EFSA Symposium

"NOVEL CHEMICAL HAZARD CHARACTERISATION APPROACHES "

Section 2: Systems biology approach and predictive toxicology

16 October 2015 – Milano at EXPO2015

Alternative and Integrated Testing Strategies

Horst Spielmann
Professor for Regulatory Toxicology
Freie Universität Berlin
& State Animal Welfare Officer, Berlin

Der Landestierschutzbeauftragte

Topics

- 2002 ECVAM - proposal for integrated testing scheme for chemicals
- 2002 OECD - sequential testing strategy eye & skin irritation/corrosion
- 2004 BfR - "Concept" for *in vitro* eye & skin irritation testing
- 2005 ECVAM - "Top-Down" & "Bottom-Up" approaches for eye irritation testing
- 2009 ECVAM - WS validation of Integrated Testing Strategies (ITS)
- 2014 OECD - GD 203 "Integrated approach on testing and assessment (IATA) for skin corrosion & irritation
- 2012 OECD - AOP for skin sensitization *an ITS approach*
- 2012 ROCHE - Embryonic Stem cell Test (EST) *an ITS approach*
- 2013 EU - ban on animal testing for cosmetics

Principles of the Future Chemicals Policy of the EU (White Paper) 2002 ►REACH !!

Action proposed in the EU White Paper

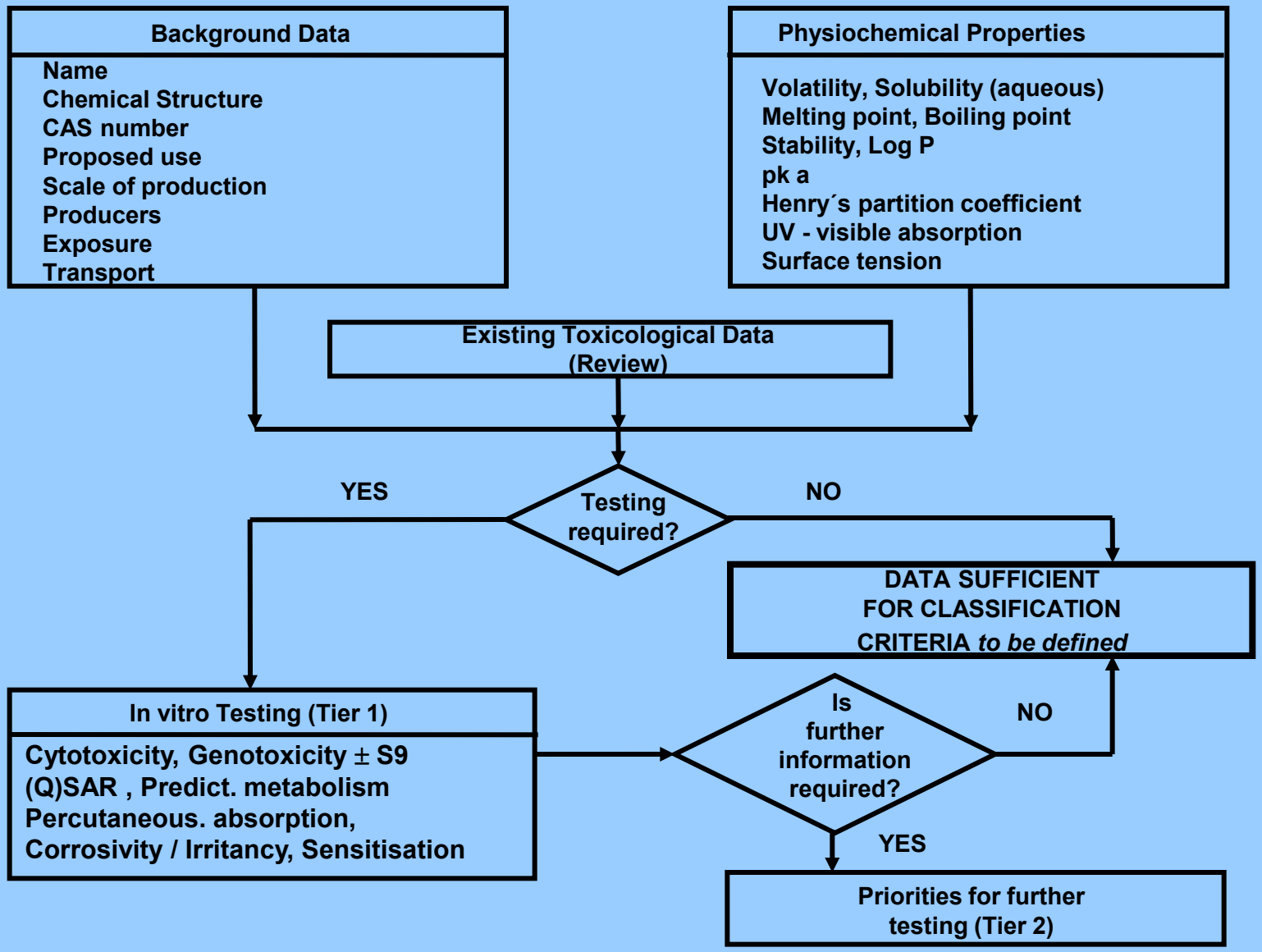
1. Identical amount of testing for new & existing chemicals
   - ►more information on existing chemcials and less on new ones
2. Steps proposed for the testing of 30.000 existing chemicals until 2012
   - ►high production volume chemicals will be tested first
3. Only in vitro/non-animal methods will be used in the basic test set
   - ► they are faster and cheaper to perform, will the information be sufficient for risk assessment for humans and the environment ?

► CONSEQUENCES in 2002:

The 6th Framework Program of the EU Commission includes funding of research for development and validation of new non-animal methods.

In 2007 the 7th FP included major funding for alternative methods

ECVAM WG proposal of an integrated testing scheme for Existing and New Chemicals in the EU 2002



OECD 2002:Sequential Testing Strategy

- Agreed stepwise decision logic
- Mandatory to follow all steps
- Increasingly more complicated
- From *in vitro* to *in vivo* approaches
- Consideration of the hazard after each step
- Works best for less complicated hazards (e.g. local effects, single endpoints)
- Is a relatively rigid approach
- Is relatively easy to harmonise internationally.

BfR
zebet

Existing human experience

↓

(Q)SAR (eye irritation/corrosion)

↓

(Q)SAR (skin corrosion)

↓

pH and buffering capacity

↓

evaluate dermal route toxicity data

↓

In vitro Test (eye irritation/corrosion)

↓

In vitro Test (skin irritation/corrosion)

↓

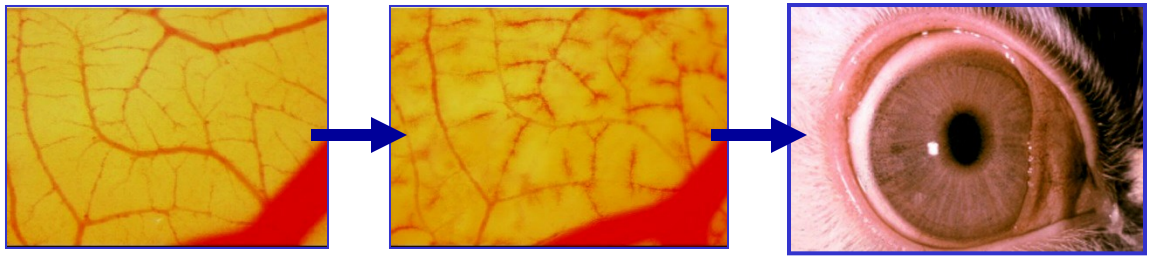
In vivo 1 Rabbit

↓

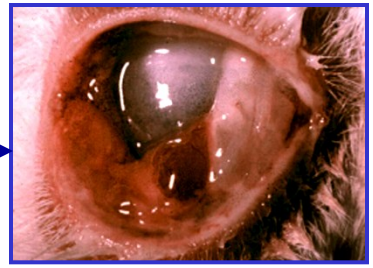
In vivo 2 Rabbits

Testing Strategy: Skin and Eye Effects (TG 404, TG 405)

(adopted 24 April 2002)



Reduces by about 90% that eye corrosives damage a rabbit eye

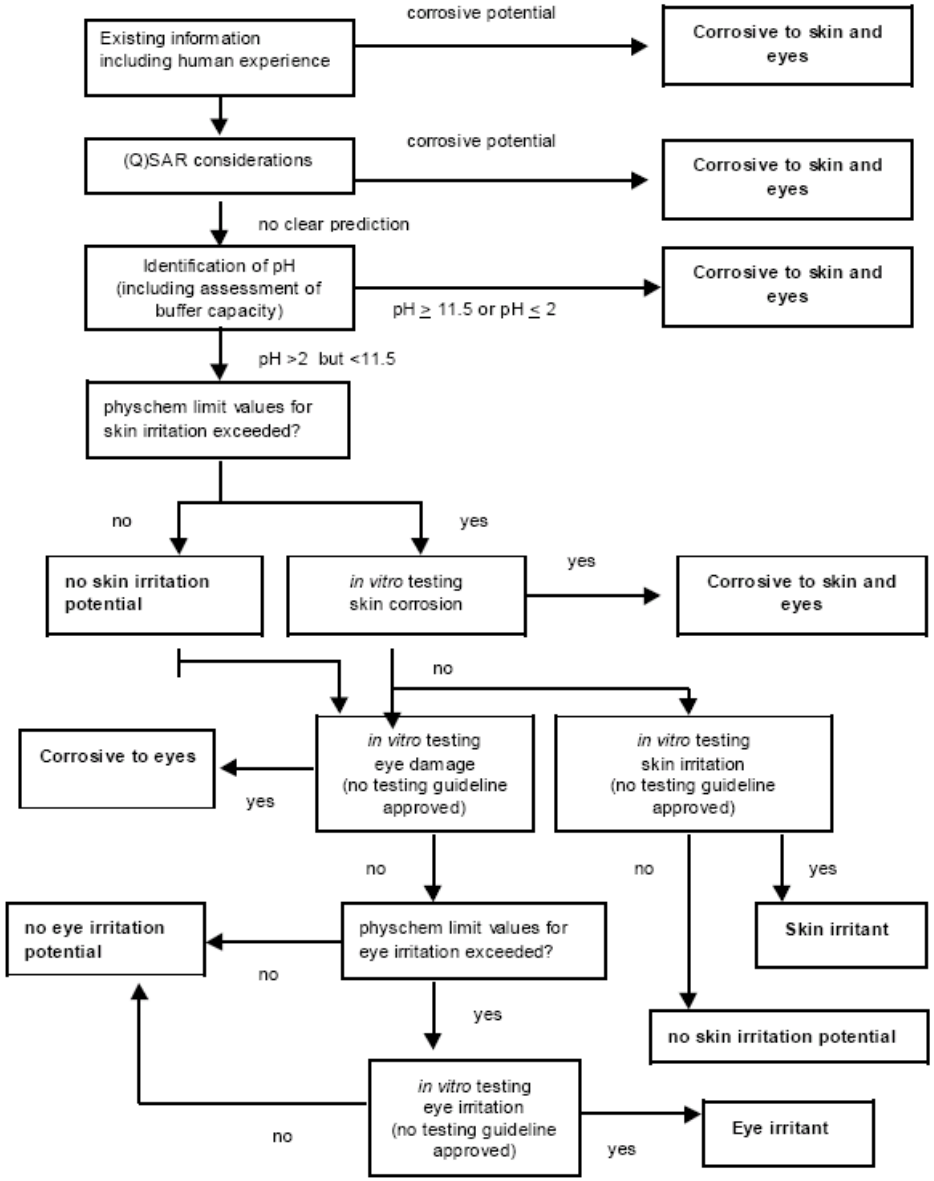


FIGURE

TESTING AND EVALUATION STRATEGY FOR EYE IRRITATION/CORROSION

	Activity	Finding	Conclusion
1	Existing human and/or animal data showing effects on eyes	Severe damage to eyes	Apical endpoint; consider corrosive to eyes. No testing is needed.
		Eye irritant	Apical endpoint; consider irritating to eyes. No testing is needed.
		Not corrosive/not irritating to eyes	Apical endpoint; considered non-corrosive and non-irritating to eyes. No testing required.
	Existing human and/or animal data showing corrosive effects on skin	Skin corrosive	Assume corrosivity to eyes. No testing is needed.
	Existing human and/or animal data showing severe irritant effects on skin	Severe skin irritant	Assume irritating to eyes. No testing is needed
	↓ *no information available, or available information is not conclusive*		
2	Perform SAR for eye corrosion/irritation	Predict severe damage to eyes	Assume corrosivity to eyes. No testing is needed.
		Predict irritation to eyes	Assume irritating to eyes. No testing is needed.
	Perform SAR for skin corrosion	Predict skin corrosivity	Assume corrosivity to eyes. No testing is needed.
	↓ *No predictions can be made, or predictions are not conclusive or negative*		
3	Measure pH (buffering capacity, if relevant)	pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)	Assume corrosivity to eyes. No testing is needed.
	↓ *2< pH < 11.5, or pH≤ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant*		
	↓		

