs) do not differ in accuracy.

Summing up the statistical analysis of HPRT assay using TK6, indicates that obtained measurements between labs do not differ in the accuracy for the CV and MF endpoint independently of the used nanomaterial. The differences in the precision between two labs is seen in the CV endpoint, however, this result is closely related to the same measured value in Nilu lab.

In overall experimental data obtained in two labs are consistent. However, in the future experiments it is necessary to refine the measurement of cell viability into two labs.

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ANNEX 5. HPRT (TK6). SU SOP

Hypoxanthine Phosphoribosyl Transferase (HPRT) gene mutation assay for use with engineered nanomaterials (ENMs)

Standard Operating Procedure for RR2

2019

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Abbreviations

HPRT - Hypoxanthine Phosphoribosyl Transferase

HAT - Hypoxanthine, Aminopterin and Thymidine

HT – Hypoxanthine and Thymidine

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

HS – Horse Serum

MMC – Mitomycin C

MMS – Methyl Methanesulphate

MNU - N-nitro-N-methylurea

P/S – Penicillin/Streptomycin

PBS – Phosphate Buffered Saline

DMSO - Dimethylsulfoxide

L-Glut – L-glutamine

Biological Setting & Test System

This SOP should be carried out under strict laboratory conditions, with all work performed under sterile conditions and in a Class II Laminar Tissue Culture Hood.

Chemicals & Reagents

PBS (ThermoFisher; 10010023) , Cell Culture Medium (cell type specific, therefore supplier information not given), HAT supplement (Sigma; H0262), HT supplement (Sigma; H0137), 6-thioguanine (6-TG) (Sigma UK, A4882), Beckman Coulter Diluent (Beckman; 628017),, P/S (ThermoFisher; 15140122), Glutamine (ThermoFisher; 25030081), DMSO (ThermoFisher; 85190), Cell Culture Serum (cell type specific, therefore supplier information not given).

Apparatus & Equipment

- Liquid Nitrogen
- Haemocytometer
- Cell Freezing Aid (Mr Frosty, 5100-0001; Thermo Fisher Scientific, UK)
- Laminar Class II Tissue Culture Hood (Scanlaf Mars)

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- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator (NUAIRE™ DHD Autoflow)
- Water Bath (37˚C)
- Glass Tanks
- Centrifuge
- T-25 (Greiner; 690175) and T-75 (Greiner; 660175) Tissue Culture Flasks
- Nunc™ Microwell 96-well (Greiner; 167008)
- 15 ml Falcon Tubes (Greiner; 188261)
- 50 ml Skirted Falcon Tubes (Greiner; 210261)
- Haemocytometer
- Light Microscope (Axiovert 40C, Zeiss, UK)

Reagent Preparation

In preparation for this SOP, it is advised that cell culture medium is prepared and pre-warmed at 37°C for 10 minutes prior to use. Cell culture medium is prepared by adding 50ml of the relevant serum (cell type specific) and 5ml of L-glutamine to 500ml of media. The full cell culture medium should then be mixed prior to use by inverting the bottle.

NB this protocol is written for undertaking the HPRT assay with suspension cells line TK6 (PHE)

Complete cell culture media for TK6 cells; RPMI 1640, HS at 10%, L-glutamine at 1%

To thaw cells from Liquid Nitrogen:

- Place a bottle of complete medium (cell line specific; TK6 media – RPMI, 10% HS, 1%L-glutamine) in the water bath at 37°C (25-30 minutes before use). Remove your vial of cells from the Liquid N_2 and place in a foam float in the water bath. Do not allow the vial to be immersed. Cells will thaw in ~1 minute, transfer them to a flask of the warm medium slowly and carefully using a Pasteur pipette.
- Label the flask with the name of the cells, the passage number, your name, the date of resuscitation and the date that the cells were previously frozen. (This is helpful because if there is a problem with them the other vials from the batch can be identified.)
- Place the flask in the CO_2 incubator (37°C & 5% CO_2).
- Check them after 24 hours for growth and contamination. They can be counted to determine when they will need splitting. Be aware some suspension cells grow fast so keep an eye on them.

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- Keep a flask of cells growing by splitting, as necessary. These split cells can seed flasks for further experiments and can also be bulked up to have cells to freeze down to replace used vials. Remember to keep note of passage number (to not freeze down very old cells). Also, keep an eye on passage number in your experiments do not let this rise beyond a reasonable range (define an acceptable passage number window to use). TK6 cells should not be grown continuously for more than 4 weeks.

- All waste media, plastics etc must be disposed of as dictated by the laboratory where work is being undertaken.

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Preparation of HPRT HAT & HT Media (for initial mutant cleansing)

- To prepare HAT media - add 1 ml of 50x hypoxanthine, aminopterin and thymidine (HAT) supplement to 50 ml complete media. Final concentration - $2x10^{-4}$M hypoxanthine / $8x10^{-7}$M aminopterin / $3.5x10^{-5}$M thymidine.

- To prepare HT media - add 2 ml of 50x HT (HAT supplement without aminopterin) supplement to 98 ml of complete media to obtain 1xHT.

Preparation of 6-thioguanine 6-TG

- To prepare working stock of 6-thioguanine (6-TG) – add 29 ml of 0.1M sodium hydroxide (Sigma, UK) to 1 ml 4.5 mg/ml 6-TG (Sigma UK, A4882). 100 ml of working stock is added per 25 ml of cell culture to achieve a final concentration of 0.6mg/ml.

Mutant purification – removal of HPRT mutants

1. To purify the cell population of existing HPRT mutants resuspend $5x10^5$ cells/ml cells in 50 ml of HAT supplemented media for 3 days (incubate at 37°C & 5% CO_2), replenish HAT media on day 1 or 2.

2. Centrifuge cells at 230g for 5 min, resuspend in 10 ml of Phosphate Buffered Saline (PBS) and centrifuge again at 230g for 5 min.

3. Resuspend cells at a concentration of $4x10^5$ cells in 50ml of HT media for 24 hours in an incubator at 37°C, 5% CO_2.

4. At this point HPRT mutant stocks can be frozen down for future use by centrifuging at 230g for 5 min, resuspending at $1x10^6$ cells/ml in freezing medium (10% dimethyl sulfoxide (DMSO) in the respective cell line serum) and aliquot into 1ml cryovials. **From this point on, HAT and HT supplements are not needed, proceed with complete cell culture media**. Cells need to be left to cool at -80°C overnight at rate of 1°C in a controlled freezing container before transferring to liquid nitrogen.

5. Cells centrifuged and resuspended in 200ml growth media for 3 days (replenished on day 2)

Mutation assay and ENM treatment protocol

6. Following HPRT mutant purification, centrifuge cells at 230g for 5 mins and resuspended in fresh culture media at a concentration of $5x10^5$ cells/ml in T25 tissue culture flasks at 10ml (one flask per replicate, per

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concentration). If reviving purified cells from liquid nitrogen add one 1 ml of thawed cells to 50 ml of normal growth media for 2 days prior to seeding for treatment.

7. Undertake ENM exposures in the 10 ml cultures (step 6) for a 24h period. Details on handling ENMs given below.

8. A suitable positive control is required to be concurrently run alongside all ENM (MMS), also given below.

Important Note: ENMs will require preparation, suspension in water/media and sonication prior to use in toxicology testing, allow time to prepare ENMs fresh on the day of dosing. Users should refer to a recognised dispersion procedure which generates a stable ENM suspension and where the final quantity of ENM suspension added to the cells does not exceed 1:100 as to avoid disturbing cell culture conditions

RR1 ENMs

JRC TiO_2

Follow RiskGONE WP4 sonication SOP. Concentration range; **0, 10, 25, 50, 100µg/ml**

Sigma ZnO. Concentration range; **0, 1, 5, 10, 20µg/ml**

- Stock ZnO is 1.2g/ml when weighed
- 20% of this 1.2g is ZnO
- (1.2/100)*20 = 0.24g/ml = 240mg/ml

12.5µl 287.5µl media for 300µl stock of 10mg/ml

ZnO Concentration (µg/ml)	Required volume of 10mg/ml stock (µl)	Required volume of media (µl)	Volume added to 10 ml cells (µl)
0	0	200	200
1	1	199	200
5	5	195	200
10	10	190	200
20	20	180	200

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RR2 ENMs

Copper Oxide (CuO), Plasmachem #YF1906191

Follow RiskGONE WP4 sonication SOP. Concentration range; **0, 0.25, 0.5, 1, 2µg/ml**

Tungsten carbide-cobalt (WC/Co), NanoAmor #5561HW

Follow RiskGONE WP4 sonication SOP. Concentration range; **0, 10, 25, 50, 100µg/ml**

Nanocyl MWCNTs

2.5, 5, 10, 25µg/ml

MMS Positive Control

Final MMS concentration of 1.5µg/ml in 10ml of cells.

9. Following the exposure period, centrifuge the cells at 230g for 5 mins and resuspend in 10 ml PBS. Repeat this wash step twice more.

10. Following the final wash step resuspend cells in 10 ml of fresh culture medium.

11. Incubate cells at 37°C with 5% CO_2 for 13 days (detailed below how to passage) treatment to allow the mutations to fully develop in both strands & the already expressed HPRT proteins to be degraded.

    For standard analysis

    a. Maintain cells at a concentration of $1.25x10^5$ cells/ml on days 1, 3, 5, 7 (of the 13-day duration) by centrifugation at 230g for 5 min and resuspension in **10ml** of fresh culture medium.

    b. On day 9 maintain concentration of $1.25x10^5$ cells/ml by centrifugation at 230g for 5 min and resuspend in **20ml** of fresh culture medium.

    c. On day 11 maintain concentration of $1.25x10^5$ cells/ml by centrifugation at 230g for 5 min and resuspend in **50ml** of fresh culture medium.

Nb. At any of these time points, the cells can be frozen down to allow staggering of 96-well plate analyses, but it is important to note of how many days after treatment cell growth is stopped as this must be continued to ensure mutations develop appropriately.

