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# DRAFT Guidance Notes on the Adaptation of the In Vitro micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials

## Study Report reflecting State of the Art Knowledge for the in vitro micronucleus assay

Enter any logistical information related to the meeting e.g. meeting date, time and location.

DRAFT circulated to the WNT for inputs by **31st December 2021**

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# Guidance Notes on the Adaptation of the In Vitro micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials

## Aim and scope of the project

1. The OECD Working Party on Manufactured Nanomaterials addresses all the components needed for thorough hazard assessment of nanomaterials (NM) for human health and the environment, including making available reliable test methods for the purpose of NMs safety assessment. At an OECD expert meeting held in Paris in October 2014 it was concluded that the scientific information available was not sufficient to fully support development of a harmonised version of the *in vitro* micronucleus test protocol because the existing or published data were not generated in a harmonised way and did not provide answers to some key questions (e.g. the uptake on NMs, dosing regimens, necessary length of exposure to NMs or scheme of treatment to cytochalasin B). The OECD WPMN approved a JRC led project with the aim to identify needed adaptations of the *in vitro* micronucleus test (Test Guideline 487) for the assessment of manufactured NMs and eventually provide appropriate guidance. The same project was subsequently included by the OECD-WNT in the work plan of the Test Guidelines Programme (Project 4.95). Therefore, work aiming to develop a Guidance Document (GD) addressing the necessary adaptations of current genotoxicity TGs for nanomaterials testing was initiated in January 2019 under the coordination of JRC. In order to share the lessons learnt from work carried out so far under this project and guide the users of the Test Guideline 487 when testing nanomaterials, it was agreed to publish this document, mindful that it will be refined in future with the testing of additional nanomaterials.

2. The whole study was divided into 3 phases:

***Phase 1: Clarification of main technical issues***

- Physical-chemical characterization of gold, silver and silica Nanoparticles.
- Cytotoxicity assessments of nanoparticles in A549, Caco-2, V79, TK6 cells
- Uptake of Nanoparticles into the various cell systems
- Define the doubling times

3. JRC coordinated this phase of work, which was completed and culminated in the following reports:

4. The Report on Physico-Chemical Characterisation of the nanomaterials chosen for the study was published in 2018 by JRC.

- Drewes, C., Ojea Jimenez, I., Mėhn, D., Colpo, P., Gioria, S., Bogni, A., Ponti, J., Kinsner-Ovaskainen, A., Gilliland, D. and Riego Sintes, J., Physicochemical characterisation of gold, silica and silver nanoparticles in water and in serum-containing cell culture media, EUR 29054 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77705-9 (online),978-92-79-77704-2 (print), doi:10.2760/818663 (online),10.2760/58721 (print), JRC110379.

5. The Report on In vitro toxicity and uptake with the selected cell lines and selected nanomaterials was published on 2020 by JRC

- Bogni A., Ponti J., Drewes C., Kinsner-Ovaskainen A., Bremer-Hoffmann S., In vitro cytotoxicity and cellular uptake evaluation of gold, silica and silver nanoparticles in five different cell lines: Caco-2, A549, CHO, V79 and TK6, European Commission, Ispra, 2020, JRC120791

### *Phase 2: Interlaboratory comparison*

- Selection, purchase and characterization of material
- Protocol development by lead laboratory
- Protocol transfer to other laboratories
- Interlaboratory study
- Study evaluation

6. The JRC coordinated this phase of work, while the experimental work was performed by BASF (Germany) and Swansea University (SU; UK). The outcomes of the experimental work conducted under this phase of the work programme are detailed within this document. Additionally, a publication detailing this phase of the study is in preparation.

7. The selection of cell lines to evaluate during this phase of study were agreed upon during an expert group meeting and included TK6 cells, V79 and human lymphocytes. These cells were selected as they are cited as appropriate for use with the *in vitro* micronucleus assay in OECD TG487, while cells such as A549 and Caco-2 (which were considered in Phase 1) have not been evaluated for use with the assay.

### *Phase 3: Guidance development*

- Set up of expert drafting group
- Guidance development
- Presentation to OECD

8. The JRC was not able to coordinate this phase of work and so the leadership was transferred to the UK and Germany.

9. The OECD guideline 487 (adopted 29. July, 2016) is the current guideline under the Mutual Acceptance of Data (MAD) agreement that instructs how an *in vitro* micronucleus assay should be performed. The procedures described in the guideline are, however, directed at testing chemicals and not nanomaterials. In the guideline this deficiency is noted, yet a recommendation of a suitable adaptation of the protocol for the nanomaterial testing is not given. Thus, the current regulatory situation for the mutagenicity testing of nanomaterials is officially to follow the current procedure of the guideline, which is not necessarily the optimal procedure from a scientific point of view. Parameters such as the treatment interval or selection of the test concentration are a few examples of aspects, which need specific consideration when testing nanomaterials; a pulse treatment of cultures with nanomaterials do not provide sufficient time for the internalization process of a

142 particular material and nanomaterials can per se only be tested at precipitating levels. Since there is no official
143 document on how to adapt the OECD 487 protocol for nanomaterial testing, there is a certain level of urgency
144 in providing a guidance on the necessary adaptations, which would allow a more relevant assessment of the
145 mutagenic potential of nanomaterials and avoid repetition of inadequately performed studies. This preliminary
146 guidance document is therefore aimed at addressing this urgent deficiency in the guidance available.

# Phase 2: Methods

## Preparation of ENMs.

10. 5nm AuNPs (#OECD-RLS-03) & 30nm AuNPs (#OECD-RLS-10)), 22nm $SiO_2$ NPs (#OECD-RLS-0102) were supplied by JRC. JRC stock ENMs were sonicated in a 90W Ultrasonic Bath (Fisher Scientific #FB15046) for 20 minutes at 37°C to encourage destabilisation of agglomerate material. Following sonication, working concentrations of particles were prepared in cell culture media using a 1:1 dilution for the highest concentration and using serial dilutions to prepare the rest of the dose range (Supplementary Information Table 1). Given each JRC ENM stock were at different concentrations, this process was ENM-specific. $CeO_2$ (Frauenhofer, Lot#NM-212 Reference Nanomaterial), $BaSO_4$ (Solvay, Lot#V106) and Tungsten-Carbide/Cobalt, (8wt% WC/Co <200nm, 99.5% LOT#5561-072018, Nanostructured & Amorphous Materials Inc., USA), was used as a trial positive particle control and was weighed, suspended and sonicated according to the NANoREG protocol (Alstrup Jensen et al., 2014).

### *Cell culture (University of Swansea protocol).*

11. The human lymphoblastoid (TK6) cells were purchased from Public Health England (PHE). The cells were cultured in RPMI 1640 supplemented with 10% Horse serum and 1% L-glutamine. TK6 cells were routinely sub-cultured for 2 weeks prior to ENM testing, cells were regularly checked for potential changes to morphology and density by light microscopy. The human liver cancer (HepG2) cell line was purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10% FBS, 1% Penicillin/Streptomycin (P/S) and 1%L-glutamine. HepG2 cells were grown to 80% confluency before being routinely sub-cultured. The V79 cells (AbbVie GmbH) were cultured in MEM Eagle media (Pan-Biotech, UK) containing 1% L-glutamine, 1% Amphotericin, 10%FBS and 1% P/S. V79 cells could grow to 80% confluency before being routinely sub-cultured.

### *Semi-automated in vitro cytokinesis-blocked micronucleus assay.*

12. Two slightly different approaches were taken with the CBMN assay depending on whether suspension or adherent cell lines were used. TK6 (suspension cells), HepG2 and V79 (adherent) cells were seeded at $1.0x10^5$ cells/ml in T25 flasks along with satellite flasks per concentration to be counted for cytotoxicity and were then treated with ENMs for 1 cell-cycle (12 hours). Mitomycin-C (MMC) at 0.01µg/ml was used as the chemical positive control, and WC/Co at 20µg/ml & 100µg/ml was tested as a potential positive particle control. The *in vitro* cytokinesis-blocked micronucleus assay was performed as described previously by Evans *et al* & Burgum and colleagues (Evans et al., 2019, Burgum et al., 2020). On the day of exposures, cells are counted at least 2 hours before exposures (with suspension cells this can be done immediately prior to exposures). When using the HepG2 cells time is required for them to re-adhere to the flasks before exposures. Cells were dosed (both the satellite flasks and the CBMN-flasks) with ENMs prepared in cell-culture media and the negative control being media only. Exposures were performed as close to sonication times as possible to avoid sedimentation. Following the exposure period, the satellite flask cells were counted for the calculation of relative population doubling (RPD), a measure of cytotoxicity (Equation 1). Cell counts were performed using a Beckman coulter

counter by adding 100µl of cells to 10ml of diluent. Following ENM exposure for 1 – 1.5 cell cycle, TK6 cells were centrifuged and washed with PBS in triplicate (removal of as much ENM as possible), satellite flasks were counted to facilitate calculation of cytotoxicity data. V79 and HepG2 cells had exposure media aspirated and were then washed twice with PBS before being counted in the same manner. TK6 cells were re-seeded into clean T25 flasks in cell-culture media containing 3µg/ml of cytochalasin-B (cyto-B), the cells were returned to the incubator for a further 1.5 cell cycles. V79 and HepG2 cells remained in the same flasks and were supplemented with fresh media containing the same concentration of cyto-B.

***Cell culture (BASF SE Protocol):***

13. **V79 cell cultures:** V79 (adherent) cells were seeded at $5.0x10^5$ cells/ml in T25 flasks (attachment period 24-28 hours) and were treated with ENMs along with satellite flasks per concentration to be counted for cytotoxicity and LA ICP MS assessment for 1 cell-cycle (14 hours). Ethyl methanesulfonate (EMS) at 500µg/ml and 600µg/mL was used as the chemical positive control, and WC/Co at 30 µg/mL & 100µg/ml was tested as a potential positive particle control. After 1 cell-cycle test substance treatment the cultures were rinsed twice with HBSS (Hanks Balanced Salt Solution). The cultures intended for mutagenicity assessment were incubated in MEM (incl. 10% [v/v] FCS) supplemented with CytB (final concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO; AppliChem, Cat.No. A7657) for 24 hours. Cultures used for LA ICP MS assessments were trypsinized, fixed twice in methanol : acetic acid (19:1; -20 °C) and spread on slides.

