Chapter 33

Luciferase Reporter System for Studying the Effect of Nanoparticles on Gene Expression

Min Ding, Linda Bowman, and Vincent Castranova

Abstract

Nanotechnology exploits the fact that nanoparticles exhibit unique physicochemical properties, which are distinct from larger particles of the same composition. It follows that nanoparticles may also express distinct bioactivity and unique interactions with biological systems. Therefore, it is essential to assess the potential health risks of exposure to nanoparticles to allow development and implementation of prevention measures. One of the biggest challenges facing the field of nanotoxicology is the huge variety of different nanoparticle types possessing a variety of properties. Genetic Luciferase Reporter System or Reporter gene assay has become an invaluable tool in studies of gene expression. This is achieved by linking the firefly luciferase gene to a promoter sequence. Luciferase assays are quick, highly sensitive, have wide dynamic range, and are cheap to perform. Because of their simplicity and versatility, and because of the absence of endogenous luciferase activity in most cell types, this test can be applied for testing a large variety of nanomaterials for their pathogenic or carcinogenetic effects on a wide range of mammalian cells. This system is an ideal early-stage toxicology tool for screening nanomaterials. Here we describe the Genetic Luciferase Reporter System as the method for detecting alteration of gene expression in response to external stimuli (e.g., nanoparticles).

Key words: Nanoparticles, Firefly luciferase, Reporter genes, Gene expression, Promoter

1. Introduction

Nanotechnology includes manipulating matter on a near-atomic scale to produce new structures, materials, and devices. It is essential to conduct hazard and exposure assessment to support risk assessment and allow the development of recommendations for exposure limits and the implementation of prevention strategies (1). A major challenge in hazard assessment in nanotechnology is the large and rapidly growing number of possible nanoparticles to be tested for biological activity. For example, carbon nanotubes (CNT) can vary in width, length, be single-walled, double-walled, or multi-walled; CNT can be functionalized with a variety of chemical

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groups. Thus, for CNT alone, there can be hundreds of different test materials with different physicochemical properties, possibly exhibiting substantially different bioactivity.

Reporter gene assay provide an easy to perform, fast, and affordable way to test nanomaterials for a wide variety of biologic effects in mammalian cells. Direct quantitation of changes in gene expression may rely on the measurement of specific mRNAs, using techniques such as northern blot hybridization, RT-PCR, or a variety of array-based techniques. These procedures are not always practical for the analysis of many different gene constructs. An alternate approach to gauge changes in transcription is to link the presumed *cis*-acting sequences from the gene of interest to the coding sequence of an unrelated reporter gene (2–6) (Fig. 1).

The most versatile and common reporter gene is the luciferase of the North American firefly *Photinus pyralis*, (see Fig. 1). The protein requires no posttranslational modification for enzyme activity; it is not toxic at high concentration (in vivo) and can be used in prokaryotic and eukaryotic cells (7–9). A reporter protein (i.e., luciferase, see Fig. 2). is easily detectable and quantifiable.

The assay procedure is simple and includes transfection of cells with luciferase expression plasmids, followed by cell lysis using Triton X-100 to release the expressed reporter protein luciferase. Both ATP and the substrate luciferin are added to the lysate in a luminometer. The enzyme catalyzes a rapid, ATP-dependent oxidation of the substrate, which then emits light (Fig. 3). Total light output is proportional to the amount of luciferase present over a wide range of enzyme concentrations (10, 11). It is important to recognize that these assays use reporter constructs to measure protein level or activity and not RNA level. The two levels are frequently, but not always, correlated with one another.

The most commonly used luciferase reporter systems in our lab are pre-oncogene/promoter-luciferase reporters, such as AP-1-luciferase, NF-κB luciferase, p53 luciferase, COX-2 luciferase, NFAT luciferase (11–14). Alterations of these promoter activation or gene expression are linked to neoplastic transformation, tumor

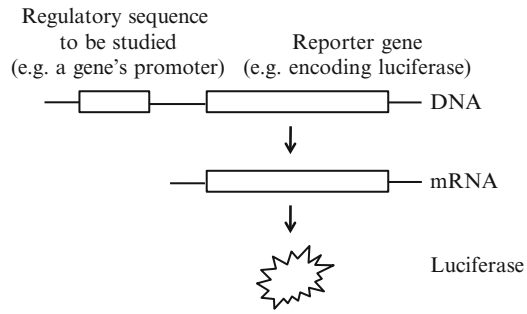

Fig. 1. The use of luciferase reporter gene system to study a regulatory sequence.

