
**Nanotechnologies — Testing
the photocatalytic activity of
nanoparticles for NADH oxidation**

*Nanotechnologies — Test de l'activité photocatalytique des
nanoparticules pour l'oxydation du NADH*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Photocatalytic activity (PCA) is the measure of capacity of a material to promote a specific photochemical reaction under defined conditions (as defined in ISO 20507:2014, 2.3.31). With the expanding use of nanomaterials in various industries, the possible impacts on human health and the environment due to the enhancement of detrimental chemical reactions in the presence of light (both natural and artificial) is an ongoing concern. The absorption of a photon with sufficient energy generates an electron-hole pair that can migrate to the nanoparticle (NP) surface and react with water and oxygen, thus forming extremely reactive radicals and reactive oxygen species (ROS). Generation of the ROS by some wide-bandgap materials, such as TiO_2 , ZnO , WO_3 , CeO_2 , carbon nanotubes, quantum dots and some metal NPs when illuminated by UV-VIS light, can cause oxidative stress, resulting in toxic effects in living organisms^[5]. Therefore, measuring the nanomaterial PCA under physiological conditions allows for an assessment of its photo-toxicity potency.

Existing standard test methods for particle and surface PCA measurement (see ISO 10676 and ISO 10678) are not directly applicable to determine nanomaterial PCA leading to photo-toxicity, as they require a large test volume and/or long measurement duration, while utilizing organic dyes as indicators that are not biocompatible.

The in vitro NP PCA test for NADH oxidation is intended to evaluate the nanomaterial photo-toxicity potency when exposed to an ultraviolet (UV) light.

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Nanotechnologies — Testing the photocatalytic activity of nanoparticles for NADH oxidation

1 Scope

This document specifies a method for the measurement of the photocatalytic activity (PCA) of nanoparticles (NPs), suspended in an aqueous environment in physiologically relevant conditions, by measuring the ultraviolet (UV)-induced nicotine adenine dinucleotide hydrate (NADH) oxidation.

The measurement is intended to assess the potential for the photo-toxicity of nanomaterials. The method is also applicable to NP aggregates and agglomerates.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms, definitions, symbols and abbreviated terms

For the purposes of this document, the terms and definitions given in ISO/TS 80004-1, ISO/TS 80004-2 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1 Terms and definitions

3.1.1

actinometry

method to measure the number of photons integrally or per unit of time

3.1.2

catalytic activity

property of a component corresponding to the catalysed substance rate of conversion of a specified chemical reaction, in a specified measurement system

[SOURCE: ISO 18153:2003, 3.2, modified — The notes have been deleted.]

3.1.3

oxidation

chemical reaction accompanying a gain of oxygen, loss of hydrogen of an organic substrate or loss of one or more electrons from a molecular entity

3.1.4

NADH equivalent specific PCA

PCA measured as the NADH *photo-oxidation* (3.1.5) rate per unit weight of nanoparticles

3.1.5

photo-oxidation

oxidation reactions induced by light

3.2 Symbols and abbreviated terms

DIW	deionized water with $\geq 18 \text{ M}\Omega\cdot\text{cm}$ resistivity
NADH	nicotine adenine dinucleotide hydrate
NaOH	sodium hydroxide
2NB	2-nitrobenzaldehyde
NP	nanoparticle
PB	phosphate buffer
PCA	photocatalytic activity
ROS	reactive oxygen species
TI	trans-illuminator
TiO ₂	titanium dioxide
UV	ultraviolet
UV-Vis	ultraviolet and visible
$A_c(i,j)$	phenolphthalein absorbance before exposure to trans-illuminator UV irradiation in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 10, 11$)
$A_e(i,j)$	phenolphthalein absorbance after exposure to trans-illuminator UV irradiation in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 10, 11$)
$\Delta A(i,j)$	change in phenolphthalein absorbance after exposure to trans-illuminator UV irradiation in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 10, 11$)
ΔA_a	average change of phenolphthalein absorbance over all wells before and after UV irradiation by using a UV trans-illuminator
C_0	starting concentration of the NP suspension for a dilution series of test solutions; the suspension absorbance at 310 nm or 365 nm (depending on the used UV trans-illuminator) is $1.4 < A < 1.6$
$C(i,j)$	light intensity correction factor of each well, which accounts for the UV irradiation intensity variation of the UV trans-illuminator at the location of each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 8, 9$)
$I_{F,0}(i,j)$	NADH fluorescence intensity measured before UV irradiation in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 8, 9$)
$I_{F,t}(i,j)$	NADH fluorescence intensity measured following the UV irradiation of t duration by using a UV trans-illuminator in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 8, 9$)
$k_{\text{app}}(i,j)$	apparent NADH photo-oxidation rate in each well, expressed in $\mu\text{mol}/\text{min}$ ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 8, 9$)
λ_{exc}	excitation wavelength used to record fluorescence in multiple well plate readers
λ_{ems}	emission wavelength used to record fluorescence in multiple well plate readers
$A \lambda(\text{max})$	maximum absorbance of NP suspension in a wavelength range from 300 nm to 800 nm
$\lambda(\text{max, TI})$	wavelength at which a UV trans-illuminator provides the maximum intensity of light
$S(i,j)$	slope of the NADH fluorescence intensity versus the UV irradiation time in each well ($i = \text{B, C, D, E, F, G}; j = 2, 3, 4, \dots, 8, 9$)
$S_c(i,j)$	$S(i,j)$ corrected for the trans-illuminator light intensity variation at each well
b	slope of k_{app} versus NP concentration in the linear range, expressed in units of $\text{mmol}/\text{min}\cdot\text{g}$

4 Description of the test method

In this document, the PCA of NPs in an aqueous suspension is measured as the photo-oxidation rate of NADH present in the NP suspension. By observing the NADH fluorescence intensity decrease before and after successive irradiation with artificial UV light, the fraction of the oxidized NADH due to the photocatalytic action of NPs can be measured. The photo-oxidation rate of NADH is determined at several NP concentrations with a dilution series and the NP concentration range showing the linear dependence of the photo-oxidation rate of NADH versus NP concentration is determined^[6]. The photo-oxidation slope in this linear range provides the NADH photo-oxidation rate per unit of NP concentration in the aqueous suspension. The multiplexed assay utilizes a 96-well plate, UV trans-illuminator and a multiple-plate optical reader leading to a fast and accurate measurement. The 96-well platform allows for a simultaneous measure of a range of NP concentrations and provides an option to compare with reference NPs as positive and negative controls. It accounts for the spontaneous NADH photo-oxidation under UV illumination in the absence of NPs and intrinsic NP fluorescence.