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12. On day 13 dilute cells to $4x10^5$ cells/ml and add 100 ml of 6-TG working stock per 25 ml of complete cell culture medium. Add 100 ml per well of the resultant cell suspension into 10 x 96 well plates (Mutation Frequency = $4x10^4$ cells/well) for standard analysis (OECD TG 476).

13. To prepare non-selective conditions to measure colony forming ability (plating efficiency) dilute cells to 200 cells/ml in cell culture media and aliquot in to 10 plates per dose for standard analysis (OECD TG 476).

For plating

- Make a stock of $4x10^5$ for x10 plates (100ml) but make extra (105ml)
- Take 1 ml from the stock to make the PE plates
- Add the 1 ml to 40ml of culture media for (10,000 cells/ml)
- Take 5ml and add to 5ml of culture media (5,000 cells/ml)
- Take 4ml and add to 96ml of culture media (200 cells/ml) this will be enough for x10 PE plates
- Now 100ul per well = 20 cells per well
- Repeat above steps for all concentrations being tested
- Repeat above steps for all concentrations being tested
- A training video demonstration for preparing the appropriate cell seeding densities can be found in TEAMS > Project RiskGONE > Communication & Dissemination > Training > HPRT

14. Incubate all plates at 37°C with 5% CO_2 for 14 days. It is CRUCIAL the incubator remain closed the entire time as colonies can be easily disrupted/broken apart by continued opening and closing of incubator doors. Plan for this by topping up the water in the incubator tray prior to the incubation period. Decontamination cycle may also be wise prior to 14-day incubation.

15. After 14 days score all plates for colony formation (whereby a colony = >20 cells in diameter, ensuring colonies are clearly apart, thereby accounting for clonal expansion). This is to be done with a light microscope, not by eye. Choose an objective which gives a view of one entire well or where you can visualise two-thirds of the well at least. The outer wells of the 96-well plate are NOT scored, therefore 60 wells per plate will be scored for colony growth. The outside wells can dry out due to long growth period in incubator these can give false-negative colony growth. The inclusion and exclusion criteria have been covered in a training video which can be found here: TEAMS > Project RiskGONE > Communication & Dissemination > Training > HPRT

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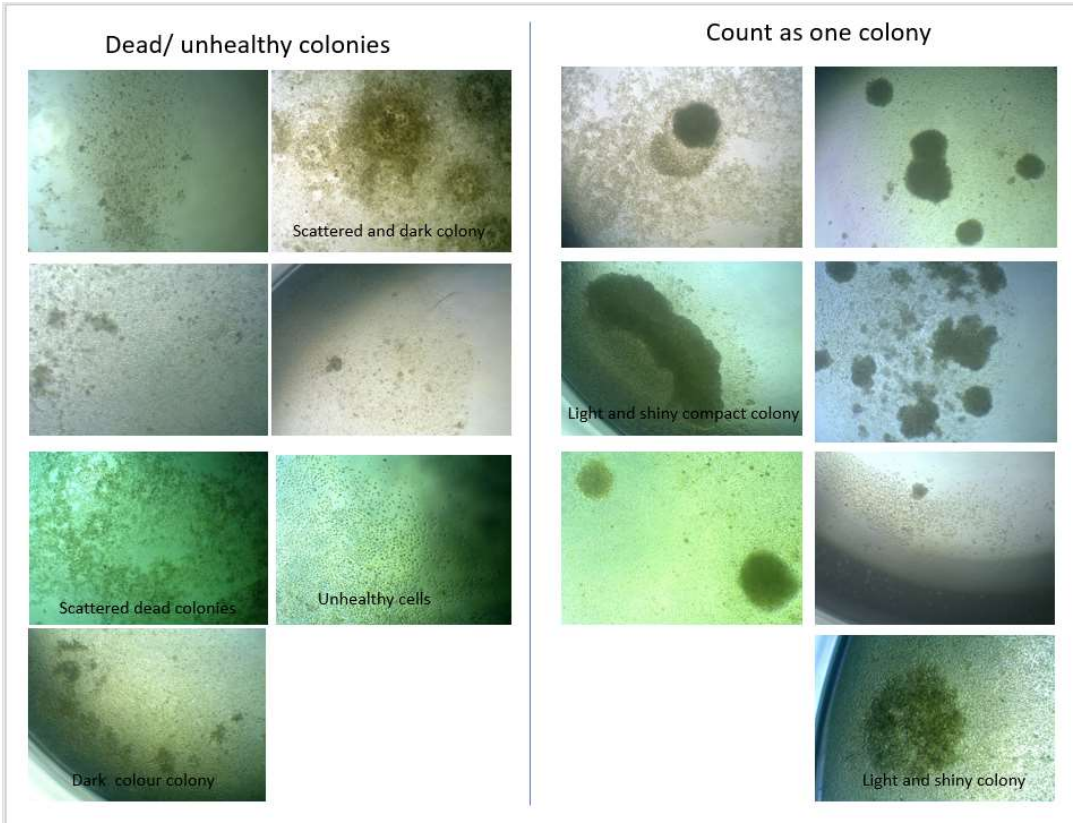
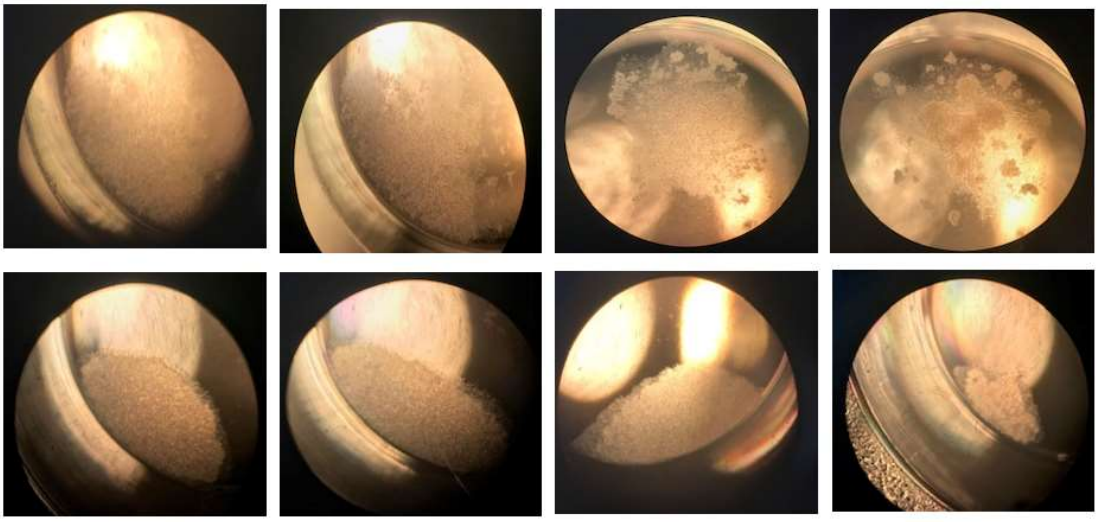
Example TK6 Colony Growth





Light, shiny, well-defined borders = Healthy Colony

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Calculating mutation frequency

The following calculations have been embedded into the Swansea University HPRT data template which has been added to ENanoMapper.

Important note: when downloading the spreadsheet; the dilution factor required is 0.0005

Plating efficiency

Plating Efficiency % (PE)= −Ln (X_o/N_o)× 100

Cell Viability (Relative PE)

$$Cell\ Viability\ (\%) = \frac{PE}{PE\ of\ control} \times 100$$

Mutant Frequency (MF)

$$MF = \frac{-Ln\left(\frac{X_S}{N_S}\right)}{-Ln\left(\frac{X_O}{N_0}\right)} \text{ x DF}$$

DF = dilution factor = $\frac{(No.of\ initial\ cells\ per\ well)\ non-selectiv\ \ conditions}{(No.of\ initial\ cells\ per\ well)\ selective\ contitions}$

Selective conditions

X_S=No. of wells without colonies

N_S=Total no. of wells

Non-selective conditions

X_0=No. of wells without colonies

N_0=Total no. of wells

Troubleshooting the assay

Low plating efficiency

- Bad batch of 96-well plates (very rare)
- Bad batch of horse serum (very rare)

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- High pH on microtiter plates; caused by incubator being frequently opened in the first 4-5 days. Low CO2 levels. Incorrect pH in media prior to serum addition.
- Slow cell growth

High negative control mutant fraction

- An artefact of low plating efficiency
- Improper HAT/HT cleansing caused by i) Thymidine starvation or ii) inadequate aminopterin
- Cells exposed to other mutagens for prolonged periods (UV)
- Inadequate selective agent
- $HPRT^+$ may be mistakenly counted as live when in fact these will be dead colonies selected by 6-TG

Interpretation of Results

TK6 HPRT colonies should be scored via light microscopy using the scanning objective. In concordance with the photo gallery images a training video has been prepared which outlines the inclusion and exclusion criteria. This video can be found on Microsoft TEAMS RiskGONE project > WP5 > Training Materials

Historical ranges

Data generated in RR1 by both Swansea & NILU has demonstrated a negative and positive control historical range of:

Swansea University TK6 Negative Control: $3.58x10^{-6}$ – $9.81x10^{-6}$

NILU TK6 Negative Control: $3.92x10^{-7}$ – $2.11x10^{-6}$

Swansea University TK6 Positive Control: $1.05x10^{-4}$ – $1.14x10^{-4}$

NILU TK6 Positive Control: $8.78x10^{-5}$ – $11x10^{-4}$

References

Johnson G.E. (2012) Mammalian Cell HPRT Gene Mutation Assay: Test Methods. In: Parry J., Parry E. (eds) Genetic Toxicology. Methods in Molecular Biology (Methods and Protocols), vol 817. Springer, New York, NY

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OECD (2016), *Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264809-en.

