**Supplemental material: Development of a standard operating procedure for the DCFH2-DA acellular assessment of reactive oxygen species produced by nanomaterials**

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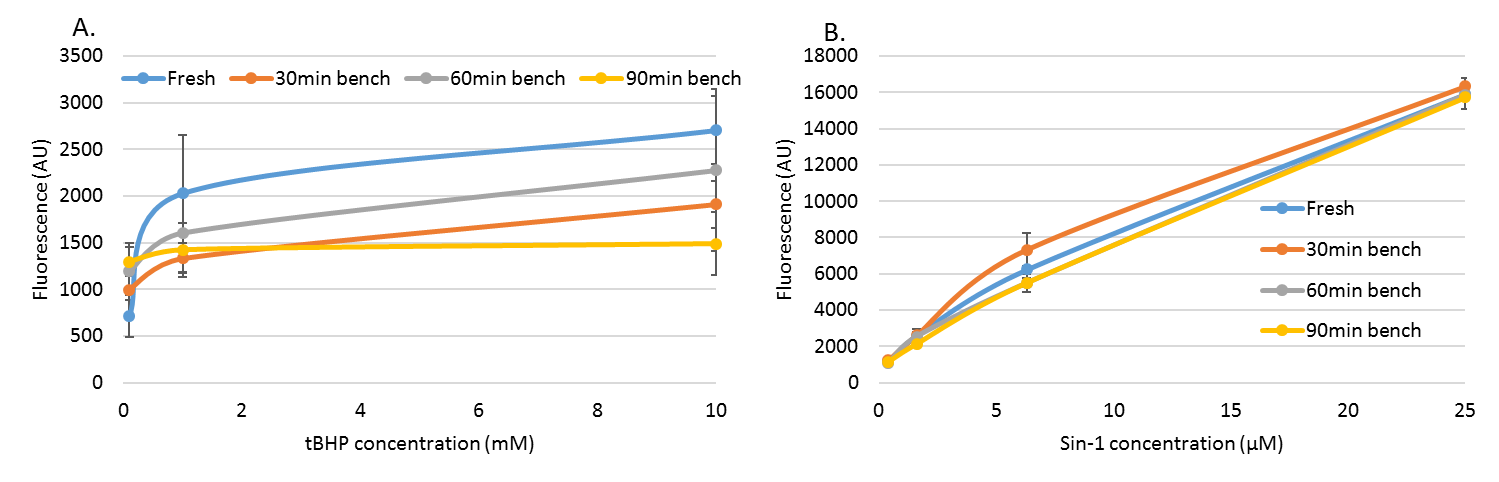
[Final Standard Operating Procedure: detection of acellular Reactive Oxygen Species (ROS) 19](#_Toc86401923)

### Development of standard operating procedure

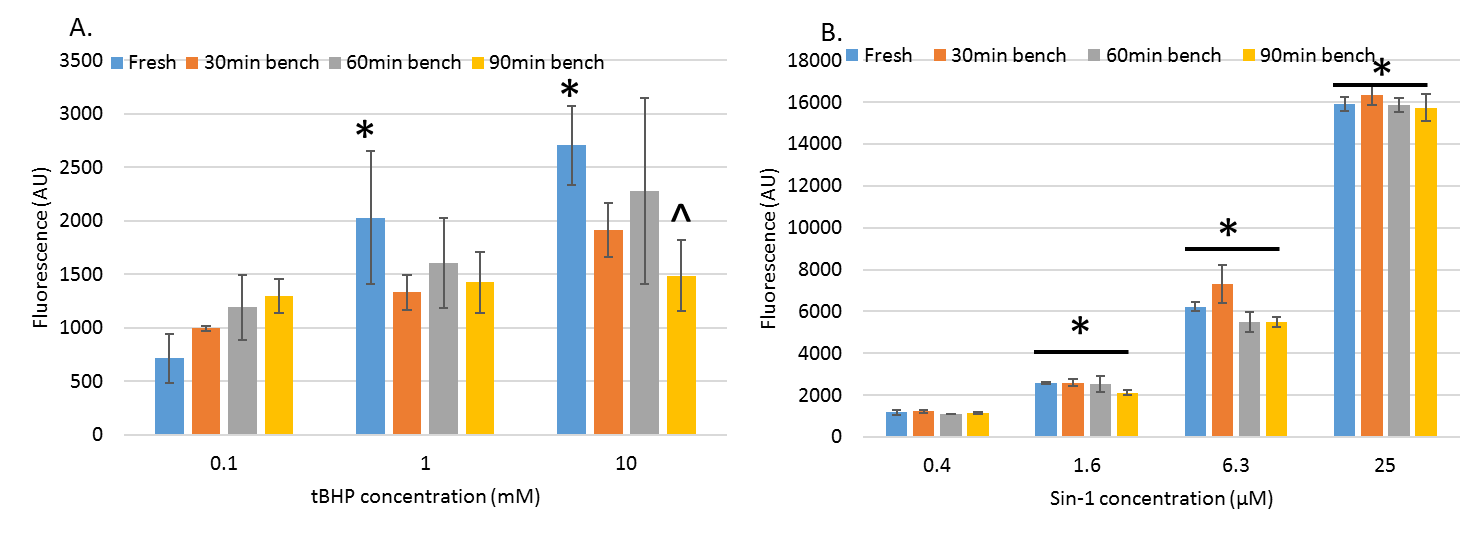
#### Choice of chemical positive control for assay: SIN-1 versus tBHP.

Comparing the oxidation of DCFH2 in response to two possible positive controls (tBHP and SIN-1) it was evident that both induced oxidation. However, a greater linearity was observed between SIN-1 concentration and fluorescence signal (Figure S1a) than that of tBHP (Figure S1a). Furthermore, the stability of SIN-1 over time (in terms of how consistent the fluorescence signal was maintained when the treatments were left after preparation) appeared better than the stability of tBHP. The signal generated by SIN-1 was relatively unchanged for the duration of the test period (90 min). The signal generated in response to tBHP, however, was shown to decrease within 30 minutes, and was lower than the signal generated from a freshly prepared sample on each of the further incubation times.

Both these observations – that a better dose-dependent fluorescence signal and better maintenance of fluorescence signal over time was shown for SIN-1 – were confirmed by statistical analysis (Figure 1b).



**Figure S1a.** Acellular ROS generation in response to A) tBHP and B) Sin-1. Each treatment was prepared at various concentrations and left for various time periods protected from light on the bench (0, 30, 60 and 90 min) prior to addition of DCFH for 90 min. Each data point represents the mean of three independent replicates.



**Figure S1b.** Acellular ROS generation in response to A) tBHP and B) Sin-1. Each treatment was prepared at various concentrations and left for various time periods protected from light on the bench (0, 30, 60 and 90 min) prior to addition of DCFH for 90 min. Each data point represents the mean of three independent replicates. Statistically significant differences are shown when p < 0.05, and identified with \* when a value is compared to the lowest concentration of its corresponding incubation time, and with ^ when a value is compared to the signal generated by the freshly prepared sample at the same treatment concentration.

#### Effect on fluorescence signal when using 10 or 50 µM DCFH

The different partners involved in the interlaboratory assessment of the acellular DCFH assay traditionally used different concentrations of DCFH2-DA. Therefore, the difference in fluorescence signal and the sensitivity to the presence of ROS was assessed at these different DCFH2-DA concentrations. SIN-1 (at 0.00, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, and 25.00 µM) was incubated with either 10 µM or 50 µM DCFH2-DA. The fluorescence signal was measured at ex/em wavelengths of 485/530 was measured after 0 minutes and then again after 30, 60 and 90 minutes incubation at 37 °C. As can be seen in Figure S2A and S2B, when comparing the absolute values generated by these two different DCFH2-DA concentrations, there is very little difference. The only noteworthy difference was after 30 minutes when the 50 µM DCFH2-DA sample allowed for a slightly higher fluorescence signal; for other time points there was no difference. The most striking difference in effect when using these different DCFH2-DA concentrations was when the data was expressed as a ratio based on the 0 µM SIN-1 concentration (Figures S2C and S2D). Due to a background signal that was considerably smaller when using 10 µM DCFH2-DA (Figure S2C) the ratio changes in fluorescence signal are much exaggerated compared to those of the 50 µM DCFH2-DA measurements (Figure S2D). This would imply that using 10 µM DCFH2-DA would allow for better sensitivity, and therefore has been chosen for this SOP.

**Figure S2.** Acellular ROS generation in response to SIN-1, incubated at different concentrations in the presence of either 10 µM (A and C) or 50 µM (B and D) DCFH2-DA, with fluorescence measured at time 0, and after 30, 60 and 90 minutes of incubation. Data is expressed as fluorescence intensity as arbitrary units achieved with ex/em wavelengths of 485/530nm (A and B), as a ratio values based on the background (no SIN-1 present) for each respective time point. Each data point represents the mean ± SD of six technical replicates.

#### Use of FCS in medium

The use of proteins within the vehicle fluid can be used to provide a greater representation of a biological environment. In this SOP, we have suggested fetal calf serum (FCS), this allows continuity with the cellular version of this assay, and as shown within the main manuscript, a better particle dispersion. The effect of including or excluding FCS in these assays, as well as the effect of using water or cell culture medium, has been assessed here in response to nanoparticle carbon black (NPCB), copper oxide NPs (CuONPs), and titanium dioxide NPs (TiO2NPs) (Figure S3). As a general rule, samples prepared in 2% FCS had a lower ROS production than those prepared without FCS, and samples prepared in water had a higher ROS formation than those prepared in cell culture medium. The resulting size distributions using these conditions are shown in Table S1.



**Figure S3.** Acellular ROS generation in response to A) NPCB, B) CuO NPs, and C) TiO2NPs, in the presence and absence of 2% foetal calf serum (FCS). Data is expressed as fluorescence intensity resulting from DCFH oxidation over an incubation period of 90 minutes. Each data point represents the mean ± SD of at least three independent replicates.

**Table S1.** Hydrodynamic diameter (by DLS) of NPCB, TiO2 and CuO when suspended in H2O or MEM, with and without 2% FCS. Data shows average particle diameter (Z.av) and uniformity of these measurements with a polydispersity index (PDI). Standard deviation used here refers to measurements taken from 5-7 independent replicates, each replicate also consisted of 3 measurement replicates.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Dispersion of NPCB | | | | Dispersion of TiO2 | | | | Dispersion of CuO | | | |
|  | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD |
| In H2O | 714.68 | 190.76 | 0.57 | 0.05 | 970.90 | 391.98 | 0.60 | 0.11 | 472.19 | 142.53 | 0.48 | 0.03 |
| In H2O 2% FCS | 221.82 | 52.78 | 0.33 | 0.06 | 284.61 | 62.12 | 0.51 | 0.13 | 227.89 | 81.01 | 0.55 | 0.10 |
| In MEM | 26339.43 | 33087.64 | 0.69 | 0.34 | 5793.27 | 8265.33 | 0.79 | 0.14 | 25228.40 | 29968.87 | 0.92 | 0.07 |
| In MEM 2% FCS | 199.00 | 106.82 | 0.54 | 0.16 | 349.91 | 107.64 | 0.59 | 0.17 | 283.39 | 311.09 | 0.60 | 0.27 |