5 Reagents and apparatus

5.1 Reagents

5.1.1 NADH, β -Nicotinamideadenine dinucleotide, reduced disodium salt, CAS Number: 606-68-8.

a) Stock solution of NADH:

- 1) dissolve approximately 35 mg of NADH in 10 ml of 5 mmol/l phosphate buffer, pH = 8.

b) Working solutions of NADH:

- 1) dilute a stock solution of NADH by a factor of 20 into 5 mmol/l, pH = 8 phosphate buffer;
- 2) the resulting concentration of the working NADH solution will be about 250 μ mol/l;
- 3) verify the working NADH concentration [NADH] by measuring the absorbance of the solution at $\lambda = 339$ nm. If necessary, adjust the NADH concentration by diluting with a 5 mmol/l phosphate buffer or by adding a NADH stock solution until absorbance $A_{339} = 1,56 \pm 0,05$.

5.1.2 2-nitrobenzaldehyde (2NB), CAS Number: 552-89-6.

- a) Prepare 50 ml 0,1 mol/l solution of 2NB by dissolving 0,756 g of the dry 2NB in 50 ml of 50/50 DIW/ethyl alcohol (by volume).
- b) Adjust the 0,1 mol/l 2NB solution to pH = $12 \pm 0,2$ by adding 0,03 mol/l NaOH.

5.1.3 Phenolphthalein, CAS Number: 77-09-8.

- a) Prepare 20 ml of stock solution of phenolphthalein by dissolving 20 mg of dry phenolphthalein in 20 ml of 50/50 DIW/ethyl alcohol.
- b) Add 100 μ l of phenolphthalein stock [prepared in step a)] to a 50 ml 0,1 mol/l 2NB solution (prepared as per 5.1.2). The solution acquires pink colour.
- c) Store the solution in light-protected bottle (amber glass or wrapped in Al foil).

5.1.4 Phosphate buffer, sodium phosphate monobasic/dibasic solution for pH buffer of pH 8 (5 mmol/l PB at pH 8).

EXAMPLE The phosphate buffer is prepared as follows.

Step 1: Dissolve 1,261 g of disodium phosphate, heptahydrate (CAS Number: 7782-85-6) in 1 l of DIW.

Step 2: Add 0,041 g of monosodium phosphate, monohydrate (CAS Number: 10049-21-5) to the solution prepared in Step 1.

Step 3: Measure the solution pH, following the complete dissolution of salts.

5.1.5 NP suspension.

- Prepare 50 ml of NP suspension in a 5 mmol/l phosphate buffer according to the recommended dispersion protocol for the particular nanomaterial (e.g. Reference [7]).
- Adjust the NP dispersion concentration C_0 so that the highest absorbance reading of the NP dispersion in a range of 300 nm to 800 nm is $1,4 < A < 1,6$. Calculate the stock solution NP concentration C_0 (in mg/l) following the adjustment.
- From the mass concentration of the prepared stock NP suspension, dilution factors for the preparation of the target concentration of working suspensions can be calculated.

5.1.6 Ethyl alcohol, anhydrous, > 99,5 % pure, less than 0,005 % water. CAS Number: 64-17-5.

5.2 Apparatus

5.2.1 UV-Vis spectrophotometer, wavelength range: 190 nm to 800 nm, absorbance range: 0,1 to 3,0.

5.2.2 Cuvette for UV-Vis absorption measurement, quartz or optical glass, 1 cm optical path length.

5.2.3 96-well plate, [flat bottom surface transparent at $\lambda(\text{max, TI})$: $T > 60 \%$], dark plastic sides preferable.

5.2.4 Microplate absorbance and fluorescence reader, capable of absorbance and fluorescence measurement in a range from 300 nm to 800 nm.

5.2.5 Multi-pipette loader, which has at least six channels with at least 300 μl channel capacity.

5.2.6 300 μl pipette tips, compatible with the multi-pipette loader.

5.2.7 UV trans-illuminator, 365 nm light source with a horizontal illumination area larger or equal to the 96-well plate.

6 Measurement procedure

6.1 Measurement of NP suspension basic properties

6.1.1 UV-Vis absorption spectrum measurement

- Measure the UV-VIS absorption spectrum of the NP suspension (see 5.1.5) in a range from 300 nm to 800 nm in a 10 mm optical path-length quartz or optical glass spectrophotometer cuvette against 5 mmol/l PB as reference.

NOTE Filling a standard 10 mm cuvette usually requires around 3 ml of sample.

Preferably, use the same cuvette for both the reference and the sample.

- If the absorbance of suspension at $\lambda(\text{max})$ [$A \lambda(\text{max})$] exceeds 1,6, dilute the suspension with a phosphate buffer (5 mmol/l PB, pH = 8) until $1,4 < A \lambda(\text{max}) < 1,6$. If it's below 1,4, increase the NP concentration accordingly.

6.1.2 NP suspension stability measurement

NOTE This is required to ensure NPs stay suspended during the measurement duration.

- Measure the baseline (reference absorbance) using 5 mmol/l PB, pH = 8,0.
- Measure the UV-Vis absorption spectrum of the NP working suspension, with the concentration adjusted in accordance with 6.1.1.
- Wait for 20 min while maintaining the cuvette in the spectrophotometer, then re-measure the UV-VIS absorption spectrum of the working suspension.
- Compare the two spectra and verify that a change in the maximum absorbance is less than 5 % at $\lambda(\text{max})$. The NP working suspension is not regarded as stable if absorbance at $\lambda(\text{max})$ decreases more than 5 % over 20 min.

6.2 UV trans-illuminator light intensity calibration based on 2NB actinometry

- Prepare a 50 ml 0,1 mol/l solution of 2NB, containing phenolphthalein in accordance with 5.1.2 and 5.1.3. Use an amber glass container to store the solution.
- Fill each well of the 96-well plate with 300 μl of solution, as prepared in a).
- Place the 96-well plate in the reader, programme it to shake the plate for 5 s, and measure and record the absorbance at 540 nm – $A_c(i,j)$.

WARNING — Observe that it is positioned at the same location and orientation as during the NADH/NP UV exposure. Follow the directions in Annex A for the plate positioning.

