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Protocol

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A template wizard for the cocreation of machine-readable data-reporting to harmonize the evaluation of (nano)materials

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Supplementary Methods

1 Templates for material characterization

1.1 Physicochemical characterization

i. Hydrodynamic particle size by DLS (PC_GRANULOMETRY template)

By using the DLS technique, the hydrodynamic particle size can be determined by measuring the time-dependent fluctuations of the scattered light when a diluted dispersion of particles is illuminated with a laser light. Assuming the Brownian motion of the particles, the translational diffusion coefficient can be determined, and the *equivalent spherical hydrodynamic diameter* (which includes the electrical double layer - EDL - around the particle surface in solution) is calculated using the Stokes-Einstein equation [1].

$$D = \frac{k_B T}{6\pi\eta R_h}$$

where D is the diffusion coefficient, k_B is the Boltzmann constant, T indicates the temperature at which the analysis is carried out, η is the kinematic viscosity of the solvent, and R_h indicates the hydrodynamic radius of the particles.

In a DLS instrument, the fluctuations of the scattered light are recorded and analyzed in correlation delay time domain. The motion of dispersed particles is described by an intensity autocorrelation function that can be expressed as an integral over the product of intensities at time t and delayed time (t + τ). The applicability of the method and its limitations are reported in detail in RiskGONE deliverable D4.2[2]

ii. *Particle size and particle counting by NTA*

NTA technique provides size characterization based on the free diffusion behavior of particles in solution. NTA measures particle diffusion by tracking the random motion of single particles in solution via high temporal-resolution video acquisition and enhanced contrast microscopy. The diffusion of the objects in suspension is measured with single-particle resolution, and hence it is particularly suitable for the characterization of size distribution for highly polydisperse nanoparticle populations [3]. The applicability of the method and its limitations are reported in detail in RiskGONE deliverable D4.6[4].

iii. Zeta Potential by ELS

The zeta (ζ) potential is the property defining the surface charge of a material dispersed in a liquid. ELS technique is used to estimate the zeta potential (ζ) of nanoparticles in suspension from their electrophoretic mobility (μ_e), defined as $\mu_e = n/E$, where n is the particle velocity, and E is the externally applied electric field. The zeta potential and the electrophoretic mobility are then related by the Henry equation:

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0 f(KA)}{3\eta}\zeta$$

where ε_r is the relative permittivity of the solution, ε_0 is the permittivity in vacuum, f(KA) is the Henry function, and η is the viscosity of the solution. Similar to the DLS technique, a laser is transmitted through the measurement cuvette during ELS measurement and an electric field is applied to the particle suspension. If the particles are charged, they experience a movement toward the electrode with an opposite charge sign with respect to their surface charge. The movement of the particles induces a shift in frequency, a so-called Doppler shift, in the scattered light, which is proportional to the particle velocity. This Doppler shift and the movement direction toward the positive or negative electrode are then used to estimate the electrophoretic mobility of the particles in suspension and, in turn, their zeta

potential [3]. The applicability of the method and its limitations are reported in detail in RiskGONE deliverable D4.6[4].

iv. Effective density by VCM

The determination of the effective density of NM by the VCM is based on the measurement of the volume of the pellet obtained by low speed, benchtop centrifugation of NM suspensions in a packed cell volume (PCV) tube. In an ideal situation, assuming the perfect stacking of NM agglomerates (i. e., with no intervening space), the total volume of the agglomerate in a sample of NM suspension is equal to the volume of the pellet as measured after centrifugation. However, in a real situation, part of the medium can be easily trapped within the empty spaces between agglomerated particles, leading to a lower effective density of agglomerates in comparison to one of the primary particles. By knowing the mass of the NM in suspension, the material density and the stacking factor (SF), the effective density (r_{EV}) of the NM can be calculated. The calculation equation allows to take into account for potential NM dissolution in the medium as follows:

$$\rho_{EV} = \rho_{media} + \left[\left(\frac{M_{ENM} - M_{ENMsol}}{V_{pellet}\,SF}\right) + \left(1 - \frac{\rho_{media}}{\rho_{ENM}}\right)\right]$$

where M_{ENM} is the mass of the original NM, M_{ENMsol} is the mass of the solubilised fraction, V_{pellet} is the volume of the pellet in the capillary after centrifugation, r_{media} is the density of the medium and r_{ENM} is the density of the NM. The applicability of the method and its limitations are reported in detail in RiskGONE deliverable D4.7.

1.2. In-chemico characterization *FRAS assay and Dynamic dissolution in lung simulant fluids*

There are two *in chimico* assays for which templates have been developed in the past few years: the Ferric reducing ability of serum (FRAS) assay [5]and Dynamic dissolution in lung simulant fluids [6,7,8]. FRAS assay is an indirect assay, based on the principle of absorbance change due to reduction of an Fe3+ complex to Fe2+ complex, by the residual antioxidants in a sample, pre-incubated with a NM (see Figure S1). The absorbance change is correlated to the antioxidant capacity of the serum: the more antioxidants species, the more intense the blue color. Antioxidants may be depleached by free radicals produced by NMs, leading to oxidative damage, shown by a reduced blue colour in the assay [9].

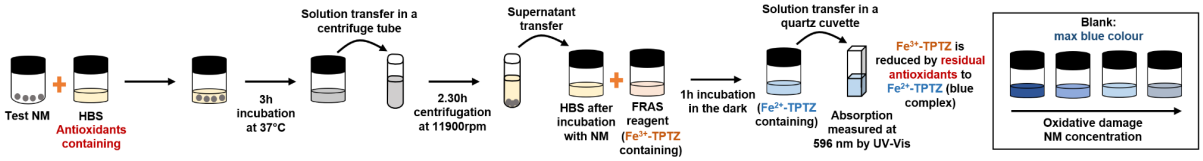

Figure S1. FRAS assay workflow

The FRAS template consists of four worksheets, namely 'Test_conditions', 'Raw data', 'Results' and 'Materials'. The Results sheet is automatically generated after filling all the necessary information (i.e., test concentration and raw absorption data) and is shown on Figure 2.

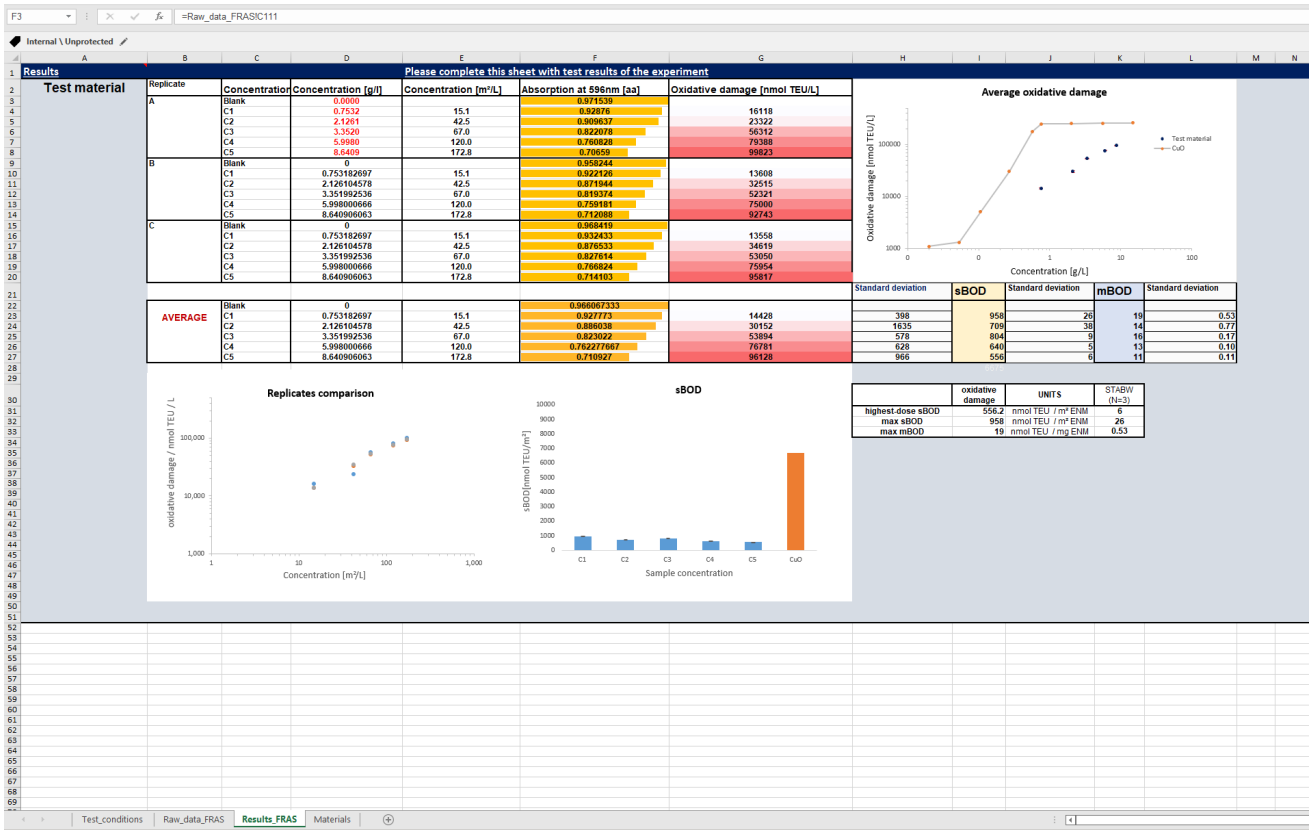

Figure S2. The results sheet from the FRAS assay template

The Dynamic dissolution of a test material, in lung simulant fluids, is monitored via a continuous flow system consisting of a heat cabinet with reservoir, flow-through cells, peristaltic pump set at 2mL/h and external autosampler. Leaching/biopersistence of relevant metals may be estimated after analysis of the samples in time by a suitable analytical method (i.e., ICP-MS) More details can be found in the available SOP[10].