4	Evaluate systemic toxicity via the dermal route	Highly toxic at concentrations that would be tested in the eye.	Substance would be too toxic for testing. No testing is needed.
	↓ *Such information is not available, or substance is not highly toxic* ↓		
5	Perform validated and accepted *in vitro* or *ex vivo* test for eye corrosion	Corrosive response	Assume corrosivity to eyes. No further testing is needed.
	↓ *Substance is not corrosive, or internationally validated in vitro or ex vivo testing methods for eye corrosion are not yet available* ↓		
6	Perform validated and accepted *in vitro* or *ex vivo* test for eye irritation	Irritant response	Assume irritancy to eyes. No further testing is needed.
	↓ *Substance is not an irritant, or internationally validated in vitro or ex vivo testing methods for eye irritation are not yet available* ↓		
7	Experimentally assess *in vivo* skin irritation/corrosion potential (see OECD Guideline 404)	Corrosive or severe irritant response	Assume corrosivity to eyes. No further testing is needed.
	↓ *Substance is not corrosive or severely irritating to skin* ↓		
8	Perform initial *in vivo* rabbit eye test using one animal 	Severe damage to eyes	Consider corrosive to eyes. No further testing is needed.
	↓ *No severe damage, or no response* ↓		
9	Perform confirmatory test using one or two additional animals 	Corrosive or irritating	Consider corrosive or irritating to eyes. No further testing is needed
		Not corrosive or irritating	Consider non-irritating and non-corrosive to eyes. No further testing is needed.



PERSPECTIVE:

BfR View of a Strategy for Skin and Eye Irritation/Corrosion Assessment without Use of Animals

Source:
Höfer et al. (2004) Animal Testing and Alternative Approaches... (Arch. Toxicol. 78: 549–564)

REGULATORY USE OF (Q)SARs IN TOXICOLOGICAL HAZARD ASSESSMENT STRATEGIES*

I. GERNER, H. SPIELMANN, T. HOEFER, M. LIEBSCH and M. HERZLER†

Federal Institute for Risk Assessment (BfR), Thielallee 88-92, D-14195 Berlin, Germany

SAR and QSAR in Environmental Research,
VOL. 15 (5–6), October–December 2004, pp. 359–366

Conclusions

Assessment of the potential of a chemical to induce local lesions

We have shown that

- **structural alerts for the prediction of a potential to cause local lesions and**
- **physicochemical limit values (DSS) for the prediction of the absence of such a potential**

provide testing and assessment strategies which use only (Q)SARs and results of in vitro testing **instead of the current employment of test animals for the purpose of classification and labelling of acute local health hazards.**

Physico-chemical limit values (DSS) for the absence of severely skin and eye irritating potential

Rules appropriate for all groups of chemicals:

Basis: Evaluation of data of 1627 chemicals with purity ≥ 95%

Attention: Rules are valid exclusively for the Risk phrases mentioned within this specific "exclusion rule". This is due to the fact that acute local tissue lesions called "irritation" or "corrosion" and specified by the respective R-phrase of the EU are in reality based on a great variety of totally different biochemical reactions (depending on the chemical reactivity of the molecule which contacts the biological medium or structure first).

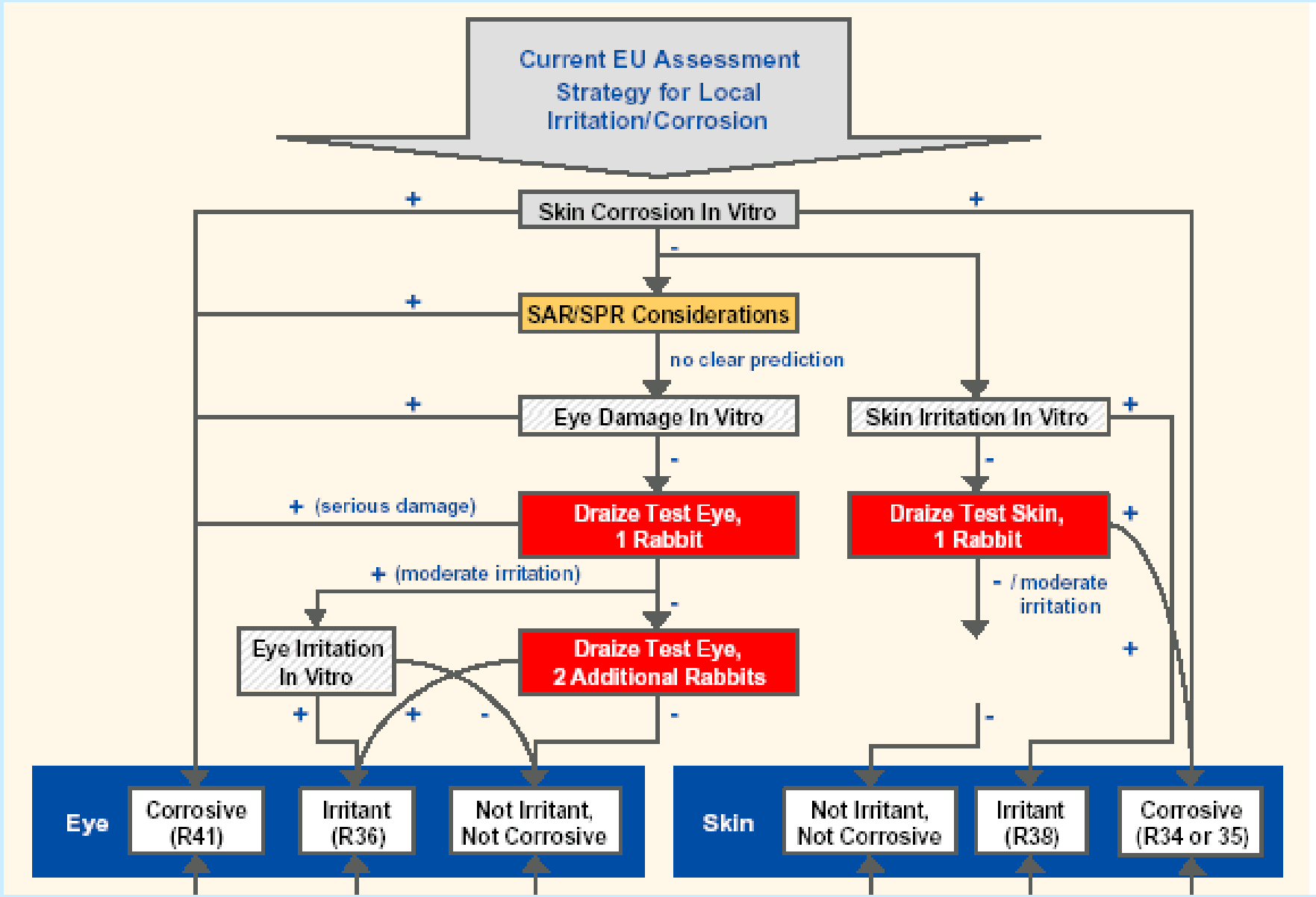
IF melting point > 200°C	**THEN NOT (skin corrosion R34 or R35) (is true for 245/252 chemicals tested = 97%)**

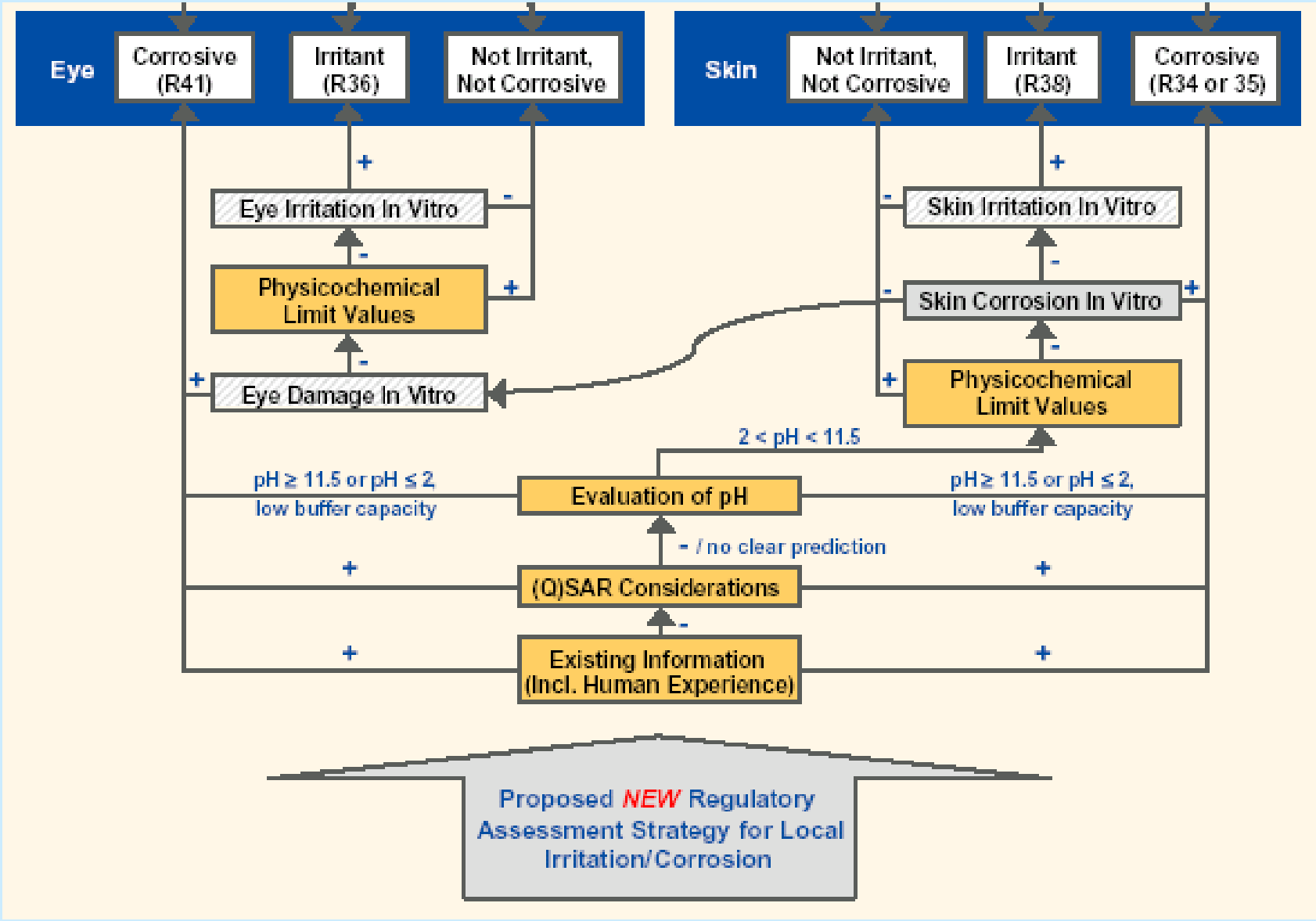
(7 skin corrosive substances are organic salts which release strong inorganic acids or bases when getting in contact with aqueous substrates/organic media)

IF log P_{ow} > 9	**THEN NOT (lesions R34,R35,R36 or R41) (is true for 32/32 chemicals tested = 100%)**
IF log P_{ow} < -3.1	**THEN NOT (skin corrosion R34 or R35) (is true for 53/53 chemicals tested) = 100%)**
IF lipid solubility < 0.01 g/kg	**THEN NOT (skin corrosion R34 or R35) (is true for 58/58 chemicals tested = 100%))**
IF aqueous solubility < 0.00002 g/l	**THEN NOT (eye irritation R41) (is true for 109/109 chemicals tested = 100%))**
IF aqueous solubility < 0.000005 g/l	**THEN NOT (eye irritation R36) (is true for 38/38 chemicals tested= 100%))**
IF molecular weight > 650 g/Mol	**THEN NOT (eye irritation R36) (is true for 139/139 chemicals tested= 100%))**

Attention:
chemicals with molecular weight > 650 g/Mol may elicit severe tissue damage resulting in local corrosion!