14. **Whole blood cultures:** After 48 hours the activated cell cultures were pooled and centrifugated in 10 mL aliquotes at 900 g for 5 minutes. After centrifugation the supernatant (culture medium) was removed and the cells suspended in ENM dilutions in culture medium along with satellite tubes for each concentration for LA ICP MS assessment. All tubes were transferred into cell culture flasks and incubated for 20 hours. Mitomycin C (MMC; Roche Diagnostics) at 0.04 µg/mL and Colchicine (Col; Roche Diagnostics) at 0.05 µg/mL were used as the chemical positive controls, and WC/Co at 10, 30, 60 and 100µg/mL was tested as potential positive particle control. At the end of the exposure period, the cells were transferred in tubes, centrifuged for 5 minutes at 900 g and resuspended in HBSS (Hanks Balanced Salt Solution). Washing of the cells was repeated at least once. Then the cells were centrifuged at (900 g, 5 min) and resuspended in DMEM/F12 medium with 10% [v/v] FCS and transferred into 25 cm² cell culture flasks. Cyt B (see 3.2.2) was added to the cultures intended for mutagenicity assessment and incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity for 20 hours. To prepare the cells for the LA ICP MS assessments, they were separated from the nanoparticles using density centrifugation (Ficoll paque) and washed once at 900 g for 5 minutes. The obtained cells were fixed twice with methanol:acetic acid (19:1; -20°C) and spread on slides.

15. **Buffy coat cell cultures:** After 48 hours the activated cell cultures were pooled and centrifugated in 10 mL aliquotes at 900 g for 5 minutes. After centrifugation the supernatant (culture medium) was removed and the cells suspended in ENMs dilution in culture medium along with satellite tube for each concentration for LA ICP MS assessment. All tubes were transferred into cell culture flasks and incubated under agitation for 20 hours (corresponding to 1 cell cycle) . Mitomycin C (MMC; Roche Diagnostics) at 0.04 µg/mL and Colchicine (Col; Roche Diagnostics) at 0.05 µg/mL were used as the chemical positive controls, and WC/Co at 10, 25 30, 60 and 100µg/mL was tested as potential positive particle control. At the end of the exposure period, the cells were transferred in tubes, centrifuged for 5 minutes at 900 g and resuspended in HBSS (Hanks Balanced Salt Solution). Washing of the cells was repeated at least once. Then the cells were centrifuged at (900 g, 5 min) and resuspended in RPMI medium with 20% [v/v] FCS and transferred into 25 cm² cell culture flasks. Cyt B was added to the cultures intended for mutagenicity assessment and incubated at 37°C, 5% (v/v) CO2 and ≥ 90% relative humidity for 20 hours. In order to prepare the cells for the LA ICP MS assessments, they were separated from the nanoparticles using density centrifugation (Ficoll paque) and washed once at 900 g for 5 minutes. The obtained cells were fixed twice with methanol:acetic acid (19:1; -20 °C) and spread on slides.

***Cell harvesting for semi-automated Metafer scoring (University of Swansea Protocol).***

16. The TK6, HepG2 and V79 cells were harvested by centrifugation (230g for 5 min), resuspended in 5ml of pre-warmed PBS and centrifuged at 230g for 10 minutes. The supernatant was discarded, this was repeated a second time. The cells were then resuspended in hypotonic solution (KCl 0.56%), before being centrifuged immediately at 230g for 10 minutes. The cells were resuspended in Fixative 1 (methanol: acetic acid: NaCl (0.09%) (5:1:6 parts)) and incubated at 4°C for 10 minutes before centrifugation at 230g for 10 minutes. Cells were resuspended in Fixative 2 (methanol: acetic acid (5:1 parts)) and incubated at 4°C for 10 minutes before centrifugation at 230g for 10 minutes, this was repeated 3 times. Cells can be maintained overnight in Fixative 2 at 4°C, tubes covered by foil. The day before making slides, freshly opened microscope slides were placed in a glass tank of Fixative 2 at 4°C, for 2 hours before slide preparation (ideally this is done overnight). On the day of preparing slides, the fixative was replaced with $ddH_2O$. On the day of slide preparation, the fixed cell suspensions were centrifuged at 230g for 10 minutes and thoroughly re-suspended in ~1ml of Fixative 2. Slides were removed from the $ddH_2O$ and wiped dry with slide tissue. A total of 100µl of the cell suspension was evenly pipetted onto the slide held at an angle. The slides were then stood near-vertical on tissue paper to dry. The cell density was checked to ensure cells were evenly distributed, without clumping. Once dried, the slides were stained with 30µl of Vectashield mounting medium with DAPI, coverslip applied and incubated in the dark for 15 minutes. Slides were scored using the Zeiss AxioCam HRc (Carl Zeiss Microscopy and Imaging, UK) semi-automated Metafer system. The details for the classifier used to support the analysis can be found in Table 1 of the Supplementary Information. All experiments were performed in triplicate (*n*=3) and 2000 binucleated (BN) cells per replicate were scored per concentration (6000 BN cells in total).

***Cell harvesting for manual scoring approach (University of Swansea Protocol).***

17. Cells were harvested by centrifugation (230g for 5 min), resuspended in 5 ml of pre-warmed PBS and centrifuged at 230g for 5 minutes. The supernatant was discarded, and this step was repeated once more before the cells were resuspended in 5ml of PBS. The cell density was checked at this stage using a practice slide. Labelled slides were 90% methanol-cleaned, dried and placed onto cyto-clips with 5mm cyto-dot filter cards and cyto-funnel attached. A total 100 µl of cell suspension was pipetted into each funnel and centrifuged at 200g for 10 minutes in a Shandon Cytospin. Slides were then examined for density. Cells were then fixed by immersing slides in 90% methanol at -20°C for 10 minutes. Once dried the slides were stained in 20% Giemsa solution Gurr buffer (pH 6.8), filtered for 10 minutes. The slides were then rinsed in pH 6.8 Gurr buffer and soaked in pH 6.8 phosphatase buffer for 1-2 minutes. Slides were dried by standing them vertically. The following stages were performed in a fume hood. Slides were dipped in xylene for 10 seconds and dried. One drop of DPX was placed on the cytodot and a 22mm x 22mm coverslip applied, the slides were then left to dry for 24 hours. Slides were viewed under a light microscope, 1000BN cells per replicate were scored per concentration (3000 BN total). Cells were scored to calculate Cytokinesis Black Proliferation Index (CBPI) as a measure of cytostasis (Equation 2).

**Equation 1)**

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

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

**Equation 2)**

% Cytotoxicity = 100 – 100 ($CBPI_T$ – 1 / $CBPI_C$ – 1); where T is treatment, C is control

CBPI = No mononucleated cells + 2x BN cells + 3 x MNCs / N; N is the total number of cells scored

***Cell harvesting for manual scoring approach (BASF SE Protocol).***

18. After the CytB treatment the adherent cells were rinsed with pre-warmed HBSS and trypsinized. The obtained cell suspensions from all different cultures were treated in the same manner, namely the cells were centrifuged and the cell pellet treated with a hypotonic solution (10 minutes with 0.4% KCl for V79 and buffy coat cells; 20 minutes with 0.28% KCl for whole blood cultures). After the hypotonic treatment, the cells were fixed twice by adding fixative (19 parts methanol and 1-part acetic acid; -20°C). Slides were prepared by immersing in deionized water followed by pipetting the fixed cells on the slide. The cells were stained with May-Grünwald (3min) and 10%[v/v] Giemsa (in Titrisol, pH7.2, 20min), mounted and scored as described above.

***Kinetochore staining of micronuclei.***

19. To determine if the DNA damage was a consequence of an aneugenic or clastogenic response, kinetochore staining of micronuclei in BN cells was performed. Following the exposure period, the cells were washed three times with PBS before being cytocentrifuged (500g, 5 minutes) onto microscope slides and fixed in ice-cold 90% methanol at −20°C. Immunofluorescent staining of kinetochore proteins was performed as previously described (Singh et al., 2012, Burgum et al., 2020). Kinetochore scoring was performed on a Zeiss AxioCam HRc (Carl Zeiss Microscopy and Imaging, UK). Only the lowest and highest significant NP concentrations were used for each particle type, with WC/Co included at 100µg/ml, of which 50 micronuclei were scored for the presence or absence of FITC fluorescence in the micronucleus indicating the presence of a whole chromosome (K+) or chromosome fragment (K-) respectively (n=1 biological replicate).

***Exposure assessment.***

20. For LA-ICP-MS analysis, a 193 nm ArF Excimer Laser (NWR193 Excimer Laser Ablation System, Elemental Scientific Lasers, Bozeman, MT USA) equipped with a two-volume cell (TwoVol$^2$ Ablation Cell, Elemental Scientific Lasers) was coupled to an ICP-MS Triple Quadrupole (8900 ICP-MS Triple Quad, Agilent Technologies, Santa Clara, CA, USA). The samples were transported in a carrier gas flow (He, 800 mL/min) and introduced *via* a Dual Concentric Injector (DCI, Elemental Scientific Lasers) to the ICP-MS. An additional gas flow (Ar, 1 L/min) was added, and the sample transferred *via* a quartz injector pipe (inner diameter: 3.5 mm) into the plasma. The ICP-MS was equipped with platinum sampler and skimmer. To resolve the issue of polyatomic interferences especially for low masses (*e.g.* $^{31}P$), it was operated in TQ modus with $O_2$ as reaction gas. The optimum RF was set to 1300 V and the set-up was tuned daily for maximum signal intensity and an oxide ratio (*m/z* 232/248) below 1.5 % with a NIST Glass standard (NIST SRM 612, National Institute of Standards and Technology, Gaithersburg, MD, USA). Each cell was ablated separately with 50 bursts and a spot size of 25 µm. A laser pulse frequency of 100 Hz and a laser energy of 0.5 J/cm$^2$ ensured a fully ablation avoiding ablation of the glass slide. The isotope $^{31}P^{16}O^+$ was monitored with a dwell time of 50 ms regardless of the type of experiment and chosen as internal standard. For Au NPs the isotope $197^+Au$, for $CeO_2$ NPs the isotope $^{140}Ce^{16}O^+$ and for WC-Co NPs the isotopes $^{59}Co^+$ and $^{184}W^{16}O^+$were detected each with a dwell time of 50 ms. To distinguish a cell event from the continuous background, a threshold three times higher than the mean signal of the background was applied. For an identified cell, an association rate was calculated by dividing the summed signal intensities of the NPs by the summed phosphorus signal intensities. This results in an association rate to what extent the NPs are allocated to the cells.

***Statistics***.

21. All data is presented as the mean +/- the standard deviation (SD). For data generated at the Swansea University lab: statistical analysis was performed in SPSS statistics software (v.20 IBM, UK) where all data sets were firstly analysed for normality (Shapiro-Wilk test, $p \leq 0.05$) and for equal variance $p \leq 0.05$). A one-way analysis of variance (ANOVA) was performed with post hoc Dunnett's multiple comparisons applied to evaluate pairwise statistical significance between control and concentrations; the alpha value was set to 0.05.

