Designation: E2526 – 08 (Reapproved 2013)

Standard Test Method for Evaluation of Cytotoxicity of Nanoparticulate Materials in Porcine Kidney Cells and Human Hepatocarcinoma Cells[1]

This standard is issued under the fixed designation E2526; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method provides a methodology to assess the cytotoxicity of suspensions of nanoparticulate materials in porcine proximal tubule cells (LLC-PK1) and human hepatocarcinoma cells (Hep G2) which represents potential target organs following systemic administration

1.2 This test method is part of the *in vitro* preclinical characterization cascade.

1.3 This test method consists of a protocol utilizing two methods for estimation of cytotoxicity, 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F813 Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity

F1877 Practice for Characterization of Particles

F1903 Practice for Testing For Biological Responses to Particles ***In Vitro***

2.2 *ISO Standard:*[3]

ISO 10993-5 Biological Evaluation of Medical Devices: Part 5 Tests for in vitro Cytotoxicity

3. Terminology

3.1 *Abbreviations:*

3.1.1 *APAP*—acetaminophen- positive control

3.1.2 *DMSO*—dimethyl sulfoxide

3.1.3 *DMEM*—Dulbelcco's modified eagles media

3.1.4 *Hep G2*—human hepatocarcinoma cells

3.1.5 *LDH*—lactic dehydrogenase

3.1.6 *LLC-PK1*—porcine proximal tubule cells

3.1.7 *LPS*—lipopolysacchride, bacterial endotoxin

3.1.8 *MTT*—3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

3.1.9 *Physiologic solution*—isotonic with a pH 7.2 ± 0.2

4. Summary of Test Method

4.1 Nanoparticulate test materials in suspension in cell culture media and appropriate controls are added to cell cultures. The release of LDH indicates membrane damage and the diminution of MTT reduction indicates loss of cell viability. These are quantitative indicators of cytotoxicity. Aseptic procedures are required.

5. Significance and Use

5.1 Assessing the propensity of a nanomaterial to cause cytotoxicity to the cells of a target organ can assist in preclinical development.

5.2 The standard historical cytotoxicity testing of materials and extracts of materials has used fibroblasts and is well documented in Practice F813, Test Method F895, and ISO 10993-5. The use of macrophages and micron size particles has also provided information on cytotoxicity and stimulation using Practice F1903.

[1] This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.03 on Environment, Health, and Safety.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

5.3 This test method adds to the cytotoxicity test protocols by using target organ cells. Two quantitative assays measuring LDH leakage and MTT reduction are used to estimate cytotoxicity.

5.4 This test method may not be predictive of events occurring in all types of nanomaterial applications and the user is cautioned to consider the appropriateness of the test for various types of nanomaterial applications. This procedure should only be used to compare the cytoxicity of a series of related nanomaterials. Meaningful comparison of unrelated nanomaterials is not possible without additional characterization of physicochemical properties of each individual nanomaterial in the assay matrix.

6. Reagents and Materials

6.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[4] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

6.2 Reagents and supplies (aseptic procedures are needed and care should be taken to use sterile reagents and supplies as necessary).

6.2.1 MTT (3-(4,5-dimethylthiazolyl-2)-2,5- diphenyltetrazolium bromide).

6.2.2 Acetaminophen.

6.2.3 Dimethyl sulfoxide.

6.2.4 Glycine.

6.2.5 Sodium Chloride.

6.2.6 Medium 199 Cell Culture Media.

6.2.7 Triton X 100.

6.2.8 LDH-Cytotoxicity Assay Kit (Biovision Cat. # K311-400 was used in developing this test method)*.

6.2.9 96 well flat bottom cell culture plates.

6.2.10 RPMI 1640.

6.2.11 L-glutamine.

6.2.12 Fetal bovine serum (FBS).

6.3 *Cell Lines:*

6.3.1 LLC- PK1 (porcine proximal tubule cell) (ATCC#CL-101)*.

6.3.2 Hep G2 (human hepatocarcinoma)(ATCC # HB-8065)*.

6.4 *Equipment:*

6.4.1 Plate reader.

6.4.2 Plate Centrifuge set at 700-800 *g*.

6.4.3 Cell Culture Microscope.

NOTE 1—Commercial sources are indicated for informational purposes only to aid laboratories initiating these test procedures. This does not indicate endorsement by ASTM. Other equivalent sources may be available.