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ANNEX 6. HPRT (V79-4 cells). NILU SOP

1. INTRODUCTION

The standard *in vitro* mutagenicity assay (mammalian cell gene mutation test) detects mutagenic effects of the test substances in mammalian cells. The test is performed according to OECD TG 476 and detects gene mutations induced by mutagenic and carcinogenic compounds. The test is performed on stable cell lines growing in suspension or attached to the surface in monolayer. Suitable cell lines include L5178Y mouse lymphoma cells, human lymphoblastoid TK6 cells and the Chinese hamster cells CHO, AS52 or V79. In these cell lines the most commonly used genetic endpoints measure mutation at hypoxanthine guanine phosphoribosyl transferase (*HPRT*), thymidine kinase (*TK*) genes or a transgene of xanthine-guanine phosphoribosyl transferase (*XPRT*). The *TK* and *XPRT* genes are located at autosomal chromosomes, and the *HPRT* gene is located at the X chromosome. The *TK*, *HPRT* and *XPRT* mutation tests detect different spectra of genetic events. *TK* and *XPRT* assays allow detection of large genetic events (e.g. large deletions), while the *HPRT* test detects point gene mutations. The test is used for assessment of the genotoxic potential of a test compound. The above mentioned cells have one functional copy of the gene which encodes the *HPRT* enzyme.

This SOP is specifically for adherent cells, specifically V79 or V79-4 cells.

2. PRINCIPLE

The *HPRT* mutation assay detects base changes (one of the 4 bases - adenine, thymine, guanine, cytosine). This mutation results in amino acid change in the enzyme HPRT which is involved in the purine salvage pathway. HPRT converts free purine bases such as hypoxanthine into the corresponding nucleotide, thus bringing them back into the cellular pool. The *HPRT* gene is X-linked so only 1 allele has to be inactivated to affect the phenotype. HPRT is a non-essential enzyme for the cell which means that mutant cells survive.

Mutants are identified by loss of activity of HPRT. Selection of mutants is based on the selective toxicity of the purine analogue 6-Thioguanine (6-TG). 6-TG is a base analogue of the purine precursors that can be metabolized by HPRT enzyme through phosphorylation to nucleoside monophosphate (MP). In the cell, 6-TGMP interferes with DNA precursor synthesis and may itself be incorporated into DNA, thus causing inhibition of DNA replication and toxicity resulting in cell death. While culture medium containing 6-TG kills normal cells, mutant *HPRT* $^{-}$ cells are unable to take up or metabolise 6-TG and so survive, grow and form colonies which are detected visually.

A very low background level of spontaneous mutants is expected in a cell population, as detected in negative control.

2.1 Experimental design

The cells are exposed to at least three analyzable concentrations of the test substance, in addition to control either without metabolic activation or both with and without metabolic activation. Either short term (up to 2-4 hrs) or long-term treatment (24, 48 hrs) can be performed.

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After the treatment, the cells are trypsinized, diluted for sub-culturing (at least 1 million cells are inoculated in each passage from each culture) and plated in small inoculums (e.g. 50-100 cells per well of a 6 well plate) for cytotoxicity testing. The control and treated cultures are sub-cultured every 2-3 days (inoculating of at least 106 cells each time) and are incubated in medium for minimum 6-8 days, to allow optimal phenotypic expression of induced mutations (and dilution of pre-existing HPRT in the cells) prior to mutant selection. Mutation frequency is determined by seeding known numbers of cells (e.g. 2x105/dish on at least 5 dishes, so that in total for each culture 1 million cells are plated) in medium containing the selective agent 6-TG for detection of mutant cells (colonies), and 50-10000 cells/well (on each plate of 6 well plates) in medium without 6-TG to determine the cloning efficiency (viability) (see experimental design scheme below). Two seedings for mutant selection are conducted, the first after at least 3 passages and the second 2-3 days later. Mutant colonies are stained after 8-10 days of culture in selection medium containing 6-TG. Cytotoxicity and viability are determined after colony formation (5-6 days for cloning efficiency in non-selective medium). Colonies visible after staining with methylene blue are counted and mutant frequency is determined per 1 million of viable cells.

Prior to the main study preliminary experiments on the same cell lines are performed to determine cytotoxicity and to select the concentration range for the mutation experiment. In the main experiment cytotoxicity is determined by plating efficiency (PE) either without, or with and without metabolic activation.

2.2 Metabolic activation system

The V79 cells have limited metabolic capacity to metabolize pre-mutagenic compounds to mutagenic metabolites. The exogenous metabolic activation system post-mitochondrial (S9) fraction, obtained from rat liver, can be used to detect indirect mutagens requiring metabolic activation for mutagenic effect.

Treatment with the test substances is carried out in the presence and absence of S9 MIX (S9 fraction with cofactors) according to SOP HEL11B004, as well as negative and positive controls.

Abbreviations

HPRT - Hypoxanthine Phosphoribosyl Transferase

HAT - Hypoxanthine, Aminopterin and Thymidine

HT – Hypoxanthine and Thymidine

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

HS – Horse Serum

MMS – Methyl Methanesulphate

P/S – Penicillin/Streptomycin

PBS – Phosphate Buffered Saline.

DMSO - Dimethylsulfoxide

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3. MATERIAL AND REAGENTS

(Vendors and products are suggestions and not mandatory)

V79 or V79-4 cells

Petri dishes Ø 10cm, Ø 5cm or/and flasks 25cm^2 or/and 75cm^2

6 well plates

12 well plates

Sterile plastic centrifuge tubes 15 ml

Sterile plastic centrifuge tubes 50ml

Microcentrifuge tubes (1.5 ml)

Pasteur pipettes

Pipette tips

Cell culture medium Fetal Bovine Serum, (26140-079, Invitrogen)

Penicillin-Streptomycin (15140-122, Invitrogen)

Other supplements of culture medium required for the specific type of cell line (e.g. additional antibiotics, non essential aminoacids, sodium pyruvate, etc.)

Trypsin-EDTA (59429C, Sigma)

Trypan blue (T6146, Sigma) (0.4%) or trypan blue kit (0.4%) (C10228, Invitrogen

Glucose-S-phosphate

PBS (ThermoFisher; 10010023),

HAT supplement (Sigma; H0262),

HT supplement (Sigma; H0137),

6-thioguanine (6-TG) (Sigma UK, A4882),

Glutamine (ThermoFisher; 25030081),

DMSO (ThermoFisher; 85190),

Countess® Cell Counting Chamber Slides or Bürker chamber

Phosphate buffer saline (PBS) 1X PBS (Invitrogen, 10010-049)

$MgCl_2$.6 H_2O (Mw=203,3; 98,0%)

$CaCl_2$.6 H_2O (Mw=147; 99%)

1% methylene blue (M9140-256, Sigma Aldrich)

CO_2

Distilled water

Ethanol

Bürker chamber

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Cover slips 22x22 mm

Chemicals for positive control

MMS (**CAS # M4016**_ Sigma Aldrich)

4. EQUIPMENT

Centrifuge Allegra X-22R / Centrifuge Galaxy 14D

Water bath Grant SUB Aqua 12

Vortex (or any vortex)

Refrigerator Matsui

Refrigerator Liebherr

Freezer Thermo Scientific -80

CO_2 incubator

Laminar Flow Hood

Cell counter Countess / Bürker chamber with cover glass

Light Microscope Leica

Autoclave Tomy SX-500E

Finn pipettes

5. PREPARATION PROCEDURES

5.1 Solutions

Preparation of methylene blue (1%)

Dissolve 1g of methylene blue in 100 ml of distilled water. Filter through filtration paper. It is not necessary to sterilize it. Keep stock solution at room temperature.

Preparation of 6-Thioguanin (6-TG)

Prepare stock solution of 6-TG at 500 µg/ml by adding 10 mg of 6-TG in 20 ml of 0.5% of Na_2CO_3 (dilute 0.1g of Na_2CO_3 in 20 ml of distilled H_2O). Sterilize in autoclave 5 min in 0.3 atm. (Solution program).

5.2 Medium, culture conditions & stocks

Cells are cultivated in complete culture medium DMEM D5546 medium (500 ml), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (5ml), 5 % (v/v) FBS (27ml) and incubated in culture dishes or flasks in humidified atmosphere in 37° C, 5%.

5.3 Cell lines and preparation of cultures

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The V79-4 are from European Collection of Cell Cultures (ECACC). Cells are with high cloning efficiency (70-90%) and are sensitive to chemical mutagens.

Cells are thawed and sub-cultured 2-4 times before used in experiments. Cells are incubated in culture medium at 37°C in humidified atmosphere of 5% CO_2. Use fresh cultures, 1-4 weeks after thawing. Ideally, cells are trypsinized twice before used in experiments. Cells that reach 50-75% of confluency in monolayer are suitable for the experiment.

5.4 Exposure conditions and treatment with test substance and controls

Concentration range for exposure of cells should be established based on expected cytotoxicity, solubility in the test system and changes in pH or osmolarity.

Control and at least four concentrations should be used according to OECD TG 467. For cytotoxic compounds, these concentrations should cover a range from low or no toxicity to maximum toxicity; this will usually mean that the concentration levels should be separated by no more than a factor between 2 and 10. For cytotoxic compounds, the maximum concentration should give approximately 10-20 % (but not less than 10 %) relative survival (relative PE) or relative total growth. For relatively non-cytotoxic compounds the maximum concentration will be 5 mg/ml, 5 ml/ml, or 0.01M, whichever is the lowest.

Proliferating cells are exposed to the test substance for 24h exposure with test substance in medium can be used, especially for testing nanomaterials. Always perform at **least one repeat of the experiment.**

6. PROCEDURE

Mutant purification – removal of HPRT mutants

16. To prepare **HAT media** - add 1 ml of 50x hypoxanthine, aminopterin and thymidine (HAT) supplement to 50 ml complete media. Final concentration - 2 x 10^{-4}M hypoxanthine / 8 x 10^{-7}M aminopterin / 3.5 x 10^{-5}M thymidine.
17. To prepare HT media - add 2 ml of 50x HT (HAT supplement without aminopterin) supplement to 98 ml of media.
18. To purify the cell population of existing HPRT mutants, resuspend 5x10^5 cells in 50 ml of HAT supplemented media for 3 days.
19. Centrifuge cells at 230g for 5 min, resuspend in 10 ml of PBS and centrifuge again at 230g for 5 min.
20. Resuspend cells at a concentration of 4x10^5 cells in 50 ml **of HT media** for 24 h.
21. HPRT mutant stocks can be frozen down for future use by centrifuging at 230g for 5 min, resuspending at 1x10^6 cells/ml in freezing medium (10% dimethyl sulfoxide (DMSO) in the respective cell line serum) and aliquot into 1 ml cryovials.