Assessment strategy for local irritation/corrosion EU (& OECD) 2004





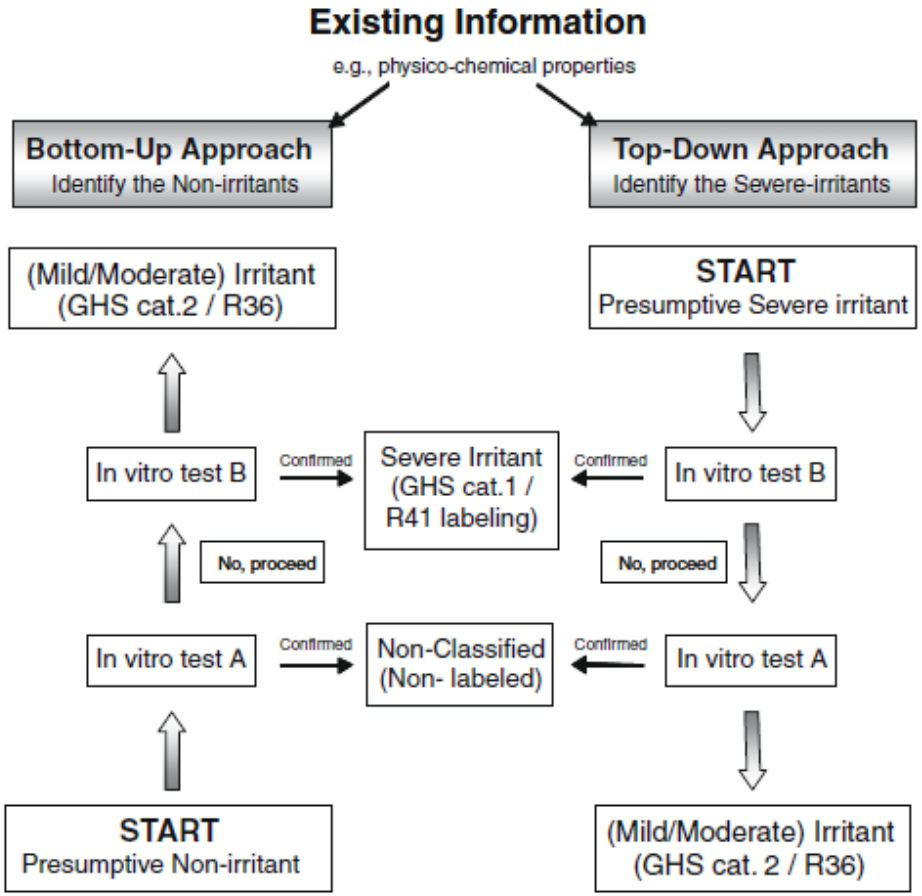
Proposed new BOTTOM-UP Approach BfR 2004

ECVAM Workshop February 2005 - Publication TIV 2010

Review

A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom–Up and Top–Down approaches ☆

Laurie Scott [a], Chantra Eskes [b], Sebastian Hoffmann [b], Els Adriaens [c], Nathalie Alepée [d], Monica Bufo [e], Richard Clothier [f], Davide Facchini [g], Claudine Faller [h], Robert Guest [i], John Harbell [j], Thomas Hartung [b], Hennicke Kamp [k], Béatrice Le Varlet [l], Marisa Meloni [m], Pauline McNamee [n], Rosemarie Osborne [o], Wolfgang Pape [p], Uwe Pfannenbecker [p], Menk Prinsen [q], Christopher Seaman [r], Horst Spielmann [s], William Stokes [t], Kevin Trouba [o], Christine Van den Berghe [d], Freddy Van Goethem [u], Marco Vassallo [e], Pilar Vinardell [v], Valérie Zuang [b,*]



ATLA 37, 1–8, 2009

Overcoming Barriers to Validation of Non-animal Partial Replacement Methods/Integrated Testing Strategies: The Report of an EPAA–ECVAM Workshop

Agnieszka Kinsner-Ovaskainen,[1] Zerrin Akkan,[2] Silvia Casati,[1] Sandra Coecke,[1] Raffaella Corvi,[1] Gianni Dal Negro,[3] Jack De Bruijn,[4] Odile De Silva,[5] Laura Gribaldo,[1] Claudius Griesinger,[1] Joanna Jaworska,[6] Joachim Kreysa,[1] Gavin Maxwell,[7],Pauline McNamee,[6] Anna Price,[1] Pilar Prieto,[1] Roland Schubert,[8] Luca Tosti,[1] Andrew Worth[1] and Valerie Zuang[1]

ATLA 40, 175–181, 2012

Report of the EPAA–ECVAM Workshop on the Validation of Integrated Testing Strategies (ITS)

Agnieszka Kinsner-Ovaskainen,[1] Gavin Maxwell,[2] Joachim Kreysa,[1] João Barroso,[1] Els Adriaens,[3] Nathalie Alépée,[4] Ninna Berg,[5] Susanne Bremer,[1] Sandra Coecke,[1] José Z. Comenges,[1] Raffaella Corvi,[1] Silvia Casati,[1] Gianni Dal Negro,[6] Monique Marrec-Fairley,[7] Claudius Griesinger,[1] Marlies Halder,[1] Eckhard Heisler,[8] Doris Hirmann,[9] André Kleensang,[1a] Annette Kopp-Schneider,[10] Silvia Lapenna,[1] Sharon Munn,[1] Pilar Prieto,[1] Len Schechtman,[11] Terry Schultz,[12] Jean-Marc Vidal,[13] Andrew Worth[1] and Valérie Zuang[1]

EPAA ECVAM workshop definitions of ITS (Integrated Testing Strategy)

2009

In the context of safety assessment, **an ITS is a methodology which integrates information for toxicological evaluation from more than one source**, thus facilitating decision-making. This should be achieved whilst taking into consideration the principles of the **Three Rs** (*reduction*, *refinement* and *replacement*)". It was also agreed that, in line with the proposal put forward during the **OECD Workshop** on *Integrated Approaches to Testing and Assessment,* held in December 2007, **a good ITS should be structured, transparent and hypothesis driven**..

2012

During this workshop it was recognized that there is a fundamental difference between:

a) **Type 1 ITS**, i.e. strategies to gather and analyze a broad range of data coming from different sources (epidemiological studies, animal data, in vitro data, read-across methodologies, etc) and used to draw conclusions based on weight-of-evidence (WoE) approaches; and

b) **Type 2 ITS**: testing strategies composed of e.g. a number of in vitro and in silico methods that, combined and weighted in a fixed way, would serve to replace some or all in vivo experimentation for a given toxicity endpoint.

This distinction is essential, when the validation of ITS is under consideration.

EPAA ECVAM workshop on ITS (Integrated Testing Strategy)

Table 2: Requirements for formal validation of ITS

	Formal validation of ITS component	Formal validation of ITS
Screening	Not required	Not required
Hazard classification & labelling	Not required	Not required
Replacement of Test Guideline used for regulatory purposes	Required *(data requirements are different than in validation of 1-to-1 replacement methods)*	Required *(the principles of ITS validation need to be established)*
Risk assessment	Not required	Not required

OECD GD 203 - ENV/JM/MONO (2014)19
COMPOSITION OF THE IATA FOR SKIN CORROSION AND IRRITATION

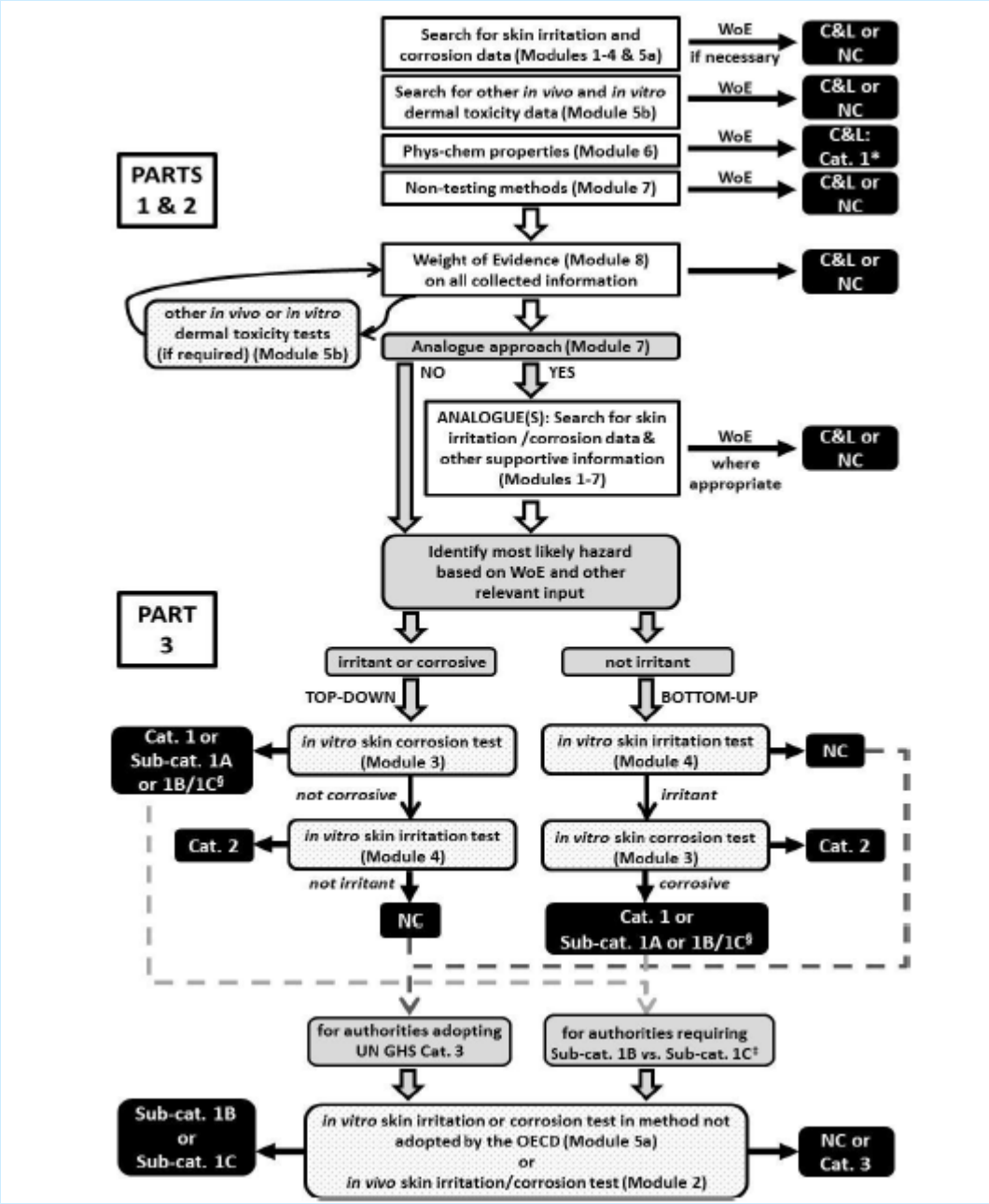
2014

Table 1: Parts and Modules of the IATA.

