UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI FARMACIA

METODI IN VITRO CONVALIDATI E IN CONVALIDA

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DiSFeB

SCHEMA DELLA PRESENTAZIONE

- **Perchè sviluppare metodi alternativi**
- **Definizione di metodo alternativo**
- **Convalida di un metodo alternativo**
- **Metodi convalidati**
- **Metodi in convalida**

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PERCHE' LO SVILUPPO DI METODI ALTERNATIVI

1. Validita' scientifica dei test in vivo.

2. Alti costi della sperimentazione.

3. Tempi lunghi della sperimentazione.

4. Opinione pubblica contraria per ragioni 'etiche' alla sperimentazione animale.

5. Divieto dell'uso nei prodotti cosmetici di ingredienti o di combinazioni di ingredienti sperimentati su animali: 11/9/2004 per i prodotti finiti e 11/3/2009-2013 per gli ingredienti.

6. REACH (Registration, Evaluation and Authorization of CHemicals): new legislation on chemicals.

7. TOX21

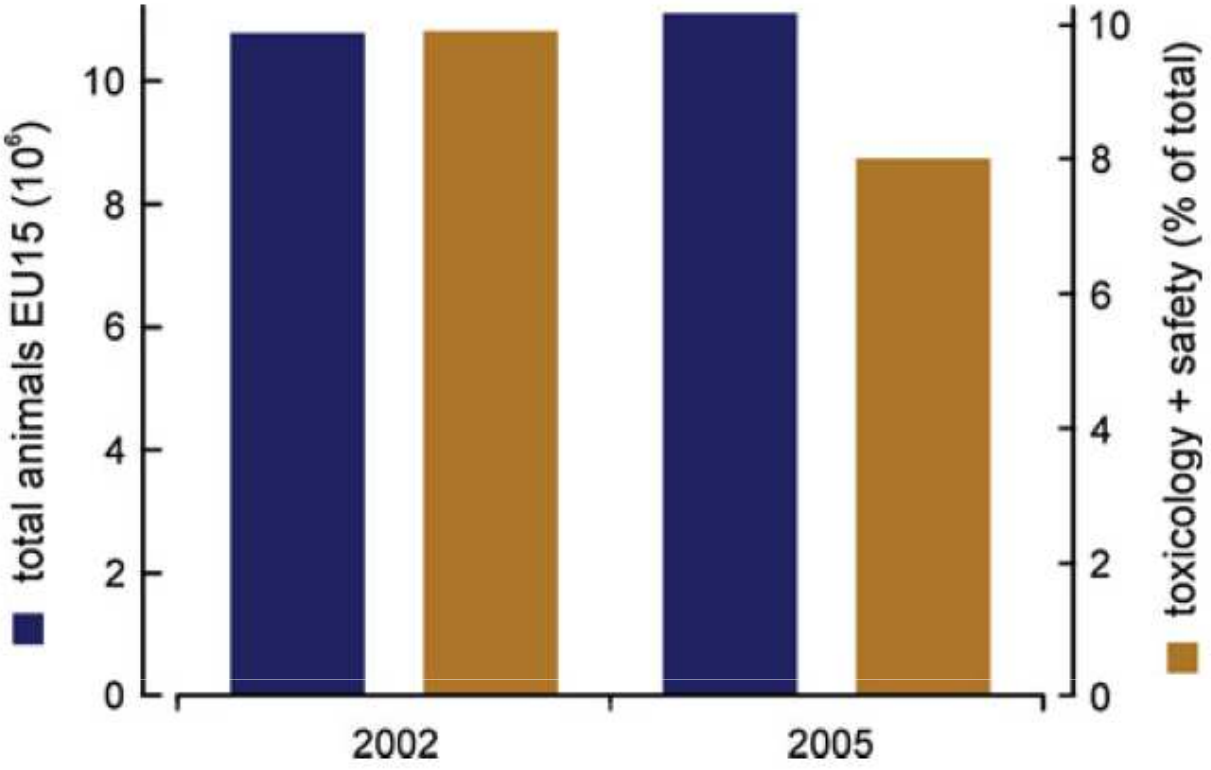

Fig. 5 Comparison of animal numbers used for scientific purposes in Europe. *Blue bars* depict total animal numbers in million animals for the 15 European member states reporting in 2002 and 2005. *Orange bars* show percentages of animals used for toxicology and other safety evaluation purposes in the 15 reporting members for 2002 and the 25 reporting states for 2005. Animal numbers are according to the fifth report on the statistics on the number of animals used for experimental and other scientific purposes in the member states of the European Union (Commission of the European Communities 2007)

