MEDWIN PUBLISHERS
Committed to Create Value for Researchers

Advances in Pharmacology and Clinical Trials
ISSN: 2474-9214

Progressive Paradigms: Non-animal Testing Approaches in Scientific Discovery

Umesh MC[1]*, Mukundan GK[2], Seekallu S[1] and Doreswamy CM[1]

[1]Department of Preclinical Research, Anthem Biosciences Limited, India

[2]BGS Global Institute of Pharmaceutical Sciences, BGS Health & Education City, India

***Corresponding author:** Mohan Cheluru Umesh, Department of Preclinical Research, Anthem Biosciences Limited, Bengaluru, Karnataka, India, Tel: +919611287003; Email: mohan.c@anthembio.com

Review Article

Volume 11 Issue 1

Received Date: August 04, 2026

Published Date: August 21, 2026

DOI: 10.23880/apct-16000260

Abstract

Over the past several decades, traditional animal models have been employed by the cosmetic industries for the safety testing of cosmetic products or ingredients. In recent days, due to innovation in science, the use of these animal models has been restricted and without the use of animals, many *In vitro* models such as 3D cell culture, cell-based tests, and *In vitro* cell culture models were developed and scientifically validated as alternatives to animal methods. In addition, these *In vitro* alternative models gained regulatory acceptance due to ethical and scientific concerns regarding the use of animals. The 3Rs principles—Replacement, Reduction, and Refinement were considered in the development and validation of these alternative methods. In this review article, well-established and accepted *In vitro* alternatives to animal test methods, such as testing for skin irritation/corrosion, skin sensitization, eye irritation or serious eye damage/eye hazard identification, and phototoxicity for various categories of chemicals are discussed.

Keywords: Alternatives to Animal Testing; *In vitro* Methods; Reconstructed Human Epidermis; Reconstructed Human Cornea-Like Epithelium; Skin Sensitization

Abbreviations

ADRA: Amino acid derivative reactivity assay; ARE-Nrf2: Antioxidant Response Element-Nuclear erythroid 2-related factor 2; DPRA: Direct peptide reactivity assay; HaCaT: Human keratinocyte; J/cm^2: joules per centimeter square; kDPRA-Kinetic Direct peptide reactivity assay; MPE: Mean Photo Effect; NAC: N-(2-(1-naphthyl) acetyl)-L-cysteine (NAC) or NAL: α-N-(2-(1-naphthyl) acetyl)-L-lysine (NAL) amino acids; OECD: Organisation for Economic Co-operation and Development; PIF: Photo Irradiation Factor; SCT: Skin Corrosion Test; SIT: Skin Irritation Test; TER: Transcutaneous electrical resistance; TEER: Transepithelial electrical resistance; TG: Test guideline; UVA: Ultraviolet light A.

Introduction

In preclinical research, laboratory animals had increasingly been relied upon as test systems in early drug discovery testing. Since, there was a lack of non-animal alternative models created more need for laboratory animals, especially for toxicology testing for analyzing clinical pathology parameters in biological fluids such as blood and urine coupled with histopathology examination in altered tissues [1].

Some of the standard animal toxicity tests are listed in Table 1.

CC BY

Standard Animal Toxicity Tests		
1	Acute, Sub-acute, and Chronic Toxicity Tests	To determine the effect of drugs/chemicals on health and mortality during several lengths of exposure.
2	Reproductive Toxicity Tests	To determine the effect of drugs/chemicals on fertility and fecundity
3	Developmental Toxicity Tests	To determine the effect of drugs/chemicals to cause abnormalities in an embryo, fetus, or newborn.
4	Genotoxicity Tests	To determine the ability of drugs/chemicals to cause damage to genetic material.
5	Ocular and Skin Irritation/ corrosion Tests	To determine the ability of drugs/chemicals to irritate or corrosive to eyes or skin
6	Hypersensitivity Tests	To assess the tendency of drugs/chemicals to elicit rashes and other allergic responses.
7	Phototoxicity Tests	To determine the extent to which drugs/chemicals are activated by sunlight, thereby enhancing their toxicity.
8	Toxicokinetic Tests	To determine the absorption, distribution, metabolism, and excretion of drugs/ chemicals.
9	Behavioral Tests	To monitor the effect of drugs/chemicals on cognitive function during development and in the adult.

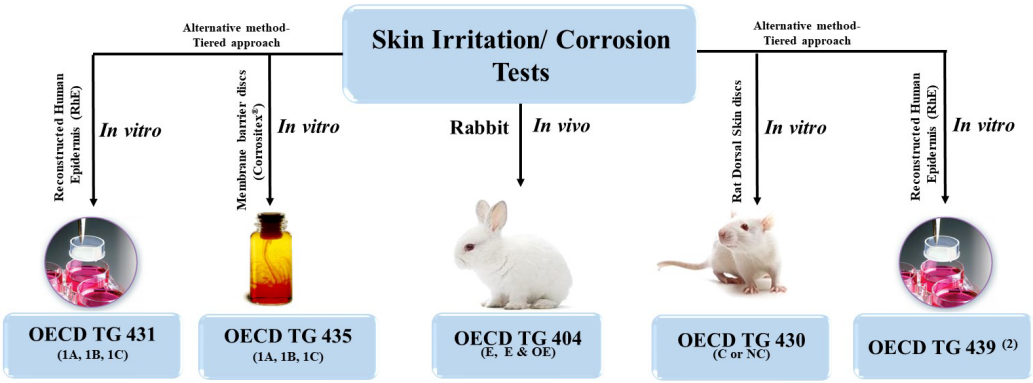
Table 1: Standard Animal Toxicity Tests.