The main input file sheet named 'Dissolution_Dynamic', which reports the experimental parameters, the sample details and the main results in a format similar to other eNanoMapper templatest; the second sheet is the single component results summary. This is relevant for the assay expert only; graphs plotting relevant assay parameters are automatically generated. Each sheet is specific for one component (i.e., Zn). If the material in analysis contains both Zn and Cu, then the template will include two 'ion release' sheets as shown on Figure S3 (left). The third sheet is the material results overview, specifically relevant to compare the behavior of different metal components in a single material (Figure S3 right).

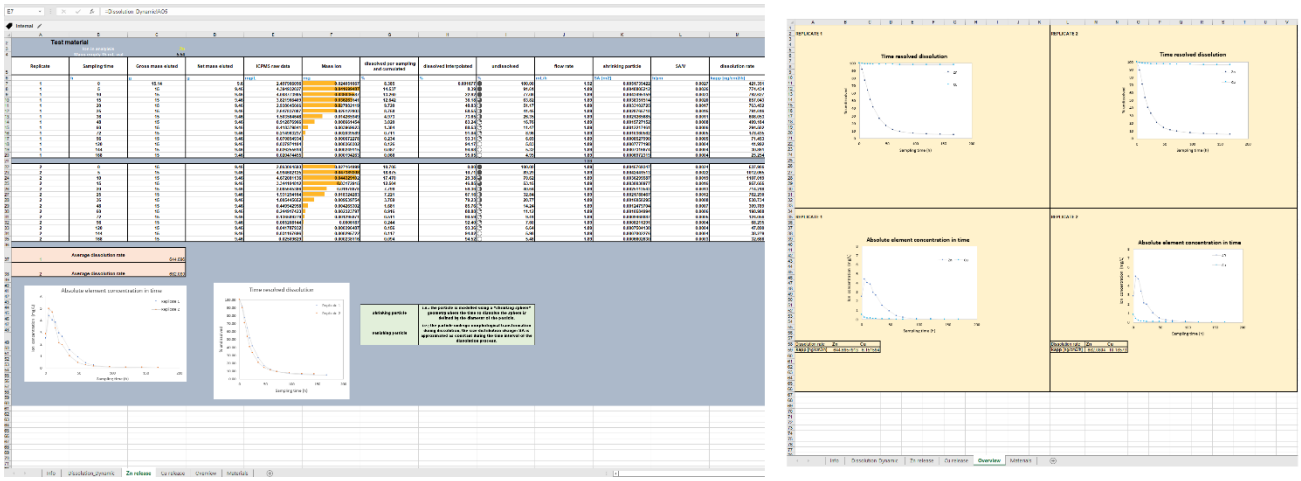

Figure S3. Dynamic dissolution template. On the left, the single component results summary (here for Zn ion); on the right, the overview panel where multiple metal components are displayed simultaneously for a single test material

1.3 Biological characterization: Endotoxin template

To characterize NMs for potential endotoxin contamination is of pivotal importance for the interpretation of *in vitro* and *in vivo* investigations, as endotoxins can bind to the surface of NMs and alter their toxicological potential, for example by inducing the release of inflammatory mediators from exposed cells[11] The chromogenic version of the Limulus Amebocyte Lysate (LAL) assay is a commonly used fast, sensitive, and endotoxin-specific method based on an aqueous extract of amebocyte blood cells from Atlantic horseshoe crab. Bacterial endotoxins catalyze the activation of a proenzyme in the extract, which in turn catalyzes the splitting of yellow-colored p-Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA. The released pNA can be photometrically measured at 405-410 nm. The activation rate is proportional to the endotoxin concentration in the sample that can be thus calculated as endotoxin units (EU) per volume of reaction (EU/mL), based on the linear regression equation obtained from a standard curve obtained from an *E. coli*-derived Endotoxin Standard. Within the RiskGONE project (Deliverable 4.4), an SOP and data collection template for the LAL assay applied to NMs were developed based on the kit manufacturer instructions, the European Standard EN ISO 29701, previously gained knowledge and experience from research projects for hazard assessment of NMs (e.g., NANoREG Deliverable 5.06) and relevant literature[11].

2 Dose response templates

2.1 Cell viability: Alamar Blue

The AB assay is a high throughput, cell metabolism-based method largely applied in toxicology and nanotoxicology to investigate cell viability (cytotoxicity), cell proliferation, and cellular metabolic activity in response to chemicals and NMs. The test is based on the chemical resazurin and its intracellular conversion to resorufin due to the reducing conditions found in the cytosol of metabolically active cells [12]. As a result of this reaction, the compound turns from blue-colored and non-fluorescent to purple-fluorescent, and this conversion is easily quantified as fluorescence intensity or based on absorbance properties. Less metabolically active/dying cells have less capability to convert resazurin, resulting in a lower fluorescent/colorimetric signal. We recently discussed the application of this assay to NMs, which has proven to be robust, simple to perform and relatively cheap. However, specific challenges, e.g., interference of the particles with the assay, should be properly addressed. A detailed step-by-step procedure to apply the AB assay for testing of NMs, from NM preparation, cell

exposure, inclusion of interference controls, and analysis and interpretation of the results was presented [13]. The procedure presented is based on the use in 96 well plates, which allows to increase the throughput of the method, but it can easily be adjusted to the use with different exposure plates.

2.2 Cell viability: Colony Forming Efficiency

The CFE (also called clonogenic or plating efficiency assay) measures the ability of cells to survive and form colonies, which is an ultimate index of cytotoxicity.
The CFE assay is based on the treatment of individual, adherent mammalian cells in a small inoculum. Briefly, the cells are exposed to a test compound, positive and negative controls, and cultured to allow for colony formation, generally for 10-12 days, depending on the proliferation rate of the cells. Colonies are then stained and counted manually or by automatized systems (e.g. GelCount). Cytotoxicity of the test compound is then measured as relative CFE (RCFE), which is the ratio of viability of treated cells to negative control cells, as only surviving cells will divide and form a colony. Besides a cytotoxic effect of the test compound (reduction of the number of colonies formed), a potential cytostatic effect can be detected by measuring the colony size. A reduced colony size indicates a delay in the cell cycle and thus a cytostatic effect.

Being non-colorimetric and non-fluorescent, the method is specifically suitable for the assessment of toxicity of NMs *in vitro* to avoid interference with the readout of the test method, which is commonly seen with many 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) [14,15].

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 CFE assay for assessing the cytotoxicity of NMs[16]. The SOP has been developed based on the JRC report and optimized for increased throughput by moving from petri dishes to 6-well plates, and further to 12-well plates. The SOP for application of the 12-well format was standardized and validated by an interlaboratory comparison study in four laboratories within the H2020 NMBP-13 project RiskGONE, and is described in [17] .
An example of the CFE assay template and the results are shown in Figure S4.

a)

IN-VITRO TEST CONDITIONS	Please ensure you also complete a Test Method Description Form (TMDF) for this test type* .		
Project Work Package:	WP5-HUMAN HAZARD ASSESSMENT		
Partner conducting test/assay:	NILU		
Test facility - Laboratory name:			
Lead Scientist & contact for test:		E-mail address:	
Assay/Test work conducted by:		E-mail address:	
Full name of test/assay (add OECD Test ref-ID if app.):	CFE		
Short name or acronym for test/assay:	CFE		
Type or class of experimental test as used here:			
End-Point being investigated/assessed by the test:	PE	rCFE	
Metric(s) used to assess End-Point outcome/response:	Colonies per well		
SOP(s) for test - ref. project or other doc. - Title/ID:			
Path/link to sop/protocol on proj. server/web where applic.:		TBD	
Test start date (YYYY-MM-DD):	2020-09-28		
Test end date (YYYY-MM-DD):	2020-09-28		
TEST MATERIAL DETAILS	Material 1		
Select item from Project Materials list:	ERM00000063		
Material Name	Zinc oxide (Sigma Aldrich 721077)		
NM Core chemistry:	ZnO		
CAS No:	1314-13-2		
Material Supplier:	Sigma Aldrich		
Material State:			
Batch	MKCJ4155		
Vial			
Date of preparation (YYYY-MM-DD):	2020-09-28		

Test_conditions | Raw_data_CFE | Results_CFE | Materials

b)

Raw data	Please complete with the raw data generated by your experiment					
	T1					
Experiment 1	Start date	2020-09-28				
	Conc	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
	0	26	25	26	24	19	21
	1	21	19	15	21	18	23
	3	18	17	21	24	21	14
	10	24	23	22	24	21	20
	30	0	0	0	0	0	0
	100	0	0	0	0	0	0
	SC	21	29	27	23	19	25
	PC	0	0	0	0	0	0
Experiment 2	Start date	2020-09-28					
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
	0	17	10	21	17	13	19
	1	12	21	22	13	21	12

Test_conditions | Raw_data_CFE | Results_CFE | Materials

Figure S4. CFE assay template and corresponding results. a) Representative example of a Data data entry template for the colony forming efficiency (CFE) assay . b) Representative example of a Data template for entering raw data from the colony forming efficiency assay, with data on the number of colonies from the different replicate wells.

