

JRC SCIENCE AND POLICY REPORTS

Interlaboratory comparison study of the Colony Forming Efficiency assay for assessing cytotoxicity of nanomaterials

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2014

Report EUR 27009 EN

European Commission

Joint Research Centre
Institute for Health and Consumer Protection

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JRC92910

EUR 27009 EN

ISBN 978-92-79-44677-1 (PDF)

ISSN 1831-9424 (online)

doi:10.2788/406937

Luxembourg: Publications Office of the European Union, 2014

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Abstract

Nanotechnology has gained importance in the past years as it provides opportunities for industrial growth and innovation. However, the increasing use of manufactured nanomaterials (NMs) in a number of commercial applications and consumer products raises also safety concerns and questions regarding potential unintended risks to humans and the environment. Since several years the European Commission's Joint Research Centre (JRC) is putting effort in the development, optimisation and harmonisation of *in vitro* test methods suitable for screening and hazard assessment of NMs. Work is done in collaboration with international partners, in particular the Organisation for Economic Co-operation and Development (OECD). This report presents the results from an interlaboratory comparison study of the *in vitro* Colony Forming Efficiency (CFE) cytotoxicity assay performed in the frame of OECD's Working Party of Manufactured Nanomaterials (WPMN). Twelve laboratories from European Commission, France, Italy, Japan, Poland, Republic of Korea, South Africa and Switzerland participated in the study coordinated by JRC. The results show that the CFE assay is a suitable and robust *in vitro* method to assess cytotoxicity of NMs. The assay protocol is well defined and is easily and reliably transferable to other laboratories. The results obtained show good intra and interlaboratory reproducibility of the assay for both the positive control and the tested nanomaterials.

In conclusion the CFE assay can be recommended as a building block of an *in vitro* testing battery for NMs toxicity assessment. It could be used as a first choice method to define dose-effect relationships for other *in vitro* assays.

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Executive summary

The European Commission's Joint Research Centre (JRC) provides scientific support to European Union policies, including nanotechnology. Nanotechnology has gained importance in the past years, it provides opportunities for industrial growth and innovation, and has an enormous potential to solve future challenges to the society. Nanotechnology innovation will be seen in many sectors including public health, employment, occupational safety and health, information society, industry, innovation, environment, energy, transport, security and space. However, the increasing use of manufactured nanomaterials (NMs) in a number of commercial applications and consumer products raises also safety concerns and questions regarding potential unintended risks to humans and the environment. Since several years the JRC is putting effort in the development, optimisation and harmonisation of test methods suitable for hazard assessment of NMs. International harmonisation of these test methodologies is ensured, among others, through JRC's participation in the Organisation for Economic Co-operation and Development (OECD) Working Party of Manufactured Nanomaterials (WPMN). The OECD WPMN's programme on the safety of manufactured NMs addresses all the different components including the availability of adequate methods needed for thorough manufactured NMs risk assessments. One of the objectives of this programme is to explore the potential application of alternative (*in vitro*) methods for testing of manufactured NMs.

This report presents the results from an interlaboratory comparison study of the Colony Forming Efficiency cytotoxicity assay performed in the frame of the OECD WPMN. The Colony Forming Efficiency (CFE) assay is a label-free method for the assessment of basal cytotoxicity. Being non-colorimetric and non-fluorescent the method avoids possible interferences of NMs with the toxicity assessments. The CFE assay has been optimised and standardised for NMs testing by the JRC's Nanobiosciences Unit. The aim of this project was to evaluate intra and interlaboratory reproducibility of the CFE assay, to identify possible factors that could influence results and to propose measures to increase harmonisation of the protocol used for testing NMs. Twelve laboratories from European Commission, France, Italy, Japan, Poland, Republic of Korea, South Africa and Switzerland participated in the study. A positive control (sodium chromate) and 9 NMs were tested. The results of the study show that the CFE assay protocol is easily and reliably transferable to other laboratories. The intra and interlaboratory reproducibility of the CFE assay was good with a percentage of Coefficient of Variation (CV %) less than 20% for the intralaboratory and less than 30% for the interlaboratory variation. We conclude that the CFE assay is a suitable method to assess cytotoxicity of NMs and has several advantages over commonly used *in vitro* cytotoxicity assays (e.g. MTT, Lactate Dehydrogenase (LDH) release, Neutral Red Uptake, etc.), as it allows avoiding test interferences while being often more sensitive. The assay was considered useful as an early screening method. It could be also used in combination with other *in vitro* assays to define the subtoxic doses for further *in vitro* testing.

List of abbreviations

CFE	Colony Forming Efficiency
CLS	Centrifugal Liquid Sedimentation
CV	Coefficient of Variation
CVD	Chemical Vapour Deposition
DLS	Dynamic Light Scattering
EC	European Commission
IC	Inhibitory Concentration
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IHCP	Institute for Health and Consumer Protection
LDH	Lactate dehydrogenase
L-M	Levenberg-Marquardt
JRC	Joint Research Centre
MDCK	Madin Darby Canine Kidney cell line
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NM	Nanomaterial
NP	Nanoparticle
NMs	Nanomaterials
OECD	Organisation for Economic Co-operation and Development
PC	Positive Control
PE	Plating Efficiency
PBS	Phosphate Buffered Saline
SOP	Standard Operating Procedure
swCNTs	Single walled Carbon NanoTubes
TEM	Transmission Electron Microscope
TEOS	Tetraethyl orthosilicate
UV-Vis	UV-Visible
WPMN	Working Party for Manufactured Nanomaterials

1. Introduction

Nanomaterials (NMs) are used in a variety of industrial sectors and in numerous consumer products, such as paint, catalysts, sports items, surface treatment products, textiles, cosmetic products, food additives and packaging, vehicle tyres, electronic items and analytical chemical equipment. Some applications are new, while others, such as paint and catalysts, have been around for many years.

According to the European Commission's Recommendation on the definition of a nanomaterial adopted in 2011 a "Nanomaterial means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm" (EU 2011).

NMs are not intrinsically hazardous per se but there may be a need to take into account specific considerations in their risk assessment. They are often well-known chemical substances that demonstrate new physical and chemical characteristics when in nano form. Gold, for example, is known for being very inert but if produced in nano form, it changes colour, reacts chemically, and can even be used to catalyse chemical reactions. Other examples of new characteristics materials may possess in nanoscale are electrical conductivity and magnetic properties. The enormous complexity of nanotechnology comes from the fact that the same chemical substance can be used in a variety of different nano forms that have wide range of properties including size, shape, surface functionalization, surface charge, that affect drastically their way of interacting with biological systems such as biological fluids (protein interactions) and living cells (cellular uptake, mechanisms of toxic response). This renders their safety assessment a real challenge.

Nanotechnology is a quickly developing field and it is expected that a large number of new NMs will be produced and put to use in the near future. To enable a quick and reliable safety assessment of these NMs used in consumer products, there is an urgent need to provide robust, standardized test methods for toxicity screening.

In vitro test methods are an essential tool for toxicity testing of NMs. They are used for basic research in mechanistic toxicity studies, for toxicity screening purposes, as well as for regulatory testing. However, some properties of materials in nano form can hinder the outcome of an experiment through assay interference, leading to a risk of false positive or false negative results. Recent research has shown that a good knowledge of the physico-chemical characteristics of the NMs being tested is essential. Each NM may pose specific challenges especially in terms of sample preparation and dosimetry. The assessment of NMs toxicity *in vitro* has to consider the NMs physicochemical characterisation of the stock suspensions measured by different analytical techniques, physicochemical characterisation of NMs behaviour in biological media (e.g. cell culture medium) and NMs interaction with proteins. The possible interactions between the NMs and the assay systems should be systematically analysed. Some problems reported in the literature include interference of

NMs with optical detection methods (light absorption, fluorescence), chemical reaction between the NMs and the *in vitro* assay components, adsorption of assay molecules (e.g. antibodies, enzymes) on the particle surface (Casey et al., 2007; Kroll et al. 2012; Han et al., 2011; Holder et al., 2012; Monteiro-Riviere et al., 2009; Oostingh et al., 2011; Stone et al., 2009). Therefore, the conventional and well-established *in vitro* assay protocols used to test chemicals or pharmaceuticals should be employed with caution and adapted when applied for NMs. Consequently, there is a need to develop and standardize novel, well-controlled and possibly label-free *in vitro* methods, with reduced possibility of interferences between NMs and the assays readouts.

Since several years, the JRC is working on the development and optimization of *in vitro* test methods suitable for hazard assessment of NMs. As part of this effort, existing *in vitro* test methods are adapted for NMs testing, by introducing appropriate controls to account for potential artefacts resulting from interaction of NMs with the assays. This work is done in close collaboration with the OECD WPMN (<http://www.oecd.org/science/nanosafety>).

Under the OECD WPMN, the Sponsorship Programme for the Testing of Manufactured Nanomaterials was launched to investigate an agreed set of representative MNMs using appropriate test methods, preferably the OECD Test Guidelines, for a number of endpoints relating to physico-chemical characterisation, human health and environmental safety. The need for adaptation of existing Test Guidelines for the purpose of NMs testing is also being assessed.

Based on the work of the WPMN, the OECD Council issued a recommendation (C(2013)107 from September 2013) stating that in the testing of manufactured NMs current OECD Test Guidelines should be applied and adapted as appropriate to take into account the specific properties of manufactured NMs in the testing of these materials. The recommended tools for the adaptation of the existing chemical regulatory frameworks or other management systems to the specific properties of manufactured NMs include two documents: Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials [ENV/JM/MONO(2009)21] and Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [ENV/JM/MONO(2012)40].

Within its objectives, the WPMN aims also at exploring specific applications of *in vitro* methods to manufactured NMs including the use of existing alternative methods that could be incorporated in harmonized testing approaches. In a series of OECD WPMN Expert Consultation Meetings on Alternative Test Methods in Nanotoxicology, several *in vitro* methods have been identified as particularly relevant for hazard assessment of NMs and sufficiently developed to be proposed for round-robin and/or validation studies, and possible future adaptation in Test Guidelines. These include methods for assessing cytotoxicity (Colony Forming Efficiency assay), genotoxicity (*in vitro* micronucleus test, Comet assay), mutagenicity, as well as *in vitro* models for biokinetic studies and methods for topical toxicity assessment (skin and eye tolerance).

One of the methods that have been successfully adapted and standardised by the European Commission (EC) JRC Nanobiosciences Unit for the assessment of basal cytotoxicity of NMs is the label-free Colony Forming Efficiency assay. In June 2012 the WPMN officially included in its work plan a project on the interlaboratory comparison of the CFE assay.

This report presents the results and conclusions from the CFE interlaboratory comparison study. Chapter 2 outlines the study design and its organisation. A detailed description of the CFE assay is presented in Chapter 3. Information about the synthesis and physico-chemical characterisation of the NMs used during the project is given in Chapter 4. The statistical approach used for the analysis of the CFE assay results, i.e. calculation of Inhibitory Concentration (IC_{50}) values and assessment of intra and interlaboratory variability, is presented in Chapter 5. Chapter 6 describes and summarises the results obtained from the CFE assay in all laboratories for each of the NMs tested and provides an analysis of the assay reproducibility. The conclusions from the study are presented in Chapter 7. A list of abbreviations has been included before the Introduction. Furthermore, the protocols used for cell culturing and for performing the CFE assay are given in Appendices 1 and 2.

2. Organisation of the interlaboratory comparison study

The main objectives of the interlaboratory comparison study were:

- To evaluate the suitability of the CFE assay for cytotoxicity screening of NMs
- To assess the transferability, intralaboratory and interlaboratory reproducibility of the CFE assay
- To identify possible factors that influence results
- To propose measures to further increase harmonisation of the assay protocol
- To deliver a final protocol for the assay suitable for testing a large range of NMs.

The project was officially included in the OECD WPMN work plan in June 2012. The study started in September 2012 and was coordinated by the JRC. A total of twelve laboratories from Europe, Japan, South Korea and South Africa participated in this work (see Table 2.1).

JRC was in charge of the experimental design of the study, prepared a detailed project plan, performed the selection, synthesis and physicochemical characterisation of all tested NMs (except single walled carbon nanotubes (swCNT) that were synthesised, characterised and provided to all labs by AIST, Japan), supplied the participants the Standard Operating Procedures (SOPs, see Appendix 1 and 2 to this report), the NMs and the cell line.

The Madin Darby Canine Kidney (MDCK) cell line used in this project was selected based on its ability to form well-defined colonies that are relatively easy to count. All laboratories received a flask of cells in culture, from the same passage, and were asked to propagate and freeze a stock of the cells for the purpose of the study, according to a well-defined protocol (see Appendix 1).

The selected NMs were gold nanoparticles (Au NPs, three sizes), silver nanoparticles (Ag NPs, two sizes) and silica nanoparticles (SiO₂ NPs, two sizes), single-walled carbon nanotubes (swCNTs) and zinc oxide nanoparticles (ZnO NPs, one size) (Table 2.2). The physico-chemical characterisation of the NMs used during the project, including the stability testing, was performed at JRC and is extensively presented in Chapter 4 of this report. All the NMs were found to be stable for at least 6 months, except NPs code I (ZnO) that is unstable and had to be used within 15 days after suspension in aqueous media.

A positive control (sodium chromate, Na₂CrO₄) and 9 NMs were tested in the CFE assay. Cells were exposed to selected concentrations of NMs and positive control for 72 h. In the first step, a complete dose-response range of sodium chromate (positive control) was tested by each laboratory. This step was used for training and problem-solving purposes. It also allowed evaluating the CFE protocol transferability to each laboratory. In the second step, all 9 NMs were tested in the 12 laboratories.

The experimental part was concluded in May 2014. All the results (raw data) were submitted by the laboratories to JRC for analysis. The statistical analysis was carried out by an

independent statistician from an external company (StatBio, Enskild, Sweden). The approach used for the analysis is outlined in Chapter 5.

Table 2.1. List of laboratories participating in the study

Country	Institution	Acronym	Laboratory number
Japan	National Institute of Advanced Industrial Science and Technology	AIST	1
France	CEA Life Science Division	CEA	2
Switzerland	Swiss Federal Laboratories for Materials Science and Technology	EMPA	3
Italy	Italian National Agency for New Technologies, Energy and Sustainable Economic Development	ENEA	4
France	Institut Pasteur de Lille	IPL	5
Italy	Istituto Superiore di Sanità	ISS	6
European Commission	Joint Research Centre, Institute for Health and Consumer Protection, Nanobiosciences Unit	JRC	7
Republic of Korea	Korea Research Institute of Standards and Science	KRISS	8
Republic of Korea	National Institute of Environmental Research	NIER	9
Republic of Korea	National Institute of Food and Drug Safety Evaluation	NIFDS	10
South Africa	National Institute for Occupational Health	NIOH	11
Poland	Nofer Institute of Occupational Medicine	NIOM	12

Table 2.2. List of nanomaterial tested in the study

NPs name	Nominal size (nm)	Batch number	Supplier	NPs code used in the study
Au NPs	5	RLS 57	JRC in-house synthesis	A
Au NPs	35	RLS 70	JRC in-house synthesis	B
Au NPs	15	NM 330*	JRC NMs Repository	C
Ag NPs	30	RLS 213	JRC in-house synthesis	D
Ag NPs	20	NM 300*	JRC NMs Repository	E
swCNTs	-		AIST	F
SiO ₂ NPs	20	I0102A	JRC in-house synthesis	G
SiO ₂ NPs	90	I0102C	JRC in-house synthesis	H
ZnO	240	NM110*	JRC NMs Repository	I

* Starting from May 2014 the Repository Materials are named as follow: NM330 is JRCNM03300a; NM300 is JRCNM03000a and the corresponding dispersant is JRCPD03001a; NM110 is JRCNM01100a.

3. Colony Forming Efficiency Assay (CFE)

3.1 Description of the assay

The CFE is a clonogenic assay that measures the ability of a single cell to form a colony. This *in vitro* assay can be used to determine cytotoxicity induced by NMs. It can be performed with any adherent cells that are able to form colonies (e.g. Balb/3T3, MDCK, HaCaT, HepG2, A549). The CFE assay cannot be used with non-adherent cells or cells unable to form colonies.

In the CFE assay the cells are seeded in a dish at a low cell density, treated with the investigated potential toxicant and cultured for several days to allow the formation of colonies. Changes in cell viability after treatment with a toxicant will result in a decreased number of colonies formed in comparison to the negative control (untreated cells) or solvent control (cells exposed to the solvent used for NMs synthesis and dispersion or for dissolution of chemicals). Cytotoxicity (inhibition of colony formation) is expressed as percentage of CFE with respect to the negative or solvent control.

The conventional *in vitro* cytotoxicity tests widely used for testing soluble chemicals and drugs may be difficult to apply for NMs due to possible interferences of NMs with the assay readouts and/or the assay components. There are several problems that can arise when using *in vitro* assays, including interference with optical detection methods (light absorption, fluorescence), chemical reaction between the NMs and the assay components, adsorption of assay molecules on the particle surface, shading effects (Kroll et al., Casey et al., 2007, Monteiro-Riviere et al., 2009). The great advantage of the CFE assay is that it is a label-free test (non-colorimetric, non-fluorescent) that reduces the possibility of the occurrence of interferences. This test has already been used in different *in vitro* systems to assess cytotoxicity of a wide range of NMs e.g. gold NPs (Coradeghini et al., 2013), silver NPs (Locatelli et al., 2012), titanium oxide NPs (De Angelis et al., 2013; Fenoglio et al., 2013), zinc oxide NPs (De Angelis et al., 2013), silica NPs (Ubaldi et al., 2012), or mwCNTs (Ponti et al., 2010). By using this assay it is also possible to distinguish between cytotoxic effects (reduction of the number of colonies formed) or cytostatic effects (reduction in the colony area). In this interlaboratory comparison study, however, only the cytotoxic effects were assessed.

The procedure used for performing the CFE assay in the present study is described concisely below and schematically presented in Figure 3.1. All methodological details can be found in the complete SOPs for the MDCK cell maintenance and for the CFE assay presented in Appendices 1 and 2, respectively.

Briefly, on Day 1, 200 MDCK cells are seeded in 3 mL of fresh complete medium in 60-mm Petri dishes (six replicates per treatment condition). After 24 h (Day 2), the treatment suspensions of NMs (Table 3.1) and the positive control ($\text{Na}_2\text{CrO}_4 \cdot 6\text{H}_2\text{O}$, 100 μM) are added to the cells. The cells are kept in contact with the treatment suspension for 72 h (from Day 2 to Day 5), then on Day 5 the test compounds are removed and replaced with complete fresh medium and the cells are cultured for additional 3 days until Day 8. At the end of each

treatment (Day 8), the medium is removed; the colonies are first fixed using a solution of 4% (v/v) formaldehyde in PBS, then stained using 0.4% (v/v) of Giemsa in MilliQ water. After drying, colonies (composed of at least 50 cells) are counted under a stereomicroscope.

The results are normalised to the negative control (cells exposed to fresh complete culture media) or solvent control (cell exposed to solvent used for NM synthesis or dispersion) and expressed as:

% CFE = (average of number of colonies in treatment / average of number of colonies in negative control or solvent control) * 100.

A reduction in the number of colonies formed in the treatment with respect to the negative or solvent control is a measure of cytotoxicity.

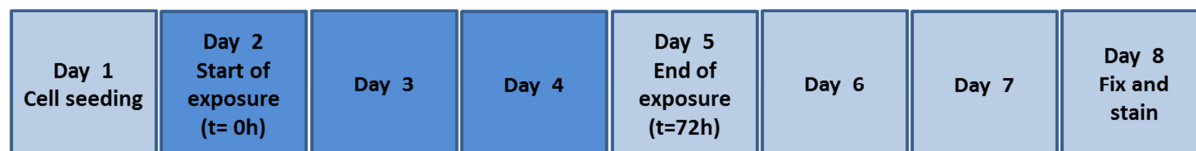


Figure 3.1. Schematic presentation of the protocol for the CFE assay.

Table 3.1. Concentrations of NPs code A-E and G-I. The concentrations are expressed as μM , $\mu\text{g/mL}$ (of Au, Ag, Si or Zn) and number of NPs/mL. The number of NPs was calculated based on size distribution obtained from Centrifugal Liquid Sedimentation (CLS) measurements. Note that for NPs code F (swCNTs) concentrations were expressed only in $\mu\text{g/mL}$ and three concentrations were tested: 1, 10 and 100 $\mu\text{g/mL}$. For each NM a solvent control corresponding to the solution in which NPs are dispersed was tested. For each NM the concentration of solvent was the same in each treatment suspension and corresponded to the concentration present in the highest dose tested.

NPs concentrations tested				
(μM)	($\mu\text{g/mL}$)	Number of NPs/mL		
A – B – C	A – B – C	A	B	C
1	0.20	3.28E+11	7.29E+08	1.16E+10
5	0.98	1.64E+12	3.64E+09	5.78E+10
10	1.97	3.28E+12	7.29E+09	1.16E+11
50	9.85	1.64E+13	3.64E+10	5.78E+11
100	19.70	3.28E+13	7.29E+10	1.16E+12
200	39.93	6.57E+13	1.46E+11	2.31E+12
300	59.09	9.85E+13	2.19E+11	3.47E+12
400	78.78	1.31E+14	2.92E+11	4.62E+12
D – E	D – E	D	E	
0,1	0.01	1.06E+08	1.19E+09	
0,5	0.05	5.32E+08	5.96E+09	
1	0.11	1.06E+09	1.19E+10	
1,5	0.16	1.60E+09	1.79E+10	
2,5	0.27	2.66E+09	2.98E+10	
3,5	0.38	3.72E+09	4.17E+10	
5	0.54	5.32E+09	5.96E+10	
10	1.08	1.06E+10	1.19E+11	
G – H	G – H	G	H	
16,6	1	7.79E+10	1.98E+09	
166,4	10	7.79E+11	1.98E+10	
1664,3	100	7.79E+12	1.98E+11	
I	I	I		
1	12.3	5.23E+04		
10	123.0	5.24E+05		
15	184.2	7.84E+05		
20	245.6	1.05E+06		
22,5	276.3	1.18E+06		
25	307.0	1.31E+06		
50	614.0	2.61E+06		

3.2 Catalogue of images of colonies formed by MDCK cells

During the course of the project images of representative colonies formed by MDCK cells and observed under the stereomicroscope were collected. This catalogue of images is an important tool providing instructions for counting of colonies obtained in the CFE assay. When establishing the CFE protocol in a laboratory one has to be aware of possible problems that can occur (e.g. related to wrong cell seeding) and which may influence the colony formation and/or the colony counting and thus change the final result of the assay. Examples of such typical errors were also included in the image catalogue.