These animal models are costly, time-consuming, and increasingly criticized by animal welfare groups. In recent days, the use of laboratory animals in the safety assessment of a chemical has been limited by scientific, regulatory restrictions, and social concerns [2]. Many toxicology experts reported animal testing as an unsatisfactory outcome to toxicology problems because of the non-mechanistic results from the exposure [3-4]. Hence, these limitations have prompted the development of non-animal approaches. European Centre for the Validation of Alternative Methods (ECVAM) has developed and completed the prospective validation study for *In vitro* skin and eye irritation/corrosion tests, phototoxicity tests, and, skin sensitization tests. Nowadays, these *In vitro* test methods become very popular for their acceptance of outcomes by regulatory bodies for testing various categories of products such as cosmetics, pharmaceutical, nutraceutical, or agrochemical products. Brief information on these tests is discussed in this review article.

In vitro Alternative Methods for Toxicity Testing

Skin Irritation and Corrosion Tests

Acute dermal irritation/corrosion test in rabbits (OECD TG 404) is one of the traditional models for testing skin irritancy of chemicals. However, this model has been replaced nowadays with *In vitro* alternative models (Figure 1).



'1A, 1B, 1C' indicates UN GHS Category 1A, 1B & 1C for skin corrosive chemicals
'E, E & OE' indicates Grading Skin reactions for Erythema, Eschar and Oedema formations
'C or NC' indicates Corrosive (No subcategory classification can be made) or Non-corrosive
'2' indicates UN GHS Category 2 for skin irritant chemicals

Figure 1: Skin Irritation/ Corrosion Test Models.

Copyright© Umesh MC et al.

OECD TG 439 [5] describes an *In vitro* skin irritation test method for the identification of irritant chemicals substances or mixtures under category 2 as per the United Nations Globally Harmonized System (UN GHS) of classification and labeling of Chemicals. Similarly, OECD TG 431 [6] describes an *In vitro* skin corrosion test method for the identification of corrosive chemicals substances or mixtures to categorize as per UN GHS classification. European Centre for the Validation of Alternative Methods (ECVAM) developed and completed the prospective validation study of these *In vitro* skin irritation and corrosion tests. These methods utilize Human reconstructed epidermis (RhE) tissues as test systems which closely mimic the biochemical and physiological properties of the upper parts of the human skin, i.e., the epidermis. EpiSkin™ and EpiDerm™ SIT test models are considered validated reference models for both tests. Currently, there are additional RhE models also accepted by the OECD. These RhE models mainly differ in the surface area of tissue units (cm^2), the quantity of test chemicals for exposure (solids / liquids /semisolids or waxy), and the duration of exposure (Table 2). These RhE models should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria.

Test name	Test model name	Surface area (cm^2)	Exposure amount (solids/liquids/ waxy)	Exposure Duration (min)	Negative and Positive Control	End-point
In vitro Skin Corrosion test	EpiSkin™ (VRM) (*)	0.38	20 mg/ 50 µL/ 50 mg	3, 60 and 240	0.9% NaCl and Acetic acid	MTT Assay
	EpiDerm™ SCT (EPI-200) (VRM)	0.63	25 mg/ 50 µL/ 50 µL	3 and 60	Water and 8N KOH	
	SkinEthic™ RHE	0.5	20 mg/ 40 µL/ 20 mg	3 and 60	Water and 8N KOH	
	epiCS®	0.3	25 mg/ 40 µL/ 50 µL	3 and 60	Water and 8N KOH	
	LabCyte EPI-MODEL 24 SIT	0.6	25 mg/ 50 µL/ 50 µL	3 and 60	Water and 8N KOH	
In vitro Skin Irritation test	EpiSkin™ (VRM) (*))	0.38	10 mg/ 10 µL/ 10 mg	15	DPBS and 5% SDS	MTT Assay
	EpiDerm™ SCT (EPI-200) (VRM)	0.63	25 mg/ 30 µL/ 25 mg	60	Water and 1% Triton	
	SkinEthic™ RHE	0.5	16 mg/ 16 µL/ 16 mg	42	DPBS and 5% SDS	
	LabCyte EPI-MODEL 24 SIT	0.3	25 mg/ 25 µL/ 25 mg	15	Water and 8N KOH	
	epiCS®	0.6	30 mg/ 30 µL/ 30 mg	20	DPBS and 0.01% CPZ	
	Skin+ ®	0.5	16 mg/ 16 µL/ 16 mg	42	Water and 5% SDS	
	KeraSkin™ SIT	0.6	20 mg/ 50 µL/ 50 mg	30	DPBS and 5% SDS	

DPBS-Dulbecco's Phosphate Buffer Saline; NaCl-Sodium chloride; SDS-Sodium dodecyl sulfate; KOH-Potassium hydroxide. (*)-the EpiSkin™ method is no longer commercially available from December 2024.

Table 2: Reconstructed Human Epidermis Models for Skin Irritation and Corrosion Tests.