3 Genotoxicity assays

3.1 Comet assay

The comet assay, also called SCGE (Single Cell Gel Electrophoresis), is a rapid and informative method to detect DNA damage at a single cell level, employed in *in vivo* and *in vitro* genotoxicity testing on many different cell types. Cells embedded in agarose gels on a microscope slide are lysed to remove membranes, proteins, etc., leaving supercoiled DNA loops attached to the nuclear matrix, known as nucleoids. Strand breaks (SBs) relax the supercoiling and allow DNA to move toward the anode under electrophoresis, forming comet-like structures observed by fluorescence microscopy. The relative intensity of DNA in the comet tail indicates the frequency of (single or double) SBs [18,19]. SBs may result from direct damage or occur as intermediates in DNA repair. The enzyme-modified version of the comet assay, incorporating digestion with a lesion-specific endonuclease after the lysis step, detects diverse types of DNA lesions (such as oxidized bases) by converting them to abasic sites and

SBs [20],[21,22]. The most used enzymes are formamidopyrimidine DNA glycosylase (Fpg) [23], [22] or the mammalian counterpart, 8-oxoguanine DNA glycosylase (OGG1), which cleave oxidized purines, and endonuclease III (Endo III) for oxidized pyrimidines [24], [18].

The *in vitro* enzyme-modified version of the comet assay has been miniaturized to increase its robustness and throughput; the standard layout of 1 or 2 gels per slide has been expanded to 12 mini-gels per slide, or 96 mini-gels on a GelBond film ([25]; [26]). A commercial 'microarray' assay (CometChip) is also available [27]. Semi-automated and automated image analysis systems speed up the process. The method is thoroughly described in a recent Nature Protocols paper (Collins et al., 2023)[18]. Another dedicated protocol paper addresses all relevant points that need to be taken into consideration when assessing NM genotoxicity using the comet assay [28].

An OECD TG exists for the *in vivo* but not the *in vitro* comet assay.

Results — Please complete this sheet with test results of the experiment

MEDIAN	DNA Strand breaks					Fpg + Strand breaks					Net Fpg sites				
	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %	Tail intensity %
	Experiment 1	Experiment 2	Experiment 3	Average	SD	Experiment 1	Experiment 2	Experiment 3	Average	SD	Experiment 1	Experiment 2	Experiment 3	Average	SD
0	1,1	1,8	1,0	1,3	0,4	0,9	0,6	0,8	0,8	0,2	-0,2	-1,1	-0,2	-0,5	0,6
10	0,7	0,5	1,1	0,8	0,3	0,2	1,3	0,9	0,8	0,6	-0,5	0,7	-0,2	0,0	0,6
25	1,1	0,9	0,5	0,8	0,3	0,6	0,4	1,0	0,7	0,3	-0,5	-0,5	0,6	-0,2	0,6
50	1,0	0,8	0,2	0,7	0,4	1,3	1,9	0,9	1,4	0,5	0,3	1,1	0,7	0,7	0,4
100	0,7	0,8	0,1	0,6	0,4	0,5	0,2	0,6	0,4	0,2	-0,3	-0,6	0,5	-0,1	0,6
PC_MMS	1,2	2,0	9,4	4,2	4,5	4,8	19,4	40,9	21,7	18,2	3,6	17,4	31,6	17,5	14,0
PC_KBrO3	0,6	0,7	0,5	0,6	0,1	0,3	4,3	0,6	1,7	2,3	-0,3	3,7	0,1	1,1	2,2
SC	0,3	1,1	0,1	0,5	0,6	0,2	1,0	0,4	0,5	0,4	0,0	-0,2	0,3	0,1	0,2
PC_H2O2	37,1	53,1	64,8	51,7	13,9	68,0	56,5	70,9	65,1	7,6	30,8	3,4	6,1	13,5	15,1
INT	0,9	0,8	0,9	0,9	0,1	1,1	0,9	0,5	0,8	0,3	0,2	0,1	-0,5	-0,1	0,3

Test_conditions | Raw_data_COMET | Results_COMET | Materials

Figure S5. Example of results output for the enzyme-modified version of the comet assay. SD, standard deviation; SC, solvent control; PC, positive control; INT, interference control.

3.2 Micronucleus assay

The micronucleus assay is considered the gold standard for detecting chromosomal damage *in vitro*. The assay is designed so that cells exposed to a genotoxic test agent result in chromosomal breakage, forming small spherical nuclei (micronuclei) and being detected as fixed DNA damage. The protocol is based on cell exposure to the test agents for a minimum of 1.5 cell cycles followed by a two cell cycle incubation with cytochalasin B to induce binucleated cells, as described in the OECD Guidance Document on adaptation of the *in vitro* micronucleus assay (OECD TG 487 [29]) for testing of manufactured NMs (Series on Testing & Assessment No. 359; ENV/CBC/MONO(2022)15). The entire methodology is known as the cytokinesis-blocked micronucleus (CBMN) assay. As described in OECD TG 487, several methods can be used to determine cytotoxicity, including cytokinesis-block proliferation index (CBPI), relative population doubling (RPD), Relative Increase in Cell Count (RICC) or replication index (RI). It is crucial to include an appropriate cytotoxicity test in parallel to the measurement of micronucleus frequency to ensure that the genotoxicity evaluation is conducted within an appropriate dose range.

The assay methodology has evolved in cell preparations, staining, and scoring methods: from quantifying the DNA damage in mononucleated cells and binucleated cells, stained with Giemsa or fluorescence dyes, whilst evaluation may be by manual or automated microscopy scoring or flow cytometry. The preference is the evaluation of micronuclei in binucleated cells in this version of the assay ensures that scoring is restricted to those cells that have divided in the presence of the test agent. When microscopic techniques are used, in addition to measuring micronuclei, the assay permits identifying other changes such as nucleoplasmic bridges, nucleoplasmic bud, necrosis, or apoptotic cells and deriving a nuclear division index.

3.3 *In vitro* mammalian cell gene mutation assay OECD TG 476– Swansea University Layout

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. The *in vitro* mammalian cell gene mutation assay is significantly important for detecting point mutations induced by engineered NMs as the bacterial reverse gene mutation assay (Ames test) is not appropriate for use with these materials [30]. The HPRT methodology, specifically, 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 compound 6-thioguanine (6-TG), resulting in HPRT - mutants being seen as live colonies when cultured in media. The HPRT assay takes advantage of the fact that large mutations, and the subsequent alterations to the X-chromosome lead to cell lethality, therefore even small point mutations and exon deletions can be detected, with spontaneous mutation frequency (MF) being lower at the *Hprt* loci than the thymidine kinase (*Tk*) loci [31]. 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 NMs. A version of the *in vitro* mammalian cell gene mutation test (OECD TG 476)[32] with suspension human lymphoblastoid (TK6) cells was utilized to analyse gene mutants in the *Hprt* gene. The method has been pre-validated in interlaboratory study withing RiskGONE project.

4. Novel methods

4.1 Label-free Cell monitoring by Electrical Impedance (bioimpedance)

The need for label-free detection methods has emerged, especially in nanotoxicity studies, because of the possible interferences of NMs with labeling-substances and detection systems [15,14,33]). Impedance-based assays that measure the electrical properties of cells are label-free and have recently emerged as a reliable alternative with the potential to become a method of choice for the initial screening of NMs' toxic effects. Impedance-based monitoring takes advantage of the passive properties of an object, which occur when the object is composed of dissipative elements, such as ohmic resistors or conservative elements, such as capacitances and inductances [34]. It measures the opposition to the flow of electrical current (impedance) through tissues, cell monolayers and single cells when an external alternating current (AC) field is applied. The impedance magnitude is governed by the specific characteristics of the AC field and properties of the biological system under investigation. In short, at low AC frequencies (kHz range), healthy cells dwelling in or passing the AC field impede the flow of current because the cell membrane constitutes a significant barrier to current flow. Thus, the impedance of the system is high. On the contrary, the impedance is low when cells have a compromised membrane, e.g., when undergoing cell death. Therefore, this method can assess cell viability, proliferation, cell-cell and cell-substrate interaction of adherent cells growing onto a microelectrode array [35]. Single cells in suspension can also be investigated using impedance-based flow cytometry [36]Real-time nanotoxicity screening can be run in medium and high throughput settings using multiple daisy-chained analyzers able to monitor 16-, 96- or 384-well plates carrying a microelectrode array at the bottom of each well. The limitation of impedance-based devices is that they give little information about mechanisms behind

the above-mentioned cellular responses. However, a significant advantage is that real-time observation facilitates the identification of key time-points and concentrations for further, more targeted, in-depth mechanistic studies [37]

4.2 Enzyme activity inhibition test

The idea of measuring activity of isolated acetylcholinesterase (AChE) upon exposure to a suspension of NPs is based on the knowledge that interplay of NM properties, such as the size, surface chemistry, crystallinity, and hydrophobicity, govern the reactivity of NMs [38]. Effects of surface curvature and surface characteristics of carbon-based NMs on the adsorption and activity of acetylcholinesterase. [38]This particular enzyme was chosen as the model system, since it has a well-known structure and well studied activity. The adsorption and inhibition of AChE activity is a result of interaction with NMs either in a suspension or interaction with a surface of a material. The measurement of AChE activity is done according to the most widely applied method by Ellman (1961), adapted for microplates. AChE hydrolyzes the substrate acetylthiocholine chloride to produce thiocholine and acetate. The thiocholine in turn reduces the color indicator (5,5'-dithiobis-(2-nitrobenzoic acid)) acid liberating 3-thio-6-nitrobenzoate. The formation of this chromogenic product is followed at 405 nm and the rate of formation is considered related to the activity of the material.