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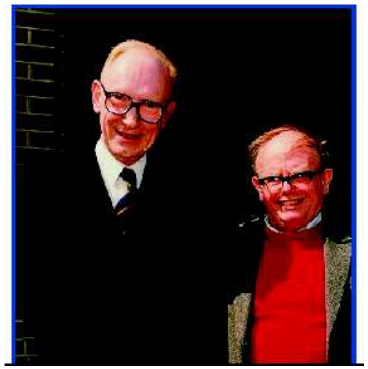
DEFINIZIONE DI METODO ALTERNATIVO

D. Smyth nel libro "Alternative to Animal Experiments" (1978):

...*ALTERNATIVES*...all procedures which can completely replace the need for animal experiments, reduce the number of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs for man and other animals..



L'utilizzo dei metodi alternativi in tossicologia rientra nell'applicazione del concetto delle "R", espresso da Russel e Burch nel 1959.

- **REDUCTION:** qualsiasi mezzo utile al fine di ridurre il numero di animali necessari per la sperimentazione, garantendo però la qualità del risultato.

- **REFINEMENT:** qualsiasi miglioramento delle tecniche sperimentali, che consente la riduzione delle eventuali sofferenze inflitte agli animali.

REPLACEMENT: la sostituzione di un metodo che prevede l'uso di animali con un metodo che prevede l'uso di 'materiale non dotato di senso' (es. metodi *in vitro*).

Assoluto: l'animale non viene usato in nessuna fase dell'esperimento

Relativo: l'animale viene usato per prelevare un organo o un tessuto per preparare la coltura primaria

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Regulators will only accept alternatives to animal tests in toxicology, if they will allow them to classify and label chemicals in the same way as the current tests.

The OECD has therefore decided that **in vitro toxicity tests can be accepted for regulatory purposes only after a successful experimental validation study**.

OECD = Organization for Economic Cooperation and Development

Chi si occupa in Europa della validazione?