In vitro, skin irritation and corrosion tests using RhE models are cytotoxicity-based assays. Preliminary tests (MTT reduction test and coloring potential test) to identify test chemical is an MTT reducer or coloring potential agent are performed. The RhE tissue inserts are pre-incubated under a CO_2 incubator before exposure. Then test chemical is applied on the surface of RhE tissue inserts and incubated as per recommended duration by the developer. For the irritation test, the reversibility of the viability is done during the post-incubation period. For the corrosion test, a direct MTT assay is performed. The extent of irritancy or corrosivity of tested chemicals are determined by performing an MTT assay by determining the %cell viability compared to the negative control.

However, the other two *In vitro* models as per OECD TG 430 [7] and OECD TG 435 [8] also describe *In vitro* skin corrosion test methods. Where OECD TG 430 utilizes rat dorsal skin discs (20 mm diameter) which is placed between two compartments. Magnesium sulfate solution ($MgSO_4$) is used as receptor fluid. The test is conducted by application of test chemicals (solids/liquids) onto the epidermal surface of the skin discs for 24 hours at 20-23 °C followed by removal of test chemicals by washing with water. The corrosive nature of chemicals are identified by the loss of integrity and barrier function of the skin by measuring the Transcutaneous

Copyright© Umesh MC et al.

electrical resistance (TER) below the threshold level (5 k). In the case of OECD TG 435, where an artificial membrane is used as a test system for corrosive assessment. The Corrositex® test model is considered a validated reference model. The testing should be performed as per the protocol defined by the kit supplier. The corrosive nature of chemicals are identified by the loss of barrier function of the artificial membrane by identifying the change in the indicator solution i.e., barrier penetration is identified with time. However, this method has certain limitations for the chemicals with a pH range of 4.5 to 8.5 do not qualify for the test. For all these described methods, the UN GHS classification is done as per the prediction model of the test (Table 3).

A. **Skin Irritation Prediction - Reconstructed Human Epidermis Model (OECD TG 439)**	
***In vitro* result**	**Classification (*In vivo* Prediction)**
Mean tissue viability ≤ 50%	Category 2 (Irritant)
Mean tissue viability > 50%	No Category (Non-Irritant)
B. **Skin Corrosion Prediction - Reconstructed Human Epidermis Model (OECD TG 431)**	
Viability measured after an exposure time points (t=3, 60 and 240 minutes)	**Prediction to be considered**
35% after 3 min exposure	**Corrosive**: Optional Sub-category 1A
= 35% after 3 min exposure AND < 35% after 60 min exposure OR = 35% after 60 min exposure AND < 35% after 240 min exposure	**Corrosive:** A combination of optional Sub-categories 1B-and-1C
≥ 35% after 240 min exposure	**Non-corrosive**
C. **Skin Corrosion (Corrositex®) Prediction- *In Vitro* Membrane Barrier Test Method (OECD TG 435)**	
Mean breakthrough time (min.)	**UN GHS prediction**
Category 1 test chemicals (determined by the method's categorization test)	Category 2 test chemicals (determined by the method's categorization test)
0-3 min	0-3 min	Corrosive: optional Sub-category 1A
> 3 to 60 min	> 3 to 30 min	Corrosive: optional Sub-category 1B
> 60 to 240 min.	> 30 to 60 min	Corrosive: optional Sub-category 1C
> 240 min	> 60 min	**Non-corrosive**
D. **Skin Corrosion Prediction - Transcutaneous Electrical Resistance Test Method (TER) (OECD TG 430)**	
TER Value	**UN GHS Classification**
Mean TER Value is >5 kΩ or mean TER Value is ≤5 kΩ and if the skin discs show no obvious damage; mean disc dye content is less than (<) the mean disc dye content of the 10M HCl positive control	Non-Corrosive
Mean TER Value is ≤5 kΩ and the skin discs are obviously damaged; or Mean TER Value is ≤5 kΩ and the skin discs show no obvious damage but the mean disc dye content is greater than or equal to (≥) the mean disc dye content of the 10M HCl positive control	Corrosive

Table 3: Skin Irritation and Skin Corrosion Prediction Model.

Eye Irritation Test

The rabbit model for testing acute eye irritation/corrosion potential of chemicals is one of the traditional models. OECD introduced TG 405 on *In vivo* Eye Irritation/Serious Eye Damage in 1981. Later the other *In vitro* methods have been validated, considered scientifically valid, and adopted as OECD TG 437 [9], OECD TG 438 [10], OECD TG 460 [11], OECD TG 467 [12], OECD TG 491 [13],

Copyright© Umesh MC et al.

OECD TG 492 [14], OECD TG 492B [15] and, OECD TG 494 [16] to address the human health endpoint of serious eye damage. An overview of eye irritation/corrosion models is represented in Figure 2.