22. For data generated at the BASF lab: An appropriate statistical analysis was performed. The proportion of cells containing micronuclei was calculated for each test group. A comparison of the micronucleus rates of each test group with the concurrent vehicle control group was carried out for the hypothesis of equal proportions (i.e. one-sided Fisher's exact test, BASF SE). If the results of this test were statistically significant compared with the respective vehicle control ($p \leq 0.05$), labels (s) were printed in the tables. In addition, a statistical trend test (SAS procedure REG) was performed to assess a possible dose-related increase of micronucleated cells. The used model is one of the proposed models of the International Workshop on Genotoxicity Test procedures Workgroup Report. The dependent variable was the number of micronucleated cells and the independent variable was the concentration. The trend was judged as statistically significant whenever the one-sided p value (probability value) was below 0.05.

# Phase 2: Results

23. The data generated by SU with TK6 cells revealed no cytotoxic effects following 1 cell-cycle exposure to either of the AuNPs. Some significant (*) cytotoxicity was observed following the $SiO_2$ exposure but only at the highest test concentration of 100μg/ml. Chromosomal breakage, measured by the *in vitro* CBMN assay revealed some significant (*) genotoxicity following exposure to each JRC particle type, the significant responses were most apparent at the highest test concentration of each material where 2-fold increases over background levels (of %Mn/BN) were observed. The WC-Co particle control was observed to be positive following 1 cell-cycle exposures to TK6 cells, with 2-fold increases in micronuclei induced at the two test concentrations of 20 and 100μg/ml (**Figure 1**).

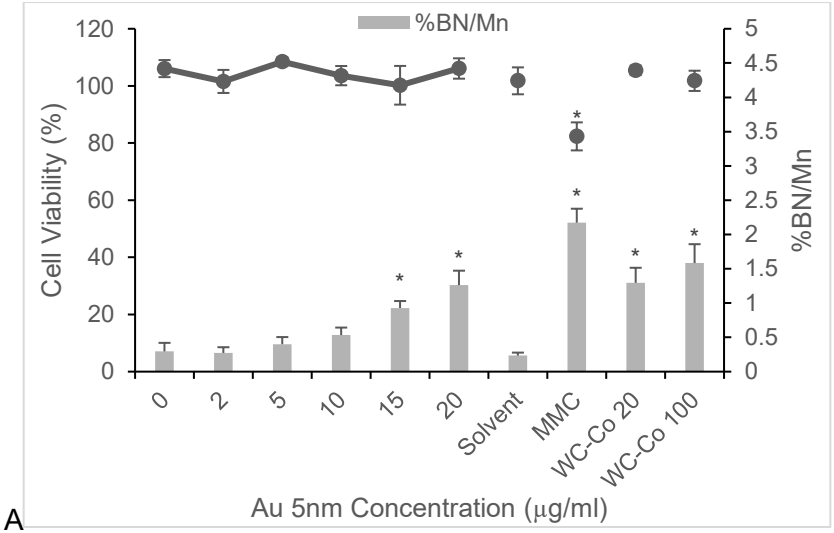
24. HepG2 cell line data generated by SU showed a more muted response to the JRC materials as compared to the TK6 suspension cells. Firstly, no significant cytotoxicity was observed across any test material. Secondly, the HepG2 cells showed no variation in %Mn/BN following 1 cell-cycle exposure to 30nm AuNPs. Whilst the 5nm AuNPs and the $SiO_2$ NPs did induce significant (*) %Mn/BN, this had limited biological significance as there was no increase in micronuclei that was greater than 1-fold elevation. Unlike the TK6 response to WC-Co particles, the HepG2 cell line only showed significant (*) %Mn/BN at the highest test concentration of the material, 100μg/ml (**Figure 2**).

25. The BASF-generated data on buffy coat cells showed no significant cytotoxic response to the JRC materials. This muted response to the materials was mirrored in the genotoxicity data whereby the only significant (*) data was reported by the positive chemical controls and the WC-Co at 10, 60, and 100μg/ml (**Figure 3**). Whole blood exposures to JRC nanomaterials showed largely corresponding data sets with no significant (*) cytotoxic or genotoxic damage with exception of the positive chemical controls and the WC-Co particle control, however only at the highest test concentration of 100μg/ml (**Figure 4**). BASF-generated data on V79 cells using the JRC reference materials showed no significant (*) cytotoxicity or genotoxicity following 1 cell cycle exposures. The exception being the positive chemical control, MMC (**Figure 5**).

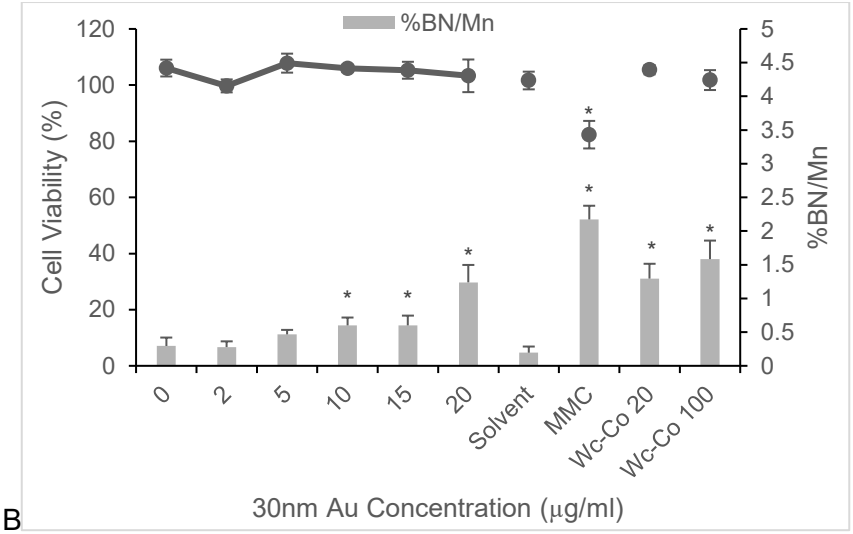
26. While in the above data sets, SU and BASF evaluated the response of differing cell lines to the test nanoparticles, one data set was generated using the same cell line, evaluated in both labs. This final data set involved exposure of the test nanoparticles ot the V79 cells by SU, utilising the BASF protocol to determine the transferability of the protocol and ability to generate harmonised data sets. Following the 1 cell-cycle exposures to the JRC reference materials the V79 cells showed a muted response with no significant (*) cytotoxicity or genotoxicity with the only exception being the positive chemical control, EMS (**Figure 6**). Thus, there was no difference in the data generated by both the SU and BASF labs using this cell line; demonstrating the transferability and reproducibility of this SOP.

## Phase 2: SU TK6 Results

**Figure 1. TK6 cytotoxicity & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). MMC (0.01µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 20 and 100µg/ml. Results were considered statistically significant (*) when p<0.05.**




A

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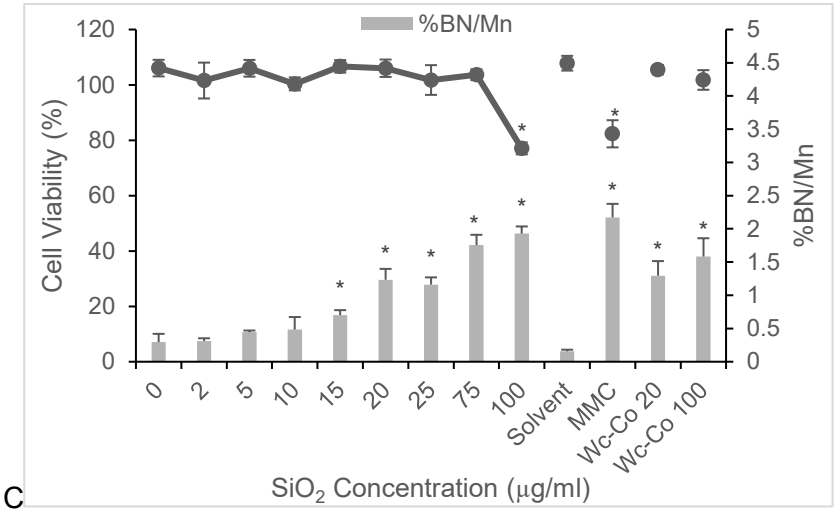
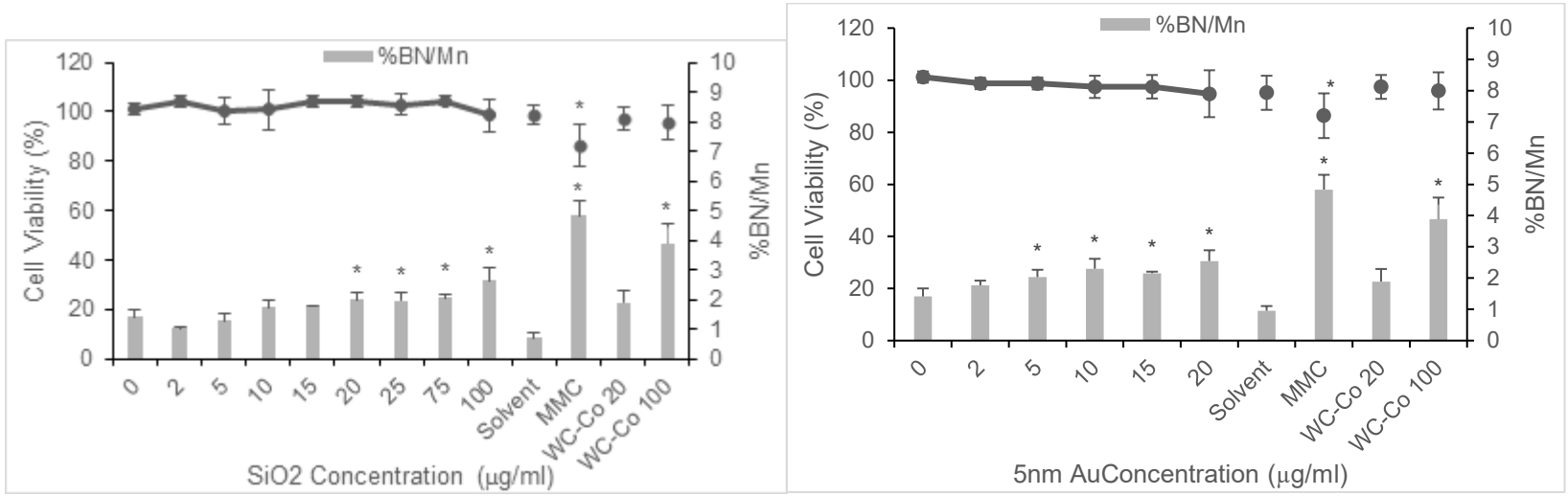