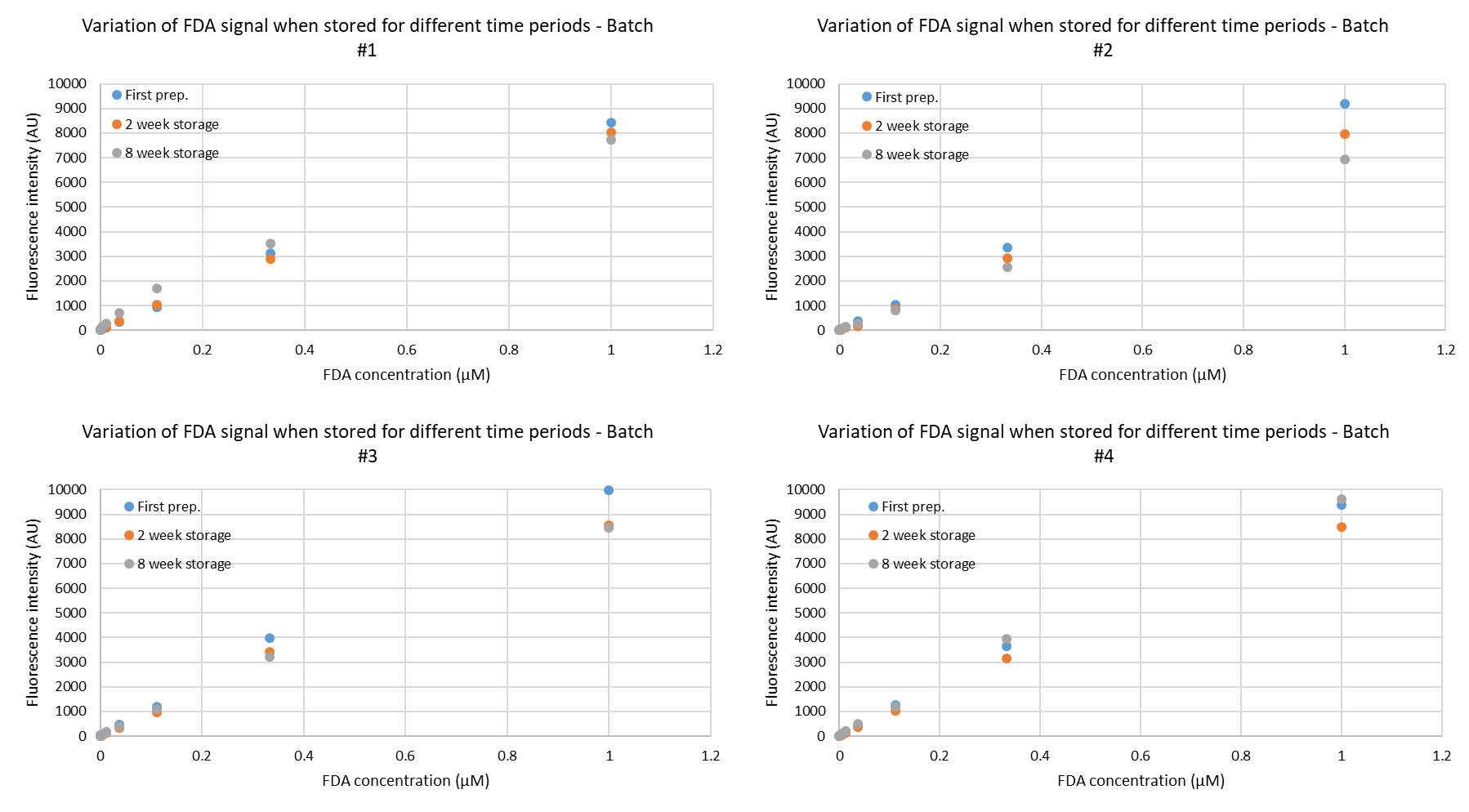
Furthermore, various dispersion protocols were evaluated in a Ultrawave QS25 sonicating water bath operating at 400 J/sec for 15 min (Table S2) to identify a suitable process to use during interlaboratory comparisons; three dispersion methods were compared. Method one suspended particles at 1 mg/ml in phenol red free MEM plus 2% FCS, followed by sonication for 15 minutes in a sonicating water bath, and diluted to 50 µg/ml. Method two suspended particles using MQ water containing 0.5% EtOH at 2.56 mg/ml in MQ water, sonication for 15 minutes in a sonicating water bath, and dilution to 50 µg/ml in phenol red free MEM plus 2% FCS. Method three was similar to method two, except a pre-wetting of particles with EtOH was used (with EtOH volume limited to achieve a final concentration equal to 0.5%), followed by suspension at 2.56 mg/ml in MQ water, sonication for 15 minutes in a sonicating water bath, and finally diluted to 50 µg/ml in phenol red free MEM plus 2% FCS. In addition, different preparation vessels were assessed for their impact on particle dispersion, including preparing samples in glass, plastic bijous tubes, Eppendorf tubes and an initial preparation (up to sonication) in glass with further preparation (performing dilutions) in Eppendorf tubes; resting periods after sonication were also evaluated, and multiple sonication steps were tested at different stages of the dispersion process. NPCB was used to define all these conditions, with selected methods further qualified using BaSO4 and ZnO were assessed by DLS.

**Table S2.** Hydrodynamic diameter (by DLS) of NPCB, BaSO4 and ZnO when suspended using different vessels, and with different handling procedures. Data shows average particle diameter (Z.av) and uniformity of these measurements with a polydispersity index (PDI). Standard deviation used here refers to at least 3 measurement replicates.

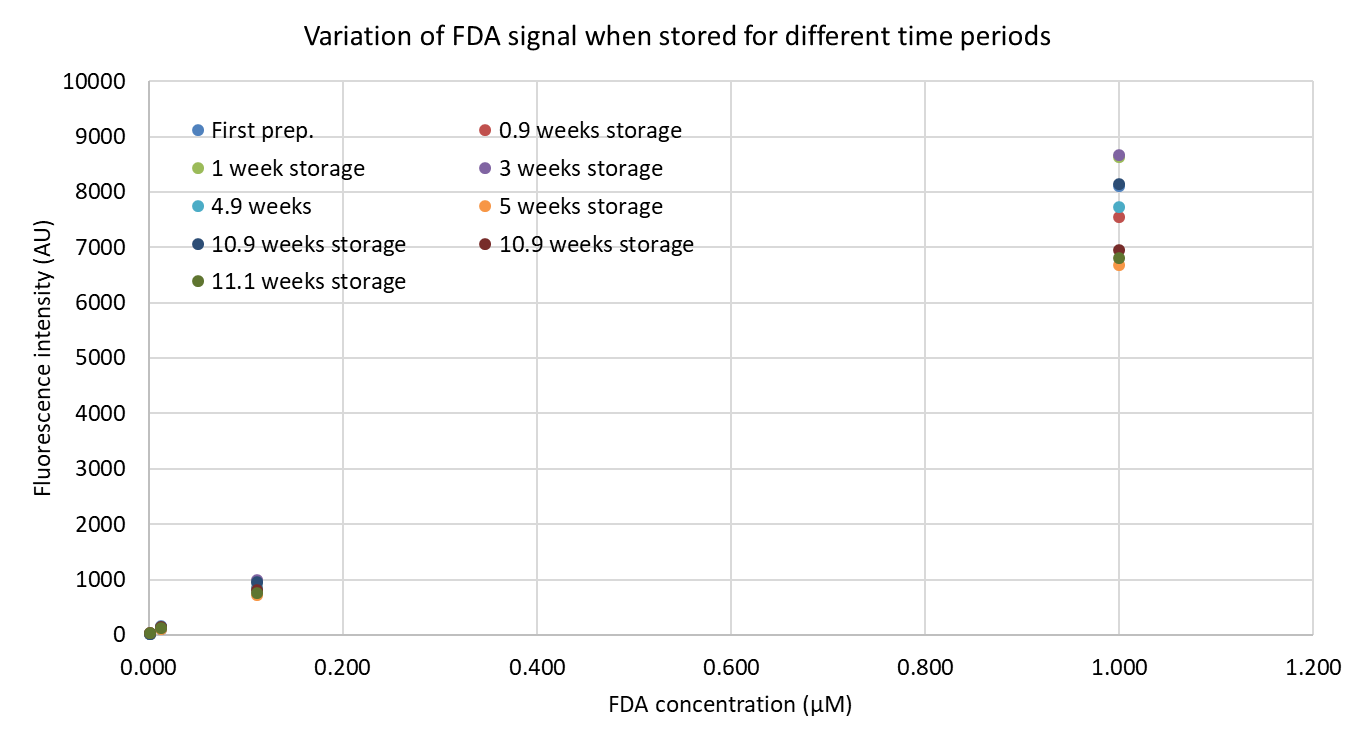
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Dispersion of NPCB | | | | Dispersion of BaSO4 | | | | Dispersion of ZnO | | | |
| Method #1 | | | | | | | | | | | | |
|  | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD |
| Bijous | 292.73 | 9.73 | 0.48 | 0.01 | 376.50 | 42.08 | 0.63 | 0.09 | 471.10 | 9.62 | 0.34 | 0.04 |
| Glass | 512.93 | 75.35 | 0.80 | 0.08 | 525.67 | 82.80 | 0.65 | 0.12 | 483.20 | 15.99 | 0.38 | 0.07 |
| Glass to eppendorf | 765.73 | 139.40 | 0.69 | 0.08 | 421.23 | 68.18 | 0.62 | 0.11 | 735.30 | 206.75 | 0.61 | 0.06 |
| Eppendorf | 689.33 | 11.30 | 0.62 | 0.02 | 459.30 | 41.38 | 0.58 | 0.04 | 484.97 | 13.78 | 0.38 | 0.03 |
| Method #1 - bijous tube sample measured for a second time approx. 20 min after first preparation. | | | | | | | | | | | | |
|  | Z-Av | SD | PDI | SD |  |  |  |  |  |  |  |  |
| Bijous | 316.87 | 28.62 | 0.47 | 0.06 |  |  |  |  |  |  |  |  |
| Method #2 | | | | | | | | | | | | |
|  | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD | Z-Av | SD | PDI | SD |
| Bijous | 1175.37 | 319.46 | 0.86 | 0.13 | 549.00 | 51.15 | 0.67 | 0.08 | 2629.33 | 151.14 | 0.92 | 0.04 |
| Glass | 1591.67 | 452.04 | 0.88 | 0.10 | 493.90 | 50.25 | 0.71 | 0.09 | 2281.33 | 285.94 | 0.80 | 0.17 |
| Glass to eppendorf | 3625.67 | 1577.20 | 1.00 | 0.00 | 536.73 | 58.65 | 0.65 | 0.07 | 2909.33 | 296.39 | 0.87 | 0.12 |
| Eppendorf | 1342.00 | 49.11 | 0.95 | 0.05 | 453.70 | 73.22 | 0.56 | 0.03 | 1920.33 | 286.07 | 0.95 | 0.05 |
| Method #2 with addition sonication after final test concentration obtained | | | | | | | | | | | | |
|  | Z-Av | SD | PDI | SD |  |  |  |  |  |  |  |  |
| Bijous | 360.07 | 36.39 | 0.64 | 0.03 |  |  |  |  |  |  |  |  |
| Glass | 311.57 | 6.90 | 0.34 | 0.02 |  |  |  |  |  |  |  |  |
| Glass to eppendorf | 362.07 | 15.86 | 0.57 | 0.08 |  |  |  |  |  |  |  |  |
| Eppendorf | 698.63 | 150.34 | 0.64 | 0.02 |  |  |  |  |  |  |  |  |
| Method #3 | | | | | | | | | | | | |
|  | Z-Av | SD | PDI | SD |  |  |  |  |  |  |  |  |
| Bijous | 2466.33 | 1345.01 | 0.96 | 0.07 |  |  |  |  |  |  |  |  |
| Glass | 4735.00 | 1267.96 | 1.00 | 0.00 |  |  |  |  |  |  |  |  |
| Glass to eppendorf | 6838.00 | 1264.00 | 1.00 | 0.00 |  |  |  |  |  |  |  |  |
| Eppendorf | 5191.00 | 1616.43 | 1.00 | 0.00 |  |  |  |  |  |  |  |  |

#### Effect of cold storage of FDA on fluorescence signal

A 10 mM stock of fluorescein (12.5 mM fluorescein diacetate) was stored at -20 °C for a period of 12 weeks, during this time aliquots were periodically removed and tested for strength of their fluorescence signal. Each sample was diluted as per the DCFH acellular SOP protocol to provide a dose range between 0 and 1 µM. The data represented in Figure S4a shows the variation in signal from measurements taken of four batches of F-DA which had been stored for different periods of time at 20 °C; three separate aliquots were tested of each batch during an 8 week period. Figure S4b. shows the variation in signal from measurements taken of one batch of F-DA which had been stored for different periods of time at -20 °C; nine separate aliquots were tested during the 12 week period. It was noted that the dilution of fluorescein provides a consistently linear relationship to fluorescence signal, and the intensity was not shown to greatly reduce upon storage time.



