May 16-17, 2018
IDEA Workshop on the replacement of animal testing in QRA for skin sensitization

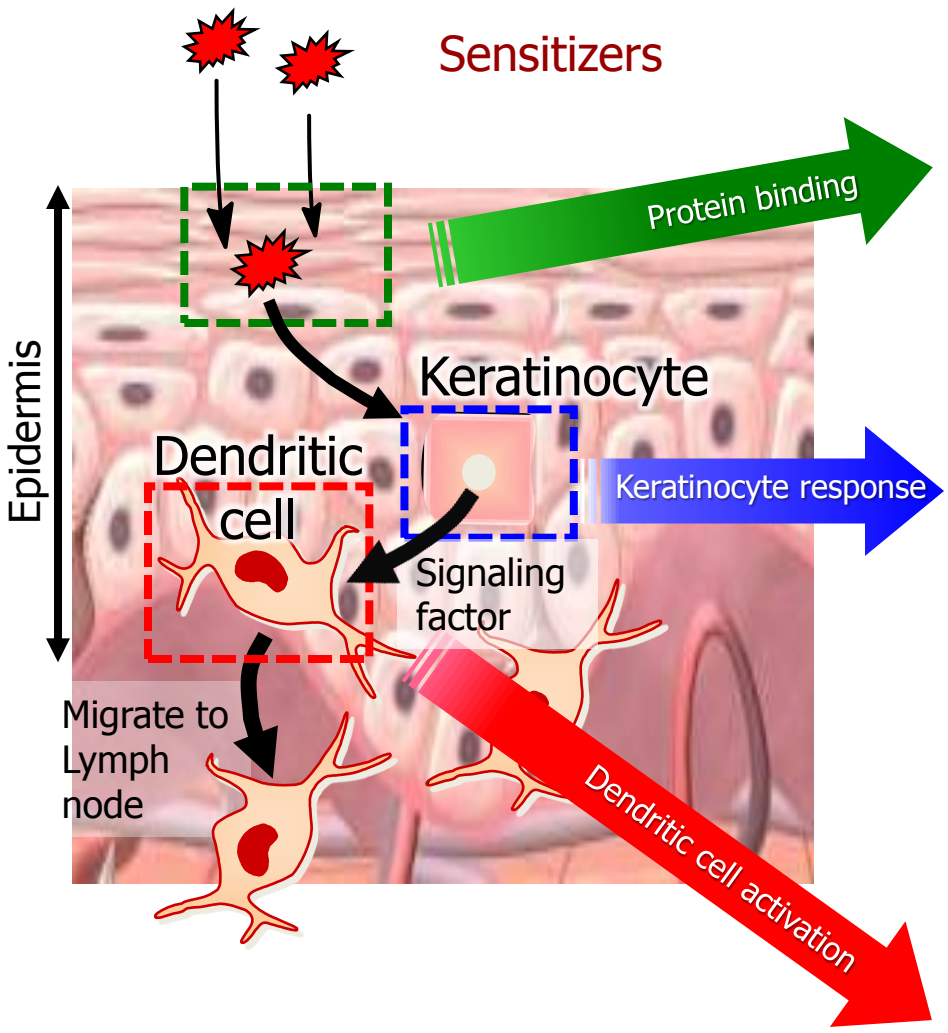
Kao's Case Study
(hydroxycitronellal, coumarin)

Masaaki Miyazawa

kao

Enriching lives, in harmony with nature.

Regulatory Accepted Non-animal Test Methods



Key Events in AOP of Skin Sensitization

Protein binding; KE1

DPRA (TG442C)

Keratinocyte response; KE2

KeratinoSens (TG442D)

Dendritic cell activation; KE3

h-CLAT (TG442E)

Regulatory Accepted Non-animal Test Methods

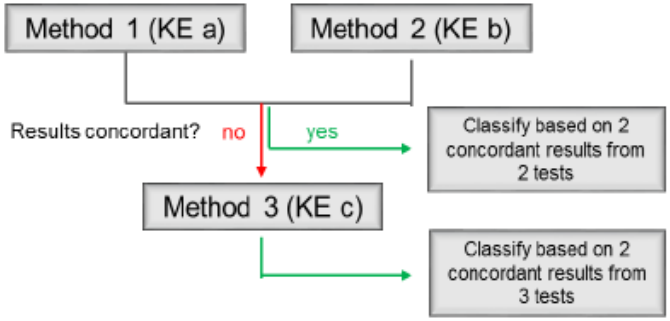
203 chemical dataset using the currently available dataset (Urbisch et al, 2015; Takenouchi et al., 2015; Jaworska et al, 2015)