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22. Centrifuge the cells and resuspend in 200 ml growth media and incubate for 3 days (replenished on day 2)

Cell culture preparation and treatment:

- Confluent cells (75% confluent) are trypsinized, and $1x10^6$ cells are inoculated per dish f10cm, 2 dishes **per sample**. If 5 concentrations will be used (including negative control) then 10 dishes without S9 mix and 10 dishes with S9 mix will be prepared, as well as 2 dishes for positive controls (one directly acting mutagen and one pre-mutagenic compound added metabolic activation, e.g. S9 fraction).

In the case of 5 concentrations additionally to controls, altogether for one tested compound 12 cultures with and 12 cultures without metabolic activations will be prepared. Cells are seeded on Petri dishes at a density allowing to reach 50-75% confluency after 24-48h in culture. Label each dish properly.

- Expose the cells with test substance as well as positive, solvent and negative controls, for the required time. At **least $1x10^6$** cells should be exposed with each concentration (including the controls). Prepare dilutions from stock solution immediately before exposure of the cells.
- After the treatment, remove the medium and wash the cells 2x with PBS and trypsinize the cells
- Count the cell suspension and calculate the number of cells in a Bürker chamber under light microscope or using Countess device.
- Prepare a suspension at $1x10^5$ cells/ml in falcon tubes (15 or 20ml). For example, if there are $2.5x10^5$ cells/ml, take 1 ml of the suspension of cells plus 1.5 ml of medium in a 15 ml tube. Vortex (not too vigorously) to make a homogenous suspension. Use the cells for step A (Determination of cytotoxicity) and step B (Determination of mutant frequency).

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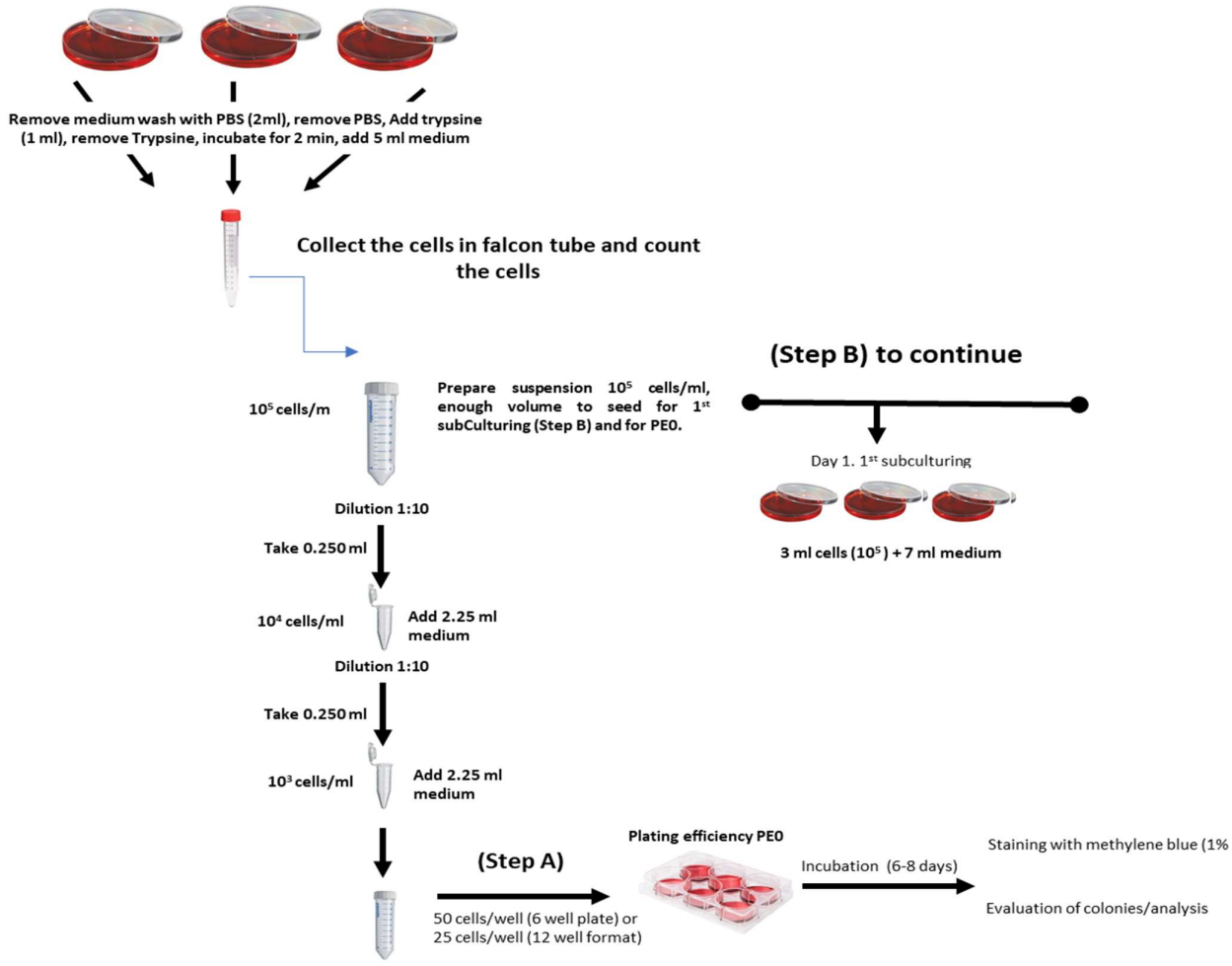

A: Determination of cytotoxicity – Plating efficiency (PE) after the treatment

- Prepare cell suspension of 1x10^4 cells/ml by 10 x dilution of the 10^5 suspension
  e.g. take 0.1 ml of suspension of 1x10^5 cells/ml plus 0.9 ml of medium to make total 1 ml of suspension of 1x10^4 cells/ml (alternatively 0.25 ml suspension plus 2.25 ml of medium, total 2.5 ml of 1x10^4 cells/ml.

- Vortex to achieve homogenous suspension.
- Prepare suspension of 1x10^3 cells/ml by 10x dilution from suspension of 1x10^4 cells/ml, e.g 1 ml of suspension and 9 ml of media or 0.25 ml of suspension plus 2.25 ml of media = of 1x10^3 cells/ml.
- Vortex to achieve homogenous suspension.
- Add 0.5 ml of 1x10^3 cells/ml suspension (50 cells) into each well of 6-well plate for PE (6 replicates for each concentration). If 12-well plate format is used, seed 25 cells/well. In case of dishes of diameter 5 cm, use 100 cells (0.2 ml from the cell suspension) for controls. If high cytotoxicity is expected, double the number of cells seeded. Note that total of 300 cells per plate is seeded so keep this number even when you change the layout.
- It is important to mix the suspension prior to plating to ensure equal dispersion of cells. Shake each dish/plate horizontally on the surface by moving dishes/plates right, left, up and down to achieve homogenous spread of cells on dish before placing back in the incubator.

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- Culture for 7-10 days allowing cells to form colonies. The time may vary depending on the cell line and cultivation conditions. The colonies should be visible by eye but should not be joined together. Each colony should have at least 50 cells.
- Stain the colonies with 1% methylene blue (20 µl per well for 6-well plate format, or 10 µl for 12-well plate format) for 30 min, rinse with water and let dry, then count and record total number of colonies per well (use separate template or write directly to an excel spreadsheet to collect the data).

B: Determination of mutant frequency

Procedure for phenotypic expression of HPRT mutants:

Immediately after the treatment, the cells from each treatment are trypsinized, inoculated and cultivated in culture medium for additional 6-10 days to allow phenotypic expression of induced mutations. Cells need to grow in monolayer and should not overgrow, 75% confluence is ideal. 7-10 days is necessary for mutations to be fixed and for cells to express the mutant phenotype. Mutated cells can still have functioning enzyme HPRT in their pools, and these cells are therefore important to get rid in this period. Inoculate cells as follows:

- After the treatment, split the rest of the cells into large Petri dishes at least f10cm diameter or surface 80-100 cm^2. Inoculate at least 0.9-1x10^6 cells from each sample into 3 dishes at cell density 3-4 x10^5cells/dish or 5 dishes at 2 x10^5 cells/dish (the 5 dishes option is good for cultivation over the weekend). When nearly confluent (75%), trypsinize them and inoculate again at least 1x10^6 from each culture.

For those samples with high cytotoxic concentrations, seed at least 1x10^7 cells and cultivate them for 2-3 days.

- Cells are generally split every 2-3 days, which means trypsinizing 3-4 times before performing the mutation assay.

