Appendix N- *In vitro* genotoxicity studies from OECD dossier (OECD, 2016)

The evaluation of the studies has been performed according the approach set in Appendix D

Test system/Test object	Exposure conditions (concentration/ duration /metabolic activation	Information on the characteristics of the test substance	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E	Result	Reliability/ Comments	Relevance of the result	Reference authors_year
Micronucleus test Peripheral blood lymphocytes (PBL)	20, 50, 100 µg/ml TiO2NPs Treatment 24h after lymphocytes stimulation 20h later CytoB, 72 h harvesting.	TiO_2NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 Protocol for dispersion including sonication in the media, but no results presented.	**Positive** concentration-related increase Cytotoxicity: concentration- and time-dependent decrease in cell viability	Reliability: 2 no positive control No concurrent measurement of cell proliferation (CBPI or RI)	Limited	Kang et al. (2008)
Comet assay in human peripheral blood mononuclear cells (PBMC) isolated by buoyancy density centrifugation (Ficoll-paque)	0, 50, or 100 µg /mL for 0, 6, 12, and 24 hr To determine the protective effects of antioxidants, lymphocytes were pre-treated with 1 mM N-acetylcysteine (NAC; for 1 hr prior to TiO2 NPs treatment Measure of Olive tail moment (OTM)	TiO_2NPs (P25), anatase/rutile, 15-24 nm	NSC: 2 Protocol for dispersion including sonication in the media, but no results presented.	**Positive** Statistically significant and concentration-related increase of OTM. NAC significantly decreased TiO_2 NPs-induced DNA breakage. Cytotoxicity: concentration- and time-dependent decrease in cell	Reliability: 3 No positive control, only OTM is reported. Methodology insufficiently described. Not clear how many samples (hence cells) per concentration were analysed. No data on stability of the	Low	Kang et al. (2008)

	Cell viability: trypan blue ROS: DCFDA probe fluorescence.			viability ROS increase, the level is reduced by NAC treatment	NPs		
Neutral and alkaline comet assay L-02 cell line (human fetus hepatic cells) 8-OHdG Analysis (HPLC/EC)	0, 0.01, 0.1 and 1.0 µg/mL 24 hr exposure. All experiments were performed in triplicate and repeated three times (-S9) Measure of OTM Negative control: DMSO Positive control: none Viability: determination of intracellular ATP level Apoptosis: flow cytometry ROS generation and lipid peroxidation: flow cytometry	TiO_2NPs (P25), anatase/rutile, 15-24 nm	NSC:3 no specific indications on dispersion or need for considering agglomeration and no indications of ultrasonication	**Negative** TiO_2 NPs did not increase statistically significantly OTM level in alkaline or neutral versions. No effect on cell viability by both ATP level or apoptosis assessment. TiO2NPs increased cellular 8-OHdG levels at 1 µg/ml. ROS generation: TiO_2 NPs induced significant increase in ROS level at 1 µg/ml. All concentrations of TiO_2 NPs increased MDA levels significantly.	Reliability: 3 No positive control was used. Only tail moment is reported. No data on stability of the NPs in culture medium. No proof of the NPs internalisation. Method for double strand breaks measurement (neutral comet assay procedure described) is not adequately reported.	Low	Shi et al. (2010)

					Concentrations in tables are in µg/L, while in the text µg/mL.		
Micronucleus test L-02 cell line (human fetus hepatic cells)	0, 0.01, 0.1 and 1.0 µg/L 24 hr exposure. All experiments were performed in triplicate and repeated three times (-S9)	TiO_2NPs (P25), anatase/rutile, 15-24 nm	NSC: 3 no specific indications on dispersion or need for considering agglomeration and no indications of ultrasonication	**Negative**	Reliability: 3 No positive control was used. Low concentration range tested ("trace TiO_2 NPs " according to the study authors). Concentrations in tables are in µg/L, while in the text µg/mL	Low	Shi et al. (2010)
Comet assay NRK-52E rat kidney proximal cells (CRL-1571)	cells exposed for 24h (Comet) or 48 h (cytotoxicity) to TiO_2NPs (from CEA) from 20 to 200 µg/mL Cytotoxicity: MTT and LDH assay ROS generation: spectrofluorimetry with 2′,7′-dichlorodihydro-	TiO_2NPs, anatase, 12 nm (TEM)	NSC: 1 specific dispersion protocol, different levels of agglomeration observed, but cell internalisation confirmed (Fig 3 reports onlyTiO_2NPs (12nm), but text indicates confirmation for all NPs)	**POSITIVE** Comet: Tail moment is reported, statistically significant and concentration-dependent increase. Cytotoxicity: low, dependent on NPs size (smallest NPs are more cytotoxic)	Reliability: 2 No results on positive and negative controls reported. Only tail moment is reported.	Limited	Barillet et al. (2010)