Figure 3.2 A-C shows representative colonies obtained after the treatment with the negative control in the case of a correct cell seeding (Figure 3.2 A) or when an error in the calculation of cells to be seeded occurs (Figure 3.2 B and C). When the number of cells seeded is higher than needed, colonies are overlapping and it is not possible to distinguish them between each other, which compromises the counting (Figure 3.2 B). In case of a too low cell seeding density (Figure 3.2 C), not enough colonies are formed and the test results are not acceptable (according to acceptance criteria defined in the SOP, see Appendix 2). A lower number of colonies could be also due to an error in mixing the cell suspension before or during the seeding, or to bacterial or mycoplasma contamination of the cell culture.

Figure 3.2 D shows a negative control with good cell colonies formed but with an empty zone in the dish (highlighted with a dashed line) where cells were not able to attach and proliferate. This phenomenon occurs randomly and could be due to a failed treatment of the cell culture dishes during production, or if the dish was not swirled efficiently after cell seeding leaving a dried space on the surface which prevented the attachment of cells.

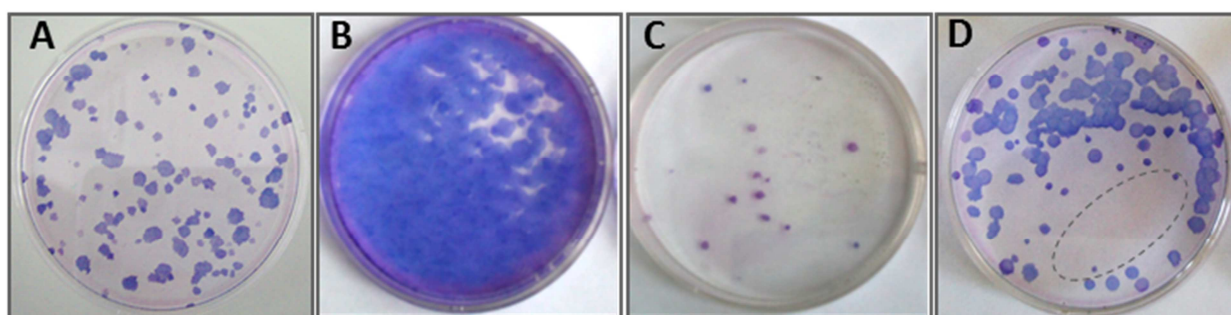


Figure 3.2. Examples of MDCK cell colonies. The images represent colonies in the negative control at the end of the CFE assay in (A) correct conditions, (B) when the number of cells seeded is higher or (C) lower than the required one (200 cells/dish). A situation where the number of colonies formed is correct but a part of the dish is free of colonies is shown in (D).

Single colonies can also have a different morphology and density as reported in Figure 3.3. Colony nr 1 has a cell density typical for MDCK cells; colony nr 2 and 3 are less dense and single cells can be distinguished. Colony number 4 and 5 represent a mix of the two situations. However, all these colonies have a well-defined perimeter and contain more than 50 cells (see acceptance criteria in the SOP, Appendix 2).

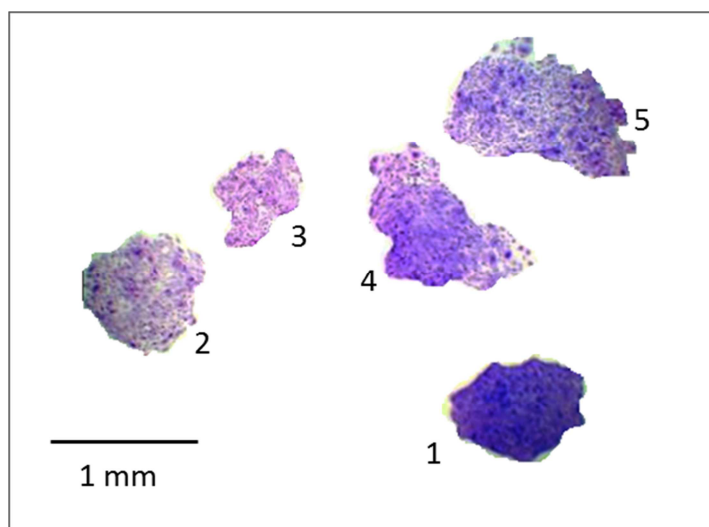


Figure 3.3. Different morphology and cell density of single colonies formed by MDCK cells after 8 days of culture (negative control treatment).

During their growth colonies can sometime overlap making the colony counting difficult; some examples are shown in Figure 3.4. The dashed lines encircle the perimeter of the colonies. Examples nr 1 and nr 3 show two overlapping colonies but with a well-defined center. In examples nr 2 and 4, three colonies are overlapping. Example nr 5 is difficult to analyse since the centers of the colonies are not well-defined. In this case one, two (as suggested here by dashed lines) or even three colonies could be counted. In the example nr 6 one could consider counting two colonies, but since the division between them is not evident and the two cannot be easily distinguished, we count it as only one colony. Example nr 7 shows two colonies of different cell density with well-defined centers. Finally, in the example nr 8 six colonies are counted since the perimeter of all of them can be well defined.

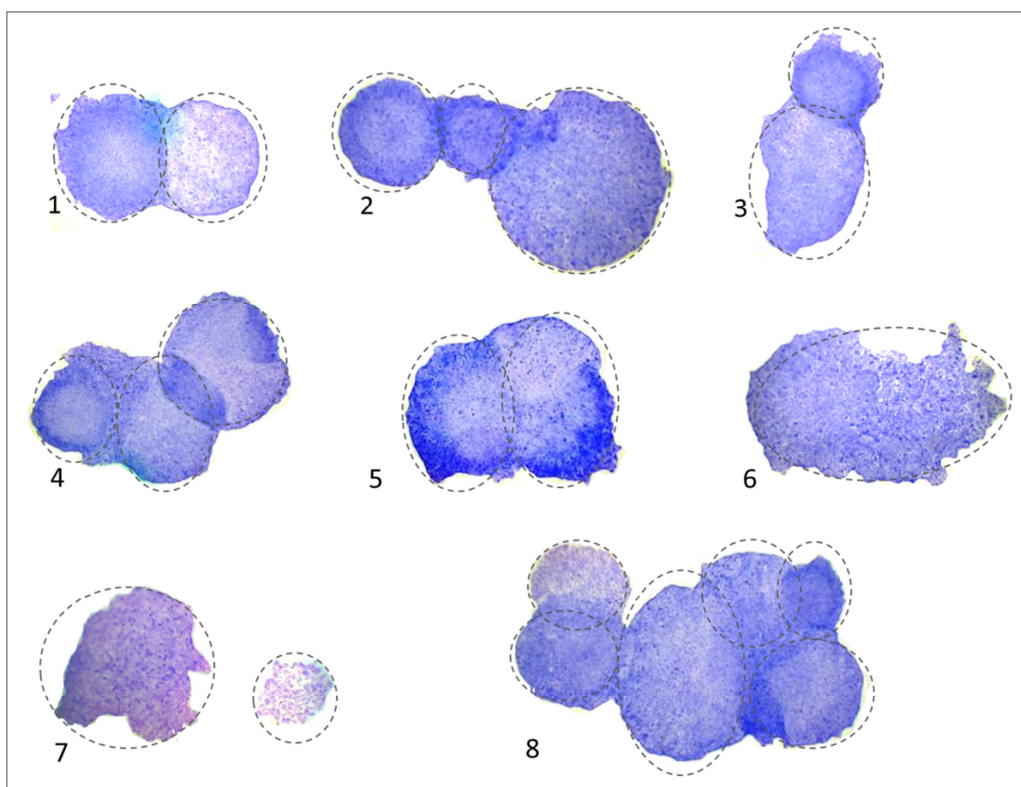


Figure 3.4. Examples of colonies that can be formed by MDCK cells; colonies are defined by dashed lines. Examples show overlap of two colonies (nr 1, 3 and 5); overlap of three colonies (nr 2 and 4); single colonies with different cell density (nr 6 and 7), and six overlapping colonies (nr 8).

Images of colonies presented in Figure 3.4 were used in a small exercise that was aimed to evaluate differences in the colony counting between various laboratories and operators. Each laboratory was asked to count the colonies (images were provided to them without the dashed lines) and the results are presented in Figure 3.5.

In most cases the laboratories counted the same number of colonies, with some exceptions in particular, in the case of examples nr 3, 5 and 8, where four/five laboratories among twelve counted significantly different number of colonies.

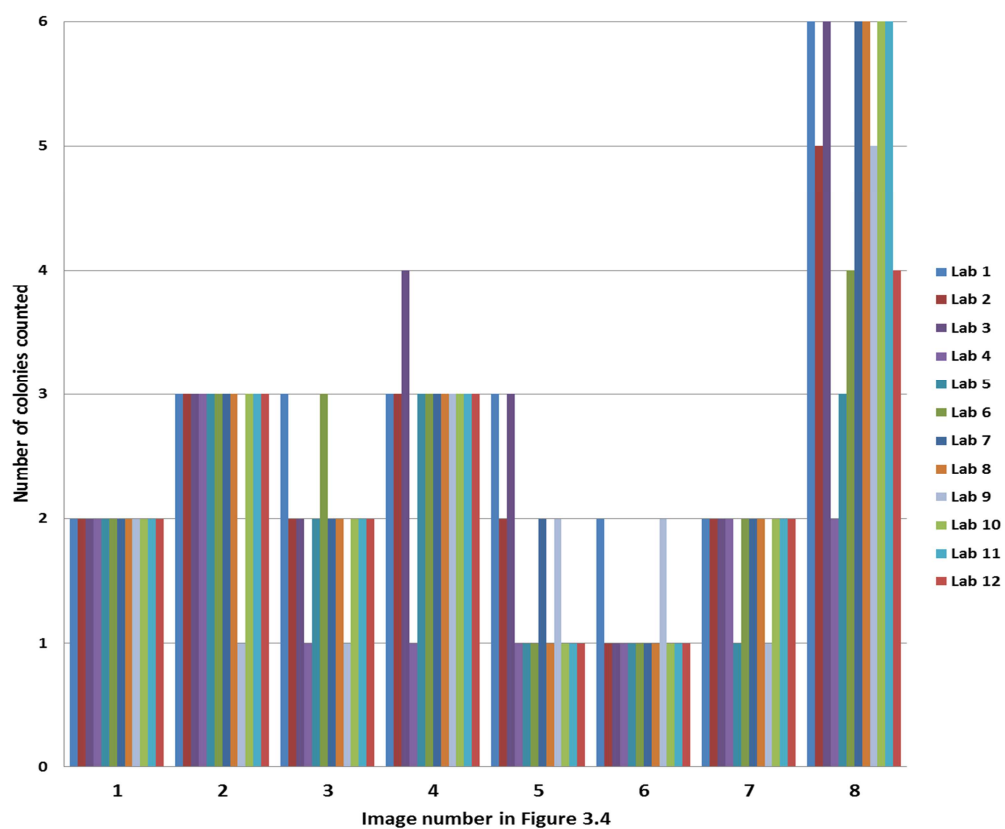


Figure 3.5. Differences in colony counting between various laboratories. Laboratories were asked to count and report the numbers of colonies presented in Figure 3.4 (without dashed lines).

4. Nanomaterials synthesis, sourcing and physicochemical characterisation

The materials used in this study were obtained by in-house synthesis (NPs code A, B, D, G, H), from the JRC repository of representative NMs (NPs code C, E, I) or supplied by AIST (NPs code F). In the case of the in-house synthesised materials the following sections detail the synthesis method and principle physicochemical characteristics of the materials as determined shortly after synthesis. In the case of the materials which were sourced from the JRC repository the synthesis routes are not detailed but physicochemical properties are reported.

For the materials A-B-C-D-E-G-H it was also possible to verify the long term colloidal stability by conducting Centrifugal Liquid Sedimentation (CLS) and/or spectroscopic analysis on samples which have been held for 6 months under ideal conditions (4°C under inert atmosphere and away from prolonged light exposure).

The swCNT (code F) were supplied by AIST. ZnO NPs (code I) were initially in dry powder but the characterisation refers to the materials after re-dispersion into biocompatible solvents.

Stock suspensions of each NP were characterised using different techniques such as Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), CLS. The main particle size and surface charge characteristics are summarised in Table 4.4 at the end of this chapter.

4.1 *Description of characterisation methods*

4.1.1 **Transmission Electron Microscopy**

TEM analysis of the liquid dispersed particles was done following liquid spotting on copper support grids. A drop of undiluted product (4 μ L) was placed onto ultrathin Formvar-coated 200-mesh copper grids (Tedpella Inc.) and left to dry in air at 4°C. For each sample, the sizes of at least 100 particles were measured to obtain the average and the size distribution. NPs were visualized using TEM (JEOL 2100, Japan) at an accelerating voltage of 200 kV. Digital images were analysed with the ImageJ software and a custom macro performing smoothing (3x3 or 5x5 median filter), manual global threshold and automatic particle analysis provided by the ImageJ.

4.1.2 **DLS and Zeta-potential measurement of NPs**

Particle size distribution was determined by DLS using a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd, UK) with temperature control (25°C). Each sample was recorded in duplicate with an equilibration step of 120 sec. Acquisition time was 80 sec. Software was set to automatic acquisition mode. Hydrodynamic diameters were calculated using the internal

software analysis from the DLS intensity-weighted particle size distribution. Z-potential was measured using the same instrument and recorded in a DTS1060C disposable cell with an equilibration time of 120 sec. Measurements were done just after pH measurement using a Smulochowski model with a $F(Ka)$ of 1.5.

4.1.3 CLS measurement of NPs

In order to assess the particle size distributions of the nanoparticles CLS measurements were performed on the as synthesised nanoparticle dispersions. Measurements were made using a CLS instrument (model DC24000UHR CPS Instruments) in an 8 wt% - 24 wt% sucrose density gradient with a disc speed of 22.000 rpm. Each sample injection of 100 μ L was preceded by a calibration step performed using certified poly(vinyl chloride) (PVC) particle size standards with mean size of 380 nm.

4.2 Gold NPs by in-house synthesis (NPs code A and B) and sourced from JRC repository (NPs code C)

The **synthesis of Au NPs code A** sodium citrate stabilised (2.5 mM of sodium citrate at pH 6.7) was carried out by reduction of gold (III) chloride trihydrate salt with the strong reducing agent sodium borohydride (NaBH_4). Briefly, 95 mL of MilliQ water were stirred in an ice bath for 2 h. Then 5 mL of aqueous solution of gold (III) chloride trihydrate (10 mM) and 2.5 mL of trisodium citrate dihydrate (100 mM) were added to the water solution during the stirring. Afterwards, 1 mL of aqueous sodium borohydride (100 mM) was added to the solution under vigorous stirring. The reduction of gold salt to gold nanoparticles by the NaBH_4 produced a change in colour of the solution from pale yellow to dark red. The suspension was then stirred in the ice bath for further 10 min and then left to warm to room temperature. The nominal concentration calculated by the stoichiometry was 0.5 mM.

For the biological testing, NPs code A were concentrated by centrifugal ultrafiltration using AMICON ULTRA-15 10KDa filter tubes (Millipore, Milan, Italy). 10 mL NPs code A suspension was added on each filter and tubes were centrifuged for 20 min at 1500 rcf at room temperature during which time the sample volume was reduced from 10 mL to 1.2 mL. The real final concentration measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent Technologies Inc., 7700 series, USA) was 4.25 mM. Stock suspension was stored at 4°C in dark remaining stable in terms of size distribution for at least 6 months.

The **synthesis of NPs code B** was carried out by a two-step seed growth method in which the reduction of gold (III) chloride hydrate salt in the presence of trisodium citrate dihydrate was used to selectively enlarge highly mono-dispersed 12 nm size gold seed particles. In the first step of the process, the 12 nm seed particles were obtained by a method adapted from that described by (Turkevich et al., 1951). Briefly, the solution was heated up using a microwave apparatus (Discover S by CEM corporation) to ensure a highly reproducible rapid heating. In

this method, 5 mL of 10 mM tetrachloroauric acid trihydrate ($\text{HAuCl}_4 \times 3\text{H}_2\text{O}$) was dissolved in 95 mL of water. The solution was rapidly heated up and held at 97°C for 5 min using a microwave power of 250 W under vigorous mechanical stirring; 2.5 mL of 100 mM trisodium citrate dihydrate was added to the solution and kept at 97°C for another 20 min. Afterwards, the solution was rapidly cooled down to 40°C and then to room temperature. The gold nanoparticles were produced by the reduction of the gold salt by sodium citrate that acted as both reducing agent and stabilizer. The final solution contained 12 nm size gold nanoparticles with 0.5 mM of gold NPs stabilized with 2.5 mM of sodium citrate at pH 6.7.

In the second stage of the synthesis, 95 mL of MilliQ water were left to stir at 60°C for 2h. 2.8 mL of sodium citrate dihydrate (100 mM) and 0.42 mL of 200 mM sodium hydroxide were added to the citrate solution and left to equilibrate for 30 min. Then, 2.24 mL of tetrachloroauric acid trihydrate 10 mM ($\text{HAuCl}_4 \times 3\text{H}_2\text{O}$) and 0.6 mL of 12 nm gold nanoparticles with a gold NPs concentration of 0.5 mM were added to the solution, under vigorous stirring. The solution was left to react for 48 h at 60°C. The final solution contained NPs code B with 0.25 mM of gold NPs stabilised with 2.8 mM of sodium citrate at pH 6.7. The nominal concentration calculated was 0.25 mM.

For the biological testing, NPs code B were concentrated by centrifugal ultrafiltration using the AMICON 10KDa filter tubes (Millipore, Milan, Italy); 10 mL NPs code B suspension was added on each filter and tubes were centrifuged for 20 min at 1500 rcf at room temperature. The final concentration was measured by ICP-MS and found to be 4 mM. This stock suspension, when stored at 4°C away from light, will normally maintain a stable particle size distribution for at least 6 months.

The NPs code C was a representative nanomaterial (NM330) stored in the JRC-Repository: The as-supplied material was initially at the concentration of approximately 0.25 mM, as measured by UV-Vis. For the biological testing, NPs code C were also concentrated using the AMICON 10KDa filter tubes (Millipore, Milan, Italy). 10 mL NPs code C suspension were added on each filter and tubes were centrifuged for 20 min at 1500 rcf at room temperature. The real final concentration measured by ICP-MS was 1 mM. The stock suspension was stored at 4°C in dark, remaining stable in terms of size distribution for at least 6 months.

4.2.1 CLS and UV-Vis characterisation of Au NPs

The particle size distributions of the Au NPs were determined in the stock suspension as-synthesised, after concentration and after long term storage. For CLS and UV-Visible (UV-vis) analysis, the samples were diluted 1:10 in solvent controls. The results of the CLS analyses are shown in

Figure 4.1, Figure 4.2 and Figure 4.3. The UV-vis spectroscopic analysis results are presented in Figure 4.4, Figure 4.5 and Figure 4.6, while the numerical data are reported in Table 4.1.