Part (*)	Module	Data
Part 1 (Existing information, physico-chemical properties and non-testing methods)	1	Existing information - Existing human data *a)* Non-standardised human data on local *skin effects* *b) Human Patch Test (HPT)*
	2	- *In vivo* skin irritation and corrosion data (OECD TG 404)
	3	- *In vitro* skin corrosion data *a) OECD TG 430* *b) OECD TG 431* *c) OECD TG 435*
	4	- *In vitro* skin irritation data (OECD TG 439)
	5	- Other *in vivo* and *in vitro* data a) *In vitro* skin corrosion or irritation data from test methods not adopted by the OECD b) Other *in vivo* and *in vitro* dermal toxicity data
	6	Physico-chemical properties (existing, measured or estimated) - e.g., pH, acid/alkaline reserve
	7	Non-testing methods - for substances: (Q)SAR, read-across, grouping and prediction systems; - for mixtures: bridging principles and theory of additivity
Part 2 (WoE analysis)	8	Phases and elements of WoE approaches
Part 3 (Additional testing)	(5b)	*Other in vivo* and/or *in vitro* dermal toxicity testing (if required by other regulations)
	(3)	*In vitro* skin corrosion testing
	(4)	*In vitro* skin irritation testing
	(5a)	*In vitro* skin irritation testing in test method not adopted by the OECD
	(2)	*In vivo* skin irritation and corrosion testing

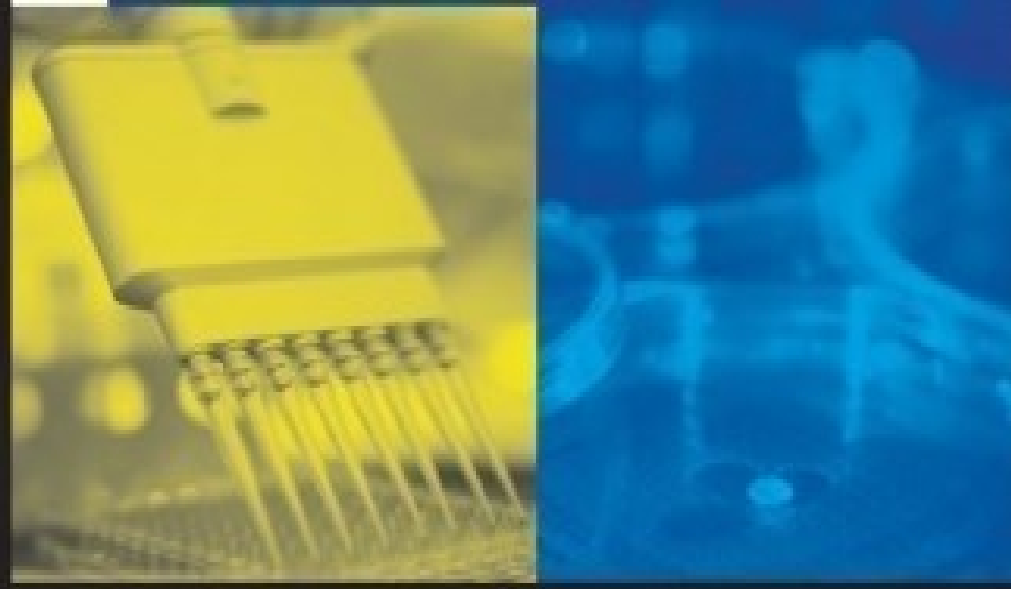
OECD GD 203 - ENV/JM/MONO (2014)19
COMPOSITION OF THE IATA FOR SKIN CORROSION AND IRRITATION

2014



US National Academy of Sciences 2007

"….. a not-so-distant future where all routine toxicity testing will be conducted in human cells or cell lines *in vitro* by evaluating perturbations of cellular responses in a suite of toxicity pathway assays….."



TOXICITY TESTING IN THE 21ST CENTURY: A VISION AND STRATEGY

Andersen and Krewski (2009). Toxicity Testing in the 21st Century: Bringing the Vision to Life. Tox. Sci., 107, 324-330.

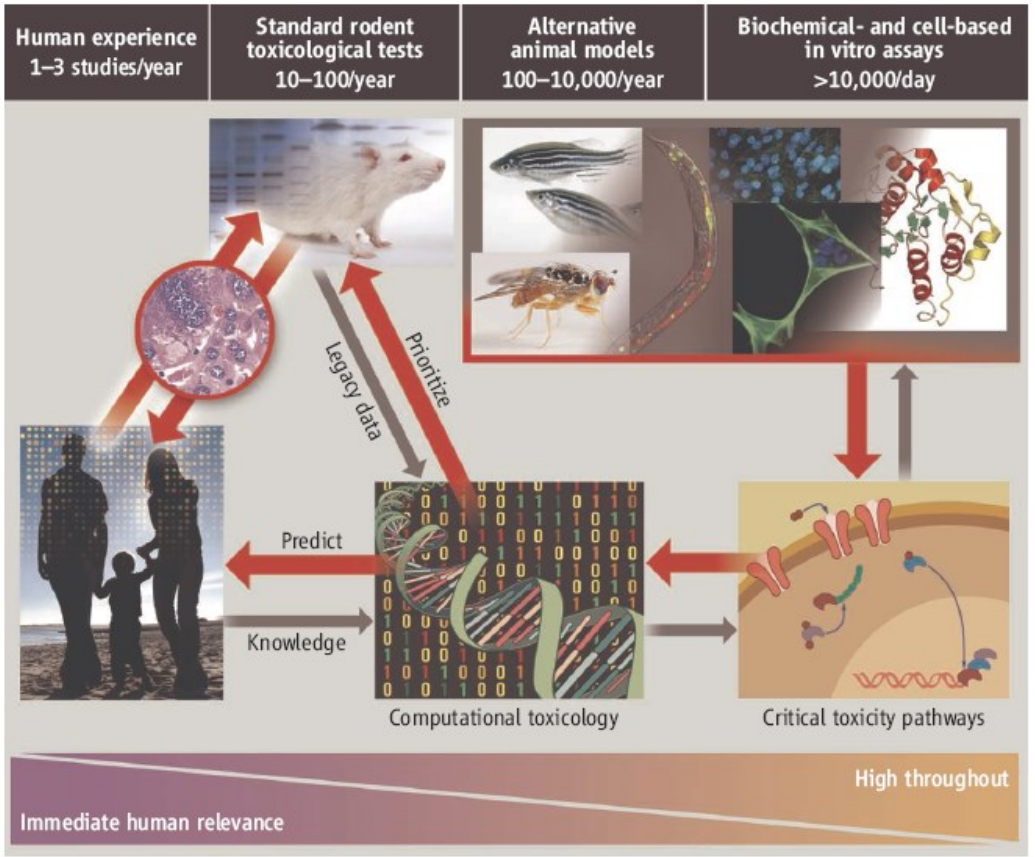
15 FEBRUARY 2008 VOL 319 SCIENCE www.sciencemag.org

TOXICOLOGY

Transforming Environmental Health Protection

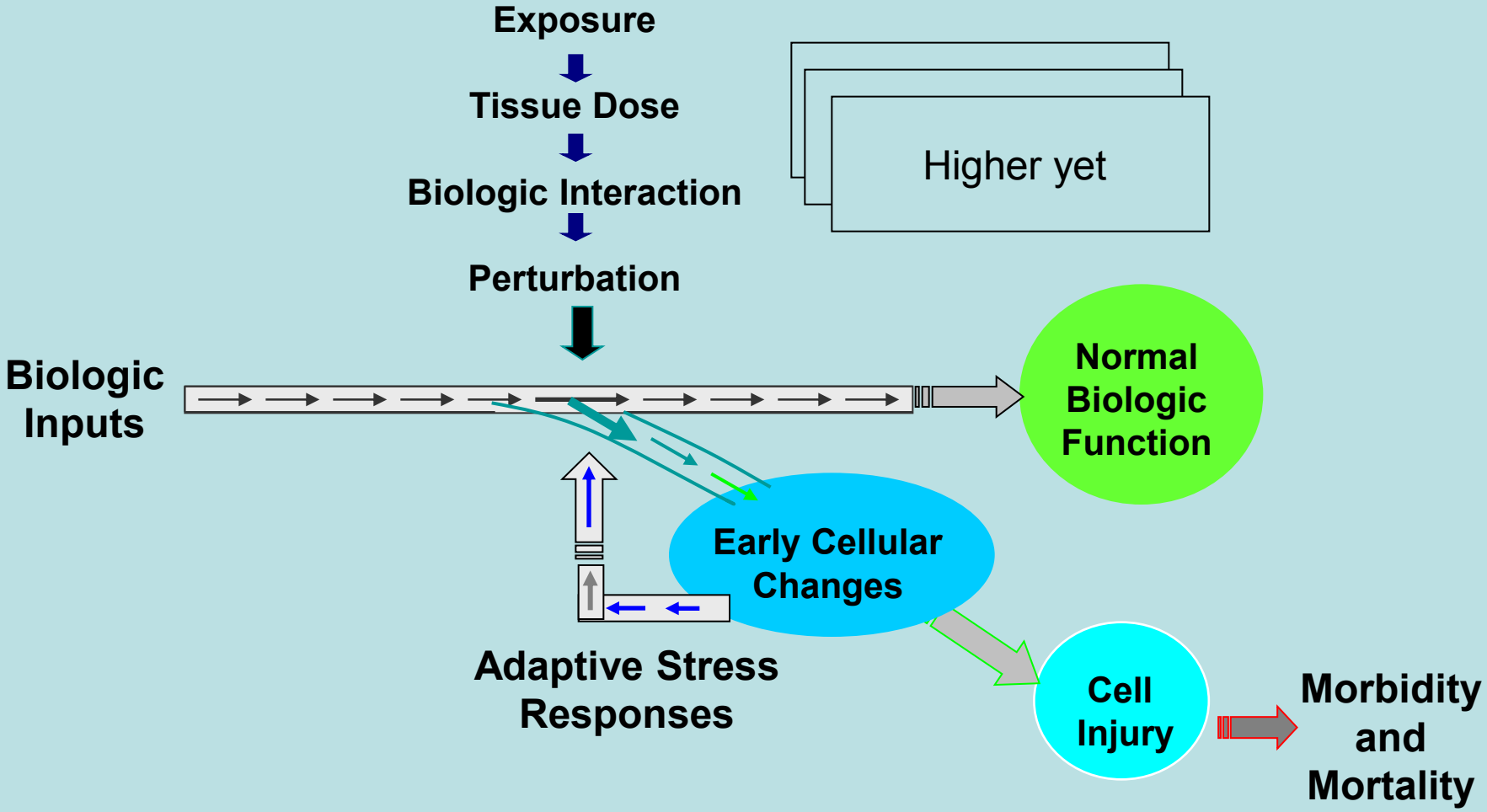
Francis S. Collins,[1*†] George M. Gray,[2*] John R. Bucher[3*]

We propose a shift from primarily in vivo animal studies to in vitro assays, in vivo assays with lower organisms, and computational modeling for toxicity assessments.



Transforming toxicology. The studies we propose will test whether high-throughput and computational toxicology approaches can yield data predictive of results from animal toxicity studies, will allow prioritization of chemicals for further testing, and can assist in prediction of risk to humans.