Missione di EURL-ECVAM

European Union Reference Laboratory for alternatives to animal testing

Direttiva 86/609/CEE

- **Studi di validazione**
- **Banca dati**
- **Comunicazione**
- **Ricerca**

ESAC

ESAC = ECVAM Scientific Advisory Committee

INTERNATIONAL CENTRES FOR ALTERNATIVES TO ANIMAL EXPERIMENTS

1981

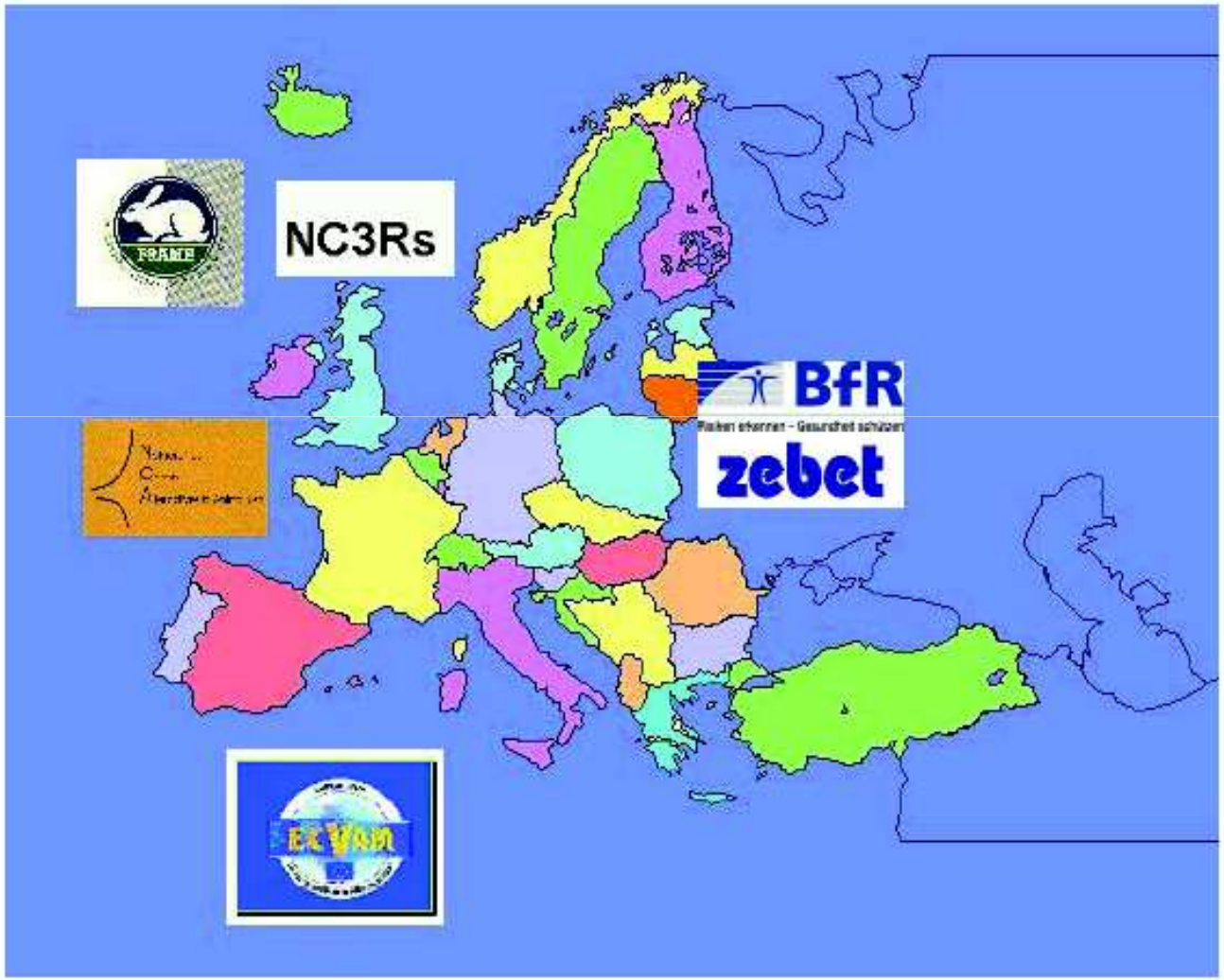
CAAT
Hopkins

1998

ICCVAM
USA

2005

JaCVAM
Japan



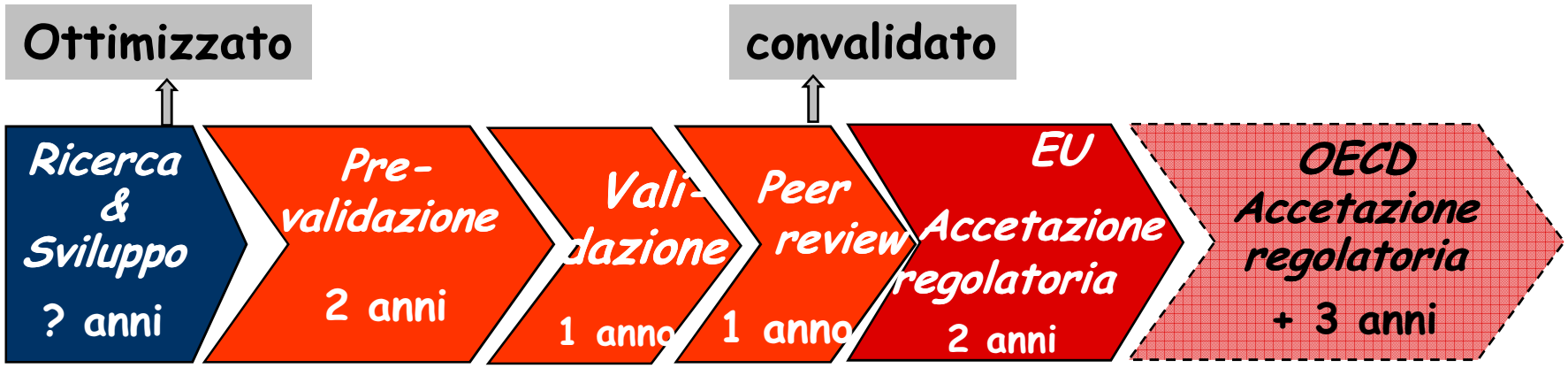
2001

vitryna

POLAND

➢ Processo di validazione

PROCESSO DI VALIDAZIONE

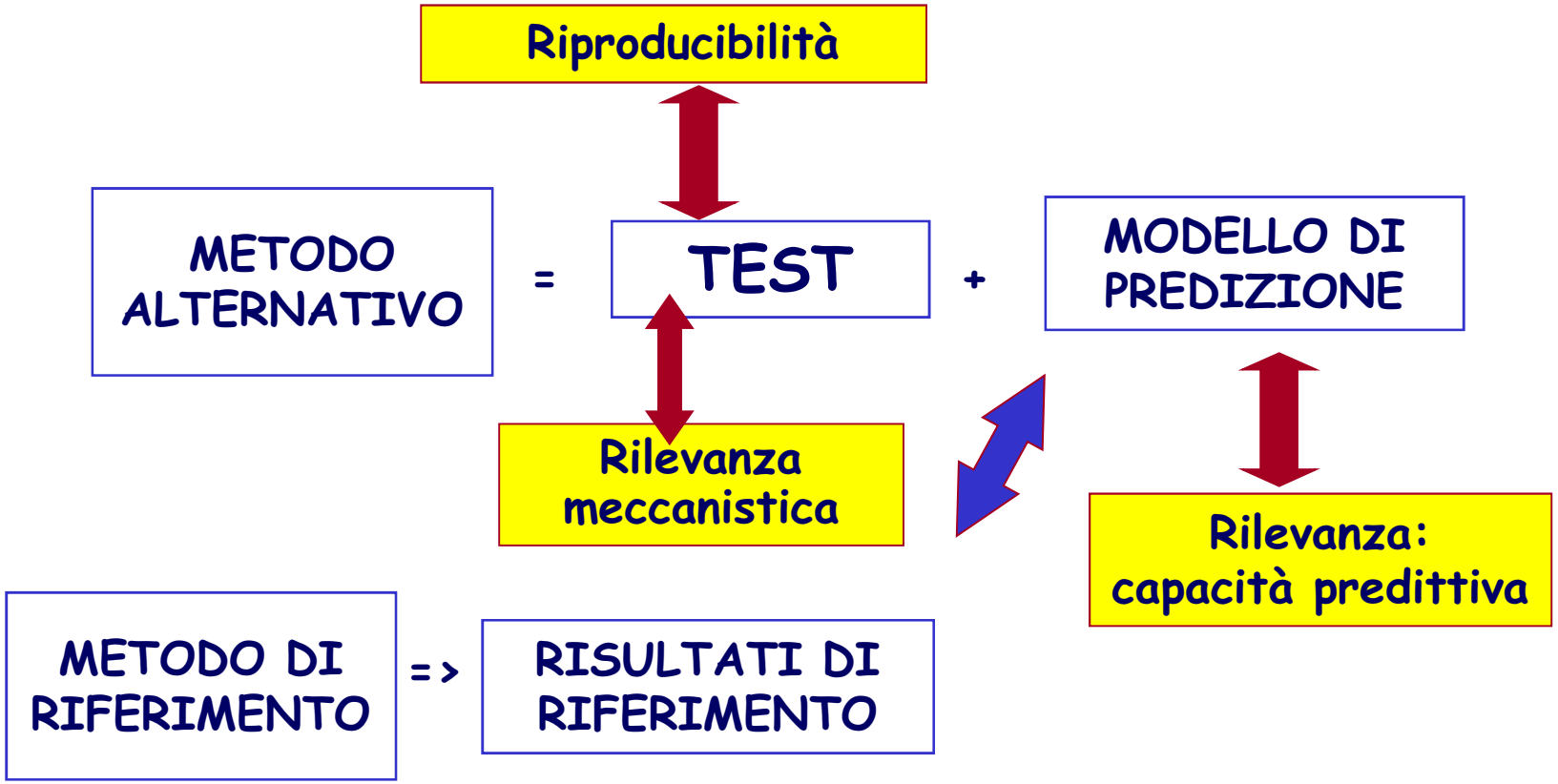


DG RTD

DG JRC
ECVAM

Policy-DGs
ECB (DG JRC)

Cos'è il processo di validazione?



Processo attaverso cui viene stabilita la riproducibilita' e la rilevanza di un test per uno scopo specifico

Tipi di Studi di Validazione

PREVALIDAZIONE studio in scala ridotta che coinvolge diversi laboratori, eseguito per valutare se il protocollo di un metodo e' sufficientemente ottimizzato e standadizzato per essere incluso in uno studio di validazione formale

VALIDAZIONE studio su larga scala che coinvolge diversi laboratori progettato per valutare la riproducibilita' e la rilevanza di un metodo ottimizzato per uno scopo preciso

VALIDAZIONE RETROSPETTIVA (weight of evidence validation) consiste in una valutazione retrospettiva di dati esistenti ottenuti da metodi in uso