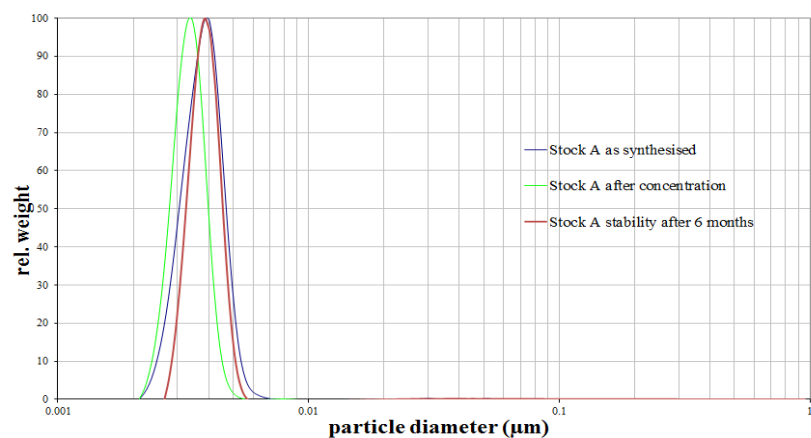


Figure 4.1. CLS analysis of Au NPs code A stock suspension

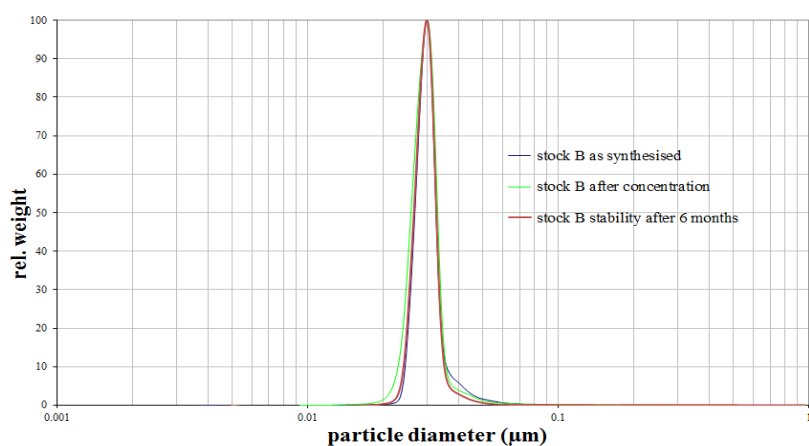


Figure 4.2. CLS analysis of Au NPs code B stock suspension

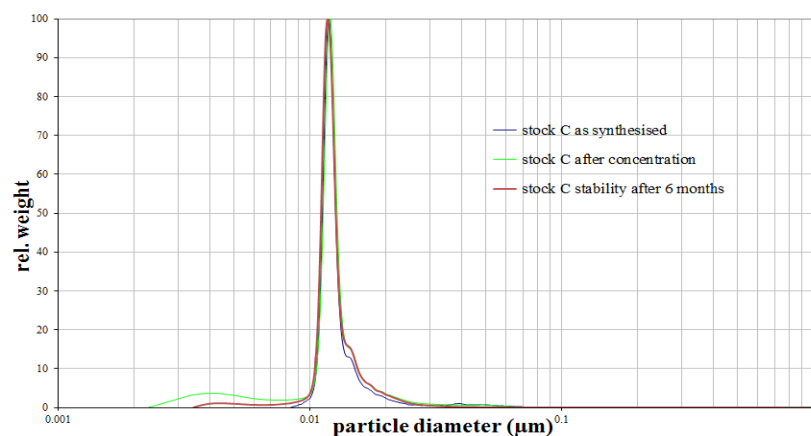


Figure 4.3. CLS analysis of Au NPs code C stock suspension

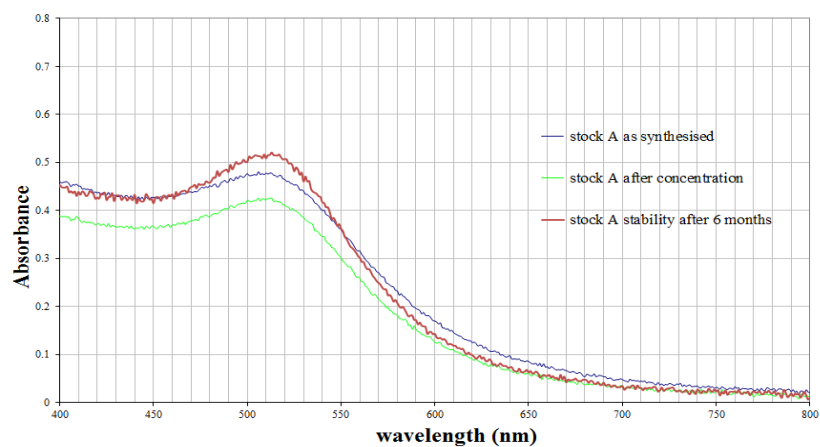


Figure 4.4. UV-*vis* spectroscopic analysis of Au NPs code A stock suspension

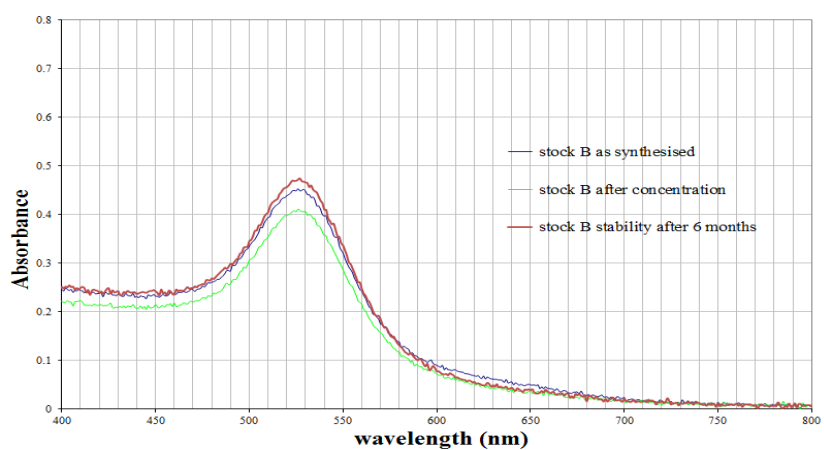


Figure 4.5. UV-*vis* spectroscopic analysis of Au NPs code B stock suspension

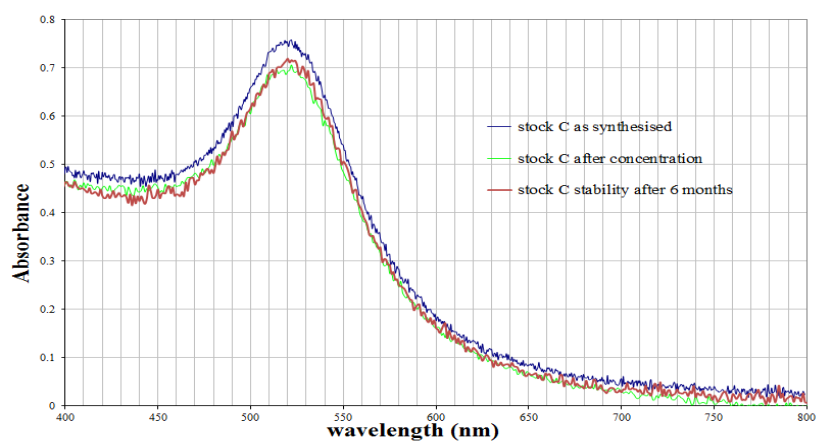


Figure 4.6. UV-*vis* spectroscopic analysis of Au NPs code C stock suspension

Table 4.1. Size distributions of NPs code A, B and C measured by CLS. The stock suspensions were diluted 1:10 in solvent controls. Measurements were done in the stocks before concentration, immediately after concentration and 6 months after concentration.

Size distribution by CLS (nm/HHW ¹ /Pdl ²)			
NPs code	Stock suspension as synthesised	Stock suspension after concentration process	Stock suspension after concentration and 6 months of storage
A	3.9/1.7/1.2	3.3/0.9/1.8	3.1/0.3/1.1
B	29.9/5.9/1.1	30.3/5.8/1.1	29.9/7.3/1.1
C	11.9/1.4/1.2	12/1.5/1.4	10.5/1.5/1.69

¹Half Height Width (HHW)

²CLS Polydispersity Index expressed as the ratio Dw/Dn where the Dw is the average particle size calculated from the particle weight-size distribution while Dn is the average particle size calculated from the equivalent number-size distribution.

The result of both CLS and UV-Vis spectroscopy show that all the gold NPs have good long term colloidal stability with no notable changes in either size or concentration occurring during a period of up to 6 months.

4.3 Silver NPs by in-house synthesis (NP code D) and sourced from JRC repository (NP code E)

The synthesis of NPs code D was carried out by reduction of silver nitrate salt with sodium citrate and tannic acid. Briefly, 6 mL of sodium citrate (28 mM) and 120 µL of tannic acid (2.24 mM) were stirred at 60°C for 15 min; then 5 mL of this solution were added to 95 mL of (0.57 mM) silver nitrate under boiling condition and vigorous stirring and kept at 97°C for further 40 min. The solution was heated up using a microwave synthesis reactor (Discover S by CEM corporation) to ensure a highly reproducible and rapid heating. On completion of the reaction the solution was cooled rapidly with compressed air down to 40°C and then by natural convection to room temperature. The final suspension contained NPs code D stabilized with 14 mM of sodium citrate and 2.7 µM of tannic acid at pH 6.8.

The nominal concentration calculated by the stoichiometry was 0.5 mM and corresponded to the real one measured by ICP-MS (Agilent Technologies Inc., 7700 series, USA). Stock suspension was stored at 4°C in dark remaining stable in terms of size distribution for at least 6 months.

In order to obtain the solvent control for biological testing, the NPs code D were filtered using the AMICON ULTRA-15 filter tubes by centrifugation for 15-10 min. at 1500 rcf (Millipore,

Milan, Italy). After centrifugation the NPs were disposed and the filtrate used as solvent control.

The **Ag NPs code E** were sourced from the JRC NMs repository (NM300) where they are stored in the form of a highly concentrated liquid dispersion containing 4% w/w of Polyoxyethylene Glycerol Trioleate and 4% w/w of Polyoxyethylene (20) Sorbitan Mono-Laurate (Tween 20). ICP-MS elemental analysis of the as-supplied silver dispersion revealed that the total silver concentration is 940 mM. Vial containing the same aqueous mixture of NM300 dispersants (NM300DIS) but without the presence of any silver was used as solvent control.

4.3.1 CLS and UV-Vis analysis of silver NPs code D and E

The particle size distributions of the Ag nanoparticles were determined in the stock suspensions as-synthesised, and after long term storage. The results of the CLS analyses are shown in Figure 4.7 and Figure 4.8. The UV-vis spectroscopic analysis results are presented in Figure 4.9 and Figure 4.10.

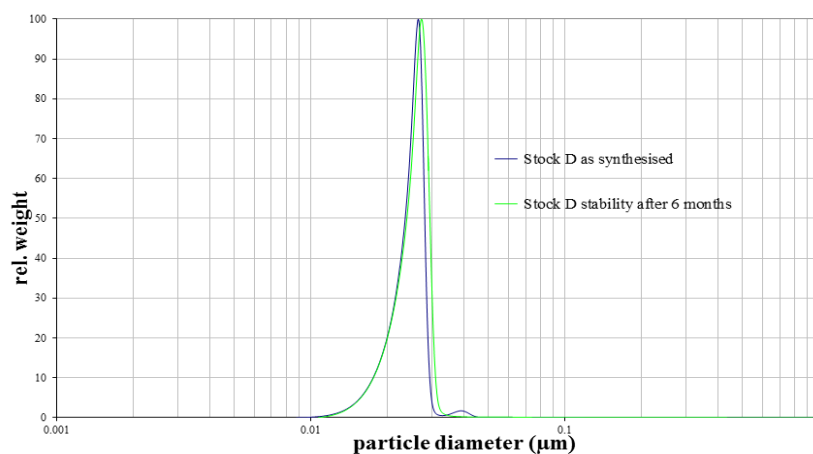


Figure 4.7. CLS analysis of Ag NPs code D stock suspension

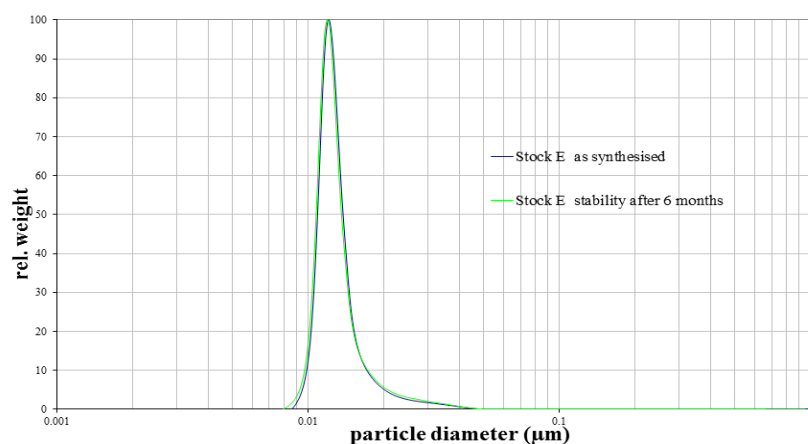


Figure 4.8. CLS analysis of Ag NPs code E stock suspension

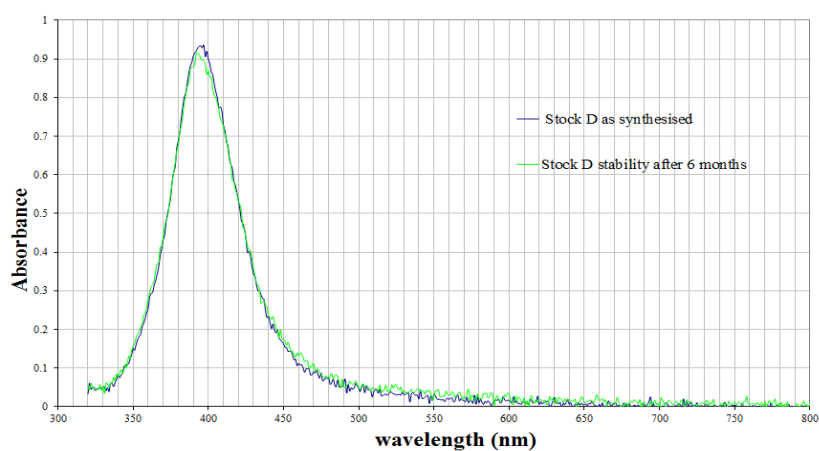


Figure 4.9. UV-Vis spectroscopic analysis of Ag NPs code D stock suspension

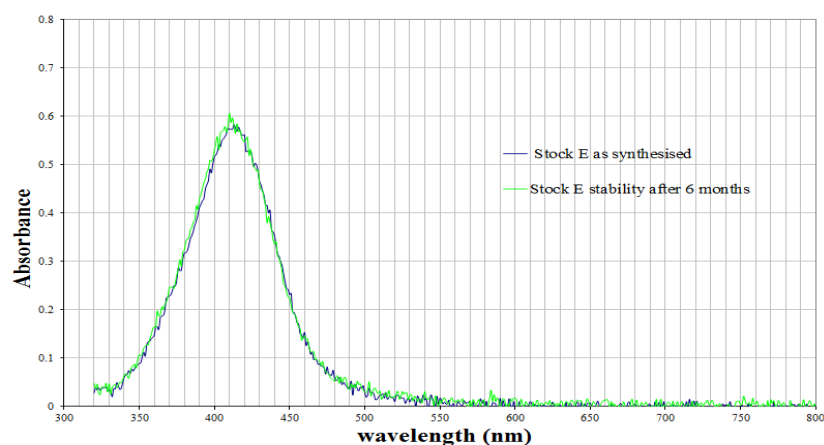


Figure 4.10. UV-Vis spectroscopic analysis of Ag NPs code E stock suspension

The results of both CLS and UV-Visible spectroscopy show that the particles have good long term colloidal stability with no notable changes in either size or concentration occurring during a period of up to 6 months.

Table 4.2. Size distributions of NPs code D and E measured by CLS. The stock suspensions were diluted 1:10 in solvent controls. Measurements were done in the stocks and 6 months after synthesis.

Size distribution by CLS (nm/HHW ¹ /Pdl ²)		
NPs code	Stock suspension as synthesised	Stock suspension after 6 months of storage
D	26.9/5.8/1.2	27.4/5.9/1.19
E	12.1/3/1.2	11.9/2.8/1.45

¹ Half Height Width (HHW)

²CLS Polydispersivity Index expressed as the ratio Dw/Dn where the Dw is the average particle size calculated from the particle weight-size distribution while Dn is the average particle size calculated from the equivalent number-size distribution.

4.4 *swCNTs (code F)*

NPs code F were obtained from the Technology Research Association for swCNTs (Japan) and supplied for this project by AIST (Japan). The synthesis and physicochemical characterisation was performed at AIST and is described in detail in Fujita et al.. (2013).

Impurity-free NPs code F were synthesised by water-assisted chemical vapour deposition (CVD). A highly efficient synthesis of NPs code F with high purity was enabled by adding a small and controlled level of water to the growth ambient (Hata et al., 2004). This high efficiency resulted in a massive growth of vertically aligned swCNT (forest) from the catalyst surface. swCNT forests possess high-purity carbon, alignment and near-ideal specific surface area, and they can be patterned into arbitrary structures for applications ranging from super-capacitors to stretchable electronics.

NPs code F stock suspension was prepared in a 10 mg/mL BSA solution using an ultrasonic homogenizer for 30 min. After ultrasonic treatment, the dispersion was centrifuged at 3000 x g at 15°C for 15 min. The supernatant was filtered using a cell strainer with a 70 mm nylon mesh (Becton Dickinson & Company) and centrifuged at 22.000 x g at 10°C for 10 min. The precipitates were re-dispersed in a 10 mg/mL BSA solution using an ultrasonic bath operating for 5 min (Branson Ultrasonics Corp.). The mixtures were filtered using a cell strainer with a 100 mm nylon mesh (Becton Dickinson & Company).

4.5 *In-house synthesised silica NPs code G and H*

The **NPs code G** were synthesised using a method adapted from (Hartlen et al., 2008). Briefly, cyclohexane (16.25 mL) was mixed with a solution of L-arginine (330 mg, 1.9 mmol) in milliQ water (250 mL). The mixture was heated to 50°C at a constant stirring rate of approximately 300 rpm before tetraethyl orthosilicate (TEOS, 20 mL) was slowly added and the reaction was kept under these conditions for 24 h.

The concentration of NPs code G in the stock suspension measured by gravimetric technique and provided to the partners was 3.2 mg/mL. The solvent control of NPs code G was obtained by ultrafiltration of nanoparticles suspension. The stock suspension was stored at 4°C in dark remaining stable in terms of size distribution for at least 6 months.

Synthesis of **NPs code H** was done in two seeding-growth steps. In the first step, SiO₂ NPs of 30 nm were obtained by mixing 50 mL of as-synthesised SiO₂ NPs of 20 nm with milliQ water (180 mL) and cyclohexane (25 mL), so that the total concentration of L-arginine was 1.5 mM. The mixture was heated to 50°C at a constant stirring rate of approximately 300 rpm before TEOS (18.5 mL) was slowly added and the reaction was kept under these conditions for 24 hours. The same reaction conditions were used for the synthesis of SiO₂ NPs of 75 nm by mixing 20 mL of as-synthesised SiO₂ NPs of 30 nm with cyclohexane (10 mL) and a solution of L-arginine (12 mg, 0.07 mmol) in MilliQ water (72 mL), so that the total concentration of L-arginine was 1 mM. The mixture was heated to 50°C at a constant stirring rate of approximately 300 rpm before TEOS (7.4 mL) was slowly added and the reaction was kept under these conditions for 24 h.

The concentration of nanoparticles code H in the stock suspension measured by gravimetric technique and provided to the partners was 3.5 mg/mL. The solvent control of NPs code H was obtained by ultrafiltration of nanoparticles suspension. The stock suspension was stored at 4°C in dark remaining stable in terms of size distribution for at least 6 months.

4.5.1 CLS analysis of silica NPs

The particle size distributions of the SiO₂ nanoparticles were determined in the stock suspensions as-synthesised and after long term storage. The results of the CLS analyses are shown in Figure 4.11 and Figure 4.12 and the size distributions are presented in Table 4.3.

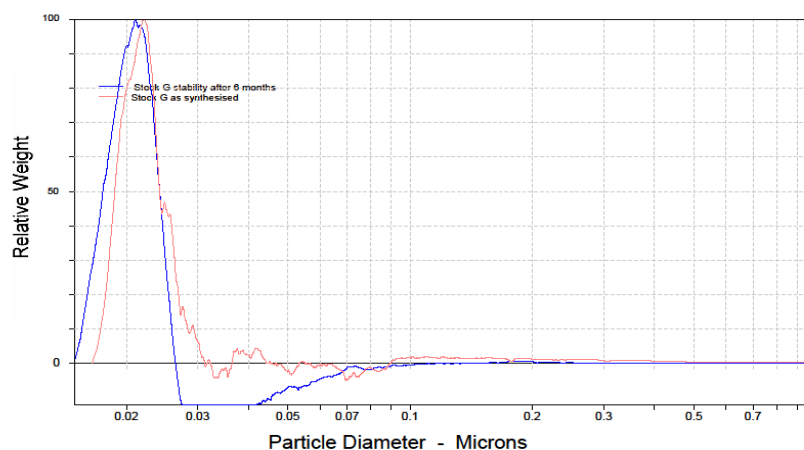


Figure 4.11. CLS analysis of SiO₂ NPs code G stock suspension as synthesised (red) and after 6 months (Blue)

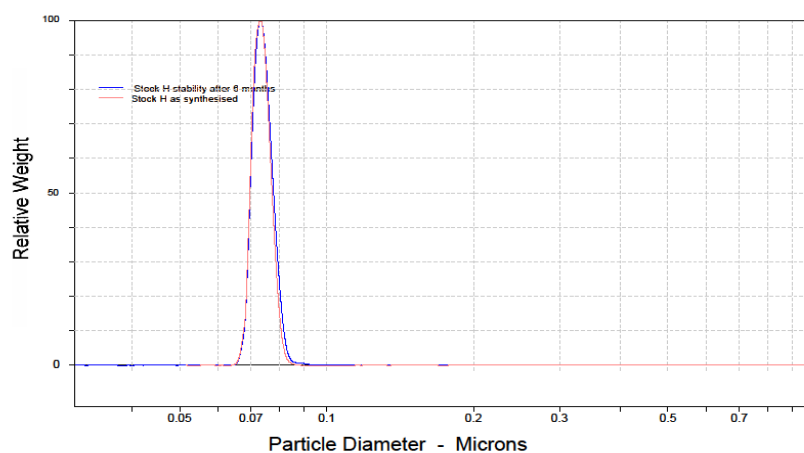


Figure 4.12. CLS analysis of SiO₂ NPs code H stock suspension as synthesised (red) and after 6 months (Blue)

Table 4.3. Size distributions of NPs code G and H measured by CLS. The stock suspensions were diluted 1:10 in solvent controls. Measurements were done in the stocks immediately after synthesis and 6 months after synthesis.

Size distribution by CLS (nm/HHW ¹ /Pdl ²)		
NPs code	Stock suspension as synthesised	Stock suspension after 6 months of storage
G	21/6.8/1.30	22.5/8.8/1.27
H	71.4/6.5/1.30	73.2/8.2/1.02

¹ Half Height Width (HHW)

²CLS Polydispersivity Index expressed as the ratio Dw/Dn where the Dw is the average particle size calculated from the particle weight-size distribution while Dn is the average particle size calculated from the equivalent number-size distribution.