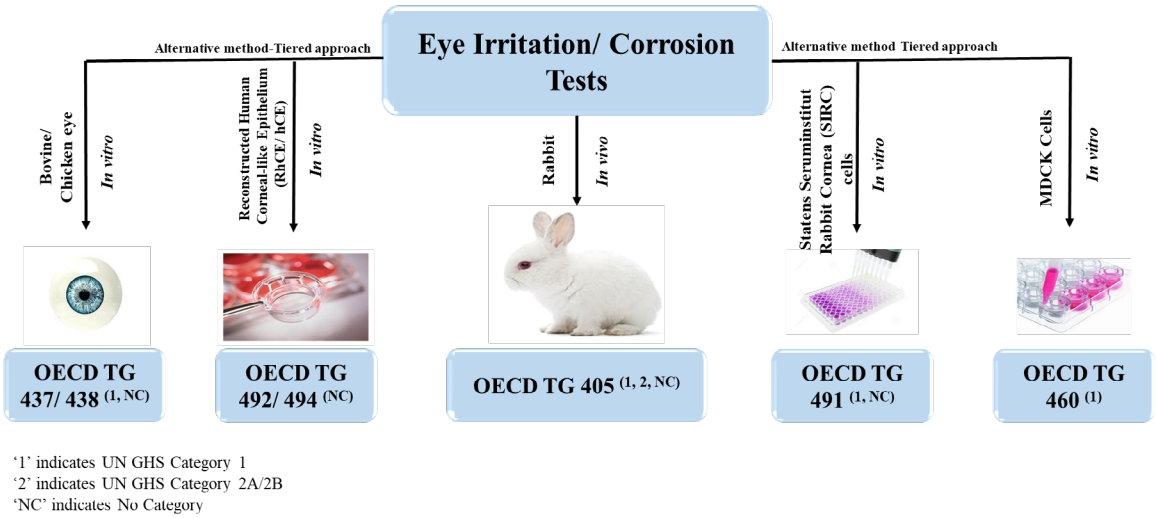


Figure 2: Eye Irritation/ Corrosion Test Models.

OECD 492 and OECD 492B are the *In vitro* test methods for eye irritation (EIT) tests, which are mainly based on the *In vitro* test system consisting of the reconstructed human cornea-like epithelium (RhCE) which closely mimics the histology, morphology, biochemical and physiological properties of the upper parts of the human corneal epithelium. European Centre for the Validation of alternative methods (ECVAM) developed and completed the prospective validation study for this test. OECD TG 492 for eye irritation test was originally adopted in 2014 and updated in 2016 and 2019 to include additional test methods using the RhCE models.

EpiOcular™-EIT and SkinEthic™ HCE EIT test models are considered validated reference models. Currently, there are additional models also accepted by OECD 492 (Table 4). Similar to skin irritation and corrosion models, the MTT assay is the endpoint measurement for eye irritation tests by RhCE models. Prediction of results shall be done based on %cell viability observed in the test chemical exposed. No UN GHS classification of chemicals are done for chemicals tested as per OECD TG 492 (Table 5).

Test model name	Surface area (cm^2)	Exposure amount (solids/ liquids)	Exposure Duration (liquids/ solids)	Negative and Positive Control	Endpoint
EpiOcular™-EIT	0.6	50 mg/50 µL	30 min / 6 hours	Water and Methyl acetate	MTT Assay
SkinEthic™ HCE EIT	0.5	30 mg/ 30 µL	30 min / 4 hours	DPBS and Methyl acetate	
LabCyte CORNEA-MODEL24 EIT	0.3	10 mg/ 50 µL	1 min / 24 hours	DPBS and Ethanol/ Lauric acid	
MCTT HCE™ EIT	0.6	40 mg/ 40 µL	10 min / 24 hours	DPBS and Methyl acetate or 2% SDS	

DPBS- Dulbecco's Phosphate Buffer Saline; SDS-Sodium dodecyl sulfate

Table 4: Models for Eye Irritation or Serious Eye Damage (OECD TG 492).

Copyright© Umesh MC et al.

Test Method	No Category	No prediction can be made
EpiOcular™ EIT (for both solids/liquids)	Mean tissue viability> 60%	Mean tissue viability ≤ 60%
SkinEthic™HCE EIT (for the liquids' protocol) SkinEthic™HCE EIT (for the solids' protocol)	Mean tissue viability> 60% Mean tissue viability> 50%	Mean tissue viability ≤ 50% Mean tissue viability ≤ 40%
MCTT HCE™ EIT (for the liquids' protocol) MCTT HCE™EIT (for the solids' protocol)	Mean tissue viability > 35% Mean tissue viability > 60%	Mean tissue viability ≤ 35% Mean tissue viability ≤ 60%

Table 5: Eye Irritation Tests Prediction Model (OECD TG 492).

However, if the UN GHS classification of test chemicals is required then the test can be performed as per OECD TG 492B. SkinEthic HCE TTL (for liquids) and SkinEthic HCE TTS (for solids) test models are considered validated reference models (Table 6). Incubation of test chemicals are performed with two different time points and an MTT assay is performed as endpoint measurement for this test. Results are predicted based on the %cell viability observed in the test chemical exposed. UN GHS classification of test chemicals is done for chemicals tested as per OECD TG 492B (Table 7).

Test model name	Surface area (cm^2)	Exposure amount	Exposure Duration	Negative and Positive Control	Endpoint
SkinEthic HCE TTL (for liquids protocol)	0.5	80 µL	5 min, 16 min and 120 min	DPBS and Methyl acetate	MTT Assay
SkinEthic HCE TTS (for solids protocol)	0.5	80 mg	30 min and 120 min	DPBS and Lactic acid	

Table 6: Models for Eye Hazard Identification (OECD TG 492B).

Test Method	No Category	Category 2	Category 1
SkinEthic HCE TTL (for liquids protocol)	Mean tissue viability > 50% within all-time treatments	Any other combination of values other than those defined for No Cat or Cat 1.	Mean tissue viability ≤ 50% within all-time treatments
SkinEthic HCE TTS (for solids protocol)	Mean tissue viability > 40% after 30 minutes and > 60% after 120 minutes		Mean tissue viability ≤ 40% after 30 minutes and ≤ 60% after 120 minutes

Table 7: Eye Hazard Identification Prediction Model (OECD TG 492B).