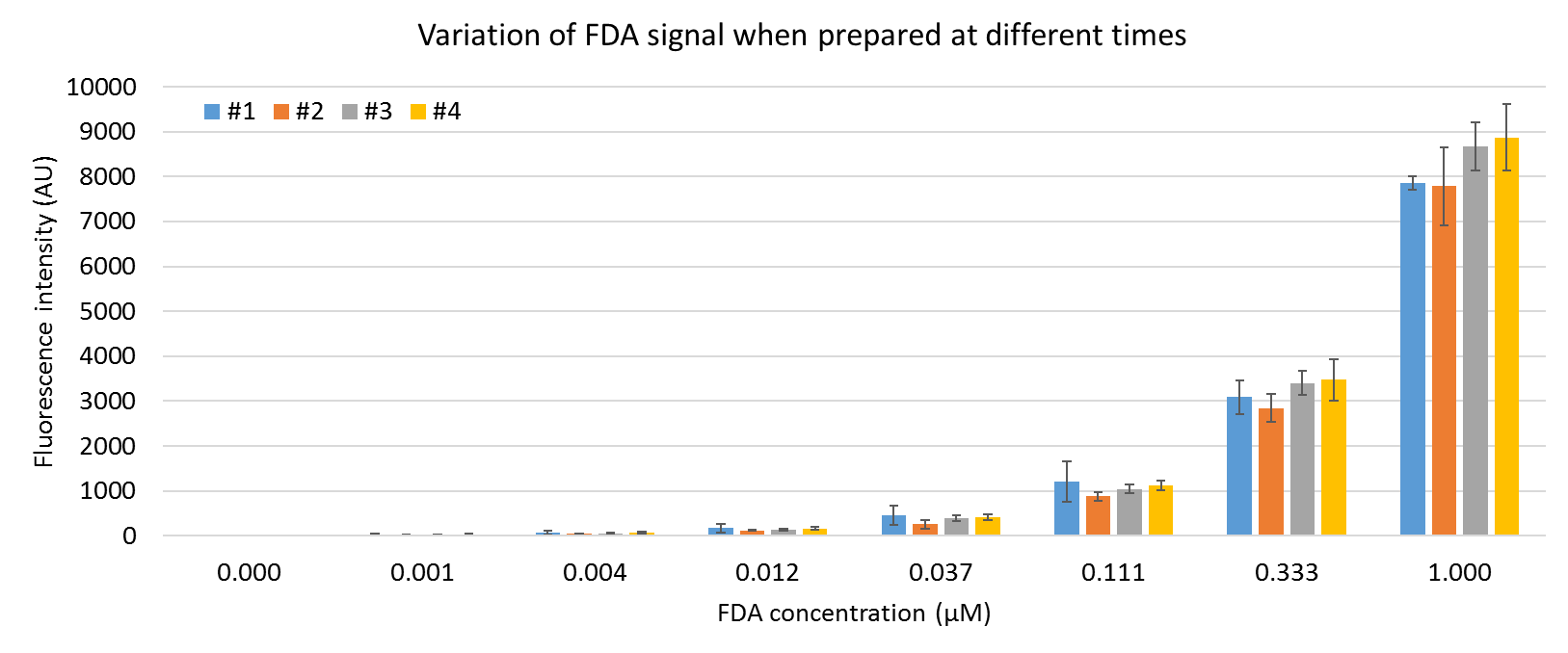
**Figure S4a. Stability of F-DA fluorescence during storage at -20 °C.** Each graph represents a different batch of F-DA stored in aliquots for different periods of time, and data shows fluorescein fluorescence intensity generated from each batch, prepared at different concentrations on day of measurement, readings were taken at ex/em wavelengths of 485/530 nm. Storage was at -20 °C for different periods of time, ranging from 0-8 weeks.

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**Figure S4b. Stability of FDA fluorescence during storage at -20 °C.** The data represents fluorescein fluorescence intensity generated from one batch of FDA stored in aliquots for different periods of time, and prepared at different concentrations on day of measurement, readings were taken at ex/em wavelengths of 485/530 nm. Samples were stored at -20 °C for different periods of time, ranging from 0-12 weeks.

#### Effect of batch preparation on fluorescein fluorescence reproducibility

A 10 mM stock of fluorescein was prepared on four separate occasions, aliquoted and stored at -20 °C. Each sample was used on day of preparation, and twice more after periods of storage. On the day of use, the 10 mM stock was prepared as per the DCFH acellular SOP, to a concentration range of 0, 0.001, 0.004, 0.012, 0.037, 0.111, 0.333 and 1.000, added to a black, clear-bottom 96-well, with fluorescence intensity measured at ex/em wavelengths of 485/530; the results of these tests can be seen in Figure S5. Very little difference was observed between the signal generated by each separate batch, across all concentrations tested, which was an assumption supported by statistical analysis, as no statistically significant differences were observed.

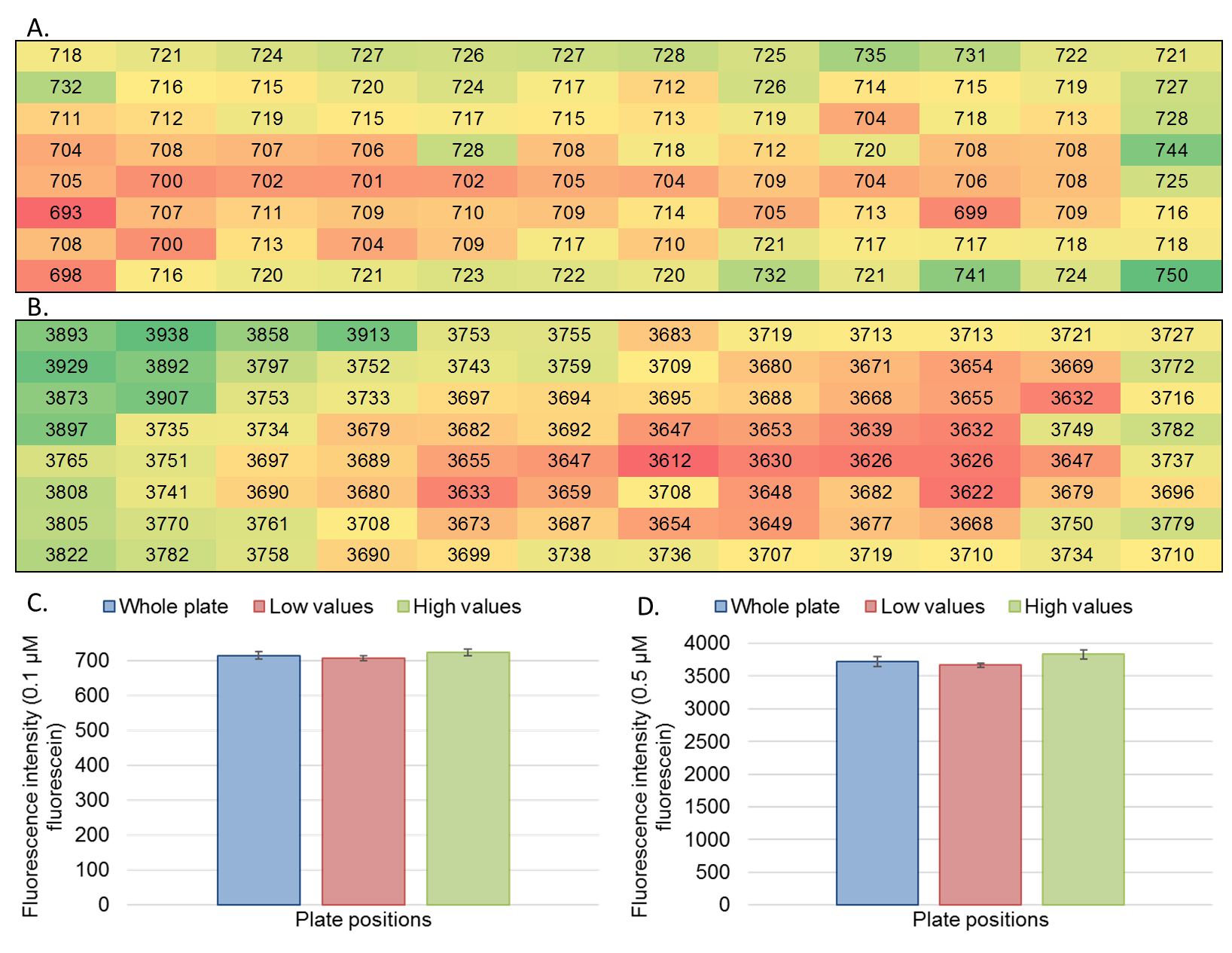


**Figure S5. Variation in FDA fluorescence, dependent on batch preparation.** The data represents fluorescence intensity generated from fluorescein at different concentrations, measured at ex/em wavelengths of 485/530 nm, and is presented as mean ± SD of three independent replicates. Each number (#1, #2, #3, and #4) represents a separately prepared batch of FDA.

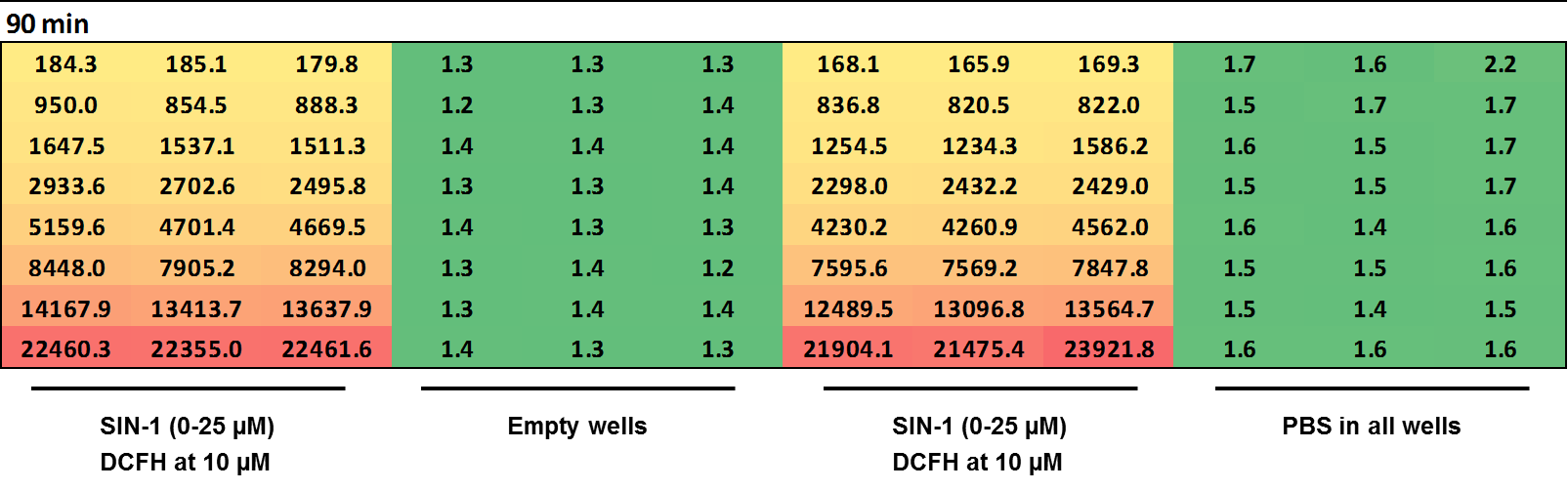
#### Fluorescence signal – plate location bias or cross-well contamination

To ensure that there is not an extreme variation in the fluorescence signal detected at different locations of a 96-well plate, fluorescein was added to every well of a 96-well plate and read at ex/em wavelength of 485/530 (Figure S6). The use of color coding identifies that there was a difference in signal detected at different plate positions. However, there was no consistent pattern relating to positions of low and high signals, when different concentrations were used. Furthermore, the standard deviation across the whole plate was very low, even when average values of low versus high signal locations were compared very little difference was observed.

