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Electronic Supplementary Information

Feasibility of using a standardized *Caenorhabditis elegans* toxicity test to assess nanomaterial toxicity

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Supplementary Methods

Media composition

S-basal: 5.85g NaCl, 1g K_2HPO_4, 6g KH_2PO_4, 10ml 1M potassium citrate, 0.33g $CaCl_2$, 0.36g $MgSO_4$, 10ml trace metals solution (1.86g disodium EDTA, 0.69g $FeSO_4$, 0.2g $MnCl_2$, 0.29g $ZnSO_4$, 0.025g $CuSO_4$, fill to 1L with ultrapure water), fill to 1L with ultrapure water

K^+ medium: 2.38g KCl, 2.98g NaCl, 0.33g $CaCl_2$, 0.36g $MgSO_4$, fill to 1 L with ultrapure water

M9 medium: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, fill to 1 L with ultrapure water

NGM agar plates: 3 g NaCl, 2.5 g bacto peptone, 17 g agar, 25 ml potassium phosphate buffer (132 ml K_2HPO_4 (1M) and 868 ml KH_2PO_4 (1M)), 1 ml $MgSO_4$ (1M), 1ml $CaCl_2$ (1M), 1ml cholesterol (5 mg ml^{-1} in ethanol), fill to 1 L with ultrapure water

Nematode synchronization

While there are several methods of synchronizing nematodes, including individually picking adults, bleaching a culture of nematodes, using dauer larvae, and filtering a mixed culture, the ISO protocol calls for the use of 5µm and 10µm filter gauze, which is not readily available. Using filter gauze will only help to remove adults from a mixed culture and will leave other stages in the filtrate. Other filter types, such as 5 µm or 10 µm polycarbonate filters, did not seem to work well because J1 nematodes did not pass through them. Bleaching will remove most contaminants, leaving only J1 nematodes for the study. However, bleach strength, time, and density of culture may all impact the quality of the resulting suspension and must be carefully controlled.

Imaging optimization and processing, nematode length measurements, and reproductive counts

Whole-well imaging improved the reliability of nematode measurements by providing a system by which a line can be placed on each nematode and their length determined through software instead of manually estimating length based on a scale bar under a microscope. However, whole-well imaging also introduced additional sources of variability and required optimization of various parameters. We optimized the amount of Rose Bengal to add to the wells by adding increasing concentrations to wells and measuring the difference in intensity between a nematode and the background. We achieved the greatest contrast at 60 mg l^{-1} of Rose Bengal. We imaged each well of the 12 well plates using a CoolSNAPHQ2 CCD camera (Photometrics, Tucson, AZ) coupled to an automated Zeiss microscope (Axio Vert.A1, Carl Zeiss Microscopy, Oberkochen, Germany) with Zen software (Carl Zeiss Microscopy, 2012 Blue Edition). The microscope was calibrated using a stage micrometer (Electron Microscopy Services) at 5x prior

to the study. Transmitted light intensity was set to 3.7 V and exposure time was 2 ms. Whole-well imaging was improved by addition of 1 ml of mineral oil to the top of the well, which reduced darkening generated by the water meniscus. The plate was calibrated by finding and focusing on the edges of the wells. A focus surface was defined by fixing five points in each well. While adding additional points would improve focus, we found that five points provided sufficient focus to allow identification and measurement of worms. Using the calibration and focal points, entire wells were imaged (see Fig. S4 A). Images were exported as .tiff files and adult hermaphrodites were measured (males, if present, were excluded) and young were counted using ImageJ (1.47v, Wayne Rasband, NIH, USA) with the scale based on the stage micrometer calibration. Total nematode length was measured using a segmented line tracing the center of the nematode from the tip of the head to the end of the tail. The difference in mean nematode length between two operator's measurements of nematodes using this procedure was 12 µm (n=45) or approximately 1.5 %, suggesting little impact of operator variability on this measurement. Additionally, this method produced similar measurements to that of using a scale bar as indicated in ISO 10872. Comparing the measurements of 50 nematodes using both methods produced a 3.7 % ± 2 % difference in length. Young were counted in one quarter of each well to estimate total well reproduction.