Yamaguchi and Takezawa developed an *In vitro* test method for eye irritancy tests where for the chemicals where no UN GHS classification is required. This model was developed and validated in association with the Japanese Centre for the Validation of Alternative Methods (JaCVAM). OECD accepted this validated model and introduced OECD TG 494 as the Vitrigel®-Eye Irritancy Test (EIT) method. In this method, the human corneal epithelium (hCE) is used as a test system. The test chemicals (Solids/ liquids) are applied on the surface of the epithelium and the transepithelial electrical resistance (TEER) values are measured to determine the irritation potential of the applied chemicals. No UN GHS classification of chemicals is done for chemicals tested as per OECD TG 494 (Table 8).

Criteria	Prediction
Time lag ≤ 180 seconds or Intensity ≥0.05 %/seconds or Plateau level >5.0 %	No Stand-alone Prediction Can Be Made
Time lag > 180 seconds and Intensity <0.05 %/seconds and Plateau level ≤ 5.0 %	No Category

Table 8: Prediction model of the Vitrigel®-Eye Irritancy Test (EIT) (OECD TG 494).

For identification of UN GHS Category 1 type of eye irritants, OECD introduced TG 491 and 460. OECD 491 describes the short-time exposure (STE) *In vitro* test method. This method is an *In vitro* cytotoxicity-based assay, which utilizes Statens Serum Institute Rabbit Cornea (SIRC) cells as a test system. Test chemicals are exposed at 5 and 0.05% concentrations. Following 5 min exposure of test chemical, cell viability is measured by MTT assay and prediction of results are done as per Table 9. Similarly, OECD 460 describes another cell-based cytotoxicity assay, which utilizes MDCK

Copyright© Umesh MC et al.

CB997 tubular epithelial cells as a test system. MDCK cells should be cultured on semi-permeable inserts. Test chemicals are added at the apical side of the inserts at 250 mg/mL to 1 mg/mL concentrations. Following 1 min exposure to test chemical, cell viability is measured by Fluorescein leakage assay. If FL20 is less than or equal to 100 mg/mL, then the test chemical is considered as Category 1.

Cell Viability		UN GHS Classification
At 5%	At 0.05%	
> 70%	> 70%	No category
≤ 70%	> 70%	No stand-alone prediction can be made
≤ 70%	≤ 70%	Category 1

Table 9: Prediction model of the STE Test method (OECD TG 491).

OECD TG 467 has been introduced to describe the top-down and bottom-up approach with the utilization of above described *In vitro* eye irritation results of tested chemicals and their physicochemical properties data to predict eye hazard identification. Defined Approaches (DAs) DAL-1 and DAL-2 shall be used for Serious Eye Damage/Eye Irritation prediction of chemicals.

Phototoxicity Tests

Currently, there is no globally accepted *In vivo* model for phototoxicity test (Figure 3). OECD TG 432 [17] for *In vitro* 3T3 NRU phototoxicity test is a well-established In vitro method for assessing phototoxicity of chemicals, which was originally adopted in 2004. UV/VIS absorption spectrum of the test chemical must be determined before biological testing is considered since a test chemical with a molar extinction coefficient of less than 1000 L mol^{-1} cm^{-1} is unlikely to be Photoreactive. This test is predictive to identify acute phototoxicity effects in animals and humans *In vivo*. However, this test is not designed to predict photo genotoxicity, photoallergy, or photocarcinogenicity effects that may arise from the combined action of a test chemical and light. Hence, OECD TG 495 [18] has been introduced to identify chemicals generating superoxide anion (SA) and singlet oxygen (SO) by Reactive Oxygen Species (ROS) assay. To obtain human relevant data, OECD has introduced recently OECD TG 498 [19], an *In vitro* phototoxicity test using the RhE method. However, currently, EpiDerm™ (EPI-200) has been considered as validated reference model for testing.

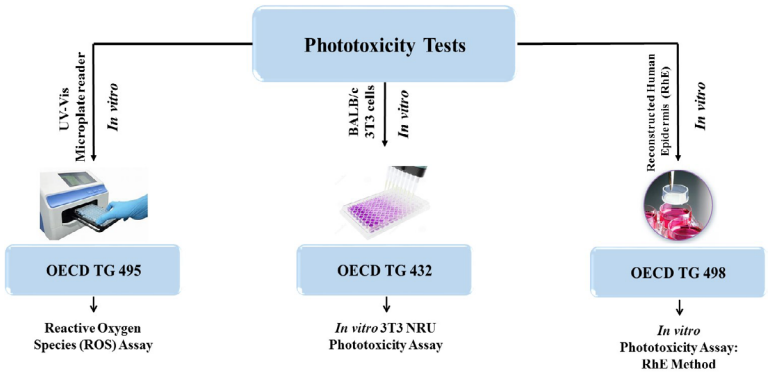


Figure 3: Phototoxicity Tests Models.

OECD TG 432 utilizes a permanent mouse fibroblast cell line, BALB/c 3T3, clone A31, as a test system. The cell line is available from the American Type Culture Collection (ATCC), Manassas, VA, USA. The cell line should be characterized by its population doubling time, absence of mycoplasma contamination and most importantly its sensitivity to UVA light. The recommended passage of less than 100 which is not sensitive to UVA light at 5 J/cm^2 (% cell viability more than 80%) is recommended for this test.