Perturbation of Toxicity Pathways



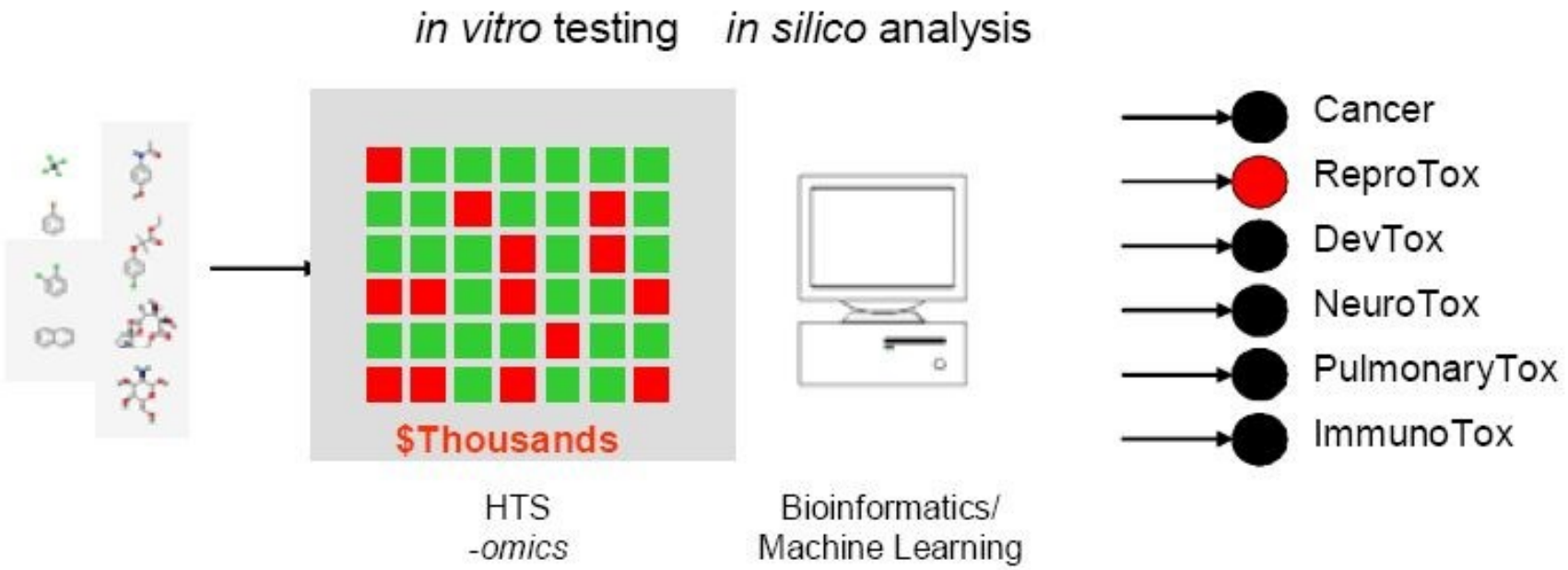
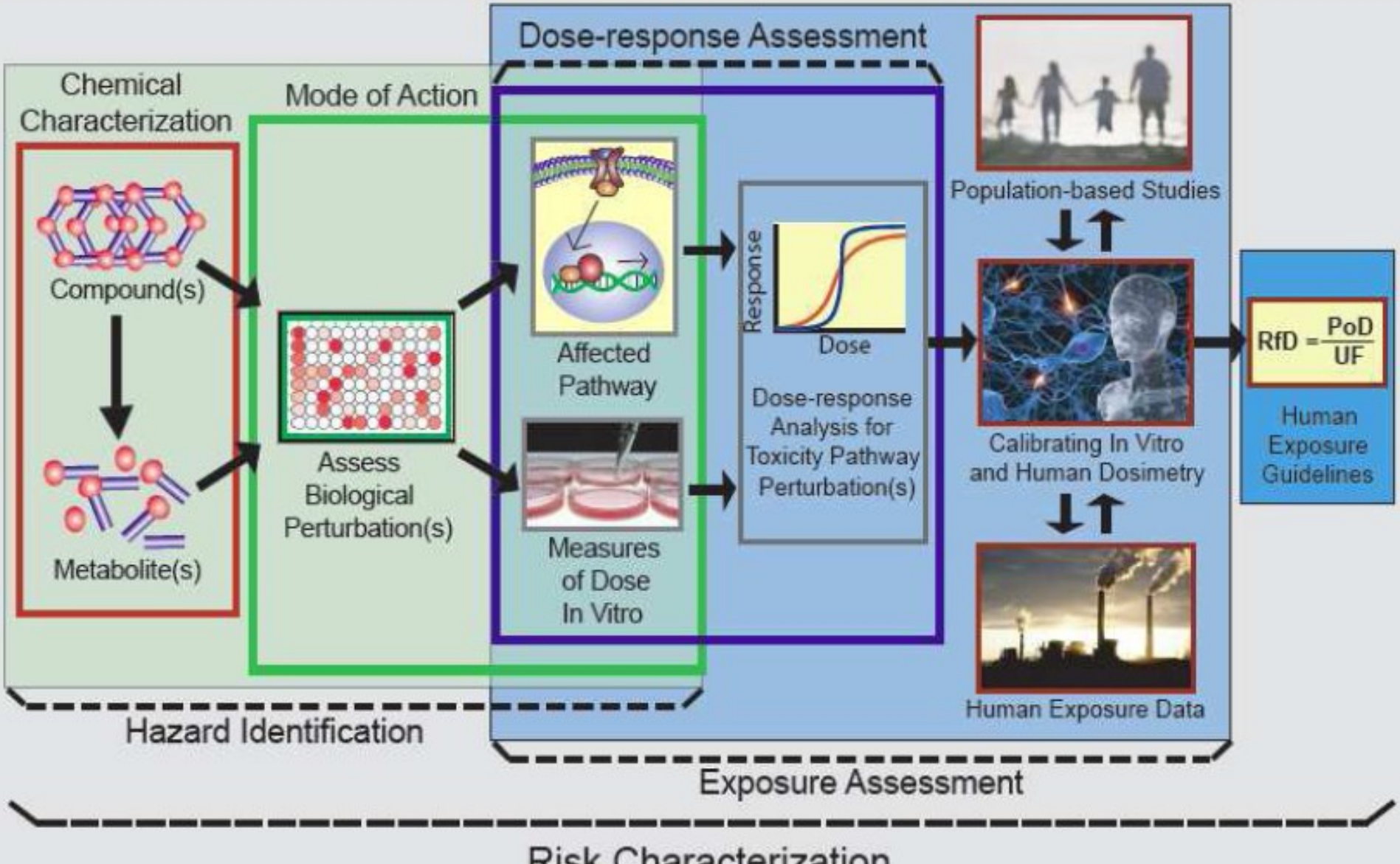


Figure 3. ToxCast™ is using a variety of HTS assays to develop bioactivity signatures that are predictive of effects in traditional toxicity testing approaches.

Toxicity Testing and Risk Assessment

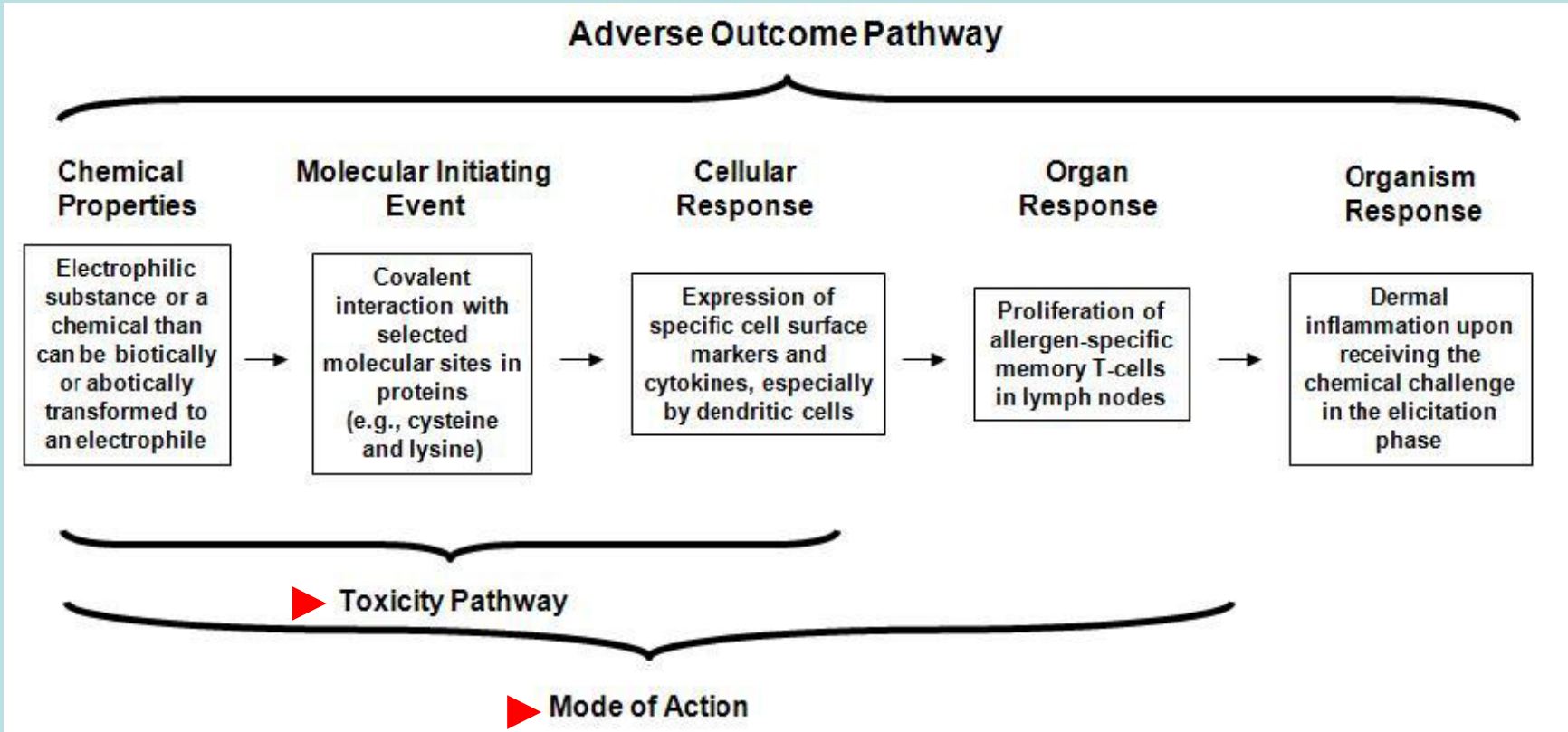
(from Krewski et al., 2010, Annual Review of Public Health, in press)



The Adverse Outcome Pathway (AOP) for Skin Sensitization

(draft; Feb 21, 2011)

- An adverse outcome pathway (AOP) is the sequence of events from chemical structure through the molecular initiating event to the *in vivo* outcome of interest.
- AOPs are representations of existing knowledge concerning the linkage(s) between a molecular initiating event and an adverse outcome at the individual or population level.
- As such, AOPs delineate the documented, plausible, and testable processes by which a chemical induces molecular perturbation and causes effects at the subcellular, cellular, tissue, organ, and whole animal levels of observation.



OECD 2012:
AOP for skin sensitization initiatiated by covalent binding to protein

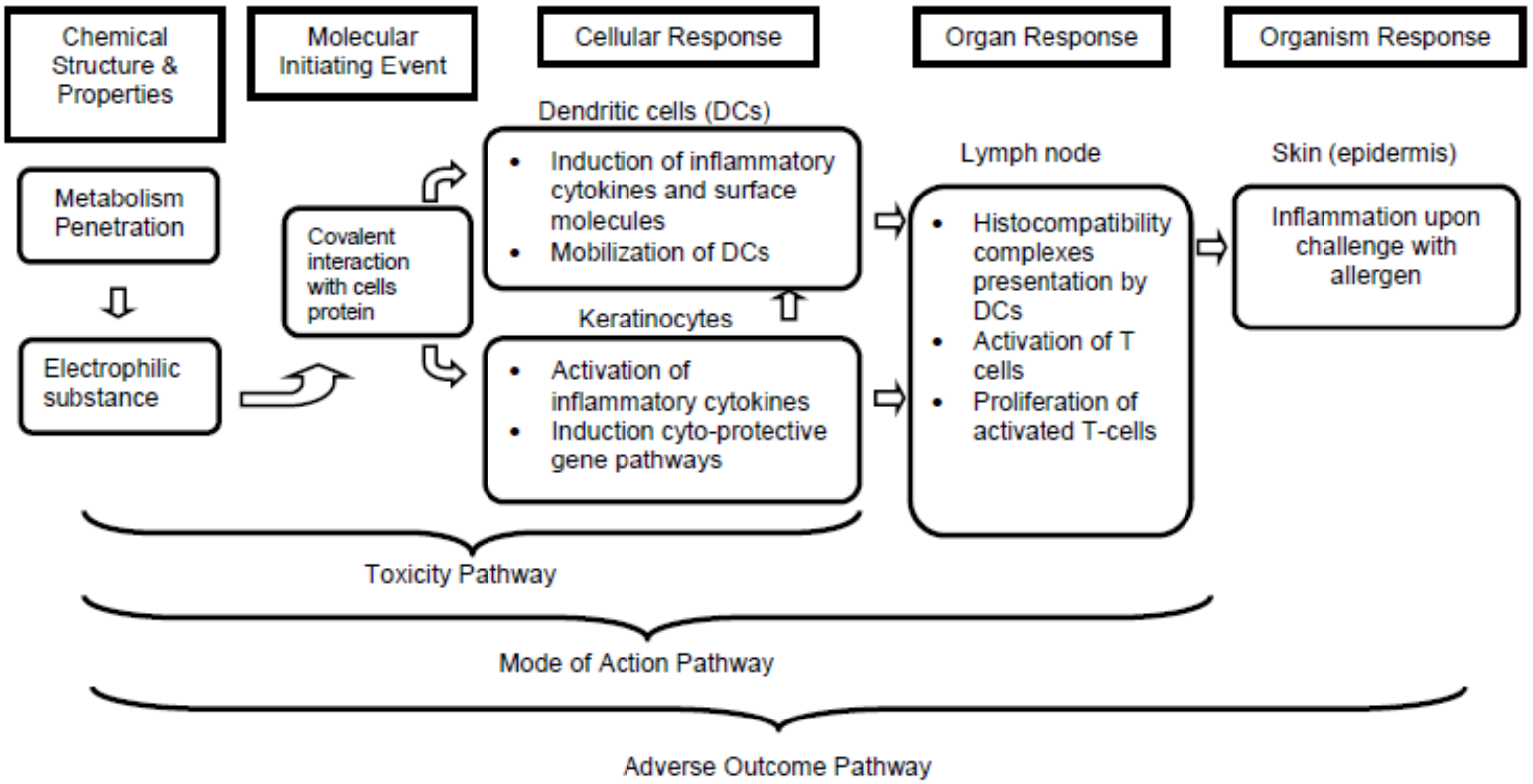


Figure 3. Flow diagram of the pathways associated with skin sensitisation.