Although the test is performed in black plates there are clear-bottoms, the volumes are high and close to well capacity. It was therefore thought necessary to determine high fluorescence signals generated could cause any cross-well contamination. If this were to occur, the plate arrangement would require further consideration. As can be seen in Figure S7, the presence of SIN-1 causes a dose dependent (0-25 µM) oxidation of DCFH2 (columns 1-3 and 7-9). Adjacent wells were either empty (columns 4-6) or contained 250 µl PBS (columns 4-6), and demonstrated no spill over of fluorescence signal. Any volatile activity of SIN-1 was not assessed.



**Figure S6.** **Variation in FDA fluorescence across a 96-well plate.** The data represents fluorescence intensity generated from fluorescein at 0.1 µM (A and C) or 0.5 µM (B and D) when measured at ex/em wavelengths of 485/530 nm. The data is presented as colour-coded raw data (A and B), or mean ± SD of the whole plate, low values (red) and high values (green) (C and D).



**Figure S7.** Cross-well contamination caused by high signals induced by SIN-1. The data represents fluorescence intensity generated from the presence of SIN-1 at 0-25 µM in 10 µM DCFH, when measured at ex/em wavelengths of 485/530 nm.

#### Assessment of fluorescence quenching

To determine if a material can interfere with the fluorescence signal through interruption of the light emitted, F-DA was used. The data presented in Figure S8 demonstrates the effect of adding NPCB (at concentrations of 0, 3, 10, 16, 78, and 390 µg/cm2) has on the fluorescence signal generated by different concentrations of fluorescein, including 0.02, 0.10 and 0.50 µM. A dose dependent increase in fluorescence was observed with increasing concentrations of fluorescein (Figure S8.A); a signal which declines as increasing concentrations of NPCB was added. The level of quenching was found to differ depending upon the starting concentration of fluorescein (Figure S8.B). There appeared to be a greater amount of NPCB-induced quenching evident when the fluorescein concentration was lower, i.e. when the fluorescence signal was lower. This difference was found to be as great as 10% (when comparing the signal generated when the three fluorescein concentrations were incubated with 16 µg/cm2 NPCB. For this reason it was decided to use the middle fluorescein concentration (0.1 µM) for interference control tested within the SOP, as it generated a sufficient fluorescence signal and provided a more sensitive record of quenching.



**Figure S8.** Quenching of fluorescein fluorescence in the presence of NPCB. Data is expressed as A) arbitrary units of fluorescence intensity or B) % fluorescence using fluorescein in the absence of NPCB as 100%. Each data point represents the mean ± SD of three technical replicates.

### Interlaboratory comparison data

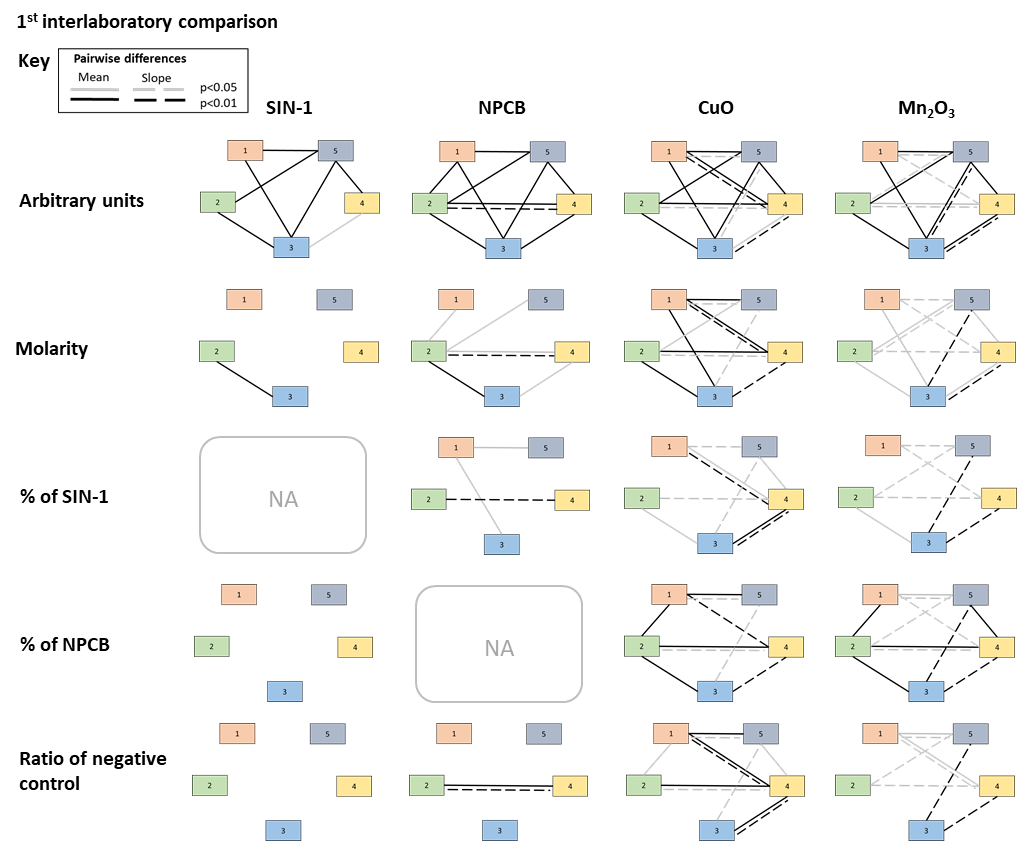
#### Interference of benchmark materials



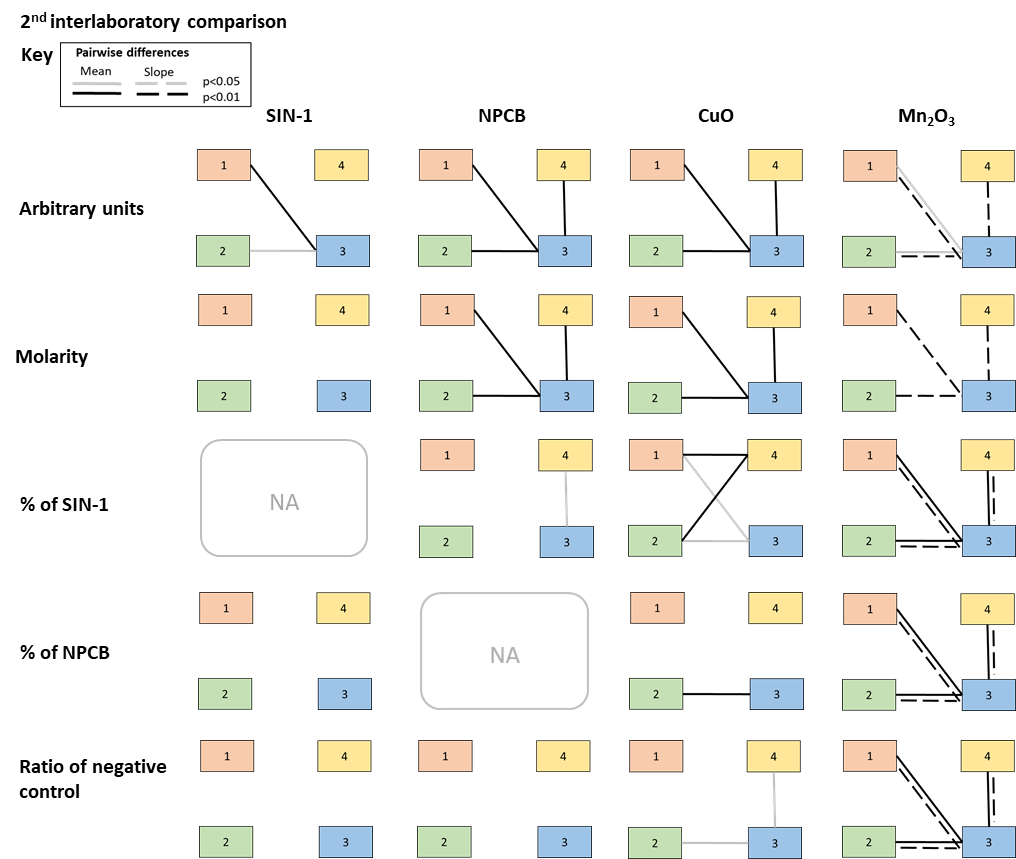
**Figure S9.** Interference (quenching of fluorescence signal or autofluorescence) of benchmark materials suspending in the presence of FCS.



**Figure S10.** Interference (quenching of fluorescence signal or autofluorescence) of benchmark materials suspending in the absence of FCS.



**Figure S11.** Pairwise differences between partners during first interlaboratory comparison study. In each column are differences shown in evaluation of SIN-1, NPCB, CuO or Mn2O3, and rows are differences found when using the various methods of data normalisation; statistically significant differences between mean values are shown with lines, and between slopes with dotted lines.



**Figure S12.** Pairwise differences between partners during second interlaboratory comparison study. In each column are differences shown in evaluation of SIN-1, NPCB, CuO or Mn2O3, and rows are differences found when using the various methods of data normalisation; statistically significant differences between mean values are shown with lines, and between slopes with dotted lines.

**Table S3.** Pairwise comparisons using Arbitrary Units

**Comparison of means (log-scale)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SIN-1 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.27 | 0.284 | -0.78 | 0.23 |
| 3 vs 1 | 0.70 | 0.004 | 0.23 | 1.18 |
| 4 vs 1 | 0.10 | 0.694 | -0.40 | 0.60 |
| 5 vs 1 | 1.51 | 0.000 | 1.00 | 2.01 |
| 3 vs 2 | 0.98 | 0.000 | 0.51 | 1.45 |
| 4 vs 2 | 0.37 | 0.144 | -0.13 | 0.88 |
| 5 vs 2 | 1.78 | 0.000 | 1.28 | 2.28 |
| 4 vs 3 | -0.60 | 0.013 | -1.08 | -0.13 |
| 5 vs 3 | 0.80 | 0.001 | 0.33 | 1.27 |
| 5 vs 4 | 1.41 | 0.000 | 0.90 | 1.91 |
| NPCB |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.46 | 0.001 | -0.73 | -0.20 |
| 3 vs 1 | 0.56 | 0.000 | 0.32 | 0.81 |
| 4 vs 1 | 0.03 | 0.797 | -0.23 | 0.30 |
| 5 vs 1 | 1.30 | 0.000 | 1.05 | 1.55 |
| 3 vs 2 | 1.03 | 0.000 | 0.78 | 1.27 |
| 4 vs 2 | 0.50 | 0.000 | 0.23 | 0.76 |
| 5 vs 2 | 1.76 | 0.000 | 1.52 | 2.01 |
| 4 vs 3 | -0.53 | 0.000 | -0.78 | -0.28 |
| 5 vs 3 | 0.74 | 0.000 | 0.51 | 0.97 |
| 5 vs 4 | 1.27 | 0.000 | 1.02 | 1.51 |
| CuO |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.06 | 0.608 | -0.31 | 0.18 |
| 3 vs 1 | 0.73 | 0.000 | 0.51 | 0.96 |
| 4 vs 1 | 0.48 | 0.000 | 0.24 | 0.72 |
| 5 vs 1 | 1.65 | 0.000 | 1.42 | 1.88 |
| 3 vs 2 | 0.80 | 0.000 | 0.57 | 1.02 |
| 4 vs 2 | 0.54 | 0.000 | 0.30 | 0.78 |
| 5 vs 2 | 1.71 | 0.000 | 1.49 | 1.94 |
| 4 vs 3 | -0.25 | 0.029 | -0.48 | -0.03 |
| 5 vs 3 | 0.92 | 0.000 | 0.71 | 1.13 |
| 5 vs 4 | 1.17 | 0.000 | 0.95 | 1.40 |
| Mn2O3 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.18 | 0.137 | -0.41 | 0.06 |
| 3 vs 1 | 0.61 | 0.000 | 0.39 | 0.82 |
| 4 vs 1 | 0.07 | 0.527 | -0.16 | 0.31 |
| 5 vs 1 | 1.52 | 0.000 | 1.30 | 1.74 |
| 3 vs 2 | 0.78 | 0.000 | 0.56 | 1.00 |
| 4 vs 2 | 0.25 | 0.035 | 0.02 | 0.48 |
| 5 vs 2 | 1.70 | 0.000 | 1.48 | 1.92 |
| 4 vs 3 | -0.53 | 0.000 | -0.75 | -0.31 |
| 5 vs 3 | 0.92 | 0.000 | 0.72 | 1.12 |
| 5 vs 4 | 1.45 | 0.000 | 1.23 | 1.67 |