- Turn on the trans-illuminator [$\lambda = \lambda(\text{max}, \text{TI})$] and warm up for 30 min.
- Position the 96-well plate on the trans-illuminator.
- Expose the plate to UV light for 10 min.
- After 10 min, turn off the trans-illuminator, and measure and record the absorbance at 540 nm using the 96-well plate reader – $A_e(i,j)$.
- Subtract the absorbance values, recorded in step c), from the absorbance values recorded in step g) for each well, as shown by Formula (1):

$$\Delta A(i,j) = A_e(i,j) - A_c(i,j) \quad (1)$$

- Calculate the average differential absorbance, as shown by Formula (2):

$$\Delta A_a = \sum \Delta A(i,j) \text{ all 60 working wells} / 60 \quad (2)$$

- Calculate the light intensity correction factors for light intensity at each well, as shown by Formula (3):

$$C(i,j) = \Delta A_a / \Delta A(i,j) \quad (3)$$

- The light intensity correction factors for individual wells will be multiplied by the slope of NADH fluorescence decrease, calculated as shown by Formula (5) in 6.3.2.3.

A sample calibration of UV trans-illuminator light intensity is given in Annex B.

6.3 Measurement of NADH solution fluorescence intensity

6.3.1 NADH photo-oxidation rate measurement at various NP concentrations

6.3.1.1 Prepare the 250 $\mu\text{mol/l}$ NADH solution in accordance with 5.1.1.

6.3.1.2 Prepare the stock NP suspension (using the appropriate dispersion procedure, see 5.1.5) $C_0 = 100\%$. The absorbance at $\lambda(\text{max})$ (in a range from 300 nm to 800 nm) should be $1,4 < A < 1,6$.

6.3.1.3 Prepare the dilution (by volume) series of NP suspension (each 2 ml using a phosphate buffer) at concentrations (relative to $C_0 = 100\%$). See 6.3.1.2: 100 %, 80 %, 60 %, 40 %, 20 %, 10 %, 8 %, 5 %.

6.3.1.4 Fill wells marked in white with 100 μl of 250 $\mu\text{mol/l}$ NADH solution, as shown in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	●	●	●	●	●	●	●	●	●	●	●	●
B	●	○	○	○	○	○	○	○	○	○	○	○
C	●	○	○	○	○	○	○	○	○	○	○	○
D	●	○	○	○	○	○	○	○	○	○	○	○
E	●	○	○	○	○	○	○	○	○	○	○	○
F	●	○	○	○	○	○	○	○	○	○	○	○
G	●	○	○	○	○	○	○	○	○	○	○	○
H	●	○	○	○	○	○	○	○	○	○	○	○

NOTE Wells marked in white contain 100 μl of 250 $\mu\text{mol/l}$ NADH solution and: a buffer for col. 2; 100 μl NP suspension at C_0 for col. 3; 100 μl NP suspension at 80 % C_0 for col. 4; 100 μl NP suspension at 60 % C_0 for col. 5; 100 μl NP suspension at 40 % C_0 for col. 6; 100 μl NP suspension at 20 % C_0 for col. 7; 100 μl NP suspension at 10 % C_0 for col. 8; 100 μl NP suspension at 8 % C_0 for col. 9; 100 μl NP suspension at 5 % C_0 for col. 10; 100 μl NP suspension at $C_0 + 100\ \mu\text{l}$ buffer (5 mmol/l PB, pH = 8) solution for col. 11.

Figure 1 — Schematic diagram of a 96-well plate for NADH photo-oxidation rate measurement at various NP concentrations

6.3.1.5 Add 100 μl of the NP suspension dilution series and fill the wells as described below. Mix the solutions in the individual wells by pipetting in and out at least three times.

- A1, B1, ..., G1, H1 (column): No use.
- A2, A3, ..., A10, A11 (row): No use.
- B2, C2, D2, E2, F2, G2: Blank buffer.
- B3, C3, D3, E3, F3, G3: NP suspension at C_0 .
- B4, C4, D4, E4, F4, G4: NP suspension at 80 % of C_0 .
- B5, C5, D5, E5, F5, G5: NP suspension at 60 % C_0 .
- B6, C6, D6, E6, F6, G6: NP suspension at 40 % C_0 .
- B7, C7, D7, E7, F7, G7: NP suspension at 20 % C_0 .
- B8, C8, D8, E8, F8, G8: NP suspension at 10 % C_0 .

- B9, C9, D9, E9, F9, G9: NP suspension at 8 % C_0 B10, C10, D10, E10, F10, G10: NP suspension at 5 % C_0 .
- B11, C11, D11, E11, F11, G11: 100 μ l NP suspension at C_0 + 100 μ l buffer (5 mmol/l PB, pH = 8) solution.
- A12, B12, ..., G12, H12 (column): No use.
- H2, H3, ..., H10, H11 (row): No use.

6.3.1.6 Measure and record the fluorescence intensity values of individual wells ($\lambda_{exc} = 340$ nm and $\lambda_{ems} = 460$ nm). The values shall be called $IF_{0(i,j)}$, where i is the row number assigned as B to G, and j is the column number assigned as 2 to 11.

6.3.1.7 Place the 96-well plate on the UV trans-illuminator and turn on the lamp for 1 min.

The UV-trans-illuminator should be switched on for 30 min prior to plate exposure.

Make sure that the plate is positioned at the same location and orientation during repetitive UV exposure. See [Annex A](#) for a description of the plate positioner.

6.3.1.8 Measure and record the fluorescence intensity values of individual wells. The values shall be called $IF_{t(i,j)}$, where t is the exposure duration to UV.

6.3.1.9 Repeat the steps in [6.3.1.7](#) and [6.3.1.8](#) until the fluorescence intensities in wells B6, C6, D6, E6, F6 and G6 decrease below 50 % of their initial values (prior to the UV exposure).

6.3.2 Calculation of NADH photo-oxidation rate at various NP concentrations

6.3.2.1 Normalize the measured fluorescence intensities of individual wells, $IF_{t(i,j)}$, as a function of UV illumination time with $IF_{0(i,j)}$ values as shown by [Formula \(4\)](#):

$$I_{F,t,N}(i,j) = I_{F,t}(i,j) / I_{F,0}(i,j) \quad (4)$$

where

i is B to G;

j is 2 to 11.

6.3.2.2 Plot the normalized intensities of each well, $I_{F,t,N}(i,j)$, as a function of the illumination time and perform a linear regression in the range where the intensity decrease is linearly dependent on the illumination time.