%BN/Mn
Cell Viability (%)
%BN/Mn
120
100
80
60
40
20
0
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
5
10
15
20
25
75
100
Solvent
MMC
Wc-Co 20
Wc-Co 100
SiO2 Concentration (μg/ml)

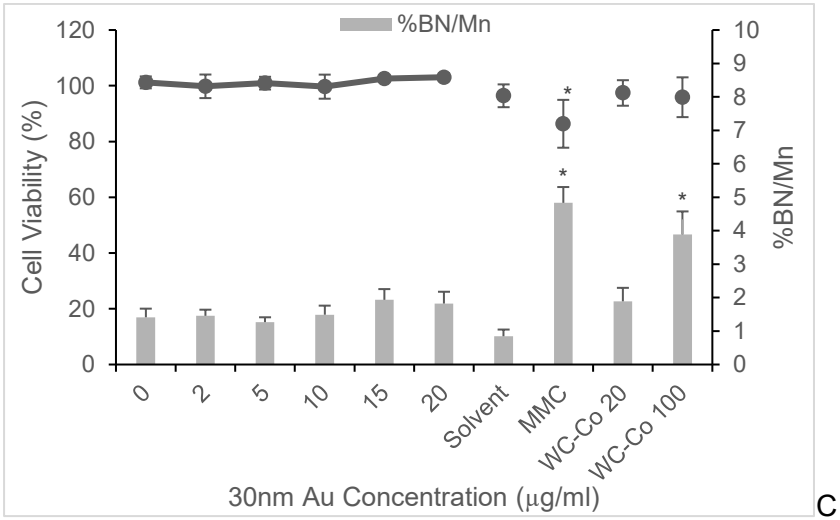
375 C

376

## SU HepG2 Results

**Figure 2. HepG2 cytotoxicity & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). MMC (0.01µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 20 and 100µg/ml. Results were considered statistically significant (*) when p<0.05**

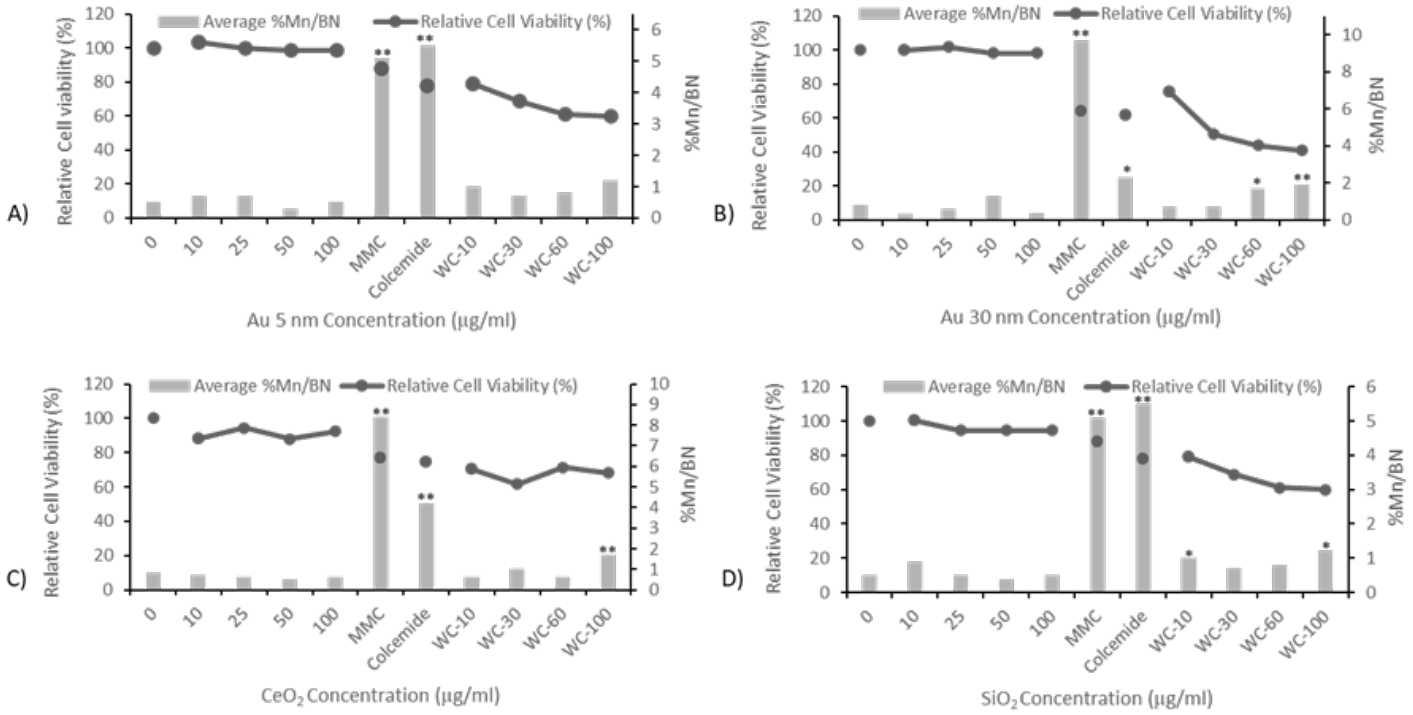



%BN/Mn
Cell Viability (%)
%BN/Mn
120
100
80
60
40
20
0
10
9
8
7
6
5
4
3
2
1
0
*
*
*
0
2
5
10
15
20
Solvent
MMC
WC-Co 20
WC-Co 100
30nm Au Concentration (μg/ml)

C

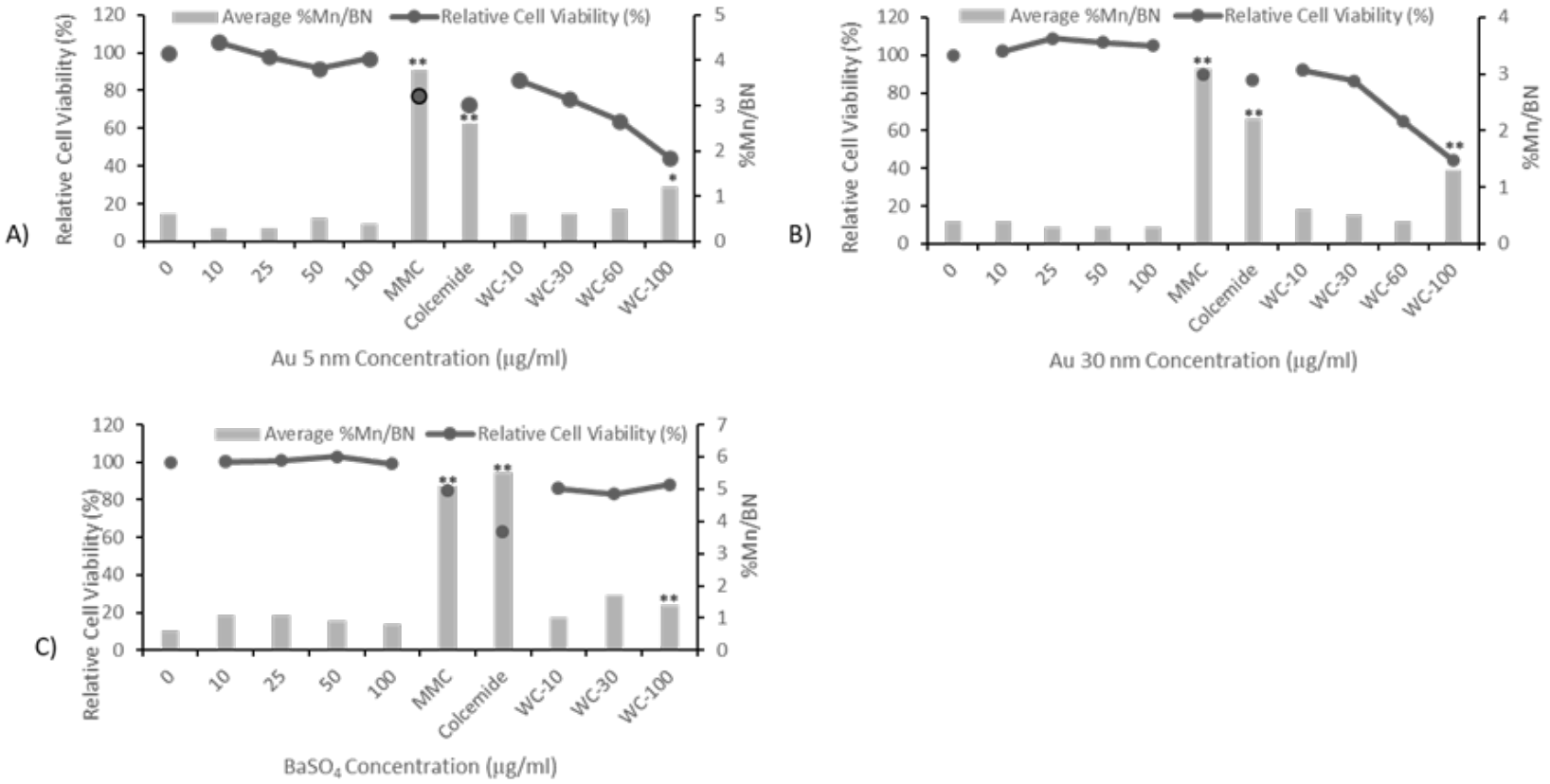
## BASF Buffy coat Results

**Figure 3.** Buffy coat CBPI and frequency of micronuclei determined by the *in vitro* CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), $CeO_2$ NPs (C), 22nm $SiO_2$ NPs (D). Positive controls used were MMC at 0.04µg/ml, Colcemide at 0.05µg/ml and WC/Co at 10, 30, 60 & 100µg/ml. Results were considered statistically significant (*) when $p \leq 0.05$ or (**) when $p \leq 0.01$.



