NON-ANIMAL SKIN SENSITIZATION TESTING UNDER REACH

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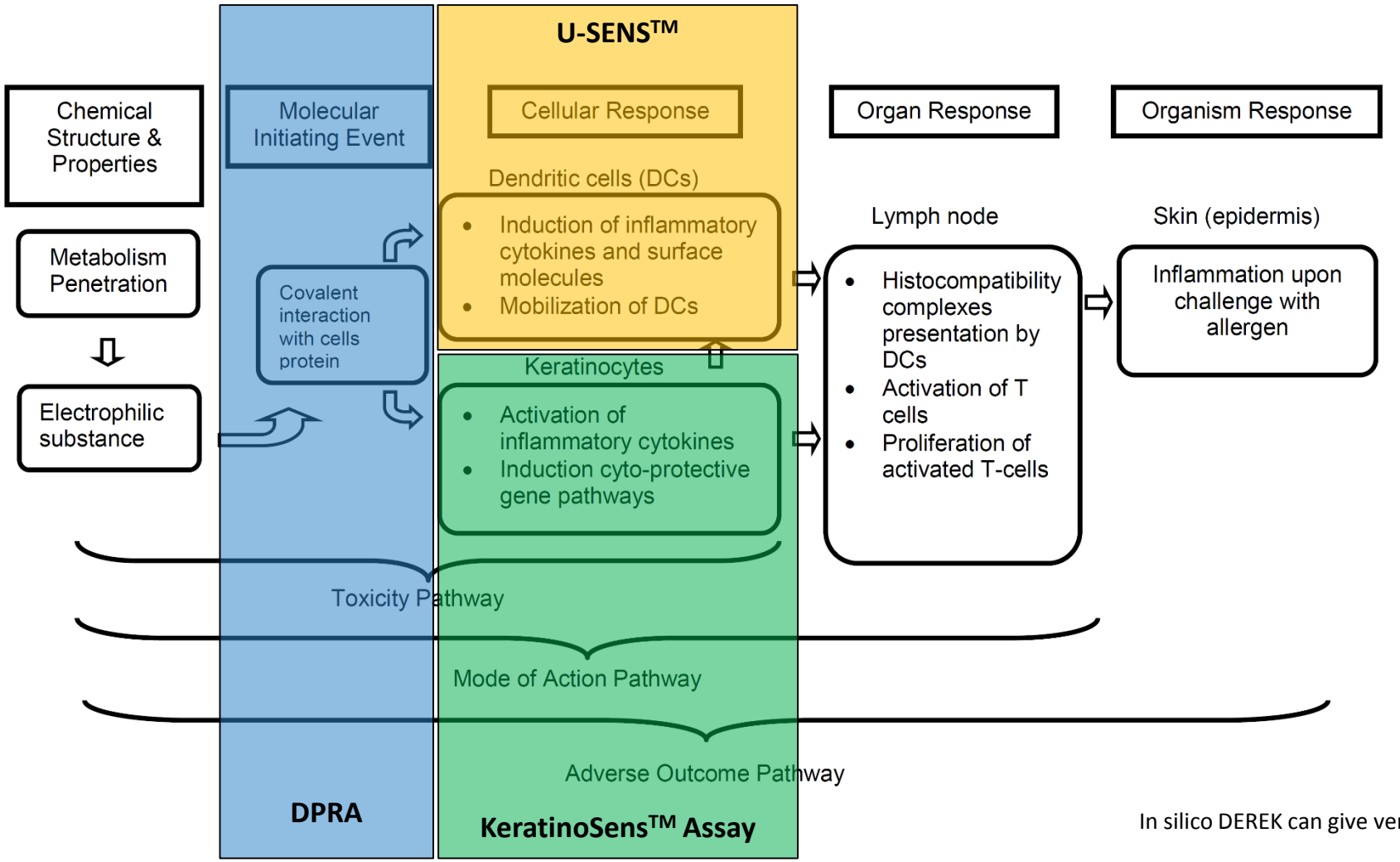
Introduction

Direct Peptide Reactivity Assay, KeratinoSens™ and U-SENS™

INTRODUCTION

Adverse Outcome Pathway and DPRA, KeratinoSens™ and U-SENS™



In silico DEREK can give very useful additional info, e.g. metabolites involved

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Direct Peptide Reactivity assay (OECD 442C)

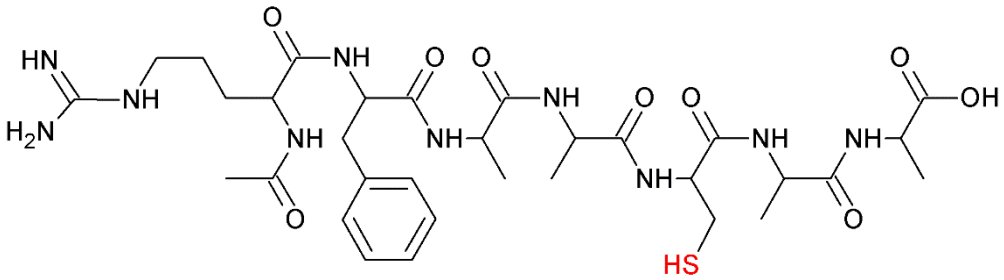
Key event 1: Protein Binding

DIRECT PEPTIDE REACTIVITY ASSAY

Principle and method in chemico DPRA assay

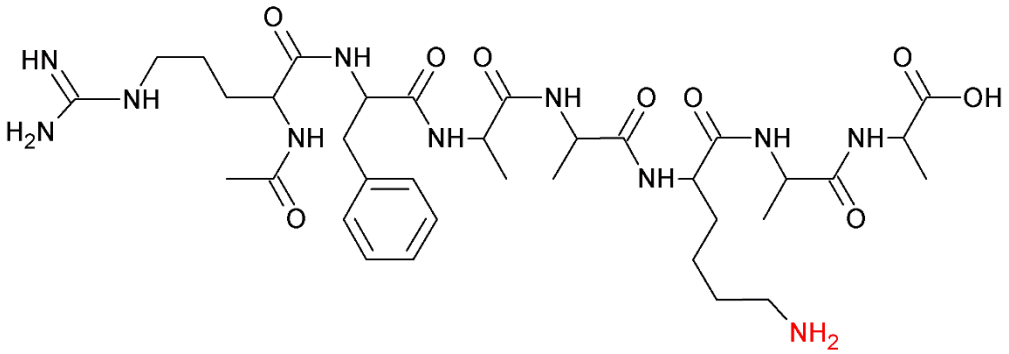
- The in chemico DPRA assay addresses the molecular initiating event of the skin sensitization AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards two model synthetic peptides containing either lysine or cysteine