4.6 Repository sourced Zinc Oxide NPs (code I)

NPs code I were supplied in powder form by the JRC NMs repository (NM110). The stock suspension at the concentration of 2.56 mg/mL of NPs code I was prepared at the JRC by weighting 2.56 mg of powder on an analytical balance and then suspending the powder in 1 mL of sterile MilliQ water. The suspension (1.5 ml volume) was sonicated for 15 min. using a Vial Tweeter sonicator UIS250v (250 watt, 24kHz; Amplitude 75, Cycle 0.5; Hielscher, Ultrasound Technology, Germany).

NPs code I stock suspension is extremely unstable and it was recommended that the 3 runs of the CFE assay should be conducted within 15 days of receiving the materials. Before the cell exposure, the NPs code I stock suspension had to be treated with vortex for 3 min. using the vortex mixer at the highest speed available and with ultra-sonication for 7 min. using the ultrasonic bath at the highest power available. After these treatments the suspension should appear homogeneous.

4.6.1 CLS analysis of Zinc Oxide NP Code I

The particle size distributions of the ZnO nanoparticles were determined in the stock suspensions after dispersion. The results of the CLS analyses are shown in Figure 4.13.

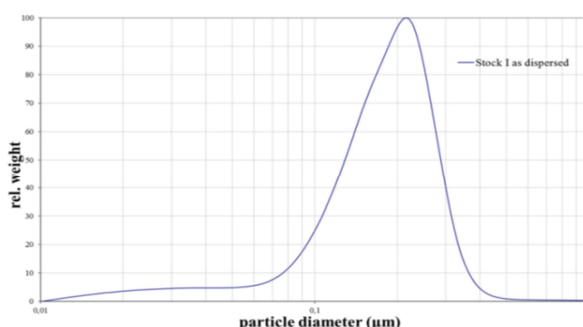


Figure 4.13. CLS analysis of ZnO NPs code I stock suspension

4.7 Summary of morphology and sizing of all NPs stock suspensions

TEM analysis was conducted on samples of all the NPs used in this study to determine the shape of the particles and to verify whether the results of particle size as derived from DLS and CLS measurements were representative of the single particles or aggregates. In all cases the TEM sample grids were prepared by grid-on-drop or drop-on-grid starting from aqueous dispersions of the particles. The DLS and CLS measurements were conducted as described above on stock dispersions of the NPs and the results obtained are summarised in Table 4.4.

Table 4.4. Characterisation of size distribution (by TEM, DLS and CLS) and Z-potential of the stock suspensions NPs code A – I. Samples for analysis were diluted 1:10 in their respective solvent controls.

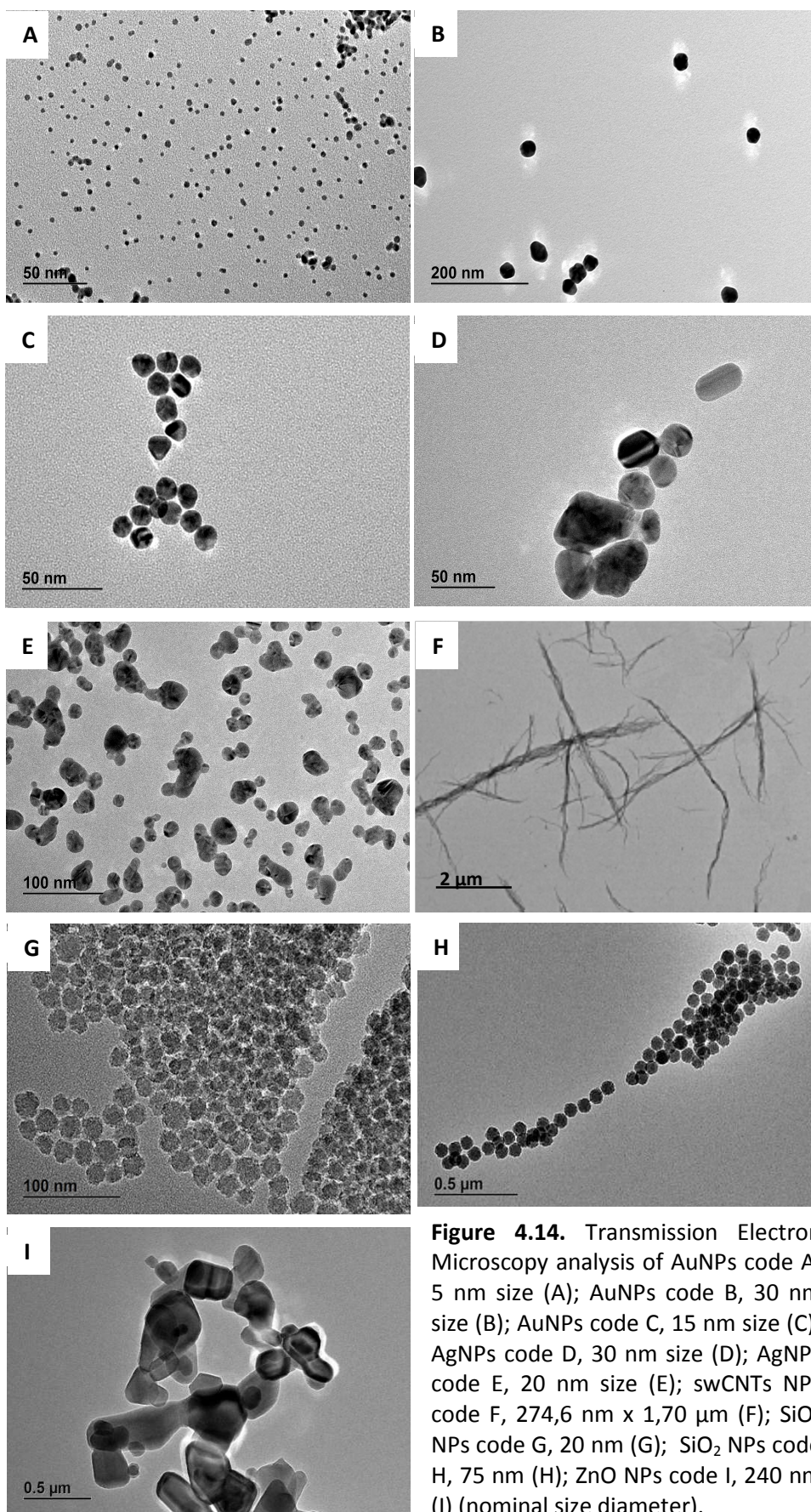
NPs code	Nominal size (nm)	Size distribution			Z-Potential (mV) \pm SD
		TEM (nm \pm SD)	DLS (nm /Pdl ¹)	CLS (nm/HHW ² /Pdl ³)	
A	5	3.6 \pm 0.8	16.6/0.5	3.3/0.9/1.8	-48 \pm 1
B	30	32 \pm 3	34.8/0.2	30.3/5.8/1.1	-43 \pm 1
C	15	13 \pm 1	28.8/0.535	12/1.5/1.4	-43 \pm 1
D	30	28 \pm 4	14.5/0.548	26.9/5.8/1.2	-33 \pm 1
E	20	14 \pm 6	27.1/0.49	12.1/3/1.2	-10 \pm 1
F	-	274.6 x 1.70 μ m	763.6/nd	nd	-13.4 \pm 0.3
G	20	19.6 \pm 7.1	26.8 / 0.012	21/6.8/1.30	- 33.0 \pm 1.5
H	75	76.8 \pm 3.8	116.8 / 0.156	71.4/6.5/1.30	- 28.0 \pm 2.1
I	240	287.04 \pm 169.75	230.2/0.168	187/144/9.9	32 \pm nd

¹DLS Polydispersivity Index

²Half Height Width (HHW)

³CLS Polydispersivity Index expressed as the ratio Dw/Dn where the Dw is the average particle size calculated from the particle weight-size distribution while Dn is the average particle size calculated from the equivalent number-size distribution.

nd: not determined.



The TEM images show that all the particles except the swCNT and ZnO can be considered approximately spherical and as such are generally suitable for use with the DLS and CLS analysis methods. When the swCNT and ZnO results are excluded, the TEM and CLS size measurements all show a good general correspondence confirming that these materials are present in the stock solutions primarily as single particles. A comparison of these results with the DLS derived size measurements shows that DLS derived sizes are generally higher than expected from TEM and CLS, but this is common in DLS measurements where results are strongly biased in the presence of a small fraction of large particles or aggregates and also because DLS measures a hydrodynamic diameter that is expected to be larger than what is seen in TEM pictures. This effect derives from the fact that the intensity of the scattered light is inversely proportional to the sixth power of the radius of the nanoparticle. As a result, a small fraction of large particles may completely mask a large fraction of smaller particles. DLS does not distinguish between constituent particles and aggregates/agglomerates. It simply gives information about all diffusing ensembles, regardless of whether they are individual particles, agglomerates or aggregates. Consequently, DLS analysis of a polydispersed mixture will produce results which tend to reflect the size of the largest particles present in a solution even when these numerically constitute only a minor fraction of the total population.

4.8 Characterisation of NPs particle size distributions in culture medium

In the previous sections the nanoparticle size distribution of the stock materials have been described as they are present in the simple dispersion or original synthesis media and without the presence of the proteins and salts which are vital components of the full cell culture media used in the validation test protocol. Under these conditions the particles are expected to either be fully dispersed (mainly single particles) as in the case of the in-house materials or, in the case of suspensions derived from dried powders, be a mixture of single particles and mechanically stable aggregates. The majority of particles in this study are likely to maintain their colloidal stability via electrostatic stabilisation which generally becomes less effective in the presence of dissolved ionic salts. At the salt concentration levels in cell culture media there is a high probability that a rapid and irreversible aggregation of normally stable nanoparticles will occur. The presence of proteins may mitigate or exasperate the effect of salt depending on the nature of the particles and the proteins involved. Consequently, it is not generally possible to reliably predict how any single type of particle will behave in any particular type of cell culture media and so it is generally advisable to determine this behaviour experimentally. In this study the colloidal stability of the test particles when exposed to the cell culture media with 10% v/v of serum has been qualitatively evaluated by using CLS. A comparison was made between the particles in the as-supplied state, immediately after dilution in water or serum-media mixture and then finally after being held

for 72 h under standard cell culture conditions (5% CO₂, 95% humidity and 37°C). These results are reported numerically in Table 4.5.

Table 4.5. NPs diluted in water or in culture medium with 10% v/v of serum at time 0 and after 72 h of incubation. Mean size distribution was measured by CLS.

NPs code	NPs conc. (μM)	Mean size in water (nm/HHW ¹ /Pdl ²)	Mean size in culture medium with 10% serum (nm/HHW ¹ /Pdl ²)	
		Incubation time		
		0 h	0 h	72 h
A	200	-	5.1/2.5/2.0	7.9/5.5/1.7
	400	3.3/0.9/1.8	4.3/2.6/2.1	9.3/6.1/1.6
B	100	-	20.4/5.9/1.2	21.0/5.6/1.2
	200	-	20.4/6.3/1.2	21.4/5.9/1.1
	400	30.3/5.8/1.1	20.7/8.3/1.2	22.0/6.6/1.2
C	100	12/1.5/1.4	9.7/nd/2.3	10.2/nd/2.4
E	10	26.9/5.8/1.2	19.7/9.2/1.5	20.4/6.4/1.4
F	100	12/1.5/1.4	12.2/0.3/1.2	10.8/0.4/2.3
G	300	21/6.8/1.30	23/44.2/1.9	136.4/103.6/2.24
H	1000	71.4/6.5/1.30	64.2/7.9/2.75	160/105/1.52
I	1280	186.8/144.0/9.9	166.7/124.6/3.2	207.7/195.3/3.4

¹Half Height Width (HHW)

²DLS Polydispersity Index

nd: not determined

The results of the CLS analysis of the eight particle types show significant evidence of aggregation/agglomeration only in the case of the ZnO and SiO₂ particles with the other materials retaining a large portion of particulates in the free un-agglomerated state. With the exception of the smallest gold nanoparticles a comparison between the CLS size distribution in water and in medium with 10% serum show a shift towards an apparently lower size. This effect is not necessarily due to any true change in particle size but is an instrumental artefact derived from the interactions between the serum proteins and the metallic nanoparticles. When citrate stabilized gold and silver nanoparticles are mixed with serum containing culture medium, the serum proteins rapidly form a protein corona surrounding the particle which stabilised them against aggregation but, at the same time, changes (reduces) the mean density of the NPs system. Since the calculation of particle size by the CLS method depends on knowing the density of the particle, any change in the actual mean density with respect to the theoretical values used by the instrument software will produce a result which will deviate proportionately from the expected physical value. In the case of gold particles with a size of around 30 nm the formation of the protein corona will produce a slight proportional increase in the Stoke's diameter of the particles but at the same time will decrease the mean density.

The overall effect on the CLS derived size is to produce a measured value which is apparent slightly lower than would normally be expected from the measurements of protein free particles. For the small gold particles the absorption of serum proteins, although reducing mean particles density, produce an increase in Stoke's diameter which is proportionally much more significant than in the case of the 30 nm particles. In this case the net effect of the greater diameter and lower density makes the particles appear larger in serum compared to particles in protein-free solution. Similar arguments are applicable to the silver nanoparticles.

The correct size of the NPs-protein complex can be determined by CLS only if the mean density of the complex is accurately known and although procedures exist to do this, they are experimentally very complex and beyond the requirements of this particular study. However, the change in hydrodynamic radius and the decreases in density have an opposite effect it is difficult to predict the CLS trend in serum for each particle's size.

In this study, CLS has been used for the characterisation of particles exposed to media principally to evaluate the extent of any particle aggregation rather than to give precise measurement of particle size in the cell culture media. In this respect the method has been able to show clearly that when mixed with serum containing media the Au and Ag particles remain largely monodispersed with only moderate aggregation/agglomeration while the ZnO and silica based materials both show evidence of some degree of aggregation during the 72 h period of incubation.

5. Approach used for the statistical analysis of data obtained from the CFE assay

5.1 Experimental design

In each experiment (run), a positive control (100 μ M sodium chromate, Na_2CrO_4), a negative (untreated) control, a solvent control and three to eight concentrations of the NP were tested using the CFE assay. For each run, six dishes per treatment condition were used. The colonies of the MDCK cells were counted manually at the end of the experiment. The raw data hence consist of the number of colonies counted in each dish for each treatment condition and for the controls. The raw data was captured in Microsoft®Excel™ templates, illustrated in Figure 5.1.

RAW DATA NP I		Number of colonies					
Treatment	Concentration	dish 1	dish 2	dish 3	dish 4	dish 5	dish 6
Positive Control	100 μ M	0	0	0	0	0	0
Control	0	122	132	120	134	123	154
Solvent Control	0.04%	152	139	132	118	148	142
I	1	145	134	144	149	138	129
I	5	137	133	143	155	141	135
I	10	129	124	135	138	146	143
I	12.5	146	113	131	138	130	145
I	15	72	75	75	82	96	101
I	20	55	28	17	77	41	10
I	25	0	0	0	0	0	0

Figure 5.1. Template showing an example of raw data: the number of colonies per treatment condition and controls.

The statistical analysis and curve fitting was executed using the software IDBS XLfit™. This software completely integrates with Microsoft® Excel™. The complete integration means that no script files with the raw data need to be inserted into IDBS XLfit™.

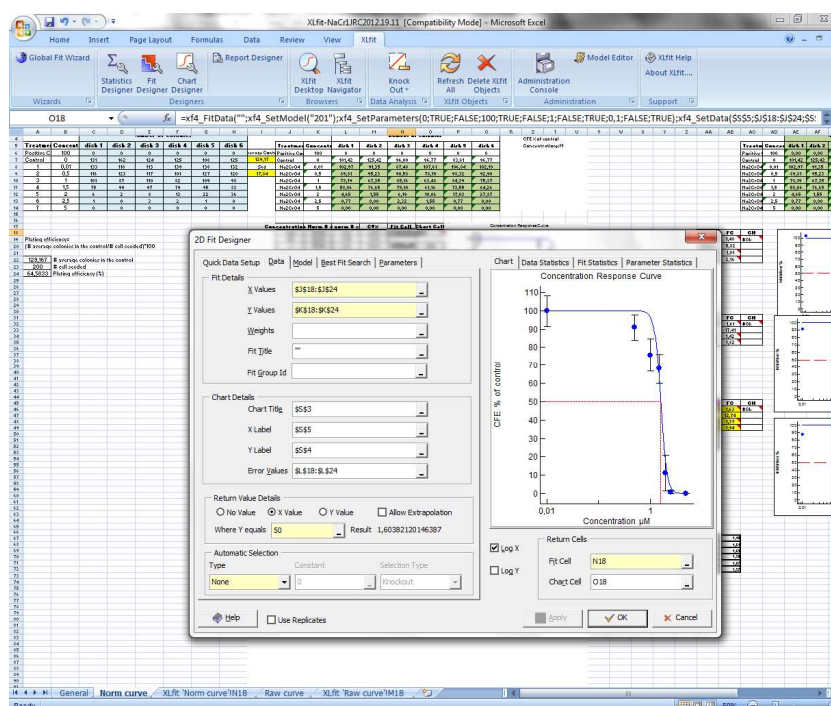


Figure 5.2. The statistical analysis and curve fitting are executed using the software IDBS XLfit™.

In the experimental phase, there are three test acceptance criteria. These criteria have to be fulfilled for a run to be accepted. The criteria are:

1. Colonies counted contain more than 50 cells.
2. The plating efficiency (PE) is higher than 45%. The PE is calculated as the average number of colonies in the negative control divided by the number of cells seeded per dish.
3. The positive control shows complete cell death (i.e. no colonies).

5.2 Control data

As introduced in the experimental design, there are three types of control data in one run:

1. The positive control (sodium chromate (Na_2CrO_4) tested at the concentration 100 μM), which shows complete cell death.
2. The negative (untreated) control.
3. The solvent control, which is NP specific.

The distribution of the average number of colonies in negative control for each laboratory is illustrated by boxplots in Figure 5.3. A red straight line (90 colonies) represents the acceptance criterion (2), $\text{PE } 45\% = 90/200$ cells seeded per dish. This criterion was not completely fulfilled in a couple of runs, as shown in the figure. The total average over all

laboratories is 129.5 colonies (illustrated as the black dotted line) with a standard deviation of 24.9. These figures are very similar to the solvent control data, with a total average of 130.3 colonies with a standard deviation of 25.5.

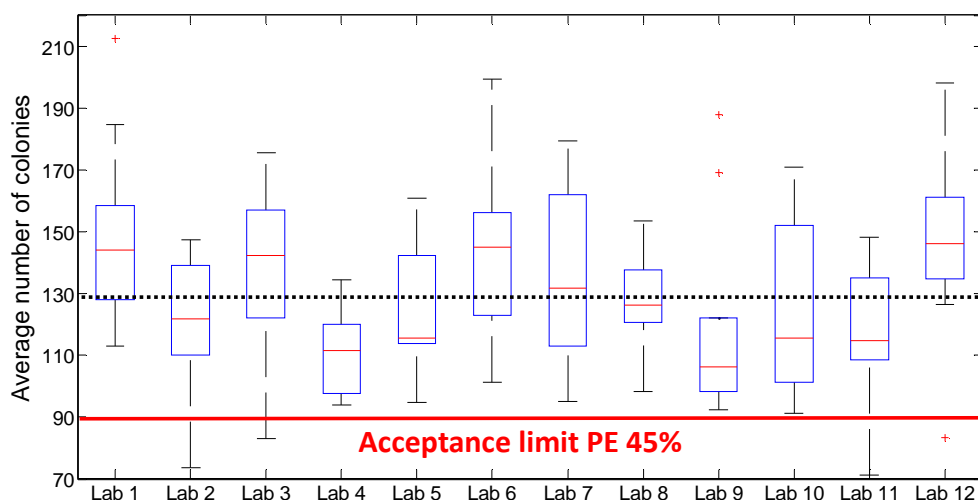


Figure 5.3. The distribution of the average number of colonies in negative control per laboratory.

The histograms in Figures 5.4 show the distributions of average number of colonies in negative and in solvent control data over all laboratories. Red straight lines (90 colonies) represent the acceptance criterion (2). Both distributions are comparable: symmetric and reasonably unimodal.

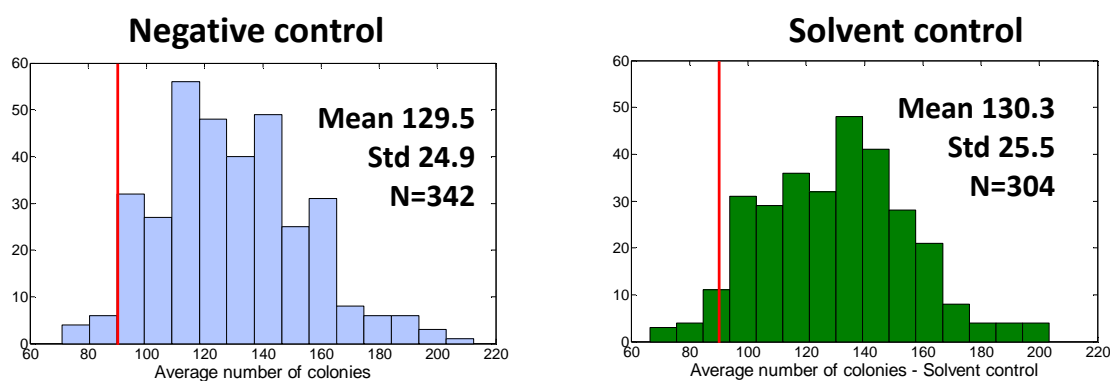


Figure 5.4. Distributions of the average number of colonies in controls.

