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Refinement and Optimization of the Photo-KeratinoSens™ Assay to Evaluate Photoallergy Potential

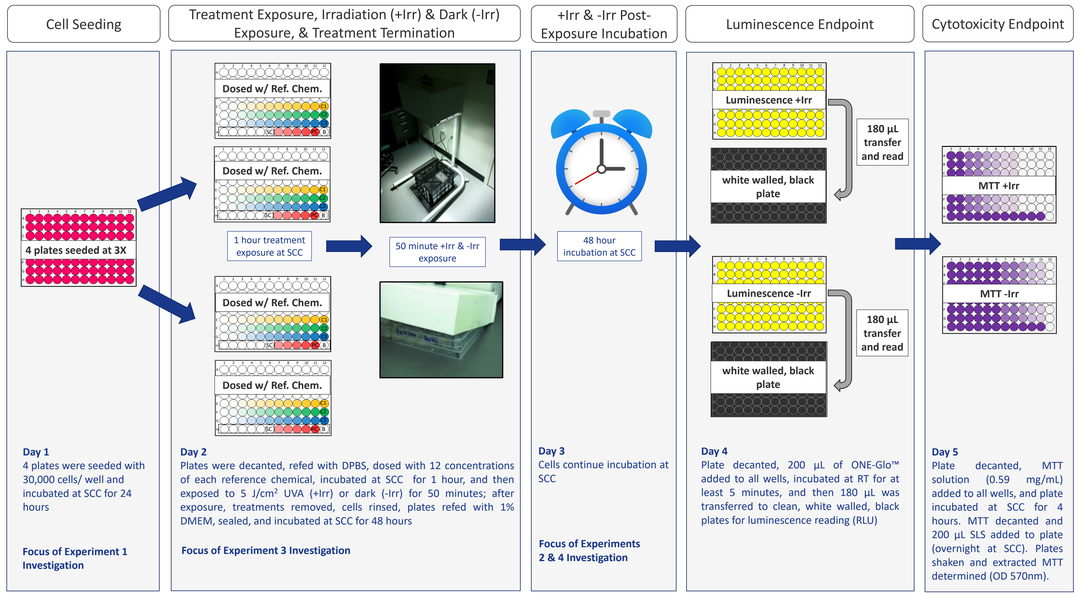
K. Bouchard, K. Cantrell, M. Madrid, K. Tran, A. Hilberer,
Institute for *In Vitro* Sciences, Inc., Gaithersburg, MD USA

Introduction

Identification of test chemicals that have the potential to become more reactive, more toxic, or may become phototoxic upon application and subsequent exposure to sunlight is integral to photosafety testing. Several regulatory-based New Approach Methodologies (NAMs) are widely used in the evaluation of phototoxicity and skin sensitization potential. More recently, there has been an increased interest in ability to identify photoallergens. To this end, an innovative photo-KeratinoSens™ assay was established with addition of an irradiation exposure (5 J/cm² of UVA) to the KeratinoSens™ Skin Sensitization Test (OECD TG 442D), as described in Tsujita-Inoue, *et al*. (2015). Twelve reference compounds were previously evaluated at IIVS under a protocol adapted from Tsujita-Inoue, *et al*. (2015), resulting in an accuracy of 58.3% (7/12 correctly predicted), with low sensitivity (50%, 5/10 positives correctly predicted) (Hilberer, *et al*., 2023).

Our work was conducted to understand the impacts of our refinements and optimizations with focus on the cell seeding density, the irradiation exposure, and the total treatment exposure time. Three reference compounds were evaluated and included two photoallergens (6-methylcoumarin (6-MC) and hexachlorophene) and a skin sensitizer (cinnamic aldehyde). Our work was conducted to optimize the current methods and create a more predictive and test system.