6.3.2.3 Calculate the slope of each well, $S(i,j)$, and multiply by $C(i,j)$, obtained from the procedure in [6.2](#), to get UV illumination intensity calibrated slopes as shown by [Formula \(5\)](#):

$$S_C(i,j) = S(i,j) \times C(i,j) \quad (5)$$

6.3.2.4 Calculate the NADH photo-oxidation rate at each well, $k_{app}(i,j)$, as shown by [Formula \(6\)](#), using [NADH] value, obtained from the absorbance measurement as in [5.1.1](#):

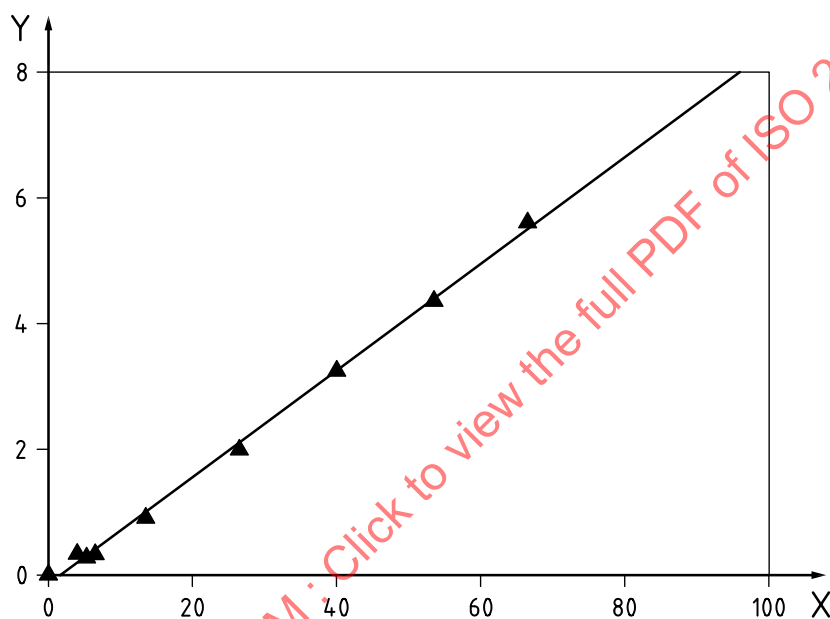
$$k_{app}(i,j) = S_C(i,j) \times [NADH] \quad (6)$$

6.3.2.5 Calculate the column averages and standard deviations of the k_{app} values for each NP concentration. Normalize k_{app} with respect to intrinsic NADH photo-oxidation by calculating a ratio $k_{app}(i,j)/k_{app}(2,j)$, where $k_{app}(2,j)$ is the value for NADH only (column 2 data). The average and the standard deviations of k_{app} at a particular NP concentration can be calculated using a single column reading.

Discard the data if the slope $Sc(i,j) > 0$. Report as $k_{app}(i,j) = 0$.

6.3.2.6 Plot the normalized k_{app} values versus NP concentrations, expressed in mg/l. An example of a rate constant versus concentration plot is shown in [Figure 2](#).

6.3.2.7 The slope of k_{app} versus NP concentration in the linear range (*b*) gives the NADH photo-oxidation rate per unit weight of NP in units of mmol/min·g. This value can be termed as the “NADH equivalent specific PCA” of NP.



Key

X NP, in mg/l

Y k_{app} , in $\mu\text{M}/\text{min}$

Figure 2 — NADH photo-oxidation rate versus NP concentration

7 Test report

7.1 Information

The test report shall include the following information:

- a reference to this document, i.e. determined in accordance with ISO 20814:2019;
- the name of the testing laboratory;
- full details concerning the sample (manufacturer's name, manufacturing date, batch no., purity, particle size, intended application, SDS);
- if used, full details concerning the negative control (manufacturer's name, manufacturing date, batch no., purity, particle size, SDS);

- if used, full details concerning the positive control (manufacturer's name, manufacturing date, batch no., purity, particle size, SDS);

NOTE Control data is acquired following the full measurement protocol as outlined above.

- the absorbance spectrum of stock NP suspension in 5 mmol/l phosphate buffer, in accordance with 6.1.2;
- the manufacturer's name and purity of NADH;
- the manufacturer's name and purity of phenolphthalein;
- the manufacturer's name and purity of 2NB;
- the manufacturer's name and purity of ethyl alcohol;
- the manufacturer's name and model number of the transilluminator;
- the manufacturer's name and model number of the 96-well plate;
- the manufacturer's name and model number of the 96-well plate reader;
- the detailed description of the 96-well plate reader settings used for absorbance and fluorescence measurements;
- records of observations;
- assessment of the results, including statistical methods.

7.2 Report data format

7.2.1 Correction factors $C(i,j)$ obtained by actinometry (see 7.2) with $\lambda(\text{max,TI})$

The $C(i,j)$ values, light intensity correction factors for each well, are shown in Table 1.

Table 1 — Light intensity correction factors

	1	2	3	4	5	6	7	8	9	10	11	12
A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	NA	$C(B,2)$	$C(B,3)$	$C(B,4)$	$C(B,5)$	$C(B,6)$	$C(B,7)$	$C(B,8)$	$C(B,9)$	$C(B,10)$	$C(B,11)$	NA
C	NA	$C(C,2)$	$C(C,3)$	$C(C,4)$	$C(C,5)$	$C(C,6)$	$C(C,7)$	$C(C,8)$	$C(C,9)$	$C(C,10)$	$C(C,11)$	NA
D	NA	$C(D,2)$	$C(D,3)$	$C(D,4)$	$C(D,5)$	$C(D,6)$	$C(D,7)$	$C(D,8)$	$C(D,9)$	$C(D,10)$	$C(D,11)$	NA
E	NA	$C(E,2)$	$C(E,3)$	$C(E,4)$	$C(E,5)$	$C(E,6)$	$C(E,7)$	$C(E,8)$	$C(E,9)$	$C(E,10)$	$C(E,11)$	NA
F	NA	$C(F,2)$	$C(F,3)$	$C(F,4)$	$C(F,5)$	$C(F,6)$	$C(F,7)$	$C(F,8)$	$C(F,9)$	$C(F,10)$	$C(F,11)$	NA
G	NA	$C(G,2)$	$C(G,3)$	$C(G,4)$	$C(G,5)$	$C(G,6)$	$C(G,7)$	$C(G,8)$	$C(G,9)$	$C(G,10)$	$C(G,11)$	NA
H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA: Not applicable.

NOTE $\lambda(\text{max,TI})$ is needed to determine which data groups have to be compared with each other. The data obtained using UV trans-illuminator having $\lambda(\text{max,TI})$ at 312 nm cannot be directly compared with those obtained using TI of $\lambda(\text{max,TI})$ at 365 nm.