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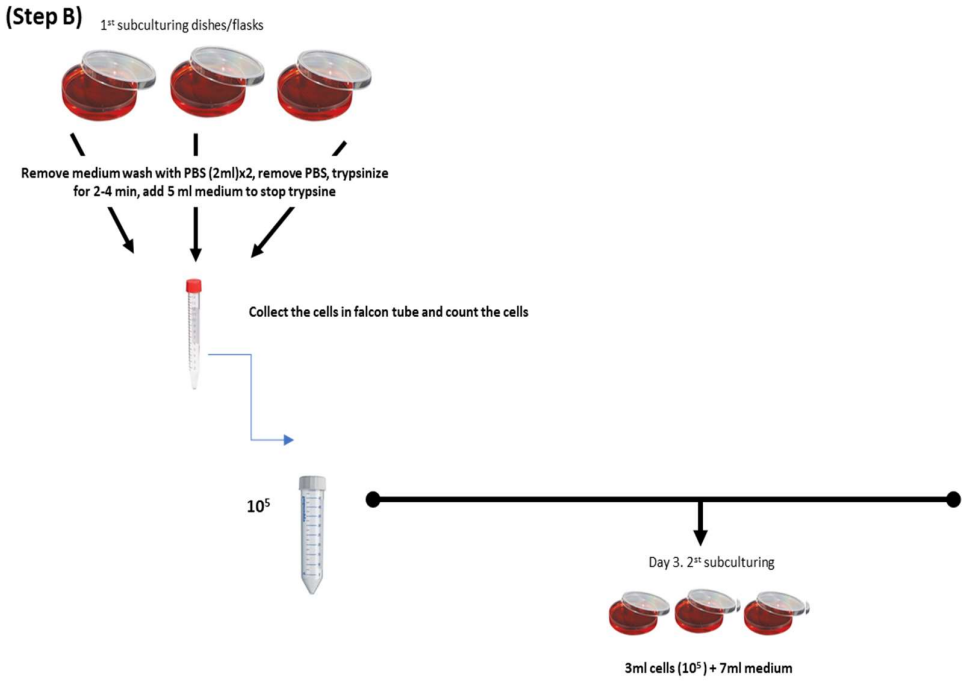

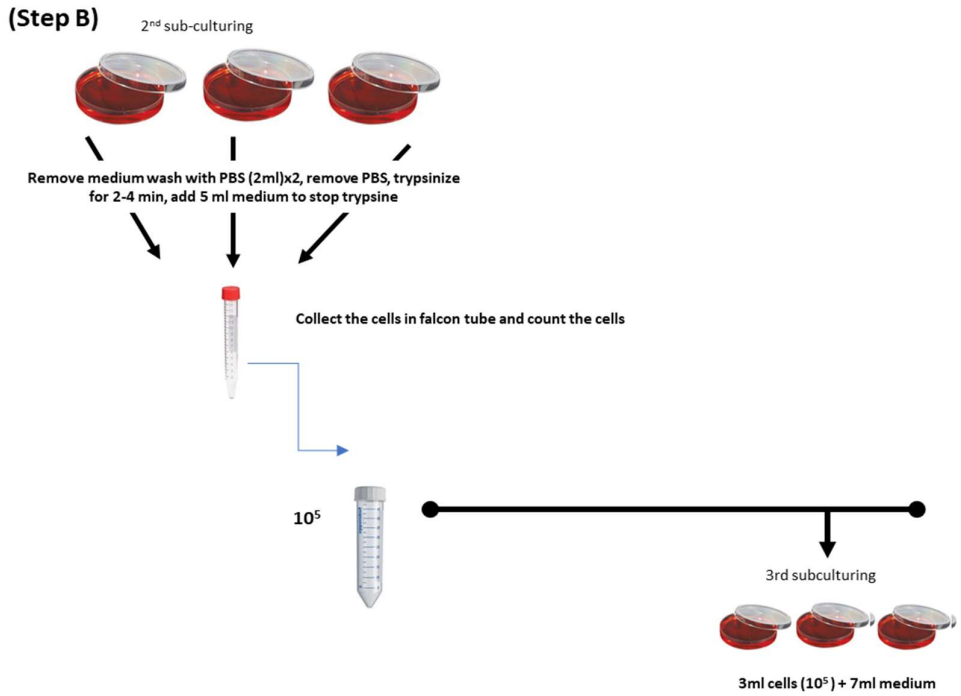

1st seeding for selection of mutants.

Usually, two mutant seedings are performed to ensure that optimal time after the exposure has elapsed for phenotypic expression of mutations. The first seeding is at 6-8 days and the second at 8-10 days after exposure. First mutation seeding:

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- Trypsinize all dishes from each sample (3-5 dishes), count cells and prepare suspension of $1x10^5$ cells/ml and $1x10^3$/ml as described above. Inoculate cells for PE, M (mutant harvest) and for culture for the second seeding:

- **For PE (viability)**
  a) Prepare suspension of $1x10^3$ cells/ml by 10x dilution from suspension of $1x10^5$ cells/ml and then by 10x dilution from suspension of $1x10^4$ /ml (see above)
  b) Vortex to achieve homogenous suspension.
  c) Add 0.1 ml of $1x10^3$ cells/ml suspension (100 cells for control samples) into each of 6 dishes (use 6-well plate or Petri dishes diameter 5cm; in that case use 200 cells as inoculate as these are bigger dishes) for PE.
  d) Shake each dish/plate horizontally on the surface to achieve homogenous spread of cells on dish.
  e) Cultivate 5-6 days to allow cells to form colonies and then stain with methylene blue. Each colony should have at least 50 cells.

- **For mutants selection**
  a) Inoculate $2x10^5$ cells per dish adding 5 ml from stock cell suspension to each of 5 dishes of at least 10 cm diameter or surface 80-100 cm^2 to allow mutant cells to form colonies. Include fresh medium to each dish to reach optimal amount of medium (10 or 15 ml depending on size of the plate).
  b) Shake each dish/plate horizontally on the surface to achieve homogenous spread of cells on dish.
  c) After 2-3h of incubation (when cells are on the surface starting to attach to it), add 0.1 ml of selective agent 6-TG from stock solution 500mg/ml to each plate (dish). This treatment kills normal cells but mutant cells survive and will form colonies.
  d) Incubate for 8-10 days to allow mutant cells to form colonies then stain with methylene blue and let dishes dry.
  e) Count mutant colonies that contain at least 50 cells.

- **Sub-culturing cells for 4th time.** Maintaining cells (all the treated cultures and controls) in culture for 2nd mutation assay by inoculating 3-5 dishes of at least f10cm or surface 80-100 cm^2, inoculum of $2-5x10^5$ cells or more per 10cm diameter dish or surface 80-100 cm^2. Incubate another 2 days.

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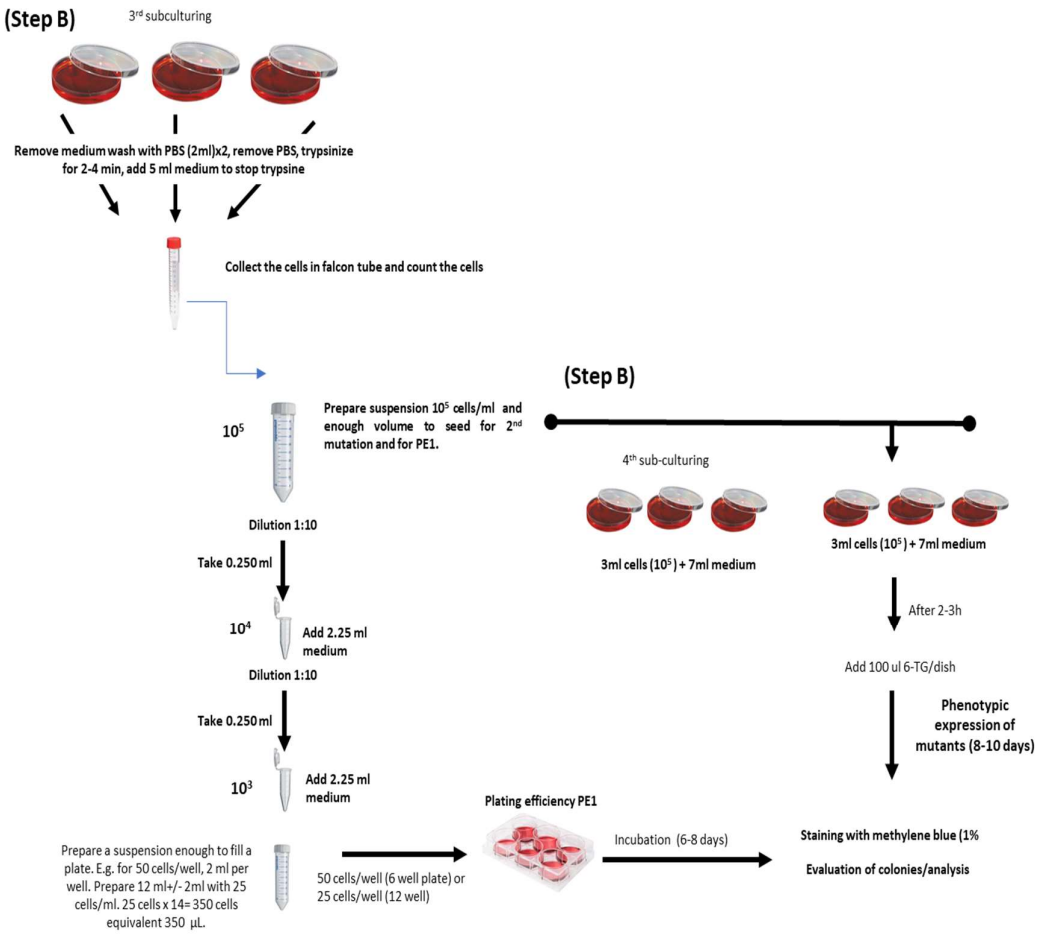

2nd seeding for selection of mutants.

Follow the same procedure is in first harvest except for sub-culturing as cells are not needed anymore.