SPCC



Cysteine residue

SPCL



Lysine residue

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DIRECT PEPTIDE REACTIVITY ASSAY

Assay setup

SPCC

- Test Item + SPCC Peptide:
  - Test Item at 100 mM

    Solvents: water, acetonitrile, 1:1 mixture water:acetonitrile, Isopropanole, acetone

- Reference line
- Reference controls
- Co-elution controls
- Positive controls

- Incubation for 24 h at 25 ° C

SPCL

- Test Item + SPLC Peptide:
  - Test Item at 100 mM

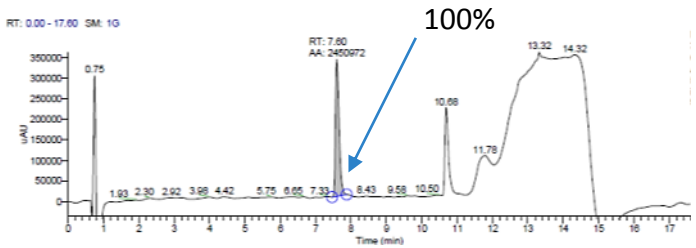
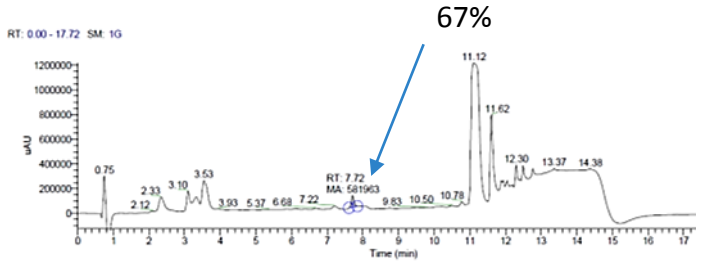
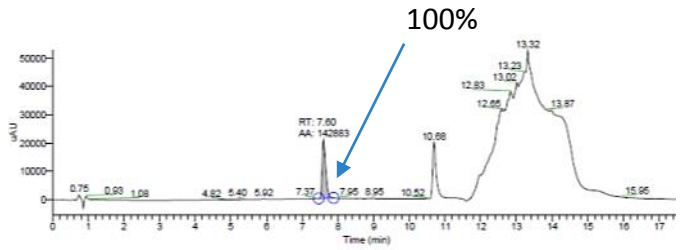
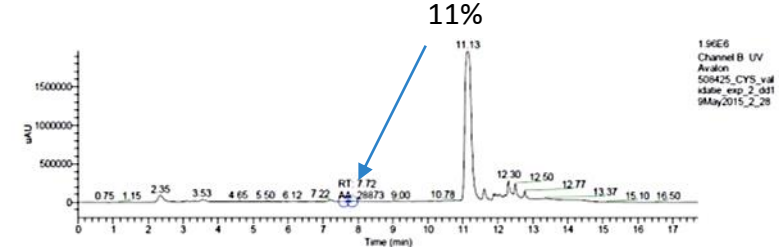
- Reference line
- Reference controls
- Co-elution controls
- Positive controls

- Incubation for 24 h at 25 ° C

DIRECT PEPTIDE REACTIVITY ASSAY

Example Result

- Relative peptide concentration left after co-incubation is measured by high performance liquid chromatography (HPLC) with gradient elution and UV detection.

	Reference Control	Test substance
SPCC		
SPCL		

Component Name	Area	RT
Cysteine_220nm	2450971.50	7.60
Cysteine_258nm	142883.36	7.60

Component Name	Area	RT
Cysteine_220nm	581963.20	7.72
Cysteine_258nm	28872.93	7.72

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak area in Replicate Injection (at 220 nm)}}{\text{Mean Peptide Peak Area in Reference Controls C (at 220 nm)}}\right)\right] \text{x } 100$$

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DIRECT PEPTIDE REACTIVITY ASSAY

Prediction Model

- Mean Cysteine and lysine peptide percent depletion values are used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

Table1: Cysteine 1:10/lysine 1:50 prediction model[1]

Mean of cysteine and lysine % depletion	Reactivity Class	DPRA Prediction[2]
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	