**Comparison of slopes (log-scale)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SIN-1 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.01 | 0.906 | -0.09 | 0.10 |
| 3 vs 1 | 0.02 | 0.567 | -0.06 | 0.11 |
| 4 vs 1 | 0.03 | 0.544 | -0.06 | 0.12 |
| 5 vs 1 | 0.02 | 0.608 | -0.07 | 0.12 |
| 3 vs 2 | 0.02 | 0.655 | -0.07 | 0.10 |
| 4 vs 2 | 0.02 | 0.625 | -0.07 | 0.11 |
| 5 vs 2 | 0.02 | 0.693 | -0.07 | 0.11 |
| 4 vs 3 | 0.00 | 0.939 | -0.08 | 0.09 |
| 5 vs 3 | 0.00 | 0.980 | -0.09 | 0.08 |
| 5 vs 4 | 0.00 | 0.924 | -0.10 | 0.09 |
| NPCB |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.03 | 0.113 | -0.06 | 0.01 |
| 3 vs 1 | 0.00 | 0.881 | -0.03 | 0.03 |
| 4 vs 1 | 0.03 | 0.115 | -0.01 | 0.06 |
| 5 vs 1 | 0.00 | 0.908 | -0.03 | 0.03 |
| 3 vs 2 | 0.03 | 0.066 | 0.00 | 0.06 |
| 4 vs 2 | 0.05 | 0.002 | 0.02 | 0.09 |
| 5 vs 2 | 0.03 | 0.071 | 0.00 | 0.06 |
| 4 vs 3 | 0.02 | 0.124 | -0.01 | 0.06 |
| 5 vs 3 | 0.00 | 0.970 | -0.03 | 0.03 |
| 5 vs 4 | -0.03 | 0.116 | -0.06 | 0.01 |
| CuO |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.00 | 0.143 | 0.00 | 0.01 |
| 3 vs 1 | 0.00 | 0.732 | 0.00 | 0.01 |
| 4 vs 1 | 0.01 | 0.001 | 0.00 | 0.02 |
| 5 vs 1 | 0.01 | 0.017 | 0.00 | 0.01 |
| 3 vs 2 | 0.00 | 0.220 | -0.01 | 0.00 |
| 4 vs 2 | 0.01 | 0.043 | 0.00 | 0.01 |
| 5 vs 2 | 0.00 | 0.399 | 0.00 | 0.01 |
| 4 vs 3 | 0.01 | 0.001 | 0.00 | 0.01 |
| 5 vs 3 | 0.01 | 0.026 | 0.00 | 0.01 |
| 5 vs 4 | 0.00 | 0.182 | -0.01 | 0.00 |
| Mn2O3 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.00 | 0.913 | -0.01 | 0.01 |
| 3 vs 1 | 0.00 | 0.514 | -0.01 | 0.00 |
| 4 vs 1 | 0.01 | 0.026 | 0.00 | 0.01 |
| 5 vs 1 | 0.01 | 0.017 | 0.00 | 0.01 |
| 3 vs 2 | 0.00 | 0.442 | -0.01 | 0.00 |
| 4 vs 2 | 0.01 | 0.034 | 0.00 | 0.01 |
| 5 vs 2 | 0.01 | 0.023 | 0.00 | 0.01 |
| 4 vs 3 | 0.01 | 0.003 | 0.00 | 0.01 |
| 5 vs 3 | 0.01 | 0.001 | 0.00 | 0.01 |
| 5 vs 4 | 0.00 | 0.992 | -0.01 | 0.01 |

**Table S4.** Pairwise comparisons with ratio of negative control (medium only)

**Comparison of means (log-scale)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SIN-1 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.04 | 0.872 | -0.46 | 0.55 |
| 3 vs 1 | 0.18 | 0.437 | -0.29 | 0.66 |
| 4 vs 1 | 0.28 | 0.265 | -0.22 | 0.79 |
| 5 vs 1 | 0.23 | 0.360 | -0.27 | 0.74 |
| 3 vs 2 | 0.14 | 0.545 | -0.33 | 0.62 |
| 4 vs 2 | 0.24 | 0.340 | -0.26 | 0.75 |
| 5 vs 2 | 0.19 | 0.450 | -0.31 | 0.70 |
| 4 vs 3 | 0.10 | 0.677 | -0.37 | 0.57 |
| 5 vs 3 | 0.05 | 0.839 | -0.42 | 0.52 |
| 5 vs 4 | -0.05 | 0.841 | -0.56 | 0.45 |
| NPCB |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.15 | 0.267 | -0.41 | 0.12 |
| 3 vs 1 | 0.05 | 0.715 | -0.20 | 0.29 |
| 4 vs 1 | 0.22 | 0.105 | -0.05 | 0.48 |
| 5 vs 1 | 0.03 | 0.826 | -0.22 | 0.27 |
| 3 vs 2 | 0.19 | 0.122 | -0.05 | 0.44 |
| 4 vs 2 | 0.37 | 0.007 | 0.10 | 0.63 |
| 5 vs 2 | 0.18 | 0.161 | -0.07 | 0.42 |
| 4 vs 3 | 0.17 | 0.169 | -0.07 | 0.42 |
| 5 vs 3 | -0.02 | 0.875 | -0.25 | 0.21 |
| 5 vs 4 | -0.19 | 0.129 | -0.44 | 0.06 |
| CuO |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.25 | 0.043 | 0.01 | 0.49 |
| 3 vs 1 | 0.21 | 0.065 | -0.01 | 0.44 |
| 4 vs 1 | 0.66 | 0.000 | 0.42 | 0.91 |
| 5 vs 1 | 0.38 | 0.001 | 0.15 | 0.61 |
| 3 vs 2 | -0.04 | 0.746 | -0.26 | 0.19 |
| 4 vs 2 | 0.41 | 0.001 | 0.17 | 0.65 |
| 5 vs 2 | 0.13 | 0.270 | -0.10 | 0.35 |
| 4 vs 3 | 0.45 | 0.000 | 0.22 | 0.68 |
| 5 vs 3 | 0.16 | 0.124 | -0.05 | 0.38 |
| 5 vs 4 | -0.28 | 0.015 | -0.51 | -0.06 |
| Mn2O3 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.13 | 0.263 | -0.10 | 0.37 |
| 3 vs 1 | 0.08 | 0.465 | -0.14 | 0.30 |
| 4 vs 1 | 0.25 | 0.033 | 0.02 | 0.49 |
| 5 vs 1 | 0.24 | 0.029 | 0.03 | 0.46 |
| 3 vs 2 | -0.05 | 0.639 | -0.27 | 0.17 |
| 4 vs 2 | 0.12 | 0.307 | -0.11 | 0.35 |
| 5 vs 2 | 0.11 | 0.316 | -0.11 | 0.33 |
| 4 vs 3 | 0.17 | 0.120 | -0.05 | 0.39 |
| 5 vs 3 | 0.16 | 0.113 | -0.04 | 0.36 |
| 5 vs 4 | -0.01 | 0.929 | -0.23 | 0.21 |

**Comparison of slopes (log-scale)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SIN-1 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.01 | 0.906 | -0.09 | 0.10 |
| 3 vs 1 | 0.02 | 0.567 | -0.06 | 0.11 |
| 4 vs 1 | 0.03 | 0.544 | -0.06 | 0.12 |
| 5 vs 1 | 0.02 | 0.608 | -0.07 | 0.12 |
| 3 vs 2 | 0.02 | 0.655 | -0.07 | 0.10 |
| 4 vs 2 | 0.02 | 0.625 | -0.07 | 0.11 |
| 5 vs 2 | 0.02 | 0.693 | -0.07 | 0.11 |
| 4 vs 3 | 0.00 | 0.939 | -0.08 | 0.09 |
| 5 vs 3 | 0.00 | 0.980 | -0.09 | 0.08 |
| 5 vs 4 | 0.00 | 0.924 | -0.10 | 0.09 |
| NPCB |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | -0.03 | 0.113 | -0.06 | 0.01 |
| 3 vs 1 | 0.00 | 0.881 | -0.03 | 0.03 |
| 4 vs 1 | 0.03 | 0.115 | -0.01 | 0.06 |
| 5 vs 1 | 0.00 | 0.908 | -0.03 | 0.03 |
| 3 vs 2 | 0.03 | 0.066 | 0.00 | 0.06 |
| 4 vs 2 | 0.05 | 0.002 | 0.02 | 0.09 |
| 5 vs 2 | 0.03 | 0.071 | 0.00 | 0.06 |
| 4 vs 3 | 0.02 | 0.124 | -0.01 | 0.06 |
| 5 vs 3 | 0.00 | 0.970 | -0.03 | 0.03 |
| 5 vs 4 | -0.03 | 0.116 | -0.06 | 0.01 |
| CuO |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.00 | 0.143 | 0.00 | 0.01 |
| 3 vs 1 | 0.00 | 0.732 | 0.00 | 0.01 |
| 4 vs 1 | 0.01 | 0.001 | 0.00 | 0.02 |
| 5 vs 1 | 0.01 | 0.017 | 0.00 | 0.01 |
| 3 vs 2 | 0.00 | 0.220 | -0.01 | 0.00 |
| 4 vs 2 | 0.01 | 0.043 | 0.00 | 0.01 |
| 5 vs 2 | 0.00 | 0.399 | 0.00 | 0.01 |
| 4 vs 3 | 0.01 | 0.001 | 0.00 | 0.01 |
| 5 vs 3 | 0.01 | 0.026 | 0.00 | 0.01 |
| 5 vs 4 | 0.00 | 0.182 | -0.01 | 0.00 |
| Mn2O3 |  |  |  |  |
| Partner | Contrast | *p*-value | Lower 95% CI | Upper 95% CI |
| 2 vs 1 | 0.00 | 0.913 | -0.01 | 0.01 |
| 3 vs 1 | 0.00 | 0.514 | -0.01 | 0.00 |
| 4 vs 1 | 0.01 | 0.026 | 0.00 | 0.01 |
| 5 vs 1 | 0.01 | 0.017 | 0.00 | 0.01 |
| 3 vs 2 | 0.00 | 0.442 | -0.01 | 0.00 |
| 4 vs 2 | 0.01 | 0.034 | 0.00 | 0.01 |
| 5 vs 2 | 0.01 | 0.023 | 0.00 | 0.01 |
| 4 vs 3 | 0.01 | 0.003 | 0.00 | 0.01 |
| 5 vs 3 | 0.01 | 0.001 | 0.00 | 0.01 |
| 5 vs 4 | 0.00 | 0.992 | -0.01 | 0.01 |

**Table S5.** Range of EC50 values for DCFH2 oxidation by SIN-1, NPCB, CuO and Mn2O3 collected during the original and revised SOP; DCFH2 oxidation was recorded over 90 min period.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Partner 1 | Partner 2 | Partner 3 | Partner 4 |
|  | **1st interlaboratory comparison - data from 90 minutes** | | | |
| SIN-1 | **10.06**  (30.20 to 40.26) | **33.56**  (21.96 to 55.52) | **16.31**  (30.52 to 46.83) | **9.97**  (30.92 to 40.89) |
| NPCB | **24.39**  (47.29 to 71.68) | **67.26**  (27.55 to 94.81) | **17.88**  (39.64 to 57.52) | **15.82**  (47.71 to 63.53) |
| CuO | **91**  (260.6 to 351.6) | **362.8**  (308.8 to 671.6) | **422.3**  (110.7 to 533.0) | **92**  (311.1 to 403.1) |
| Mn2O3 | **123.9**  (206.6 to 330.5) | **169.35**  (97.35 to 266.7) | **106.97**  (87.63 to 194.6) | **136.9**  (284.3 to 421.2) |
|  | **2nd interlaboratory comparison - data from 90 minutes** | | | |
| SIN-1 | **14.9**  (26.67 to 41.57) | **7.98**  (30.72 to 38.70) | **12.23**  (26.96 to 39.19) | **12.08**  (29.23 to 41.31) |
| NPCB | **21.03**  (51.70 to 72.73) | **21.55**  (47.96 to 69.51) | **14.15**  (21.89 to 36.04) | **16.27**  (41.50 to 57.77) |
| CuO | **91.8**  (296.6 to 388.4) | **154.8**  (305.2 to 460.0) | **127.4**  (338.8 to 466.2) | **64.6**  (213.8 to 278.4) |
| Mn2O3 | **200.1**  (175.9 to 376.0) | **156.4**  (209.5 to 365.9) | **127.98**  (38.22 to 166.2) | **128.1**  (113.4 to 241.5) |