	fluorescein diacetate acetyl ester (H_2DCF-DA) probe Positive control: etoposide Negative control: untreated cells			and on crystalline phase (anatase was the most cytotoxic). ROS generation: ROS increase, but not correlated to cytotoxicity NPs observed in cytoplasm either in vesicles or isolated, maybe NPs can enter cells via non-specific adsorptive endocytosis or by direct diffusion			
γH2AX immunostaining NRK-52E rat kidney proximal cells (CRL-1571)	NRK-52E cells exposed to concentrations from 20 to 200 µg/mL; Negative control: untreated cells Positive control: etoposide	1) TiO_2NPs, anatase, 12 nm (TEM) 2) TiO_2NPs (P25), anatase/rutile, 15-24 nm 3) TiO2, anatase, 142 nm (TEM)	NSC: 1 specific dispersion protocol, different levels of agglomeration observed, but cell internalisation confirmed (Fig 3 reports only TiO_2NPs (12nm), but text indicates confirmation for all NP)	no effect on γH2AX foci	Reliability: 1	Limited γH2AX assay is not a standardised test. The method is not validated for regulatory purposes.	Barillet et al. (2010)
Alkaline comet assay +/- Fpg Human bronchial epithelial cells, BEAS-2B	10 µg/mL Exposure for 1h in dark Measure of tail moment Negative control: untreated	1) TiO_2NPs, anatase, 10 nm 2) TiO_2NPs, anatase, 20 nm 3) TiO2, anatase, ≥200 nm, 4) TiO2,	NSC: 3 No information on dispersion, however, low concentration	**Positive:** TiO_2 NPs anatase sizes 10 nm, 20 nm, and TiO_2 rutile size 200 nm induced statically significant oxidative DNA damages (tail	Reliability: 3 Not appropriate conditions and time of exposure:	Low	Gurr et al. (2005)

	Cytotoxicity: MTT assay (0.001, 0.1, 1 and 10 µg/ml) 3 days of exposure Measurement of hydrogen peroxide: fluorogenic probe, Amplex red Measurement of lipid peroxidation: MDA	anatase, 200 nm 5) TiO2, rutile, 200 nm		moment). **Negative:** TiO_2 anatase, sizes 200 nm and >200 nm did not induce oxidative DNA damages. (A preliminary study was performed, cells treated with TiO2 particles >200 nm and with TiO2 NPs anatase (10 nm) 0, 5, and 10 µg/mL for 1 h. DNA damage was detected in treatment with 10 µg/mL TiO_2 NPs anatase (10 nm). No damage was detected with 5 µg/ml anatase TiO_2 NPs (10 nm) or with TiO_2 particles >200 nm). Treatment with anatase–rutile mixture (10 µl each) induced higher level of oxidative DNA damage than	BEAS-2B cells embedded in gel on slides were treated with TiO2 for 1h in dark. It is not clear to what extent the NPs migrated through the gels. One concentration tested for rutile. For anatase 2 concentrations positive but no concentration response. No demonstration of uptake. No positive control was used. Only tail moment is reported in the comet assay.		

				treatment with either anatase or rutile particles alone. Cytotoxicity: treatment with 10 µg/ml anatase-sized TiO_2 NPs (20 nm) for 3 days caused cell growth inhibition, IC_{50} = 6.5 µg/ml Hydrogen peroxide levels: statistically significant increase of cellular levels by TiO_2 NPs anatase sized (10 and 20 nm) and TiO_2 rutile-sized (200 nm) and not by anatase-sized (200 and >200 nm) TiO_2. Lipid peroxidation: TiO_2 NPs anatase (10 and 20 nm) increased the cellular MDA level, while TiO_2 anatase (200 nm and >200 nm), and TiO_2 rutile (200 nm) did not.			