While issues such as stitching, poor focus, and interferences may impact image quality, the automated imaging system helped to overcome many of those problems and increased the quality of our data. Additionally, this technique produces archival data which allows for additional analysis in the future. Optimal image stitching was necessary to piece together all of the images taken for each well (Fig S4A&B). This was achieved by using different settings in the Zen software package to determine which settings produced the most accurately stitched images. Poorly stitched images contained shadows of nematodes and/or unmatched or poorly matched portions of nematode bodies (Fig. S7A&B). In these images, we were unable to accurately measure the worm lengths. The following settings were changed to improve the image stitching: minimal overlap, maximum shift, comparer, and global optimizer. The settings that produced the best stitched images were: 6% minimal overlap, 2% maximum shift, best comparer, and best global optimizer (Fig. S7C). However, it should be noted that these settings differ depending on which objective is being used. After stitching, the images were exported as .tiff images. We did not try to use the stitching algorithm provided by ImageJ because this program, and many other software packages, requires that a complete rectangle of images be acquired and we only acquired images to recreate the circular well.

We attempted to improve the quality of images by washing wells with M9 several times to remove larger *E. coli* aggregates that could interfere with nematode imaging. This was accomplished by adding 1 ml of M9, allowing the nematodes to settle for 10 minutes, and then removing 1 ml from the wells; however, this step proved to be time consuming and image

improvement was not consistent. At times, washing the wells caused *E. coli* that had grown on the bottom of plates to be suspended and cover nematodes, making measurements and counting the number of offspring extremely difficult. Additionally, it was necessary to ensure that nematodes were not removed from wells during washes and, if they were, to add them back into their appropriate wells. Thus, we do not recommend this approach.

Sensitivity testing

To test the impact of *C. elegans* culturing conditions on assay results, we tested three conditions: 1) nematodes from agar plates as described above, 2) nematodes in liquid culture in S-basal and fed with *E. coli*, or 3) nematodes maintained in liquid culture and starved for 4 d prior to starting the assay. To test the impact of live or dead *E. coli*, a portion of *E. coli* were UV treated in a petri dish for 30 min to kill them; the plate was mixed every 5 min by gentle swirling to ensure that all cells were exposed to UV light. Cell mortality was confirmed by plating the cells on LB-agar and incubated overnight at 37 °C to ensure that no viable cells were present. Live and dead bacteria were then tested in tandem to determine the EC_{50} values for BAC-C16. The sensitivity of the assay to the bacterial feed density was investigated by testing a range of feed densities extending above and below the concentration stipulated in the ISO protocol. Plate shaking was tested by preparing two sets of plates with control wells and wells with 15mg l^{-1} BAC-C16. One set of plates was placed in the incubator at 20 °C on a shaker rotating at 15.7 rad s^{-1} (150 rpm) for 96 h and the other set was placed in the incubator without shaking. Based on the findings from the sensitivity testing with BAC-C16 and on the parameters of the assay that would be most useful to adapt the test for use with ENMs, feed density, bacteria viability, and different assay media were tested with PSNPs to determine if changing these parameters altered the ENM's toxicity.

Figure S1. Generalized procedure for *C. elegans* toxicity assay as outlined in ISO 10872.

