
***In Vitro* Methodologies for Assessing Cutaneous Irritation, Sensitization and Photosensitivity**

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Conflict of Interests

- None – but in the spirit of full disclosure
 - I am a member of the Cosmetic Ingredient Review's Expert Panel
 - I am a member and chair of the Expert Panel for Fragrance Safety
 - I am reimbursed for my travel & time for both from the Personal Care Products Council (Washington, DC) and the Research Institute for Fragrance Materials (Woodcliff Lake, NJ), respectively

IRRITANT CONTACT DERMATITIS

- 1) Acute primary irritation
- 2) Cumulative irritation
- 3) Corrosion
- 4) Phototoxicity

CHEMICAL IRRITANCY

- Inherent chemical properties
- Concentration
- Amount
- Duration / frequency

PHOTOIRRITATION/ PHOTOTOXICITY

- Photoexcitable chemical which absorbs
 - UVB (290-320 nm) - less frequently
 - UVA (320-400 nm) - most frequently
 - Visible (400-775 nm) - rare/endogenous

IN VITRO METHODS FOR CORROSION

❑ **rat skin transcutaneous electrical resistance (OECD TG 430)**

- liquids (150 μ l) & solids (“sufficient to cover surface”) x 24h
- TER > 5 k Ω , non-corrosive; $\leq$ 5 k Ω , corrosive
- not applicable to surfactants or neutral organics
 - need to use dye (sulforhodamine B) penetration
- http://www.oecd-ilibrary.org/environment/test-no-430-in-vitro-skin-corrosion-transcutaneous-electrical-resistance-test-method-ter_9789264203808-en.

❑ **Corrositex (reconstituted collagen matrix; OECD TG431)**

- liquids (500 μ l) & solids (500 mg)
- breakthrough time correlated to corrosivity
- applicable only to materials that produce a color or physical change in the “chemical detection system” w/in 5 mins
- http://www.oecd-ilibrary.org/environment/test-no-431-in-vitro-skin-corrosion-human-skin-model-test_9789264071148-en

IN VITRO METHODS FOR CORROSION

(OECD TG 435)

- ❑ http://www.oecd-ilibrary.org/environment/test-no-435-in-vitro-membrane-barrier-test-method-for-skin-corrosion_9789264242791-en
- ❑ **Episkin (multilayered epidermis on collagen matrix)**
 - liquids & solids (sufficient material “to cover the the skin”)
 - degree of corrosivity defined as $\leq 35\%$ cell viability at defined times (3, 60, & 240 mins)
- ❑ **EpiDerm (multilayered epidermis on cell culture inserts)**
 - liquids (50 μ l) & solids (25 mg)
 - degree of corrosivity defined as $\leq 50\%$ cell viability @ 3 mins or $\leq 15\%$ viability at 60 mins at defined times

IN VITRO METHODS FOR ACUTE IRRITATION

Acute: (OECD TG 439)

- ❑ http://www.oecd-ilibrary.org/environment/test-no-439-in-vitro-skin-irritation-reconstructed-human-epidermis-test-method_9789264242845-en
- ❑ EpiSkin™
- ❑ EpiDerm™ SIT (EPI-200)
- ❑ Modified EpiDerm (EPI-200)
- ❑ SkinEthic™ RHE

IN VITRO: ACUTE IRRITATION

- ❑ Dose: minimum of 25 $\mu\text{L}/\text{cm}^2$ (liquid) or 25 mg/cm^2 (solid)
- ❑ Incubation: 3 hrs
- ❑ Viability: mitochondrial dehydrogenases to reduce the vital dye MTT
- ❑ The test substance is considered to be:
 - irritant to skin if the tissue viability after exposure and post-treatment incubation is $\leq$ to 50 %.
 - no category if the tissue viability after exposure and post-treatment incubation is $>$ 50 %.

IN VITRO: CHRONIC CUMULATIVE IRRITATION

- Chronic cumulative irritation

- no ECVAM / OECD acceptable in vitro method

- Under development:

- Reconstructed organotypic skin model (ROSM) w/ keratinocytes, basement membrane & fibroblasts

- Cultured for varying lengths of time w/ chemical in ?