% Depletion for SPCC = 67%
% Depletion for SPCL = 11%

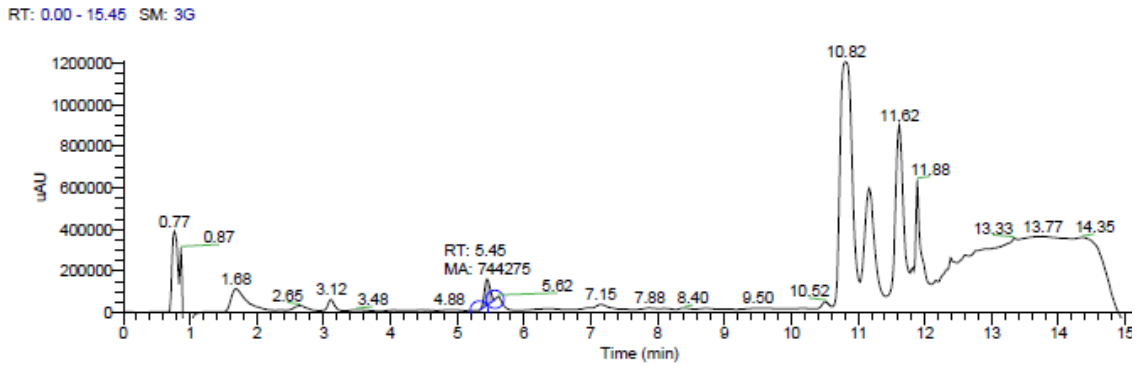
Mean = 39%

DIRECT PEPTIDE REACTIVITY ASSAY

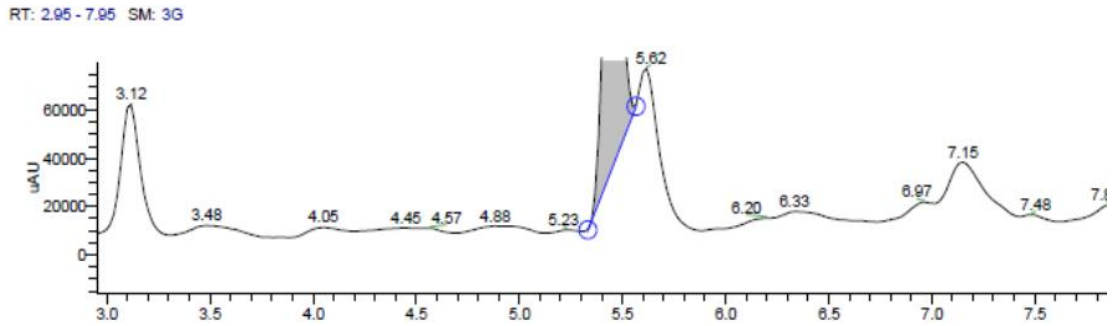
Co-elution

Test substance

SPCC



SPCL



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DIRECT PEPTIDE REACTIVITY ASSAY

Prediction model co-elution with SPCL

% Depletion for SPCC = 67%

Table2: Cysteine 1:10 prediction model[1]

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction[2]
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

DPRA

Results proficiency chemicals

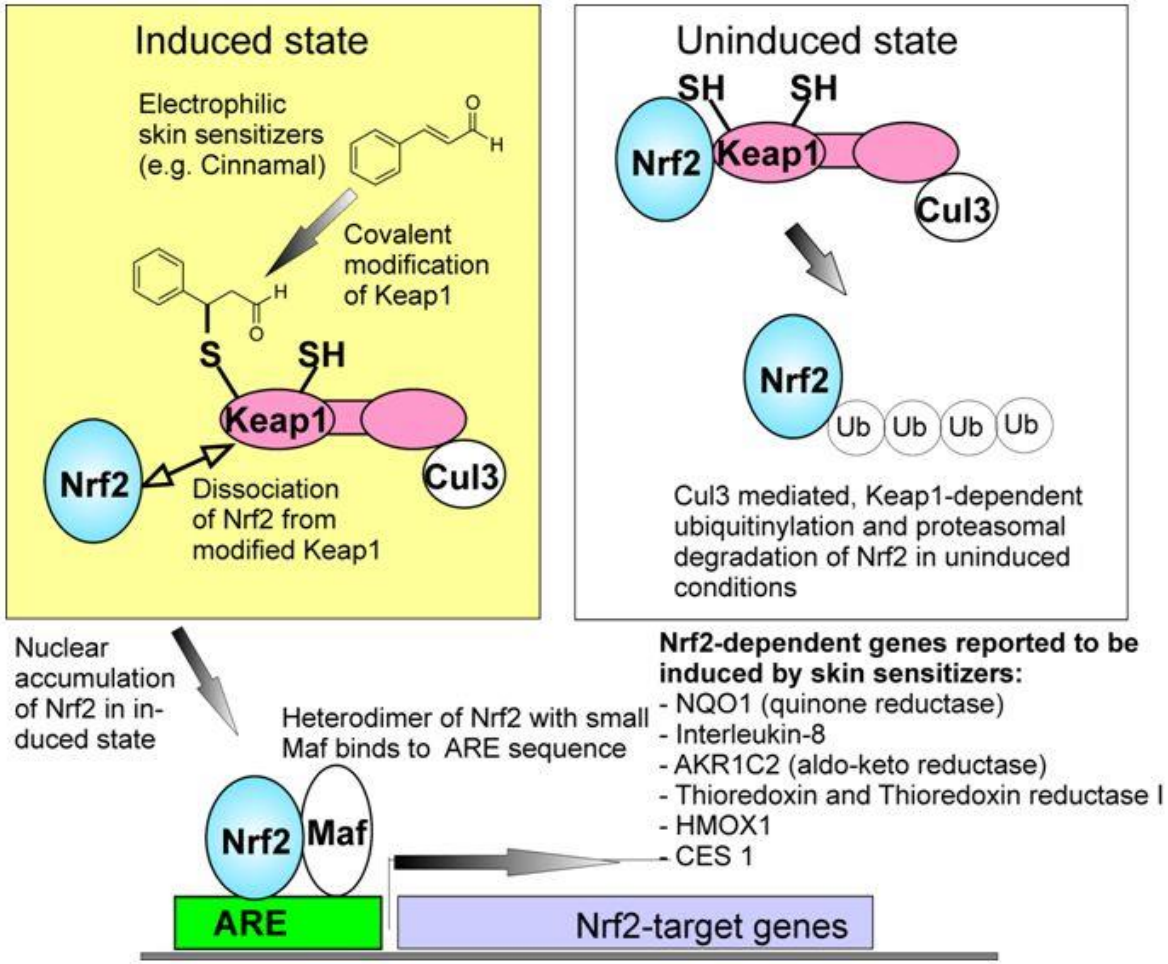
Reference Chemical	*In vivo* Classification	Reactivity Class			DPRA Classification	Correct Classification?
		Run 1	Run 2	Run 3		
p-Benzoquinone	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
2,4-Dinitrochlorobenzene	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
Oxazolone	Sensitiser (extreme)	High	High	High	Sensitiser	Yes
Formaldehyde	Sensitiser (strong)	Moderate	Moderate	Moderate	Sensitiser	Yes
2-Phenylpropionaldehyde	Sensitiser (moderate)	Moderate	High	High	Sensitiser	Yes
Diethyl Maleate	Sensitiser (moderate)	High	High	High	Sensitiser	Yes
Benzylideneacetone	Sensitiser (moderate)	High	High	High	Sensitiser	Yes
Farnesal	Sensitiser (weak)	Moderate	Moderate	Moderate	Sensitiser	Yes
2,3-Butanedione	Sensitiser (weak)	High	High	High	Sensitiser	Yes
4-Allylanisol	Sensitiser (weak)	Moderate	Moderate	Moderate	Sensitiser	Yes
Hydroxycitronellal	Sensitiser (weak)	Low	Moderate	Low	Sensitiser	Yes
Butanol	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
6-Methylcoumarin	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
Lactic Acid	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes
4-Methoxyacetophenone	Non-sensitiser	Minimal	Minimal	Minimal	Non-sensitiser	Yes

