Assessing increased sensitivity and variability issues in an established *in vitro* phototoxicity testing program

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INTRODUCTION

- The 3T3 Neutral Red Uptake (NRU) Phototoxicity assay is an established *in vitro* assay used to evaluate the potential phototoxicity hazard of a test chemical. The assay methods and prediction model are described in The Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 432 "*In Vitro* 3T3 NRU phototoxicity test"[1].
- IIVS' routine assay performance is evaluated on the comparison of the positive control, chlorpromazine, and solvent control results to our historical database. Failure to consistently meet acceptance criteria calls for examination of assay performance.
- High assay sensitivity and low optical density values have contributed to the failure of recent assay results to meet acceptance criteria.
- Storage conditions, preparation, and manufacturer lot-to-lot consistency of assay reagents (DMSO, chlorpromazine, and neutral red) were evaluated.
  - Variations in positive control or solvent control responses in different DMSO (lot-lot or catalog number)
  - Preparation, storage condition, and filtration methods affected neutral red signal
- UVA light source investigated for impacts on irradiance uniformity. Variations in UVA light intensity observed depending on plate placement under the light source.
- Several variables (reagents and light source) which likely impacted assay performance and may have contributed to increasing assay sensitivity were investigated.

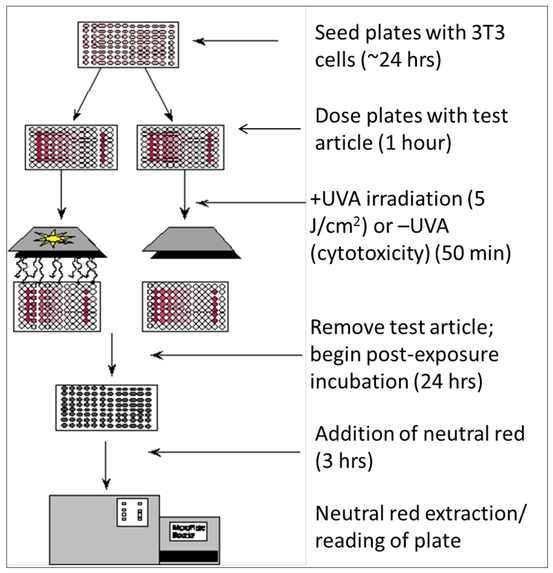


Figure 1. Generalized Overview of 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay

MATERIALS AND METHODS

3T3 NRU PT Assay Overview

The methods for the performance of the 3T3 NRU Phototoxicity Assay are a modification of the procedures outlined in OECD Test Guideline 432 "*In Vitro* 3T3 NRU phototoxicity test". A generalized outline of the assay procedures are summarized in Figure 1. Balb/c 3T3 mouse fibroblasts were seeded into 96-well plates (1.0×10^4 cells/ well) and incubated at standard culture conditions (SCC) (37±1 ºC, 5.0±1% CO_2, >80% RH) for ~24 hours. Cells were treated with chlorpromazine, Hanks' Balanced Salt Solution (HBSS), or DMSO, as described below, for 1 hour at SCC. Plates designated for the photoirritation assay were exposed to UVA for 50 minutes; plates designed for cytotoxicity (-UVA) were exposed simultaneously in the absence of UVA. The light source (Dermalight SOL 3 solar simulator), equipped with UVA H1 filter (320-400 nm), was adjusted to deliver 1.7 ± 0.1 mW/cm^2, (total irradiation dose of 5 J/cm^2). After +UVA/ –UVA exposure, treatments were removed, the cells were rinsed with HBSS, and then incubated in assay medium (culture medium supplemented with streptomycin sulfate and penicillin) for ~24 hours. The cells were then incubated in assay medium containing 33 µg/mL neutral red at SCC for 3 hours. The neutral red solution was decanted, an extraction solvent (1% acetic acid, 49% water, and 50% ethanol) was added, and the uptake of neutral red dye into the cells was measured using a spectrophotometer (optical density at 550 nm). The Mean Photo Effect (MPE) (e.g. measurement of differences in +UVA and –UVA dose response curves) was calculated using PHOTOTOX 2.0 Software (ZEBET). The mean OD_{550} value of the solvent controls exposed to UVA were analyzed as a percentage relative to the solvent controls of the cytotoxicity control (-UVA).

UVA Light Performance

- To investigate irradiance uniformity, five 96-well plates (+UVA) were treated with chlorpromazine (9.53 to 0.156 µg/mL) and placed at specific locations under the UV light source (See Figure 2) . A cytotoxicity plate not exposed to irradiance (-UVA) was treated with chlorpromazine (100 to 1.63 µg/mL.)
- Variability between plate positions was assessed by comparing MPE values for each position under the light. (Figure 2).
- A second experiment was conducted as above, with the exception that an additional plate was treated with chlorpromazine (9.53 to 0.156 µg/mL) and rotated under the light at varying locations during UVA exposure. (Figure 3)

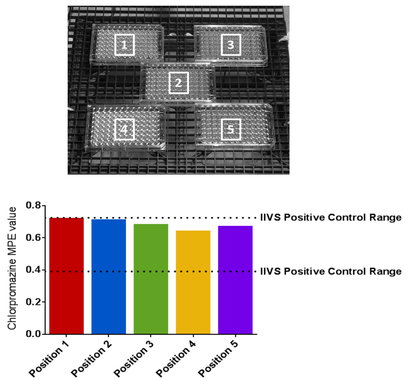
DMSO Lot Consistency and Chlorpromazine Preparation

- The performance of chlorpromazine (Batches 1-3) prepared in three different DMSO reagents was evaluated. Two different lots with identical catalog numbers (Lot 1 and Lot 2) and two different product catalog numbers were used. (Figure 4)
- Each chlorpromazine batch was prepared in its batch specific DMSO reagent at 9.53 to 0.156 µg/mL (+UVA) or 100 to 1.63 µg/mL (–UVA).
- Specific DMSO reagents used to prepare solvent control (1% DMSO in HBSS) for respective chlorpromazine-treated plate.