7. Experimental Procedure

7.1 Aseptic precautions are required.

7.2 *Positive Control Preparation:*

7.2.1 LLC-PK1 Acetaminophen (APAP) positive control: 25 mM APAP in M199 cell culture media.

7.2.2 Hepatocyte Acetaminophen (APAP) positive control: 20 mM APAP in RPMI 1640 cell culture media.

7.2.3 Triton X100 is diluted to 1 % in cell culture medium. This is the positive control for the LDH assay.

7.3 *MTT Assay Reagents:*

7.3.1 MTT solution-5mg/mL MTT in PBS, store for up to one month at 4°C in the dark.

7.3.2 Glycine Buffer-0.1M glycine (MW 75.07), 0.1 M NaCl (MW 58.44), pH 10.5, store at room temperature.

7.4 *Biovision LDH-Cytotoxicity Assay Kit Reagents:*

7.4.1 Reconstitute catalyst in 1 mL dH_20 for 10 min and vortex (stable for 2 weeks at 4°C).

7.4.2 Reaction mixture (for one 96-well plate): Add 250 mL of reconstituted, catalyst solution to 11.25 mL of dye solution (stable for 2 weeks at 4°C).

7.4.3 For other LDH Cytotoxicity assay kits, follow their instructions.

7.5 *Cell Culture:*

7.5.1 *LLC-PK1 Cell Preparation:*

7.5.1.1 Harvest cells from flasks prepared from cryopreserved cells according to the instructions from the supplier (limit passages to 20). An example of the appearance of the cells is in Fig. 1.

7.5.1.2 Count cell concentration using a Coulter type counter or hemocytometer.

7.5.1.3 Dilute cells to a density of 2.5×10^5 cells/mol in M199 (3 % FBS) cell culture media.

7.5.1.4 4 Plate 100 µL cells/well as per plate format described in Fig. 3 for 4 plates (time zero, 6 hour sample exposure, 24 hour sample exposure, 48 hour sample exposure). The format indicates no cells in rows D&E and they serve as particle controls. Each plate accommodates two samples (Rows A-C and F-H). Each nanoparticulate material is tested at 9 dilutions. Column 11 receives the positive control and column 12 receives Triton X 100.

7.5.1.5 Incubate plates for 24 hours at 5 % CO_2, 37°C and 95 % humidity (cells should be approximately 80 % confluent).

7.5.2 *Hep G2 Cell Preparation:*

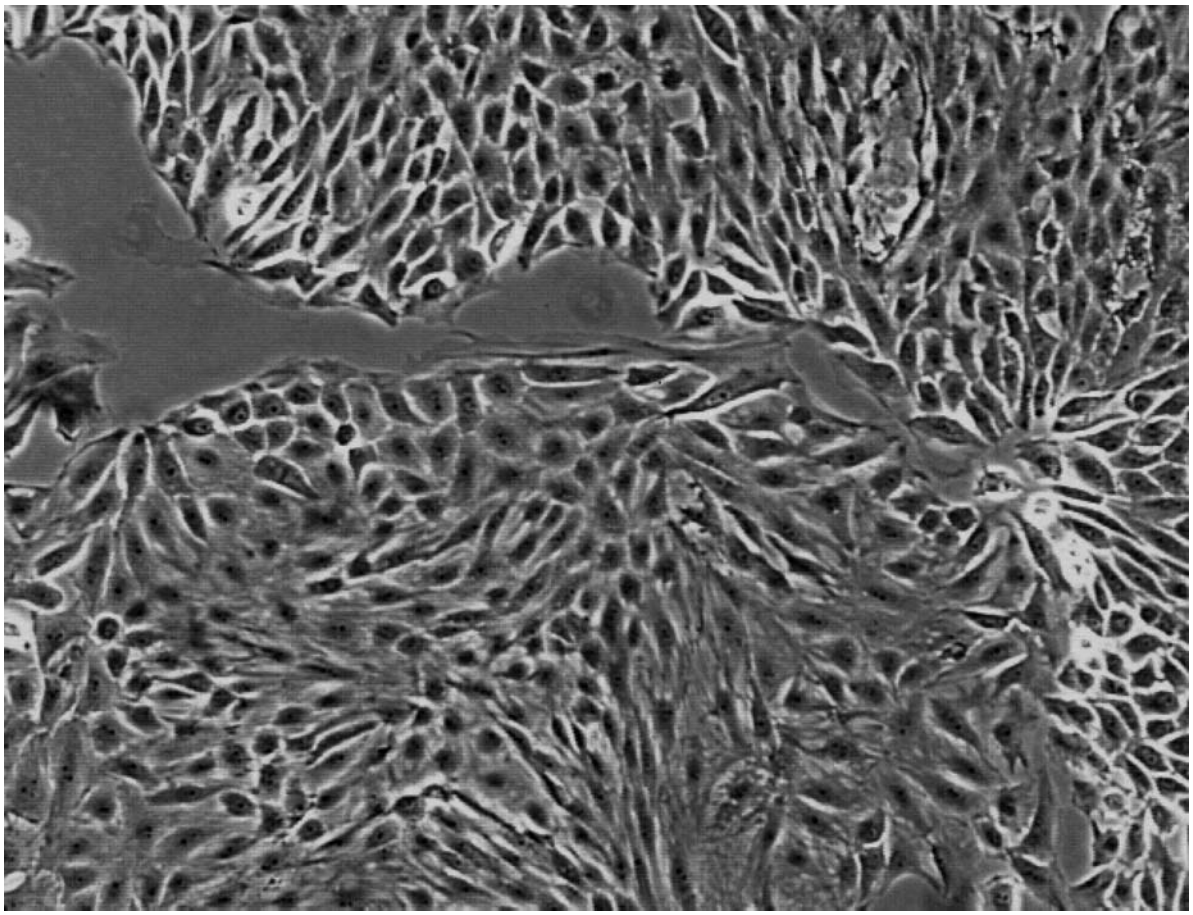
7.5.2.1 Harvest cells from flasks prepared from cryopreserved cells according to the instructions from the supplier. (limit passages to 20). An example of the appearance of the cells is in Fig. 2.