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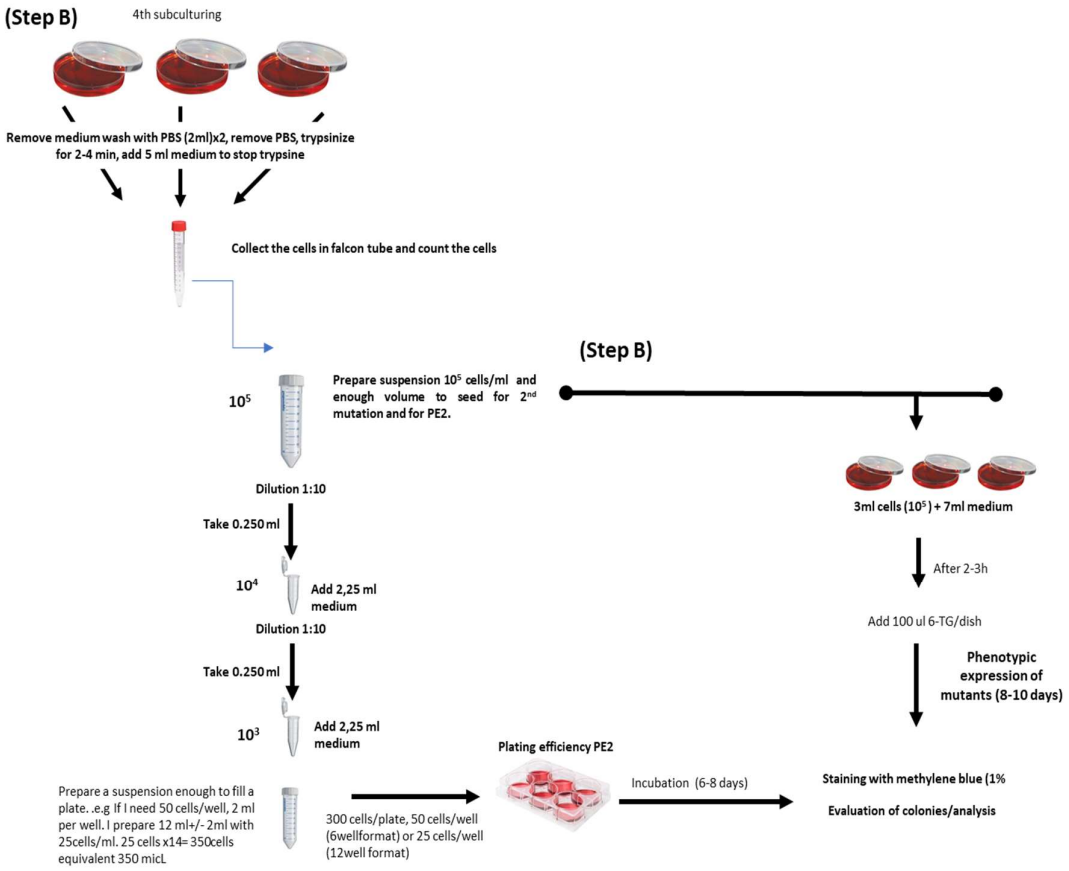

Staining with methylene blue.

- Add 1% methylene blue to each dish or plate. e.g 100 µl of methylene blue per petri dish.
- Shake each dish/plate horizontally on the surface to achieve homogenous spread of methylene blue on dish.
- Stain for approx. 20-30 min.
- When colonies are stained, remove medium and wash dishes carefully in water. Avoid direct stream of water as colonies may detach from the surface.
- Let them dry.
- Each viable cell forms a colony (at least 50 cells per colony) that can be identified by eye.
- Count colonies in each dish using counter and record in datasheet.

7. CHRONOLOGICAL PLANNING

Table :1 and 2 Examples of scheme of the experiment (Optional start on Wednesday or Friday):

Day 1 Cell inoculation for experiment

Day 2-3 Treatment with tested compound

Day 8-10 First mutation harvest

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Day 12/13 Second mutation harvest

Example of Scheme of the experiment. Exposure for 24h

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	Day 0. Cells inoculated at least 1x10^{6}cells/treatment. 2 duplicates per treatment	Day 1. Exposure to test substance+NC +Pc + SC (if used) for 24h	Day 2.,end of exposure, Seeding for cytotoxicity (PE0)+seeding for 1st culturing		Day 4 2nd culturing (passaging the cells and adjusting the number of cells/ml)		
Week 2	Day 7 3rd culturing (passaging the cells and adjusting the number of cells/ml)	Day 8 Check the cytotoxicity PE0 plates for colonies and if visible, stain with Methylene blue 1%. Count colonies PE0. If colonies are not ready you can wait 1-2 more days	Day 9, 4th culturing + 1st mutation harvest + PE1		Day 11 2nd mutation harvest + PE2		
Week 3			Day 16 Stain PE1 of 1st mutation harvest		Day 18 Stain PE2 of 2nd mutation harvest		
Week 4	Day 22 Stain Mutations First and second mutations harvest (if ready)						

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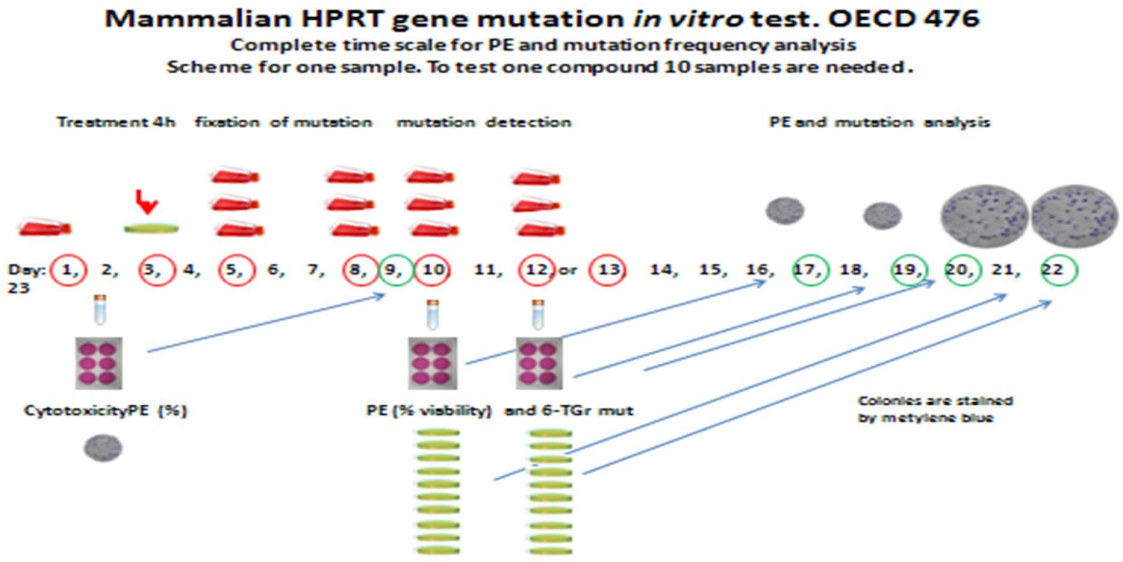

Figure 1. Scheme of Mutation experiment for one sample

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

Cytotoxicity and viability

Cytotoxicity is determined by measuring the relative plating efficiency rPE % (survival) of the cultured cells (inoculated immediately after the treatment period) after staining of visible colonies. The survival (viability) is determined in time of each mutation harvest and calculated on the basis of the number of colonies versus number of inoculated cells.

PE is calculated as % of colonies from all seeded cells following formula:

$$PE(\%)=(\text{Colonies Counted} / \text{Cells Inoculated}) \times 100$$

Cytotoxicity (rPE %) is evaluated by expressing the PE of treated cells relatively to PE of control cells, where PE of control cells is set to 100%.

Example: Cytotoxicity (in %) is determined by the number of colonies counted divided by number of cells inoculated into dish x100. For example, from 200 cells inoculated to each dish (total dishes 5) there are

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151, 155, 162, 175 and 157 colonies found. PE is calculated as sum of all colonies in this case 800/ (5 x 200 inoculated cells) x 100 = 80%.

Evaluation of mutagenicity

The frequency of spontaneous as well as induced mutations (MF1 and MF2) is determined relative to corresponding PE (viability) PE1 and PE2. Each viable cell forms a colony. Count colonies stained with methylene blue (a colony must contain minimum 50 cells), using a click counter (tally counter). Calculate the plating efficiency (PE) (survival, viability) as the number of colonies relative to the number of inoculated cells (as %) following the same formula as for cytotoxicity (see above).

Determination of viability of cells is necessary for determination of mutant frequency as it is calculated as mutant frequency per surviving cells. Viability of cells in time of mutation harvest (in %) is determined as PE (%) in the same way as for cytotoxicity by number of colonies counted divided by number of cells inoculated x100. Viability of cells treated with test compound is calculated from PE of control samples where control is considered as 100%.

Number of mutants is determined by counting and recording number of mutant colonies. The mutant frequency for the treated and control cultures is calculated as number of mutant cells (colonies) per $1x10^5$ or $1x10^6$ surviving cells (colonies) using the following formula:

Mutation frequency (%) = (Mutant Colonies / surviving inoculated cells) x 100

Example: Number of colonies in controls are: 0, 1, 0, 2, 1, i.e., 4 colonies per $1x10^6$ cells (each dish had an inoculum of $2x10^5$ cells, and there were 5 dishes). If viability is 100% then the frequency of mutations is frequency **4 per $1x10^6$ or 0.4 per $1x10^5$.** However, normally cell viability is less than 100% so actual number of surviving cells should be calculated and number of mutants should be adjusted to that number. For example, if viability is 80% then actual mutant frequency is 4 per $8x10^5$ or $5/1x10^6$ cells.

Individual culture data are provided on data sheet. All data are summarized in tabular form. Data sheet includes sample code, number of colonies per dish, values from all parallels and average survival (PE), columns with cytotoxicity relative to control (in %).

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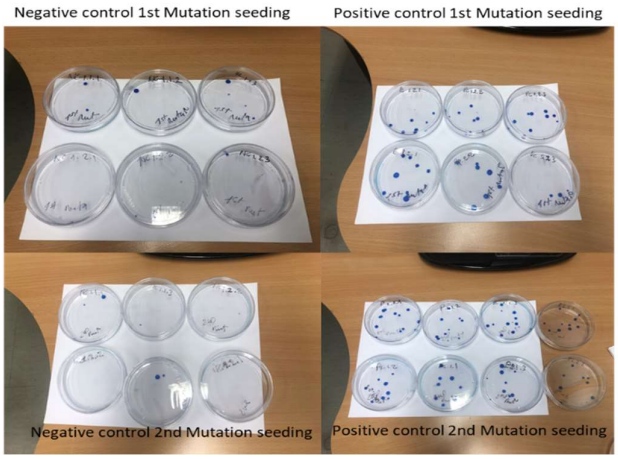

Figure 2: Representative plates with mutant colonies of V79-4 cells: spontaneous and induced mutations: negative control (Nc), positive controls MMS (methyl methane sulphonate).