Neutral Red Preparation

- Optimize neutral red preparation methods for maximal optical density signal. Different preparations of assay-use neutral red (33 µg/mL) in culture medium were added to untreated confluent 3T3 cells.
  - Stock neutral red received from vendor (3.3 g/L) was compared to neutral red prepared at IIVS (3.3 g/L) from neutral red powder diluted in Dulbecco's Phosphate Buffered Saline.
  - Storage conditions (2-8°C) of stock neutral red in large batches or smaller aliquots
  - Differences in filter type (bottle system or syringe attachment) or filter vendors

RESULTS



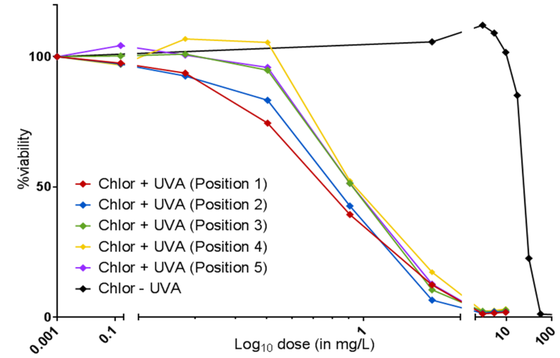
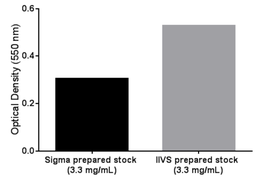


Figure 1. Positioning of UVA-exposed plates under the solar simulator indicates fluctuations in irradiance uniformity. Five chlorpromazine-treated plates were exposed to UVA light at different areas on the light surface, labeled positions 1-5 *(a)*. A cytotoxicity control was kept in the dark under identical conditions (-UVA). Mean relative viability at each chlorpromazine concentration was determined and plotted as concentration response curves on a logarithmic scale *(b)*. The mean photo effect (MPE) was calculated in PHOTOTOX Version 2.0 software (ZEBET) for each plate position and compared to the IIVS historical positive control data range (0.391-0.725) (*c*).



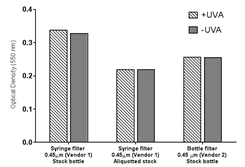
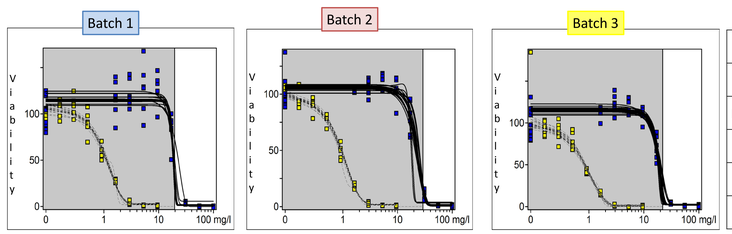


Figure 2. Optimization of neutral red preparation for the 3T3 NRU PT assay. Optical density comparison of 33 µg/mL neutral red prepared from stock solutions from vendor or prepared at IIVS (a). Comparison of filtration equipment (bottle or syringe), vendor, and storage preparation (aliquot or large batch). OD acceptance criteria OECD TG 432 is ≥0.4 and ≥0.3 for InVitox[2].



Chlorpromazine Batch	1	2	3
DMSO Lot	Lot 1	Lot 2	Catalog 2
Mean Photo Effect (MPE)	0.741	0.682	0.795
% Solvent Comparison	125.8%	117.1%	72.4%
Average OD_{550} (+UVA)	0.221	0.391	0.141
Average OD_{550} (-UVA)	0.176	0.334	0.195

Figure 3. Comparison of chlorpromazine batches prepared in varying DMSO reagents. Dose response curves for 3 different batches of chlorpromazine prepared in 3 different DMSO reagents. The data were generated using Phototox 2.0 software. The relative viability (y-axis) was plotted against the concentration of chlorpromazine (x-axis). The +UVA dose responses are represented by the small yellow squares; -UVA dose responses are represented by the small blue squares. Only the results of Batch 2 chlorpromazine prepared with lot 2 DMSO met all acceptance criteria and resulted in a mean optical density value >0.3

DISCUSSION & FUTURE CONSIDERATIONS

- Assay performance is routinely monitored using defined acceptance criteria (e.g. internal historical database). Failure to consistently meet this criteria warrants closer examination of reagents and equipment, and may require assay optimization.
- Fluctuations in light uniformity affect assay sensitivity. Modification of procedures (e.g. rotation of plate during UVA exposure) can enhance UVA-exposure uniformity.
- Changes in manufacturing, lot, storage conditions, or vendor may impact assay results. Consider verification of performance of reagents and supplies prior to assay use.
- The optical density signal may be influenced by procedures, reagents, and supplies used in preparation of neutral red.
- This work provided a better understanding of the impacts of assay-specific reagents, supplies, and equipment, and these results were used to assess and optimize assay performance. Additional R&D work is considered to fully elucidate the high assay sensitivity and potential impacts of DMSO.

[1]*OECD Test Guideline (432) for the In Vitro 3T3 NRU Phototoxicity Test, Organization of Economic Cooperation and Development. Adopted 13 April 2004*

[2]*Anon. INVITTOX Protocol 78. 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay. ECVAM DB-ALM; 2008. http://ecvam-dbalm.jrc.ec.europa.eu/*