**Table S6.** Replicate reproducibility of each partner for each independent interlaboratory comparison study, i.e. each version of the SOP. The data presented provides mean (of at least three independent replicates) DCFH2 oxidation in response to SIN-1, NPCB, CuO and Mn2O3, SD, COV and the COV of the first SOP minus the COV of the second SOP; a positive value would represent improved replicate reproducibility.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Partner 1** | | |  | | | **Partner 2** | | |  | | |
|  | **1st RR** | | | **2nd RR** | | | **1st RR** | | | **2nd RR** | | |
| **SIN-1 (µM)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 205.6 | 39.1 | 19 | 155.4 | 42.2 | 27.1 | 99.8 | 15.8 | 15.9 | 171.6 | 46.7 | 27.2 |
| **13** | 1903.7 | 130 | 6.8 | 2113.6 | 463.1 | 21.9 | 1082.9 | 513.9 | 47.5 | 2144.8 | 187.9 | 8.8 |
| **25** | 3228.4 | 114.1 | 3.5 | 3542.7 | 695.7 | 19.6 | 1843.7 | 912.1 | 49.5 | 4056.3 | 232.3 | 5.7 |
| **50** | 5562.9 | 165.8 | 3 | 5988.4 | 1122.3 | 18.7 | 3308.8 | 1580.6 | 47.8 | 7090.8 | 463.7 | 6.5 |
| **100** | 9269.9 | 305.8 | 3.3 | 9800.4 | 1619.8 | 16.5 | 5595.6 | 2545.1 | 45.5 | 11581.3 | 723.1 | 6.2 |
| **NPCB (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 205.6 | 39.1 | 19 | 155.4 | 42.2 | 27.1 | 99.8 | 15.8 | 15.9 | 171.6 | 46.7 | 27.2 |
| **16** | 375.1 | 25.1 | 6.7 | 346.8 | 43.4 | 12.5 | 189.8 | 127.6 | 67.2 | 415.2 | 46.4 | 11.2 |
| **31** | 553.6 | 28.8 | 5.2 | 548.8 | 66.4 | 12.1 | 180.2 | 62.8 | 34.9 | 609 | 69.1 | 11.4 |
| **63** | 906.8 | 88.1 | 9.7 | 937.2 | 88.2 | 9.4 | 271.4 | 100.2 | 36.9 | 955.2 | 139.5 | 14.6 |
| **125** | 1779.8 | 340.2 | 19.1 | 2043.7 | 208.9 | 10.2 | 471.3 | 184.7 | 39.2 | 1993.8 | 307.6 | 15.4 |
| **CuO (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 205.6 | 39.1 | 19 | 155.4 | 42.2 | 27.1 | 93.4 | 21.6 | 23.1 | 171.6 | 46.7 | 27.2 |
| **16** | 232 | 24 | 10.3 | 174.7 | 14.4 | 8.2 | 209.9 | 34.3 | 16.4 | 236.1 | 54.4 | 23 |
| **31** | 267.2 | 23 | 8.6 | 198.4 | 18 | 9.1 | 240.5 | 37.9 | 15.7 | 256.9 | 54.7 | 21.3 |
| **63** | 325.1 | 20.1 | 6.2 | 237.1 | 17 | 7.2 | 290.4 | 44.9 | 15.4 | 298 | 60.1 | 20.2 |
| **125** | 425.6 | 20.3 | 4.8 | 310.7 | 13.1 | 4.2 | 346.3 | 97.9 | 28.3 | 368.3 | 67.8 | 18.4 |
| **250** | 575.8 | 19.3 | 3.3 | 436.1 | 9.4 | 2.2 | 489.1 | 208.1 | 42.5 | 484.6 | 70.2 | 14.5 |
| **500** | 790.5 | 49.1 | 6.2 | 654.2 | 53.6 | 8.2 | 934.6 | 726 | 77.7 | 689.3 | 86.9 | 12.6 |
| **1000** | 1160.2 | 99.4 | 8.6 | 989.7 | 73.2 | 7.4 | 2026.3 | 1145.2 | 56.5 | 1200 | 222.5 | 18.5 |
| **Mn2O3  (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 203.2 | 43.3 | 21.3 | 155.4 | 42.2 | 27.1 | 96.7 | 23.4 | 24.2 | 171.6 | 46.7 | 27.2 |
| **16** | 457.6 | 21.3 | 4.6 | 439.8 | 104.7 | 23.8 | 322.7 | 124.4 | 38.5 | 472.6 | 62.9 | 13.3 |
| **31** | 545.3 | 17.2 | 3.2 | 519.8 | 114.2 | 22 | 364.1 | 100.7 | 27.7 | 543.7 | 63.4 | 11.7 |
| **63** | 638.6 | 1.3 | 0.2 | 618 | 133.9 | 21.7 | 445.8 | 114.3 | 25.6 | 629.5 | 59.5 | 9.4 |
| **125** | 740.8 | 5 | 0.7 | 757.9 | 187 | 24.7 | 570.6 | 172.3 | 30.2 | 724.1 | 58.4 | 8.1 |
| **250** | 932.2 | 32.7 | 3.5 | 1021.2 | 349.8 | 34.3 | 775.8 | 291.5 | 37.6 | 853.3 | 55.3 | 6.5 |
| **500** | 1417.7 | 33.8 | 2.4 | 1421.5 | 479.5 | 33.7 | 1155.1 | 523.1 | 45.3 | 1198.1 | 167.1 | 13.9 |
| **1000** | 2157.1 | 54.1 | 2.5 | 2288.8 | 571.8 | 25 | 1400.3 | 487.1 | 34.8 | 2091.4 | 244.8 | 11.7 |
|  | **Partner 3** | | |  | | | **Partner 4** | | |  | | |
|  | **1st RR** | | | **2nd RR** | | | **1st RR** | | | **2nd RR** | | |
| **SIN-1 (µM)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 679.7 | 304.4 | 44.8 | 703.3 | 107.5 | 15.3 | 134.6 | 57.2 | 42.5 | 154 | 13.8 | 8.9 |
| **13** | 10317 | 2404 | 23.3 | 9922.7 | 1396.6 | 14.1 | 2570 | 224.3 | 8.7 | 3153.7 | 723 | 22.9 |
| **25** | 18531.5 | 6111.2 | 33 | 16891.4 | 1972.4 | 11.7 | 4933.2 | 240.9 | 4.9 | 5867.7 | 1288.8 | 22 |
| **50** | 34435.7 | 8627.2 | 25.1 | 28321.6 | 2950.2 | 10.4 | 8751.9 | 473 | 5.4 | 10460 | 1693.8 | 16.2 |
| **100** | 60989 | 13550.9 | 22.2 | 45387.1 | 6371.4 | 14 | 14748.3 | 855.8 | 5.8 | 17273.5 | 1498.9 | 8.7 |
| **NPCB (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 679.7 | 304.4 | 44.8 | 703.3 | 107.5 | 15.3 | 134.6 | 57.2 | 42.5 | 154 | 13.8 | 8.9 |
| **16** | 1436.1 | 107.7 | 7.5 | 3236.3 | 603.4 | 18.6 | 392.1 | 10.3 | 2.6 | 468.5 | 15.2 | 3.3 |
| **31** | 2311.4 | 339.5 | 14.7 | 4609.3 | 273.1 | 5.9 | 664.8 | 27 | 4.1 | 705.9 | 86.3 | 12.2 |
| **63** | 3756.9 | 726.7 | 19.3 | 7057.8 | 764.6 | 10.8 | 1263.7 | 141.4 | 11.2 | 1253.9 | 116.9 | 9.3 |
| **125** | 6163.4 | 1061.4 | 17.2 | 8793.6 | 1864.2 | 21.2 | 2423.3 | 114.9 | 4.7 | 2156.3 | 206.9 | 9.6 |
| **CuO (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 679.7 | 304.4 | 44.8 | 703.3 | 107.5 | 15.3 | 134.6 | 57.2 | 42.5 | 154 | 13.8 | 8.9 |
| **16** | 1328.9 | 293.2 | 22.1 | 645.1 | 75.6 | 11.7 | 436.7 | 59.9 | 13.7 | 153.4 | 7.7 | 5 |
| **31** | 1628.8 | 476.6 | 29.3 | 667.7 | 47.8 | 7.2 | 583.6 | 75.5 | 12.9 | 183 | 9.3 | 5.1 |
| **63** | 2143.4 | 928.7 | 43.3 | 734 | 18.7 | 2.5 | 903.9 | 115.8 | 12.8 | 250.9 | 15.2 | 6.1 |
| **125** | 2811.9 | 1469 | 52.2 | 910 | 33.1 | 3.6 | 1619.8 | 216 | 13.3 | 367.4 | 27.1 | 7.4 |
| **250** | 4013.3 | 2800.5 | 69.8 | 1241.7 | 65 | 5.2 | 2948.6 | 300 | 10.2 | 520.9 | 41.1 | 7.9 |
| **500** | 5480.1 | 4366.7 | 79.7 | 1782.2 | 145 | 8.1 | 5187.3 | 573.7 | 11.1 | 693.6 | 60.6 | 8.7 |
| **1000** | 8026.8 | 6702.2 | 83.5 | 2757.7 | 467.2 | 16.9 | 8843 | 606.5 | 6.9 | 914.2 | 101.1 | 11.1 |
| **Mn2O3  (µg/ml)** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** | **Av.** | **SD** | **COV** |
| **0** | 679.7 | 304.4 | 44.8 | 703.3 | 107.5 | 15.3 | 134.6 | 57.2 | 42.5 | 154 | 13.8 | 8.9 |
| **16** | 2198.7 | 398.8 | 18.1 | 882 | 104.2 | 11.8 | 386.5 | 52.8 | 13.7 | 502.3 | 107.1 | 21.3 |
| **31** | 2613.8 | 481.7 | 18.4 | 948.2 | 130.9 | 13.8 | 469.5 | 69.9 | 14.9 | 634.7 | 143.9 | 22.7 |
| **63** | 3067.6 | 557.5 | 18.2 | 1059.8 | 159 | 15 | 616.9 | 130.8 | 21.2 | 837.7 | 177.9 | 21.2 |
| **125** | 3443.7 | 394.4 | 11.5 | 1179.4 | 186.1 | 15.8 | 914.2 | 251.4 | 27.5 | 1121.2 | 247.6 | 22.1 |
| **250** | 3904.3 | 212.2 | 5.4 | 1290.3 | 206.1 | 16 | 1602.5 | 512.9 | 32 | 1526.2 | 433.6 | 28.4 |
| **500** | 5096.3 | 931.4 | 18.3 | 1351.4 | 337.7 | 25 | 3021 | 769.4 | 25.5 | 2050.4 | 719.7 | 35.1 |
| **1000** | 7047.9 | 2098.7 | 29.8 | 1528.2 | 469.4 | 30.7 | 4832.9 | 955.2 | 19.8 | 2675.7 | 745.8 | 27.9 |

### Final Standard Operating Procedure: detection of acellular Reactive Oxygen Species (ROS)

**Assay principles**

The probe used in this assay is the lipophilic non-fluorescent molecule 2'-7'-dichlorodihydrofluorescin diacetate (DCFH2-DA). This probe was first devised to detect reactive oxygen species (ROS) in the absence of cells [[2](#_ENREF_2), [3](#_ENREF_3)]. Later, this method was developed to detect intracellular ROS production in response to membrane stimulation with phorbol 12-myristate 13-acetate (PMA) [[4](#_ENREF_4)], and then more recently as a tool to study cellular and acellular ROS produced in response to nanomaterials [[5](#_ENREF_5)]. The removal of the diacetate moieties (DA) is a requirement to allow DCFH2 to interact with ROS. In the absence of cells, the removal of diacetate is achieved by alkaline hydrolysis [[2](#_ENREF_2), [3](#_ENREF_3)]. However for the cellular system, the presence of DA not only stabilizes the probe, but also enables it to be cell permeable; de-esterification of DCFH2-DA to DCFH2 in a cellular system is performed by cell-membrane-bound esterases during probe internalization [[1](#_ENREF_1), [4](#_ENREF_4)] (Figure 1). In either event, the still non-fluorescent probe DCFH2 is subject to oxidation in the presence of ROS, becoming DCF¯ and fluorescent.