KeratinoSens™ (OECD 442D)

Key event 2: Keratinocyte activation

KERATINOSENS™

The NRF2-KEAP1-Anti Oxidant Responsive Element pathway: A stress pathway in Keratinocytes



A. Natsch, *Toxicol. Sci.* **2010,** *113.*

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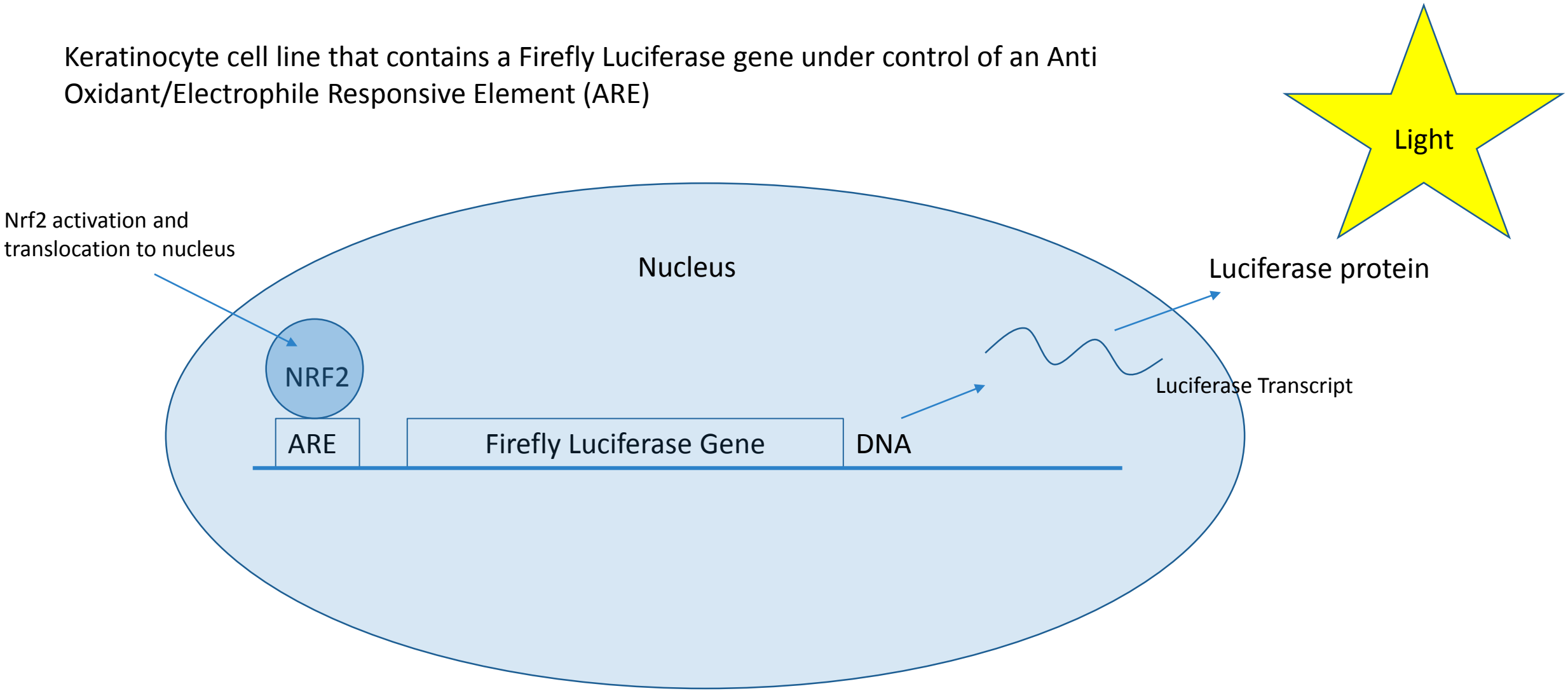
KERATINOSENS™

Principle assay

Keratinocyte cell line that contains a Firefly Luciferase gene under control of an Anti Oxidant/Electrophile Responsive Element (ARE)



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KERATINOSENS™

Method

Prior the main assay:

- Solubility test with DMSO and 100-fold dilution in exposure medium to assess the top concentration in the assay
- Other suitable solvents: water, medium, ethanol (400x diluted in assay)