Photo-KeratinoSens™ Materials & Methods



SCC- Standard Culture Conditions (*i.e.*, 5% ± 1% CO_2, 37° ± 1°C, and humidified air); DPBS-Dulbecco's Phosphate Buffered Saline; 1% DMEM- Dulbecco's Modified Essential medium containing 1% serum; Ref. Chem.- Reference Chemicals; RT- room temperature; C-compound

Figure 1. Outline of Photo-KeratinoSens™ Materials & Methods adapted from Tsujita-Inoue, *et al.* (2015), and as described by Hilberer, *et al*. (2023).

Conclusions & Future Directions

Our results showed that the cell seeding density and amount of time between seeding and luciferase endpoint impacted the overall luciferase signal (Experiments 1 & 2), and may indicate the culture conditions becoming less optimal. We further investigated both parameters (exposure and cell density) in Experiments 3 and 4 with an additional variable: treatment incubation using a phenol red free medium containing 1% serum (*i.e.*, 1% DMEM) to expand the total treatment exposure time from ~ 2 hours (when DPBS used) to ~24 or ~48 hours (when medium used). The cells with extended exposure in medium produced increases in luciferase induction with increasing light exposures (Experiment 3), but the induction by cells at the 3X seeding density were <1.5- fold up to 9 J/cm². Further, while the 1X seeding density with a 48 hour chemical exposure resulted in appropriate predictions for all reference compounds: (*i.e.,* two photoallergens (6-methylcoumarin and hexachlorophene) and one skin sensitizer (cinnamic aldehyde), the RLUs +Irr were higher than those –Irr for the solvent control (*note: amiodarone, while initially included in the abstract, was later excluded due to conflicting research on its photoallergen properties*).

While the longer total exposure time (in media without phenol red) was most promising for correct predictions, additional assay parameters required investigation, with potential consequences of increased luciferase activity in the presence and absence of irradiation, which was further increased in the presence of light. This may suggest activation of stress pathway by the cells due to the combined effects of medium, irradiation, and/or longer total exposure time.

Our optimizations of the existing methodologies resulted in more appropriate predictions on this very small data set. However, a larger subset of reference materials with clearly defined photosafety information (*e.g.,* clinical safety data to support classification as photoallergen or photoirritant), need to be considered for further assessment of these refinements and their implementation into study protocols.

IIVS would like to recognize Tara Supit and Jarett Kwiatek, who helped generate data for this poster.

References:

Organization for Economic Cooperation and Development (OECD) Test Guideline (442D) "ARE-Nrf2 Luciferase KeratinoSens™ Test Method" (2022)

Tsujita-Inoue, K., Hirota, M., Atobe, T., Ashikaga, T., Tokura, Y., & Kouzuki, H. (2015). Development of novel in vitro photosafety assays focused on the Keap1-Nrf2-ARE pathway. *Journal of applied toxicology : JAT*, *36*(7), 956–968

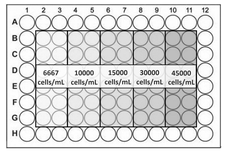
Hilberer A., Ritacco G., Bouchard K., Cantrell K., Madrid M., Oeda S., Kobayashi H., Atobe T., Suttinont C., Hirota M., Kouzuki H., Api A.M., Gerberick G.F., Ryan C. 2023. "Evaluation and Transferability of a New Approach Methodology to Address Photoallergy Potential" The Toxicologist, a Supplement to Toxicological Sciences, Abstract 3649

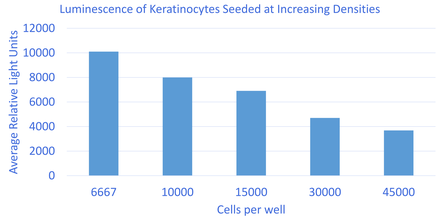
Experimental Methods and Results of Refinements and Optimizations

The following four experiments were modifications of the Photo-Keratinosens™ method presented in Figure 1. A brief description of the procedures, as well as pertinent results are presented below. Luciferase data are expressed as relative light units (RLUs) in experiments 1, 2 and 4. The fold-gene induction was determined by comparing the induction at each concentration (+Irr or –Irr) relative to the respective control (+Irr or –Irr) in experiment 3, and by dividing the induction values at 1-9 J/cm² by the induction value at 0 J/cm² in experiment 3. The fold induction ratios in experiment 4 were calculated by dividing the +Irr fold- induction by the -Irr fold- induction (at the corresponding concentration), and a fold-induction ratio ≥1.5 indicates increased gene induction in presence of irradiation and may suggest photoallergy potential.