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9. ACCEPTANCE CRITERIA OF THE STUDY

According to the OECD guidelines (OECD TG 476, 2015), acceptance of a test is based on the following criteria:

1. The negative control is considered acceptable within the laboratory historical negative control database. Negative control data should consist of mutant frequencies from single or preferably replicate cultures consisting of solvent or vehicle handled in the same way as the treated cultures. Concurrent negative controls should ideally be within the 95% CI (confident interval) which means that if 100 different samples and compute a **95**% **confidence interval**, then approximately **95** of the 100 **confidence intervals** will contain the true mean value of the distribution of the laboratory's historical negative control database. Where concurrent negative control data fall slightly outside the 95 %CI control limit they may be acceptable for inclusion in the historical control distribution **as long as these data are not extreme outliers** and **there is evidence of no technical or human failure.**
2. Concurrent positive controls should produce a statistically significant increase compared with the concurrent negative control and should induce responses that are within the 95% CI generated in the historical positive control data base and
3. In case of using metabolic activation two experimental conditions (i.e., short treatment with and 24h without metabolic activation) were tested unless one gave positive results. In case of nanoparticles the treatment should be 24h and normally no metabolic activation is needed.
4. Adequate number of cells and concentrations are analyzable.
5. The criteria for the selection of top concentration. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 10 and 20 % rPE. Care should be taken when interpreting positive results only found at 10% rPE or below. In case of nanoparticles maximum concentration used should be 100 µg/mL.
6. When determining the highest **test chemical concentration**, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity, or marked changes in pH or osmolality should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artifactual positive results and to maintain appropriate culture conditions.

10. INTERPRETATION OF RESULTS

A result is classified **as positive** if a concentration-related or reproducible increase in mutant frequency is observed when evaluated by an appropriate trend test, or **if at least one of the concentrations exhibits a statistically significant increase in mutant frequency compared with the negative control.** A positive result in the HPRT-mutation assay indicates that the test substance induces mutations in the cultured cells used. A positive concentration-response that is reproducible is most meaningful.

There is no requirement for verification of a clear positive or negative response. Equivocal results are clarified by further testing using modified experimental conditions. Negative results indicate that, under the test conditions, the test substance does not induce mutations in the cultured mammalian cells used. Negative results need to be confirmed on a case by case basis.

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According to OECD guidelines (OECD TG 476, 2015):

Providing that all acceptability criteria are fulfilled, a test chemical is considered **clearly positive** if, in all experimental conditions examined:

a) **If at least one of the concentrations exhibits a statistically significant increase in mutant frequency** compared with the negative control,

b) **The increase is concentration related when evaluated with appropriate trend test**,

c) Any of the results are **outside the distribution of the historical negative** control data.

In case of testing nanoparticles **concentration-response is not always observed**. In that case a result is classified **as positive** if **at least two concentrations are positive (significantly different from control).**

1. Providing that all acceptability criteria are fulfilled, a test chemical is **considered clearly negative if,** in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test,

c) all results are inside the distribution of the historical negative control data. The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

Biological relevance of the results will be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance will not be the only determining factor for a positive response. A test substance, for which the results do not meet the above criteria, is considered non-mutagenic in this system.

11. RECORDING OF TEST PERFORMANCE AND REPORT

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

After all results have been collected from at least one experiment and one repeat, data are analyzed and the 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. A report on Quality control should be included.

12. CRITICAL PHASES

- Preparation of dilutions of the test substance

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- Exposure of cells with test substance – be sure that right concentration is included into correctly marked vial/well with cells
- Dilution of cell suspension
- Inclusion of 6-TG in each well

13. IMPORTANT ASPECTS

- Avoid any chance for contamination. Work must be performed in sterile conditions in laminar flow hood.
- The temperature of the incubator must be within acceptable range
- Cell density during passages should be no more than 75% confluency.
- Treatment of cells is critical – concentration preparation, treatment time and washing.
- Cell counting, preparation of cell suspension, dilution in steps and plating cells in small inoculum and equal spreading on the dish.

14. REFERENCES

Dušinská M., Slameňová D.: Use of mammalian cells in screening for chemical carcinogens, Biologické, listy, 47, 264-278, 1982.

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OECD (2016), *Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264809-en

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ANNEX 7. SU MN assay SOP

Experimental plan for RiskGONE OECD TG-487: In vitro mammalian cell micronucleus ring trial

Partners involved: Swansea University (Lead), NILU, ANSES, LIST (pending training).

The ring trial will consist of each partner testing the TK6 cell line with the engineered nanomaterials (ENMs); Zinc oxide (ZnO, Sigma), titanium dioxide (TiO_2, JRC), tungsten carbide-cobalt (WC/Co). This standard operating procedure details the protocol that enables both the manual and semi-automated scoring versions of the *in vitro* cytokinesis-blocked micronucleus assay. However, only the manual scoring version will be used in the ring trial. **Experiments are to be performed in biological duplicate for an *n*=2 (Two independent experiments)**. The ENM exposure period will be for 1.5 cell cycles of TK6 cells and a cyto B time of 1.5 cell cycles.

It is important to check TK6 cells regularly, culturing past 6 weeks can cause the background levels of micronuclei to drift above 1.2% Mn/BN (beyond our historical levels). If TK6 are cultured for too long they can lose their smooth cellular appearance under light microscopy and become 'spikey' in appearance, if this does occur we advise discarding the cells and taking out a fresh vial.

ENMs:

Zinc Oxide (ZnO) – Sigma #MKCJ4155

Titanium dioxide (TiO_2) – JRC #JRCNM01005a990582

Tungsten carbide-cobalt (WC/Co) – NanoAmor #5561HW

Chemicals:

Mitomycin C (MMC) – Sigma, #M4287-2mg. Suspend in double distilled water at 1mg/ml, keep at 4°C.

***In vitro* cytokinesis-blocked micronucleus (CBMN) test protocol for use with TK6 cells and engineered nanomaterials (ENMs)**

Swansea University

Standard Operating Procedure

2020

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Abbreviations

Cyto B – Cytochalasin B

EDTA – Ethylenediaminetetraacetic Acid

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

HS – Horse Serum

KCl – Potassium Chloride

MMC – Mitomycin C

NaCl – Sodium Chloride

P/S – Penicillin/Streptomycin

PBS – Phosphate Buffered Saline

L-glutamine

Biological Setting & Test System

This SOP should be carried out under strict laboratory conditions, with all work performed under sterile conditions and in a Class II Laminar Tissue Culture Hood.

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Chemicals & Reagents

Table 1. List of chemicals and reagents required for *in vitro* CBMN assay.

Reagent	Supplier
PBS	ThermoFisher; 10010023
Cell culture medium	ThermoFisher; 21870076
Cytochalasin B	Merck #250233
Methanol	Sigma; 34860
Beckman Coulter Diluent	Beckman; 628017
Giemsa Stain	VWR; 350864
Gurr pH 6.8 Buffer tablets	VWR; 331542
Glutamine	ThermoFisher; 25030081
Horse Serum	ThermoFisher; 16050122
Trypsin-EDTA (0.25%)	ThermoFisher; 25200056
Potassium Chloride (KCl)	Sigma; P9333
Sodium Chloride (NaCl)	Sigma; S7653
Acetic acid	Sigma; 1005706
VECTASHEILD Mounting Medium with DAPI	VECTOR Laboratories; H-1200
DPX Mountant	VWR; 13510
Xylene	VWR; 214736

Cell culture preparation

In preparation for this SOP, it is advised that cell culture medium is prepared and pre-warmed at 37°C for 30 minutes prior to use. TK6 complete cell culture medium is prepared by adding 50ml of horse serum to 500ml of media (RPMI 1640), 5ml of L-glutamine is also added to complete the media. The full cell culture medium should then be mixed prior to use by inverting the bottle.

Procedure

To thaw cells from Liquid Nitrogen:

- Place a bottle of complete medium in the water bath at 37°C (25-30 minutes before use). Remove your vial of cells from the Liquid N_2 and place in a foam float in the water bath. Do not allow the vial to be immersed. Cells will thaw in ~1 minute, transfer them to a T25 flask of the warm medium slowly and

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carefully using a Pasteur pipette. TK6 cells can be left for 24 hours in the T25 before being moved to a T75 to begin growing exponentially.

- Label the flask with the name of the cells, the passage number and your name. Keeping a note in your lab notebook of the date of resuscitation and the date that the cells were previously frozen will be helpful because if there is a problem with them, the other vials from the batch can be identified.)
- Place the flask in the CO_2 incubator.
- Check them after 24 hours for growth and contamination. They can be counted to determine when they will need splitting. Cells should not be allowed to become too confluent, TK6 cells for example should be maintained below 800,000-1 million cells per ml. If required change media every 2 days.
- Monitor passage number in your experiments; do not let this rise beyond a reasonable range (TK6 cells should ideally not be routinely sub-cultured beyond 25-30 passages). TK6 morphology should be checked by light microscopy daily, they should retain a smooth spherical morphology. If the cells develop a spiked surface morphology then the cells should be discarded, and a fresh vial should be withdrawn.
- At day 4 the SOP can be performed 2 different ways, such that scoring can be conducted using a manual approach, or alternatively, a semi-automated scoring approach using the Metafer microscope.

DAY 1 – seed cells

- Count cells (e.g., using a Beckmann Coulter Counter, haemocytometer, or other cell counting system) and seed at $1x10^5$ cells/ml in 10ml of media in $25cm^3$ flasks per treatment.
- Incubate overnight at 37°C/ 5% CO_2

DAY 2 – initial cell count & dosing of cells with test agent

Concurrent positive and solvent/vehicle controls should be included in each biological replicate. For TK6 cells, the recommended negative control is cell culture media (where dispersant solutions have not been used); if a dispersant has been used then the two negative controls should be included, 1) culture media only and 2) dispersant solution only. The chemical positive control is:

MMC, dose for 1.5 cell cycles at 0.01µg/ml

This procedure must be conducted in a Class II Laminar Tissue Culture Toxic Hood, with the user wearing double gloves to ensure safety when dosing with chemicals and / or ENMs.