By looking at the scatter plots in Figure 5.5, we may conclude that the different solvents used for the NPs (NPs A-I) do not have toxic effects. The linear relationships are strong between the negative and solvent control data, the Pearson product-moment correlation coefficients are very high between 0.84 and 0.93.

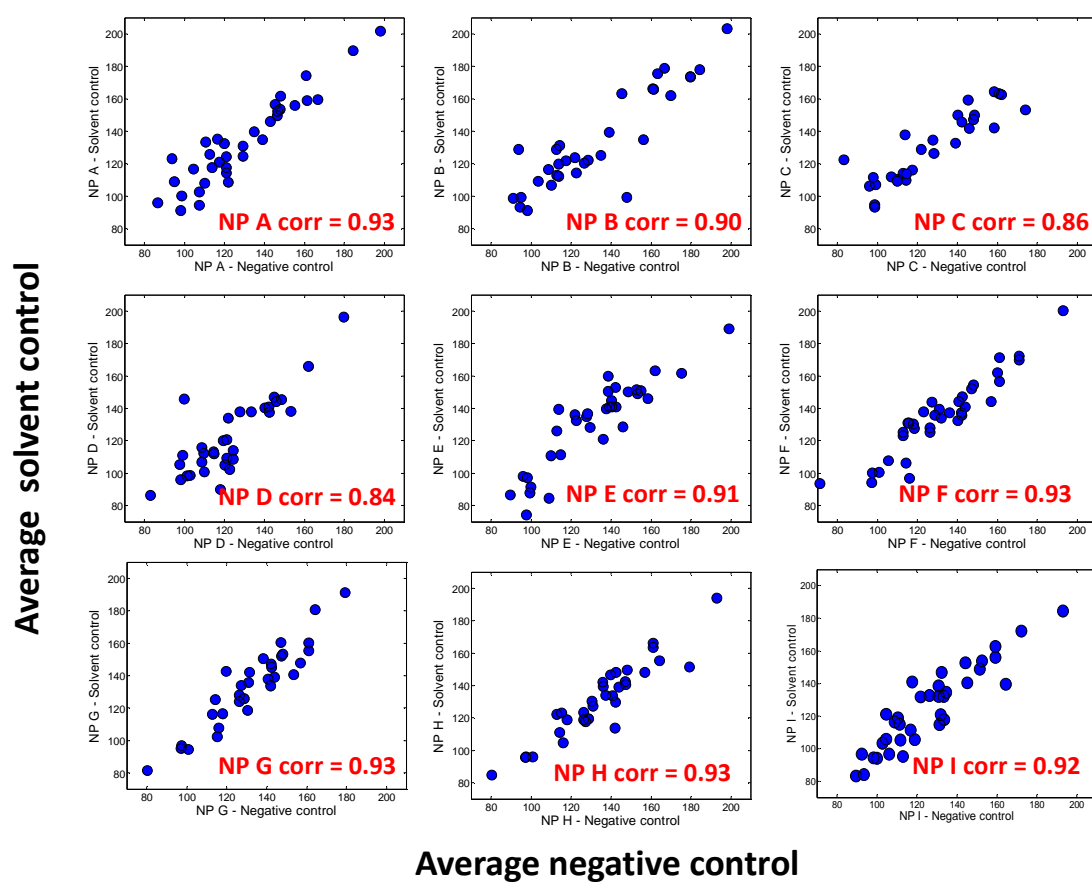


Figure 5.5. Scatter plots of average negative controls versus average solvent controls.

5.3 Run independency

For the comparison study, three independent runs are required. Independent runs are prepared on different days and/or with different passage numbers of the cell line. Thus, some laboratories were omitted from the intra and interlaboratory comparison study because the runs were not considered as independent. Some laboratories performed their runs during the same days with same cell passage number. In the following figures, laboratories which have provided less than three independent runs are marked with an asterisk ‘*’.

5.4 Concentration response curve

The chosen model for the concentration response curve is a four parameter logistic function (inhibition, y , versus concentration, x), also called the Hill function:

$$y = A + \frac{B - A}{1 + (x/C)^D}$$

where the parameters A (minimum of y), B (maximum of y), C (IC_{50}) and D (slope factor) are estimated using a Levenberg-Marquardt (L-M) algorithm, see Figure 5.6.

The L-M algorithm is an iterative numeric minimization algorithm for non-linear squares fitting with the measurements regarded as mutually independent of constant variance. The L-M algorithm interpolates between the Gauss–Newton algorithm and the method of gradient descent. We will fit concentration response models to both normalised and raw data (in the report only normalised data will be presented). When we are analysing normalised data, we are both normalising the raw data against an average negative control and an average solvent control value and our curves are constrained to go from 100 down to 0 percent. The main inhibitory concentration (IC_{xx}) parameter of interest is the IC_{50} (the concentration of an inhibitor where the response is reduced by half). The overall performance of the inhibitor is also interesting, so the IC_{10} (the concentration of an inhibitor where the response is reduced by 10%) and the IC_{90} for normalised data are also calculated. These parameters are illustrated also in Figure 5.6.

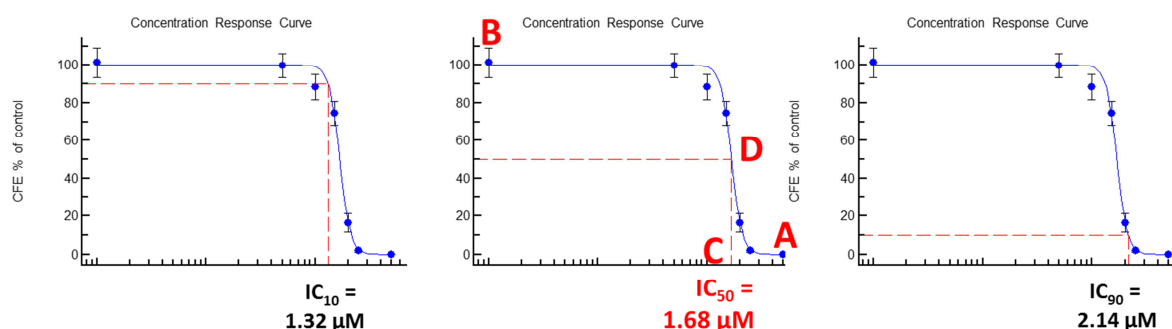


Figure 5.6. Three inhibitory concentration parameters (IC_{50} , IC_{10} , IC_{90}) are represented in the normalized concentration response curve.

The slope factor (Hill slope) quantifies the steepness in the concentration response curve. In the standard curve, the slope factor is 1. A steeper curve has a higher slope factor (and a more gradual curve has thus a lower slope factor). In this report, only the results of the IC_{50} are compared.

5.5 Comparison of results, intra and interlaboratory variation

To be able to evaluate the reproducibility and repeatability of the CFE assay, the intra (within laboratory) and interlaboratory (between laboratories) variations are assessed. There are two layers of variation within laboratories: between the six dishes within a run and between the three runs within one laboratory. The interlaboratory variation is between the 12 laboratories.

The different types of variation are illustrated in Figures 5.7 – 5.9:

4. Intralaboratory, variation within a run, Figure 5.7.

To analyse the variation within a run, a concentration response model to the normalized data for each dish is fitted. Six concentration response models are thus fitted, since there are six dishes in one run.

5. Intralaboratory, variation between runs, Figure 5.8.

For each run, concentration response curves for normalized (to negative and solvent control) data, averaged over the six dishes are generated. This averaged normalized curve is one out of the three curves used to calculate the intralaboratory variation between runs.

6. Interlaboratory, variation between laboratories, Figure 5.9.

The averaged result (of the three runs) from one laboratory is compared with the corresponding results from the participating laboratories in the study.

To assess the protocol transferability of the assay, the variations 1) - 3) are calculated for the positive control, sodium chromate (Na_2CrO_4). For the nine NPs, the variations 2) - 3) are assessed to evaluate the reproducibility and repeatability of the assay.

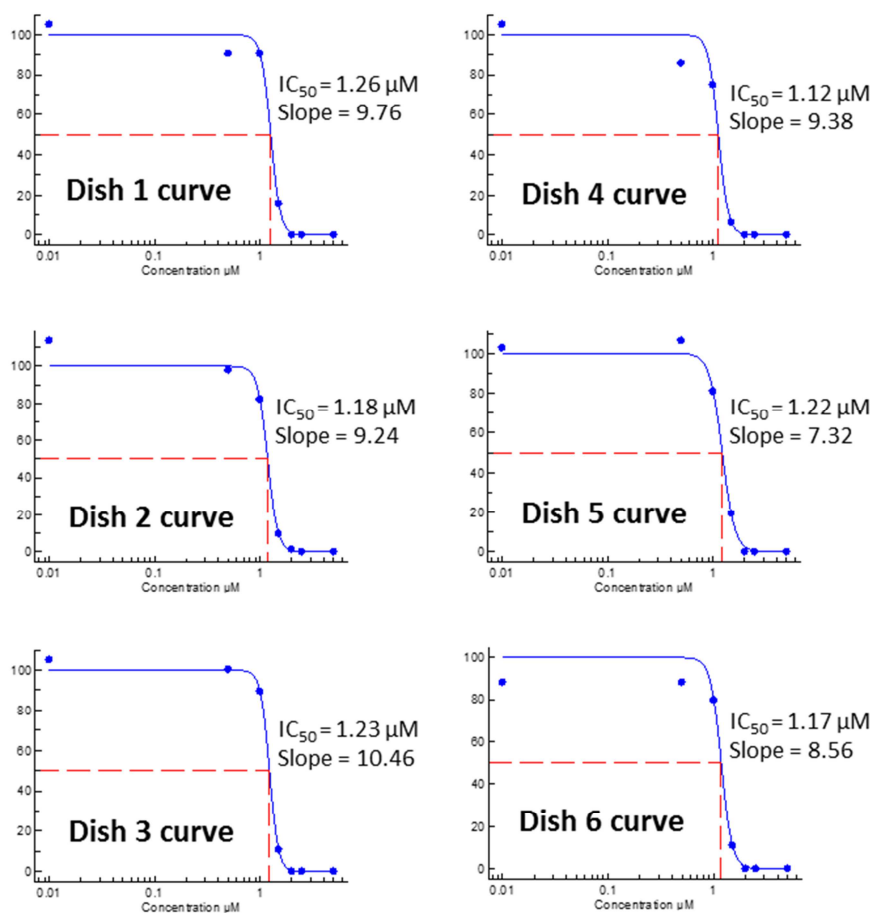


Figure 5.7. An example of intralaboratory variation within a run (6 dishes)

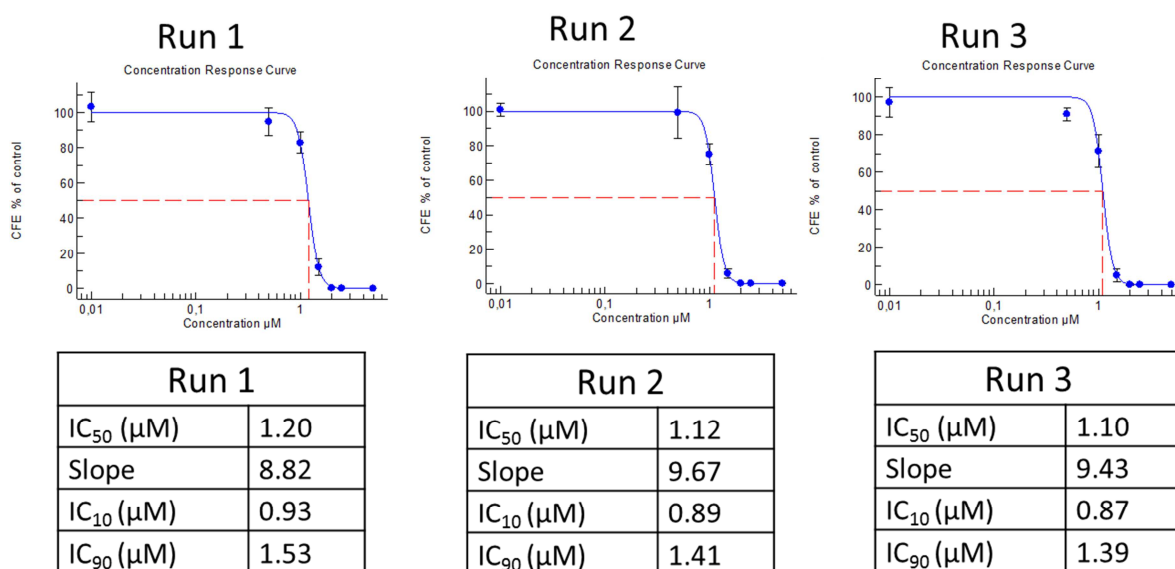


Figure 5.8. An example of intralaboratory, variation between runs (3 runs)

Laboratory 1		Laboratory 2		...	Laboratory 12	
Average IC ₅₀	1.14	Average IC ₅₀	1.22		Average IC ₅₀	1.32
Std IC ₅₀	0.05	Std IC ₅₀	0.08		Std IC ₅₀	0.02
...
...

Figure 5.9. An example of interlaboratory variation between laboratories (12 labs).

For the comparison of results, the coefficient of variation (CV) is used as a normalized measure of dispersion. The CV expresses the sample standard deviation in terms of the sample mean. Consequently, the CV provides a unitless measure of dispersion and a good measure for comparison. CVs are used as measures of intra and interlaboratory reproducibility. CVs less than 30% are often considered to be of “high quality” in that these would be judged to have a small or reasonable level of intra and interlaboratory variability (Busquet et al., 2014).

5.6 Deviating observations

Outliers may be due to variability in the measurement or may indicate experimental errors. There is no stringent mathematical definition of what defines an outlier. So determining whether an observation is an outlier depends on the question and method used. There are numerous model-based methods for outlier identification when data are assumed to come from parametric distributions (especially the normal distribution). In this study, one common non model-based outlier definition is applied. Observations which are lower than the lower quartile (Q1)-3*interquartile range (Q3 - Q1) or higher than the upper quartile (Q3)+3*interquartile range (Q3 - Q1) are considered to be outliers and these observations are highlighted. No deletion of outlier data are made unless the observations are clearly erroneous due to experimental errors. If a data point is excluded from the analysis, it is reported.

An example of an observed outlier, which was excluded from the analysis, may be found in run 4 from laboratory 5 (at concentration 1 μ M) testing the PC sodium chromate, illustrated in Figure 5.10. The set of observations coming from the six dishes are [102 105 102 92 89 36]. The lower quartile is 89, the upper quartile is 102 and the interquartile range is thus 102-89=13. Outliers are observations, which are lower than 89-3*13=50 or higher than 102+3*13=141. Figure 5.10 shows a box plot of the data set, the box has lines at the lower quartile (value 89), median (97), and upper quartile values (102). Whiskers extend from each

end of the box to the adjacent values in the data and the outlier (36) is displayed with a red plus (+) sign.

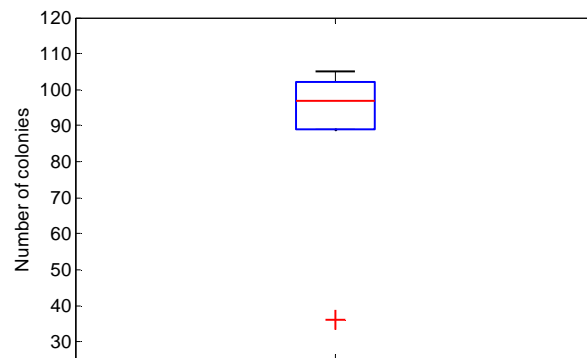


Figure 5.10. Boxplot of the number of colonies at concentration 1 μ M testing the PC sodium chromate, laboratory 5. The outlier is displayed with a red plus (+) sign.

6. Results of the interlaboratory comparison study

6.1 Na_2CrO_4 positive control

In each run, seven concentrations of the positive control sodium chromate are tested. 11 laboratories have provided at least three independent runs but lab 9 has provided two. Three independent runs have been randomly selected for laboratories which have provided more than three accepted runs. A typical concentration response curve (normalised to the negative control) for sodium chromate is shown in Figure 6.1.

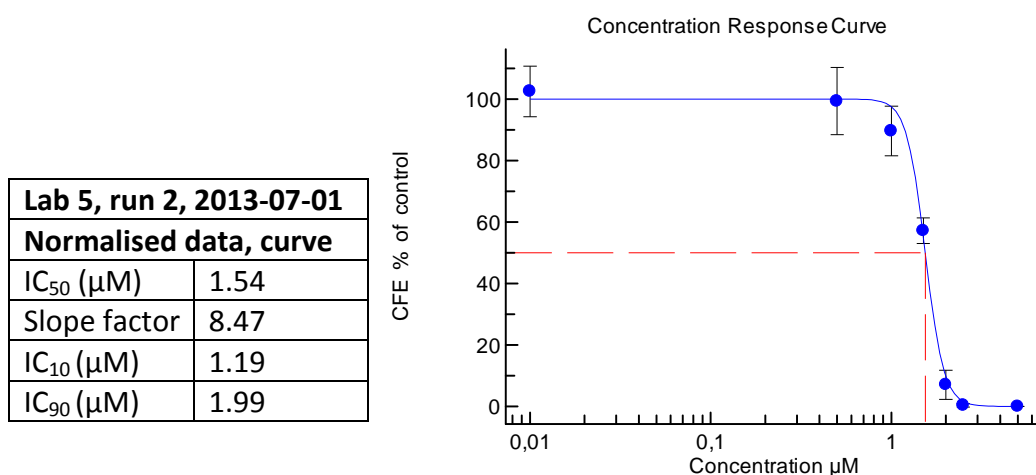


Figure 6.1. A typical concentration response curve for sodium chromate from laboratory 5, run 2.

6.1.1 Intralaboratory variation within runs

The bar charts in Figure 6.2 depict the IC₅₀ values from the three runs from the 12 laboratories. The error bar represents the standard deviation of the six dishes in a run. The corresponding scatter plot of intralaboratory CV of IC₅₀ within runs is shown below the bar chart. Laboratories 1 and 12 have little variation within runs and hence low CVs. The laboratories 4 and 8 tend to have lower IC₅₀ values compared to the other laboratories. Laboratory 8 has furthermore considerable variation within the runs, thus large CVs. Laboratory 9's last two runs have similar concentration response curves since they have the same experimental conditions and are not independent (laboratory 9's first run is differing substantially). The CVs are in the range from 1.1 to 15.5%.

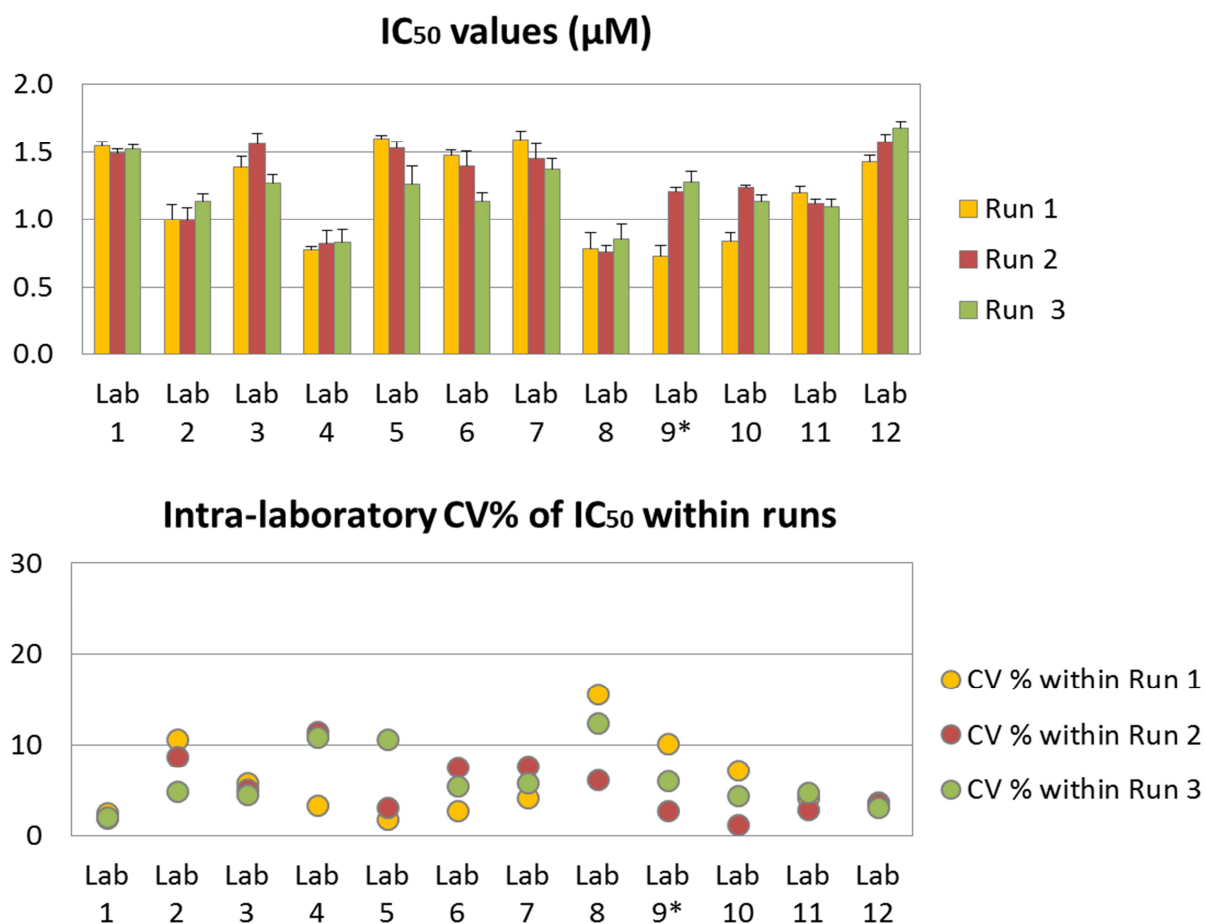


Figure 6.2. The bar charts depict the IC₅₀ values from the three runs from the 12 labs. The error bar represents the standard deviation of the six dishes in a run. The corresponding intralaboratory CVs of IC₅₀ within runs is shown in the scatter plot (below). Laboratory 9 is marked with an asterisk '*' because it has provided less than three independent runs.