This type of NM characterization is called biological characterization based on enzyme inhibition/adsorption intensity. The more biologically reactive the suspended material (has a potential to inhibit enzyme) or the surface (has a potential to adsorb to the surface) the higher the inhibition of enzyme activity is. Results show that AChE is a promising candidate for ranking different NMs according to their adsorptive and inhibitory properties [39].

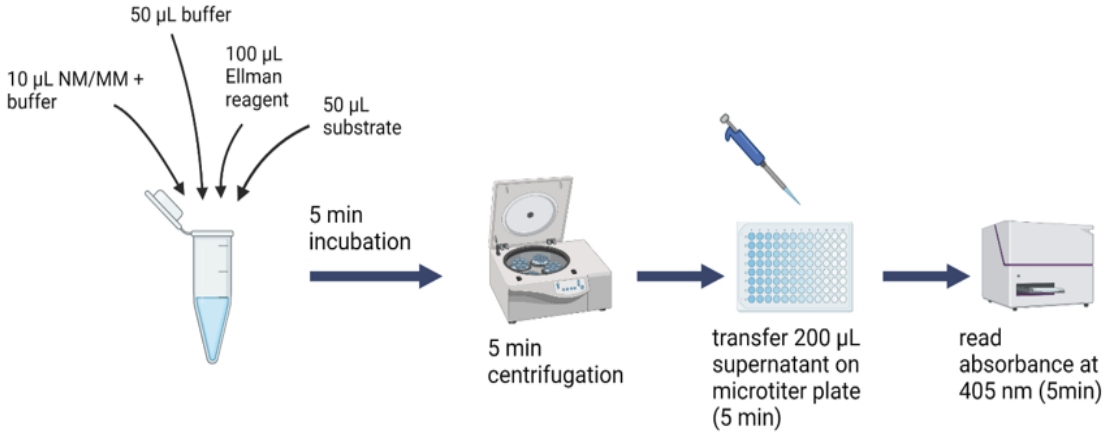

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Figure S6. Experimental set-up for Ellman control assay.

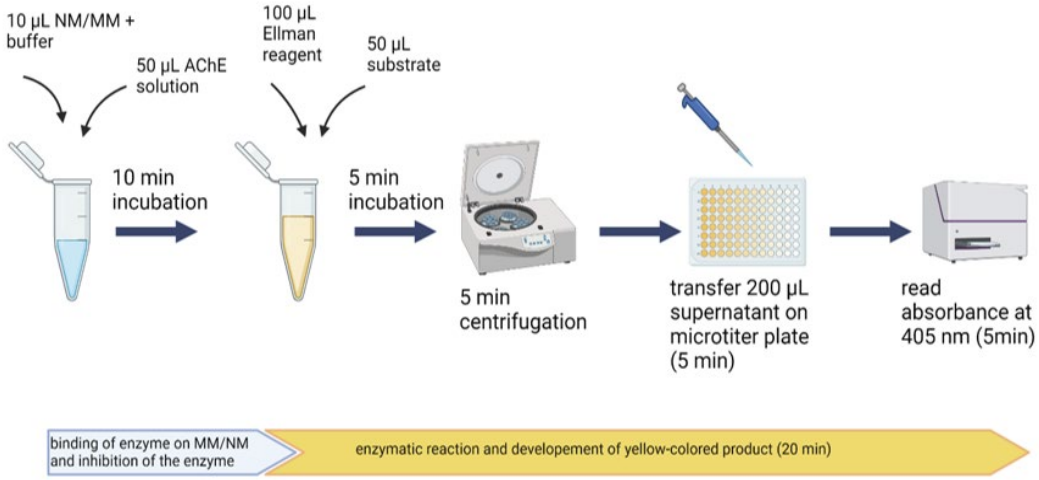

Figure S7. Experimental set-up for measurement of AChE inhibition.

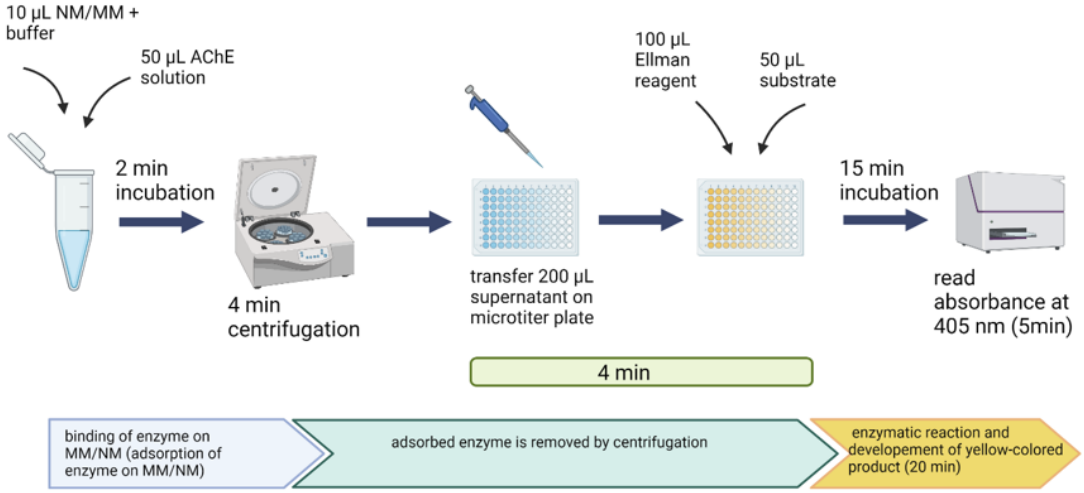

Figure S8. Experimental set-up for measurement of AChE adsorption.

5 Organism level response

5.1. Daphnia culturing, acute and chronic tests data capture

Daphnia culturing

As a model organism for ecotoxicity testing, *Daphnia magna* are cultured in a controlled environment to establish baseline health of the organisms to effectively test for toxicity response (deviations from the baseline health observations) to a range of different substances and conditions. The Daphnia facility at the University of Birmingham has a primary culturing room to maintain the daphnia cultures at 20°C (±1°C) and a 16:8 light: dark cycle. Bham 2 strain *D. magna* are used for all exposures and experimental work.

Daphnia exposures - acute

The acute exposure protocol follows the guidelines set out by the OECD 202 test for daphnia (OECD, 2004). The principle of the test is that daphnids are exposed to a series of different concentrations of a toxicant and are exposed for 48 hours. Observations are made at 24 and 48-hour intervals to assess for

"immobilisation" within the daphnia test, which is defined as a daphnid that is not moving/swimming after gently agitating the vessel for 15 seconds (disregarding any movement of their antennae). Immobilisation is used as it is hard to visually determine daphnia death without the use of a microscope, and this therefore speeds up the observations. As a result of immobilisation being used as the end point for the test, results are reported as the effect concentration (EC50) rather than a lethal concentration/dose (LC50). Results are plotted as a dose-response curve as shown in Figure S9.

Daphnia exposures- chronic

Daphnia chronic toxicity response can be established using total reproduction and growth over a 21-day testing duration. The chronic toxicity exposures are based on the OECD 211 daphnia chronic reproduction test (OECD, 2012). Observations are made over the test durations for time to first brood, time between broods, total neonates per brood (and over the whole period – see Figure S9) and growth over time, often measured from the eye to the tail spine. These observations allow for sublethal toxicity to be observed in the daphnia and the impact of the toxicant on the reproductive health of the daphnia to be determined.