7.5.2.2 Count cell concentration using a Coulter type counter or hemocytometer.

7.5.2.3 Dilute cells to a density of 5.0×10^5 cells/mol in RPMI 1640 (2 mM L-glutamine, 10 % FBS) cell culture media.

7.5.2.4 Plate 100 µL cells/well as per plate format described in Fig. 3 for 4 plates (time zero, 6 hour sample exposure, 24

[4] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.



NOTE 1—Image was taken with a phase contrast microscope at 225× magnification. LLC-PK1 cells are approximately 80 % confluent at this stage.

FIG. 1 Example of LLC-PK1 Cell Culture Appearance

hour sample exposure, 48 hour sample exposure). The format indicates no cells in rows D&E and they serve as particle controls. Each plate accommodates two samples (Rows A-C and F-H). Each nanoparticulate material is tested at 9 dilutions. Column 11 receives the positive control and column 12 receives Triton X 100.

7.5.2.5 Incubate plates for 24 hours at 5 % CO_2, 37°C and 95 % humidity (cells are approximately 70 % confluent).

7.6 *Time Zero Plate:*

7.6.1 Remove time zero plates from the incubator and replace media from Triton-X positive control wells (see plating format in Fig. 3) with 1 % Triton-X. Add 100 µL of media to the remaining wells. Let the plate set for 10 min at room temperature. Spin at 700 *g* for 3 min.

7.6.2 Remove 100 µL of media from each well and transfer to another plate on ice maintaining the plate format in Fig. 3. Use this plate for the LDH assay in 7.8 upon completion of the incubation step in 7.6.6.