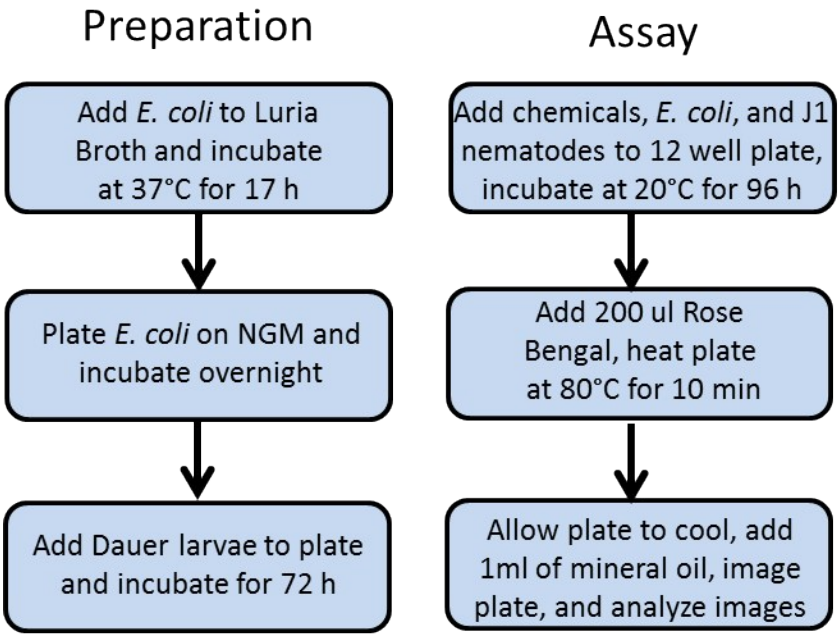


Figure S2.

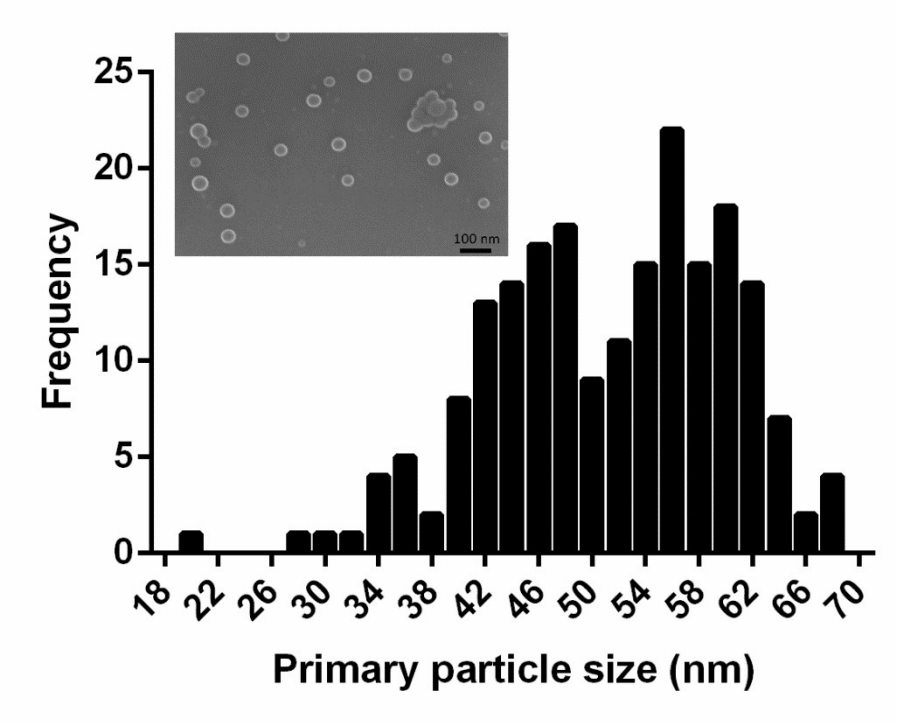
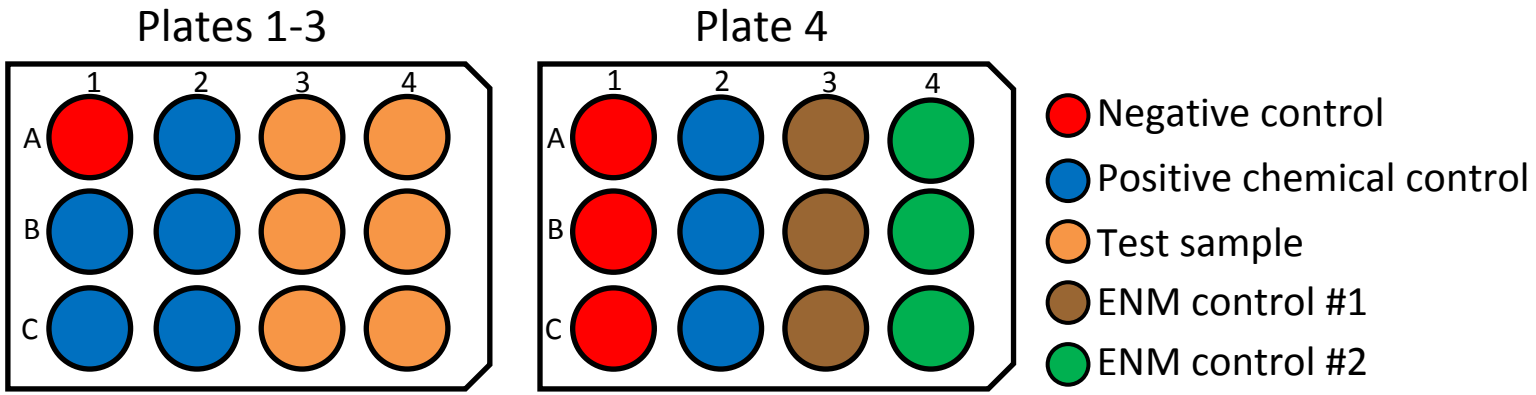