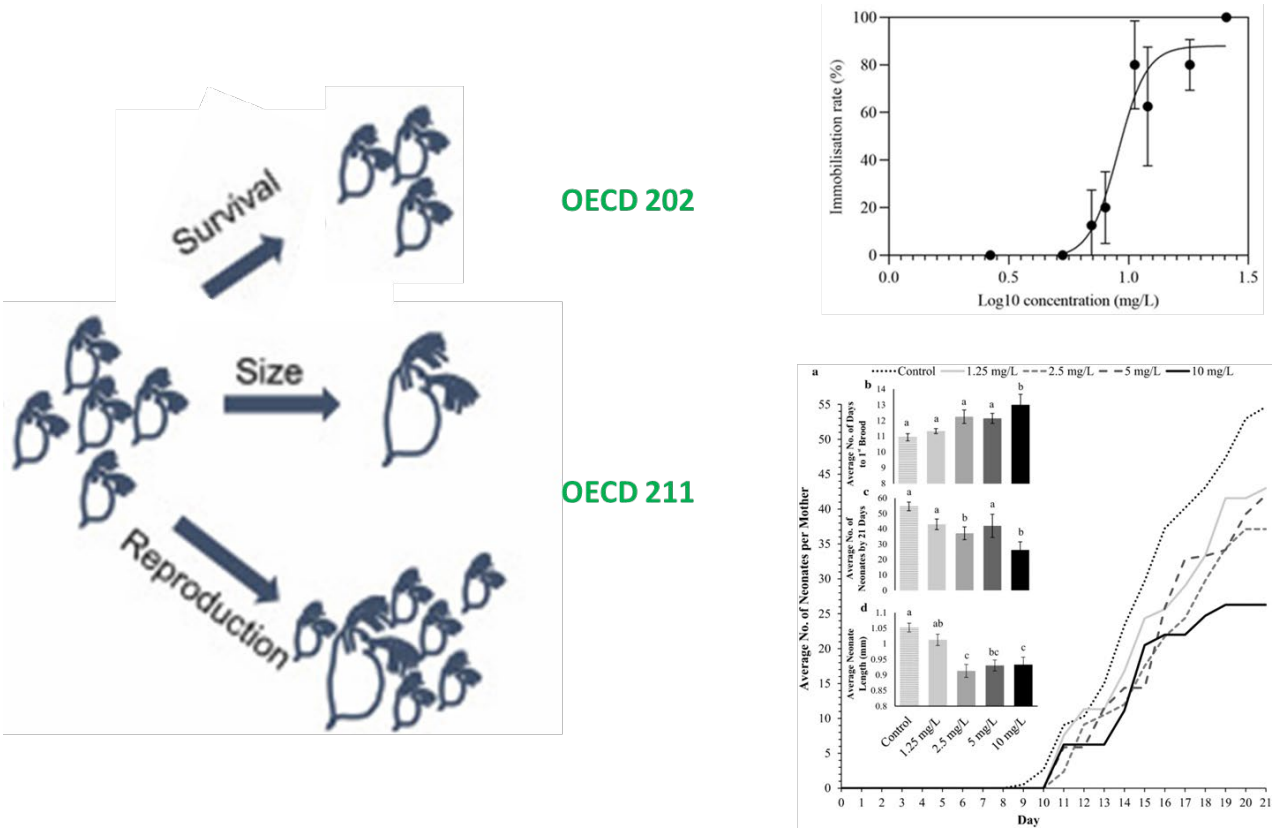

Figure S9 : Schematic illustration of the *D. magna* standard tests for acute and reproductive toxicity. **The figure shows the number of surviving organisms plotted as a dose-response curve (OECD 2020) and the growth and reproduction assays (OECD 211) showing the cumulative number of offspring per adult over the 21 days with insets showing (from top) average time to1st brood, aver number of neonates per brood and average neonate length as a function of the exposure concentration.**

The Daphnia ecotoxicity templates correspond to the existing OECD 202 (acute / immobilisation) and OECD 211 (chronic / reproduction) TG for *Daphnia magna* (*D. magna*), as developed for chemicals, and extend them slightly in line with current scientific best practice and efforts underway in RiskGONE and NanoHarmony to update the TGs for use with NMs. While the NANoREG template for the acute

Data capture templates for nanosafety

D. magna toxicity currently available to download from the Template Wizard does an excellent job of capturing the information about the NMs dispersion and characterization and notes the medium conditions at the start and end of the exposure (pH, Dissolved Oxygen and temperature), the template does not capture the raw data on the % immobilisation, does not request confirmation that <10% of the control daphnids died (were immobilised) during the test, nor does it include the calculation of the EC50 values, but includes only the summary values for EC10, EC25 and EC50. There is currently no template at all for the chronic (reproduction) assay so we have developed that one also, based on the information (metadata) captured for the acute toxicity assay.

Since the set-up of the daphnia assays requires several pre-steps before the NMs exposure step, including acclimation of the daphnids to the test medium, which is typically different from the medium used for the running cultures (as the volumes of water needed mean that most laboratories use tap water or borehole water for the running cultures), and as part of the quality control of the facility should record the time to each brood of offspring, and the number of offspring per adult in the culture, we also include a template for capturing QA/QC information on the running cultures. This also allows for analysis of seasonal changes, or early indications of infection or other problems in the running cultures that will have implications for the validity of the tests, especially for the chronic (21-day) tests and their extension to multi-generations (which is not currently requested by ECHA but which a growing body of evidence suggests is important for assessment of epigenetic changes in offspring and for ensuring population and ecosystem health).

For the chronic (reproduction) assay the template captures the same metadata as the acute test, but includes also the additional measurements that are taken at each medium change, including the number of neonates, the time to each brood, and from imaging of the daphnids information on growth such as eye-tail length. Optimal additional end-points include assessment of lipid deposits (which can be measured experimentally, or potentially from image analysis via a machine learning model that has been developed previously,), protein secretion into the medium, and any morphological or other defects observed (again from imaging).

The data templates have been designed to ensure that all relevant metadata related to the assay performance and compliance are captured, including key parameters such as dissolved oxygen and pH. The metadata directly related to the OCED TG is mandatory, while other aspects, often added to increase the amount of data that can be retrieved from the experiment and to provide some mechanistic insights, such as total proteins secreted or lipid droplets, are optional, and clearly indicated as such.

The inclusion of the daphnia culturing template is an effort to increase the comparability of data, and to allow for tracking of changes in assay performance over time. While regulatory testing laboratory running under Good Laboratory Practice (GLP) will routinely capture and report this type of quality assurance and quality control information in their information management systems, we have found that in many cases this is sort of “assumed knowledge” and passed from person to person in the lab, and that individual records are not always kept such that when asked for details about the typical number of offspring in their running cultures (for example) researches may not be able to provide this information with certainty. Thus, by explicitly recording this as part of metadata, the data become available for assessing trends over time, such as potential temporal and seasonal variations, or potential impacts from switching food type/provider for example, which could be missed in the absence of this metadata.

As noted in the previous point, the template is designed to capture key performance related aspects of the assay, such as explicit confirmation that <10% of control organisms died, which are critical for

QA/QC purposes. Additionally, the addition of the data culturing template is an attempt to document implicit knowledge or background data that is not typically reported as part of scientific publications, but which can be essential to understanding how the assay performs in different laboratories over time, and for identification of potential sources of error early (prior to publication of datasets) if unexplained draft from the assay performance occur, such as for example, an infection in the cultures which would impair their validity for the assay and negate the results. We note that such reporting of culturing conditions, which is captured in the paperwork of laboratories operating under GLP for example, it is missing for many scientific labs, as it is not known that collecting this data is recommended practice.

The template is designed to capture the requirements of the OECD TGs 202 and 211 for acute and chronic toxicity to daphnia, respectively, and to plot the data directly allowing calculation of the EC_{50} atr 24 and 48 hours and the life trait data in the reproduction assay. The template will be used to support the SPSF application to the OECD WPMN for updating of the existing daphnia reproduction (OECD 211) TG for use with NMs.

2 Template Wizard implementation

From the technical perspective, the Template Wizard online page is implemented entirely as a front-end tool, without relying on server side functionality for the template generation. The Template Wizard server side includes a set of Excel templates defining fields to be populated with a pseudo language as described in the JavaScript library https://github.com/ideaconsult/xlsx-datafill and shown in Figure S10.

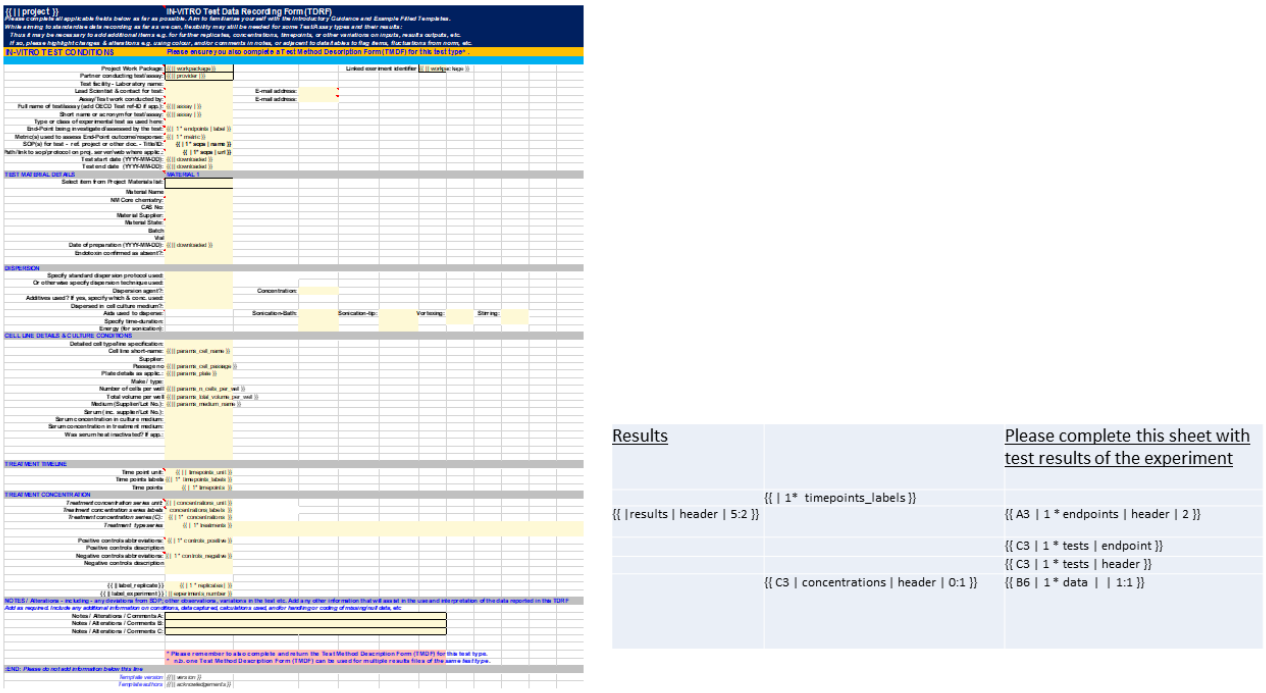

Figure S10. The internal definition of a template in the Template Wizard implementation. The Example shown here is also the Alamar Blue assay template as it is configured at the server side. left: Test information sheet; right: results sheet

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Instructions for use of templates available in the Nanosafety Data Interface

In your internet browser, type or copy https://enanomapper.adma.ai

The NanoSafety Data Interface integrates data from multiple projects, and has two different sets of sections – public and private as shown in Figure 1.