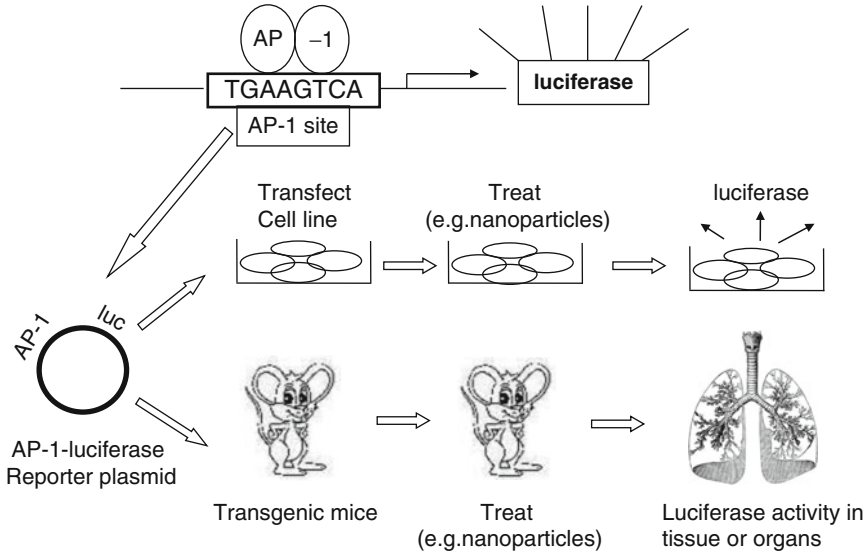

Fig. 2. Overview of luciferase reporter system. A diagram of how a reporter system is created in cell lines or animals to study a gene alteration/expression.

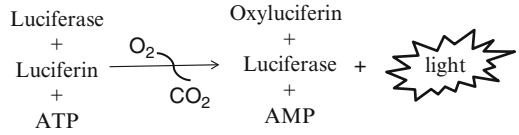

Fig. 3. The firefly luciferase catalyzes bioluminescent oxidation of the luciferin in the presence of ATP, magnesium, and oxygen.

progression, metastasis, inflammation, proliferation, and apoptosis. The assays described here would be suitable for studying the effects of nanomaterials on AP-1, NF-κB, p53 activity, or COX-2 expression and should help to assess the potential health risks of exposure to nanoparticles.

2. Materials

2.1. Development of Stable Transfected Cell Lines

All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be followed accordingly. Selection of stably transfected colonies requires that cells be grown essentially in isolation. A cell line that does not grow as an isolated cell will not be able to be stably transfected.

1. Parental adherent cell lines (e.g., JB6).
2. Minimum Essential Medium Eagle (EMEM) supplemented with FBS (5 %), Penicillin-Streptomycin (1 %), and L-glutamine (2 mM) suitable for the cell line use (JB6 in this case).
3. 37 °C, 5 % CO_2,80 % humidity incubator for cell culturing.

4. Purified plasmid DNAs: reporter plasmids containing the promoter sequences of interest (e.g., AP-1, NF-kB, p53) and the antibiotic-resistance gene that allows the transfected cells to survive in a culture containing the corresponding antibiotic. The ready to use vector plasmids are commercially available, e.g., plasmid 11783, "NFAT/AP-1 3x luciferase from Addgene Inc".
5. Dilution medium: antibiotic-free and serum-free cell culture medium suitable for lipid-mediated transfection such as Opti-MEM I (Invitrogen Inc.) yields the highest efficiency of transfection.
6. Transfection Medium as above but containing serum.
7. Cell counter or hemocytometer.
8. Cationic Lipofectamine™ reagent (Invitrogen Inc).
9. Heat-inactivated Fetal Bovine Serum (FBS).
10. Cloning cylinder.
11. Sterile vacuum grease.
12. Trypsin-EDTA: 0.5 % Trypsin, 5.3 mM EDTA. Dilute to 0.01 % in 1× PBS for JB6 cells.
13. Phorbol-12-mysristate-13-acetate (TPA): 20 μg/mL solution in dimethyl sulfoxide (DMSO). Aliquot and store at −80 °C.
14. Luciferase Assay System (Promega), containing Luciferase assay substrate, Luciferase assay buffer, and Cell culture lysis 5× reagent (30 mL/vial). Add Luciferase Assay Buffer (10 mL) to the vial containing the lyophilized Luciferase Assay Substrate. Reconstituted luciferase assay reagent should be stored in aliquots at −20 °C for up to 1 month or at −70 °C for up to 1 year. Add 4 volumes of water to 1 volume of 5× Luciferase Cell Culture Lysis Reagent to make 1× lysis buffer (see Note 1).
15. Complete medium for JB6 cells: EMEM supplemented with FBS (5 %), Penicillin-Streptomycin (1 %), and L-glutamine (2 mM) for the cell line of JB6.
16. Antibiotics for selection of transfected cells: 300 mg/mL G418 for use with JB6 cells.