7.2.2 Calibrated slope of NADH fluorescence decrease

The $S_c(i,j)$ values (see 6.3.2.3), the calibrated slope of NADH fluorescence intensity versus UV irradiation time of each well, are shown in Table 2.

Table 2 — Calibrated slope of NADH fluorescence intensity versus UV irradiation time

	1	2	3	4	5	6	7	8	9	10	11	12
A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	NA	$S_c(B,2)$	$S_c(B,3)$	$S_c(B,4)$	$S_c(B,5)$	$S_c(B,6)$	$S_c(B,7)$	$S_c(B,8)$	$S_c(B,9)$	$S_c(B,10)$	$S_c(B,11)$	NA
C	NA	$S_c(C,2)$	$S_c(C,3)$	$S_c(C,4)$	$S_c(C,5)$	$S_c(C,6)$	$S_c(C,7)$	$S_c(C,8)$	$S_c(C,9)$	$S_c(C,10)$	$S_c(C,11)$	NA
D	NA	$S_c(D,2)$	$S_c(D,3)$	$S_c(D,4)$	$S_c(D,5)$	$S_c(D,6)$	$S_c(D,7)$	$S_c(D,8)$	$S_c(D,9)$	$S_c(D,10)$	$S_c(D,11)$	NA
E	NA	$S_c(E,2)$	$S_c(E,3)$	$S_c(E,4)$	$S_c(E,5)$	$S_c(E,6)$	$S_c(E,7)$	$S_c(E,8)$	$S_c(E,9)$	$S_c(E,10)$	$S_c(E,11)$	NA
F	NA	$S_c(F,2)$	$S_c(F,3)$	$S_c(F,4)$	$S_c(F,5)$	$S_c(F,6)$	$S_c(F,7)$	$S_c(F,8)$	$S_c(F,9)$	$S_c(F,10)$	$S_c(F,11)$	NA
G	NA	$S_c(G,2)$	$S_c(G,3)$	$S_c(G,4)$	$S_c(G,5)$	$S_c(G,6)$	$S_c(G,7)$	$S_c(G,8)$	$S_c(G,9)$	$S_c(G,10)$	$S_c(G,11)$	NA
H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA: Not applicable.

7.2.3 Plot of k_{app} versus NP concentration

The plot of k_{app} versus NP concentration shall be drawn to determine linear concentration range (see 6.3.2.6). Linear regression from the lowest-used NP concentration to the highest where linearity is confirmed (see Figure 2) shall be performed for the k_{app} versus NP concentration slope calculation.

7.2.4 NADH equivalent specific PCA

7.2.4.1 The slope of k_{app} versus NP concentration in the linear range b for the test sample, expressed in mmol/min·g, is the NADH photo-oxidation rate per unit weight of NP and is termed as the “NADH equivalent specific PCA” of NP. The record should contain the average value of six technical replicates (rows B through G of the 96-well plate) $\pm$ uncertainty, expressed as one standard deviation.

7.2.4.2 The slope of k_{app} versus NP concentration in the linear range b for the negative control, expressed in mmol/min·g, is the NADH photo-oxidation rate per unit weight of NP and is termed as the “NADH equivalent specific PCA” of NP. The record should contain the average value of six technical replicates (rows B through G of the 96-well plate) $\pm$ uncertainty, expressed as one standard deviation.

7.2.4.3 The slope of k_{app} versus NP concentration in the linear range b for the positive control, expressed in mmol/min·g, is the NADH photo-oxidation rate per unit weight of NP and is termed as the “NADH equivalent specific PCA” of NP. The record should contain the average value of six technical replicates (rows B through G of the 96-well plate) $\pm$ uncertainty, expressed as one standard deviation.

8 Precision

8.1 Repeatability

The absolute difference between two independent measurements carried out within a short time period, for the same sample in the same laboratory by the same operator using the same experimental and analytical measuring device, will not exceed the repeatability limit, r , in more than 5 % of cases.

Typical precision data for six different laboratories obtained in an interlaboratory test are given in Annex C.

8.2 Reproducibility

The absolute difference between two individual measurements carried out using the same test method, for the same sample in different laboratories by different operators using different experimental and analytical measuring devices, will not exceed the reproducibility limit, R , in more than 5 % of cases.

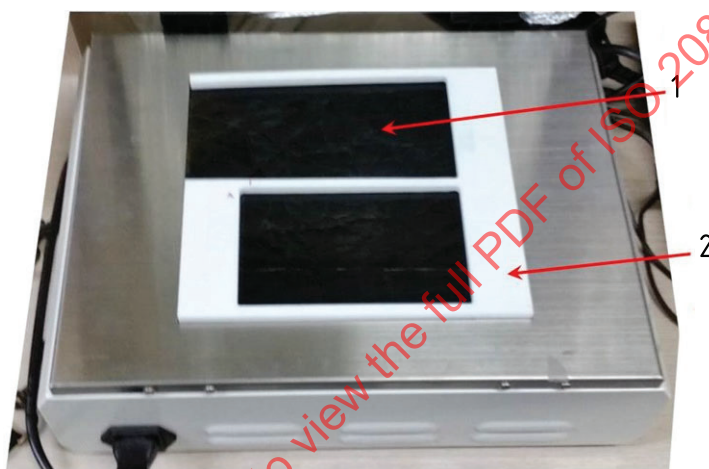
Typical precision data for six different laboratories obtained in an interlaboratory test are given in Annex C.

Annex A (normative)

Schematic diagram of 96-well positioning block

The block ensures the positioning of a 96-well plate on a UV trans-illuminator at the same location, as shown by [Figure A.1](#). Mark the plate orientation to avoid a 180-degree rotation over the vertical axis.

The mask can be polytetrafluoroethylene (PTFE), aluminium foil or thick black paper.