LLNA	DPRA		KeratinoSens™		h-CLAT	
	Positive	Negative	Positive	Negative	Positive	Negative
151 sensitizers	108	43	112	39	124	27
52 non-sensitizers	15	37	21	31	18	34
Sensitivity (%)	71.5		74.2		82.1	
Specificity (%)	71.2		59.6		65.4	
Accuracy (%)	71.4		70.4		77.8	

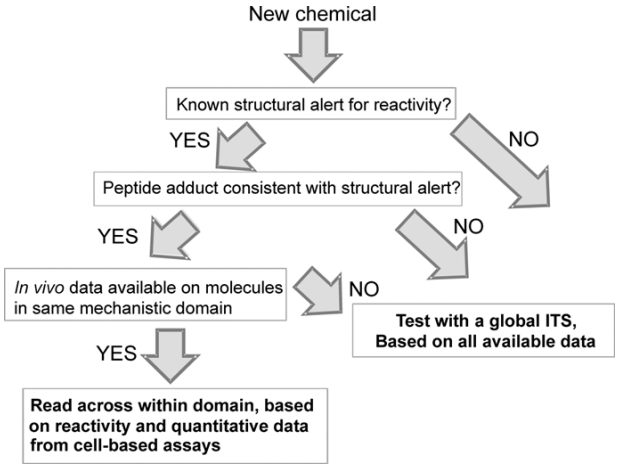
human	DPRA		KeratinoSens™		h-CLAT	
	Positive	Negative	Positive	Negative	Positive	Negative
72 sensitizers	61	11	59	13	64	8
25 non-sensitizers	5	20	7	18	8	17
Sensitivity (%)	84.7		82		88.9	
Specificity (%)	80.0		72.0		68.0	
Accuracy (%)	83.5		79.4		83.5	

One single non-animal test method is not sufficient to cover the AOP and to have 100% accuracy compared with the LLNA and human

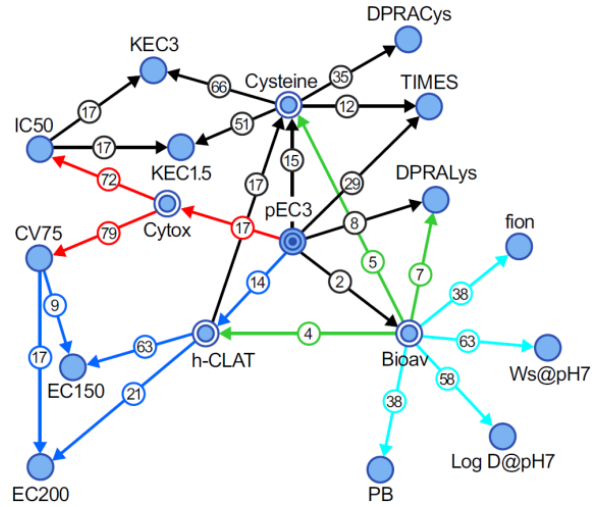
Defined Approaches in OECD IATA Guidance



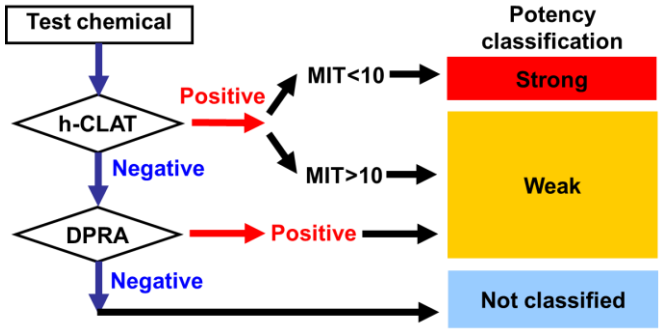
Urbisch et al. (2015) Regul Toxicol Pharmacol.:
2 out of 3 ITS



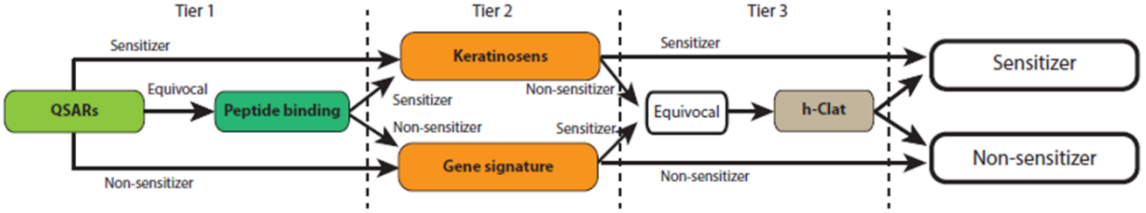
Natsch et al. (2015) Toxicol. Sci.:
Global/domain-based assessment