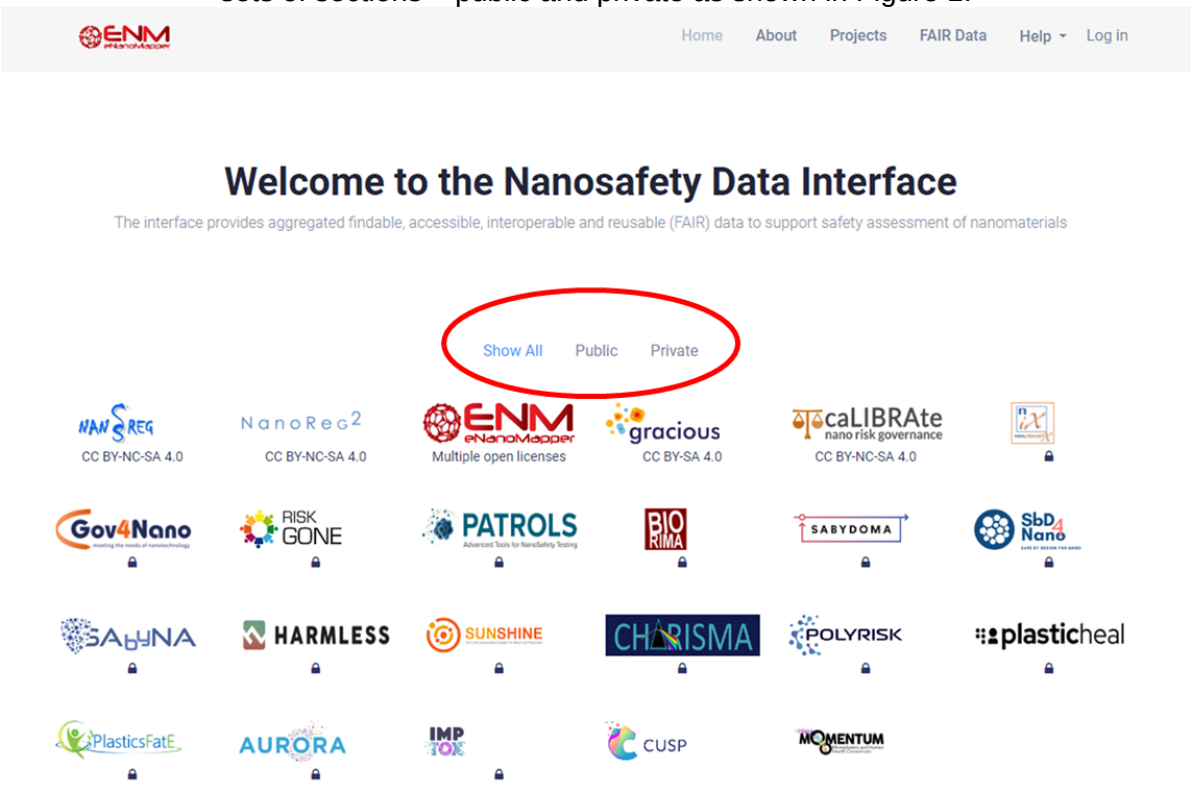

Figure 1: NanoSafety Data Interface main screen

In the “Public database” you can access data with open licenses and the various templates. If you are not a member of an ongoing project, please use the nanomaterial (NM) Database, as shown in Figure 2, to customize and download the required templates.

The “Private database” contains data and templates of ongoing projects which are currently restricted to project members. If you are part of an ongoing project listed within the database, please click on the appropriate icon (project logo).

The instruction below are for the public eNanoMapper database, but are also applicable for project databases.

Select the public eNanoMapper database by clicking on the NM icon as shown in Figure 2.

Figure 2: Users not associated with a specific running project can select the NM database which is public.

In the new window that opens, click on Template Wizard to access the tempates, as shown in Figure 3. The link will take you to the "Templates Wizard gallery", as shown in Figure 4.

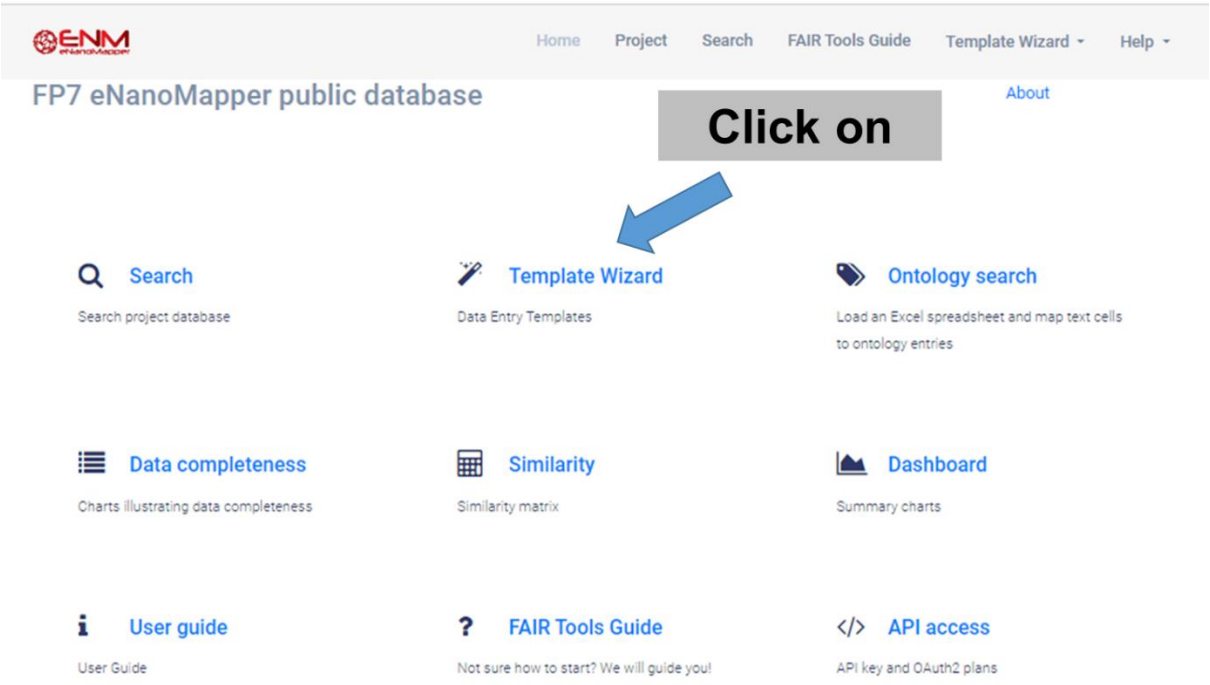

Figure 3. Click on Template Wizard to access the range of templates available – the Template Wizard Gallery (shown in Figure 4).

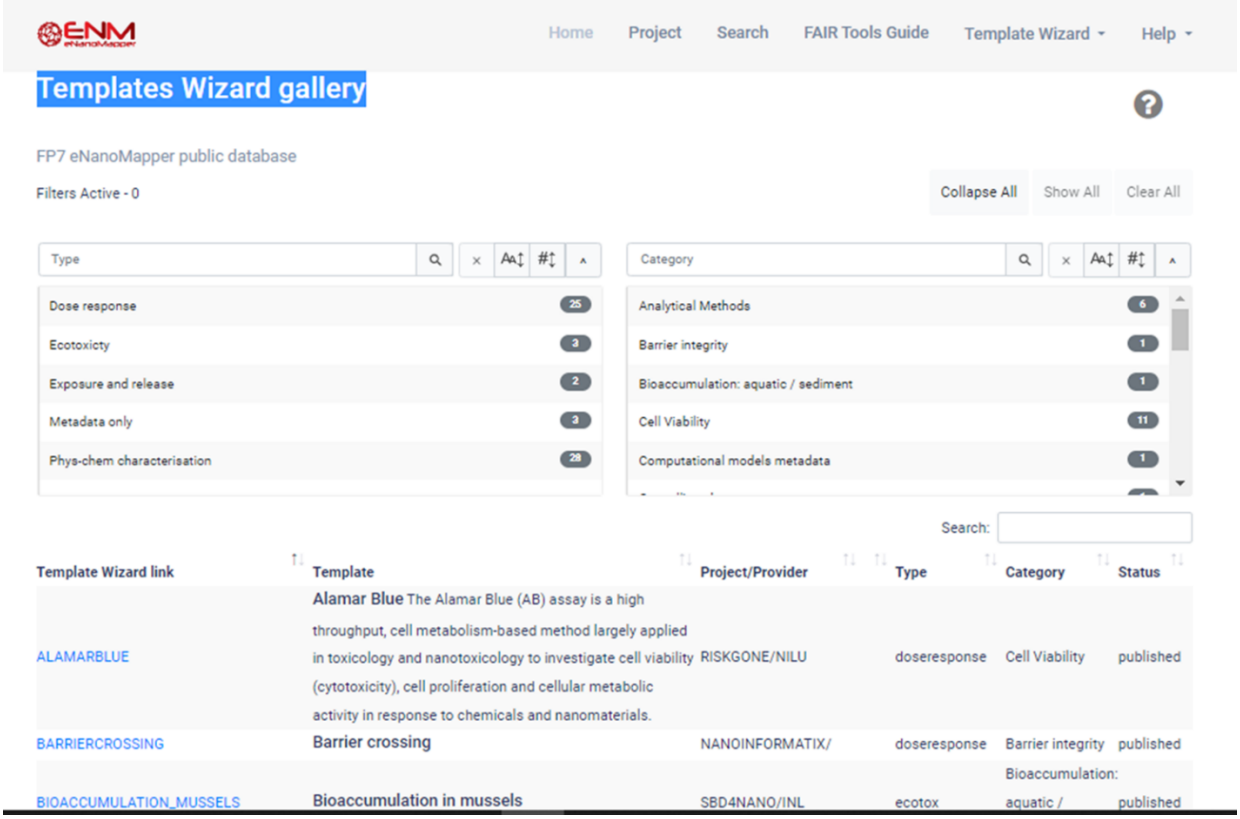

Figure 4: The Template Wizard gallery in the NM database, in which users can search for templates by type or category via the left and right searhc boxes, respectively.