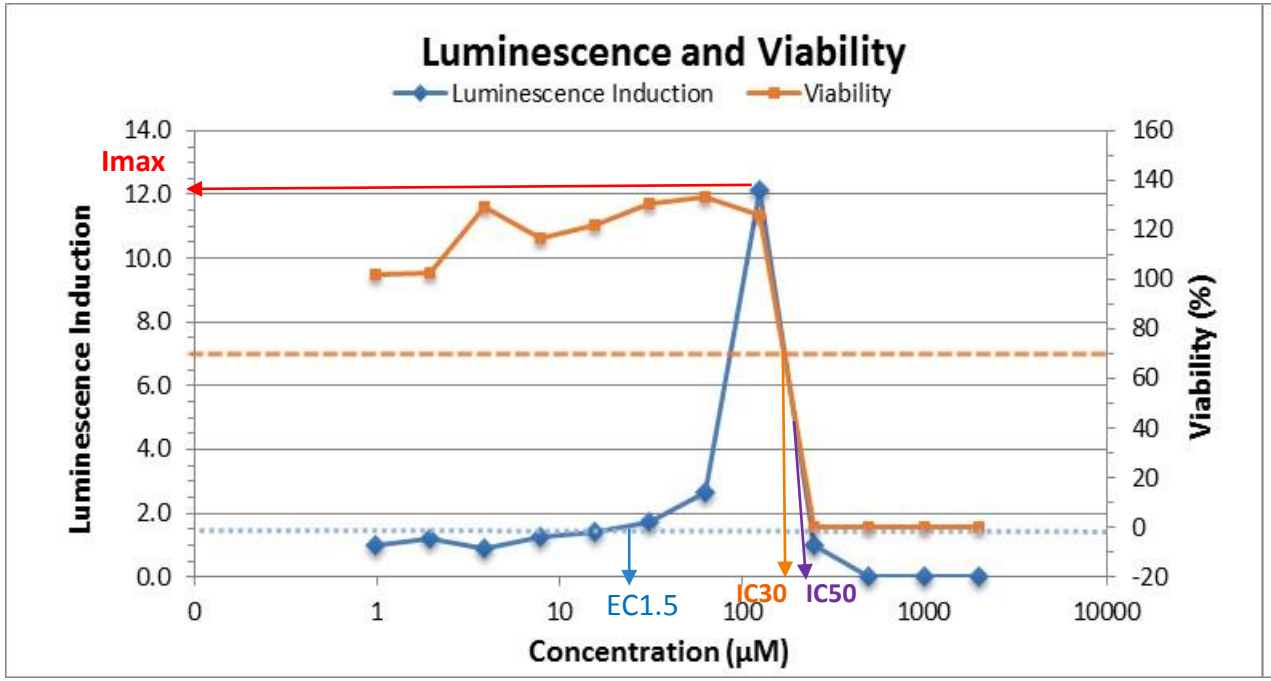
Main assay:

- Day 1: cell seeding for luciferase and MTT assay
- Day 2: Addition test items (12 concentrations), positive control (5 concentrations). Vehicle control (1% DMSO), triplicates are tested but 18 vehicle controls
- Day 4: Luciferase assay, Start MTT assay
- Day 5: Measurement MTT assay
- At least 2 independent repeats are performed
- Luminescence induction and viability is calculated for each datapoint relative to the vehicle control
- Imax, $EC_{1.5}$, IC_{30} and IC_{50} are calculated (when applicable)

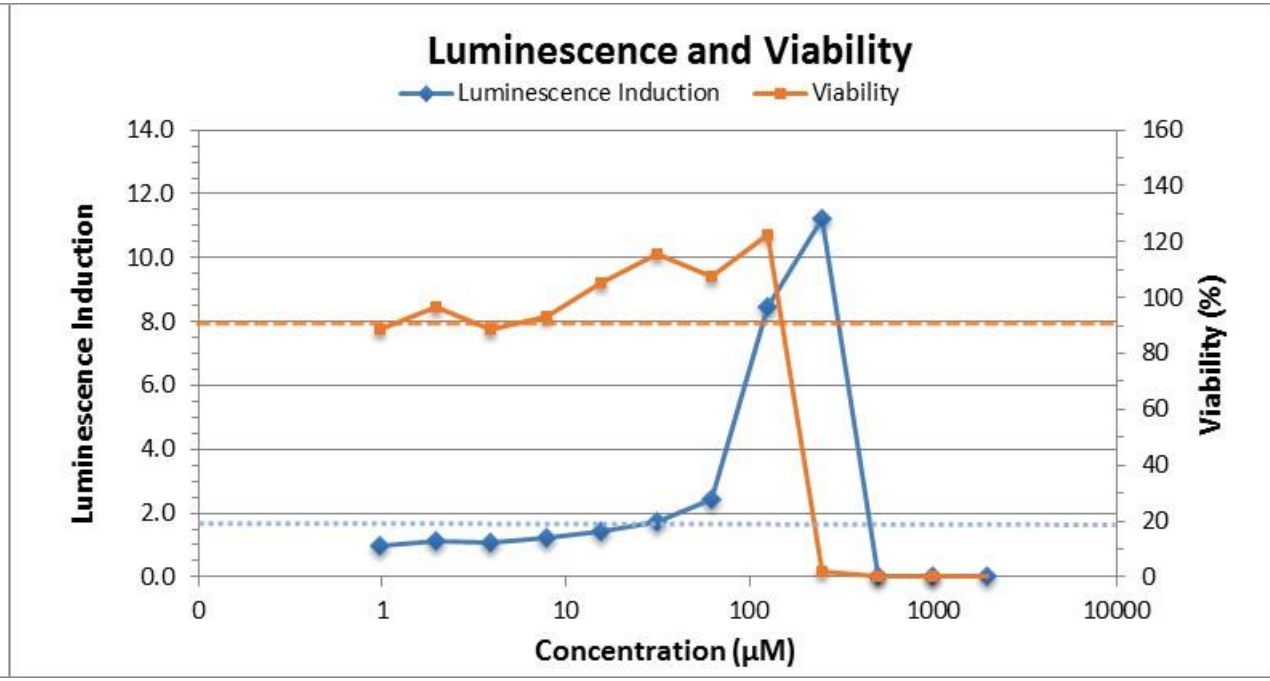
KERATINOSENS™

Example Result Test Item

Experiment 1



Experiment 2



	$EC_{1.5}$ (µM)	I_{max}	IC_{30} (µM)	IC_{50} (µM)
Test item Experiment 1	20	12.13	181	200
Test item Experiment 2	19	11.20	180	200