Jaworska et al. (2015) Arch. Toxicol.:
Bayesian Network



Takenouchi et al. (2015) J. Appl. Toxicol.:
Kao STS

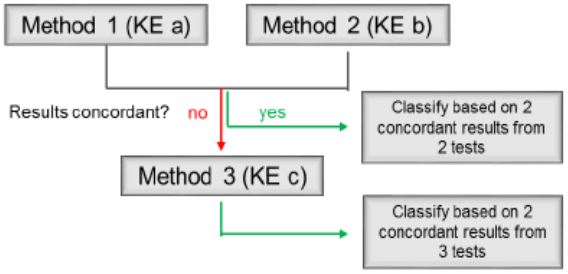


Van der Veen et al. (2014) Regul Toxicol Pharmacol.:
RIVM STS

Integrated testing strategies (ITS) that use multiple tests have been developed to evaluate the sensitizing potential and potency of chemicals

Workflow to Conclude a Non-sensitizer

Sequential Testing Strategies (STS)



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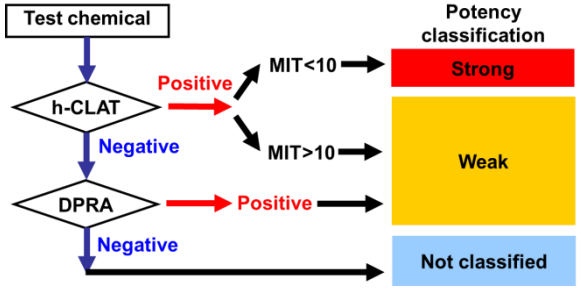

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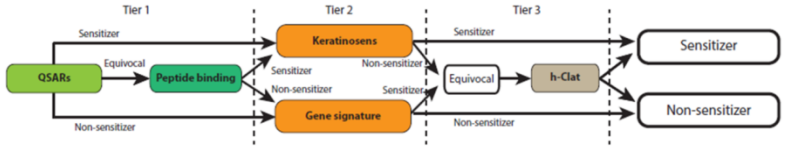

Van der Veen et al. (2014) Regul Toxicol Pharmacol.:
RIVM STS

- There is no differential weighting of the individual test methods used.
- These strategies are likely to yield low false negatives and high false positives and unlikely to be effective as a replacement strategy of LLNA.

Examine whether combination of individual test methods is optimal to conclude a non-sensitizer as a first tier of bottom-up approach

Potential Overlapping Information of Individual Test Methods

- Individual test methods qualitatively and conceptually address each KE of the AOP, rather than being their exact reproduction

- Provide overlapping information in covering KEs.
  - ✓ Both the DPRA and KeratinoSens cover the KE of protein reactivity, such as binding to thiol residues of cysteine (Natsch et al., 2010)
  - ✓ Binding to cysteine or lysine residues within proteins could drive MAPK signaling pathway modulation and subsequent up-regulating DC activation like h-CLAT (Megherbi et al., 2009; Guedes et al., 2016)

- Binary test battery of KS* (KE2) and h-CLAT (KE3) might provide sufficient information to address protein binding (KE1)

*KeratinoSens

Predictive capacity of binary test battery with KS and h-CLAT was examined with 203 chemical dataset when compared with 2 out of 3 ITS

Binary Test Battery of KS and h-CLAT

LLNA	Binary test battery of KS and h-CLAT		2 out of 3		3 out of 3	
	Positive	Negative	Positive	Negative	Positive	Negative
151 sensitizers	141	10	119	32	146	5
52 non-sensitizers	33	19	15	37	37	15
Sensitivity (%)	**93.4**		**78.8**		**96.7**	
Specificity (%)	36.5		71.2		28.8	
Accuracy (%)	78.8		76.8		79.3	

human	Binary test battery of KS and h-CLAT		2 out of 3		3 out of 3		LLNA	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
72 sensitizers	68	4	64	8	72	0	66	6
25 non-sensitizers	14	11	4	21	16	9	9	16
Sensitivity (%)	**94.4**		**88.9**		**100**		**91.7**	
Specificity (%)	44.0		84.0		36.0		64.0	
Accuracy (%)	81.4		87.6		83.5		84.5	

Binary test battery of KS and h-CLAT has higher sensitivity than 2 out of 3 ITS when compared with LLNA and human

False Negative Chemicals in Binary Test Battery

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- LLNA (Pos/Neg), Human (Pos/Neg/No data), DPRA (Pos)

Chemical name	LLNA EC3	Human	Discussion
Benzoyl peroxide	0.22	Positive	Acyl transfer agent, amine reactive chemical
Squaric acid	4.3	Positive	Amine reactive chemical
Phthalic anhydride	0.16	Negative	Acyl transfer agent, amine reactive chemical
1,2-cyclohexane dicarboxylic	0.84	No data	Acyl transfer agent, amine reactive chemical
Diethylenetriamine	5.8	Positive	Pro(pre)-hapten
Kanamycin	- (Negative)	Positive	Allergen in human after considerable exposure