Within the two tables above, you can search for templates by “type” or “category.”

For example, if you move the slider under the right hand side “Category” search box you will see all of the available templates.

If you click “cell viability”, a list with 11 templates will be shown, as illustrated in Figure 5 below.

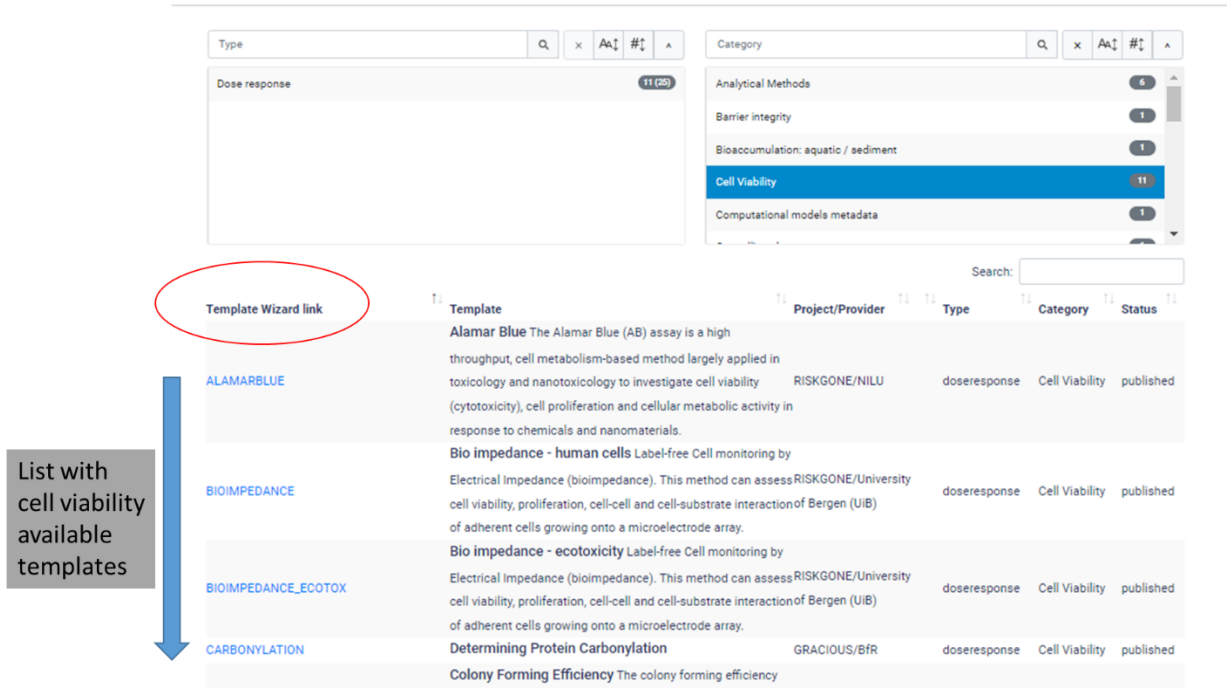

Figure 5: The list of currently (publicly) available templates of *in vitro* dose-repsonse studies, including the Alamar Blue cytotoxicity assay which is used for the subsequent illustration of the template use.

The next steps are illustrated for an *in vitro* dose-response assay, but are similar for any selected template.

Click on the name of the specific (cell viability) assay that you have performed. Let's usee **the Alamar Blue (AB) assay**, for example.

A new window will open, as shown in Figure 6.
Before downloading the template, you must fill in all positions marked with red.
Then click on the blue Download template button (at the top right in Figure 6), and save the file (test data recording template, TDRF).

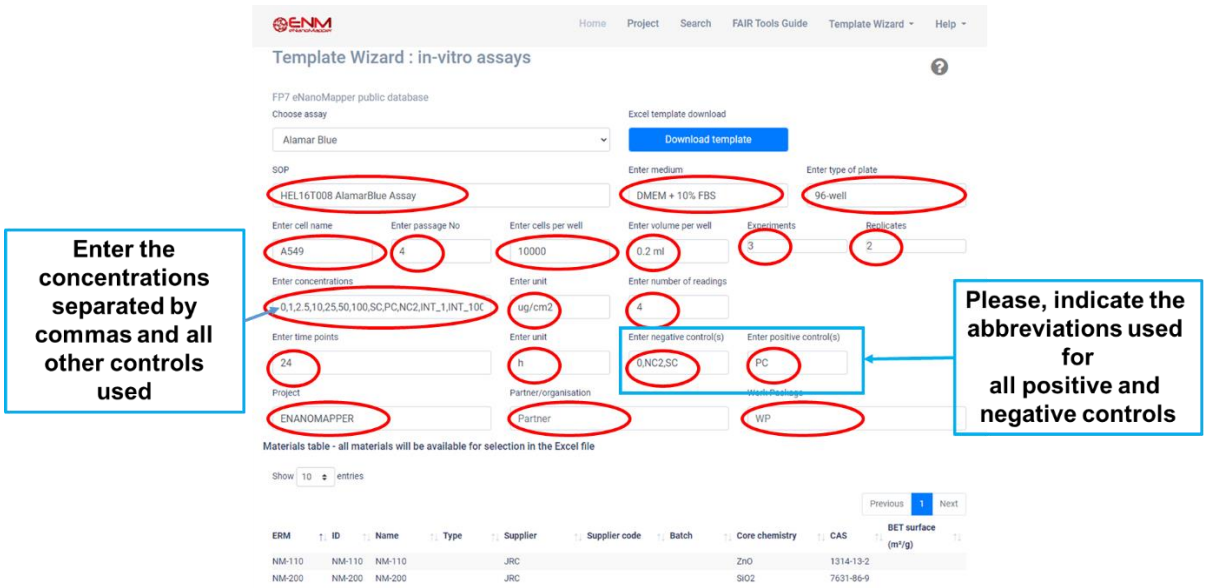

Figure 6: The Template Wizard for Alamar blue assay

When opening the saved file in Excel, you will see the following (Figure 7):

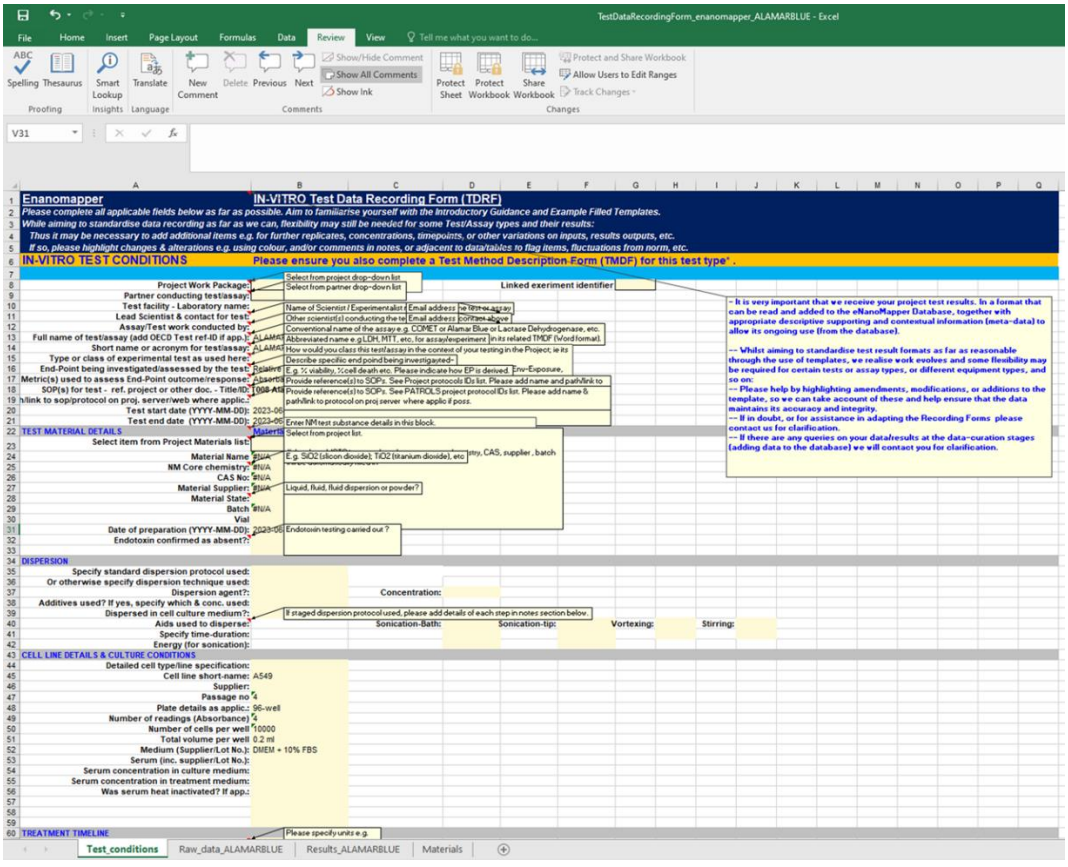

Figure 7: The test data recording template, after downloading the selected template from the NM template Wizard.

Under the “Review” window, deselect “Show all comments,” and the explanations will be deactivated. They can be re-activated with the same procedure. These comments help you by describing how to fill the necessary information in the rows (marked in yellow), in case you are not sure what to write.