Frequency
25
20
15
10
5
0
100 nm
18
22
26
30
34
38
42
46
50
54
58
62
66
70
Primary particle size (nm)

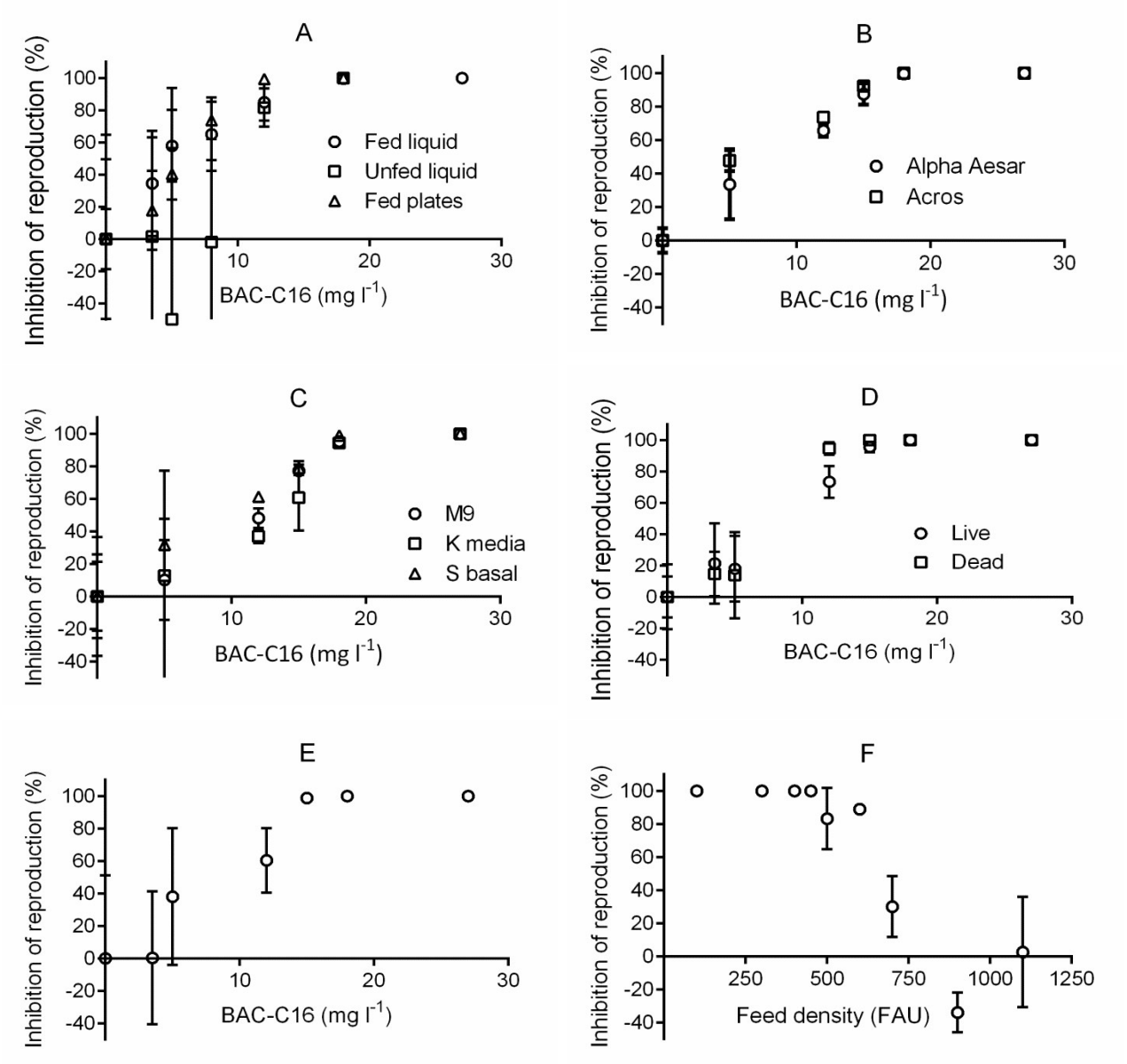
Figure S3. Toxicity assay 12 well plate design to examine toxicity of ENMs and potential artifacts associated with dispersion and coatings associated with ENMs. The negative control is water, positive control is BAC-C16, solvent control is the solvent that the ENMs are suspended in, and the ENM control can be a filtrate, sonication, coating, or other control associated with the ENM being used.