- Assay for ↑ heat shock protein (HSP)-27

- Methodology assessed w/ SLS and acute irritants (acids & bases)

- Chen, et al. Toxicology Letters. 226: 124 = 131, 2014

PHYSICOCHEMICAL METHODS FOR ASSESSING PHOTOTOXICITY

- Physicochemical:
 - Is it a photoabsorber?
 - Henry, et al. J Photchem Photobiol B. 2009; 96: 57 – 62.
- Reactive Oxygen Species (ROS) assay
 - Excitation of molecules by light → ROS
 - superoxide anion (SA) and singlet oxygen (SO)
 - does not measure phototoxicity directly
 - 100% sensitivity; some false positives
 - Not validated by ECVAM / OECD
 - Haranosono, et al. J Toxicol Sci. 2014; 39: 655-64.
- Both systems fail to account for biotransformation & autoxidation

ROS (Japanese Center for the Validation of Alternative Methods): PHOTOTOXICITY

Solvents: DMSO or 20 mM of NaPB

Controls: Quinine hydrochloride (+); Sulisobenzon (-)

Irradiation: Solar stimulator x 1h @ controlled temperature

Judgment	Conc.	SO (mean X 3) SA (mean X3) (% ↑, irradiated / non-irradiated)		
Photoreactive	200 μM	≥25	and	≥70
		<25	and	≥70
		≥25	and	<70
Weakly photoR	200 μM	<25	and	≥20, <70
Non-photoR	200 μM	<25	and	<20

Inconclusive: The results do not meet the above-mentioned criterion.

IN VITRO METHODS FOR ASSESSING PHOTOTOXICITY

□ Phototoxicity

- 3T3 Neutral Red phototoxicity test (OECD TG 432)
- <http://www.oecd-ilibrary.org/content/book/9789264071162-en>
 - 3T3 Balb/c fibroblasts incubated for 1 hr w/ test substance and irradiated w/ 5 J/cm²
 - Photoirritation factor (PIF) = ratio of cytotoxicity with and without UV light (prefer solar simulator)
 - PIF < 2: no potential for phototoxicity
 - PIF > 5: phototoxic
 - PIF 2- 5: inconclusive

DRAWBACKS TO 3T3

- ❑ false negatives

- toxicological hazard Primarily due to a mechanism of action not captured by the cell line used
- the cell lines lack of metabolic capacity, compounds metabolized in vivo to biologically active forms may be missed

- ❑ false positive

- limited bioavailability in vivo due to poor absorption and distribution, or rapid biotransformation and excretion

IN VITRO METHODS FOR ASSESSING PHOTOTOXIC METABOLITES

- Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS) -- Portes, et al. Toxicol In Vitro. 2002; 16: 765-70.
- Enzymatic reactive oxygen species assay (eROS) -- Kato, et al. Enzymatic reactive oxygen species assay to evaluate phototoxic risk of metabolites. Toxicol Lett. 2017; 278: 59-65.

Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS)

- 100 µl test substance directly to tissue surface; incubated at 37°C x 18-24 h
- UV Irradiation (solar simulator): 6 J/cm²; control = non-irradiated
- Tissues and incubated x 18-24 h at 37°C
- Tissue viability measured using the MatTek MTT Viability Assay protocol
 - Currently uses cell viability ($\leq 30\%$) and $PIF \geq 2$
 - PGE₂ levels as alternative to viability
- Prevalidation suggests under-prediction and a precautionary factor of 10 for extrapolation to man

eROS

- 10 mM chemical in DMSO incubated at 37 °C in 20 mM NaPB containing human liver S9 fractions (0.2 mg-protein/mL) and “typical cofactors” x 5 min
- 100 µM of “activated” substrate in DMSO irradiated w/ solar stimulator x 1h @ controlled temperature
- Controls: Fenofibrate w/ S9 (+); Fenofibrate w/ denatured S9 (-)
- Readings [chemical w/ S9 / chemical w/ denatured S9] as per ROS f
- Favorable intra-/inter-day reproducibility
- eROS assay provided false predictions for some compounds
- Material must be water soluble

ALLERGIC CONTACT DERMATITIS

□ Allergic

- Pre-haptens
- Pro-haptens

□ Photoallergic

- Pre-haptens
- Pro-haptens

IN VITRO METHODS FOR ASSESSING ALLERGY: AOP

1: Molecular Initiation

- Direct Peptide Reactivity Assay (DPRA)

2: Keratinocyte Activation

- KeratinoSens™ (ARE-Nrf2 Luciferase Test Method)
- IL-8 Luc Assay (not ECVAM; +JaCVAM)

3: Dendritic Cell Activation

- Human Cell line Activation Test (h-CLAT)
- Myeloid U937 skin sensitization test (MUSST; ECVAM, not OECD)

4: T cell activation

- none

DPRA (OECD TG 442C)

- peptide to chemical ratio used is 1:10 (cysteine) and 1:50 (lysine) [controls: cinnamic aldehyde (+); vehicle (-)]

● **Cys & Lys:** **% depletion (mean)**

- Minimal Reactivity $< 6.38\%$
- Low Reactivity: $\geq 6.38 - < 22.62\%$
- Moderate Reactivity: $\geq 22.62 - < 42.47\%$
- High Reactivity: $\geq 42.47\%$

● http://www.oecd-ilibrary.org/environment/test-no-442c-in-chemico-skin-sensitisation_9789264229709-en