Experiment 1: Impact of Cell Seeding Density

Five densities ranging from 6667 to 45000 cells/well were seeded into a 96 well clear plate (12 replicate wells per seeding density). After 24 hours, the plates were refed with 1% DMEM and were incubated at standard culture conditions for 48 hours. The luciferase endpoint was performed as presented in Figure 1, Day 4.





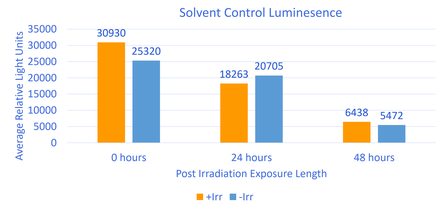
Luminescence signals, presented as Relative Light Units (RLUs), of KeratinoSens™ cells seeded at 6667, 10000, 15000, 30000 and 45000 cells per well. The luciferase signal decreases as the cell density increases.

Experiment 2: Impact of Post-Exposure Incubation Time

Six 96-well plates seeded at 30,000 cells/well were treated with the solvent control (DPBS) in twelve replicate wells and exposed to 5 J/cm² of UVA irradiation (+Irr) (3 plates) or protected from light at RT (-Irr) (3 plates).

Following the +/-Irr exposure, the DPBS was decanted. The cells were immediately assayed for luminescence (2 plates), referred to as 0 hours post-irradiation, or received 1% DMEM for 24 hours (2 plates) or 48 hours (2 plates). Each group had one plate exposed to light and one plate protected from the dark.

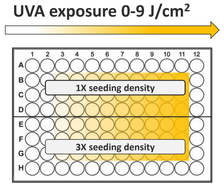
After each respective post-exposure incubation, the plates were processed as described in Figure 1, Day 4.

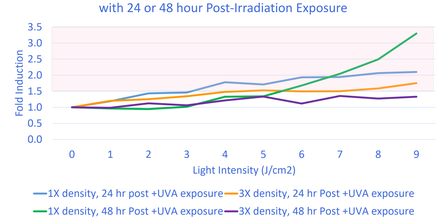


Luminescence signals, presented as Relative Light Units (RLUs), of irradiated (+Irr) and non-irradiated (-Irr) solvent control with post-exposure incubation period of 0, 24 and 48 hours. The luciferase signal decreases as the post-exposure incubation time increases.

Experiment 3: Impact of Irradiation Exposures

The inner 60 wells of 2 clear 96 well plates were seeded with KeratinoSens™ cells at 10,000 cells/well (1X) on the top half and at 30,000 cells/well (3X) on the bottom half. The plates were refed with 1% DMEM without phenol red (media), and then one plate exposed to 0-9 J/cm² of UVA (+Irr) by using moving an opaque paper over the wells at specified time intervals to expose cells to the respective UVA exposure, and one plate retained at RT, protected from light (-Irr). The cells received a 24 or 48 hour post-exposure incubation period before luciferase assessment as described in Figure 1, Day 4.