This assay is a cytotoxicity-based assay performed in duplicate 96-well plates that utilizes BALB/c 3T3 mouse fibroblasts to measure the dose-dependent reduction in neutral red uptake by the cells after exposure to a test chemical in the presence and absence of UVA light. Two 96-well plates cultured with a monolayer of 3T3 cells are exposed to 8 different dilutions of a test chemical using Earl's Balanced Salt Solution (EBSS). The first plate is exposed to 5 J/cm^2 of UVA light for 50 min (10 min = 1J/cm^2 of UVA light)

Copyright© Umesh MC et al.

using a solar simulator chamber while the second plate is kept in the dark condition for 60 min. Neutral red uptake (NRU) by cells exposed to the test chemical in the presence of UVA exposure is compared to the NRU by cells exposed to the test chemical in the absence of UVA exposure to evaluate Phototoxicity.

Photoirradiation factor (PIF) and Mean Photo Effect (MPE) is calculated by phototox version 2.0 software. The prediction model is considered for the interpretation of the results (Table 10).

***In vitro* 3T3 Phototoxicity Assay (OECD TG 432)**			
Prediction	**PIF**	**or**	**MPE**
No phototoxicity	PIF < 2	or	MPE < 0.1
Equivocal phototoxicity	PIF > 2 and <5	or	MPE > 0.1 and < 0.15
Phototoxicity	PIF > 5	or	MPE > 0.15
***In vitro* Phototoxicity Assay: Reconstructed Human Epidermis Phototoxicity test method (OECD TG 498)**			
Prediction	**Results**		
No phototoxic	No of treated inserts (+UVA) show decrease in mean tissue viability of ≥30% compared to (-UVA) inserts		
Phototoxic	One or more number of treated inserts (+UVA) show decrease in mean tissue viability of ≥30% compared to (-UVA) inserts		
Reactive Oxygen Species (ROS) Assay for Photoreactivity (OECD TG 495)			
Prediction	**Concentration**	**SO (Mean 3 wells)**	**SA (Mean 3 wells)**
Photoreactive	200 µM	≥25	≥70
		<25	≥70
		≥25	<70
Weakly photoreactive	200 µM	<25	≥20, <70
Weakly photoreactive	20 µM	≥25	≥20
Non-photoreactive	200 µM	<25	<20

Table 10: Phototoxicity Test Prediction Model.

In the OECD TG 495 [18] ROS assay, the SA and SO assay reactions are performed on 96 well plates. For SA detection, the test chemical (10 to 1 mM, N=3) is incubated with Imidazole (0.2 mM) and p-nitrosodimethylaniline (0.2 mM RNO) using sodium phosphate buffer. For SO detection, the test chemical (10 to 1 mM, N=3) isincubated with nitroblue tetrazolium chloride (0.2 mM NBT) using sodium phosphate buffer. After exposure to UV light for 1 h, the photoexcited molecules generate type I photochemical reactions i.e., superoxide anion (SA), and type II photochemical reactions i.e., singlet oxygen (SO) which shall be determined by spectrophotometric measurement (absorbance at 440 nm and 560 nm, pre and post-exposure of light). The measured mean values of SA and SO levels in test chemical test concentrations shall be used for interpretation of the results as per the prediction model.

In OECD TG 498 [19] phototoxicity assay, three to five test concentrations of test chemical is applied onto the surface of Human reconstructed epidermis (RhE) tissues (EpiDerm™, EPI-200) half of the treated RhE inserts are irradiated with 6 J/cm^2 of UVA light and remaining half of the treated RhE inserts are kept at room temperature. After a post-incubation period of 18-24 hours, an MTT assay is performed to determine the % cell viability and interpretation of the results shall be done as per the prediction model.

Skin Sensitization Tests

Several *In vivo* models are available for identification of skin sensitization of chemicals. Among these, OECD TG 406 [20] identified classical methods (*In vivo*) that use Guinea pigs, the GPMT of Magnusson and Kligman, and the Buehler Test. However, Murine tests (*In vivo*) such as LLNA as per OECD TG 429 [21] and other tests of LLNA: DA as per OECD TG 442A [22], LLNA: BrdU-ELISA and BrdU-FCM as per OECD TG 442B [23] to assess induction response have gained acceptance and have an advantage over Guinea pig tests.

Copyright© Umesh MC et al.

Recently, OECD introduced several test guidelines based on adverse outcome pathways (AOP) (Figure 4) involved such as OECD TG 442C [24], OECD TG 442D [25], and OECD TG 442E [26] based on recent innovations and acceptance of validated methods (*In vitro*).

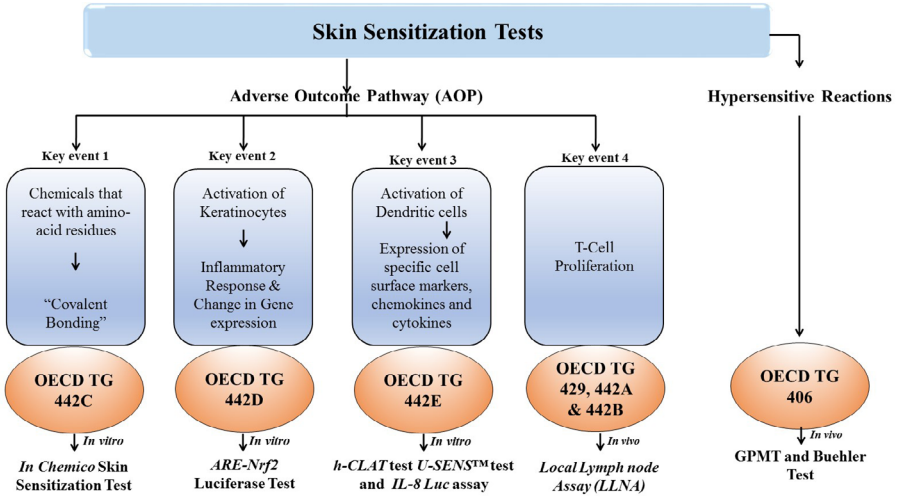


Figure 4: Skin Sensitization Tests Models.

