

GHS-Based Product Safety Assessment: Tier-Based Toxicology Testing

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PROJECT GOAL

The ultimate goal of this Tier-Based Toxicology Testing Program is to reassess the safety of various product(s) for purposes of reclassification under the Globally Harmonized System of Classification and Labeling. A process, otherwise known as, GHS Hazard Inversion.

The safety assessments described herein utilize a tier-based toxicology testing approach. Specifically, assays are performed in sequence to determine the relative toxicological response to live cells in culture. Upon completion, finished products are compared against a known positive control (Anatase Crystalline TiO_2 , or equivalent) and a known negative control (Amorphous SiO_2 or equivalent). All experiments are performed to derive a dose-response model as pictured in Exhibit 1.

The first assay in this sequence of work measures MTT using a colorimetric plate assay that determines cellular proliferation via quantification of the Enzyme Mitochondrial Dehydrogenase (typical assay profiling and microscopic analysis are shown in Exhibit 2).

Exhibit 1. Dose-response relationship.

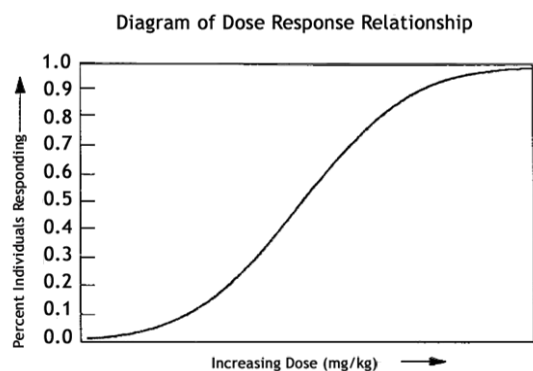
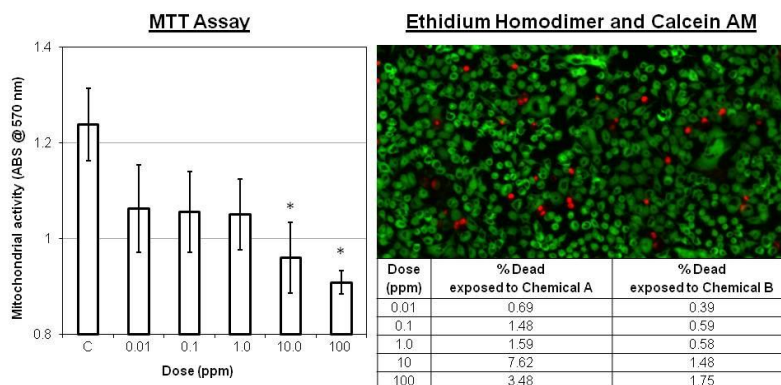


Exhibit 2. MTT assay.



Once the results of this assay demonstrate a non-toxic effect, further testing is performed following the Tier-Based Toxicology Testing Regimen described herein (Exhibit 3, continued on the next page).

Exhibit 3. Tier-Based Toxicology Testing Regimen

Tier	Assay Technique			Information Obtained
(1) Cell viability	Primary approach	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Colorimetric plate assay	Cellular proliferation via quantification of the enzyme mitochondrial dehydrogenase
(2A) Endocrine Disruption	Primary approach	CALUX bioassays	Immunosorbent assay	Potency of the antagonist
(2B) Mutagenic Activity	Primary approach	Ames Test	Colorimetric plate assay	Cancer-causing ability
(2C) Cytokine/ Chemokine Expression	Primary approach	Enzyme-linked immunosorbent assay (ELISA)	Immunosorbent assay	Detect specific proteins
(3) DNA Damage	Primary approach	Comet assay	Fluorescent microscopy	Degree of DNA strand breakage

PROJECT PLANNING

These analytical services encompass a phased research approach and allow us to determine the toxicity of product(s) on a sequential basis with execution of subordinate testing initiated only after each successful milestone has been reached. While this approach offers reduced expenses for those products which prove to be hazardous, it does result in slightly higher costs for products producing non-hazardous findings. This is due to the loss of efficiency which occurs with sequential testing and the need to restart basic study parameters after each benchmark finding. More specifically, control preparation is repeated at each Tier rather than employing a single set of controls to establish parameter definitions across all three Tiers simultaneously.