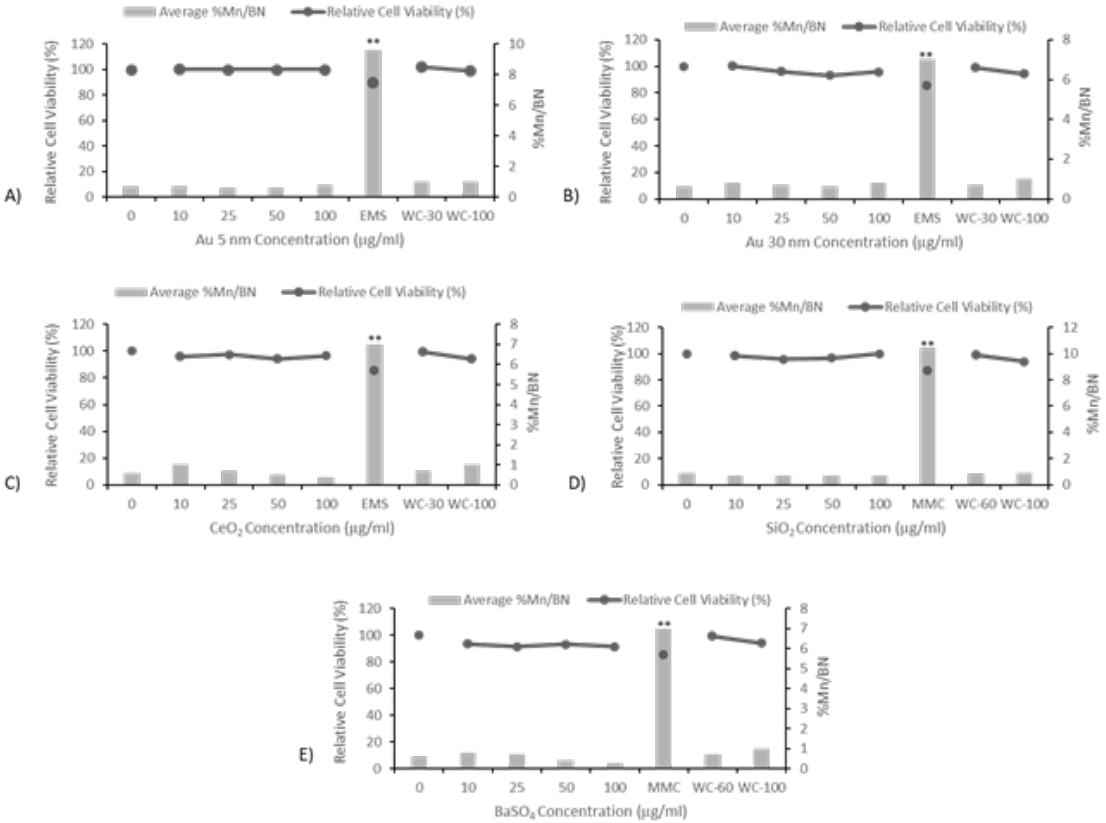
## BASF whole blood Results

**Figure 4. Whole blood CBPI and frequency of micronuclei determined by the in vitro CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), BaSO4 NPs (C), 22nm SiO2 NPs (D). Positive controls used were MMC at 0.04 µg/ml, Colcemide at 0.05 µg/mL and WC/Co at 10, 30, 60 & 100µg/ml. Results were considered statistically significant (*) when p≤0.05 or (**) when p≤0.01.**



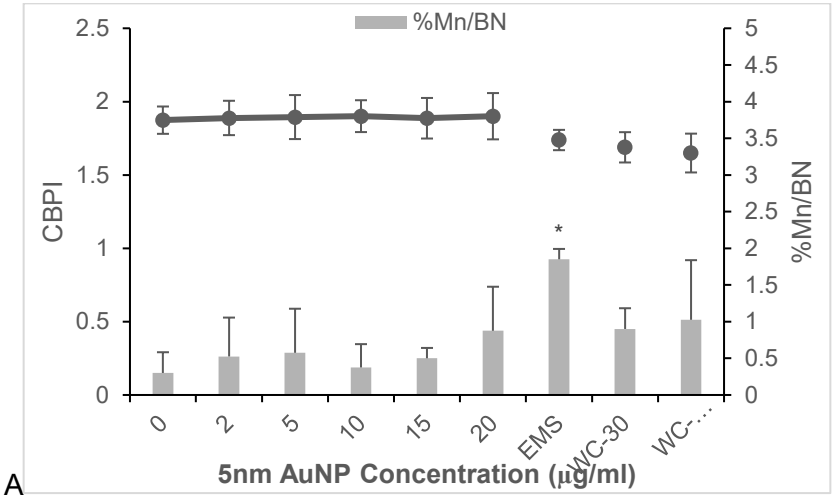
## BASF V79 Results

Figure 5. V79 CBPI and frequency of micronuclei determined by the in vitro CBMN assay following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B), CeO2 NPs (C), 22nm SiO2 NPs (D), BaSO4 NPs (E). Positive controls used were EMS 600 µg/ml and WC/Co at 60 & 100µg/ml. Results were considered statistically significant (*) when p≤0.05 or (**) when p≤0.01.

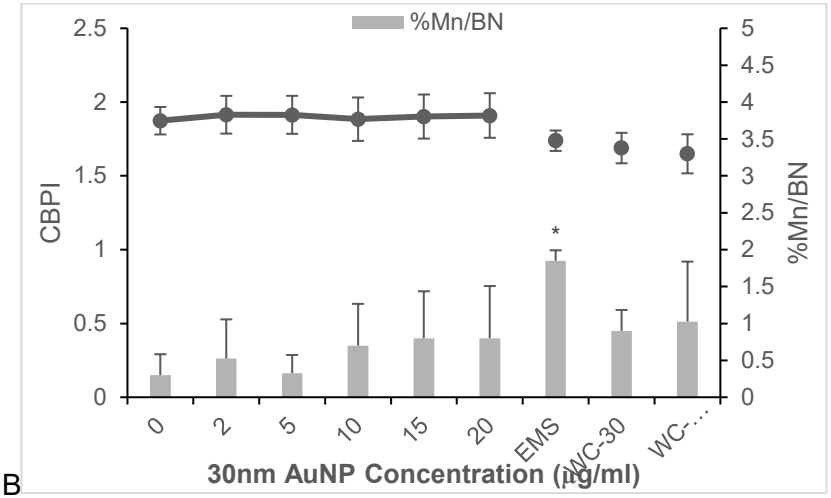


## SU V79 Results following BASF SOP

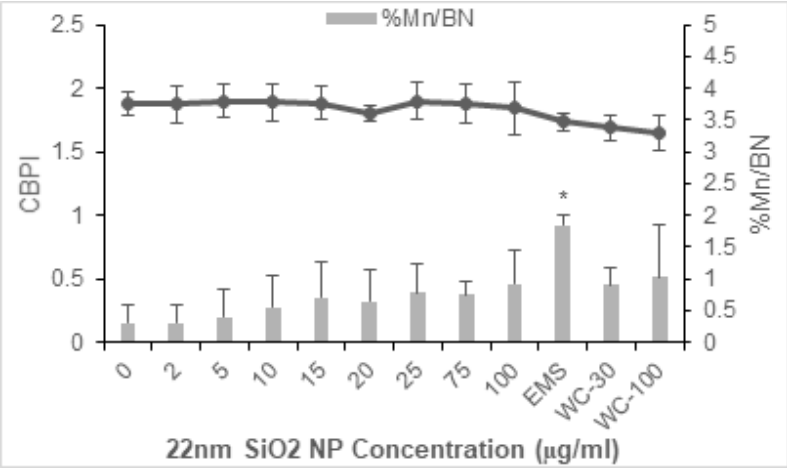
Figure 6. V79 cytostasis & genotoxicity data following 1 cell-cycle exposure to 5nm AuNPs (A), 30nm AuNPs (B) and 22nm SiO2NPs (C). EMS (600µg/ml) was used as the positive chemical control, WC-Co was used as the particulate control at 30 and 100µg/ml. Results were considered statistically significant (*) when p<0.05.




A




B




## Exposure assessment

27. In addition to the cytotoxicity and genotoxicity analysis, BASF evaluated the association of the test nanoparticles with the buffy coat cells, whole blood cultures and V79 cells by LA-ICP-MS analysis. The ratio of the respective elements Au, Ce, Ba and Co to Phosphorus is given as a semi-quantitative measure for the association of the tested nanoparticles with the cells. The data given in **Table 1** show that ratio of the individual element to the intracellular phosphorus increased concentration relatedly in each individual experiment. A comparison of the ratios between the elements is not possible. However, the ratios between the cell culture type used can be compared. Thus, it can be observed that V79 cells in all cases have a higher ratio of the element to their intracellular phosphorus levels as compared to buffy coat or whole blood cells. Consequently, despite the lack of genotoxicity in V79 cells, this data demonstrates that cell association did occur following exposure to the test nanoparticles.

**Table 1.** The ratio of the indicated elements to Phosphorus in buffy coat, whole blood and V79 cells after treatment with the indicated concentrations of the given nanoparticles.

| µg/mL | Buffy coat cells | | | | Whole blood cultures | | | | V79 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $Au_{5nm}$ | $Au_{30nm}$ | $CeO_2$ | WC/Co | $Au_{5nm}$ | $Au_{30nm}$ | $BaSO_4$ | WC/Co | $Au_{5nm}$ | $Au_{30nm}$ | $CeO_2$ | $BaSO_4$ | WC/Co |
| | Au/P | Au/P | Ce/P | Co/P | Au/P | Au/P | Ba/P | Co/P | Au/P | Au/P | Ce/P | Ba/P | Co/P |
| 0 | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | 0.02 | <LOD | <LOD | <LOD | <LOD | 0.03 | <LOD |
| 10 | 0.01 | 0.003 | 0.04 | -- | 0.008 | 0.01 | -- | -- | 0.23 | 0.46 | 57.53 | -- | -- |
| 25 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 30 | -- | -- | -- | 0.08 | -- | -- | 1.24 | 0.019 | -- | -- | -- | 27.32 | 0.45 |
| 50 | 0.02 | 0.010 | 0.36 | -- | 0.035 | 0.03 | -- | -- | 0.48 | 2.01 | 219.86 | -- | -- |
| 60 | -- | -- | -- | 0.1 | -- | -- | 1.61 | 0.045 | -- | -- | -- | 32.53 | 0.95 |
| 100 | 0.04 | 0.058 | 3.81 | 0.16 | 0.089 | 0.16 | 1.97 | 0.072 | 0.76 | 4.56 | 334.19 | 35.71 | 1.26 |

# Recommendations

28. From the Phase 2 experimental work presented here, the following recommendations can be made to answer the original questions posed during the expert meeting held in Paris in October 2014:

1. *Appropriate cell lines to use in the in vitro micronucleus assay when testing nanomaterials:*

29. Suspension cells are recommended (e.g. TK6 cells, buffy coat cells). Within the Phase 2 work, it was evident that these cells were more sensitive as compared to adherent V79 and HepG2 cells.

30. It is important to note that the most sensitive cell system, preferably of human origin (regardless the expected route of exposure to the nanomaterial in the final consumer product) and p53 competent should be used. Intracellular uptake of the NMs should be demonstrated, and only cells that can efficiently internalise NMs should be used.

31. As this Phase 2 study only included a small number of nanomaterials, it is recommended that expanding the sensitivity comparison of TK6 versus V79 cells is continued through further inter-laboratory trials using a wider range of nanomaterials.

2. *Positive controls:*

32. Currently, there are no suitable particle positive controls that have been identified for the *in vitro* micronucleus assay. Thus, in the absence of particle positive controls, it is recommended that chemical positive controls continue to be used (e.g. MMC, EMS).