Key

- 1 position for a 96-well plate (UV irradiation)
- 2 masking block

Figure A.1 — 96-well plate

Annex B (informative)

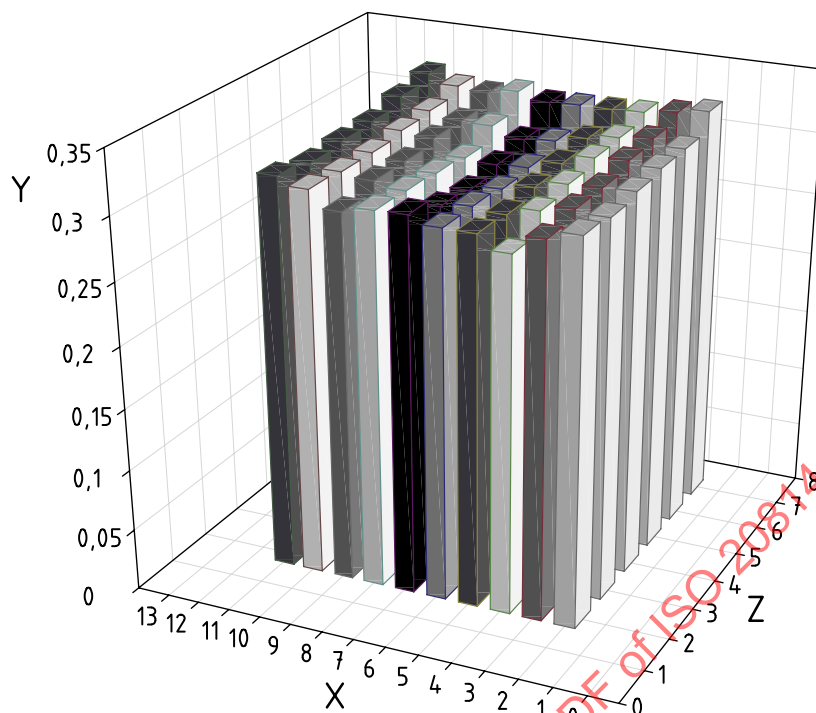
Sample calibration of UV trans-illuminator light intensity

The following steps show a sample calibration of UV trans-illuminator light intensity.

- a) 50 ml of 100 mmol/l solution of 2NB is used, as prepared in accordance with [5.1.2](#). The solution is stored in an amber glass container.
- b) Each well of the 96-well plate is filled with 300 µl of solution, as prepared in a).
- c) The 96-well plate is placed in the reader, shaken for 5 s, and the absorbance at 540 nm is recorded for each of the 60 wells. An example of the initial 2NB absorbance shown in [Table B.1](#). A graphical representation is shown in [Figure B.1](#).

Table B.1 — Initial absorbance of the 2NB

$A_c(i,j)$	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0,308 0	0,301 0	0,286 0	0,297 0	0,297 0	0,303 0	0,302 0	0,297 0	0,310 0	0,317 0	
C		0,305 0	0,307 0	0,301 0	0,295 0	0,297 0	0,293 0	0,301 0	0,305 0	0,308 0	0,312 0	
D		0,309 0	0,307 0	0,306 0	0,300 0	0,294 0	0,290 0	0,299 0	0,305 0	0,308 0	0,314 0	
E		0,311 0	0,312 0	0,311 0	0,303 0	0,296 0	0,295 0	0,296 0	0,308 0	0,311 0	0,314 0	
F		0,311 0	0,315 0	0,312 0	0,308 0	0,302 0	0,300 0	0,307 0	0,308 0	0,313 0	0,321 0	
G		0,326 0	0,320 0	0,318 0	0,317 0	0,317 0	0,316 0	0,321 0	0,317 0	0,319 0	0,326 0	
H												

**Key**

X columns

Y absorbance at 540 nm, cm⁻¹

Z rows

NOTE Each well contains 300 µl of 2NB solution.

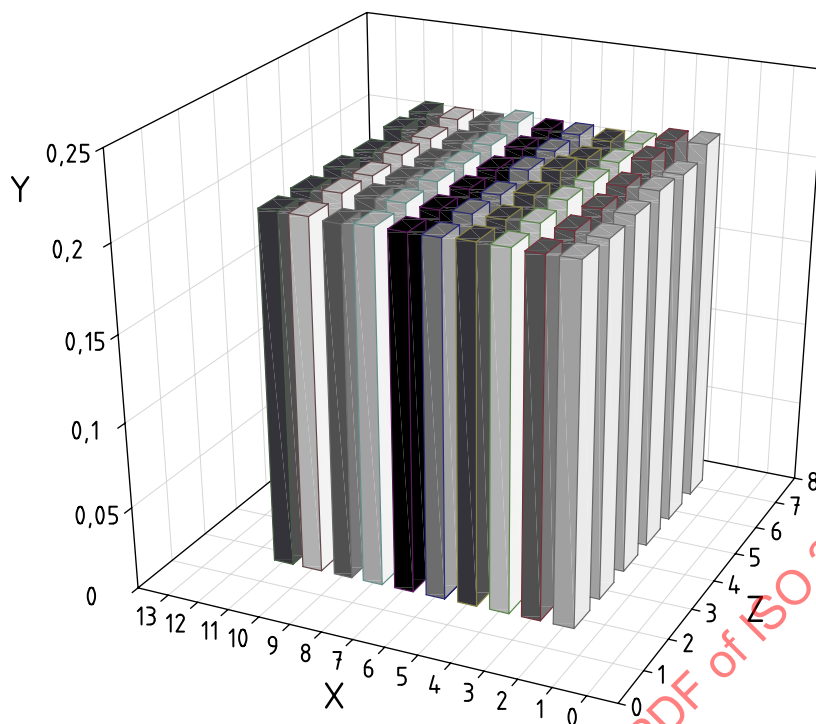
Figure B.1 — Graphical representation of the initial absorbance of a 100 mmol/l solution of 2NB in a 96-well plate ($\lambda = 540$ nm)

The 96-well plate is positioned on the trans-illuminator using a plate positioner.

- d) The trans-illuminator is turned on ($\lambda = 365$ nm) and the plate is exposed for 10 min.
- e) After 10 min, the trans-illuminator is turned off and absorbance is measured at 540 nm using the 96-well plate reader (see [Table B.2](#)). A graphical representation is shown in [Figure B.2](#).

Table B.2 — Absorbance of 2NB following a 10-min exposure to UV light

$A_e(i,j)$	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0,208 0	0,207 0	0,208 0	0,208 0	0,207 0	0,207 0	0,207 0	0,206 0	0,207 0	0,207 0	
C		0,207 0	0,208 0	0,209 0	0,208 0	0,208 0	0,208 0	0,208 0	0,208 0	0,208 0	0,207 0	
D		0,208 0	0,208 0	0,210 0	0,210 0	0,207 0	0,208 0	0,209 0	0,207 0	0,207 0	0,208 0	
E		0,210 0	0,209 0	0,209 0	0,213 0	0,210 0	0,208 0	0,208 0	0,207 0	0,208 0	0,207 0	
F		0,208 0	0,213 0	0,209 0	0,208 0	0,210 0	0,209 0	0,208 0	0,207 0	0,207 0	0,210 0	
G		0,214 0	0,213 0	0,209 0	0,208 0	0,209 0	0,209 0	0,211 0	0,208 0	0,208 0	0,209 0	
H												

**Key**

X columns

Y absorbance at 540 nm, cm⁻¹

Z rows

NOTE Each well contains 300 µl of 2NB solution.