2.2. Nanoparticles-Induced Alterations of Gene Expression

2.2.1. Preparation of Nanomaterials

1. Nanoparticles to be investigated, e.g., TiO_2 nanoparticles. To make stock solutions, dissolve nanoparticles in sterile PBS at 10 mg/mL, and sonicate the solution. Working solutions are prepared by diluting the stock solution of nanoparticle with cell growth medium for cell treatment. The final concentration of the particle should be 0–400 μg/cm^2. Use 1× sterile PBS to dilute the stock solution of nanoparticles for animal.
2. Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH of 7.4.

3. 15 mL Sterile conical centrifuge tubes.
4. Sonicator with microprobe set at 5 output (approximately 150 W output) and constant duty cycle.

2.2.2. Firefly Luciferase Reporter Gene Assay

1. Eagle's Minimum Essential Medium (EMEM) supplemented with FBS (5 %).
2. 1 % Penicillin-Streptomycin solution (10,000 units/mL penicillin G sodium and 10,000 μg/mL streptomycin sulfate) in 0.85 % saline.
3. 200 mM L-glutamine solution.
4. Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH of 7.4.
5. Trypsin-EDTA: 0.5 % Trypsin, 5.3 mM EDTA. Dilute to 0.01 % in 1× PBS for JB6 cells.
6. Cell counter or hemocytometer.
7. Triton/glysine luciferase cell culture lysis buffer (1×): 25 mM Tris-phosphate (pH 7.8), 2 mM DTT 2 mM 1, 2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 % glycerol, 1 % Triton® X-100. This buffer can be prepared from commercially available 5× concentrated stocks. Equilibrate the lysis buffer at room temperature before use.
8. Single-Tube Luminometers with auto injectors and printer.
9. Luminometer cuvettes 12×75 mm.
10. Luciferase Assay System (Promega), containing Luciferase assay substrate, Luciferase assay buffer, and Cell culture lysis 5× reagent (30 mL/vial). Add Luciferase Assay Buffer (10 mL) to the vial containing the lyophilized Luciferase Assay Substrate. Reconstituted luciferase assay reagent should be stored in aliquots at –20 °C for up to 1 month or at –70 °C for up to 1 year. Add 4 volumes of water to 1 volume of 5× Luciferase Cell Culture Lysis Reagent to make 1× lysis buffer (see Note 1).

3. Methods

3.1. Development of Stable Transfected Cell Lines

3.1.1. Titrating G418, Hygromycin, or Puromycin

Since each parental cell line has a different sensitivity to G418, hygromycin, or puromycin, one should determine the optimal concentration of drug for selection. We typically use 300 mg/mL of G418 for selecting the transfected clones using JB6 cells.

1. Culture JB6 cells in a 75 cm^2 flask with EMEM containing FBS (5 %), glutamine (2 mM), and penicillin/streptomycin (1 %) and incubate at 37 °C, 80 % humidity, 5 % CO_2 until 80 % confluent.
2. Remove medium, rinse 1× with 10 mL 1× PBS and aspirate.