6.1.2 Intralaboratory variation between runs

Figure 6.3 illustrates the bar charts of the average IC₅₀ values from the three runs with the corresponding scatter plot of intralaboratory CV of IC₅₀ between runs (blue dots). Laboratories 1, 3, 5, 6, 7 and 12 have similar IC₅₀ values, around 1.5 μM. Moreover, laboratory 1 has the lowest variation between runs, with a CV of 1.8% (illustrated in Figure 6.3). Laboratories 9 and 10 have substantial variation between runs, with CVs of 28.3 and 19.4%, respectively. In the case of laboratory 9, the last two runs are not independent (as already described in the previous sections) and the first run from laboratory 10 has a lower IC₅₀ value than the other two more similar runs. The CVs are in the range from 1.8 to 19.4% (28.3% if the non-independent result is included).

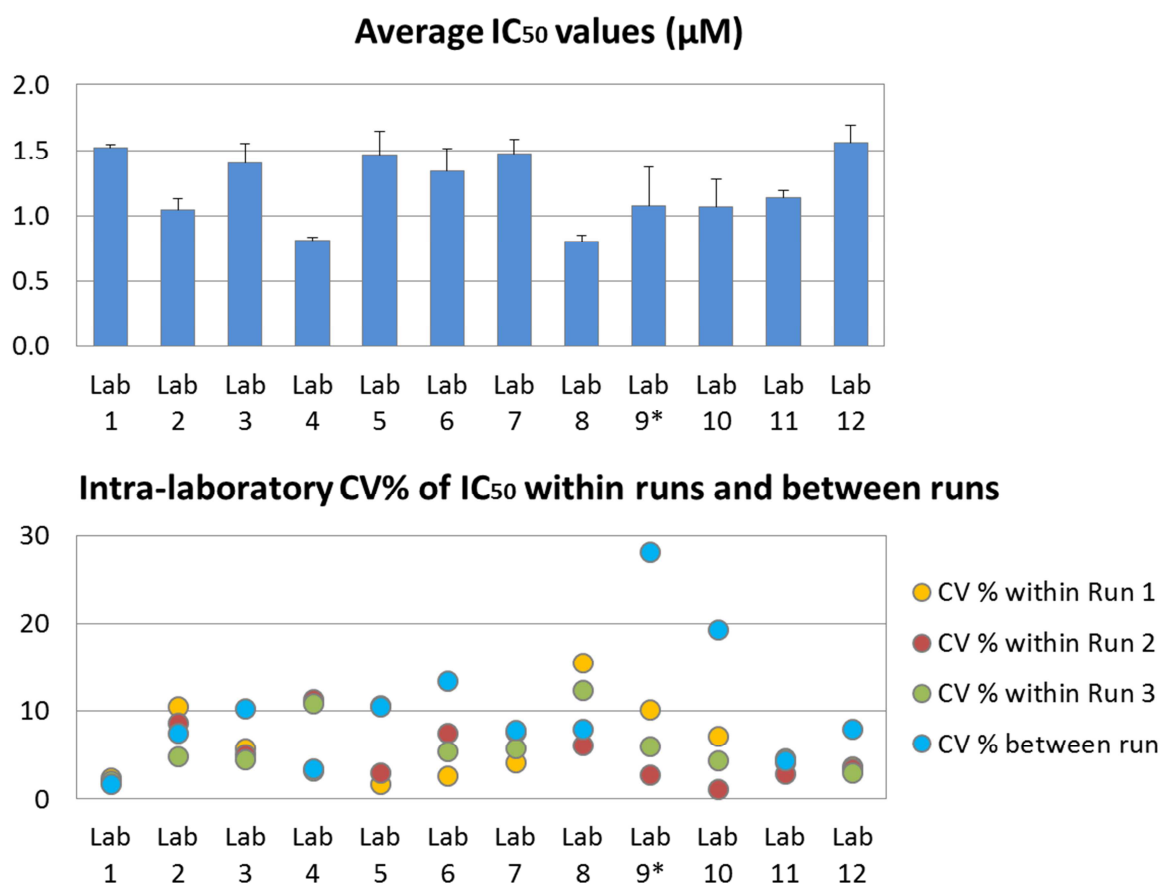


Figure 6.3. The bar charts depict the average IC₅₀ values from the three runs from the 12 laboratories. The error bar represents the standard deviation of the three runs. The corresponding intralaboratory CV% of IC₅₀ between runs is shown in the scatter plot (below). Laboratory 9 is marked with an asterisk “*” because it has provided less than three independent runs.

6.1.3 Interlaboratory variation

The overall average of the 12 laboratories is 1.22 μM with a standard deviation of 0.27 μM. When the results from laboratory 9 are omitted to only have independent results, the overall average is 1.24 μM with a standard deviation of 0.28 μM. The interlaboratory CV is 22.5%.

6.2 Au NPs code A-B-C

The gold NPs code A - C differ in size. NP A has the nominal size of 5 nm, NP B 35 nm and NP C 15 nm. Most laboratories show no response (no toxicity) in the concentration response experiments. The typical concentration response curves for NPs A-C are illustrated in Figure 6.4.

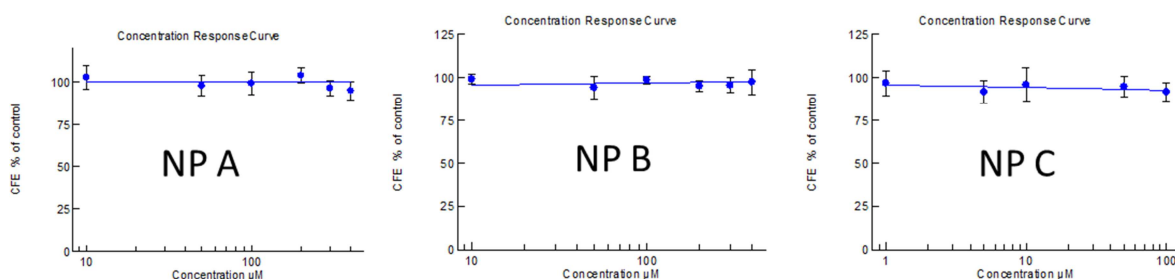


Figure 6.4. Typical concentration response curves with no toxicity for NPs A-C.

NP code A. The response “no toxicity” is found in 10 laboratories, all of which have provided three independent runs. Laboratories 8 and 10 are the exceptions; they have provided less independent runs and the concentration response curves exhibit toxicity. Laboratory 8 has one independent run (the three runs may be seen as one run with 18 replicate dishes because the runs were done during the same days with the same cell passage number) and laboratory 10 has two independent runs. The runs from laboratory 8 have responses at the highest tested concentration. The runs from laboratory 10 show a clear concentration response relationship. Examples of the concentration response curves from these laboratories are shown in Figure 6.5.

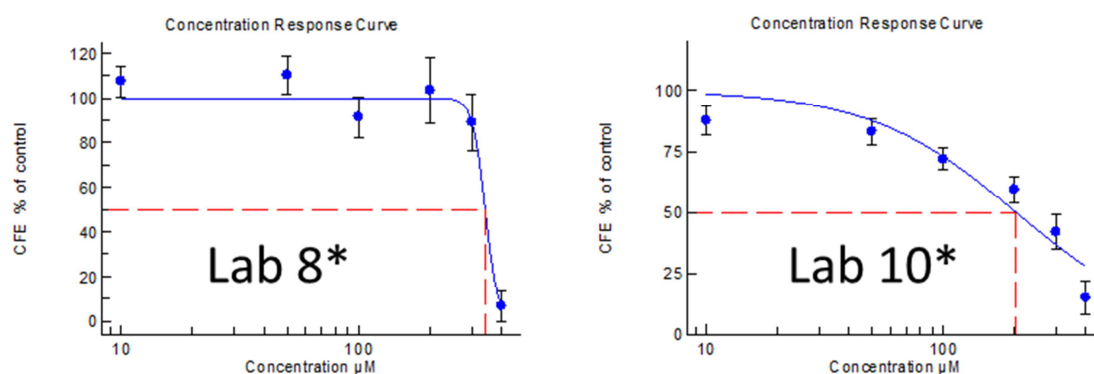


Figure 6.5. Concentration response curves for laboratories 8 and 10 for NP code A, marked with an asterisk, ‘*’ because they have provided less than three independent runs.

NP code B. The response “no toxicity” is found in 9 laboratories, which have provided three independent runs. Laboratory 8 did not provide any results and laboratory 9 provided results from two independent runs. The runs from laboratory 10 exhibit concentration responses as illustrated in Figure 6.6.

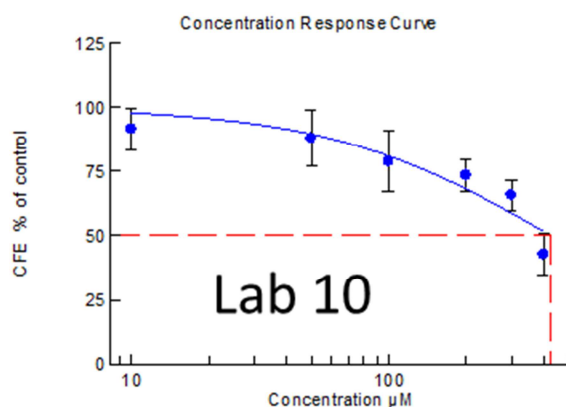


Figure 6.6. Concentration response curve for laboratory 10 for NP code B.

NP code C. No toxicity is detected for seven of eight laboratories, which have provided three independent runs. Thus four laboratories have provided less than the agreed number of independent runs; laboratory 8 did not provide any results, laboratories 9 and 10 have provided two independent runs and laboratory 6 has only provided results from one run because of a (bacterial) contamination problem.

Laboratory 11’s runs exhibit toxicity; two runs have responses only at the highest tested concentration and one run has a clear concentration response relationship. Laboratory 10’s runs also show concentration response relationships. Examples of the concentration response curves from laboratories 10 and 11 are shown in Figure 6.7.

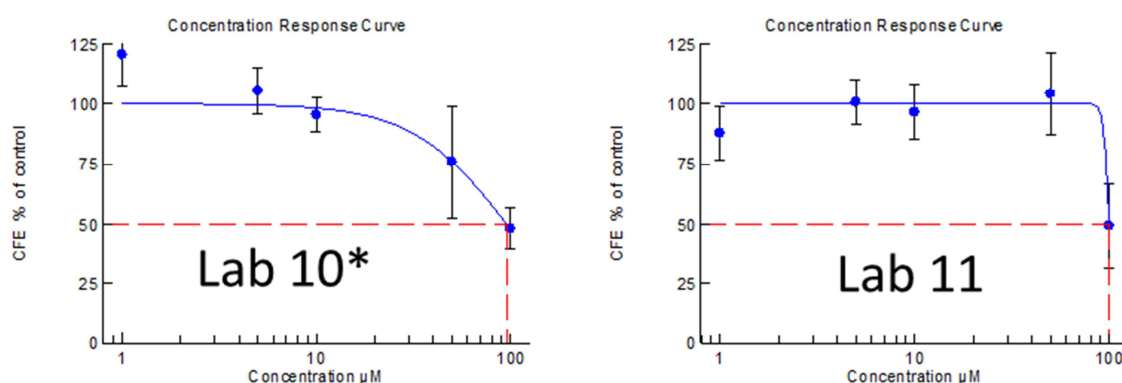


Figure 6.7. Concentration response curves for laboratories 10 and 11 for NP code C. Laboratory 10 is marked with an “*” because it provided less than three independent runs.

6.3 Ag NPs code D-E

The silver NP code D has a nominal size of 30 nm and NP code E a nominal size of 20 nm. All laboratories produced concentration responses. Standard concentration response curves for NPs D and E are illustrated in Figure 6.8.

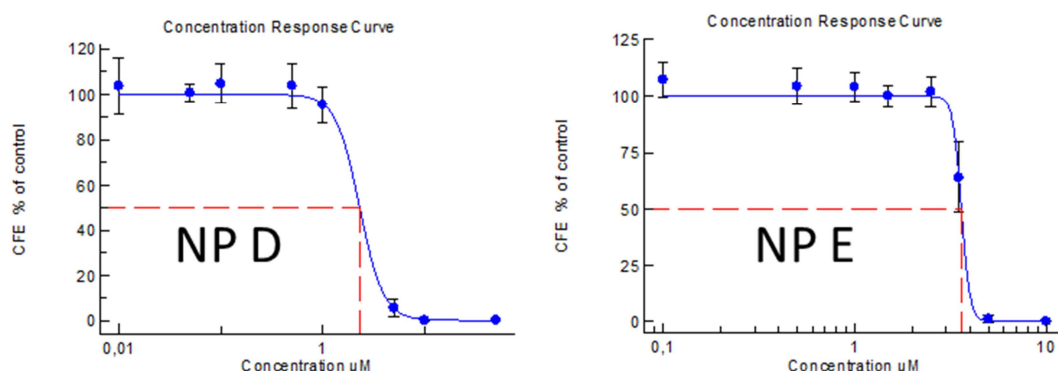


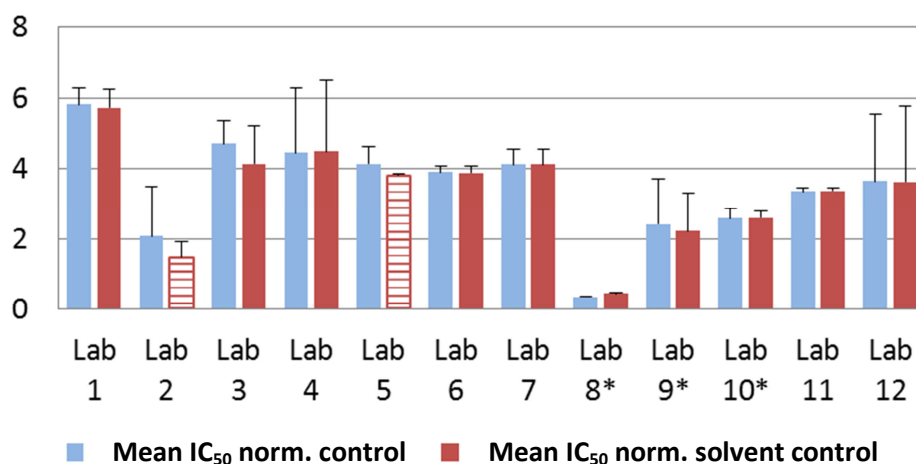
Figure 6.8. Typical concentration response curves for NPs D and E.

NP code D. The bar charts in Figure 6.9 depict the average IC_{50} values from three runs. The blue bars represent data normalised to negative controls and red bars normalised to solvent controls. The error bar represents the standard deviation of the three runs. Three laboratories which have provided less than three independent runs are marked with an asterisk, '*'. Laboratory 9 provided results from two independent runs. Laboratories 8 and 10 provided results from one independent run each. Laboratories 2 and 5 did not provide solvent control data for one run each and the related bars are marked with stripes. Laboratories 4 and 12 have considerable variation between the runs. Laboratory 8 has remarkably low IC_{50} values.

For the comparison, the accepted results for 9 laboratories the nine accepted laboratories for negative control normalised data and 7 laboratories for solvent control normalised data are considered. The intralaboratory CVs are in a majority of cases low and under 20% (six out of nine laboratories for negative control normalised data and five out of seven laboratories for solvent control normalised data). Laboratories 2, 4 and 12 have high intralaboratory CVs, in the range from 43-69%.

The interlaboratory CVs are low and under 30%, 25.4% in the case when the data are normalised to the negative controls and 18.4% when the data are normalised to solvent controls.

NP D – Average IC₅₀ (μM)



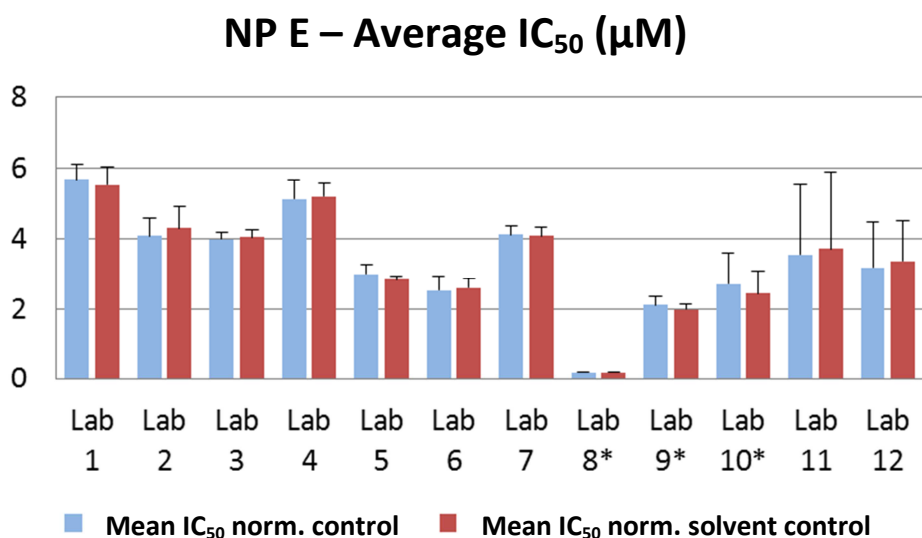
Results from laboratories with 3 independent runs		
NP D	Norm. Neg Cont	Norm. Solv Cont
# Labs	9	7
Mean IC ₅₀	4.0	4.2
Std	1.0	0.8
CV %	25.4%	18.4%

Figure 6.9. The bar charts depict the average IC₅₀ values from three runs. The blue bars represent normalised negative control data and red bars normalised solvent control data. The error bar represents the standard deviation of the three runs. Laboratories with less than three independent runs are marked with an asterisk '*'. Striped bars contain results from two runs.

NP code E. Figure 6.10 illustrates the corresponding bar charts of the average IC₅₀ values from NP E. The results for NPs D and E are very similar; the size of the silver NP does not seem to have an impact on its toxicity. Nine laboratories have provided results from three independent runs. Laboratories 9 and 10 have provided results from two independent runs and laboratory 8 from one independent run. Laboratory 11 has substantial variation between the runs. Again laboratory 8 has remarkably low IC₅₀ values.

The intralaboratory CV is low for seven out of the nine accepted laboratories. In these cases the CV is below 20% for both types of normalisations (to negative and solvent controls). Laboratories 11 and 12 have higher intralaboratory CVs, in the range from 34-58%.

The interlaboratory CVs are low also for NP E: 25.7% in the case when the data are normalised to the negative controls and 24.6% when the data are normalised to solvent controls.



Results from laboratories with 3 independent runs		
NP E	Norm. Neg Cont	Norm. Solv Cont
# Labs	9	9
Mean IC ₅₀	3.9	4.0
Std	1.0	1.0
CV %	25.7%	24.6%

Figure 6.10. The bar charts depict the average IC₅₀ values from the three runs. The blue bars represent data normalised to negative controls and red bars data normalised to solvent controls. The error bar represents the standard deviation of the three runs. Laboratories with less than three independent runs are marked with an asterisk '*'.

6.4 *swCNTs code F*

For the swCNT, nine laboratories observed no toxicity in three independent runs. As for NPs D-E laboratories 8, 9 and 10 provided less than three independent runs. Laboratories 9 and 10 provided results from two independent runs and laboratory 8 from one independent run. Furthermore, laboratory 9's runs established concentration response relationships (responses were already detected at the lowest tested concentration, Figure 6.11). This response was not observed for laboratories 8 and 10.

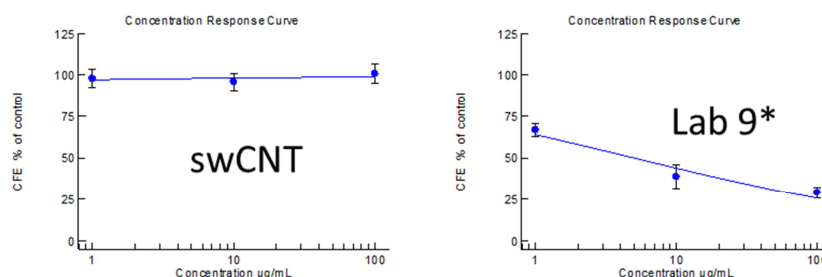


Figure 6.11. Typical concentration response curve with no toxicity (to the left) and a curve with a concentration response for laboratory 9 (to the right) which provided two independent runs, thus marked with an asterisk ‘*’.

6.5 SiO₂ NPs code G-H

NPs code G-H are silicon dioxide (SiO₂) NPs with the nominal sizes of 20 nm and of 90 nm, respectively. As for the previous NPs, nine laboratories have tested three independent runs. The exceptions are laboratories 9, 10 (two independent runs) and laboratory 8 (one independent run).

NP code G. For NP G, no concentration response relationship was observed in any of the laboratories.

NP code H. Most laboratories show no response to the tested NP code H, but at the highest tested concentration the following laboratories observed same toxicity: two runs from laboratory 4 and one run from laboratory 12. In addition, the three non-independent runs from laboratory 8 and one run from laboratory 9 show responses at the highest tested concentration. Figure 6.12 illustrates the standard curve for NP G and H and two examples of curves with responses.