Acylating agents or amine-reactive chemicals

Pre/pro-hapten

- LLNA (Pos), Human (No data), DPRA (Neg)

Chemical name	LLNA EC3	Human	Discussion
Clotrimazole	4.8	No data	LogKow=6.26
1-Cyclohexylethyl 2-butenoate	5.53	No data	LogKow=4.32
N,N-Dibutylaniline	19.6	No data	LogKow=5.12
1-Octen-3-yl acetate	30	No data	LogKow=3.6
Methyl pyruvate	2.4	No data	Undergo hydration in aqueous assay solution

Lipophilic chemicals (logKow >3.5)

Acylating agents, pre/pro-haptens, and lipophilic chemicals are considered predictive limitations of binary test battery

→ **TIMES and EPI suite** are useful to identify chemicals falling into the predictive limitations

False Negative Chemicals in Binary Test Battery

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- LLNA (Pos/Neg), Human (Pos/Neg/No data), DPRA (Pos)

Chemical name	LLNA EC3	Human	Discussion
Benzoyl peroxide	0.22	Positive	Acyl transfer agent, amine reactive chemical
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Kanamycin	- (Negative)	Positive	Allergen in human after considerable exposure

Acylating agents or amine-reactive chemicals

Pre/pro-hapten

Additional testing with DPRA might be useful for acylating agents and pre/pro-haptens.

- LLNA (Pos), Human (No data), DPRA (Neg)

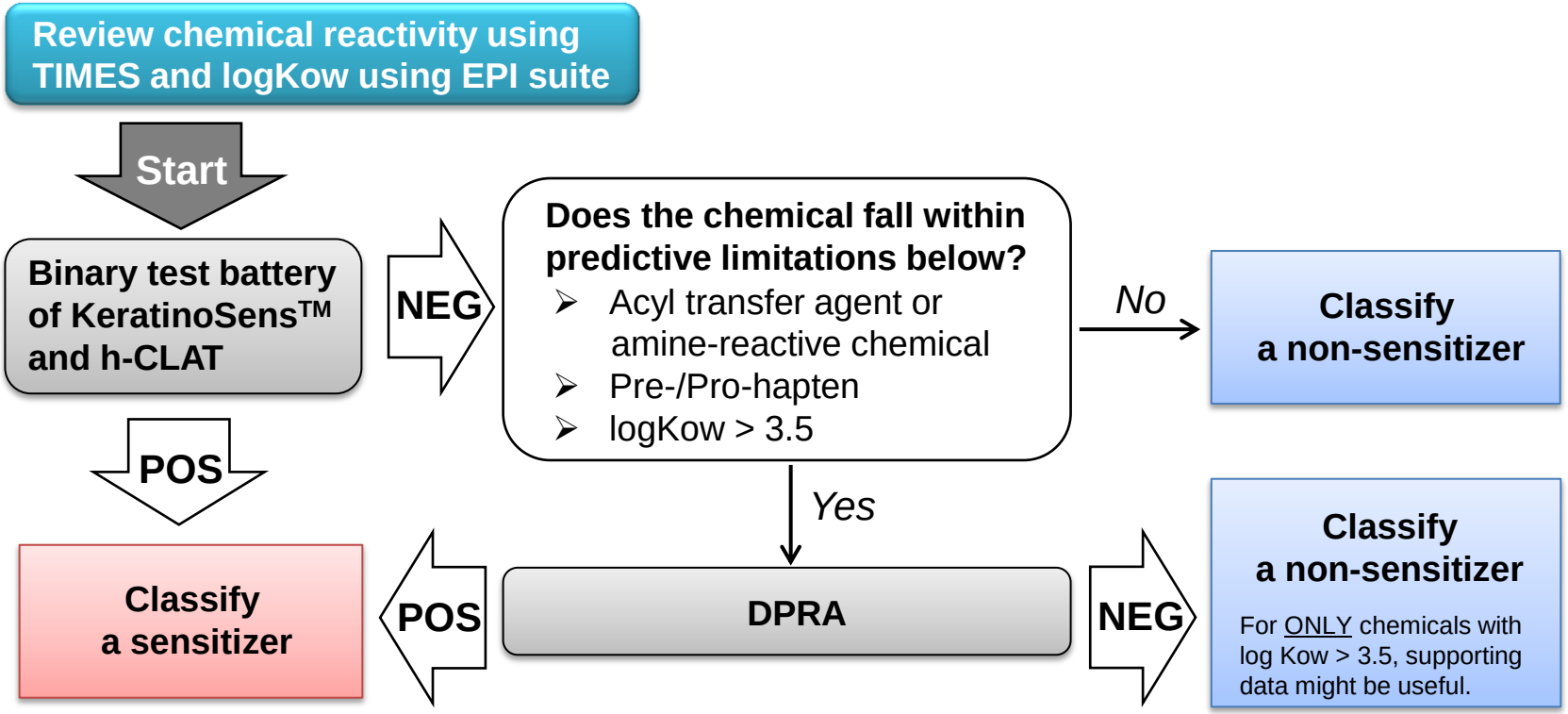
Chemical name	LLNA EC3	Human	Discussion
Clotrimazole	4.8	No data	LogKow=6.26
1-Cyclohexylethyl 2-butenoate	5.53	No d	
N,N-Dibutylaniline	19.6	No d	
1-Octen-3-yl acetate	30	No data	LogKow=3.6
Methyl pyruvate	2.4	No data	Undergo hydration in aqueous assay solution