33. Previously, there has been a suggestion in the scientific literature that tungsten carbide nanoparticles could be considered as a particle positive control for the *in vitro* micronucleus assay with human lymphocytes[1]. Thus, within the Phase 2 study, the nanoparticle tungsten carbide was included for further evaluation. This study demonstrated that tungsten carbide nanoparticles have the potential to be a suitable particle positive control, but only when used with TK6 or buffy coat cells (however, the *in vivo* relevance and the biological significance, i.e. germ cell mutagenesis or somatic cell carcinogenicity have not yet been demonstrated).

3. *Top dose:*

34. It is recommended that the top dose should be restricted to 100 µg/mL or 100 µg/cm$^2$. Doses higher than this are not physiologically relevant, but more importantly, can result in interference with scoring due to high deposition on cells. Lower concentrations can be considered if they are justified by *in vivo* organ burdens. Furthermore, agglomeration dynamics need to be carefully considered. Typically, at high doses, agglomeration is more predominant with lower dispersion stability than at lower doses when a stable dispersion of the particles can be more readily achieved.

[1] Moche H, Chevalier D, Barois N, Lorge E, Claude N, Nesslany F. Tungsten carbide-cobalt as a nanoparticulate reference positive control in in vitro genotoxicity assays. Toxicol Sci. 2014 Jan;137(1):125-34.

*4. Treatment time:*

35. One cell cycle treatment time, followed by 1.5 cell cycle exposure to cytochalasin B is recommended for the cytokinesis blocked micronucleus assay.

36. It should be noted that this experimental time can be challenging to conduct within normal working hours for the TK6 cells, which have a cell cycle time of 12-14h. Swansea University have therefore repeated the Phase 2 *in vitro* micronucleus assay experiments using 1.5 cell cycle exposures. The data demonstrated that there was no significant differences in the data generated when using either 1 or 1.5 cell cycle exposure with the TK6 cells. Thus, to enable the assay to be conducted during standard working hours, for TK6 cells, a 1.5 cell cycle exposure is recommended and is detailed within the SOP in Annex 1.

*5. Dispersion:*

37. Several methods can be utilised to disperse nanomaterials, however within the Phase 2 trial conducted, the NANoREG protocol (Alstrup Jensen et al., 2014) was implemented as this is a well-established method.

*6. BASF and Swansea University have generated a harmonised SOP for the in vitro micronucleus assay for testing of manufactured nanomaterials (**Annex 1**).*

# Limitations

38. The described project has addressed several key issues regarding the testing of nanomaterials in the *in vitro* micronucleus assay, nevertheless there are some issues which have either not been addressed in this study or not resolved.

39. The recommendations in this guidance document are derived from data obtained from a small number of compounds performed in two laboratories using different cellular test systems. A cross validation of the protocols was only partly performed by Swansea University using V79 cells. A larger scale inter-laboratory validation comprising of several independent laboratories would definitely add value to the obtained results. It should be noted that if future ring-trials are to be conducted, it is critical to ensure that all laboratories use an identical approach to dispersing their materials; if harmonisation in dispersion approach across the participating laboratories cannot be achieved then the data may not align. For example, if sonication is being applied to disperse nanomaterials, then delivery of specific energy levels is required.

40. The number and type of nanomaterials used for this project were more or less predefined by the previous work carryout out by JRC[2]. A follow up trial should focus on a broader range of nanomaterials representing a larger portion of the existing nanomaterials. In this context, the assessment of the *in vivo* relevance of the obtained results with the tested nanomaterials is very limited. In this study $CeO_2$ was the only compound with relevant *in vivo* follow up data (Ma-Hock et al[3]). Thus, a selection of further nanomaterials with solid *in vivo* data would provide a better opportunity for interpretation of *in vitro* data.

41. During the meeting where the structure of the protocol was decided upon by the expert team the subject of the highest concentration to be tested was thoroughly discussed. Thus, 100 µg/mL was selected as the optimal highest concentration to be used in the experiments. Despite the unanimous decision by the expert team, it is recommended to discuss this topic in a broader community to establish a range finding workflow for the selection of the top concentration to be used for mutagenicity assessment.

42. Inclusion of S9 mix in the study protocol was not considered as relevant, since all selected nanomaterials were inorganic and not likely subject to metabolization for induction of mutagenicity. However, ECHA recommends the use of S9 mix for organic nanomaterials. The issues not addressed in this document relate to firstly identify the plausibility of use of S9 for organic nanomaterials, the protocol on how to incorporate an S9 Treatment within a test substance treatment period corresponding to one cell cycle as well as the identification of a corresponding positive nanomaterial requiring metabolization. Future

[2] Drewes, C., Ojea Jimenez, I., Mėhn, D., Colpo, P., Gioria, S., Bogni, A., Ponti, J., Kinsner-Ovaskainen, A., Gilliland, D. and Riego Sintes, J., Physicochemical characterisation of gold, silica and silver nanoparticles in water and in serum-containing cell culture media, EUR 29054 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77705-9 (online),978-92-79-77704-2 (print), doi:10.2760/818663 (online),10.2760/58721 (print), JRC110379.

3 Ma-Hock, L., Gröters, S., Strauss, V., Keller, J., Wiench, K., van Ravenzwaay B. and Landsiedel, R., Long-term inhalation study with $CeO_2$ and $BaSO_4$ nanomaterials. Manuscript in preparation.

metabolism considerations with respect to the replacement of animal derived S9 mix could include in vitro metabolism assays such as TGP project 4.150.

# Additional Supporting Interlaboratory Trial

43. Since completion of the Phase 2 experimental work, the Horizon 2020 project RiskGONE has agreed to undertake an interlaboratory trial using the harmonised SOP generated through this Phase 2 project. This interlaboratory trial will include three laboratories: Swansea University (UK), Norwegian Institute for Air Research (NILU; Norway) and the French Agency for Food, Environmental and Occupational Health & Safety (ANSES; France). The interlaboratory trial will involve each partner exposing the TK6 cell line to the engineered nanomaterials:

- Zinc Oxide (ZnO) – Sigma #MKCJ4155
- Titanium dioxide ($TiO_2$) – JRC #JRCNM01005a990582
- Tungsten carbide-cobalt (WC/Co) – NanoAmor #5561HW
- Chemical positive control: Mitomycin C (MMC) – Sigma, #M4287-2MG. Suspend in double distilled water at 1mg/ml, keep at 4°C.

44. The ring trial will consist of each partner testing the TK6 cell line with the selected engineered nanomaterials (ENMs), following the SOP in **Annex 1**. 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.

45. This trial started in September 2021 and the data collation is planned for completion by January 2022. *[note from the Secretariat: analysis of the data and refinement to the considerations provided in this document will be considered in a future version of this document*].

# Annex 1 – Harmonised SOP generated by BASF & SU

46. This annex includes the finalised SOPs for testing engineered nanomaterials with the *in vitro* micronucleus assay using TK6 and buffy coat cells, as these were the most sensitive cell lines identified.

## Experiments for characterization of nanoparticles, recommended by both BASF & Swansea University

47. The interpretation of *in vitro* genotoxicity requires a minimum physical-chemical characterisation of the test item, including both intrinsic properties (size, shape, specific surface area) and extrinsic properties (agglomeration and solubility in the genotoxicity test medium). The following (non-GLP) technologies can be applied to generate this information:

1. Transmission Electron Microscopy (TEM) to determine the particle size distribution and shape distribution. These are ECHA requirements to identify the Nanoform (or non-nano-form) of the test item. (European_Chemicals_Agency_(ECHA), 2019) The method is selected accordingly to the NanoDefine Method Manual, published by JRC in 2020 (Mech et al., 2020). Specifically, a tip of a spatula of material is dispersed in 5ml of ethanol. The dispersion is treated for 5min in an ultrasonic bath. 5 drops of the dispersion are applied between two glass slides to create a thin liquid film. A carbon coated TEM grid is dipped on the film to transfer the particles to the TEM substrate. The TEM samples are analysed using a Tecnai Osiris machine (Thermo-Fisher) operated at 200 keV under bright-field (BF) conditions (Spotsize; 100µm condenser aperture; 20µm objective aperture). About 10 Images per sample are acquired using a Gatan 1000XP 2K CCD camera with an acquisition time of 2s at magnifications of 13500x and 26500x. The particle size distribution and aspect ratio distribution are evaluated using the free ParticleSizer plugin for ImageJ developed and validated within the EC FP7 project NanoDefine. Automated evaluation is faster, reproducible and limits operator bias to a minimum compared to manual image analysis. The report includes distributions, median D50 and statistics on particle size and aspect ratio.
2. Brunauer-Emmett-Teller (BET) method to determine the specific surface area. This is an ECHA requirement to identify the Nanoform (or non-nano-form) of the test item (European_Chemicals_Agency_(ECHA), 2019). The method is selected accordingly to the NanoDefine Method Manual, published by JRC in 2020 (Mech et al., 2020). Specifically, the method conforms with ISO_9277:2014 “Determination of the specific surface area of solids by gas adsorption – BET method. If the test item is organic, then the degassing temperature is limited to 80°C, or at least 50°C below melt temperature.
3. Analytical Ultracentrifugation (AUC) method to determine the agglomeration in genotoxicity test medium. Characterise two concentrations (0.03 mg/mL and 0.1 mg/mL) and repeat one

concentration at 20h (conforming with the duration of the genotoxicity testing) to assess the stability throughout the incubation time. AUC implements an optical system that is synchronized with the centrifugal frequency to monitor the radial concentration profile during centrifugal separation. The samples are prepared by the identical protocol as for the genotoxicity testing in the identical medium. Suspensions are then directly loaded in AUC cells without further preparation. All measurements are done on a Beckman Coulter XLI Proteome Lab (Beckman Coulter Inc., Brea, USA). The cells are mounted on a 4-sample or 8-sample titanium rotor, and data is acquired during a gravitational sweep from 73g to 116000g over 2h. The samples are measured using either interference or absorbance optics to detect sedimentation as described by the NanoDefine project (Mehn et al., 2018). Both types of sedimentation data are evaluated with sedfit v15.0 software and are represented in particle size distributions in mass metrics and in number metrics.