3. Add 5 mL trypsin/EDTA (0.01 %).
4. Incubate at 37 °C for 2–5 min until cells become detached.
5. Separate cells by pipetting up and down several times.
6. Add 5 mL fresh medium.
7. Count cells using cell counter or hemocytometer and adjust volume to 1×10^5 cells/mL.
8. Seed cells into a 24-well plate (0.5 mL, 5×10^4 cells per well) and incubate overnight.
9. On day 2, Pre-warm the complete media: EMEM + FBS (5 %) + Penicillin-Streptomycin (1 %) + L-glutamine (2 mM).
10. Make serial dilutions of G418 in complete media yielding eight concentrations of G418: 0, 50, 100, 200, 300, 350, 400, 500 mg/mL. Run triplicate wells for each concentration (see Note 2).
11. Remove cells from incubator.
12. Remove media from the cells.
13. Replace media with antibiotic containing media and return the plate to incubator.
14. The minimum concentration of G418 resulting in complete cell death after 3 days of titration should be used for selection (see Note 3).

3.1.2. Cationic Lipid-Mediated Transfection of JB6 Cells and Selection of Stable Transfected Clones (See Note 4)

1. Grow JB6 cells in 60-mm culture dishes using complete medium until ~ 80 % confluence by the day of transfection (see Note 5). Make at least four dishes for each plasmid to allow for sufficient number of transfected colonies to grow.
2. On the day of transfection, add 10 μg plasmid DNA into 250 μL dilution medium in a polystyrene tube, mix well. This amount is sufficient for use with one 60 mm culture dish (see Note 6).
3. In another tube mix 50 μL of cationic lipid reagent with 250 μL of dilution medium (see Note 6).
4. Combine diluted DNA and diluted cationic lipid reagent, mix, and incubate for 15 min at room temperature.
5. While DNA-lipid complexes are forming, replace the cell medium in the culture dishes with 3.5 mL of fresh transfection medium per 60 mm dish.
6. Slowly and gently add DNA-lipid complexes to the culture dish(es) containing cells. Mix complexes into the medium gently, holding the plate at an angle.
7. Incubate cells for at least 5 h at 37 °C in 5 % CO_2 (up to overnight).
8. Following the incubation add 6 mL fresh medium and serum (to reach final concentration of 5 % serum) per dish (each dish

already contains ~4 mL transfection mixture with plasmid DNA). Incubate cells at 37 °C in 5 % CO_2.

9. One day after the start of transfection passage cells onto fresh culture medium containing 300 mg/mL G418.
10. Two days post-transfection, change medium and add G418 to 300 mg/mL final concentration to select for expression of the transfected antibiotic-resistance gene (see Note 2).
11. Observe cell growth every 2–3 days and change medium containing the selected antibiotic once every week. Only stably transfected cells will survive and grow to form colonies in the presence of the selective antibiotics. Isolated colonies should begin to appear under a microscope after about 2–4 weeks (see Note 7).
12. Find large, healthy, and well separated colonies containing ~ 500–1,000 cells. Select the colonies to be picked. Circle the colonies with a laboratory marker to determine where to place the cloning cylinders.
13. Coat one end of cylinder with sterile vacuum grease by touching the cylinder to grease that has been autoclaved in a glass Petri dish. Gently place colony cylinder around the colony to be picked (see Fig. 4).
14. Using a sterile Pasteur pipet, rinse the colony with trypsin/EDTA by filling and emptying the cloning cylinder.
15. Add three drops of trypsin/EDTA to the cloning cylinder. Wait for 1–2 min. Fill the cloning cylinder with medium and repeatedly run the contents of the cylinder in and out through a Pasteur pipet in order to remove the trypsinized cells from the dish and disperse them.
16. Plate the collected cells in a 6-well plate (one colony per well) and add fresh medium with the selective antibiotic (300 mg/mL G418).
17. Maintain the cells in culture and split the cells every 4–5 days, until they reach 80 % confluency (see Note 8).
18. Split clones that reach ~80 % confluence into triplicate 6-well plates. Use one or two plates for checking protein expression/induction and save one plate for expansion of selected positive clone.
19. Check the clones for the highest luciferase protein expression by treating with inducing reagent (a positive control). We recommend using 20 ng/mL TPA to treat JB6 cells for inducing expression of AP-1, NF-κB, or COX-1 genes. Following 24 h incubation with the inducing reagent pick six colonies to check for protein expression using luciferase activity test.
20. Expand the best clones to create a stable transfected cell line. The cell line can be stored in liquid N_2 (see Note 9).