OECD 2012: AOP for skin sensitization initiatiated by covalent binding to protein

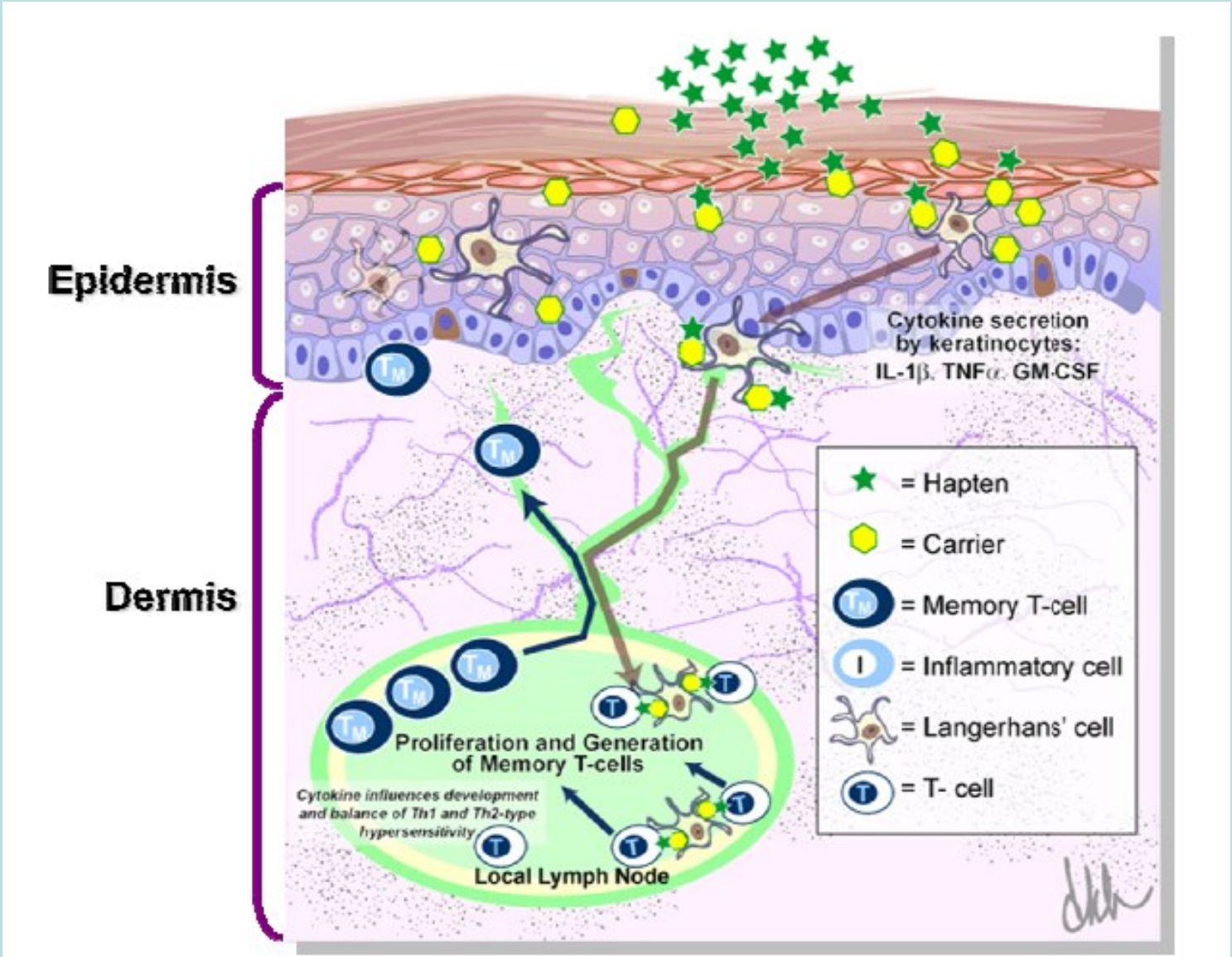


Figure 1. The Induction Phase of Skin Sensitisation.

OECD 2012: AOP for skin sensitization initiatiated by covalent binding to protein

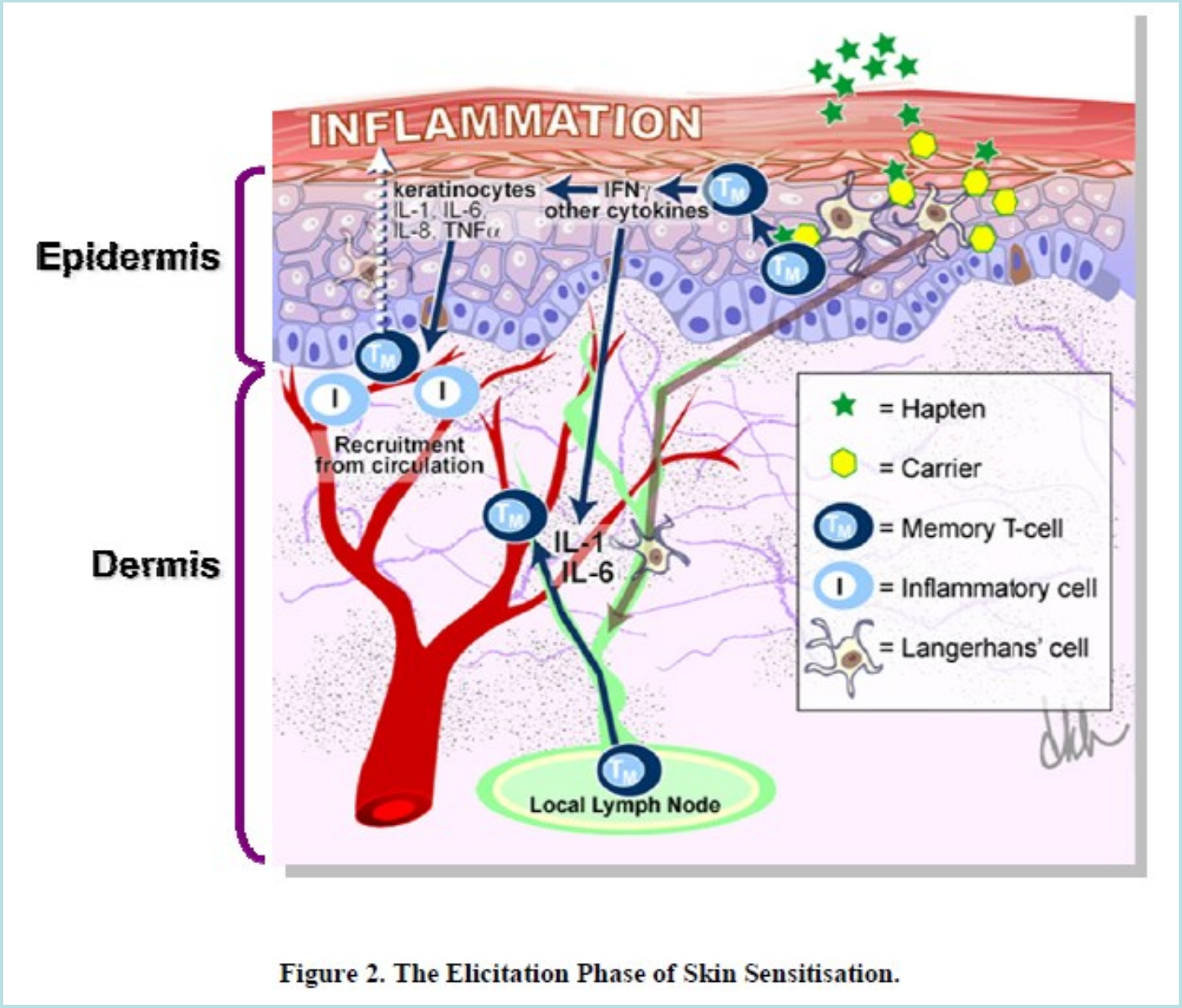
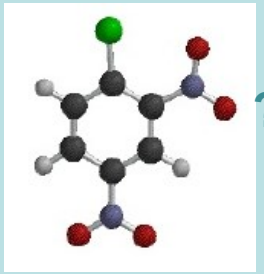


Figure 2. The Elicitation Phase of Skin Sensitisation.

Adverse Outcome Pathway (AOP) for Skin Sensitization



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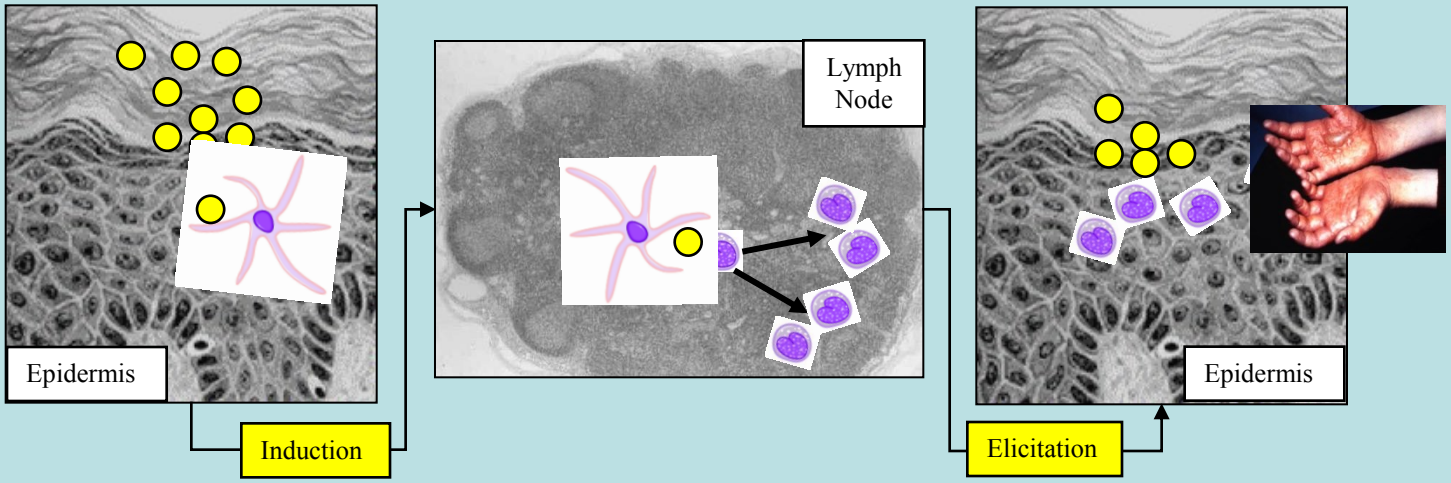
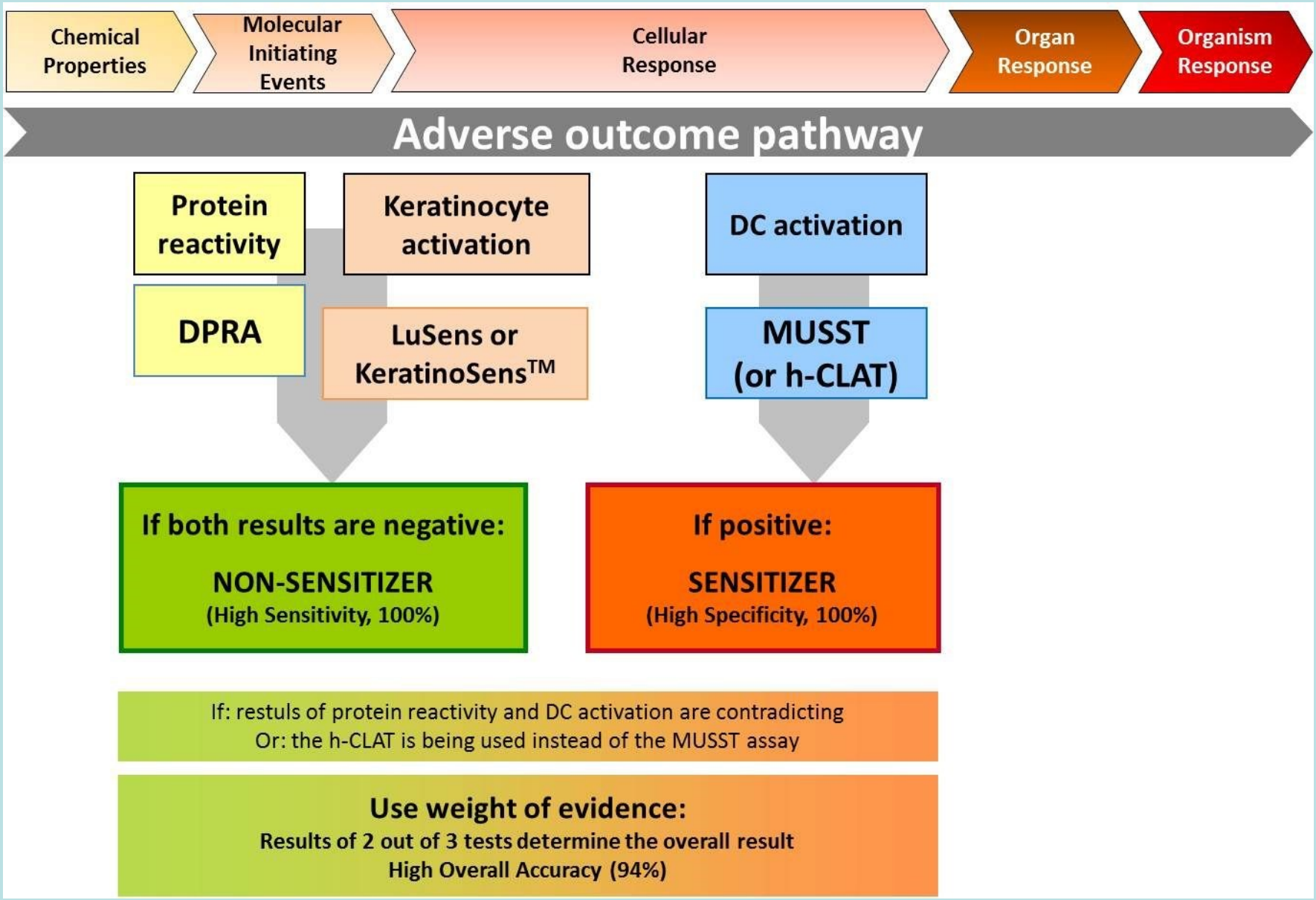