VALIDAZIONE CATCH-UP e' una validazione in cui la performance di un metodo viene valutata in base alla comparazione con un metodo che e' gia' stato validato

ECVAM's Criteria For Validation

1. Un test alternativo viene considerato valido solo se le seguenti due condizioni sono soddisfatte:
   a) Il metodo e' riproducibile
   b) Il metodo e' rilevante

2 Il modello predittivo deve essere stato sviluppato prima dello studio di validazione

3. La performance del test viene valutata usando sostanze codificate

4. Ci deve essere indipendenza nella:
   a) gestione dello studio
   b) selezione, codifica e distribuzione delle sostanze chimiche
   c) raccolta dei dati e analisi statistica

5. Gli studi devono essere eseguiti in conformita' ai principi di GLP e GCCP

CRITERIA FOR PREDICTION MODELS

Un modello predittivo viene considerato adeguato quando:

1) e' associato ad un protocollo specifico

2) e' associato ad un effetto tossicologico specifico

3) ha le sue limitazioni ben definite

4) e' associato ad un indicazione dell'accuratezza della sue predizioni

Un modello predittivo viene considerato valido quando:

1) Viene valutato con dati indipendenti (le sostanze usate per derivare i dati per la valutazione del modello # da quelle usate per derivare i dati utilizzati per la definizione del modello)

2) Soddisfare o eccedere la capacita' predittiva definita dal management team di uno studio di validazione

Bruner *et al*. (1996). *Toxicology in Vitro* **10**, 479-501.
Worth & Balls (2001). *ATLA* **29**, 135-143.

Parametri per la valutazione della performance di un test

- **Riproducibilita'**

  quantitativa

  qualitativa: accordo tra la classificazione ottenuta nei diversi laboratori

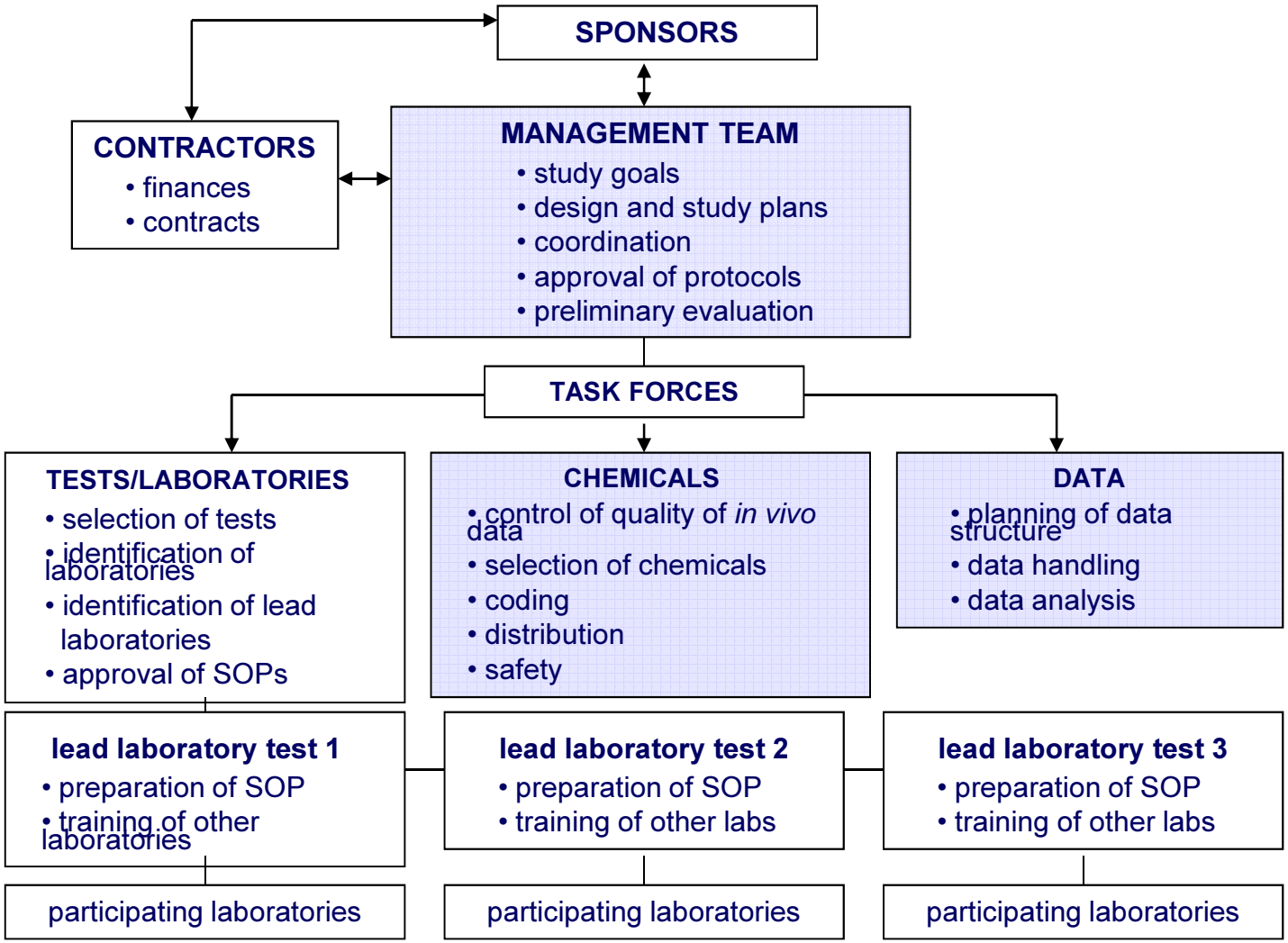
- **Capacita' predittiva**
  - **Sensitivity**: la percentuale delle sostanze positive/attive correttamente identificate come tali dal test
  - **Specificity**: la percentuale di sostanze negative/inattive correttamente identificate dal test