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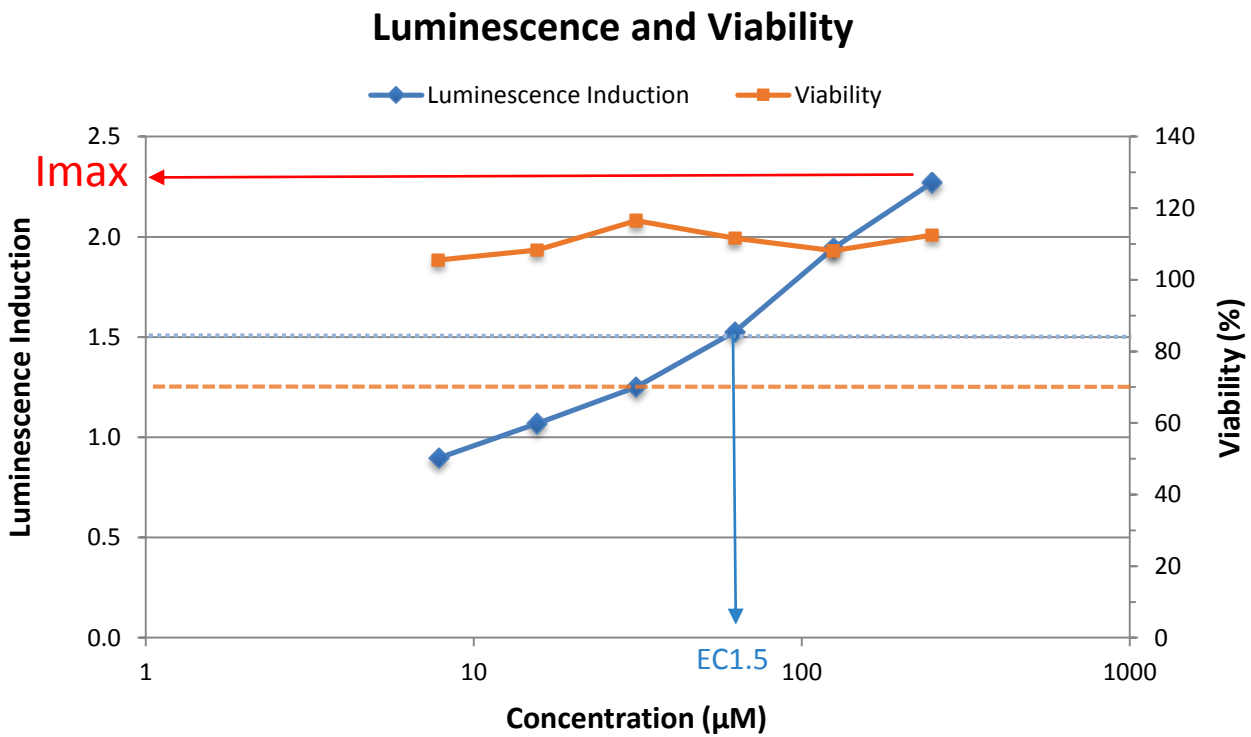
KERATINOSENS™

Example Result Positive control



EC1.5	Imax	IC30	IC50
59.4	2.27		
		NA	NA

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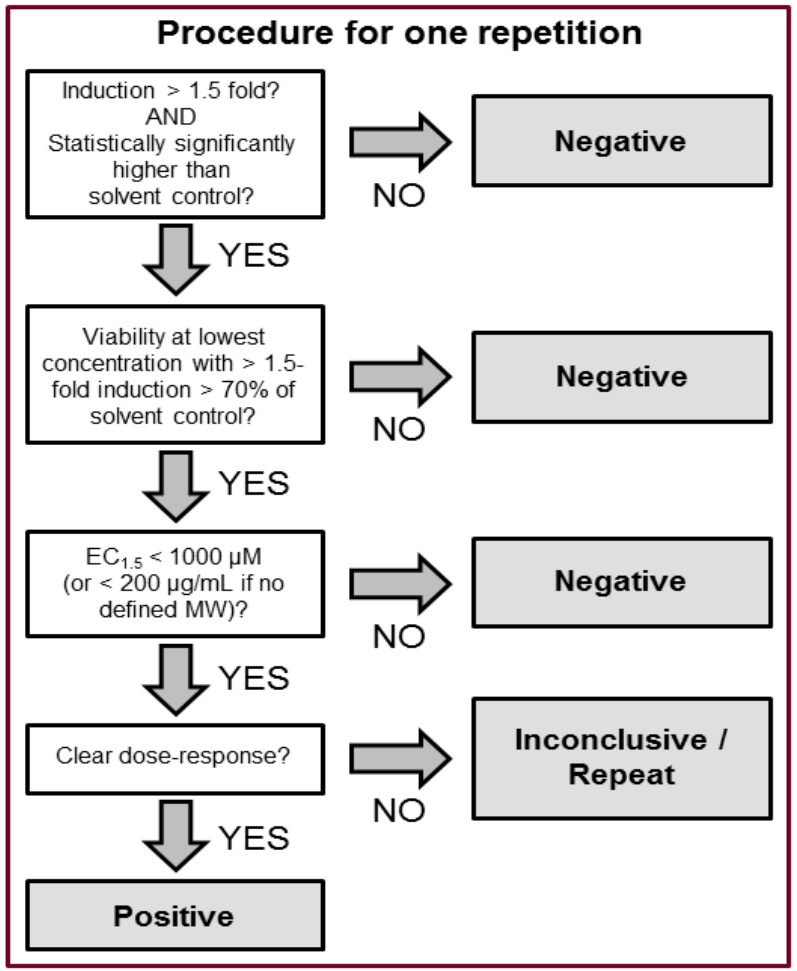
KERATINOSENS™

Prediction model



Perform at least two independent repetitions

- **If the two repetitions are positive, final outcome is: POSITIVE**
- **If the two repetitions are negative, final outcome is: NEGATIVE**

In case the first two repetitions are not concordant, perform a third repetition and conclude on the basis of the mode of the outcomes (i.e., 2 out of 3).