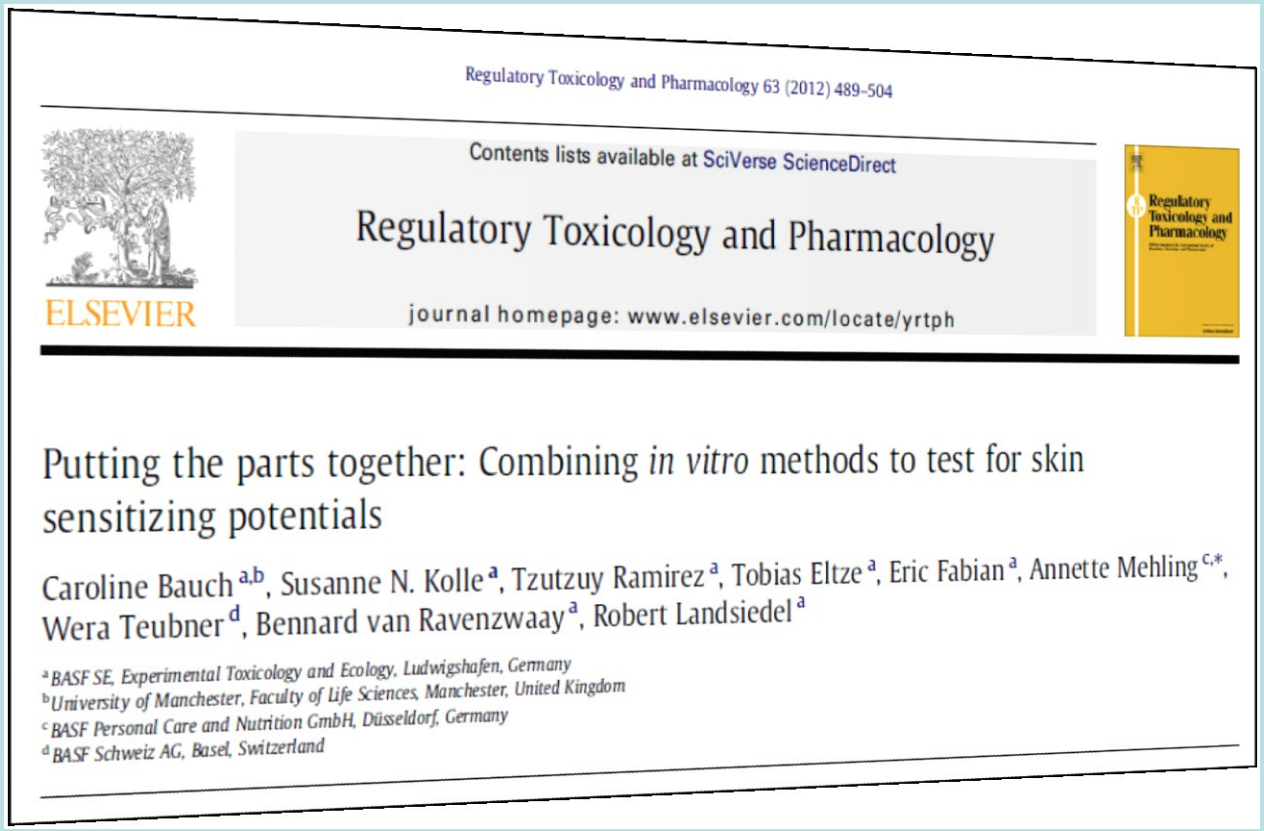
Chemical Structure & Properties	Molecular Initiating Event	Cellular Response	Organ Response	Organism Response
1. Skin Penetration 2. Electrophilic substance: directly or via auto-oxidation or metabolism	3-4. Haptenation: covalent modification of epidermal proteins	5-6. Activation of epidermal keratinocytes & Dendritic cells	7. Presentation of haptenated protein by Dendritic cell resulting in activation & proliferation of specific T cells	8-10. Allergic Contact Dermatitis: Epidermal inflammation following re-exposure to substance due to T cell-mediated cell death
	Key Event 1	**Key Event 2 + 3**	**Key Event 4**	**Adverse Outcome**

Strategy for Testing Skin Sensitisation Potential Without Animals Combining Different Methods Addressing the Adverse Outcome Pathway

Susanne Kolle and colleagues 2012 BASF



Test Battery and Weight of Evidence Assessment (Bauch *et al.* 2012)



Regulatory Toxicology and Pharmacology 63 (2012) 489–504

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potentials

Caroline Bauch [a,b], Susanne N. Kolle [a], Tzutzuy Ramirez [a], Tobias Eltze [a], Eric Fabian [a], Annette Mehling [c,*], Wera Teubner [d], Bennard van Ravenzwaay [a], Robert Landsiedel [a]

[a] BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany
[b] University of Manchester, Faculty of Life Sciences, Manchester, United Kingdom
[c] BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany
[d] BASF Schweiz AG, Basel, Switzerland

Test strategy compared to human data

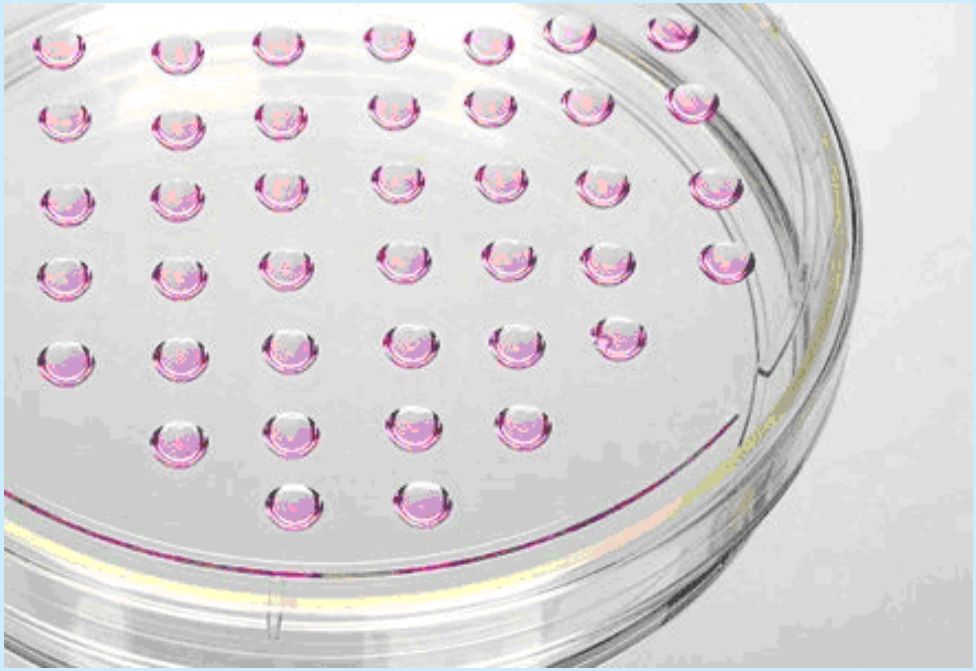
Sensitivity	93%
Specificity	95%
Accuracy	**94%**

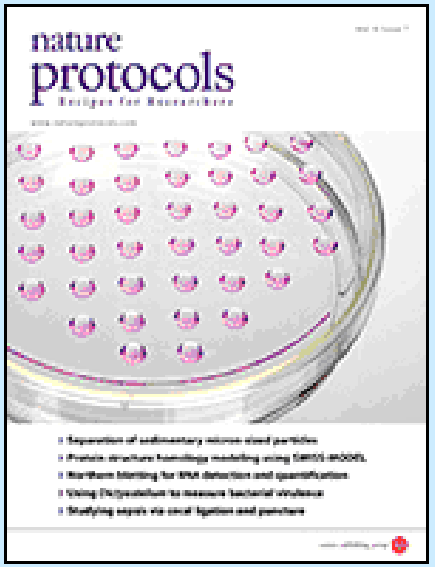
nature protocols

Nature Protocols Vol. 6, June 2011

Seiler A & Spielmann H

The validated embryonic stem cell test to predict embryotoxicity *in vitro*

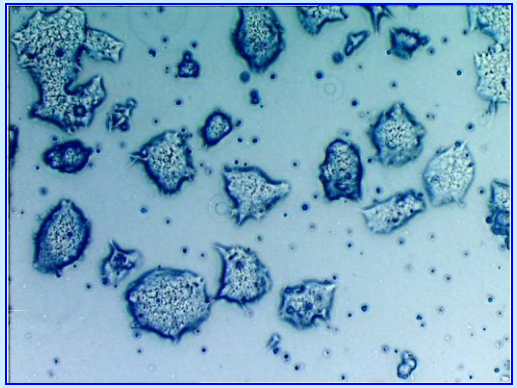




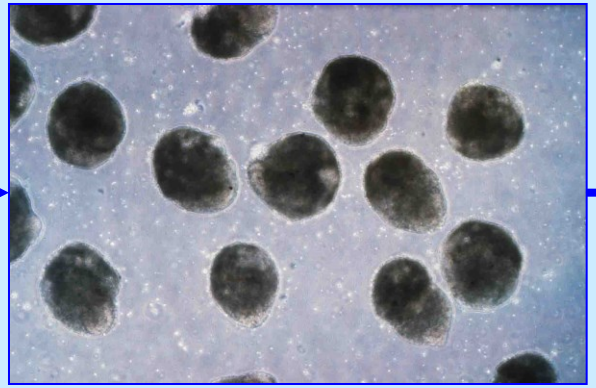
The validated embryonic stem cell test to predict embryotoxicity *in vitro* hanging drop culture. Embryoid bodies for the differentiation of embryonic stem cells in the embryonic stem cell test are generated by pipetting a single-cell suspension onto the lid of a cell culture dish. The cells aggregate at the bottom of the drop by gravitational force, thereby forming the embryoid body.

