National Institute of Environmental Health Sciences
*Division of the National Toxicology Program*

# Skin Sensitization Testing of Mixtures Without Animals


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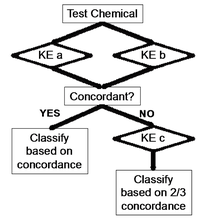
## Introduction

- The assessment of skin sensitization potential is included in international regulatory safety evaluations of pesticides.
- No single internationally accepted non-animal test is recommended as a complete replacement for existing animal tests.
- Defined approaches (DAs) based on the adverse outcome pathway (AOP) for skin sensitization (see diagram below) that integrate data from multiple non-animal test methods have been accepted to replace animal use for skin sensitization testing (OECD 2021).
- However, these DAs (see diagram to the right) have not been evaluated for mixtures or formulations (i.e., end-use products, multi-constituent substances with defined compositions).
- To fill this data gap, we tested 27 agrochemical formulations using three non-animal methods accepted as test guidelines by the Organisation for Economic Co-operation and Development (OECD) to support evaluating them for skin sensitization hazard and potency classification.
  - Direct peptide reactivity assay (DPRA; OECD 2020), KeratinoSens™ (OECD 2018a), and human cell line activation test (h-CLAT; OECD 2018b).
- Using United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS) hazard classifications based on historical in vivo local lymph node assay and guinea pig assay data, formulations included:
  - 12 sensitizers, including 1 GHS category 1A and 11 GHS category 1B.
  - 15 non-sensitizers.

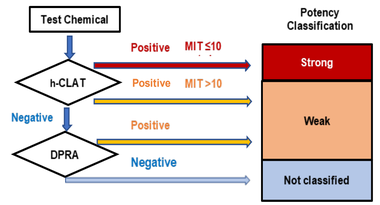
## Defined Approaches for Skin Sensitization

### 2 out of 3




- Uses two of three concordant outcomes from the first three key events (KE) of the AOP in any order (here, labeled a, b, or c) (Bauch et al. 2012) to provide a hazard classification.
- Uses the DPRA, KeratinoSens, and the h-CLAT.
- Borderline results were not used.
- The 2 out of 3 DA does not categorize substances for GHS potency.

### KE 3/1 STS




- Uses h-CLAT (KE3) and DPRA (KE1) for hazard and potency prediction (Nukada et al. 2013).
- A chemical with a positive result in h-CLAT is classified as a strong (GHS 1A) or weak (GHS 1B) sensitizer based on the minimum induction threshold, the lowest concentration that produces a positive result for either the CD54 or CD86 marker.
- Negative h-CLAT results require testing in DPRA.

### ITSv2

| Score | h-CLAT MIT (µg/ml) | DPRA Mean Cysteine and Lysine Depletion (%) | DPRA Cysteine Depletion (%) | QSAR Toolbox Hazard Prediction |
|---|---|---|---|---|
| 3 | ≤10 | ≥42.47 | ≥98.24 | - |
| 2 | >10, ≤150 | ≥22.62, <42.47 | ≥23.09, <98.24 | - |
| 1 | >150, ≤5000 | ≥6.38, <22.62 | ≥13.89, <23.09 | Positive |
| 0 | Negative | <6.38 | <13.89 | Negative |

- Applies scores to h-CLAT (KE3), DPRA (KE1) (mean depletion is preferred when available), and a hazard prediction from QSAR Toolbox (modified from Takenouchi et al. 2015).
- Scores are summed: a total score of 0-1 predicts a non-sensitizer result, 2-5 predicts GHS 1B sensitizer, and 6-7 predicts GHS 1A sensitizer.