LIMITATIONS of DPRA

DPRA test method does not contain a metabolic/bioactivation system, therefore pro-haptens & pre-haptens are not detected

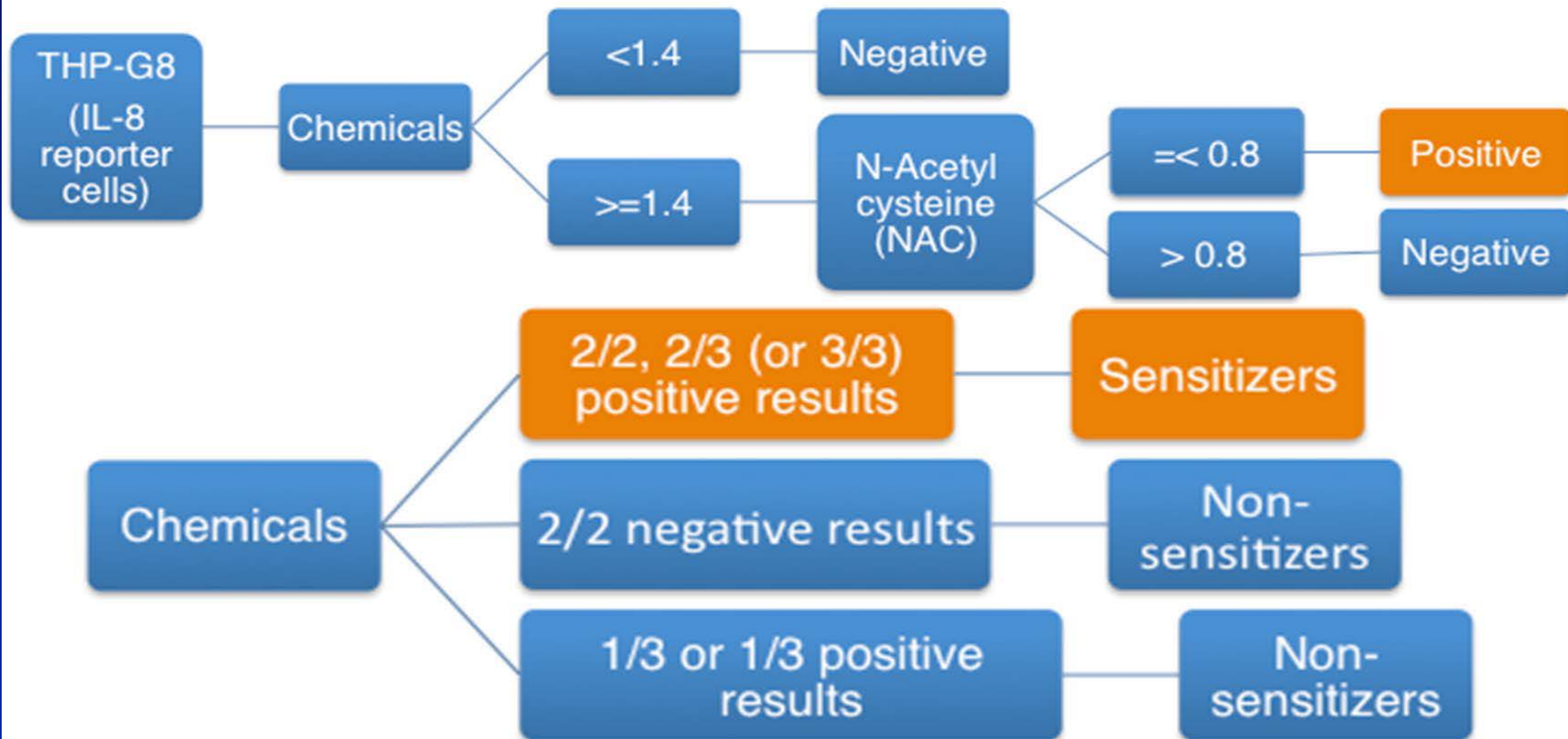
- ❑ pre-haptens (i.e. simple chemical transformation, for example oxidation by air or photo-activation in the presence of UV light)
- ❑ pro-haptens (i.e. chemicals requiring enzymatic activation)
 - peroxidase peptide reactivity assay “PPRA” identifies pro-haptens via the use of peroxidase in the DPRA
 - Gerberick. Altern Lab Anim. 2016; 44: 437-442.