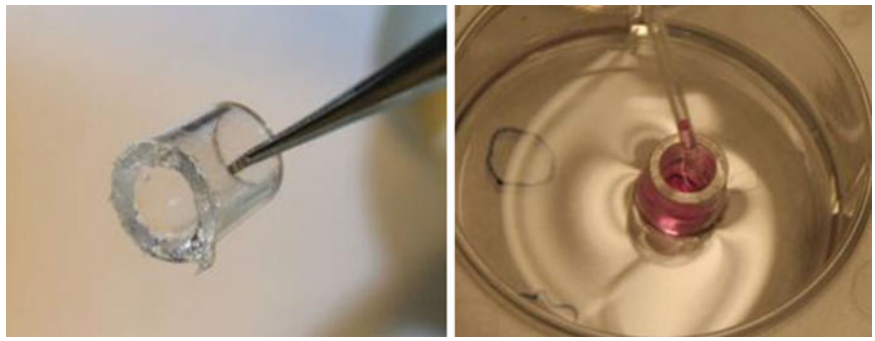

Fig. 4. Isolation of transfected colonies with a cloning cylinder.

3.2. Nanoparticles-Induced Alterations of Gene Expression

3.2.1. Preparation of Nanoparticles

1. Add 10 mg of nanoparticles (see Note 10) to 1 mL sterile PBS in 15 mL sterile conical centrifuge tube. Keep on ice.
2. While on ice, sonicate for 30 s, then pause for 15 s.
3. Repeat the sonication cycle five more times (see Note 11).
4. Dilute the nanoparticles with medium or PBS to desired concentration for cells or animal exposure (see Notes 10 and 11).

3.2.2. Firefly Luciferase Reporter Gene Assay

1. Culture the JB6 cell line stably transfected with a gene of interest in luciferase reporter plasmid (e.g., AP-1, p53, or NF-kB etc.) at 37 °C in a humidified atmosphere of 5 % CO_2 using complete medium, until 90–100 % confluency.
2. Remove culture medium. Rinse once with 10 mL of 1× PBS and aspirate. Add 5 mL trypsin/EDTA (0.01 %). Incubate at 37 °C for 2–5 min until cells become detached. Separate cells by pipetting up and down several times. Add 5 mL EMEM media containing FBS (5 %), glutamine (2 mM) and penicillin/streptomycin (1 %) to neutralize trypsin.
3. Count cells using cell counter or hemocytometer.
4. Resuspend 5×10^4 viable cells per 1 mL of Eagle's MEM Media (see step 2). Add 1 mL of cell suspension per well to a 24-well plate.
5. Incubate plates at 37 °C in a humidified atmosphere of 5 % CO_2 overnight.
6. Prepare nanoparticle solutions in fresh growth media. To find optimal dose test a range of nanoparticle concentrations (see Note 10).
7. Remove growth medium from the cells. Add 1 mL of nanoparticles resuspended in fresh growth medium per well. Incubate the cells for 12–24 h.
8. Remove growth medium from cells, rinse cells with PBS being careful not to dislodge attached cells. Remove as much PBS as possible.

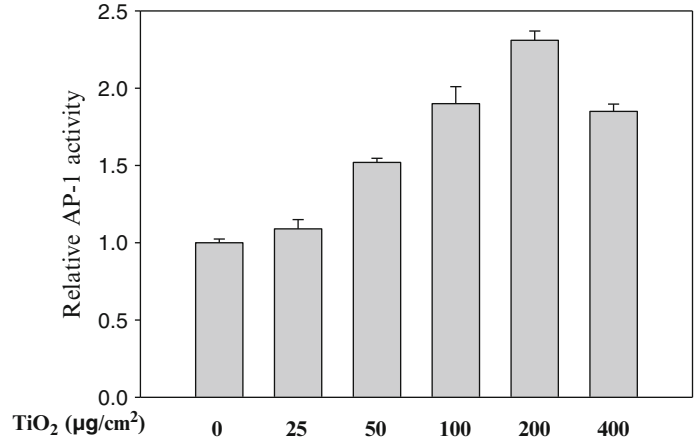

Fig. 5. Dose-dependent induction of AP-1 activation by TiO_2 nanoparticle. JB6 P^+ cells, stably transfected with AP-1 luciferase reporter plasmid, were exposed to various concentrations of TiO_2 for 24 h and the luciferase activity was measured. Results are means ± SEM of four assay wells.