## Conclusions

- Non-animal test methods have potential utility for evaluating the skin sensitization potential of agrochemical formulations.
  - Of the individual test methods evaluated for this project, KeratinoSens had the highest performance for predicting in vivo hazard outcomes and had higher balanced accuracy than any of the DAs (**Table 1**).
  - The DAs had overall concordance rates of 43-52% (**Table 2**) for GHS potency classification.
  - Based on the current set of limited data, KeratinoSens and DPRA in the 2 out of 3 approach had the highest concordance with in vivo data for skin sensitization hazard.
- **Future directions for predicting in vivo sensitization hazard of pesticide formulations:**
  - **Can DAs outperform individual assays such as KeratinoSens?**

## References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Nukada et al. 2013. Toxicol In Vitro. 27: 609–618.
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OECD. 2018a. Test Guideline 442D.
OECD. 2018b. Test Guideline 442E.
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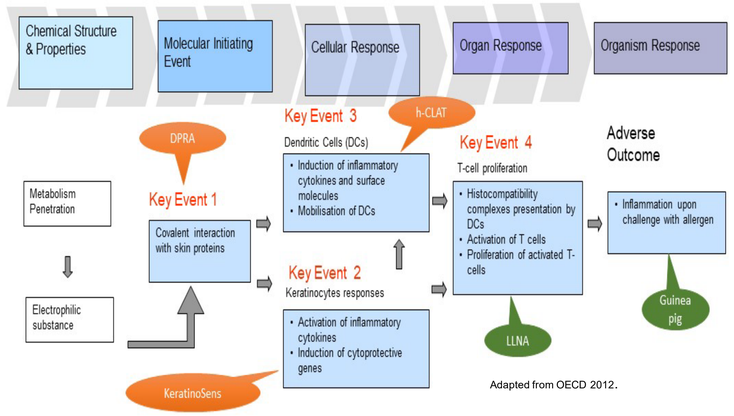
Takenouchi et al. 2015. J Appl Toxicol. 35: 1318–1332.


## Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins




Adapted from OECD 2012.

- In vitro (orange) and in vivo (green) test methods map to various key events in the skin sensitization AOP.

## Table 1. Performance of Non-animal Methods and Defined Approaches for Skin Sensitization Hazard

| Performance Statistic | DPRA (n=25) | Keratino Sens (n=27) | h-CLAT (n=27) | 2 out of 3 (n=19) | STS (n=27) | ITSv2 (n=24) |
|---|---|---|---|---|---|---|
| Accuracy (%) | 64 (16/25) | 81 (22/27) | 52 (14/27) | 79 (15/19) | 52 (14/27) | 54 (13/24) |
| Sensitivity (%) | 45 (5/11) | 75 (9/12) | 92 (11/12) | 90 (9/10) | 92 (11/12) | 91 (10/11) |
| Specificity (%) | 79 (11/14) | 87 (13/15) | 20 (3/15) | 67 (6/9) | 20 (3/15) | 23 (3/13) |
| Balanced Accuracy (%) | 62 | 81 | 56 | 78 | 56 | 57 |

- Balanced accuracy for the DAs for predicting skin sensitization hazard in vivo ranged from 56% to 78%.
- Of the individual in chemico and in vitro test methods, KeratinoSens had the highest performance for predicting in vivo hazard outcomes (balanced accuracy = 81% vs. 62% for DPRA and 56% for h-CLAT) and had higher balanced accuracy than any of the DAs.

## Table 2. Performance of Defined Approaches for GHS Potency Categorization

| Performance Statistic | STS (n=27) | | | ITSv2 (n=23) | | |
|---|---|---|---|---|---|---|
| | Not Classified (n=15) | 1B (n=11) | 1A (n=1) | Not Classified (n=13) | 1B (n=9) | 1A (n=1) |
| Concordance (%) | 20 (3/15) | 91 (10/11) | 100 (1/1) | 23 (3/13) | 67 (6/9) | 100 (1/1) |
| Underpredicted (%) | NA | 9 (1/11) | 0 (0/1) | NA | 11 (1/9) | 0 (0/1) |
| Overpredicted (%) | 80 (12/15) | 0 (0/11) | NA | 77 (10/13) | 22 (2/9) | NA |

- Overall concordance with in vivo data was 52% for the STS and 43% for the ITSv2. Thus, the STS had the better performance for GHS potency classification.
- The GHS 1A substance was not underpredicted by any DA; however, both DAs overpredicted a high proportion of the non-sensitizers.
- A recently accepted international guideline on DAs for skin sensitization (OECD 2021), which included the ITSv2 but not the STS, prescribes that only high-confidence predictions should be used. Here, results for four formulations were inconclusive and thus not included in the analysis.

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