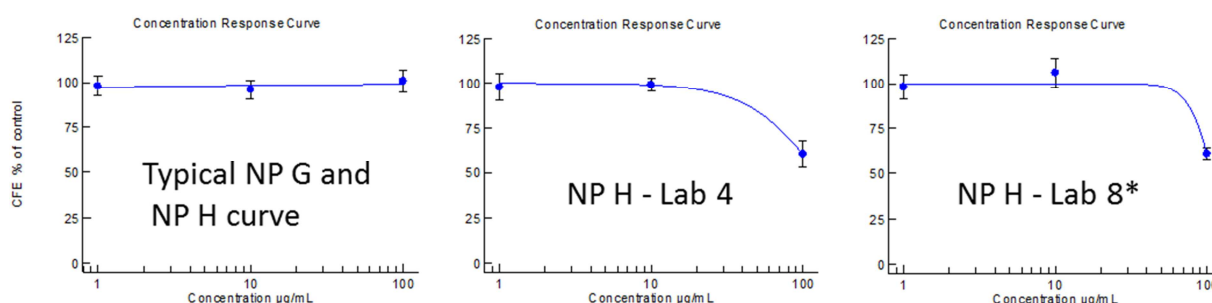
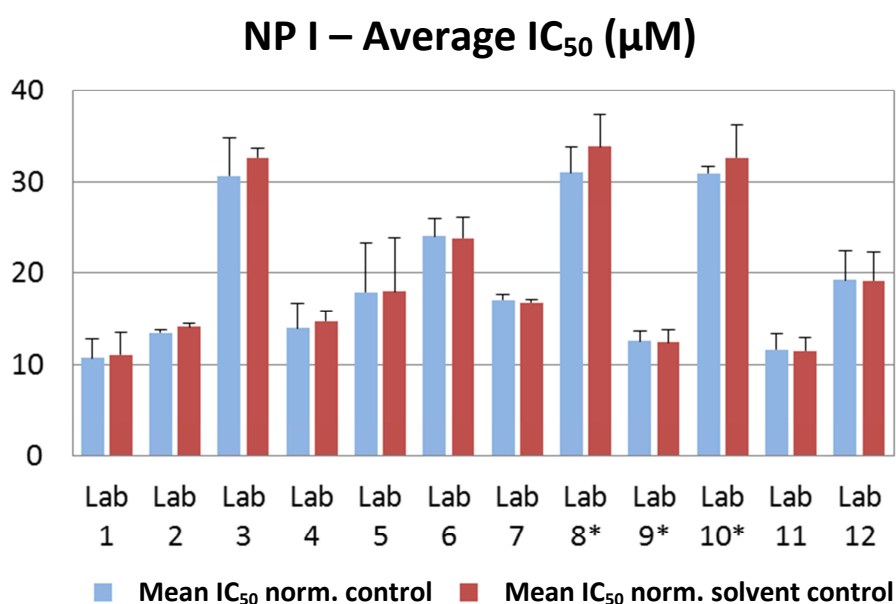


Figure 6.12. Typical curve with no toxicity for most laboratories and curves with concentration responses for NP H for laboratories 4 and 8. Laboratory 8 marked with an asterisk, ‘*’ because it provided one independent run.

6.6 ZnO NPs code I

The coded NP I is a zinc oxide (ZnO) NP with the nominal size of 240 nm. As for the previous NPs, nine laboratories have tested three independent runs. Laboratories 9 and 10 have provided results from two independent runs and laboratory 8 has provided results from one independent run. All laboratories generated concentration responses. The average IC_{50} values from NP I are depicted in Figure 6.13. For the nine accepted laboratories the intralaboratory CVs are in general low. In eight cases they are around or below 20% for both types of normalisations. The exception is laboratory 5, where the CVs are just on the 30% limit for both types of normalisations.



Results from laboratories with 3 independent runs		
NP I	Norm. Neg Cont	Norm. Solv Cont
# Labs	9	9
Mean IC_{50}	17.6	17.9
Std	6.4	6.8
CV %	36.5%	37.8%

Figure 6.13. The bar charts depict the average IC_{50} values from the three runs. The blue bars represent data normalised to negative controls and red bars data normalised to solvent controls. The error bar represents the standard deviation of the three runs. Laboratories with less than three independent runs are marked with an asterisk '*'.

The interlaboratory CVs are somewhat higher than for NPs D and E; 36.5% when the data are normalised to the negative controls and 37.8% when the data are normalised to solvent controls, see Figure 6.13. These high figures are partly due to the deviating results from laboratory 3, see Figure 6.14.

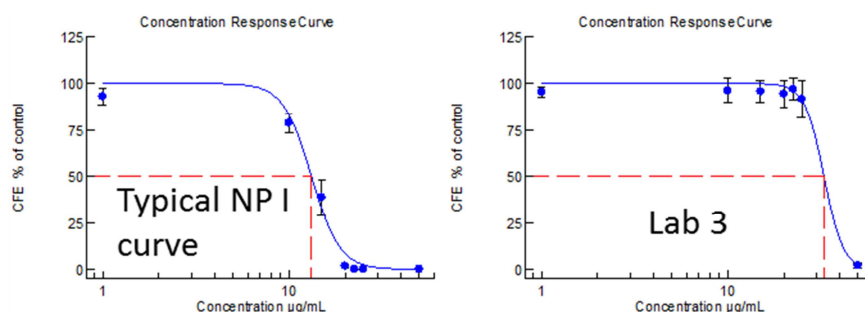


Figure 6.14. Typical concentration response curve for NP I (to the left) and curve for laboratory 3 (to the right).

6.7 Deviations from the Standard Operating Procedure

Deviations from the Standard Operating Procedure (SOP) were encountered, mainly concerning the cells and counting of the colonies. One laboratory incubated the cells for longer time than specified in the SOP (one extra day) and one laboratory seeded more cells than stated in the SOP (300 cells instead of 200 cells per dish). In some runs from one laboratory, two operators counted three dishes each. For consistency all dishes in one run should be counted by one operator to avoid an operator bias.

7. Conclusions and Recommendations

- ✓ The CFE assay is a suitable and robust *in vitro* method to assess cytotoxicity of NMs
- ✓ The assay protocol is well defined and is **easily and reliably transferable** to other laboratories
- ✓ The results obtained show **good intra and interlaboratory reproducibility** of the assay for both the positive control and the tested nanomaterials
- ✓ The assay can be **recommended as a building block of an *in vitro* testing battery**, as a first choice method to define dose-effect relationships for other *in vitro* assays

The reliability of the CFE assay for testing of NMs has been assessed through a large comparison study with 12 participating laboratories testing nine NPs in at least three independent runs.

First, the protocol transferability of the CFE assay was assessed, by testing the positive control chemical sodium chromate (Na_2CrO_4). The participating laboratories considered that the CFE assay protocol, as used in the study is well defined and is easily and reliably transferable to other laboratories. This was confirmed by the analysis of the intralaboratory variations (within a run as well as between runs) and the interlaboratory variation. The results from the participating laboratories show a very good intra and interlaboratory reproducibility and repeatability. For all laboratories, the CVs (of IC_{50}) are less than 20% for both types of intralaboratory variations and 23% in the case of the interlaboratory variation. CVs less than 30% are frequently considered to be an indicator of small or reasonable intra and interlaboratory variability.

Secondly, the nine NPs were tested in the CFE assay and the intralaboratory variation (between runs) and the interlaboratory variation were assessed. Table 7.1 gives an overview of the results for the nine NPs tested for laboratories, which have provided three independent runs. All laboratories have obtained concentration responses for NPs D, E and I and no responses (no toxicity) for NPs A, G and NM F. For the gold NPs B and C all, except for one laboratory, have observed no toxicity. The exceptions are laboratory 10 for NP B and laboratory 11 for NP C. For NP H, all laboratories, except for two runs from laboratory 4 and one run from laboratory 12, find no toxicity. Only at the highest tested concentration, these runs have shown some response.

For NPs D, E and I, the results obtained confirm good intra and interlaboratory reproducibility and repeatability. In most cases, the CVs (of IC_{50}) are less than 20% for the intralaboratory variation. In the case of the interlaboratory variation the CVs are less than 30% for NPs D and

E and less than 40% for NP I. Furthermore, the results for NPs D and E are very similar and show that in the conditions of the test, the size of the silver NP does not have an impact on its toxicity.

Table 7.1. Summary of test results. For NP H, the number in the table is marked with the caret sign '^' since some results from the accepted laboratories were not congruent. All accepted laboratories, except for two runs from laboratory 4 and one run from laboratory 12, find no toxicity. Only at the highest tested concentration, these runs have shown some response.

Laboratories with three independent runs					
NP code	NP name	NP nominal size (nm)	Results # labs	Result	# concordant labs / # labs
A	Au NP	5	10	No toxicity	10/10
B	Au NP	35	10	No toxicity	9/10
C	Au NP	15	8	No toxicity	7/8
D	Ag NP	30	8	Concentration response	9/9
E	Ag NP	20	9	Concentration response	9/9
F	swCNT	-	9	No toxicity	9/9
G	SiO ₂ NP	20	9	No toxicity	9/9
H	SiO ₂ NP	90	9	No toxicity	8 [^] /9
I	ZnO NP	240	9	Concentration response	9/9

A drawback identified by the laboratories was that the assay is quite laborious and has a relatively low throughput. Therefore, in order to fully recommend it as a screening assay, its throughput should be increased. Efforts to downscale the assays (e.g. to 6-well or 24-well plates) and adapt it for high-content image analysis platforms are already ongoing.

Based on the result of this interlaboratory study it could be concluded that the CFE assay is a suitable method to assess cytotoxicity of NMs and it has several advantages over the conventional cytotoxicity assays, as it avoids test interferences. Furthermore, the experience gained at the JRC shows that the test is particularly sensitive, as compared to other types of cytotoxicity assays (Ponti et al., 2006). Therefore, it could be included in a testing battery as an early screening method. It may well be used in combination with other *in vitro* assays (e.g. genotoxicity *in vitro* assays, such as micronucleus TG 487) to define the subtoxic doses *in vitro*. It has to be noted that for suspension cells or cells not forming colonies a similarly sensitive cytotoxicity assay will need to be defined and validated for NMs testing.

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APPENDIX 1 Standard Operating Procedure (SOP) for culturing MDCK cells

I. INTRODUCTION

The MDCK cell line was derived from a kidney of an apparently normal adult female cocker spaniel, September, 1958, by S.H. Madin and N.B. Darby.II. This line is hyperdiploid and there is a bi-modal chromosome number distribution. There are no consistent identifiable marker chromosomes. One normal X chromosome is present in most spreads. The cells are positive for keratin by immunoperoxidase staining. MDCK cells have been used to study processing of beta amyloid precursor protein and sorting of its proteolytic products. Cells form colonies when cultured in subconfluent state.

II. PURPOSE

To maintain MDCK cells in culture for a defined period in order to perform subsequent experiments. Cells are maintained in a sub-cultured state (less than 70% confluence) in Corning® 75 cm² culture flasks.

III. LIMITATIONS

MDCK cells have to be passaged at least three times after thawing, before being used in the Colony Forming Efficiency assay (passage 1 is the first passage after thawing). A new batch of cells from the frozen stock has to be thawed when the old batch in culture reaches passage number 6. The Colony Forming Efficiency assay has to be performed using cells in culture at maximum passage 10.

IV. METHOD OUTLINE

MDCK cells are routinely grown as a monolayer in tissue culture grade flasks (Corning® 75 cm² culture flasks, cat. number 430641, Corning, USA) at 37°C, 95 % humidity, and 5% CO₂. The cells have to be sub-cultured in a new culture flask when cells are sub-confluent (~70% confluence). Stocks of MDCK cells can be stored in sterile cryo vials, in liquid nitrogen. Dimethyl sulfoxide (DMSO) is used as a cryo protective agent. The cells under culture condition are always cultured at a density of 1x10⁵ cells/75 cm² culture flask. Only cells frozen in cryo vials contain 1x10⁶ cells/cryo vial.

V. MATERIALS

Cell culture type

MDCK cells European Collection of Cell Cultures (ECACC, Sigma catalogue number 84121203, Lot. number 06F015) passage 10 were supplied by JRC to each partner.

Technical Equipment and Culture plates

[Note: Suggested brand names/suppliers are listed in parentheses]

- Incubator: 37°C, 95 % humidity, and 5% CO₂
- Laminar flow clean bench/cabinet (standard: "biological hazard")
- Water bath: 37°C
- Inverse phase contrast microscope
- Centrifuge
- Cell counter or haemocytometer (e.g. Bürker Chamber)
- Pipetting aid
- Pipettes, pipettors
- Cryo vials
- Tissue culture flasks (Corning® 75 cm² culture flasks, Corning, cat. number 430641)

Reagents, Medium and Serum

- Dulbecco's Modified Eagle's Medium 1X (DMEM) [+] 4.5g/L Glucose [+] L-Glutamine, [-] Pyruvate (Gibco, cat. number 41965 used by JRC, ENEA, ISS, NIOM, NIOH, EMPA, CEA and IPL; Gibco, cat. number 11965 used by KRISS, NIER, NIFDS and AIST) (store at +4°C)
- Fetal Clone II, Bovine serum product, Optimised for CHO cells (Thermo Scientific HyClone, cat. number SH30066.03 lot. number AYB58974 used by JRC, CEA, IPL, NIOH and NIOM; cat. number SH30066.03 lot. number AUJ35588 used by AIST; cat. number SH30066.03 lot. number AWJ22309 used by ENEA (or lot. Number AUE34894), ISS and EMPA; Gibco/Invitrogen cat. number 16000 lot. number 1365348 used by KRISS; cat. number 16000 lot. number 1221293 used by NIER; cat. number 30067 lot. number 8190771 used by NIFDS)(store at -20°C)
- Non-essential amino acids MEM NEAA 100X (Gibco, cat. number 11140) (store at +4°C)
- Penicillin/streptomycin solution 10000 Unit/mL Pen.; 10000 Unit/mL Strep. (Gibco, cat. number 15140) (store at -20°C)
- Phosphate Buffered Saline (PBS) without Ca²⁺ and Mg²⁺ (Gibco, cat. number 20012) (store at Room Temperature)
- 0.05% Trypsin-EDTA (Ethylenediaminetetraacetic acid)(1X), Phenol red solution (Gibco, cat. number 25300) (store at -20°C)
- Dimethyl sulfoxide (DMSO) ≥99.5%, CAS. 67-68-5 (Sigma-Aldrich, cat. number D5879) (store at Room Temperature)
- Trypan Blue solution 0.4% (Gibco, cat. number 15250) (store at Room Temperature)

List of materials that are mandatory to use:

- Tissue culture flasks (Corning® 75 cm² culture flasks, Corning, cat. number 430641)
- Dulbecco's Modified Eagle's Medium 1X (DMEM) [+] 4.5g/l Glucose [+] L-Glutamine, [-] Pyruvate (Gibco, cat. number 41965)
- Fetal Clone II, Bovine serum product, Optimised for CHO cells (Thermo Scientific HyClone, cat. number SH30066.03 lot. number AYB58974 used by JRC, CEA, IPL, NIOH and NIOM; cat. number SH30066.03 lot. number AUJ35588 used by AIST; cat. number SH30066.03 lot. number AWJ22309 used by ENEA (or lot. Number AUE34894), ISS and EMPA; Gibco/Invitrogen cat. number 16000 lot. number 1365348 used by KRISS; cat. number 16000 lot. number 1221293 used by NIER; cat. number 30067 lot. number 8190771 used by NIFDS)(store at -20°C)
- Non-essential amino acids MEM NEAA 100X (Gibco, cat. number 11140)
- Penicillin/streptomycin solution 10000 Unit/mL Pen; 10000 Unit/mL Strep (Gibco, cat. number 15140)

Guideline for preparation of aliquot Pen/Strep, Trypsin and Serum

- Pen/strep: Under sterile conditions prepare 5 mL aliquots in 15 mL Falcon tubes
- Trypsin: Under sterile conditions prepare 10 mL aliquots in 15 mL Falcon tubes.
- Serum:
 - Defreeze the serum in a thermostatic bath at 37°C
 - Heat the thermostatic bath to 56°C and put the bottle of the serum inside the bath for 30 min.
 - Mark 50 mL Falcon tubes with the name of the serum and the origin (e.g. Fetal Clone II U.S. origin), serum batch, catalogue number, date of the aliquots preparation
 - After 30 min of incubation remove the bottle from the thermostatic bath and prepare, under sterile conditions, aliquots of 50 mL serum in each tube
 - Close the Falcon tubes and wrap the caps with parafilm
 - Store the aliquots at -20°C

VI. METHODS

Preparation of Complete Culture Medium and Freezing Medium

[Note: All solutions, glassware, pipettes, etc. have to be sterile where adequate. All methods and procedures will be adequately documented]

- Complete Culture Medium for routine culture (store at + 4°C)
Complete cell culture medium is prepared, under sterile conditions as follow:
 - Open a 500 mL Dulbecco's Modified Eagle's Medium 1X (DMEM) [+] 4.5g/l Glucose [+] L-Glutamine, [-] Pyruvate (Gibco, cat. number 41965)

- Add 50 mL of Fetal Clone II, Bovine serum product, Optimised for CHO cells (Thermo Scientific HyClone, cat. number SH30066.03) (10 % v/v)
- Add 5 mL of Non Essential a.a. MEM NEAA 100X (Gibco, cat. number 11140) (1 % v/v)
- Add 5 mL Penicillin/streptomycin solution 10000 Unit/mL Pen.; 10000 Unit/mL Strep. (Gibco, cat. number 15140) (1 % v/v)

Complete cell culture medium should be stored at +4°C for no longer than three weeks.

- Freezing Medium (freshly prepared)
 - 80 % (v/v) complete culture medium
 - 10 % (v/v) serum
 - 10% (v/v) DMSO (Sigma-Aldrich, cat. number D5879) (final conc. in cryovial 5%)

Cell maintenance and culture procedures

Routine culture

MDCK cells are routinely cultured in a sub-confluent state (maximum 70% confluency) in 75 cm² culture flasks (Corning, cat. number. 430641, USA) at 37°C, 95 % humidity, and 5% CO₂. The cells have to be examined daily under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

Receipt of cells in culture from the JRC

The MDCK cells will be shipped in culture flasks completely full of culture medium. Upon receipt completely remove the culture medium, wash once with 10 mL of PBS pre/warmed to room temperature and add 10 mL of freshly prepared complete cell culture medium pre-warmed to room temperature, under sterile conditions. Leave cells in the incubator (37°C, 95 % humidity, and 5% CO₂) overnight and the following day, either change the medium or start splitting them following “subculture of cells” instructions, if cells are already subconfluent (50-70% confluence).

Preparation of the working stock and freezing of the cells

A working stock of MDCK cells will have to be prepared once cells arrive to each laboratory from the JRC. The cells that arrive from the JRC will have passage number 10; the working stock will have passage number 11. Cells used right after thawing might show adverse effects due to the thawing process, therefore an adaptation time of three passages (passage number 11 (+1) to passage number 11 (+3)) should be given to the cells before using them in an experiment. Additionally, cells should not be used in the Colony Forming Efficiency assay after the 10th passage after the start of culturing the cells (passage 11+10). Therefore, to assure a

constant supply of cells suitable for experiments, new cells should be thawed, when the culture in use is at passage 11 (+6) (Figure S1).