4. Incubation and filtration with analysis by Inductively-Coupled-Plasma Mass Spectrometry (ICPMS) or by UV Vis-Spectrometry, to determine the static solubility in genotoxicity test medium. Characterise two concentrations (0.03 mg/mL and 0.1 mg/mL). The samples are prepared by the identical protocol as for the genotoxicity testing in the identical medium. Suspensions are then incubated at 37°C for 20h, conforming with the conditions of the genotoxicity testing. After 20 hours, the suspensions are filtered through a 1 µm glass filter directly followed by a 0.02 µm (= 20 nm) alumina membrane. Thereafter, the filtrates are analysed by UV-Vis (for organic pigments) or ICP-MS (for metal-based particles). If the test item consists of Si or Al, polymeric filters are used instead.

    a) Calibration of UV-Vis at peak pigment wavelength for quantification of the dissolved material is performed by dissolving the pigment in concentrated sulfuric acid to get the mass attenuation coefficient. The UV-Vis instrument Agilent Cary is used with 50mm quartz cells to optimize detection down to 0.001 mg/L. Blank controls (no test item in the dissolution setup) and medium controls (pure medium) are conducted. The general procedure is following “UV-VIS Absorption Spectra (spectrophotometric method)”, OECD guideline for Testing of Chemicals, guideline 101, adopted 12th May 1981. For pigments containing metals, the dissolved ions are quantified by ICP-MS (Perkin Elmer Nexion 2000b). The samples are diluted by a factor of 10 to 100 in 1% $HNO_3$, then measured with 50ms integration time on each mass. External calibration uses concentrations of 0 / 0.01 / 0.1 / 1 / 10 µg/L with matrix-matched standards. The ultra-high purity quartz nebulizer has an argon flow of 0.94 mL/min and a sample flow of 0.29 mL/min at a pump rate of 35rpm.

# *In vitro* micronucleus test on primary human lymphocytes; Cytochalasin B method with nanoparticles

# BASF

# Standard Operating Procedure 2020

## Preparation of the cells

48. The blood used for testing comes from volunteer test persons, the administration of personal data as well as the sampling procedure are described in the SOP “Organization of blood sampling for genotoxicological tests”. The selection of possible test persons is based on the criteria specified in OECD guideline 487.

- Included are; Men and women aged 18 to 35, non-smokers, do not depend on regular medication, and do not use drugs.
- Excluded are; pregnant women, people with known diseases or exposure to genotoxic substances (chemicals / ionizing radiation).
- The cells will be incubated at 37°C, 5% (v/v) $CO_2$ and ≥ 90% relative humidity in the incubator. The culture medium RPMI contains stable glutamine and is supplemented with 20% [v/v] FCS and 1% (v/v) Penicillin / Streptomycin. The culture medium is preheated to 37°C.

## Stimulation of the cells with phytohemagglutinin

49. Erythrocytes are first isolated from the whole blood. For this purpose, the blood is first diluted 1:1 with culture medium. The erythrocytes are then isolated from the remaining nucleated cells (buffy coat) by means of a Ficoll separation.

50. For the Ficoll separation, 25 mL Ficoll-PaqueTM (GE Healthcare Bio-Sciences AB) are placed in 50 mL centrifuge tubes and carefully covered with 20 mL of the diluted blood. The centrifuge tubes are centrifuged at 1055g for 20 minutes. The centrifuge's braking function should be switched off. The “buffy coat” layer (the layer between the medium and Ficoll) is carefully collected and centrifuged again at 491g (15 minutes with braking function). The pellet is then washed twice with medium and centrifuged (5 minutes at 900g). The pellet is taken up in a volume of cell culture medium (RPMI; 20% FCS, 0.6 mg / mL PHA) that corresponds to 5 times the blood volume (e.g. for 20 mL whole blood, the cells are taken up in 100 mL).

51. 50 mL of each batch are placed in a 175 cm² cell culture flask and cultivated in the incubator for 48 hours.

## Test substance preparation and preparation of the particulate positive control

52. Tungsten carbide-Cobalt will be used as particulate positive control. The nanoparticle test substance and particulate positive control will be prepared as described by the NANoREG protocol (Alstrup Jensen et al., 2014).

53. In summary, a certain amount of the respective nanoparticle is weighed and mixed with ethanol (pre-wetting) so that the final ethanol concentration is 0.5 vol%. Then the dispersion medium (0.05% w / v BSA water) is added so that a particle concentration of 2.56 mg / mL is reached. The dispersion is treated with a probe ultrasonicator (Branson Sonifier 550 W) for 16 minutes at 10% amplitude (approx. 7 W). This stock dispersion is used to create the different testing concentrations.

## The test substance is tested with the following concentrations:

- 1, 3, 10, 30, 60, 100 µg/mL

**Tungsten-Carbide/Cobalt (8wt% WC/Co <200nm, 99.5% LOT#5561-072018, Nanostructured & Amorphous Materials Inc., USA) particulate positive control is tested with the following concentrations:**

- 30, 60, 100 µg/mL

## Preparation of the chemical positive control

54. The positive controls are prepared fresh on the day of the test shortly before the substance is administered or prepared in larger quantities at an earlier point in time, stored in ready-to-use portions at 80°C, and thawed and used shortly before use.

55. Mitomycin C (0.04µg/ml) and colchicine (0.05µg/ml) are used as chemical positive controls.

56. Several concentrations of a positive control substance can be used, it being enough if only one concentration used is evaluated if it shows a sufficiently strong genotoxic effect in the experiment.

## Preparation and treatment of test cultures

57. The substance preparations and the preparations of the particulate positive control are diluted to the final concentration in culture with culture medium, the total volume (for two cultures (duplicate determination) is 30mL. The pipetting scheme for dilution is shown in Table 1.

**Table 1**. Pipetting scheme of test substance preparations

| | dilution in BSA | | | dilution in culture medium RPMI incl. 20% FCS | |
|---|---|---|---|---|---|
| Dose [µg/mL] | [mL] | from Dosis | + vehicle [mL] | [mL] aus Ansatz | + vehiclel [mL] |
| 100* | 5 | 2560 | 7.8 | 3 | 27 |
| 60* | 6 | 100 | 4 | 3 | 27 |
| 30* | 5 | 60 | 5 | 3 | 27 |
| 10 | 0.5 | 100 | 4.5 | 3 | 27 |
| 3 | 0.5 | 30 | 4.5 | 3 | 27 |
| 1 | 0.5 | 10 | 4.5 | 3 | 27 |

*: applies to both test substance and particulate positive control

58. The blood cultures are removed from the incubator, suspended and pooled. For each culture (two cultures per test group = double determination), 10 mL are pipetted into a prepared 15 mL centrifuge tube. The cells are centrifuged at 900g for 5 min and the supernatant is removed.

59. For each culture (double determination) 10 mL of the prepared substance dispersion are added to the cells. The cells are suspended with a 10 mL serological pipette and transferred into prepared 25 cm² cell culture flasks. The cells are incubated on a shaker (approx. 150 rpm) in the incubator for 20 hours (1-cell cycle).

## Checking or determining further parameters

60. Changes in the pH value can be recorded by the colour change of the indicator dye of the culture medium (phenol red: no colour change from pH 6.7 - 8.3).

61. The pH value is measured at the start of treatment at least for the highest dose and for the untreated control or the vehicle control (pH 6.7-8.3).

## Test substance removal

- The exposure phase is ended by washing the cells several times with HBSS
- Transfer the cultures into prepared 15 mL centrifuge tubes
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 5 mL HBSS
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 5 mL HBSS
- Centrifugation (5 min at 900g)
- Remove the supernatant
- Suspend the cells in 10 mL fresh medium
- Transfer of the cultures into prepared 25 cm² cell culture flasks

## Cytochalasin B treatment

- Following a 1-cell cycle exposure to the test nanoparticles, each culture is treated with 30 µL initial solution (final concentration 6 µg/mL culture medium). The cultures are incubated for an additional 20h (1-cell cycle) in the incubator.

## Cell harvest and preparation of slides

- The preparation takes place under a fume hood under non-sterile conditions.
- The fixative (19 parts methanol: 1 part acetic acid) is freshly prepared every working day, during the incubation steps the fixative is stored at -20°C.
- The hypotonic solution (0.65% KCl) is pre-warmed to 37°C in a water bath, it can be prepared the day before and stored in the refrigerator until use.

## Hypotonic treatment and fixation of the cells:

62. The cells will be transferred into 15mL tubes, centrifugated at 900g for 5 min (room temperature) and washed with HBSS. Then the cells will be treated with a hypotonic solution and fixative:

- Centrifugation (5 min, 900g, at room temperature)
- Suspend in 5 mL KCl, cover with Parafilm
- Incubate for 10 min at 37°C
- Fix the cells by adding 1 mL of cold fixative and suspending them in the solution
- Centrifugation (5 min at 900g and 4°C)
- Remove the supernatant

- Suspend in 5 mL cold fixative
- Incubate for 20 min at 4°C
- Repeat the fixation step with 5 mL fixative (incubation for 20 min at 4°C)
- Centrifugation (5 min at 900g and 4°C)
- Suspend in 3 mL fixative
- After the last fixation step, the cells can be stored for up to six hours at 4°C or applied directly to the slide

## Preparation of the cells on microscope slides

- Three preparations are made per culture as follows:
- Centrifuge the cells (5 min at 900g and 4°C) and suspend in 1-2 mL cold fixative. Depending on the pellet size / cloudiness of the cell suspension, additional fixatives can be added.
- Moisten the slide by immersing it in deionized water, allow it to drain briefly
- Apply 250 µL of the cell suspension evenly to the microscope slides
- Pull the slide through the flame of the Bunsen burner
- Extinguish the flame after approx. 1 second
- The cell density can already be assessed microscopically on the native preparation and, if necessary, adjusted (by varying the amount of fixative). The cells should be isolated and not overlapping on the slide.
- Let the preparations dry at least overnight.

## Staining of the slides

63. The cells were stained with May-Grünwald (3 min) and 10% [v/v] Giemsa (in Titrisol, pH 7.2, 10 min) and mounted.

## Cytotoxicity – proliferation index (CBPI)

64. Micronuclei can only arise in proliferating cells. The cytokinesis block proliferation index (CBPI) is a direct measure for determining the cell division activity. The number of mononuclear, binuclear and multinucleated cells is determined in 500 cells per culture (culture = slide), in two cultures per test group = 1000 cells. The CBPI can be evaluated without coding.

65. If only isolated cells are found, no analysis is carried out. This is noted on the evaluation form (n.s. = not scorable).

66. The CBPI calculated from this distribution indicates the average number of cell cycles that a culture has undergone in the presence of the actin polymerization inhibitor Cyto B. The CBPI is given as an absolute value without a unit.

$$\text{CBPI} = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

67. The CBPI was used to calculate the % cytostasis (relative inhibition of cell growth compared to the respective vehicle control group) - a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

$$\% \text{ Cytostasis} = 100 - 100 \{(\text{CBPIT} - 1) / (\text{CBPIC} - 1)\}$$

T = test substance treated culture C = vehicle control culture

## Micronucleus analysis

68. The selection of the dose groups that are used to record the micronucleus frequency depends on the quality of the preparations or cells, the cytotoxicity and possible other physiological parameters (e.g. pH value). This dose selection is made considering the applicable international test regulations. The evaluation of the cells for micronucleus frequency is carried out manually with a transmitted light microscope (e.g. Axio Scope.A1, Zeiss) on coded preparations. A magnification of 400 times is used. The findings are recorded manually on an evaluation form.