KeratinoSens ASSAY (OECD TG 442D)

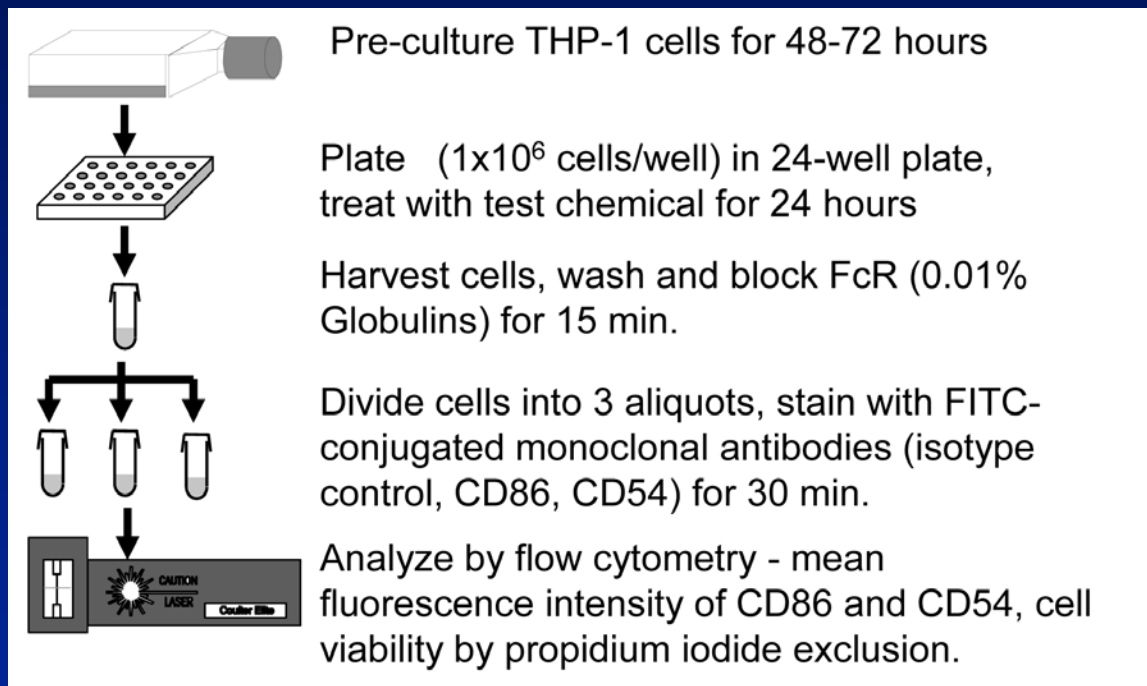
- Based on a stable reporter construct consisting of a luciferase gene under the control of the antioxidant response element (ARE) in a HaCaT (immortalized keratinocyte) cell line.
- The luciferase gene allows for the detection of sensitization potential based on the bioluminescent activity of ATP: light is produced after the breakdown of the protein luciferin by luciferase.
- Inserted genes allow for the exploitation of the signaling pathway of Keap1-Nrf2, which has previously been shown to be activated during skin sensitization events (Natsch & Emter, 2008; Natsch et al., 2009)
- Cell viability also assessed
- $\geq 2/3$ trials show 50% increased induction of luciferase gene; viability not a criterion; (-) control, solvent; (+) control, cinnamic aldehyde
 - http://www.oecd-ilibrary.org/environment/test-no-442d-in-vitro-skin-sensitisation_9789264229822-en.

IL-8 Luc Assay

THP-1 cell line is a human monocyte line; not keratinocyte



H-CLAT (OECD TG 442E) / MUSST*



<http://www.oecd-ilibrary.org/content/book/9789264264359-en>

DNCB = (+) control;
Medium = (-) control

CD86 >150% or CD54: >200% = positive;

*MUSST similar; uses Myeloid U937 cell line and only CD86;
ECVAM validated; awaiting OECD TG.

IN VITRO METHODS FOR ASSESSING Pro-HAPTEN PHOTSENSITIZATION

- No accepted methodology; no photo-activated DPRA has been proposed.
- in vitro testing methodologies utilizing KeratinoSens and h-CLAT with the addition of exposure to 5J/cm² of UVA
 - photo-KeratinoSensTM assay
 - ▣ Tsujita-Inoue et al. J Appl Toxicol. 2016; 36: 956-968
 - photo-h-CLAT assay
 - ▣ Hoya, et al. Toxicol In Vitro. 2009; 23: 911-918.
- In vitro systems to assess photosensitization are critical
 - EU ban on testing cosmetic ingredients in animals (sunscreens are cosmetic in the EU!)
 - testing for photoallergenicity in humans unethical due to potential for the induction of persistent light reactivity (PLR): extreme sensitivity to UVB in absence of inducing photoallergen

Sensitization Hazard (not risk)

- Integration of results of testing on the first three steps of AOP to predict sensitization hazard not resolved
- Various models proposed
 - Bayesian network
 - Artificial Neural Network
 - Weight of Evidence
 - Weight of Evidence or best “two out of three” to assess sensitization hazard AOP has found the greatest success
- But what about risk??

Thank you!



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