Lipophilic chemicals (logKow >3.5)

Additional supporting info. might be needed. Nevertheless, DPRA has flexibility of available solvents and allows to add up to 20% acetonitrile compared with 1% DMSO for KS and 0.2% for h-CLAT.

DPRA is recommended only for chemicals falling into predictive limitations after testing KS and h-CLAT

Workflow to Conclude a Non-sensitizer



AOP-based "binary test battery" and "additional test with DPRA" are effective as a first tier to conclude a non-sensitizer

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Binary test battery with KeratinoSens™ and h-CLAT as part of a bottom-up approach for skin sensitization hazard prediction

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ABSTRACT

Skin sensitization is one of the key safety endpoints for chemicals applied directly to the skin. Several integrated testing strategies (ITS) using multiple non-animal test methods have been developed to accurately evaluate the sensitizing potential of chemicals, but there is no regulatory-accepted ITS to classify a chemical as a non-sensitizer. In this study, the predictive performance of a binary test battery with KeratinoSens™ and h-CLAT compared to the local lymph node assay (LLNA) and human data was examined using comprehensive dataset of 203 chemicals. When two negative results indicate a non-sensitizer, the binary test battery provided sensitivity of 93.4% or 94.4% compared with the LLNA or human data. Taking into account the predictive limitations (i.e. high log Kow, pre-/pro-haptens and acyl transfer agents (or amine-reactive)), the binary test battery had extremely high sensitivity comparable to that of the 3 out of 3 ITS where three negative results of the DPRA, KeratinoSens™ and h-CLAT indicate a non-sensitizer. Therefore, the data from KeratinoSens™ or h-CLAT may provide partly redundant information on the molecular initiating event derived from DPRA. Taken together, the binary test battery of KeratinoSens™ and h-CLAT could be used as part of a bottom-up approach for skin sensitization hazard prediction.

Workflow to Classify Potency of a Sensitizer

Review chemical reactivity using TIMES and logKow using EPI suite

Start

Binary test battery of KeratinoSens™ and h-CLAT

NEG

Does the chemical fall within predictive limitations below?

- Acyl transfer agent or amine-reactive chemical
- Pre-/Pro-hapten
- logKow > 3.5

No → **Classify a non-sensitizer**

Yes ↓

DPRA

NEG → **Classify a non-sensitizer**

For ONLY chemical with log Kow > 3.5, supporting data might be useful.

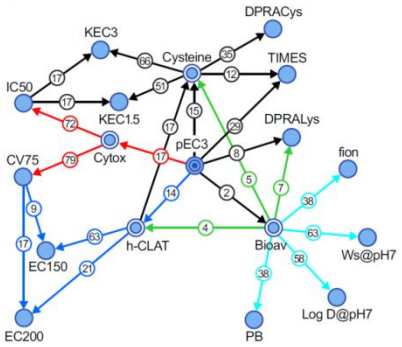
POS

Classify a sensitizer

POS

Potency classification

Bayesian Network ITS-3
(using full datasets)



- Developed by Procter & Gamble
- Potency prediction can be made as a probability distribution

Examine how BN ITS-3 can be used to classify sensitizing potency

Potency Prediction by BN ITS-3

(LLNA data set of 203 chemicals)

LLNA category	Number* (176)	BN ITS-3 Prediction			
		Strong	Moderate	Weak	NS
Extreme – Strong	30	22	**6**	**2**	0
Moderate	56	14	27	**13**	**2**
Weak	56	6	6	38	**6**
Negative	34	1	2	2	29
Accuracy(%)		**73.3**	**48.2**	**67.9**	**85.3**

Sensitivity ; 94% (134/142)

Specificity ; 85% (29/34)

: Under-predicted

: Concordant

*Excluded metals, salts, and chemicals negative in all three test methods

- 20% (29 / 142 chemicals) of under-prediction for potency classification.
- For 93% of tested chemicals, the prediction falls within one potency class mis-prediction.
- Hexyl salicylate and benzoyl peroxide fall into strong potency in LLNA, but weak potency in BN ITS-3.
- Diethylenetriamine and squaric acid fall into moderate potency in LLNA, but negative in BN ITS-3.