69. At least 1000 binuclear cells are analysed per culture (slide) and the number of micronucleated cells is recorded. A total of 2 slides per test group, i.e. at least 2000 binuclear cells evaluated. The criteria for evaluating a cell are:

- The plasma border must be complete (otherwise main cores or MN may be missing)
- The plasma must not be too dark in colour
- The main nucleus should not differ too much in size
- The cell must be clearly distinguishable from the neighbouring cell
- Apoptotic cells with a fragmented nucleus are not assessed

## The following criteria must be evaluated for the assessment as a micronucleus

- The micronucleus must be round (no angular / crystalline structures)
- The diameter of the micronucleus was less than 1/3 of the main nucleus
- The micronucleus was not linked to the main nucleus and was located within the cytoplasm of the cell
- Only binucleated cells were scored
- The micronucleus must lie completely in the plasma

## Appendix 1 for BASF SOP

## Materials and chemicals

| Materials | Manufacture | Order-no. |
|---|---|---|
| Cell culture flasks, 25 $cm^2$ | TPP | P 90025 |
| Cell culture flasks, 75 $cm^2$ | Greiner | 661160 |
| Centrifuge tubes 15 mL | TPP | TPPA 91015 |
| Centrifuge tubes 50 mL | TPP | TPPA 91050 |
| microscope slide, free of grease | Superfrost | 631-1320 |

| Reagents und medium | Hersteller | Best.-Nr. |
|---|---|---|
| RPMI 1640, w: Stab. Glutamine, w: NaHCO3, ohne Phenolrot | PAN biotech | P04-16520 |
| Hanks' Balanced Salt Solution ohne $Ca^{2+}$, $Mg^{2+}$ (HBSS) | PAN biotech | P04-34500 |
| Fetal Calf Serum (FCS) | Biowest | - |
| Penicillin/Streptomycin (10 000 E/10 000 µg/mL) | PAN biotech | P06-07100 |
| Mitomycin C (MMC) | Roche | 10107409001 |
| Colchicin | Roche | 10295892001 |
| KCl | Bernd Kraft | 4016056 |
| methanol | Bernd Kraft | 34940 |
| glacial acetic acid | Bernd Kraft | 4146307 |
| Mounting Pertex® | Medite | MEDT 41-4011-00 |

70. A product or supplier of comparable quality can be used for all articles at any time. Reagents not listed here are described in more detail in the test plan if required. Other chemicals used are customary in the laboratory and are obtained from common manufacturers or are available in the laboratory.

# Appendix 2 for BASF SOP

## Recipe

### *Culture medium*

**71.** RPMI 1640, w: Stab. Glutamine, w: NaHCO3, ohne Phenolrot unter Zusatz von:

- fetal calf serum (FCS), ca. 20% (v/v)
- Penicillin/Streptomycin (10 000 E/10 000 µg/mL), ca. 1% (v/v)

72. All components are mixed under sterile conditions in the medium bottle and marked with the ingredients, date and stored for a maximum of 6 weeks at approx. +4°C.

### *Phytohämagglutinin*

73. The PHA (1.2 mg) is dissolved in 2 mL of ultrapure water in a container and can be stored in the refrigerator for up to 6 weeks. The documentation is carried out in the raw data across studies.

### *Cytochalasin B – solution*

74. The cytochalasin B (10 mg) is dissolved in the container in 5 mL DMSO (final concentration 2 mg/mL) and can be stored in the refrigerator for up to 6 weeks.

### *Hypotonic treatment*

75. The hypotonic solution (0.65% KCl) can be prepared on the day before and stored in the refrigerator.

76. Approach: Weigh of 0.65g KCl/ 100 mL with ultrapure water

### *Fixative*

77. The fixative is freshly applied on a working day and stored at -20°C during the fixation steps.

78. Approach: 19 parts methanol (cooled overnight at -20°C) + 1-part glacial acetic acid

# *In vitro* cytokinesis-blocked micronucleus (CBMN) test protocol for use with TK6 cells and engineered nanomaterials (ENMs)
# Swansea University
# Standard Operating Procedure 2020

**Abbreviations**

- Cyto B – Cytochalasin B
- EDTA – Ethylenediaminetetraacetic Acid
- ENM – Engineered Nanomaterial
- FBS – Foetal Bovine Serum
- HS – Horse Serum
- KCl – Potassium Chloride
- MMC – Mitomycin C
- MMS – Methyl Methanesulphate
- MNU - N-nitro-N-methylurea

- NaCl – Sodium Chloride
- P/S – Penicillin/Streptomycin
- PBS – Phosphate Buffered Saline

## Biological Setting & Test System

79. 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

**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 | Sigma; C6762 |
| 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

80. 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

81. 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 flask of the warm medium slowly and carefully using a Pasteur pipette. (The amount of medium depends on cell type and on rate of cell division; 40-50ml for suspension cell lines and 10-15ml for adherent).

- 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.
- 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 (split once 70-80% confluent). 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 not be grown continuously for more than 4 weeks.
- 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 using a Beckmann Coulter Counter and seed at $1x10^5$ cells/ml in 10ml of media in $25cm^3$ flasks per treatment. Two sets of flasks are required per treatment dose: one for genotoxicity (micronucleus frequency analysis) and a second additional satellite flask per treatment for cytotoxicity calculations.
- Incubate overnight at 37°C/ 5% $CO_2$

**DAY 2 – cytotoxicity count 1 & dosing of cells with test agent**

Concurrent positive and solvent/vehicle controls should be included in each replicate. For TK6 cells, the recommended chemical positive control is:

### MMC CAS 50-07-7 (Fisher Scientific; BP2531-2), dose for 1 – 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.

### Preparation of ENMs

ENMs will require preparation, suspension and sonication prior to use in toxicology

testing, allow time to prepare ENMs fresh on the day of dosing. User's 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 (OECD TG487). For this protocol we use the NANoREG guidance document for ENM suspension and sonication (Alstrup Jensen et al., 2014).

- Count satellite 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 a Beckmann Coulter Counter.
- Dose cells in both sets of flasks (for genotoxicity and cytotoxicity evaluation) for 1 – 1.5 cell cycles (for TK6 cells, 1 cell cycle time is typically 12-15 hours) with the ENM at the desired final concentration; this is to be performed in triplicate per concentration, including triplicate negative control (media only, or vehicle used to suspend ENM) and positive controls.

**DAY 3 – cytotoxicity count 2 & addition of cytochalasin B**

- Count satellite flasks (post-treatment cell number) to calculate cytotoxicity. Add 100µl of cell suspension directly from satellite flasks to a cuvette containing 10ml of diluent for cell concentration determination using a Beckmann Coulter Counter.
- Transfer the content of the genotoxicity flasks 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 3-6 μg/ml cytochalasin B and place into new T25 flasks.
- Incubate for 1.5 cell cycles (18 hours)

**DAY 4 – harvesting cells for automated scoring protocol using the Metafer system**

- 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.
- 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 dd$H_2O$.
- Centrifuge (230g for 10 minutes) the fixed cell suspensions and thoroughly re-suspend in ~1ml Fixative 2
- Take a slide out of the dd$H_2O$ 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.

**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.
- 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 100μl of cell suspension into each funnel and centrifuge at 200g for 10 min in a Shandon Cytospin.
- 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.
- Fix slides for 10min in ice cold 90% methanol and leave to air dry (at this point slides can be stored at -20°C)
- Stain slides in 20% Giemsa solution (5ml of Giemsa + 20ml pH 6.8 Gurr buffer, filtered) for 10 minutes
- Rinse slides in pH 6.8 Gurr buffer, then soak in pH 6.8 phosphatase 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 22x22 mm 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 1000 BN cells per replicate per concentration of ENM for the presence of micronuclei (3000 BN cells in total).

### Cytotoxicity Calculations

As described in OECD TG487, CBPI, RPD or RICC are the most suitable for quantifying cytostasis and cytotoxicity (paused growth and cell death respectively).

**Relative Population Doubling (RPD):**

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

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

**Relative Increase in Cell Counts (RICC):**

$$\text{RICC} = \frac{\text{Increase in number of cells in treated cultures (final – starting)}}{\text{Increase in number of cells in control cultures (final – starting)}} \times 100$$

**Cytokinesis Block Proliferation Index (CBPI):**

% Cytotoxicity = $100 – 100 (CBPI_T – 1 / CBPI_C – 1)$; where T is treatment, C is control

CBPI = No mononucleated cells + 2x BN cells + 3 x MNCs / N; N is the total number of cells scored

**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: 20.0$\mu m^2$
- Maximum Area: 400.0$\mu m^2$

- Maximum Relative Concavity Depth: 0.900
- Maximum Aspect Ratio: 1.500
- Maximum Distance: 30.0$\mu m^2$
- Maximum Area Asymmetry: 70%
- Region of Interest Radius: 40.0$\mu m^2$
- Maximum Object Area in ROI: 50.0$\mu m^2$

## Micronuclei

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

## References

ALSTRUP JENSEN, K., KEMBOUCHE, Y., LOESCHUNER, K. & CORREIA, M. 2014. SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing, version 1.0. NANoREG.

EUROPEAN_CHEMICALS_AGENCY_(ECHA) 2019. Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification. In: ECHA (ed.) ECHA-19-H-14-EN.

MECH, A., RAUSCHER, H., RASMUSSEN, K., BABICK, F., HODOROABA, V.-D., GHANEM, A., WOHLLEBEN, W., MARVIN, H., BRÜNGEL, R. & FRIEDRICH, C. M. 2020. The NanoDefine Methods Manual - Part 2: Evaluation of methods. The NanoDefine Methods Manual. Luxembourg: Publications Office of the European Union.

MEHN, D., RIO-ECHEVARRIA, I. M., GILLILAND, D., KAISER, M., VILSMEIER, K., SCHUCK, P. & WOHLLEBEN, W. 2018. Identification of nanomaterials: A validation report of two laboratories using analytical ultracentrifugation with fixed and ramped speed options. NanoImpact, 10, 87-96.

SEAGER, A. L., SHAH, U. K., BRÜSEHAFER, K., WILLS, J., MANSHIAN, B., CHAPMAN, K. E., THOMAS, A. D., SCOTT, A. D., DOHERTY, A. T., DOAK, S. H., JOHNSON, G. E. & JENKINS, G. J. 2014. Recommendations, evaluation and validation of a semi-automated, fluorescent-based scoring protocol for micronucleus testing in human cells. Mutagenesis, 29, 155-64.