Test ideale: 100% sensitivity, 100% specificity

<sensitivity > falsi negativi

<specificity > falsi positivi

Validation Study: Management e Organizzazione



Reference: Balls *et al*. (1995). *ATLA* **23,** 129-147.

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UNA PREMESSA AI METODI IN VITRO

MESSA A PUNTO DI UN METODO *IN VITRO*

- Tipo di tossicita': es. irritazione oculare, irritazione cutanea, epatotossicita', neurotossicita'.
- Livello di tossicita': es. potenziale (tossicita' intrinseca), potenza (relativa alla tossicita'), pericolo (tossicita' in determinate condizioni d'esposizione), rischio (sicurezza nell'uso).
- Tipo di test: es. screening, aggiuntivo/complementare, sostituzione al test in vivo.
- Spettro chimico: es. tutte le sostanze, particolari classi.

CARATTERISTICHE IDEALI DI UN METODO *IN VITRO*

- ☒ Rispondere a differenti classi chimiche
- ☒ Misurare parametri rilevanti per la tossicita' *in vivo*
- ☒ Buona riproducibilita' intra e interlaboratorio
- ☒ Di facile esecuzione, ecc..

UTILIZZO DELLE COLTURE CELLULARI IN TOSSICOLOGIA

- studi sul metabolismo
- studi sul meccanismo d'azione
- messa a punto di metodi alternativi in vitro