In the Excel file, you must fill data in the “Test conditions” and “Raw_data_ALAMARBLUE” sheets. Please, look at the “Materials” sheet (by moving between Tabs on the bottom left of the Excel file) and if needed, add the information about the nanoparticles or nanomaterials used. We note that the same templates can be utilised for other materials (e.g., micro or nanoscale plastics) or chemicals in general.

In the **“Test condition sheet”**, please fill in all necessary information in the rows highlighted with yellow and type the name of the project you are working on, as shown in Figures 8 and 9 below. This information forms part of the metadata that accompanies the data and makes the data more findable once it is uploaded to the eNanoMapper or other database.

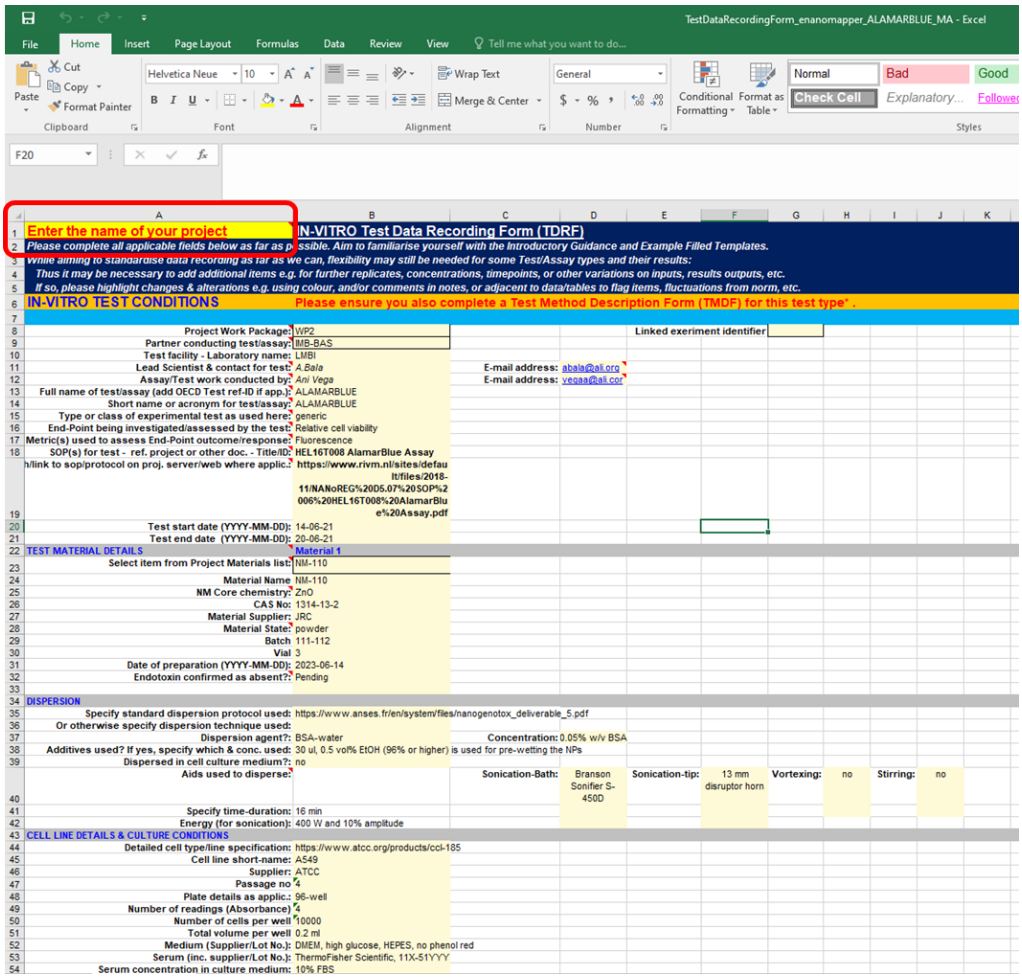

Figure 8: The **“Test condition sheet”** allows the user to add the study conditions (cells highlighted in yellow in the template), which form part of the metadata (data about the data). The project name allows data to be aggregated with other data from the same project, or for

users not aligned with a specific research project it acts as an identifier to faciliate data search.

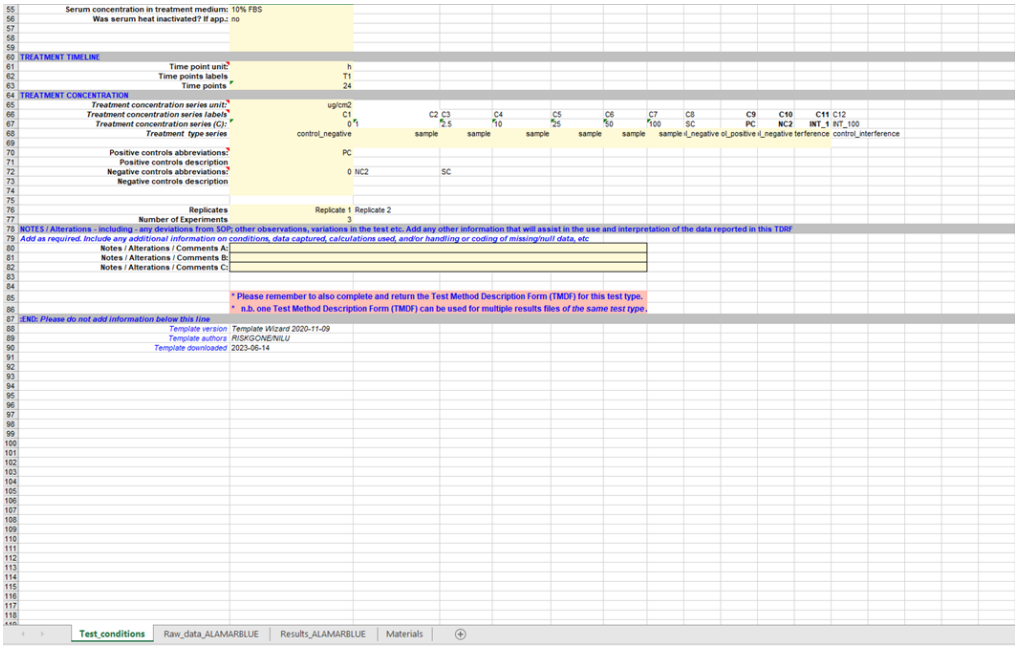

Figure 9: Continuation of the **“Test condition sheet”** allows the user to add the study conditions (cells highlighted in yellow in the template).

Now your template is ready for use to capture your experimental data. Once you have performed your experiment, please fill in the raw data obtained for the AB assay in your Lab into the sheet “**Raw_data_ALAMARBLUE**”, shown in Figure 10.

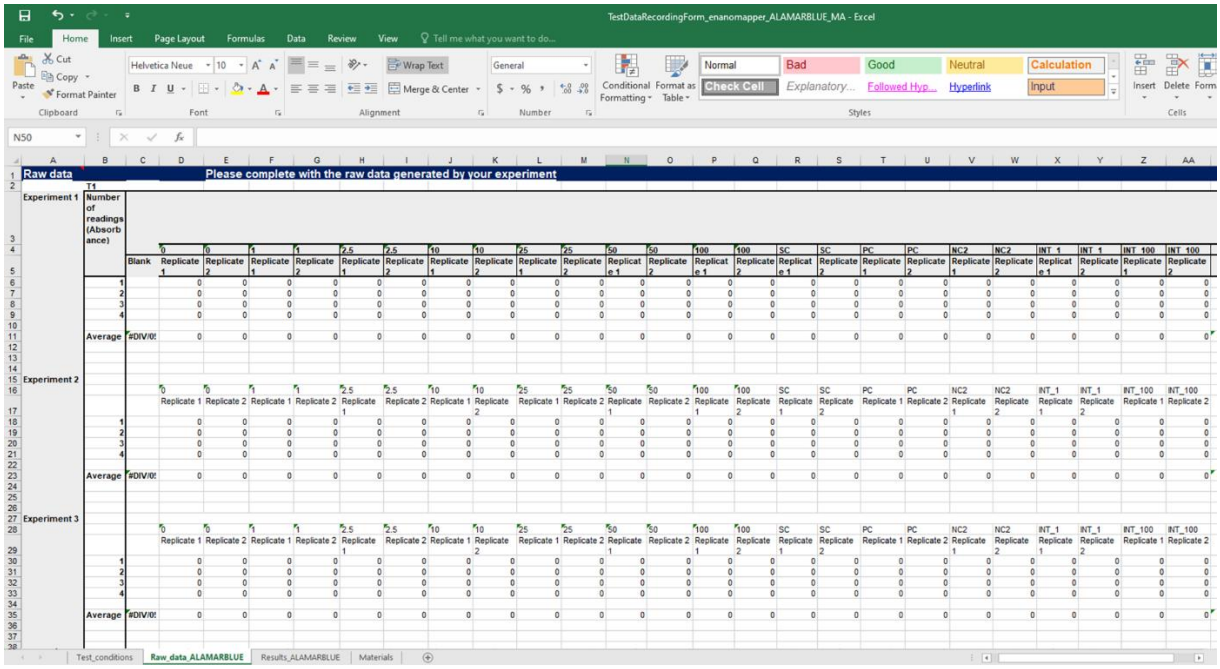

Figure 10: Results sheet ready for the experimental data to be captured. The replicates, concentrations, timepoints etc. are exactly as was specified in the **Test Condition Sheet** in the previous step. The automatic calculations inclucing average and normalisation formulae are coded into the Excel file also, reducing the risk of error in calculation.

All results will be automatically calculated and shown in the "**Results_ALAMARBLUE**" sheet.