TIMELINES

The scope of work, conducted by Reddwall Solutions' Toxicology Group, is accomplished within 60 days. The cost associated with Tier 1 analysis and MTT assessment are material dependant and include sample preparation, testing of one pre-determined negative control and one pre-determined positive control. All sample materials are analyzed at 5 different concentrations to establish the dose-response relationship. If interferences between the product and MTT colorimetric indicators exist, an alternative approach is utilized.

Specific expectations and accomplishments which make up the Tier 1 testing regimen and which form a part of this laboratory-based research include:

- ◆ *Deliverable 1:* Assess the dose-response relationship of positive and negative controls
- ◆ *Deliverable 2:* Assess the dose-response relationship of product(s) using the MTT assay
- ◆ *Deliverable 3:* Graph, interpret, and present results.

TIERED TESTING REGIMEN

Once the product(s) have demonstrated non-hazardous findings within the Tier 1 testing routine, Tier 2 testing is initiated. This cluster of subordinate tests, performed as a single analysis group, includes an evaluation of endocrine disruption, mutagenic activity and cytokine or chemokine expression. Taken together, these three studies serve to describe the non-lethal effects and provide data needed to populate GHS documentation.

The costs for Tier 2 analysis are again material dependant and involve a non-lethal mechanistic assessment. Analysis includes sample preparation and testing of one pre-determined negative control along with one pre-

determined positive control. All samples are analyzed to establish a dose-response relationship using 5 different material concentrations. If interferences between the product and colorimetric indicators exist, an alternative approach will be used.

Specific expectations and accomplishments which make up the Tier 2 testing regimen and which form a part of this laboratory-based research include:

- ◆ *Deliverable 1:* Assess the dose-response relationship of positive and negative controls
- ◆ *Deliverable 2:* Assess the dose-response relationship of product(s) using the suite of non-lethal mechanistic assays [i.e. CALUX bioassays, Ames Test and Enzyme-linked Immunosorbent assay (ELISA)]
- ◆ *Deliverable 3:* Graph, interpret, and present results.

Upon successful completion of Tier 2 testing, Tier 3 analysis is initiated. This level of evaluation involves an assessment of the potential for DNA damage, occurring as a result of direct or indirect cellular exposure to the product(s) of interest. The cost for Tier 3 analysis, including DNA damage assessment of one sample, is material dependant and incorporates sample preparation and testing of both one pre-determined negative control and one pre-determined positive control. All samples are again analyzed using up to 5 different material concentrations to establish a dose-response relationship. If interferences between the product and Comet assay exist, an alternative approach will be used.

Specific expectations and accomplishments which make up the Tier 3 testing regimen and which form a part of this laboratory-based research include:

- ◆ *Deliverable 1:* Assess the dose-response relationship of positive and negative controls
- ◆ *Deliverable 2:* Assess the dose-response relationship of product(s) using the Comet assay
- ◆ *Deliverable 3:* Graph, interpret, and present results.

TOXICITY CERTIFICATION

Once each product has conclusively demonstrated a non-hazardous finding within the Tier 1 testing regimen, a communication is provided to the client and Tier 2 testing begun. Similarly, once the product has conclusively demonstrated non-hazardous findings within the Tier 2 testing regimen, results are communicated and Tier 3 testing is initiated.

Upon successful completion of all three testing Tiers, a brief summary report will be generated specifying the toxicity testing regimen employed and outlining the results. Based on successful toxicological outcomes, summaries of the data will be written into the applicable GHS documentation and rationalized against a knowledge-based hazard assessment for regulatory purposes.

Exhibit 4. Certification

Where tiered toxicity testing demonstrates a negative result relative to positive controls, a “seal” will be added to each of the applicable analysis results.