MMC stock 1mg/ml

1:100 = 10ul stock MMC + 990ul double distilled water gives 0.01mg/ml

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Take 10ul of 0.01mg/ml and add to 10ml cells

OR

1:1000 = 1ul stock MMC + 999ul water gives; 0.001mg/ml

Take 100ul of 0.001mg/ml and add to 10ml cells

Preparation of ENMs

Tungsten carbide-cobalt (WC/Co): for this protocol we use the NANoREG guidance document for ENM suspension and sonication (Alstrup Jensen et al., 2014). Concentrations; **20 & 100µg/ml.**

RiskGONE ENMs

ZnO (Sigma): Vortex the bottle as described by Sigma operating procedure. Concentrations; **0, 1, 5, 10, 20µg/ml.**

Vortex the ZnO dispersion from Sigma vigorously for 30 seconds. **This step is very important** in order to get a good dispersion and maintain 240 mg/ml concentration (stock concentration inside the bottle). Add the required volume of ZnO batch dispersion to obtain the desired final concentration in complete exposure medium. Vortex the vial before exposure. We recommend diluting stock down to a manageable concentration of 10mg/ml using the following dosing plan below in **Table 2**.

- Stock ZnO is 240mg/ml
- 12.5µl + 287.5µl media for 300µl stock of 10mg/ml (adjust if you need more than 300µl)

Table 2. Example dosing plan for TK6 cells in T25 flasks seeded in 10ml of media.

ZnO Concentration (µg/ml)	Required volume of **10mg/ml stock (µl)**	Required volume of media (µl)	Volume added to 10 ml cells (µl)
0	0	200	200
1	1	199	200
5	5	195	200
10	10	190	200
20	25	175	200

TiO_2 (JRC): Use the RiskGONE WP4 SOP for suspension and sonication. Concentrations; **0, 10, 25, 50, 100µg/ml.**

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- Count flasks for cytotoxicity (initial cell number) 1h before dosing. 100µl of cell suspension is added to a cuvette containing 10ml of diluent for cell concentration determination using e.g., a Beckmann Coulter Counter, haemocytometer, or other cell counting system.
- Dose cells for 1.5 cell cycles with the ENM at the desired final concentration.

DAY 3 – post-treatment cell count & addition of cytochalasin B

- Count flasks (post-treatment cell number) to later calculate cytotoxicity. Add 100µl of cell suspension directly from flasks to a cuvette containing 10ml of diluent for cell concentration determination using a Beckmann Coulter Counter. Alternative cell counting method is also fine.
- Transfer the cells to a 15 ml centrifuge tube, centrifuge at 230g for 5 min and discard the supernatant. Re-suspend the pellet in pre-warmed PBS and re-centrifuge at 230g for 5 min, repeat this wash step a second time and discard the supernatant.
- Resuspend cell pellets in fresh media containing 3mg/ml cytochalasin B and place into new T25 flasks. Cytochalasin B is dissolved in DMSO and stored at -80°C.
- Incubate for 1.5 cell cycle

DAY 4 – harvesting cells for manual scoring

- Harvest cell pellets by centrifugation (230g for 5 min), resuspend in 5 ml pre-warmed PBS and centrifuge cells at 230g for 5 min. Discard the supernatant and repeat this wash step a second time.
- **If Cytospin is available in laboratory**: Resuspend cells in 10ml of PBS (if the cell pellet looks small adjust the volume as necessary). Place labelled slides (90% methanol-cleaned) into cyto-clips, place a filter card (with 5 mm cyto-dot hole) on top of the side and clip in place a cyto-funnel. Load 100ml of cell suspension into each funnel and centrifuge at 200g for 10 min in a Shandon Cytospin.
    a. Examine slides for correct cell density and adjust the volume of cell suspension as required. If the cells are too sparse, centrifuge the cell suspension at 230g for 5 min and resuspend in a smaller volume of PBS.
    b. Fix slides for 10min in ice cold 90% methanol and leave to air dry (at this point slides can be stored at -20°C)
    c. **If Cytospin is unavailable in laboratory**: Resuspend cells in 10ml 90% methanol (if the cell pellet looks small, adjust volume (reduce volume to 5ml)). Pipette 100µl of cell suspension

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onto a clean microscope slide. Pass the slide through a Bunsen burner flame (collar fully open, blue flame) to flame-fix the cells.

- Slides can be stained using acridine orange (AO) if the assay operator is experienced.
- Stain slides in 20% Giemsa solution 6.8 Gurr buffer, filtered) for maximum 10 minutes (minimum of 6 minutes required), intensity of stain can vary depending on preparation.
- Rinse slides in pH 6.8 Gurr buffer, then soak in pH 6.8 Gurr buffer for 1-2min. Note: two slide tanks can be set up simultaneously to improve efficiency
- Leave slides to air dry standing vertically
- Dip slides in xylene for 10 seconds using tweezers and drain off the excess
- Drop DPX over the area of cells
- Place 22x22mm coverslip over the DPX, ensuring there are no air bubbles by pressing lightly on the coverslip
- Leave slides to dry for 24h in the fume hood
- View slides under a light microscope, evaluate *1000BN cells per biological replicate* per concentration of ENM for the presence of micronuclei.
- Two biological replicates are needed.
- Therefore, a total of 2000BN cells will be scored for the negative control, each test dose of ENM applied and the positive control.

DAY 4 – harvesting cells for automated scoring protocol

- Harvest cell pellets by centrifugation (230g for 5 min), resuspend in 5 ml pre-warmed PBS and centrifuge cells at 230g for 10 min. Discard the supernatant and repeat this wash step a second time.
- Re-suspend cell pellets with hypotonic solution (KCl 0.56%), then centrifuge immediately at 230g for 10min
- Re-suspend pellets in Fixative 1 (methanol: acetic acid: 0.9% NaCl (5:1:6 parts)) and incubate at 4°C for 10min before centrifugation at 230g for 10min.
- Re-suspend pellets in Fixative 2 (methanol: acetic acid (5:1 parts)) and incubate at 4°C for 10 min before centrifugation at 230g for 10 min. Repeat this wash step a further 3 times. Maintain cells in the last fix wash overnight at 4°C.

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- Place freshly opened microscope slides in a glass tank of Fixative 2 at 4°C, at least a 2 hours before slide preparation (ideally overnight). On the day of preparing slides, replace the fix with ddH_2O.
- Centrifuge (230g for 10 minutes) the fixed cell suspensions and thoroughly re-suspend in ~1ml Fixative 2
- Take a slide out of the ddH_2O and wipe the water off the upper side with slide tissue, ideally with one movement (the surface should be dry or with only a faint film of water remaining). Pipette 100µl of the cell suspension evenly onto the slide
- Wait a few seconds until the suspension is evenly spread over the slide, and then put it in a vertical position for drying
- Check cell density of binuclear cells and if required adjust the final re-suspension volume by either lowering or increasing the volume of Fixative 2 added. Cells should not be overlapping, densely packed or too sparse.
- Stain slides with 30µl (3 dots of 10µl) of Vectashield mounting medium with DAPI, apply coverslip and incubate in the dark for 15 min
- Score slides on a TK6 cell classifier using the automated Metafer microscope (Axio-imager Z2 fluorescent microscope, Carl Zeiss UK) Metafer 4 software version 3.5 (MetaSystems, Germany). Not all cell lines will be suited to this classifier – it is specific to TK6 cells.
- Score 2000 BN cells per ENM concentration/per replicate, (6000 BN cells in total per ENM concentration). Classifier information can be found in the work by Seager and colleagues (Seager et al., 2014). We have also provided nuclei and micronucleus classifier settings in the Appendix.

CYTOTOXICITY CALCULATIONS

Relative Population Doubling (RPD):

$$RPD = \frac{\text{No. of population doublings in treated cultures}}{\text{No. of population doublings in control cultures}} \times 100$$

Where population doubling = [log (post-treatment cell number/ Initial cell number)]/ log 2

Acceptance Criteria

Selection of binucleated cells:

- The binucleates should have intact nuclear membranes within the cytoplasm boundary

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- The two binucleates should be approximately equal in size, similar levels of stain intensity and pattern
- The two binucleates can be connected to one another by a very fine nucleoplasmic bridge no wider than 1/4th of the nuclear diameter.
- The two binucleates may be in contact but not overlapping, however if the nuclear boundaries of both can be seen it can be scored.
- The cytoplasmic boundary of a binucleated cell must be intact and distinguishable from adjacent cells.

Scoring of micronuclei on manually prepared slides: A micronucleus must have the following features to be scored correctly.

- Small round (sometimes slightly oval), additional nucleus not connected to the main nucleus (by nuclear-plasmic bridges) which will now be visible as a binucleate (pair of nuclei) within the same cell.
- Micronucleus should have a smooth circumference.
- Size of the micronucleus should be 1/16th to 1/3rd the size of the binucleate within the cell.
- The micronucleus should have the same shape, colour as binucleated nuclei

Historical control range for TK6 cells in recent experiments

TK6 cells exposed to complete cell culture media only (negative control) have observed background micronuclei frequencies in binucleated cells between 0.3-1%. Following exposures to MMC for 1.5 cell cycles we have observed TK6 micronuclei between 2-5%Mn/BN. We would therefore consider concurrent negative and positive controls to be within this range.

Appendices for Swansea University SOP

TK6 classifier settings on the Metafer microscope (Axio-imager Z2 fluorescent microscope, Carl Zeiss UK) Metafer 4 software version 3.5 (MetaSystems, Germany):

Nuclei

- Image Processing Operations: Sharpen (3,2) Median V (3) Median H (3)
- Object Threshold: 30%
- Minimum Area: 20.0µm^2
- Maximum Area: 400.0µm^2
- Maximum Relative Concavity Depth: 0.900
- Maximum Aspect Ratio: 1.500
- Maximum Distance: 30.0µm^2

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- Maximum Area Asymmetry: 70%
- Region of Interest Radius: 40.0µm^2
- Maximum Object Area in ROI: 50.0µm^2

Micronuclei

- Image Processing Operations: Median V (3) Median H (3) Sharpen (5,3) SB Histomax
- Object Threshold: 8%
- Minimum Area: 1.50µm^2
- Maximum Area: 55.0µm^2
- Maximum Relative Concavity Depth: 0.900
- Maximum Aspect Ratio: 4.000
- Maximum Distance: 25.0µm^2

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