Mouse Embryonic Stem cell Test (mEST)

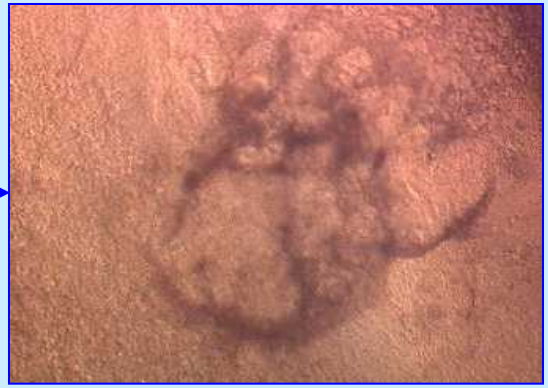
➔**Embryonic stem cells develop spontaneously into contracting myocard, this endpoint of prenatal differentiation is used in the mouse EST.**



Day 0:
undifferentiated D3-mouse ES cell line



Day 3-5:
in vitro differentiation into „embryoid bodies“



Day 5-10:
in vitro differentiation into contracting myocard

Endpoints of the Embryonic Stem Cell Test (mEST)

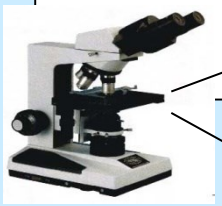
mouse ES cell line D3

differentiated cells

Endpoint 1:

inhibition of differentiation

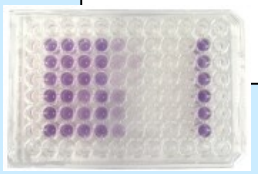
ES cells



Endpoint 2:

cytotoxic effects

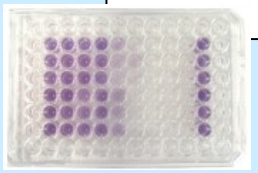
ES cells



Endpoint 3:

cytotoxic effects

3T3 cells



Endpoints: assessment from concentration response curves

1. inhibition of differentiation in ES cells ➔ ID_{50}
2. cytotoxic effects on ES cells ➔ IC_{50} D3
3. cytotoxic effects on 3T3 cells ➔ IC_{50} 3T3

Roche EST assay & Automation Project

Manual mEST assay implemented as routine screen since 2004

In Vitro - In Vivo Concordance of Proprietary and Marketed Compounds

- 36 Roche **proprietary compounds with in house in vivo data** (10+/26-)
  - *Diverse set of structures from 24 different projects*
- 16 **Marketed drugs**: known teratogens/non teratogens (11+/5-)
  - *HIV drugs, 5HT2 antagonists, kinase inhibitor, anticoagulants, statins...*

Specificity	100.00	%
Sensitivity	76.19	%
Concordance	**87.04**	%

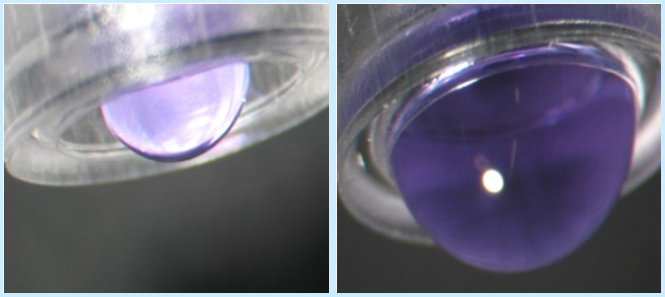
Overall, satisfactory performance of manual EST with > 85% concordance

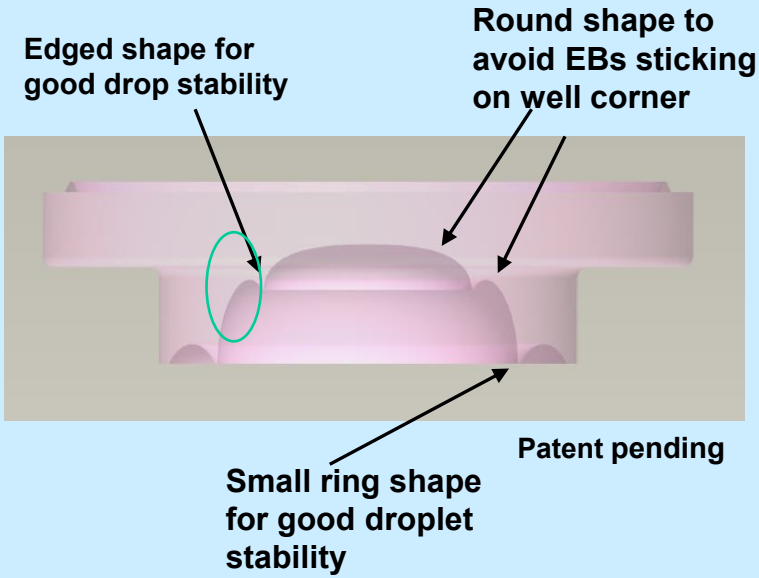
Findings from EST assay used to

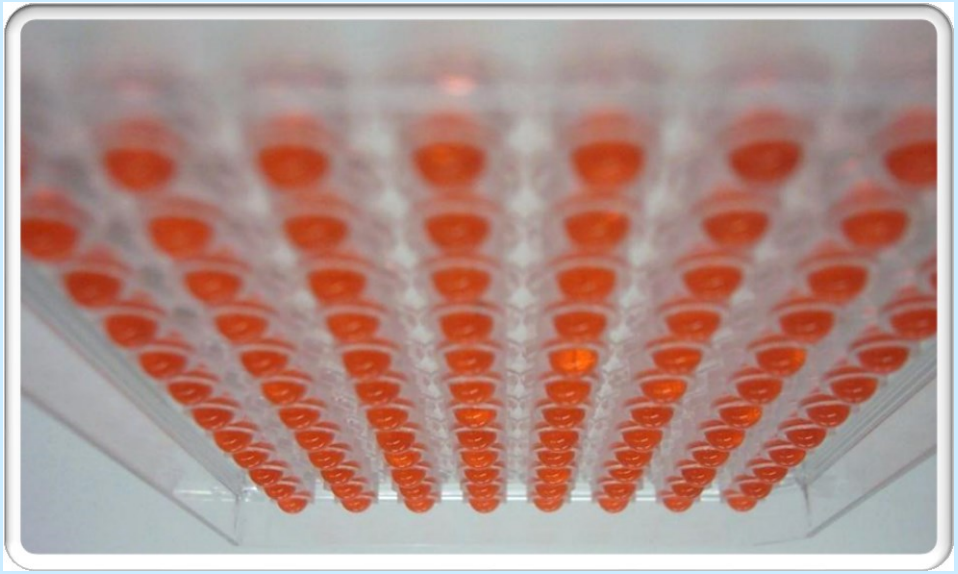
- *rank-order compounds during lead optimization*
- *follow up on in silico flags*
- *frontload in vivo studies*

Roche Hanging Droplet Culture Plate [HDCP]

- In-house developed injection-molded proprietary labware for culturing cells in 3D
- Precise engineering allows excellent droplet stability
- 24well currently used for automated EST assay.
- 96well design finalized & manufactured. Implementation ongoing.







Patent pending

Slide Claudia McGinnis

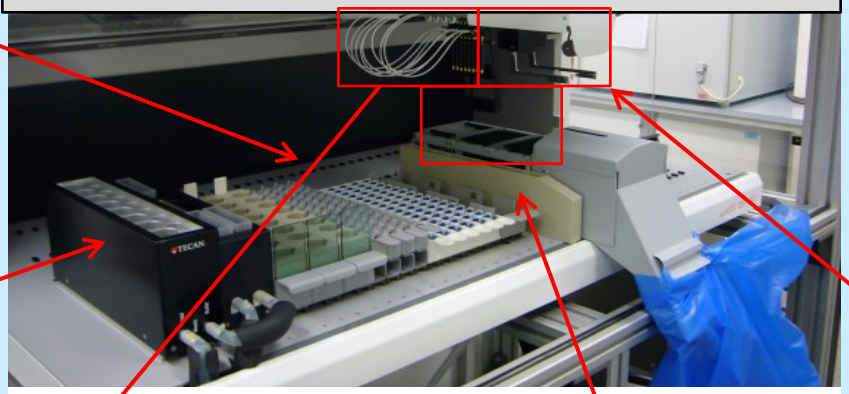
Automated EST Platform Set-up at Roche

Implemented since Q1, 2012

Centrifuge spins down EB into well for adherence and further differentiation

Staeubli Robotic Arm turns HDCP lid over for liquid handling /media changes

Compound liquid handling for preparing serial dilutions and compound plates



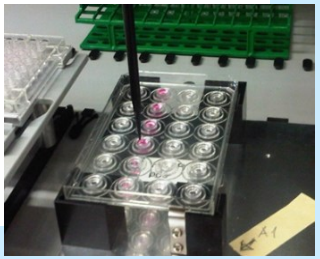
Cytomax Incubator for 10 d cytotox/ differentiation incubations

Warming rack for buffer, media, cells

RoMa Arm moves plates in/out of incubator

8-channel pipettor uses sterile, disposable tips

Position for HDCP plate for EB culture/differentiation



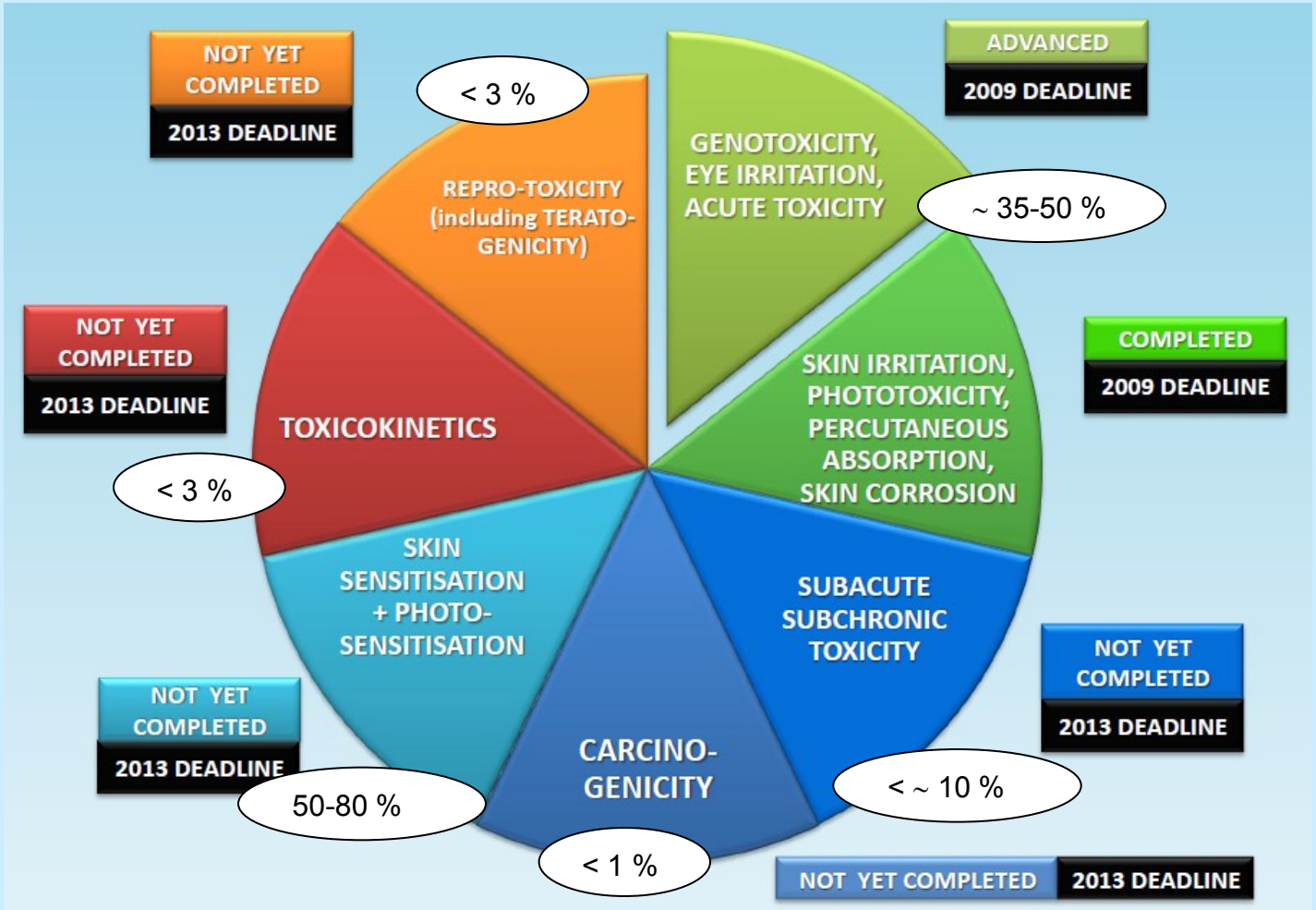
Slide Claudia McGinnis

Roche

pRED Non-Clinical Safety

Pharma Research & Early Development

Status of Science 2013



EUROPEAN COMMISSION

PRESS RELEASE

Brussels, 11 March 2013

Full EU ban on animal testing for cosmetics enters into force

Today the last deadline to phase out animal testing for cosmetic products in Europe enters into force. As of today, cosmetics tested on animals cannot be marketed any more in the EU.

A Communication adopted by the Commission today confirms the Commission's commitment to respect the deadline set by Council and Parliament in 2003 and outlines how it intends to further support research and innovation in this area while promoting animal welfare world-wide.

European Commissioner in charge of Health & Consumer Policy, Tonio Borg, stated: *"Today's entry into force of the full marketing ban gives an important signal on the value that Europe attaches to animal welfare. The Commission is committed to continue supporting the development of alternative methods and to engage with third countries to follow our European approach. This is a great opportunity for Europe to set an example of responsible innovation in cosmetics without any compromise on consumer safety."*

The Commission has thoroughly assessed the impacts of the marketing ban and considers that there are overriding reasons to implement it. This is in line with what many European citizens believe firmly: that the **development of cosmetics does not warrant animal testing**