Figure B.2 — Graphical representation of a 100 mmol/l solution of 2NB in a 96-well plate at $\lambda = 540$ nm following a 10-min exposure at the trans-illuminator ($\lambda = 365$ nm)

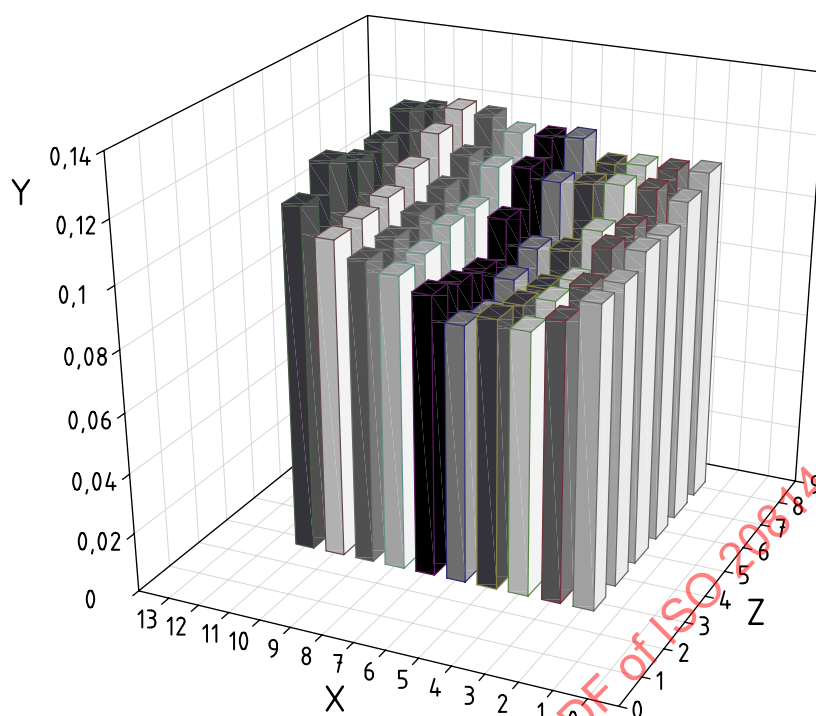
- f) Absorbance values, recorded in step e), are subtracted from the absorbance values recorded in step c) for each well, as shown in [Formula \(B.1\)](#):

$$\Delta A(i,j) = A_c(i,j) - A_e(i,j) \quad (\text{B.1})$$

The results are given in [Table B.3](#). A graphical representation is shown in [Figure B.3](#).

Table B.3 — Differential absorbance of 2NB following a 10-min exposure to UV light

$\Delta A(i,j)$	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0,099 0	0,092 0	0,087 0	0,089 0	0,085 0	0,093 0	0,097 0	0,100 0	0,105 0	0,114 0	
C		0,099 0	0,096 0	0,090 0	0,087 0	0,082 0	0,090 0	0,098 0	0,101 0	0,106 0	0,122 0	
D		0,103 0	0,102 0	0,090 0	0,086 0	0,087 0	0,088 0	0,101 0	0,103 0	0,107 0	0,117 0	
E		0,102 0	0,103 0	0,100 0	0,092 0	0,091 0	0,099 0	0,101 0	0,106 0	0,111 0	0,118 0	
F		0,107 0	0,109 0	0,109 0	0,108 0	0,107 0	0,110 0	0,109 0	0,111 0	0,117 0	0,123 0	
G		0,111 0	0,110 0	0,110 0	0,109 0	0,116 0	0,115 0	0,115 0	0,119 0	0,120 0	0,118 0	
H												



Key

X columns
Y absorbance at 540 nm, cm^{-1}
Z rows

NOTE Each well contains 300 μl of 2NB solution.

Figure B.3 — Change in absorbance $\Delta A(i,j)$ of a 100 mmol/l solution of 2NB in a 96-well plate at $\lambda = 540 \text{ nm}$ following a 10-min exposure at the trans-illuminator ($\lambda = 365 \text{ nm}$)

The average differential absorbance is calculated as shown in [Formula \(B.2\)](#):

$$\Delta A_a = \sum \Delta A(i,j) \text{ all 60 working wells} / 60 = 0,102 \text{ 1} \quad (\text{B.2})$$

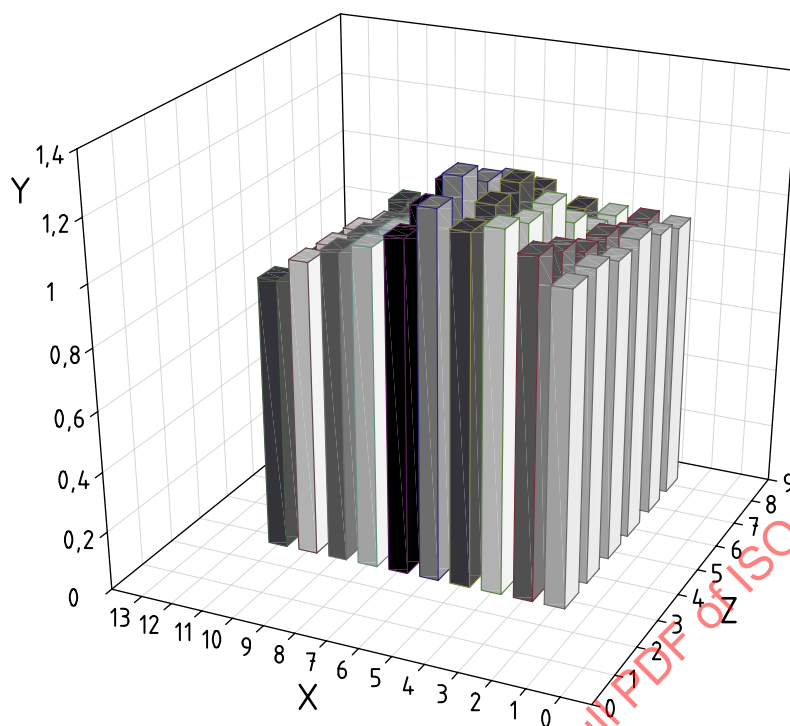
- g) The light intensity correction factors are calculated for each working well in accordance with [Formula \(B.3\)](#):

$$C(i,j) = \Delta A_a / \Delta A(i,j) \quad (\text{B.3})$$

The results are given in [Table B.4](#). A graphical representation is shown in [Figure B.4](#).