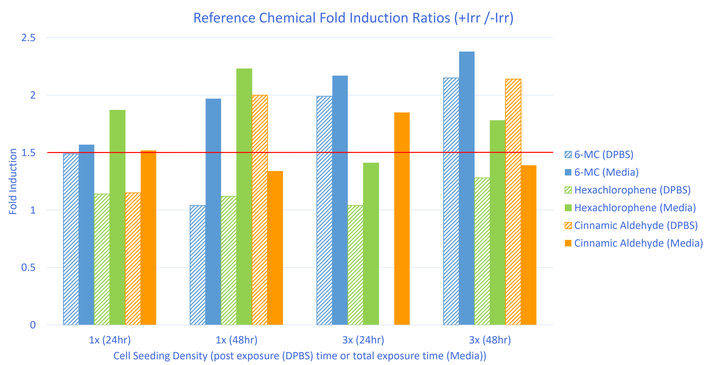
Fold-induction with increasing UVA exposures (0-9 J/cm²). The shaded area highlights fold induction ≥ 1.5. As the light intensity increased, with the greatest fold induction increases were seen in the 1X seeding density at both 24 and 48 hours. The 3X density with 48 hour post +UVA exposure was the only condition where fold induction was less than 1.5 at 0-9 J/cm². At 5 J/cm², only the 48 hour post-exposure induction ratio was <1.5 for cells seeded at 10,000 (1X) and 30,000 (3X) cells per well.

Experiment 4: Impact of Cell Seeding Density in Combination with Exposure Time

The solvent control and three reference compounds: 6-Methylcoumarin (6-MC), hexachlorophene, and cinnamic aldehyde were evaluated at 1X (10,000 cells/well) and 3X (30,000 cells/well) seeding densities with either a 24 or 48 post-exposure incubation period (prepared in DPBS) or total exposure period (prepared in Media). The reference compounds marked as "DPBS" were prepared in DPBS, decanted following the +/- irradiation exposure, refed with 1% DMEM, and returned to the incubator at standard culture conditions for 24 or 48 hours (total treatment exposure of ~ 2 hours: 1 hour exposure + 50 minute +/- UVA). The reference compounds marked as "Media" were prepared in 1% DMEM without phenol red, were not decanted following the +/- irradiation exposure, and returned to the incubator at standard culture conditions for 24 or 48 hours (*i.e.,* total treatment exposure of ~26 or ~50 hours: 1 hour exposure + 50 minute +/- UVA with 24 or 48 hour post-exposure incubation). After the 24 or 48 hour post-exposure incubation, the plates were processed under the luminescence (Day 4) or cytotoxicity (Day 5) endpoints described in Figure 1.

	DPBS Average RLUs				Media Average RLUs			
	24 hour post exposure		48 hour post exposure		24 hour exposure		48 hour exposure	
Seeding Density	1X	3X	1X	3X	1X	3X	1X	3X
+Irr exposure	15926	19804	12018	7230	69359	43814	31760	16835
Dark (-Irr) exposure	17834	19509	11170	6660	40450	25036	21243	13341
% control comparison (-/+Irr)	112%	99%	93%	92%	58%	57%	67%	79%

Summary of the solvent control average RLU values for DPBS (24 or 48 hour post-exposure) or Media (24 or 48 hour total exposure) at corresponding seeding densities. A % control comparison ((-Irr RLU/ +Irr RLU) *100) was calculated to assess the difference between the +Irr and –Irr responses. While the RLUs are overall higher with the Media procedure, the % control comparisons showed an increased response under irradiation exposure when Media used instead of DPBS.



Fold induction ratios of the reference compounds at 1X and 3X seeding densities with a 24 and 48 hour post-exposure (DPBS) or total treatment exposure (Media). Photoallergy is suggested if the fold- induction ratio is ≥ 1.5 (demonstrated by the red line in graph). Known photoallergens, 6-MC and hexachlorophene, produce fold induction ratios ≥ 1.5 at 1X (24 and 48 hrs) and 3X seeding density (48 hrs only) when prepared in medium (total exposure of 24 or 48 hours); and when prepared in DPBS, only 6-MC is correctly predicted at 3X seeding density. Cinnamic Aldehyde is a skin sensitizer, not a photoallergen, and the fold induction ratio was expected to be < 1.5; however, the ratio was >1.5 (*i.e.,* false positive) when prepared in medium at 24 hours (1X and 3X seeding density) and when prepared in DPBS at 48 hours (1X and 3X seeding density). *Note: Cinnamic Aldehyde (DPBS) at 3X density (24 hrs) was cytotoxic at all concentrations.*