Control features	Purpose of control feature
Negative control wells	To assess the growth of *C. elegans* in wells without chemicals. If the growth rate, reproduction rate, or nematode size is below the specifications from control charting, this would indicate that there may be an issue with nematode health or feed concentration.
Positive chemical control wells, dose response curve	To assess the performance of the assay relative to a positive control reference chemical by obtaining an EC_{50} value response for each plate. If the EC_{50} values are not within the specifications from the control charting for each plate, this could indicate an issue with nematode health or with the chemical control.
Positive chemical control toxicity at 15 mg l^{-1} (plate 4)	The purpose of this control is to assess the performance of the assay relative to the EC_{50} value specified in the ISO document. If the growth inhibition values are not within the specifications from the control charting, this could indicate an issue with worm health or with the chemical control. This is the only control specification in the ISO method which specifies that the growth should be between 20 % and 80 %.

Test sample wells	To measure the EC_{50} value response of the test sample in three separate plates.
ENM control #1 and #2 in plate 4	The purpose of this control in Plate 4 is to make a control measurement specific to the test sample. For ENMs, this could include a filtrate, dispersant, solvent control, etc. and is dependent on the ENM characteristics.

Figure S4. Sensitivity analysis of ISO 10872 conducted by altering test conditions (shown in Figure 1) and comparing the outcome to the original protocol. The test parameters altered were (A) the culture from which the nematodes were harvested for the assay, (B) the manufacturer of the positive control BAC C16, (C) the media that the test was performed in, (D) bacterial viability, (E) the assay performed in a 24 well plate instead of 12 well (F) the amount of feed used in the assay (all exposures include 15 mg l^{-1} BAC-C16), and (G) whether the plates were shaken or left undisturbed (at 15 mg l^{-1} BAC-C16 reproduction is normally very low or nonexistent, for shaken plates no young were found and for those not shaken the average reproduction was 0.29 ± 0.4 young per adult). For each plot, reproductive data shown are mean ± one standard deviation, n=3 for each data point.