Micronucleus test Human bronchial epithelial cells, BEAS-2B	10 µg/ml of TiO2 for 24 h in presence of CytoB	1) TiO_2NPs, anatase, 10 nm 2) TiO_2NPs, anatase, 20 nm 3) TiO2, anatase, ≥200 nm, 4) TiO2, anatase, 200 nm 5) TiO2, rutile, 200 nm	NSC: 3 No information on dispersion, however, low concentration	**Positive:** anatase-sized (TiO_2 NPs 10 nm and TiO_2 200 nm) **Negative:** anatase-sized (>200 nm) and rutile-sized (200 nm) TiO2 Cell cycle progression (% of binucleated cells): Treatment with 10µg/ml TiO_2 NPs and TiO_2 particles for 24 h did not cause observable cell cycle delay.	Reliability: 3 The experimental protocol, with co-exposure of cells to TiO2 and cytoB, is inadequate for nanomaterials. A single concentration was tested. No positive control was used.	Low	Gurr et al. (2005)
Alkaline comet assay pulmonary (bronchial epithelial BEAS 2B and 16 HBE; alveolar A549) intestinal (Caco-2, primarily undifferentiated cells used)	BEAS 2B, A549, Caco-2: 0, 50, 100 and 256 µg/ml 16 HBE: 0, 2, 8, 32, 128 and 512 µg/ml NHEK: 0, 15, 33 and 65 µg/ml 3D-skin: 0, 82, 164 and 246 µg/cm^2 Exposure: 3h and 24h Positive control for SBs: H_2O_2 or methyl	1) TiO_2 (NM-100), anatase, 50-150 nm 2) TiO_2NPs (NM-102), anatase, 21-22 nm 3) TiO_2NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 Nanogenotox Project dispersion protocol	**Positive** TiO_2 particles (NM-100) was only tested in 16 HBE cells and was positive; TiO_2 NPs (NM-102 and NM-105) were tested in all cell lines and were positive, with the 3-h or 24-h treatment. Both TiO_2 NPs, NM-102 and NM-105 were **negative** in	Reliability: 1	High	NANOGENOTOX Project, 2013 (Documentation provided to EFSA No. 7,8 and 10)

epidermal (NHEK keratinocytes) 3-dimensional human reconstructed full thickness skin model	methane sulphonate (MMS) No positive control for Fpg			3-dimensional human reconstructed full thickness skin model.			
Micronucleus test pulmonary (bronchial epithelial BEAS 2B and 16 HBE; alveolar A549) intestinal (Caco-2, primarily undifferentiated cells used) human primary lymphocytes epidermal (NHEK keratinocytes)	BEAS 2B, NM-102 and NM 105: 0, 32, 64, 128 and 256 µg/ml 16 HBE, NM-102: 0, 20, 40 and 60 µg/ml 16 HBE, NM-105: 0, 8, 12 and 16 µg/ml A549, NM-102: 0, 16, 32, 64 and 128 µg/ml A549, NM-105: 0, 16, 32, 64, 128, 256 and 512 µg/ml Caco-2, NM-102: 0, 9.5, 28, 85 and 128 µg/ml Caco-2, NM-105: 0, 28, 85, 128 and 256 µg/ml Lymphocytes, NM-	1) TiO_2NPs (NM-102), anatase, 21-22 nm 2) TiO_2NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 Nanogenotox Project dispersion protocol	**Positive** TiO_2 NPs (NM-102 and NM-105) In NHEK cells **Equivocal:** TiO_2 NPs NM-102 in lymphocytes **Negative** TiO_2 NPs NM-105 in lymphocytes **Negative** TiO_2 NPs (NM-102 and NM-105) In: BEAS 2B, 16 HBE A549 Caco-2 cell lines	Reliability: 1	High	NANOGENOTOX Project, 2013 (Documentation provided to EFSA No. 7 and 8)

	102 and NM-105: 0, 15, 45, 125 and 250 µg/ml NHEK, NM-102 and NM-105: 0, 20, 40, 60 and 80 µg/ml Exposure: 1.5-2 cell cycles CytoB added 24h after the start of the treatment for Caco-2 cells and 6h after the start of the treatment for other cells Positive control: mitomycin C						
Mouse lymphoma gene mutation assay L5178Y TK+/- cells	NM-102: two series of concentrations: 1) 0, 32, 64, 128 and 256 µg/ml 2) 0, 312.5, 625, 1250 and 2500 µg/ml NM-105: two series of concentrations: 1) 0, 32, 64, 128 and 256 µg/ml 2) 0, 625, 1250, 2500 and 5000 µg/ml	1) TiO_2NPs (NM-102), anatase, 21-22 nm 2) TiO_2NPs (NM-105), anatase/rutile, 15-24 nm	NSC: 1 Nanogenotox Project dispersion protocol	**Negative** for all forms of TiO_2 NPs tested	Reliability: 1 Only minor deficiency in data reporting.	High	NANOGENOTOX Project, 2013 (Documentation provided to EFSA No. 7 and 8)

	Positive control: MMS						

CytoB: cytochalasin B; DCFH-DA or DCFDA: 2', 7'-dichlorofluorescein diacetate; Fpg: enzyme formamidopyrimidine glycosylase; LDH: Lactate dehydrogenase; MDA: Malonaldehyde; MMS: methyl methane sulphonate; NAC: N-acetylcysteine; NSC: Nanoscale considerations; RI: replication index; OTM: Olive Tail Moment; ROS: reactive oxygen species; SB: strand breaks; TEM: Transmission electron microscopy.