Under-predicted Chemicals

Chemical	LLNA	EC3 (%)	BN ITS-3	Discussion
Undec-10-enal	moderate	6.8	weak	
p-Isobutyl-α-methyl hydrocinnamaldehyde	moderate	9	weak	
Hexyl salicylate	Strong	0.18	weak	**Lipophilic chemicals (LogKow>3.5)**
Farnesol	moderate	4.1	weak	
2-Nitro-4-phenylenediamine	strong	0.5	moderate	
Dihydroeugenol	moderate	6.8	weak	
Dibenzyl ether	moderate	6.3	weak	
4-chloroaniline	moderate	6.5	weak	
Diethylenetriamine	moderate	5.8	NS	**Pre/Pro-hatptens**
Phthalic anhydride	strong	0.16	moderate	
Maleic anhydride	strong	0.16	moderate	
1,2-cyclohexane dicarboxylic anhydride	strong	0.84	moderate	
Benzoyl peroxide	Strong	0.22	weak	**Acylating agents or amine reactive chemicals**
Squaric acid diethyl ester	strong	0.9	moderate	
Squaric acid	moderate	4.3	NS	
Formaldehyde	strong	0.61	moderate	
1-Phenyl-1_2-propanedione	moderate	1.3	weak	
Allyl phenoxyacetate	moderate	3.1	weak	
6-Methyl-3 5-heptadien-2-one	moderate	5	weak	
trans-2-Hexenal	moderate	5.5	weak	
Perillaaldehyde	moderate	8.1	weak	
Methyl methanesulphonate	moderate	8.1	weak	
3-Methyl-1-phenylpyrazolone	moderate	8.5	weak	
Oxalic acid anhydrous	weak	15	NS	
Benzocaine	weak	22	NS	
Pyridine	weak	72	NS	
Diethyl acetaldehyde	weak	76	NS	
Aniline	weak	89	NS	
Methylmethacrylate	weak	90	NS	

Under-predicted Chemicals

Chemical	LLNA	EC3 (%)	BN ITS-3	Discussion
Undec-10-enal	moderate	6.8	weak	
p-Isobutyl-α-methyl				
Hexyl salicylate				
Farnesol				
2-Nitro-4-phenylenec				
Dihydroeugenol				
Dibenzyl ether				
4-chloroaniline				
Diethylenetriami				
Phthalic anhydride				
Maleic anhydride				
1,2-cyclohexane dica				
Benzoyl peroxide				
Squaric acid diethyl ester	strong	0.9	moderate	
Squaric acid	moderate	4.3	NS	
Formaldehyde	strong	0.61	moderate	
1-Phenyl-1_2-propanedione	moderate	1.3	weak	
Allyl phenoxyacetate	moderate	3.1	weak	
6-Methyl-3 5-heptadien-2-one	moderate	5	weak	
trans-2-Hexenal	moderate	5.5	weak	
Perillaaldehyde	moderate	8.1	weak	
Methyl methanesulph				
3-Methyl-1-phenylpyr				
Oxalic acid anhydrous	weak	15	NS	
Benzocaine	weak	22	NS	
Pyridine	weak	72	NS	
Diethyl acetaldehyde	weak	76	NS	
Aniline	weak	89	NS	
Methylmethacrylate	weak	90	NS	

c chemicals
ow>3.5)

-hatptens

agents or
amine reactive chemicals

- Lipophilic chemicals (logKow>3.5)
- Pre/pro-haptens
- Acylating agents or amine reactive chemicals