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KERATINOSENS™

Results proficiency chemicals

Reference Chemical	*In Vivo* Classification	Experiment 1				Experiment 2				KeratinoSens™ Classification	Correct Classification?
		$EC_{1.5}$ (µM)	I_{max}	IC_{30} (µM)	IC_{50} (µM)	$EC_{1.5}$ (µM)	I_{max}	IC_{30} (µM)	IC_{50} (µM)		
2,4-Dinitro-chlorobenzene	Positive (Extreme)	1.64	11.6	6.96	8.34	1.59	14.0	6.04	15.54	Positive	Yes
4-Methylaminophenol sulfate	Positive (strong)	3.22	3.46	11.9	15.3	3.02	8.24	10.06	10.93	Positive	Yes
Methyldibromo glutaronitrile	Positive (strong)	18.8	1.71	26.1	33	4.22	4.93	15.5	17.5	Positive	Yes
2-Mercaptobenzothiazole	Positive (moderate)	586	3.5	2353	>2400	371	2.43	689	1310	Positive	Yes
Cinnamyl alcohol	Positive (weak)	49.2	12.8	2195	>2310	14.9	6.95	1316	2013	Positive	Yes
Ethylene glycol dimethacrylate	Positive (weak)	38.9	137	579	793	22.7	56.5	548	724	Positive	Yes
Isopropanol	Negative	NA	1.37	NA	NA	NA	1.34	NA	NA	Negative	Yes
Salicylic acid	Negative	NA	1.41	NA	NA	NA	1.22	NA	NA	Negative	Yes
Lactic acid	Negative	NA	1.24	NA	NA	NA	0.97	NA	NA	Negative	Yes
Glycerol	Negative	NA	1.32	NA	NA	NA	1.06	NA	NA	Negative	Yes

U-SENS™ OECD 442E (annex II)

Key event 3: Activation of dendritic cells

U-SENS™

Principle

- Measures the expression of the CD86 cell surface marker which is a measure for the activation of dendritic cells (key event 3) by flow cytometry
- Cytotoxicity is measured in parallel (propidium iodide)
- **Test system:** human myeloid cell line U937
- **Protocol:**
  - Solubility test
  - Cells are exposed for 45 h to the test item at 6 concentrations in the first assay run (1.0, 10, 20, 50, 100 and 200 µg/ml).
  - At least four concentrations are tested in each following run (2 concentrations similar to the previous run)
  - Positive control, negative control, controls for non-specific binding
- **Classification**: inductions of CD86 above 150% at non toxic concentrations (<30% tox, CV70) are considered positive.

U-SENS™

Example Result

Test items	Dose (µg/mL)	% Viability (Mean)		CD86-IgG1 S,I,	
		Experiment		Experiment	
		1	2	1	2
Diethyl Maleate					
	1	99	99	87	99
	5	-	99	-	123
	10	99	99	181	149
	20	98	97	157	170
	30	-	90	-	168
	40	-	84	-	244
	50	95	76	322	155
	60	-	63	-	97
	70	-	59	-	51
	80	-	63	-	78
	90	-	54	-	10
	100	45	66	132	-30
	200	19	14	205	3

		CV70 Exp 1	CV70 Exp 2	EC150 Exp 1	EC150 Exp 2
Diethyl Maleate	Sensitiser (moderate)	75.07	54.42	7.02	10.64

Controls

Test items	% Viability (Mean)		CD86-IgG1 S,I,	
	Experiment		Experiment	
	1	2	1	2
LA1	98	99	101	91
LA2	98	99	95	98
LA3	99	99	102	90
TNBS1	96	98	641	386
TNBS2	97	99	521	361
TNBS3	97	98	434	142

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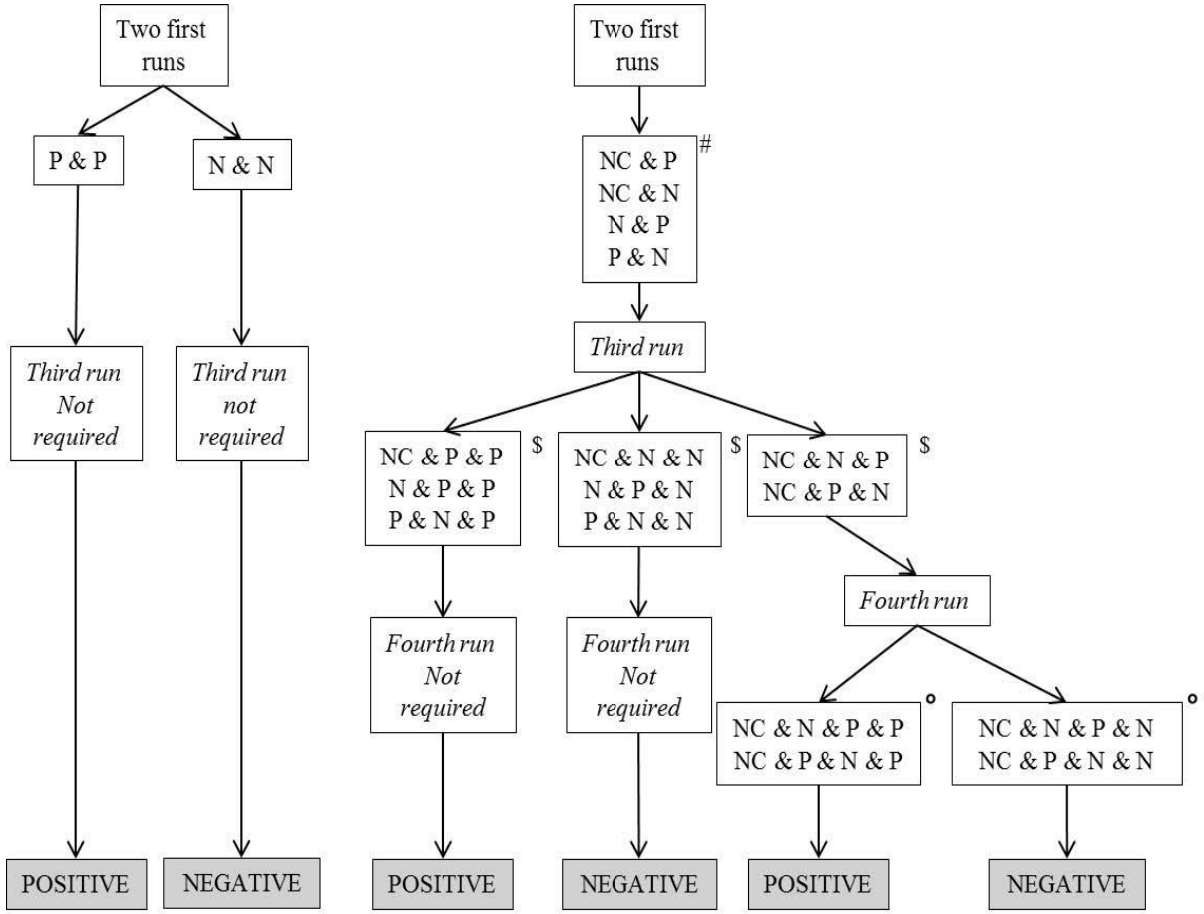
U-SENS™