To prepare the working stock follow the procedure below:

- Once the cells arrive to the laboratory remove all the culture medium from the flask, wash once with 10 mL of PBS pre-warmed to room temperature and add 10 mL of freshly prepared complete cell culture medium pre-warmed to room temperature. Incubate the cells overnight in the incubator and detach them as described below in section "Subculture of cells" the day after
- After detachment, re-suspend all the cells in 60 mL of complete culture medium (without counting them) and distribute into 6 culture flasks (75 cm²) by adding 10 mL of cell suspension into each flask
- Cells will reach a subconfluent state (~ 70% confluence) in approximately 3-5 days (if not, wait longer until sub-confluence is reached, changing medium twice a week)
- Once the cells have reached subconfluent state, freeze them as follow:
 - Detach cells from all the 6 culture flasks using trypsin-EDTA and collect them in a single 50 mL tube and count as described below in "Cell counting" section
 - Calculate the number of cryo vials to be prepared, considering that each vial should contain 1×10^6 cells. Mark each cryo vial with cell name, passage number and date
 - Prepare the freezing medium as described above (0.5 mL for each cryo vial)
 - Centrifuge the cell suspension in a 50 mL tube (200xg, 10 min.)
 - Remove the supernatant leaving the amount of complete medium in which the cell pellet will be re-suspended for freezing (e.g. 1 mL for 2 vials). The density of this cell suspension should be 2×10^6 cell/mL (since it will be diluted 1:1 with freezing medium to a final number of 1×10^6 cells in each cryo vial)
 - Open the cryo vials under the biological hood and distribute 0.5 mL of freezing medium into each vial and place them on ice
 - Gently re-suspend cells in the culture medium which remained in the 50 mL tube, and distribute 0.5 mL of this cells suspension into each cryovial
 - Mix the cell suspension with the freezing medium. This step is extremely important in order to avoid the formation of two phases solution during the freezing
 - Place the cryo vials in an isolated container (e.g. styrofoam trays) and store at -80°C for 24 h
 - After 24 h place the frozen cryo vials into liquid nitrogen (or -150°C freezer) for long term storage

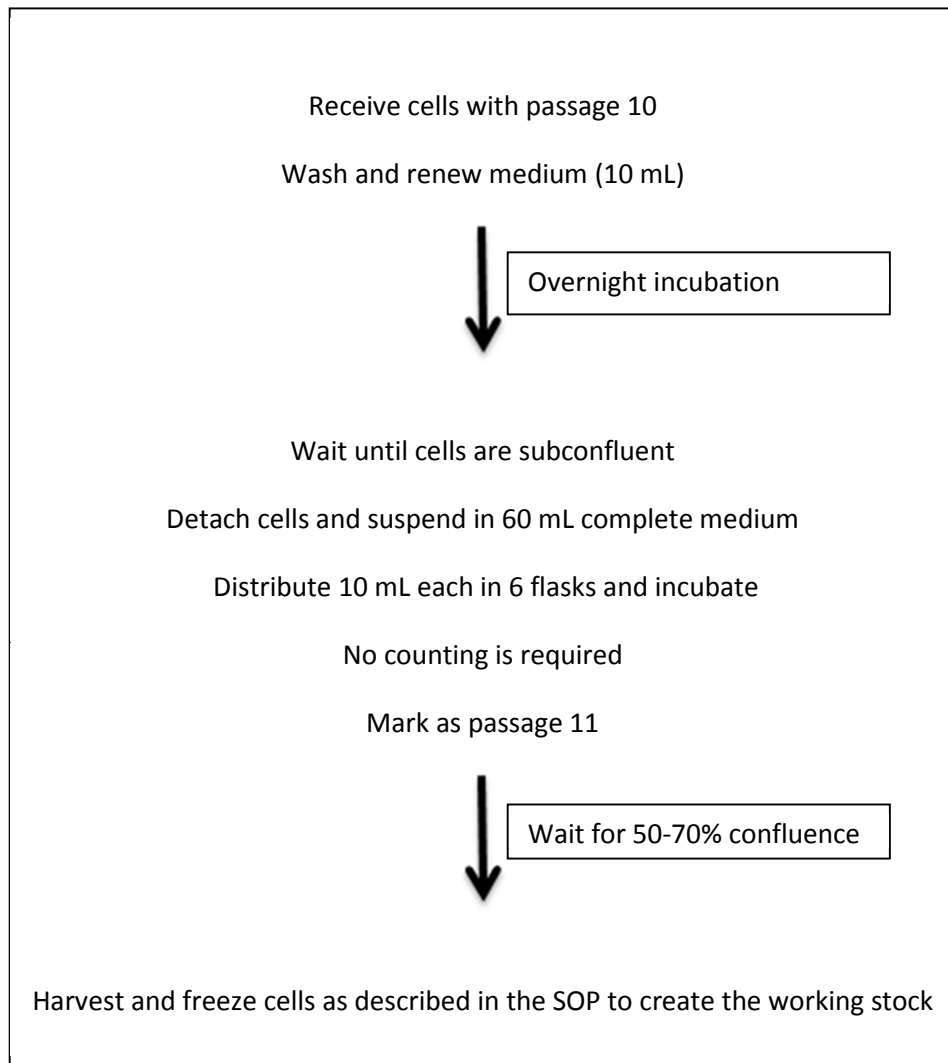


Figure S1. Preparation of the MDCK working stock

Thawing of the working stock of cells

- Remove cryo vials from the liquid nitrogen and thaw cells by putting vials into a water bath at 37°C
- Re-suspend the cells in 9 mL of pre-warmed complete cell culture medium and transfer in a 75 cm² culture flasks
- Incubate at 37°C, 95 % humidity, and 5% CO₂ for 24 h
- After 24 h change culture medium and replace with fresh pre-warmed (37 °C) complete cell culture medium and culture as described above
- After thawing, passage the cells at least three times before using in the CFE assay

[Note: A fresh batch of frozen cells from the working stock should be thawed out and cultured when the working stock in culture reaches passage number 11 (+7). The CFE experiment will

at least after 3 passages of cells in culture 11 (+3) and cells can be used until passage (+10)_n as described in Figure S2]

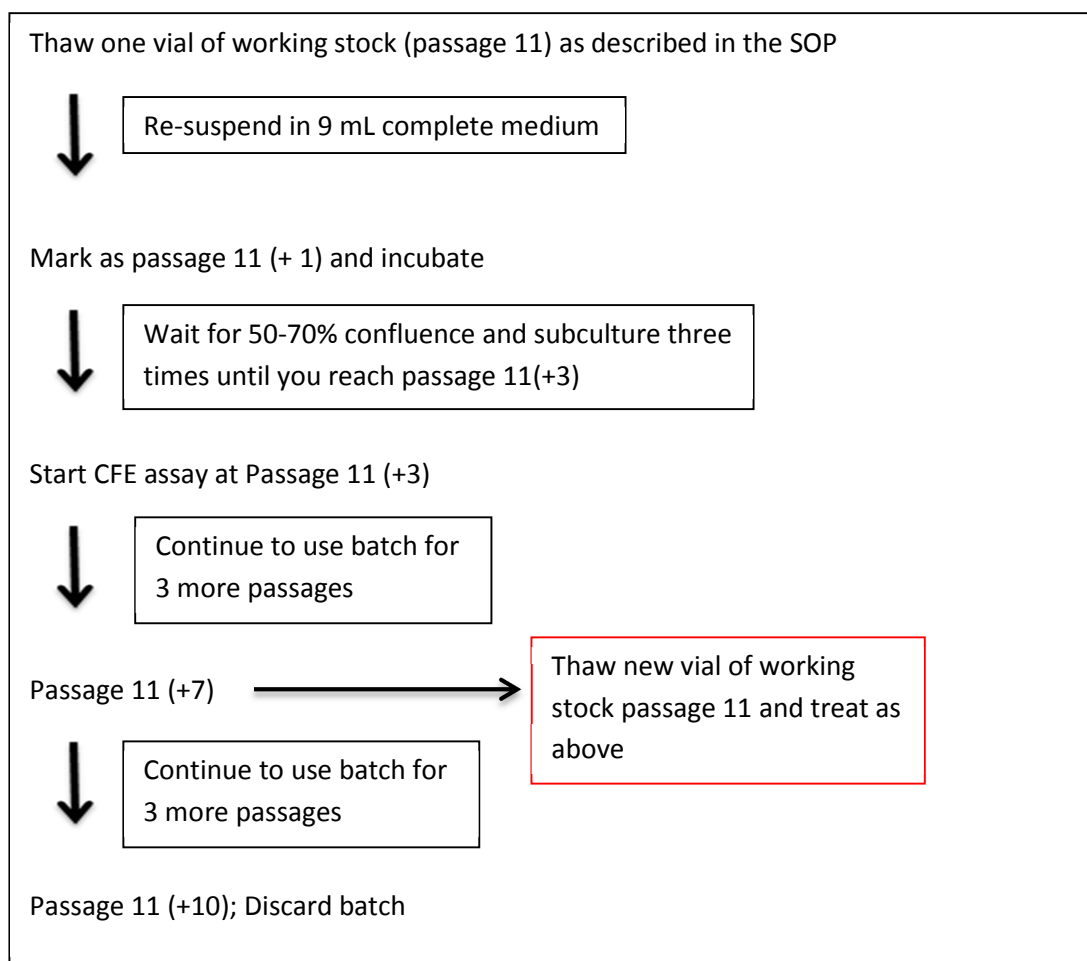


Figure S2. Maintenance of MDCK cells in the laboratory.

Subculture of cells

When cells exceed 50% confluence (but are less than 70% confluent), they have to be split by trypsinization, as follow:

- Warm-up the complete culture medium at 37 °C in the thermostatic water bath for at least 15 min
- Under sterile conditions, wash the cells twice with 10 mL of PBS
- Add 1 mL trypsin-EDTA solution into each 75 cm² culture flask
- Place the flask in the incubator for 2-5 min
- Verify under the microscope that the cells are detached. If necessary, release the cells by manually tapping on the sides of the flask until detachment is observed
- Add 9 mL of complete culture medium and disperse the cells by gentle pipetting

- Count cells as described in section "Cell counting" below
- Seed 1×10^5 cells into each 75 cm² culture flask with 10 mL of complete culture medium. Cells will reach 50% confluence in approximately 3-4 days

Cell counting

It is important to obtain a single cell suspension for exact counting, so gently disperse the cell pellet using a 10 mL pipet.

- Take 30 µL of cell suspension and add 30 µL of 0.4% Trypan Blue solution (Gibco, cat. number 15250) in a 2 mL micro-centrifuge tube (dilution factor = 2). Mix the solution well by pipetting with 200 µL pipette
- Count a sample (~10 µL) of the cell suspension using a haemocytometer or cell counter (e.g., Bürker Chamber)
- For a Bürker Chamber the number of cells/mL is calculated using the following formula:

$$N = \{[(a + b)/2] / 9 \times 10^4 \times DF\}$$

where:

N = number of cells/mL

a = number of cells counted in 9 squares of the first chamber

b = number of cells counted in 9 squares of the second chamber

10^4 = Conversion factor of the chamber volume

DF = Dilution factor of the cell suspension (usually equal to 2)

The appropriate formula has to be applied for other counting devices with taking the dilution factor into account.

- Re-suspend the required number of cells (i.e. 1×10^5 taken directly from the cell suspension) in 10 mL of complete culture medium and culture in a new 75 cm² culture flask
- After detaching the cells into a single cell suspension for subculture, cell viability, determined by staining with vital dye Trypan blue, should be higher than 80%

VII. HEALTH SAFETY AND ENVIRONMENT

All procedures have to be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). Gloves and laboratory coat must be worn by operators. Only sterile equipment must be used in cell handling. Discard all the materials used following the appropriate procedure for special biological waste. During handling of cryogenic vials in liquid nitrogen, a full-face mask and appropriate gloves must be worn.

APPENDIX 2 Standard Operating Procedure (SOP) for CFE assay

I. MATERIALS

Cell Culture type

MDCK cells, (European Collection of Cell Cultures (ECACC), Lot. number 06F015). Passage 10 is supplied by JRC.

Technical Equipment

- Incubator: 37°C, 95% humidity, 5% CO₂
- Class II biological safety cabinet
- Water bath: 37°C
- Inverse phase contrast microscope
- Stereomicroscope
- Centrifuge
- Analytical balance
- Cell counter or haemocytometer (e.g. Bürker Chamber)
- Pipetting aid
- Pipettes, pipettors
- Vacuum pump
- Aspirator
- Tissue culture flasks (Corning® 75 cm² culture flasks, cat. number 430641, Corning, USA)
- Tissue culture dishes (BD Falcon 60x15 mm Style, 20/bag, cat. number 353004, BD Falcon , USA)
- Filter Unit -500 or 1000 mL (Nalgene cat. number 166-0045) or equivalent

Reagents, Medium, Serum

- Dulbecco's Modified Eagle's Medium 1X (DMEM) [+] 4.5g/L Glucose [+] L-Glutamine, [-] Pyruvate (Gibco by Life Technologies Cat. number 41965) (store at +4°C)
- Fetal Clone II, Bovine serum product, Optimised for CHO cells (Thermo Scientific HyClone, cat. number SH30066.03 lot. number AYB58974 used by JRC, CEA, IPL, NIOH and NIOM; cat. number SH30066.03 lot. number AUJ35588 used by AIST; cat. number SH30066.03 lot. number AWJ22309 used by ENEA (or lot. Number AUE34894), ISS and EMPA; Gibco/Invitrogen cat. number 16000 lot. number 1365348 used by KRISS; cat. number 16000 lot. number 1221293 used by NIER; cat. number 30067 lot. number 8190771 used by NIFDS)(store at -20°C)

- Non-essential a.a. MEM NEAA 100X (Gibco by Life Technologies cat. number 11140) (store at +4°C)
- Penicillin/streptomycin solution 10000 Unit/mL Pen.; 10000 Unit/mL Strep. (Gibco by Life Technologies cat. number 15140) (store at -20°C)
- Phosphate Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco by Life Technologies cat. number 20012) (store at Room Temperature)
- Trypsin-EDTA (Ethylenediaminetetraacetic acid) solution (Gibco by Life Technologies Cat. number 25300) (store at -20°C)
- Dimethyl sulfoxide (DMSO), CAS. 67-68-5 (Sigma-Aldrich cat. number D5879) (store at Room Temperature)
- Trypan Blue stain 0.4% (Gibco, cat number 15250 or equivalent) (store at Room Temperature)
- Formaldehyde solution, ACS reagent, 37 (w/v) % in H_2O (Sigma-Aldrich cat. number 25254-9 or equivalent)
- Giemsa Stain Modified solution (Sigma-Aldrich cat. number GS500-500mL)
- Sodium Chromate, Na_2CrO_4 (Sigma Aldrich cat. number 307831; CAS number 7775-11-3, m.w. 161.97)

List of materials that are mandatory to use:

- Tissue culture flasks (Corning® 75 cm^2 culture flasks, Corning, cat. number 430641)
- Tissue culture dishes (BD Falcon 60x15 mm Style, 20/bag, cat. number 353004, BD Falcon, USA)
- Dulbecco's Modified Eagle's Medium 1X (DMEM) [+] 4.5g/L Glucose [+] L-Glutamine, [-] Pyruvate (Gibco, cat. number 41965)
- Fetal Clone II, Bovine serum product, optimized for CHO cells (Thermo Scientific HyClone, cat. number SH30066.03)
- Non-essential amino acids MEM NEAA 100X (Gibco, cat. number 11140)
- Penicillin/streptomycin solution 10000 Unit/mL Pen.; 10000 Unit/mL Strep. (Gibco, cat. number 15140)

II. IDENTIFICATION OF CONTROL SUBSTANCES

- Positive control: Sodium Chromate, Na_2CrO_4 (Sigma Aldrich cat. number 307831; CAS Number 7775-11-3, m.w. 161.97), 100 μM
- Solvent Control: Complete cell culture medium with the addition of the solvent that is used for synthesis and suspension of nanomaterials. The concentration (v/v) of solvent in the Solvent Control medium corresponds to that present in the highest tested concentration of nanomaterial and is the same in each NMs concentration tested.

III. METHODS

[Note: All solutions, glassware, pipettes, etc. have to be sterile and all procedures have to be carried out under aseptic conditions and in the sterile environment of a class II biological safety cabinet until when cells are ready for fixation and staining. All methods and procedures will be adequately documented]

Method outline:

Day 1	Cell seeding
Day 2	Exposure to nanoparticles
Day 5	End of exposure (72 h), change of medium
Day 8	Fixation and Staining
Day 9	Counting of the colonies and data elaboration

Day 1. Cell seeding

Preparation of cells for seeding

When cells cultured in 75 cm² flasks exceed 50 % confluence, but are less than 70% confluence, they are ready to be used for CFE assay.

Detach cells from the 75 cm² culture flasks as follow:

- Warm up the complete cell culture medium at 37°C in the thermostatic water bath for at least 15 min
- Under sterile conditions, wash the cells in 75 cm² culture flask twice using 10 mL of PBS
- Add 1 mL trypsin-EDTA solution into each 75 cm² culture flask
- Place the flasks in the incubator for 2-5 min
- Verify under the microscope if cells are detached. If necessary, release the cells by manually tapping on the sides of the flask until when complete detachment is observed
- Add 9 mL of complete culture medium and disperse the cells by gentle pipetting. Since it is important to obtain a single cell suspension for exact counting, gently disperse the cell pellet using a 10 mL pipette

Cell counting

- Take 30 µL of cell suspension and add 30 µL of 0.4% Trypan Blue solution (Gibco, cat number 15250) in a 2 ml microcentrifuge tube (dilution factor = 2). Mix the solution well by pipetting with 200 µL pipette
- Count a sample (~10 µL) of the cell suspension using a haemocytometer or cell counter (e.g., Bürker Chamber)

- For a Bürker Chamber the number of cells/mL is calculated using the following formula:

$$N = \{[(a + b)/2] / 9 \times 10^4 \times DF\}$$

where:

N = number of cells/mL

a = number of cells counted in 9 squares of the first chamber

b = number of cells counted in 9 squares of the second chamber

10^4 = Conversion factor of the chamber volume

DF = Dilution factor of the cell suspension (usually equal to 2)

After detaching the cells into a single cell suspension for subculture, cell viability determined by staining with vital dye Trypan blue, should be higher than 80%.

Calculation of the number of cells to be seeded for CFE assay and volume in which cells are suspended

- Each BD Falcon 60x15 mm dish must contain 200 cells suspended in 3 mL of complete culture medium. For each test concentration (NPs test suspensions) and control, 6 dishes are seeded (6 replicates)
- Calculate the total number of dishes used for the experiment:

Example:

- 3 test concentrations + 1 positive control + 1 negative control + 1 solvent control correspond to 6 treatment conditions in total x 6 replicates = 36 dishes in total
- Calculate at least 4 dishes more than needed (36 + 4 = 40)
- Multiply 200 cells/dish x 40 dishes = 8000 cells
- Multiply 40 dishes x 3 mL of culture medium = 120 mL

Preparation of the 60x15 mm dishes

- Open the dishes bags under the class II biological safety cabinet biological hood, under sterile conditions
- Write on each dish the name and concentration of the NPs to which the cells will be exposed; indicate C for control; C+ for positive control; C solv. for solvent control

Cell seeding

- Prepare the seeding cell suspension that contains the number of cells needed for seeding (e.g. 8000 cells) in the necessary volume (e.g. 120 mL), as previously calculated
- Volume of 'initial' cell suspension (X) needed to prepare the 'seeding' cell suspension is calculated using the following formula: $X = M / N$

Where:

- N = number of cells/mL in the 'initial' cell suspension obtained after detachment of cells from 75 cm² flasks
- M = number of cells needed for cell seeding in all BD Falcon 60x15 mm dishes (e.g. 8000 cells as in our example presented above)
- Add into a sterile bottle the calculated volume of complete culture medium needed to fill all dishes (e.g. 120 mL as in our example presented above)
- Remove X mL of medium from the bottle and add X mL of the 'initial' cell suspension.
- Gently mix the obtained cell suspension using a 10 mL pipette
- Aspirate 9 mL of cell suspension using a 10 mL pipette
- Fill 3 BD Falcon 60x15 mm dishes with 3 mL suspension each (9 mL in total)
- Put the dishes in the incubator
- Repeat the procedure until when all the dishes have been filled. It is important to mix (aspirating at least 3 times) the cell suspension before taking the new 9 mL aliquot of cells for seeding
- Leave the cell cultures in the incubator for 24 h

To have an accurate and reproducible cell seeding in all dishes, it is extremely important to be precise in all the steps described above.

Day 2: Exposure to nanoparticles and controls

- Twenty four hours after cell seeding, on Day 2, proceed to the exposure of the cells to the tested compounds/nanoparticles
- Prepare the suspensions of nanoparticles and the dilutions of solvent and positive controls
- For each treatment condition, remove an adequate amount of volume from the dish
- Add directly to the medium in the dish the adequate volume of previously prepared suspension of nanoparticles, or solution of solvent or positive controls. Nothing is added to the medium in the negative control dishes
- Assure a homogenous distribution by swirling the dish slightly in a circular way

Day 5: End of exposure (72 h)

- Remove the exposure medium from the dishes (3 mL/dish)
- Add 3 mL/dish of fresh complete cell culture medium

Day 8: Fixation and staining

- Prepare the fixing solution in PBS by adding 10% (v/v) of the Formaldehyde solution (ACS reagent, 37 (w/v) % in H₂O)
e.g. 50 mL Formaldehyde solution + 450 mL PBS
- Prepare the staining solution in MilliQ water by adding 10% (v/v) of the Giemsa Stain Modified solution

e.g. 50 mL Giemsa Stain Modified solution + 450 mL MilliQ water)

[Note: This solution has to be freshly prepared each experiment]

- Filter the 10% (v/v) staining solution using a 75 mm Filter Unit -500 or 1000 mL (Nalgene nat. number 166-0045) or equivalent
- Remove the culture medium from the dishes aspirating it using a vacuum pump connected with an aspirator
- Add 3 mL of the fixing solution into each dish and incubate at room temperature for 15 min
- Remove the fixing solution
- Add 3 mL of the staining solution into each dish and incubate at room temperature for 30 min
- Remove the staining solution and leave the dishes to dry under the chemical hood overnight

Day 9: Counting of the colonies

- Count the number of all colonies present in each dish under a stereomicroscope.
- Verify whether the test acceptance criteria presented below are met:
 - The colonies have to contain more than 50 cells/each
 - The number of colonies in the negative control dish have to be not less than 90
 - The plating efficiency has to be not less than 45%. The plating efficiency is calculated as follows: $\text{Plating efficiency} = (\text{average number of colonies in the negative control or solvent control}) \times 100 / (\text{number of cells seeded})$
 - Exposure to the positive control must result in complete cell death (no colonies in the dish)

[Note: All acceptance criteria must be met for a test to be acceptable]

Data analysis

The raw data (i.e. number of colonies counted in each dish for each treatment condition and controls) are filled in a template provided by JRC and sent back to JRC for further analysis.

IV. HEALTH SAFETY AND ENVIRONMENT

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

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European Commission

EUR 27009 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: Interlaboratory comparison study of the Colony Forming Efficiency assay for assessing cytotoxicity of nanomaterials

Author(s): Jessica Ponti, Agnieszka Kinsner-Ovaskainen, Hedvig Norlén, Sandrine Altmeyer, Cristina Andreoli, Alessia Bogni, Sylvie Chevillard, Isabella De Angelis, Seung-Tae Chung, Igchun Eom, Katsuhide Fujita, Douglas Gilliland, Maria Giuseppa Grollino, Mary Gulumian, Cordula Hirsch, Kayo Ichiraku, Takuya Igarashi, Jayoung Jeong, Eunhye Jo, Dang-Young Kim, Jean-Pierre Kaiser, Doris Lagache, Rita La Spina, Jong Kwon Lee, Jieun Lee, Annalisa Lovera, Xenia Mäder-Althaus, Fabrice Nesslany, Isaac Ojea Jimenez, Francesca Pacchierotti, Francesca Pianella, Vincent Paget, Tae Rim Kim, Joanna Roszak, Philipp Rosenkranz, Sophie Simar, Maciej Stępnik, Melissa Vetten, Nam Woong Song, Jun-Young Yang, François Rossi

Luxembourg: Publications Office of the European Union

2014 – 76 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424 (online)

ISBN 978-92-79-44677-1 (PDF)

doi:10.2788/406937

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ISBN 978-92-79-44677-1