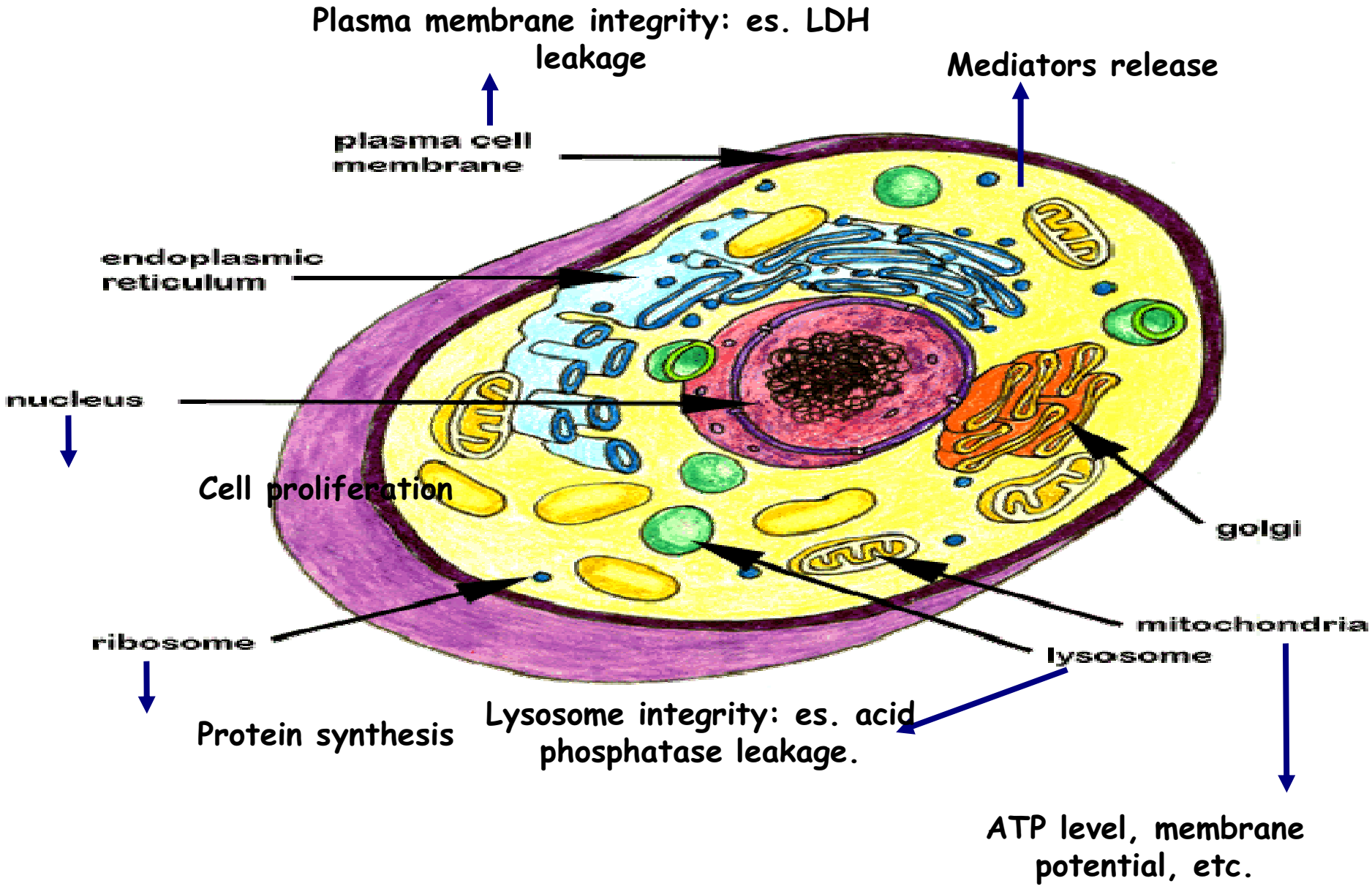
VANTAGGI

- *Riproducibilita'*
- *Sensibilita'*
- *Semplicita'*
- *Costi ridotti*
- *Controllo delle condizioni di coltura*

SVANTAGGI

- *Metabolismo*
- *Solubilita'*
- *Estrapolazione*
- *Organizzazione tissutale persa*

TOSSICITA' IN VITRO: cosa fare?