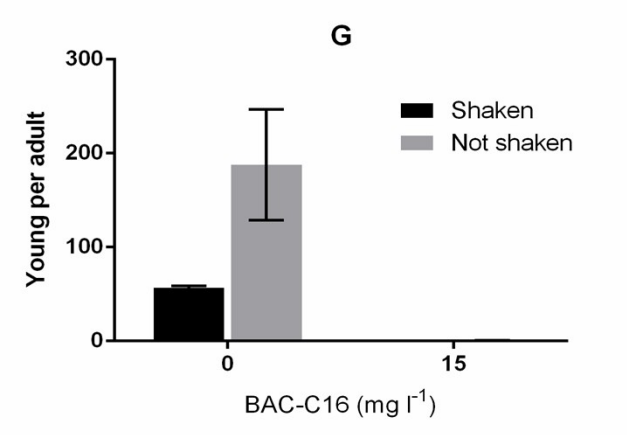
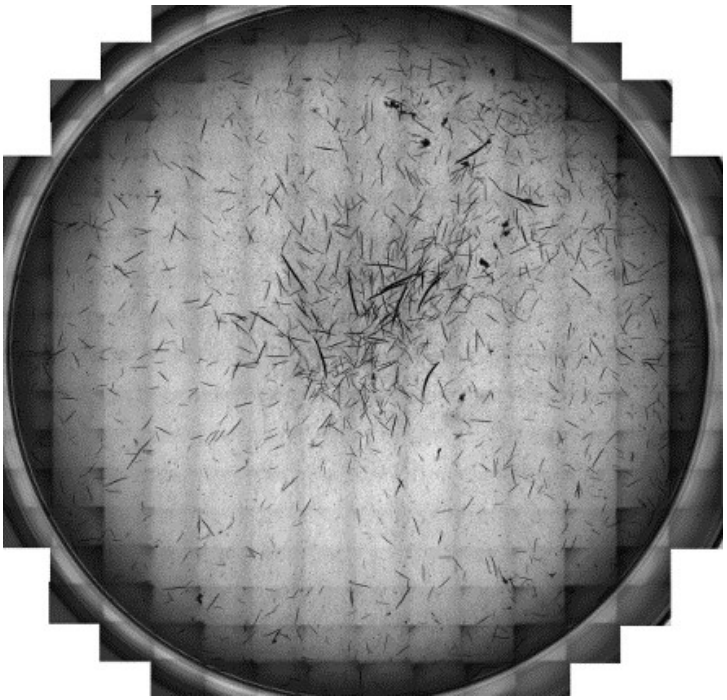

G
300
200
100
0
Young per adult
Shaken
Not shaken
0
15
BAC-C16 (mg l-1)

Figure S5. Brightfield images of individual wells of a 12 well plate containing the negative control water (A) and the PSNPs (B). *E. coli* appeared to aggregate heavily in wells containing PSNPs.