**Figure 1. Conversion of DCFH-DA into its fluorescent form, DCF-**. (image adapted from [[1](#_ENREF_1)]) DCFH2-DA passively enters cells, is cleaved by intracellular esterases, or chemically hydrolysed with NaOH, leaving DCFH2, which can be oxidised in the presence of ROS, becoming fluorescent.

**Safety** **precautions**

* In-house procedures for safe handling of nanomaterials and other potentially hazardous compounds should be followed.
* All work should be performed in a laminar flow Biosafety cabinet to ensure sterile handling of cell cultures.

**Equipment and reagents**

Reagents

* 2’,7’-dichlorodihydrofluoresin diacetate (DCFH2-DA) (Sigma D6883)
* Fluorescein diacetate (F-DA) (Sigma F7378)
* 0.01 M NaOH (Sigma S5881)
* Methanol (analytical grade) (Sigma 34860)
* 0.1M (x10) PBS (Gibco DPBS (10x) 14200-059)
* phenol red free MEM (Gibco 51200-046)
* FCS (heat inactivated: Gibco 10500-064)
* 3-Morpholinosydnonimine hydrochloride (SIN-1 hydrochloride) (Abcam #ab141525)
* Acetone

Equipment

* Black, flat-bottom 96-well plates (Corning 353219)
* Pipettes, micropipettes and tips
* 37 °C and 5% CO2 incubator
* Laminar flow hood
* Freezer (-20 °C)
* 1.5 ml microcentrifuge tubes
* 50 ml conical tubes or equivalent
* Microtiter plate reader with fluorescence capability (ex. 485 nm, em. 530 nm)

**Experimental protocol - storage**

F-DA storage

* Make 10 mM stock (of fluorescein) solution, dissolve 52.17 mg F-DA in 10 ml acetone
* make about 100 aliquots of 100 µl stock in eppendorf tubes, protected from light (black microcentrifuge tubes are recommended)
* keep at -20 °C for a maximum of 3 months, storage of F-DA stocks are stable for a period of 12 weeks; note that longer periods have not been tested).

DCFH2-DA storage

* Make 10 mM stock solution of DCFH2-DA, dissolve 48.75 mg in 10ml methanol
* make about 50 aliquots of 200 µl stock in eppendorfs (one aliquot is sufficient reagent for a full 96-well plate experiment) protected from light (black microcentrifuge tubes are recommended)
* keep at -20 °C for a maximum of 3 months. NB: Longer periods have not been tested.
* NB – DCFH2-DA is air, light and temperature sensitive.

**Experimental protocol – interference**

Particle interference

* To assess if autofluorescence can be attributed to test particles, resulting in the emitted signal being falsely interpreted as DCFH2 oxidation, a range of particle concentrations are used in the experimental setup of the acellular method, with the omission of DCFH2. Any dose-dependent fluorescence based on particles being present can be observed. If a signal is observed the particle concentration must be lowered until a suitable level is attained, if this is not possible it should be considered that the assay is not suitable for that particular material.
* To assess if particles quench a signal generated by DCFH2 oxidation, and thus provide a reduced or a false-negative result, a range of particle concentrations are used in an experimental setup using fluorescein diacetate (F-DA). If substantial quenching is observed, the concentrations responsible cannot be used for that particular material.

Preparation of F-DA for interference testing

The following conditions should be used. In order to obtain a 0.1 µM fluorescein solution:

* Add 50 µl of 10 mM stock to 450 µl acetone = 1mM F-DA solution
* Protect from light and keep on ice – use within 4 hours
* Add 2 ml 0.01 M NaOH to 500 µl of 1 mM F-DA (= 200 µM)
* Incubate 5 minutes at room temperature, protected from light
* Add 7.5 ml of 0.1 M PBS solution pH7.4 (= 50 µM)
* Dilute to 0.1 µM: 50 µl of 50 µM in 25 ml PBS

**Test for particle interference**

Particle concentrations are prepared by performing 2-fold serial dilutions according to the Table 1.

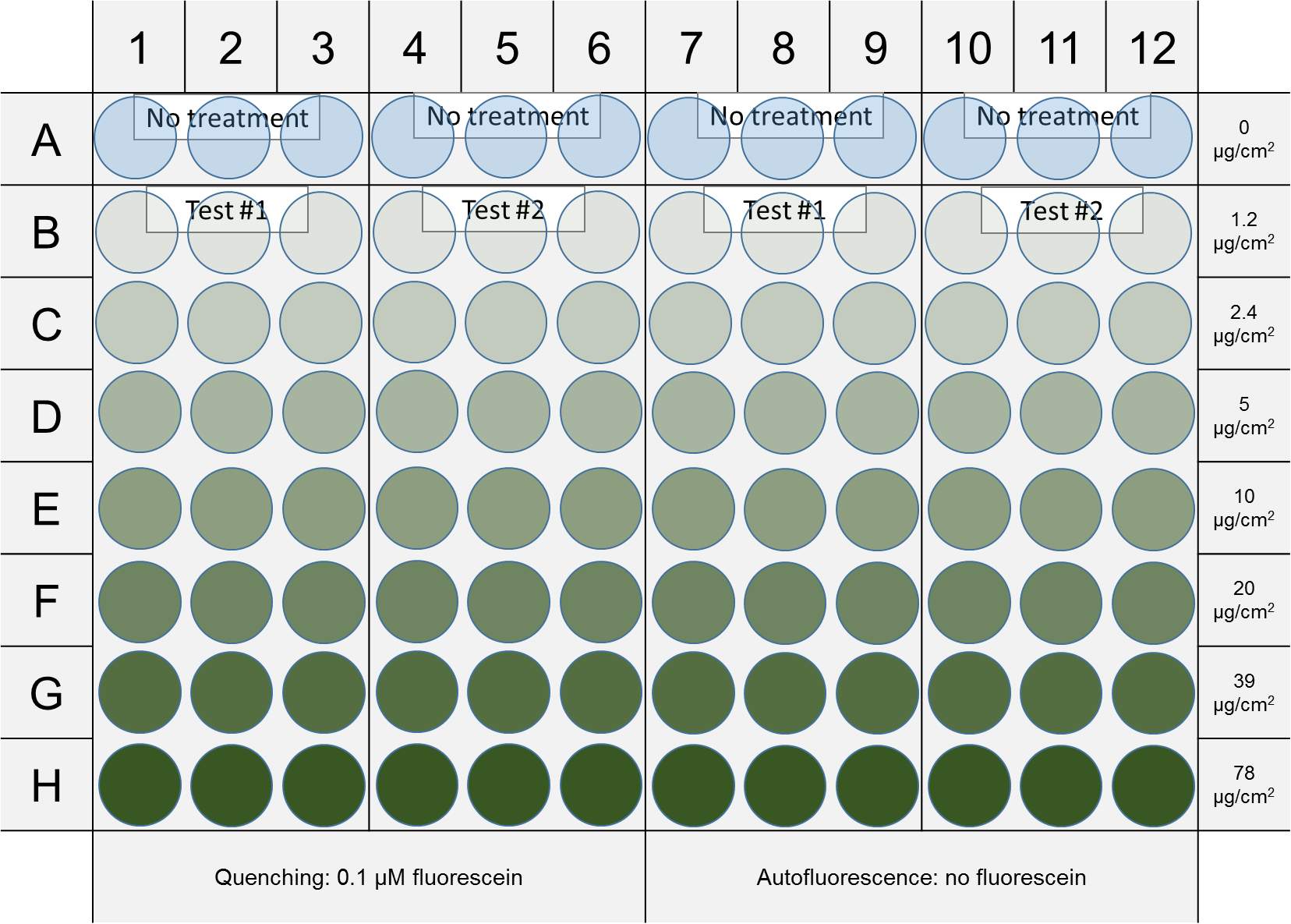
Following the plate layout provided in Figure 2, in a black clear bottom 96-well plate add, in triplicate:

* 25 µl Particles (or 25 µl vehicle fluid (MEM or MEM + 2% FCS for “no treatment” wells)
* 225 µl 0.1 µM fluorescein to columns 1-6
* 225 µl PBS to columns 7-12
* Read immediately using ex/em 485/530

Note that the fluorescence intensity of 0.1 µM should not cause a saturation of signal detection. However, if saturation does occur, it is required that instrument settings are adjusted to reduce intensity and avoid saturation.

**Table 1. Concentrations of particles used for interference test.**

|  |  |
| --- | --- |
| Treatment | Concentration of test material |
| Suspended concentration (µg/ml) | 16, 31, 63, 125, 250, 500, 1000 |
| Final concentration (µg/cm2) | 1.2, 2.4, 5, 10, 20, 39, 78 |
| Final concentration (µg/ml) | 1.6, 3.1, 6.3, 12.5, 25, 50, 100 |



**Figure 2. Plate layout for interference test.**

**Experimental protocol – DCFH2-DA assay**

**Timing/order of preparation**

Due to differences in stability, it is suggested that the following order of preparation is taken: DCFH, F-DA, particles, SIN-1 – note that substance preparation can overlap as one may start during incubation periods of another, e.g. F-DA, particles suspensions and SIN-1 can all be initiated during the 30 minutes hydrolysis of DCFH2-DA.

**Hydrolysis of DCFH2-DA**

In order to obtain a 10 µM DCFH2 solution:

* Add 50 µl of 10 mM stock to 450 µl methanol = 1mM DCFH2-DA solution
* Add 2 ml 0.01 M NaOH to 0.5 ml of 1 mM DCFH2-DA (= 200 µM)
* Incubate 30 min at room temperature, protected from light
* Add 7.5 ml of 0.1 M PBS solution pH7.4 to stop reaction (= 50 µM)
* For 1 full 96-well plate of 10 µM final concentration:
  + Add 5 ml 50 µM DCFH to 20 ml 0.01 M PBS
* Keep on ice and use rapidly

**Preparation of F-DA for generation of a standard curve**

To allow for more robust interlaboratory comparisons, the fluorescence of fluorescein at different concentrations is used as a standard curve, to control for differences in signalling intensities collected by different instrumentation. The signal intensity of fluorescein is reliably stable when the some batch is stored for various periods of time, is prepared for use on different occasions, and when different starting batches were used.

* Add 50 µl of 10 mM stock to 450 µl acetone = 1mM F-DA solution
* Add 2 ml 0.01 M NaOH to 500 µl of 1 mM F-DA (= 200 µM)
* Incubate 5 minutes at room temperature, protected from light
* Add 7.5 ml of 0.1 M PBS solution pH7.4 (= 50 µM)
* Dilute to top standard concentration (1 µM) – 100 µl of 50 µM plus 4.9 ml PBS
* Perform 3-fold serial dilutions (400 µl previous stock plus 800 µl PBS) to obtain the following concentrations: 0.001, 0.004, 0.012, 0.037, 0.111, 0.333, 1.000 µM
* PBS alone is used as 0 µM F-DA
* Protect from light and use within 4 hours

**Prepare control suspensions**

Treatments are prepared in phenol red-free medium (MEM), with 2% foetal calf serum (FCS).