Table B.4 — Light intensity correction factors

$C(i,j)$	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1,031 3	1,109 8	1,173 6	1,147 2	1,201 2	1,097 8	1,052 6	1,021 0	0,972 4	0,895 6	
C		1,031 3	1,063 5	1,134 4	1,173 6	1,245 1	1,134 4	1,041 8	1,010 9	0,963 2	0,836 9	
D		0,991 3	1,001 0	1,134 4	1,187 2	1,173 6	1,160 2	1,010 9	0,991 3	0,954 2	0,872 6	
E		1,001 0	0,991 3	1,021 0	1,109 8	1,122 0	1,031 3	1,010 9	0,963 2	0,919 8	0,865 3	
F		0,954 2	0,936 7	0,936 7	0,945 4	0,954 2	0,928 2	0,936 7	0,919 8	0,872 6	0,830 1	
G		0,919 8	0,928 2	0,928 2	0,936 7	0,880 2	0,887 8	0,887 8	0,858 0	0,850 8	0,865 3	
H												



Key

X columns

Y correction factor

Z rows

NOTE Each well contains 300 µl of 2NB solution.

Figure B.4 — Light intensity correction factors for light intensity at each well

Annex C (informative)

Interlaboratory comparison study of TiO₂ NP PCA

C.1 Study format

A comparison study was conducted by six laboratories: NIST, KRISS, AIST, Thai Metrology Institute, University of Arizona and Chung Ang University (KOREA). KRISS provided three independent data sets for repeatability analysis (KRISS1, KRISS2 and KRISS3). Each participating laboratory was supplied with 10g of NIST SRM 1898 (titanium dioxide nano-powder) and dispersed the nanomaterial in an aqueous solution according to the protocol, published in Reference [7]. This procedure yields monomodal TiO₂ particle dispersion in PBS with a mean diameter of 75 nm. The material is a mixture of anatase (bandgap $E_g = 3,2$ eV) and rutile ($E_g = 3,02$ eV) phases in a ratio of about 3:1. Therefore, both components are activated with the UVA light from the trans-illuminator (peak wavelength $\lambda = 365$ nm, corresponding photon energy $E = 3,4$ eV). All other reagents and equipment were sourced locally.

NADH fluorescence intensity data, as measured at 1 min intervals by the 96-well plate reader, was collected and analysed by the NIST Statistical Engineering Division.

All six laboratories used the same layout on the 96-well plate. [Table C.1](#) shows a sample of the fluorescence intensity raw data at $t = 0$.

Measurements of fluorescence were taken of each well in six rows (technical replicates): columns 2 to 10 at time 0, and then at time 1, 2, 3, ..., 10 min for all six laboratories. A sample of the measurements at time 1 min is given in [C.2](#).

Table C.1 — Sample of the NADH fluorescence intensity, measured in a 96-well plate following a 1-min exposure to a 365 nm light

NADH	C_0	$0,8C_0$	$0,6C_0$	$0,4C_0$	$0,2C_0$	$0,1C_0$	$0,08C_0$	$0,06C_0$
11 667	4 844	5 356	6 173	7 216	8 842	9 718	10 012	10 245
11 649	4 963	5 505	6 275	7 218	8 956	9 885	10 078	10 877
11 509	5 018	5 575	6 067	7 119	8 811	9 948	10 218	10 178
11 484	4 965	5 498	5 925	7 168	9 209	9 702	9 941	10 205
11 528	4 823	5 371	5 973	7 254	9 046	9 950	9 676	10 239
11 456	4 445	5 165	6 007	7 052	8 621	9 886	9 674	10 391
NOTE C_0 refers to a stock TiO ₂ NP concentration prior to serial dilution.								

C.2 Data analysis

At each time point starting with minute 1, a proportion of fluorescence in the well with respect to the 0 min value in the same well was computed. This is called (p_{ijk}) , $i = 1, \dots, 9$, $j = 1, \dots, 6$, $k = 1, \dots, 10$. For example, at 1 min [where i is column(concentration), j is row(replicate), $k = 1$], the data in [Table C.1](#) becomes the data shown in [Table C.2](#).

Table C.2 — Sample of the NADH fluorescence intensity data at $t = 1$, divided by the data at $t = 0$

0,963 6	0,881 8	0,901 2	0,907 9	0,935 7	0,972 6	0,957 8	0,968 2	0,963 7
0,968 2	0,866 6	0,920 9	0,928 5	0,943 8	0,960 4	0,963 0	0,974 3	0,924 2
0,971 7	0,907 6	0,910 4	0,911 1	0,932 8	0,953 5	0,965 9	0,970 4	0,971 6
0,970 0	0,883 9	0,901 3	0,896 8	0,924 9	0,955 4	0,971 4	0,976 2	0,974 5
0,977 4	0,901 8	0,889 5	0,898 3	0,936 8	0,955 9	0,967 1	0,975 9	0,977 6
0,974 5	0,818 9	0,839 7	0,862 1	0,940 0	0,955 6	0,971 3	0,973 4	0,985 2

C.3 Data fitting

The measurements in each column (NP concentration) as a function of time can be fitted either by a change-point regression, i.e. two separate linear regressions: one which fits the data up to the change point, and one that fits after the change point. The slope of the early regression line is the parameter of interest.

$$i = 1, \dots, 9, j = 1, \dots, 6, k = 1, \dots, 10$$

$$p_{ijk} = (\alpha_i - \beta_{1i} \times \text{time.change}) + \beta_{1i} \times \text{time}_{ijk} \text{ for } \text{time}_{ijk} < \text{time.change},$$

$$= (\alpha_i - \beta_{2i} \times \text{time.change}) + \beta_{2i} \times \text{time}_{ijk} \text{ for } \text{time}_{ijk} \geq \text{time.change},$$

$$= \lambda_{1i} + \beta_{1i} \times \text{time}_{ijk} \text{ for } \text{time}_{ijk} < \text{time.change},$$

$$= \lambda_{2i} + \beta_{2i} \times 0_{ijk} \text{ for } \text{time}_{ijk} \geq \text{time.change}$$

The slopes of the first linear fit, as shown in [Figure C.1](#), were divided by the col 2 (NADH only) fluorescence decay slope and multiplied by the NADH concentration. The standard uncertainty of the result is obtained by the propagation of the standard uncertainty of the two slopes obtained in the fitting (see [Table C.3](#)).