A



B

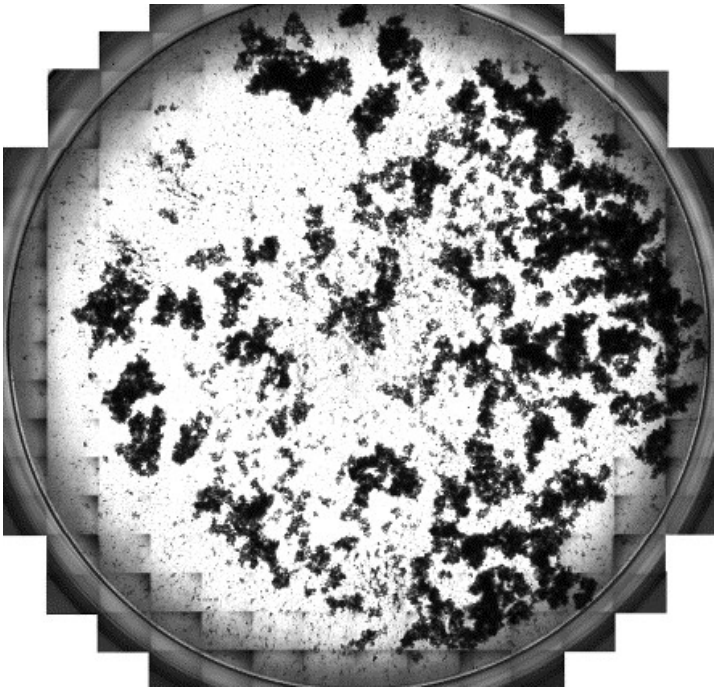
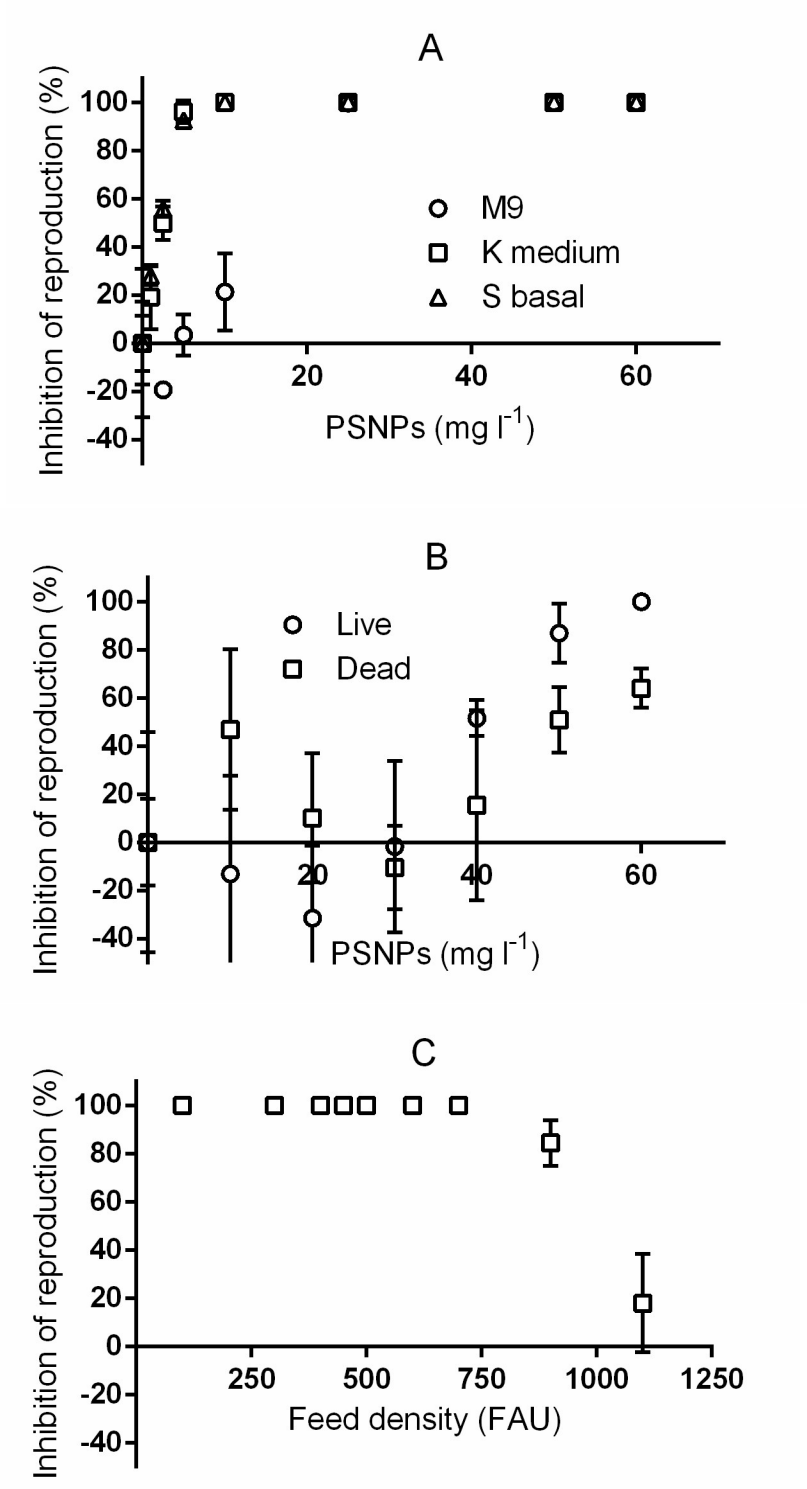


Figure S6. Sensitivity analysis of ISO 10872 containing PSNPs. The test parameters altered were (A) The feed density, (B) the media that the test was performed in, and (C) the bacterial density. Reproductive data presented as mean ± one standard deviation. N = 3 wells, each with 10 nematodes.