7.6.3 Remove remaining media from wells and discard.

7.6.4 Add 200 µL of fresh media to all wells.

7.6.5 Add 50 µL of MTT to all wells.

7.6.6 Cover in aluminum foil and incubate for 37°C for 4 hours.

7.6.7 Remove plate from incubator and spin at 700 *g* for 3 min.

7.6.8 Aspirate media and MTT.

7.6.9 Add 200 µL of DMSO to all wells.

7.6.10 Add 25 µL of glycine buffer to all wells.

7.6.11 Read at 570 nm on plate reader.

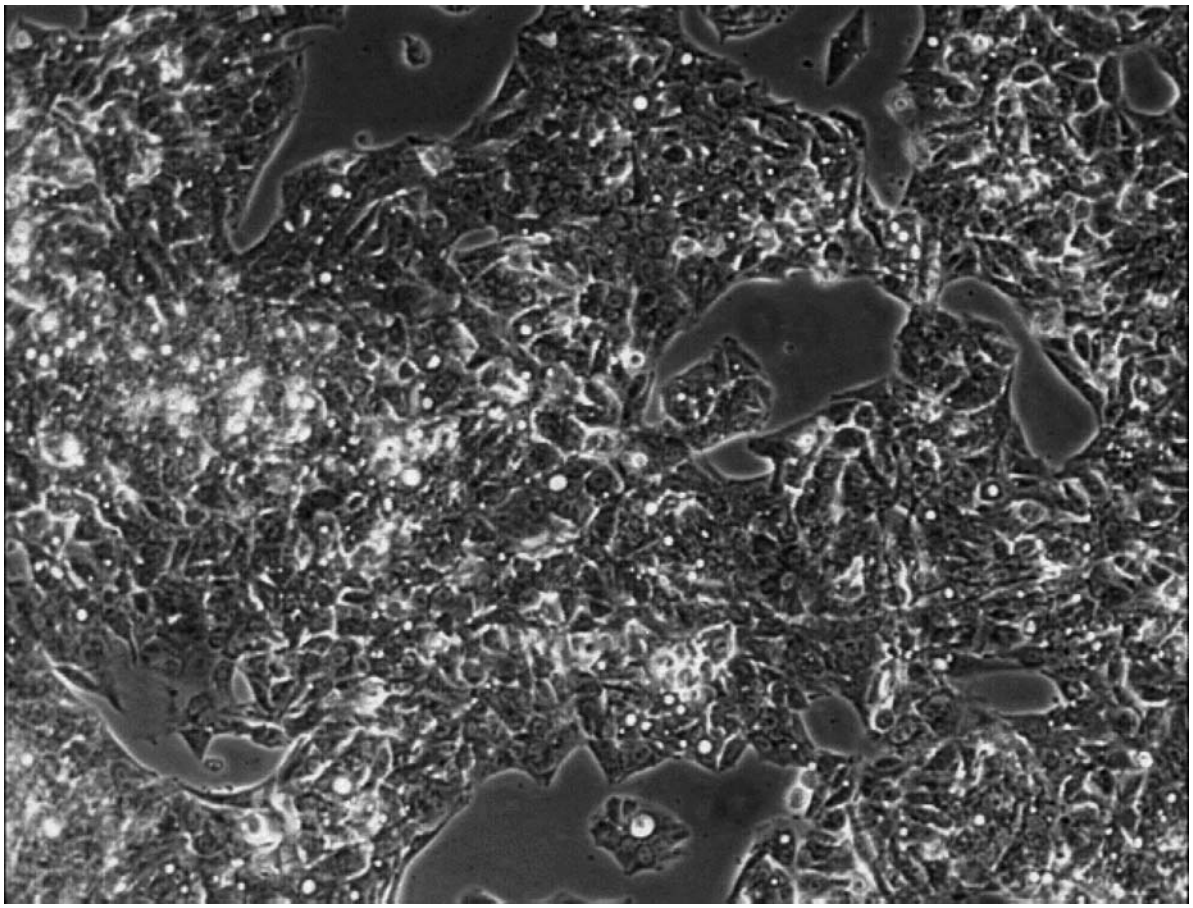
7.7 *Test Sample and Positive Control Addition:*

7.7.1 *Preparation of Nanoparticulate Material*—The nanoparticulate material should have undergone previous characterization as appropriate to determine the physiochemical state, to permit adequate data interpretation and to allow prediction of biological responses. For example, lot-to-lot variations in particle size and surface characteristics could lead to different assay results. The suspension shall be sterile and the level of LPS provided or determined by the testing lab. The assay concentrations should be expressed as mg/mL.

7.7.2 *Interferences:*

7.7.2.1 This test method involves the use of a spectrophotometer with readings at 490, 570, and 680 nm. If the particle suspension interferes at these wave lengths, a method to eliminate the particles from the solution to be analyzed shall be used. If there is no method to eliminate the particles or correct the readings with an appropriate blank, this test method is not applicable.

7.7.2.2 Suitable controls are included to determine interference with the MTT or LDH assays.



NOTE 1—Image was taken with a phase contrast microscope at 225× magnification. Hep G2 cells are approximately 80 % confluent at this stage.

FIG. 2 Example of Hep G2 Cell Culture Appearance

7.7.3 Dilute the test material in media, making a total of nine 1:4 dilutions.

7.7.4 Add 100 µL of each dilution and positive control to 6 hour, 24 hour and 48 hour exposure plates as per the plate format (Fig. 3).

7.7.5 Following the 6, 24 and 48 hour exposures, test plates should be prepared for the MTT and LDH assays as described in 7.6.2 – 7.6.11 and 7.8.

7.8 *Test Plates—LDH Assay:*

7.8.1 Add 100 µL of the Reaction Mixture prepared in 7.4.2 to each well of transfer plate prepared in 7.6.2. Shake plate on an orbital shaker briefly.