Prediction Model



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U-SENS™

Proficiency Chemicals

Proficiency Substance	In vivo Classification	CV70 (µg/ml)			EC150 (µg/ml)			Chemical classification	Correct Classification
		Exp.			Exp.				
		1	2	3	1	2	3		
4-Phenylenediamine	Sensitiser (strong)	4.19	4.81	8.48	<1	2.78	2.84	Positive	Yes
Picryl Sulfonic acid	Sensitizer (strong)	>200	>200	>200	*83.14*	48.61	12.39	Positive	Yes
Diethyl Maleate	Sensitiser (moderate)	75.07	54.42	-	7.02	10.64	-	Positive	Yes
Resorcinol	Sensitiser (moderate)	>200	>200	-	22.42	54.53	-	Positive	Yes
Cinnamic Alcohol	Sensitiser (weak)	>200	>200	-	11.03	20.86	-	Positive	Yes
4-Allylanisole	Sensitiser (weak)	>200	>200	>200	<1	>200	73.33	Positive	Yes
Saccharin	Non-sensitiser	>200	>200	-	>200	>200	-	Negative	Yes
Glycerol	Non-sensitiser	>200	>200	-	>200	>200	-	Negative	Yes
Lactic Acid	Non-sensitiser	>200	>200	-	>200	>200	-	Negative	Yes
Salicyclic acid	Non-sensitiser	>200	>200	>200	131.48	>200	>200	Negative	Yes

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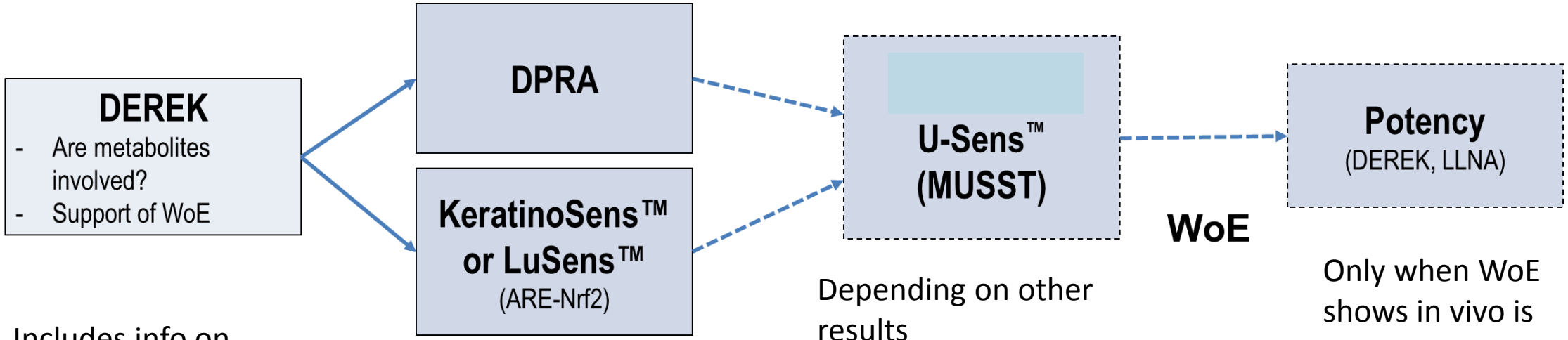
Testing strategy CRL

UVCBs and non-UVCBs

TESTING STRATEGY

Non-UVCBs (mono-constituents, mixtures with known composition)



Includes info on metabolism and potency

Results can be positive, negative or equivocal

Depending on other results

This supports the findings, and why no further *in vitro* or *in vivo* testing is required. It may also support *in vivo* testing to be performed

Only when WoE shows in vivo is necessary

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TESTING STRATEGY

UVCBs (Chemical Substances of Unknown or Variable Composition, Complex Reaction Products or Biological Materials)

Proposed test strategy when DEREK and/or DPRA are not feasible

Step	Assay/Action
1	KeratinoSens™ - Evaluation Results: - KeratinoSens™ positive: Strategy in consultation with Sponsor (vivo test required) - KeratinoSens™ equivocal: Strategy in consultation with Sponsor (vivo test required) - KeratinoSens™ negative: U-SENS™ + Evaluation results
2	WoE (assessment report)

Summary and Discussion

SUMMARY AND DISCUSSION

Summary:

- DPRA, KeratinoSens™ and U-SENS™ test methods for skin sensitization have been implemented at CRL Den Bosch
- The three methods are together with the in silico DEREK used in a test strategy for UVCBs and non-UVCBs
- Tests give reproducible results

Discussion:

- Solubility is often an issue with chemicals (testing is often possible at lower concentrations but in case of a negative results in DPRA and KeratinoSens this results in the classification equivocal and in vivo testing)
- KeratinoSens™: No criteria for the MTT assay (what to in case of an increase?)
- KaratinoSens™ : False positives due to activation of nuclear receptors
- U-SENS™: toxicity, solubility and color interference result in a positive classification
- Different terminology is used in the guidelines:
    - KeratinoSens $EC_{1.5}$ and IC_{30} ←→ U-SENS EC_{150} and CV_{70}
- Testing of UVCBs is difficult with the new methods
- Skin penetration

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ANY
QUESTIONS
?