Potential under-predicted chemicals in BN ITS-3

No specific reason for under-predictions

Modified Potency Classification by BN ITS-3

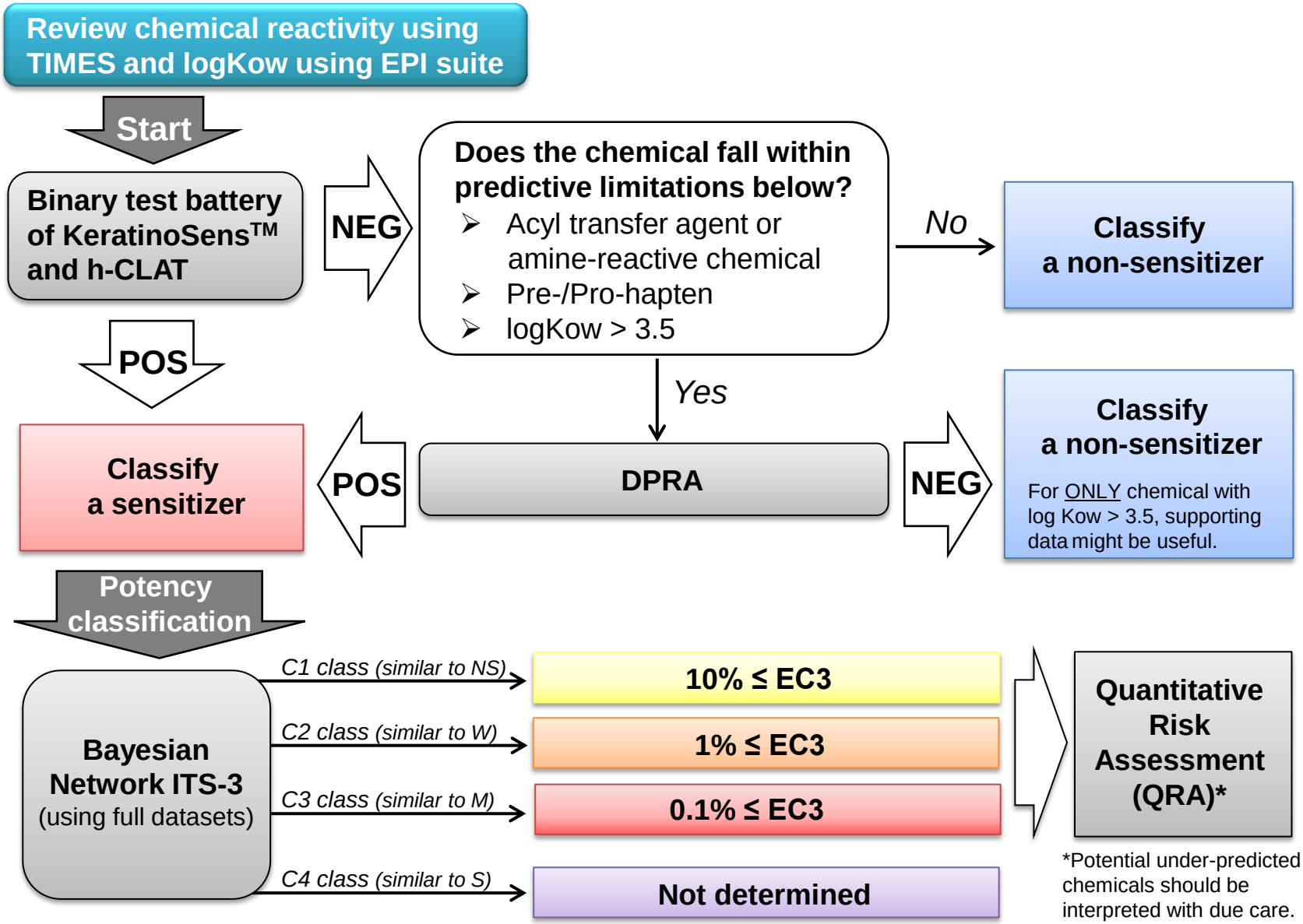
LLNA category	Number (94)	BN ITS-3 Prediction			
		Strong	Moderate	Weak	NS
Extreme – Strong	13	12	**1**	0	0
Moderate	30	7	16	**7**	0
Weak	25	2	3	14	**6**
Negative	26	0	0	1	25
Accuracy(%)		92.3	53.3	56.0	96.2

*Excluded metals, salts, chemicals negative in all three test methods, chemicals with logKow>3.5, pre/prohaptens, and acylating agents or amine reactive

By excluding potential under-predicted chemicals,
BN ITS-3 predictions fall within one potency class mis-prediction.

BN ITS-3 Decision		Modified potency classification
NS	⇒	**Minimal potency → 10% ≤ EC3**
Weak	⇒	**Low potency → 1% ≤ EC3**
Moderate	⇒	**Moderate potency → 0.1% ≤ EC3**
Strong	⇒	**High potency → Not defined**

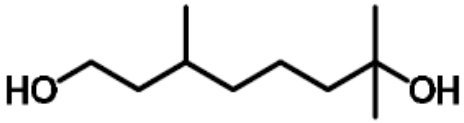
Workflow to Evaluate Sensitizing Potential and Potency



This workflow supports a practical skin sensitization assessment!

Hydroxycitronellal, Coumarin

➢ Hydroxycitronellal (CAS# 107-75-5)

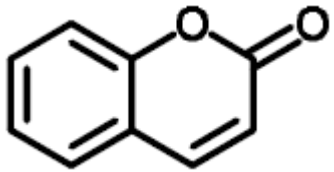


47th amendment of IFRA standard

LLNA weighted mean EC3 values ($\mu g/cm^2$) [no. studies]	Potency Classification Based on Animal Data[1]	Human Data			WoE NESIL[3] ($\mu g/cm^2$)
		NOEL – HRIPT (induction) ($\mu g/cm^2$)	NOEL – HMT (induction) ($\mu g/cm^2$)	LOEL[2] (induction) ($\mu g/cm^2$)	
5612 [9] **(EC3=33%)**	Weak	5000[4]	NA	5906	5000