In chemico Skin Sensitization

OECD TG 442C describes *in chemico* assays that address mechanisms described under the first key event (KE1) in the AOP for skin sensitization- "Covalent Binding to Proteins". *In chemico* sensitization of test chemicals can be assessed by following three test methods;

- Direct Peptide Reactivity Assay (DPRA)
- Amino acid Derivative Reactivity Assay (ADRA)
- The kinetic Direct Peptide Reactivity Assay (kDPRA)

The DPRA, ADRA, and kDPRA tests are *in chemico* assays, the first key event in the skin sensitization Adverse Outcome Pathway (AOP).

Test chemicals causing allergic contact dermatitis (ACD) are generally electrophilic. DPRA and ADRA assays identify dermal sensitizers based on their reactivity with synthetic peptides (Cysteine or Lysine peptides) or amino acids [N-(2-(1-naphthyl) acetyl)-L-cysteine (NAC) or α-N-(2-(1-naphthyl) acetyl)-L-lysine (NAL) amino acids] containing the nucleophilic amino acid residues. Conjugation of the test chemical with peptides/amino acids can be analyzed using HPLC. Whereas kDPRA assay is a modified DPRA method, which utilizes only Cysteine peptide and the identification of peptide depletion can be made by using a Fluorescence microplate reader. A brief description and prediction model of DPRA, ADRA, and kDPRA tests are presented in Table 11.

DPRA: Cysteine/ Lysine prediction model		
Mean of cysteine and lysine % depletion	**Reactivity Class**	**DPRA Prediction**
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	
DPRA: Only Cysteine prediction model		
Mean of cysteine % depletion	**Reactivity Class**	**DPRA Prediction**
0% ≤ mean % depletion ≤ 13.89%	No or minimal reactivity	Negative

Copyright© Umesh MC et al.

13.89% < mean % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < mean % depletion ≤ 98.24%	Moderate reactivity	
98.24% < mean % depletion ≤ 100%	High reactivity	
ADRA : NAC/ NAL prediction model		
Mean of NAC and NAL % depletion	ADRA Prediction	
Less than 4.9%	Negative	
4.9% or Higher	Positive	
ADRA : Only NAC prediction model		
Mean of NAC % depletion	ADRA Prediction	
Less than 5.6%	Negative	
5.6% or Higher	Positive	
kDPRA : Prediction model		
Reaction rate	kDPRA Prediction	
log kmax ≥ -2.0	UN GHS subcategory 1A	
Non-reactive or log kmax < -2.0	Non-subcategory 1A	

Table 11: *In chemico* Skin Sensitization Test- Prediction Model.

In vitro Skin Sensitization (Activation of keratinocytes)

OECD TG 442D describes *In vitro* skin sensitization by two test methods. These methods address the second key event (KE2) "activation of keratinocytes" of the skin sensitization AOP. This KE2 type of test can be analyzed by following tests;

- The ARE-Nrf2 Luciferase KeratinoSens™ Test Method
- The ARE-Nrf2 Luciferase LuSens Test Method

Both the test methods utilize the test system KeratinoSens™ / LuSens™ cell line, which was created from an immortalized human keratinocyte line called HaCaT. These cells were transfected with a plasmid containing the luciferase gene under the transcriptional control of an antioxidant response element (ARE) from a gene that is known to be up-regulated by contact sensitizers.

These assays are luminescence-based assays where luciferase enzymes convert marker substrates into light-emitting products that can be quantified by luminescence. The luciferase activity signal measured in the test reflects the activation by sensitizers of endogenous Nrf2-dependent genes in the presence of sensitizing test chemicals.

In vitro Skin Sensitization (Activation of Dendritic cells)

OECD 442E describes *In vitro* skin sensitization by four test methods. These methods address the third key event (KE3) "activation of dendritic cells" of the skin sensitization AOP. This KE3 type of skin sensitization can be analyzed by the following methods.

- Human Cell Line Activation test (h-CLAT)
- U937 cell line activation Test (U-SENS™)
- Interleukin-8 Reporter Gene Assay (IL-8 Luc assay)
- Genomic Allergen Rapid Detection (GARD™) for assessment of skin sensitizers (GARD™skin)

h-CLAT assay is a cell-based assay, which utilizes human monocytic leukemic cell lines (THP-1 cells) as a test system. Following 24 hours exposure to the test chemical, determination of skin sensitizers using flow cytometry by examining changes in the expression of cell surface markers CD54 and CD86 implicated in dendritic cell activation. For the prediction of a positive response, relative fluorescence intensity (RFI) and EC150 are considered.

U-SENS™ assay is a cell-based assay, which utilizes a human histiocytic lymphoma cell line (U937 cells) as a test system. Following 48 hours exposure of to test chemical, determination of skin sensitizers using flow cytometry by examining changes in the expression of cell surface marker CD86 implicated in dendritic cell activation. For the prediction of a positive response, Cell viability and EC150 are considered.