9. Add 150 μL of (1×) luciferase cell culture lysis buffer per well to cover the cells.
10. Rock culture dishes several times to ensure that all cells are covered with the lysis buffer. Leave at room temperature for 30 min (see Note 12).
11. Mix well by pipetting up and down several times.
12. Program the luminometer to inject 60 μL luciferase assay substrate and perform a measurement cycle containing a 2-s measurement delay followed by a 10-s measurement read for luciferase activity.
13. Connect the reconstituted luciferase assay reagent vial (10 mL) to the injector of luminometer. Prime the luminometer injector at least three times with Luciferase Assay Reagent or as recommended in the owner's manual.
14. Dispense 40 μL of samples into luminometer cuvette, one cuvette per sample.
15. Place the tube in the luminometer and initiate reading as recommended in the owner's manual (see Note 13).
16. Express the readings as relative values (activity in treated samples compared to untreated controls) (see Fig. 5).

4. Notes

1. Do not store Luciferase Assay Reagent with dry ice. Thaw Luciferase Assay Reagent at temperatures below 25 °C and mix

well before use. Store Luciferase Assay Substrate in the dark. Reporter Lysis Buffer may be stored at room temperature and should be stored away from direct sunlight. Luciferase Cell Culture Lysis Reagent should be stored at −20 °C.

2. Optimal concentration for resistant clones selection in mammalian cells depends on the cell line used as well as on the plasmid carrying the resistance gene. Therefore antibiotic titration should be done to find the best condition for every experimental system. Optimal concentrations, as recommended by Clontech are 400 mg/mL G418 for HeLa cells and 200 mg/mL hygromycin for CHO cells. We use 300 mg/mL G418 for JB6 cells. In mammalian cells the optimal level of puromycin is typically around 1 mg/mL.
3. The chosen selective conditions (the minimum antibiotics level) may still allow mammalian cells to divide once or twice but will eventually kill them.
4. Different transfection techniques might be suitable for different cell lines. We recommend that DEAE-dextrans, calcium phosphate, or liposome-mediated transfection are used with adherent cell lines and that electroporation or liposome-mediated transfection is used for nonadherent cell lines.
5. Avoid antibiotics at the time of plating and during transfection.
6. Prepare a master mix if multiple identical transfection are performed.
7. Densely plated cells will require more frequent media changes. Un-transfected cells will not survive beyond ~3 days following the addition of antibiotic. Unstably transfected cells will survive for a while but will eventually die if they lost the transfected plasmid. Only stable transfected cells will survive and proliferate to form a clone and a cell line. In our experience, only a few cells are left in each dish after 2–3 weeks of incubation with antibiotic. One to two months later, each stably transfected cell becomes a colony. Reduce the frequency of medium changes to once per 2 weeks to promote the growth of colonies.
8. The clones need to be grown in order to obtain enough cells for further tests and/or storage. It may take 2–4 weeks for a clone (~50–100 cells) to reach 80 % confluence in a 6-well plate.
9. Development of a stable transfected mammalian cell line takes 2–3 months. Stable cell line development services are also available by GenScript Inc.
10. The amount of nanoparticles used will depend on the particular nanomaterial. The nanoparticle doses should be relevant to anticipated workplace exposures or to the amounts used in cellular or animal test systems. We used 0.5–100 μg/cm^2 for JB6 cells.

11. Nanoparticles need to be suspended in physiological solutions or medium use in the study. Since nanoparticles in physiological media tend to form coarse agglomerates, sonication is necessary to help nanoparticles disperse in solution. Many commercially available nanoparticles, such as Titanium dioxide (TiO_2) nanoparticle, Single-walled carbon nanotubes (SWCNT), and Tungsten carbide-cobalt nanoparticles (WC-Co) are water insoluble. For the tests with adherent cells, we recommend using these at 0–400 μg/cm^2 concentration.
12. At this point the plates can be transferred to 4 °C and stored for 2–3 days. The supernatant/cell lysate also can be stored at −70 °C for weeks.
13. If the samples were stored at 4°C or −70 °C, then Luciferase assay reagent and samples should be brought to ambient temperature prior to performing a luciferase assay. Generally, luciferase activity is stable for several hours at room temperature in 1× Luciferase Cell Culture Lysis Reagent. Assaying cold samples (0–4 °C) using standard assay volumes may result in a 5–10 % decrease in enzyme activity.

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Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for occupational Safety and Health.

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