NOEL = No observed effect level; HRIPT = Human Repeat Insult Patch Test; HMT = Human Maximization Test;
LOEL = lowest observed effect level; NA = Not Available

➢ Coumarin (CAS# 91-64-5)



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		NOEL – HRIPT (induction) ($\mu g/cm^2$)	NOEL – HMT (induction) ($\mu g/cm^2$)	LOEL[2] (induction) ($\mu g/cm^2$)	
>12 500 [2]	Weak	3 543[4]	5517[4]	8 858	3 500

Negative at 10, 25, 50% in LLNA
(Vocanson *et al*., 2006)

Well-known contact allergens listed in 26 fragrance substances

Hydroxycitronellal, Coumarin

Hydroxycitronellal

- ✓ log Kow = 2.11 → Not lipophilic
- ✓ TIMES ; Weak to Strong (Parent), NS (Metabolite), non-acylating agent
- ✓ KeratinoSens, h-CLAT ; both Positive **→ Judge as a sensitizer**
- ✓ DPRA ; Positive
- ✓ BN ITS-3 prediction ; Moderate potency

→ Kao's modified potency ; 0.1% ≤ EC3 → NESIL* =9.59 μg/cm^2

Coumarin

- ✓ log Kow = 1.51 → Not lipophilic
- ✓ TIMES ; Non-sensitizer for parent and metabolite, non-acylating agent
- ✓ KeratinoSens ; Positive, h-CLAT ; Negative **→ Judge as a sensitizer**
- ✓ DPRA ; Negative
- ✓ BN ITS-3 prediction ; Non-sensitizer

→ Kao's modified potency ; 10% ≤ EC3 → NESIL* =2003 μg/cm^2

*Safford *et al*., Regul Toxicol Pharmacol. 2008 Jul;51(2):195-200.

Skin Sensitization Quantitative Risk Assessment

Hydroxycitronellal (HC)	Shampoo	Face Cream
Predicted EC3	≥0.1%	≥0.1%
NESIL*	9.59 [µg/cm^2]	9.59 [µg/cm^2]
SAF of QRA2**	300	100
AEL	0.032 [µg/cm^2]	0.096 [µg/cm^2]
CEL of Product***	72.6 [µg/cm^2][1)]	5451 [µg/cm^2][2)]
Conc. limit. of HC in the product based on AEL/CEL (%)	**0.044% (440ppm)**	**0.00175% (17.5ppm)**
Conc. limit. of HC in the product based on current IFRA standard (47th Amendment)	**1% (Category 9)**	**1% (Category 5)**

Coumarin (CM)	Shampoo	Face Cream
Predicted EC3	≥10%	≥10%
NESIL*	2003 [µg/cm^2]	2003 [µg/cm^2]
SAF of QRA2**	300	100
AEL	6.67 [µg/cm^2]	20.0 [µg/cm^2]
CEL of Product***	72.6 [µg/cm^2][1)]	5451 [µg/cm^2][2)]
Conc. limit. of CM in the product based on AEL/CEL (%)	**9.19%**	**0.367%**
Conc. limit. of CM in the product based on current IFRA standard (47th Amendment)	**5% (Category 9)**	**0.8% (Category 5)**

*Safford *et al*., Regul Toxicol Pharmacol. 2008 Jul;51(2):195-200.
** IDEA Project, Final report on the QRA2 (2016)
***SCCS Notes of Guidance, 9th revision, 2016

1) 10.46g x 0.01 (RF) / 1440cm^2 = 72.6 µm/cm^2
2) 1.54g x 1 (RF) x 2 (Frequency of application)/ 565cm^2 = 5451 µm/cm^2

Summary

- Binary test battery of KeratinoSens and h-CLAT is first used to classify S/NS as a part of bottom-up approach. A positive result in either KeratinoSens™ or h-CLAT is a sensitizer.
- The majority of false neg. in the binary test battery were found to be acylating chemicals, pre/pro-haptens, and lipophilic chemicals (Log Kow >3.5). The additional test of DPRA is effective to minimize uncertainty for false negatives.
- It was proposed to initially use TIMES (commercially available) and EPI suite (freely available) to identify the above chemicals falling within predictive limitations.
- For potency prediction on risk assessment, the BN ITS-3 (P&G) is used as a second step.
- 20% (29 / 142 chemicals) of under-prediction for potency classification. For 93% of tested chemicals, the predictions of BN ITS-3 fall within one potency class mis-prediction, when compared with LLNA. The mis-prediction created uncertainty.
- The four modified potency classes were defined as worst case scenario, incl. minimal (10% ≤ EC3), low (1% ≤ EC3), moderate (0.1% ≤ EC3), and high potency (EC3 not defined). The lowest EC3 in each class is used to derive NESIL.

THANK YOU FOR YOUR ATTENTION!