IL-8 assay is a cell-based assay, which utilizes THP-G8 cells as a test system. Following exposure to test chemical, determination of luciferase gene induction in IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in THP-G8 using a luminometer.

Whereas, in the GARD™ assay expression levels of the GARDskin GPS in the SenzaCell cell line is measured following

Copyright© Umesh MC et al.

exposure to test chemicals using NanoString nCounter mRNA analysis. For the prediction of positive results, cell viability, and mRNA fold induction is considered. An overview of these assays is described in Table 12.

h-CLAT Assay	U-SENS™ Assay	IL-8 Luc assay	GARD™ Assay
Principle: Quantification of changes of CD86 and CD54 cell surface marker expression on a human monocytic leukemia cell line, THP-1 cells, following 24 hours of exposure to the test chemical.	Principle: Quantification of changes of CD86 cell surface marker expression on a human histiocytic lymphoma cell line, U937 cells, following 45 ± 3 hours exposure to the test chemical.	Principle: Quantitative measurement of luciferase gene induction in IL-8 and GAPDH in THP-G8 cells following exposure to test chemicals.	Principle: Quantitative measurement of gene expression levels of the GARDskin GPS in SenzaCell cell line following exposure to test chemicals.
Test system: THP-1 cells	Test system: U937 cells	Test system: THP-G8 cells	Test system: SenzaCell
Positive control:	Positive control:	Positive control:	Positive control:
2,4-dinitrochlorobenzene	2,4,6-Trinitro-benzene-sulfonic acid	4-nitrobenzyl bromide	p-Phenylenediamine
Data Analysis: Flow cytometry analysis	Data Analysis: Flow cytometry analysis	Data Analysis: Luminometer	Data Analysis: NanoString nCounter mRNA analysis
Data output: Relative fluorescence intensity (RFI) and EC150	Data output: Cell Viability and EC150	Data output: Measured values of IL8LA, GAPLA, nIL8LA, Ind-IL8LA and Inh-GAPLA	Data output: Cell viability, mRNA fold induction.

Table 12: Overview of *In vitro* Skin Sensitization Tests.

Conclusions

The development and regulatory acceptance of non-animal methods have significantly advanced toxicity testing and contributed to the implementation of the 3Rs principles. Validated *In vitro* and *In chemico* methods can provide complete or partial replacement of specific *In vivo* tests, depending on the toxicological endpoint, applicability domain, test guideline and regulatory requirements. Integration of multiple new approach methodologies, including *In vitro*, *In chemico* and *In silico* approaches through defined approaches and integrated testing strategies, is expected to further improve the human relevance and predictive performance of non-animal testing. Nevertheless, the limitations and applicability domains of individual methods should be carefully considered when selecting and interpreting test results.

References

1. Anadón A, Martínez MA, Castellano V, Martínez Larrañaga MR (2014) The role of *In vitro* methods as alternatives to animals in toxicity testing. Expert Opin Drug Metab Toxicol 10(1): 67-79.

2. Bhanushali M, Bagale V, Shirode A, Joshi Y, Kadam V (2010) An *in-vitro* toxicity testing - a reliable alternative to toxicity testing by reduction, replacement and refinement of animals. Int J Adv Pharm Sci 1(1): 15-31.

3. May JE, Xu J, Morse HR, Avent ND, Donaldson C (2009) Toxicity testing: the search for an *In vitro* alternative to animal testing. Br J Biomed Sci 66(3): 160-165.

4. Goldberg AM, Frazier JM (1989) Alternatives to animals in toxicity testing. Sci Am 261(2): 24-30.

5. OECD Test No. 439: *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

6. OECD Test No. 431: *In vitro* skin corrosion: reconstructed human epidermis (RHE) test method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

7. OECD Test No. 430: *In vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test Method (TER). OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

Copyright© Umesh MC et al.

8. OECD Test No. 435: *In vitro* Membrane Barrier Test Method for Skin Corrosion. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

9. OECD Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

10. OECD Test No. 438: Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

11. OECD Test No. 460: Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

12. OECD Test No. 467: Defined Approaches for Serious Eye Damage and Eye Irritation. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

13. OECD Test No. 491: Short Time Exposure *In vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

14. OECD Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labeling for eye irritation or serious eye damage. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

15. OECD Test No. 492B: Reconstructed Human Cornea-like Epithelium (RHCE) Test Method for Eye Hazard Identification. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

16. OECD Test No. 494: Vitrigel-Eye Irritancy Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

17. OECD Test No. 432: *In vitro* 3T3 NRU Phototoxicity Test. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

18. OECD Test No. 495: ROS (Reactive Oxygen Species) Assay for Photoreactivity. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

19. OECD Test No. 498: *In vitro* Phototoxicity - Reconstructed Human Epidermis Phototoxicity test method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

20. OECD Test No. 406: Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

21. OECD Test No. 429: Skin Sensitisation: Local Lymph Node Assay. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

22. OECD Test No. 442A: Skin Sensitization: Local Lymph Node Assay: DA. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

23. OECD Test No. 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA or –FCM. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

24. OECD Test No. 442C: *In chemico* Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

25. OECD Test No. 442D: *In vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

26. OECD Test No. 442E: *In vitro* Skin Sensitisation: *In vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, France.

Copyright© Umesh MC et al.