**NPCB preparation:**

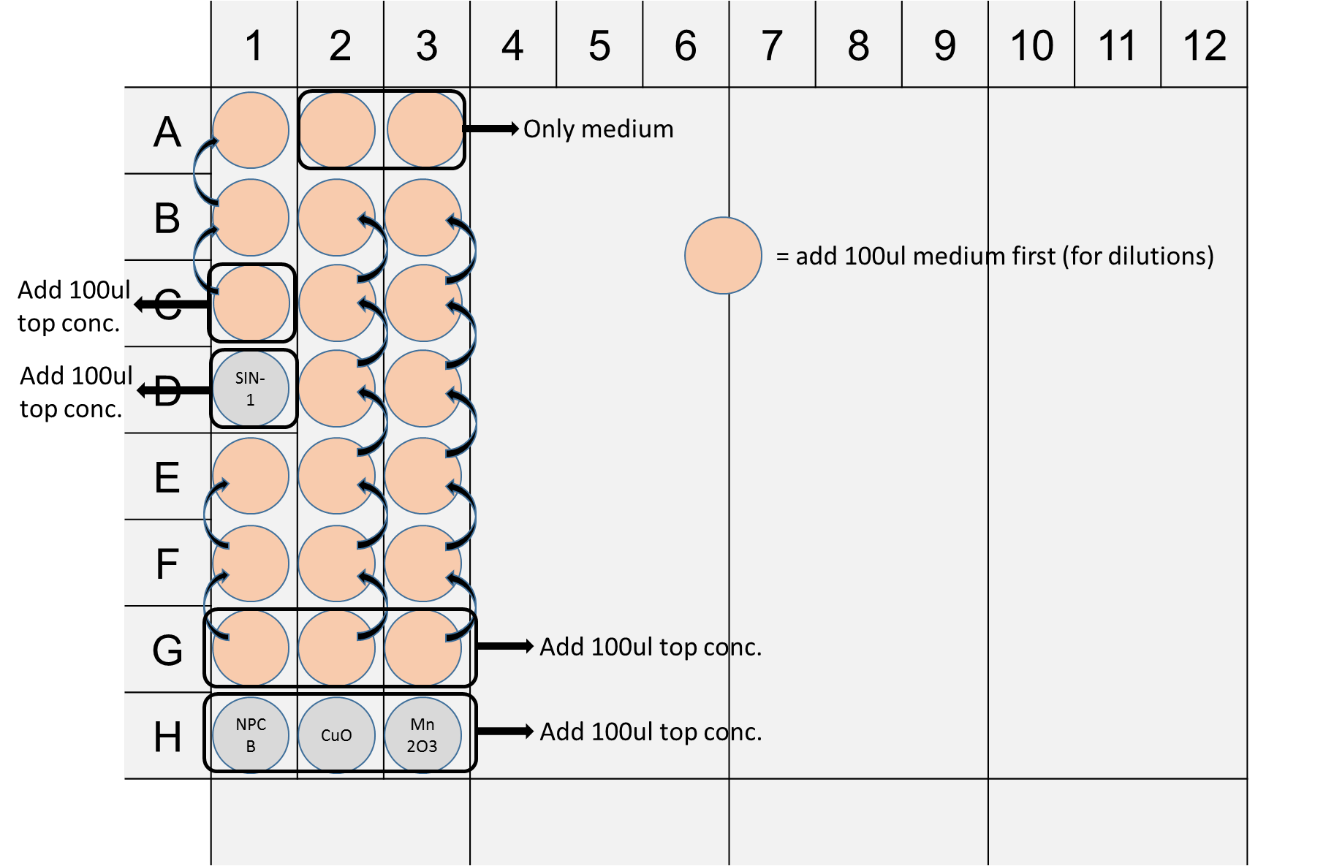
* NPCB is weighed into bijous at between 4-6 mg, and suspended in the treatment medium at 1 mg/ml
* The sample is briefly vortexed and then ultra-sonicated in a sonicating water bath for 15 minutes prior to dilution to the required highest concentration (125 µg/ml) in the same treatment medium.
* Sample is vortexed before use, and dilutions are made using a 96-well, round-bottomed ‘loading’ plate as depicted in Figure 3; concentrations align with those given in Table 2.

**SIN-1 preparation (for Abcam #ab141525 (MW: 206.63)):**

* When required, thaw and perform the following dilutions:
* 1 in 200 in treatment medium (5 µl of 200 mM SIN-1 + 1000 µl medium) = 1000 µM
* 1 in 10 in treatment medium (100 µl of 1000 µM SIN-1 + 900 µl medium) = 100 µM
* Sample is vortexed before use, and dilutions are made using a 96-well, round-bottomed ‘loading’ plate as depicted in Figure 3; concentrations align with those given in Table 2.

**Table 2. Concentrations of positive controls used in acellular ROS generation.**

|  |  |  |
| --- | --- | --- |
| Treatment | Sin-1 | NPCB |
| Suspended concentration | 12.5, 25, 50, 100 µM | 16, 31,63,125 µg/ml |
| Final concentration | 1.25, 2.5, 5, 10 µM | 1.2, 2.4, 5, 10 µg/cm2 |



**Figure 3. Preparation of sample dilutions within a 96-well, round-bottom loading plate.**

**Prepare test material suspensions**

Treatments are prepared in phenol red-free medium (MEM), with 2% foetal calf serum (FCS).

In general, the concentration range of the test material can be selected based on two aspects: a quantified sub-lethal concentration (to represent a biologically relevant dose, for which the half-LC20, LC20, and 2 x LC20 are used), and/or concentrations identified to be below a level to cause substantial interference with the fluorescence signal (as previously identified).

NB. For the purposes of an inter-laboratory comparison study, a large concentration range will be used, see Table 3.

**Particle preparation:**

* Each test material is weighed into bijous at between 4-6 mg, and suspended in the treatment medium at 1 mg/ml
* The sample is briefly vortexed and then ultra-sonicated in a sonicating water bath for 15 minutes prior to dilution to required concentrations in the same treatment medium.
* Each sample is vortexed before use, and dilutions are made using a 96-well, round-bottomed ‘loading’ plate as depicted in Figure 3; concentrations align with those given in Table 3.

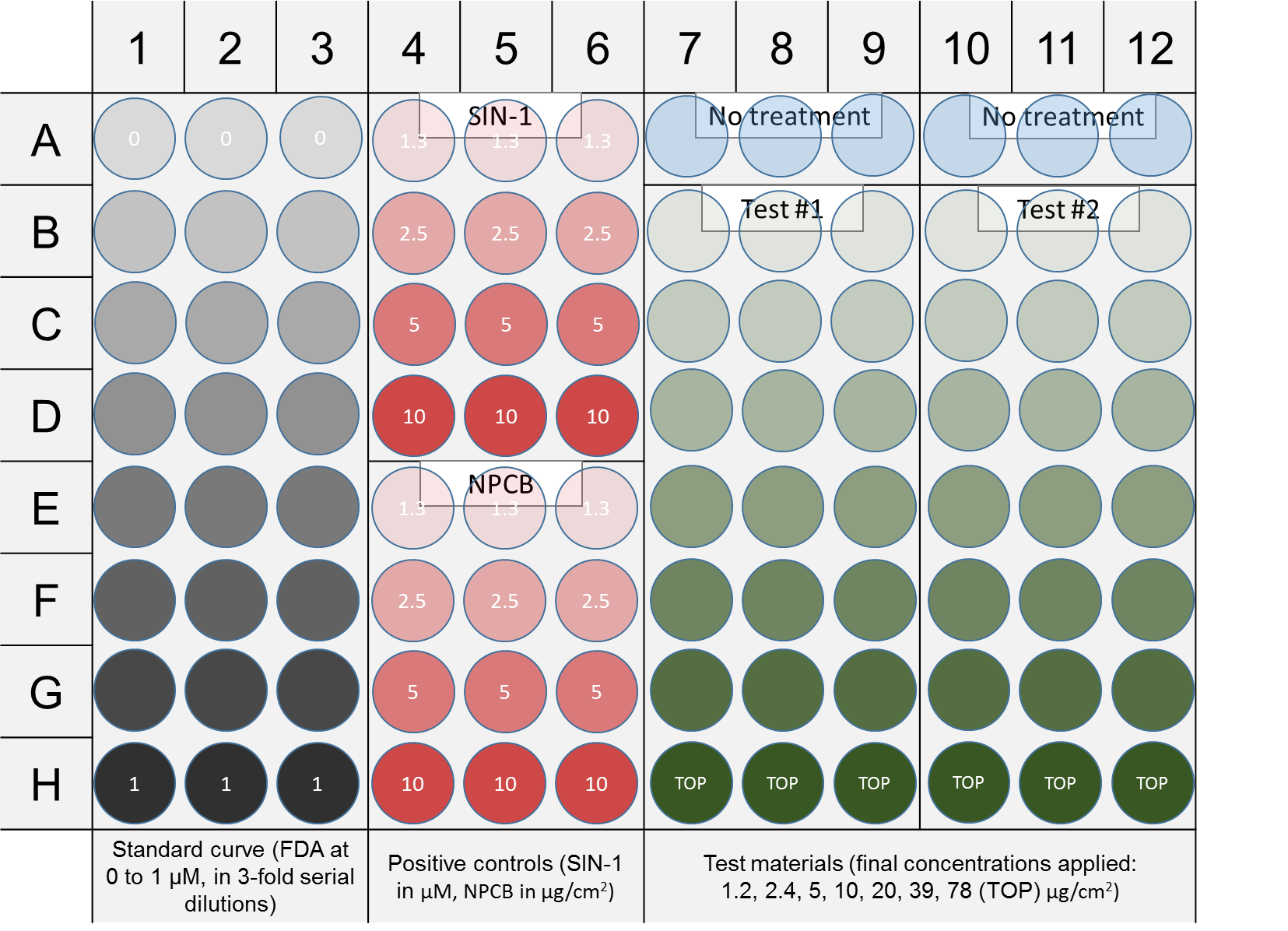
**Table 3. Concentrations of particles used in acellular ROS generation.**

|  |  |
| --- | --- |
| Treatment | Test material |
| Suspended concentration (µg/ml) | 16, 31, 63, 125, 250, 500, 1000 |
| Final concentration (µg/cm2) | 1.2, 2.4, 5, 10, 20, 39, 78 |
| Final concentration (µg/ml) | 1.6, 3.1, 6.3, 12.5, 25, 50, 100 |

**Assay**

This assay should be conducted with at least three independent replicates. As no substantial plate bias of fluorescence signal has been observed, the plate layout provided in Figure 4 can be followed during each repeat of this assay. In a black clear bottom 96-well plate add, in triplicate:

* For standard curve (columns 1-3), add 250 µl of relevant concentration to each well.
  + For no treatment, positive controls and test materials (columns 4-12), add 25 µl using a multi-channel pipette from loading plate in triplicate (pipetting prior to transfer to ensure adequate dispersion), followed by 225 µl DCFH2 reaction mix.
* Read immediately, and then after 30, 60, and 90 minutes.
* At ex/em 485/530
* Between measurements store plate at 37 °C and protect from light



**Figure 4. Plate layout for acellular DCFH assay.**

**Data analysis**

**Acceptance criteria**

The data obtained is accepted only if the following criteria are met:

* No interference of NP with the assay is observed during the interference pre-test.
* The coefficient of variation of each set of experimental triplicates is below 30%
* The positive control treatments (SIN-1 and NPCB) induce a significant increase in DCFH2 oxidation

**Data handling – test for particle interference**

The raw data obtained is transferred to a simple spreadsheet-based analysis template for data handling. This spreadsheets includes the following:

* Assessment of variance (standard deviation and COV) between technical replicates.
* Calculation of percent fluorescence quenching based on fluorescein signal in the absence of particles – quenching up to 30% is considered acceptable.
* Calculation of signal increases in the presence of particles when fluorescein is absent, based signal in the absence of particles – increases up to 1.3 x background is considered acceptable.

**Data handling – test for ROS generation**

The raw data obtained is transferred to a simple spreadsheet-based analysis template for data handling. This spreadsheets includes the following:

* Assessment of variance between technical replicates.
* Fitting of the standard curve based on fluorescein fluorescence.
* Calculation of particle-induced ROS generation by subtracting time zero values from each subsequent time measurement, and converting the arbitrary fluorescence values to molar values of oxidised fluorescein through use of the standard curve.

**References**

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