7.8.2 Incubate at room temperature for up to 20 min in the dark.

7.8.3 Read the plate on plate reader at 490 nm using a reference wavelength of 680 nm.

8. Calculation or Interpretation of Results

8.1 For the LDH and MTT assays, rows D&E are used as sample blanks which are subtracted from the corresponding sample and control columns (see Fig. 3).

8.2 Columns 1 (rows A-C) and 12 (rows A-C) correspond to the media control and Triton X positive control wells, respectively, for sample columns 2 (rows A-C) – 11(rows A-C). Columns 1(rows F-H) and 12 (rows F-H) are the media control and Triton X positive control wells, respectively, for sample columns 2 (rows F-H) – 11 (rows F-H) (see Fig. 3).

8.3 *LDH Assay:*

$$\%\ \text{Total LDH Leakage} = \left(\frac{(\text{sample abs} - \text{media control abs})}{(\text{triton X positive control abs} - \text{media control abs})} \right) \times 100 \quad (1)$$

8.4 *MTT Assay:*

$$\%\ \text{Cell Viability} = \left(\frac{\text{sample abs}}{\text{media control abs}} \right) \times 100 \quad (2)$$

8.5 Mean, SD and %CV should be calculated for each blank, positive control, negative control and unknown sample.

8.6 *Acceptance Criteria:*

8.6.1 The 48 hour % cell viability and % total LDH leakage for the APAP positive controls should be less than 75 % and greater than 15 %, respectively, for the kidney cytotoxicity assay, and less than 50 % and greater than 50 %, respectively, for hepatocyte cytotoxicity assay.

8.6.2 The positive and sample replicate coefficient of variations should be within 50 %.

8.6.3 If conditions 8.6.1 and 8.6.2 are not met, repeat the assay. If they are met, determine the results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
B	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
C	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
D	Media Media	Media Sample 1 Dilution 9	Media Sample 1 Dilution 8	Media Sample 1 Dilution 7	Media Sample 1 Dilution 6	Media Sample 1 Dilution 5	Media Sample 1 Dilution 4	Media Sample 1 Dilution 3	Media Sample 1 Dilution 2	Media Sample 1 Dilution 1	Media APAP	Media Triton X 1%
E	Media Media	Media Sample 2 Dilution 9	Media Sample 2 Dilution 8	Media Sample 2 Dilution 7	Media Sample 2 Dilution 6	Media Sample 2 Dilution 5	Media Sample 2 Dilution 4	Media Sample 2 Dilution 3	Media Sample 2 Dilution 2	Media Sample 2 Dilution 1	Media APAP	Media Triton X 1%
F	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP	Cells Triton X 1%
G	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP	Cells Triton X 1%
H	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP	Cells Triton X 1%

FIG. 3 Example of Plate Format

8.6.4 Determine the highest concentration of the nanoparticulate material that does not interfere with the assay system indicated in rows D and E (see also 7.6.2).

8.6.5 Generate a concentration-response curve, and from this curve estimate LC50.

9. Rationale

9.1 *MTT Assay*—MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells to a water-insoluble purple formazan. The amount of formazan can be determined by solubilizing it in DMSO and measuring it spectrophotometrically. Comparisons between the spectra of treated and untreated cells can give a relative estimation of cytotoxicity.[5]

9.2 *LDH Assay*—LDH is a cytoplasmic enzyme that is released into the cytoplasm upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity. The basis of the LDH assay: *(1)* LDH oxidizes lactate to pyruvate, *(2)* Pyruvate reacts with the tetrazolium salt INT to form formazan, and *(3)* the water-soluble formazan dye is detected spectrophotometrically.[6,7]

9.3 This procedure should only be used to compare the cytoxicity of a series of related nanomaterials. Meaningful comparison of unrelated nanomaterials is not possible without additional characterization of physicochemical properties of each individual nanomaterial in the assay matrix.

10. Precision and Bias

10.1 Precision and bias have not been determined for this test method and will be determined within 5 years of publication of the standard. At this time there is no commercially available test article of sufficient quantity and reproducible quality to conduct intra- or interlaboratory comparisons of precision and bias.

11. Keywords

11.1 cytotoxicity; hepatocytes; kidney cells; nanoparticles

[5] Alley, M.C., et al., *Cancer Research*, Vol 48, 1988, pp. 589–601.

[6] Decker, T., and Lohmann-Matthes, M. L., *Journal of Immunological Methods*, Vol 15, 1988, pp. 61–69.

[7] Korzeniewski, C., and Callewaert, D. M., *Journal of Immunological Methods*, Vol 4, 1983, pp. 313–320.