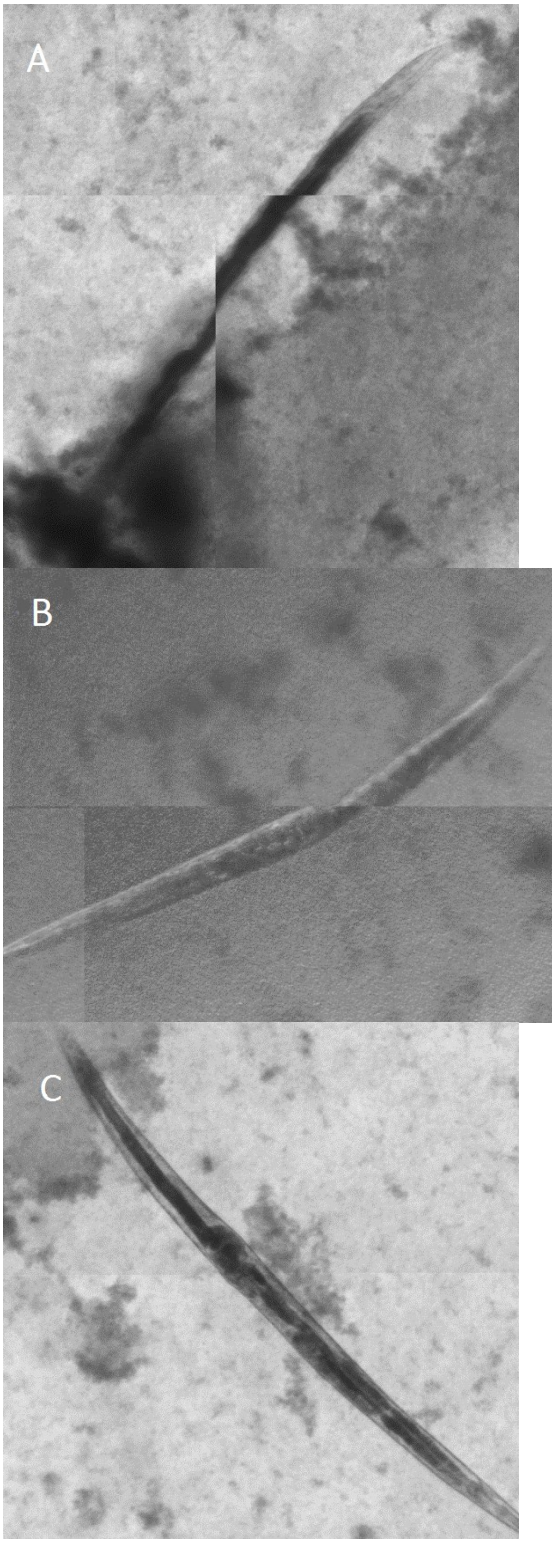


Figure S7. Optimization of image stitching parameters was required to enable reliable measurements. (A) Poorly stitched tiles showing multiple breaks in nematode body and poorly focused sections of nematode, (B) questionably stitched tiles showing single break in nematode body, and (C) optimized stitching of tiles showing intact nematode.

Table S1. Details of Branches of the Cause-and-Effect Analysis for ISO 10872.

Branch in Figure 2	Description	ENM specific relevance
1) Organism maintenance	Includes the type of culture from which nematodes are harvested which may impact their health and size	
2) Reference chemical	Addresses issues associated with the type and concentration of BAC-C16 as well as the solubility and potential error involved in pipetting the solution	
3) Bacteria	Associated with bacterial concentration, its measurement, how it changes during the assay, the strain used, and the viability of bacteria	Bacterial concentration as a result of heteroagglomeration and/or bactericidal effects of ENM may impact ENM growth inhibition
4) Protocol	This branch applies to the specific steps in the assay protocol as well as potential adaptations for ENMs including the media used, plate shaking, the size of the assay container, volume of the suspension in each well, and the number of nematodes added to each well	Media characteristics may impact ENM stability, dissolution, transformations, and aggregation
5) Microscopy	Addresses issues associated with imaging and measuring the nematodes and can include the calibration of the microscope, identifying the nematodes, and whether any steps were taken to remove bacteria from the wells	ENM stability or interactions with nematodes or *E. coli* may impact imaging of nematodes and ability of measuring length
6) ENM specific	Includes how the ENMs were dispersed, whether they dissolve and/or aggregate and settle, their interaction with the media, bacteria, plate material, or the nematodes	Same as for the “Description” column