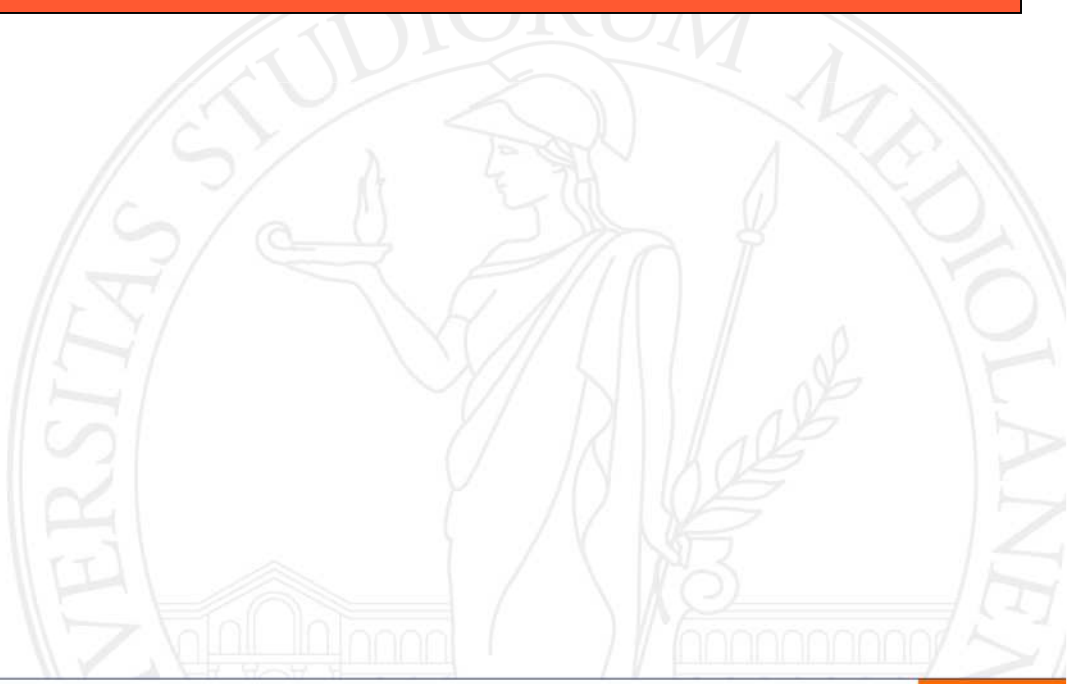


Strategy for the development of *in vitro* tests

- **Exploiting current knowledge:**
  translating *in vivo* data into *in vitro* assays

- **In search of the unexpected:**
  identification of new genes selectively induced by skin toxicants

METODI CONVALIDATI



Test convalidati utilizzabili nella valutazione del rischio a livello europeo
(Annex V, Directive 67/548/EEC)

- Corrosione (ATLA 26: 275-280, 1998):

  1) TER, resistenza elettrica transcutanea in espianti di cute di ratto;

  2) epidermide umana ricostituita e valutazione della vitalità cellulare (vitalità a 1h < 15% la sostanza viene classificata come corrosiva);

  3) CORROSITEX™ (ICCVAM), che valuta cambiamenti di colore e/o fisici in una matrice di collagene.

- Fototossicità (ATLA 26: 7-8, 1998): (fotoirritazione)

  1) Uptake del rosso neutro nelle 3T3;

  2) epidermide ricostituita e vitalità cellulare (se si osserva una riduzione del 30% dopo esposizione a UVA, allora la sostanza è potenzialmente fototossica).

- Irritazione cutanea: epidermide umana ricostituita

- Irritazione oculare

Metodi alternativi scientificamente validati e accettati

AltTox.ORG

End-point and Method Name		Test Type[1]	Endorsement of Scientific Validity		Regulatory Acceptance	
			Lead Authority	Subsequent Endorsement	National/ Regional (for methods not yet accepted internationally)	International acceptance
Acute aquatic toxicity						
	Upper threshold concentration step-down approach	*In vivo*	ESAC (2006)			
Acute mammalian toxicity (oral)						
	Acute toxic class method	*In vivo*		ESAC (2007)		OECD TG 423 (2001)
	Fixed dose procedure	*In vivo*		ESAC (2007)		OECD TG 420 (2001)
	Up-and-down procedure	*In vivo*	ICCVAM (2001)	ESAC (2007)		OECD TG 425 (2006)
	Normal human keratinocyte neutral red uptake (NHK NRU) assay	*In vitro*[2]	ICCVAM (2006)		US agencies (2008)	Draft OECD TG
	Balb/c 3T3 neutral red uptake assay	*In vitro*[2]	ICCVAM (2006)		US agencies (2008)	Draft OECD TG
Acute mammalian toxicity (inhalation)						
	Acute toxic class method	*In vivo*				OECD TG 436
	Fixed concentration procedure	*In vivo*				Draft TG OECD 433
Chronic toxicity						
	Ending 1-year dog studies of pesticides	*In vivo*	ESAC (2006)		Revised US EPA Pesticide Data Requirements	

With minor modification from ALTTOX

End-point and Method Name		Test Type[1]	Endorsement of Scientific Validity		Regulatory Acceptance National	International
Dermal penetration						
	In vitro skin absorption methods	*In vitro ex-vivo*	OECD Expert Group (2002)			OECD TG 428(2004)
Endocrine mechanistic screens						
	Androgen receptor binding assay (rat prostate)	*In vitro*			OPPTS TG 890.1150 (EPA, 2009)	
	Aromatase inhibition assay (human recombinant)	*In vitro*			OPPTS TG 890.1200 (EPA, 2009)	
	ER-alpha transcriptional activation assay for estrogen agnoists[1]	*In vitro*				OECD TG 455 (2009)
	Estrogen receptor binding assay	*In vitro*			OPPTS TG 890.1250 (EPA, 2009)	
	Steroidogenesis (H295R human cell line)	*In vitro*			OPPTS TG 890.1550 (EPA, 2009)	Draft OECD TG
	US EPA Tier 1 Screening Battery	*In vitro/In vivo*			US EPA (2009)	
Eye corrosion						
	Bovine corneal opacity permeability (BCOP) test	*Ex-vivo*	ICCVAM (2007)	ESAC (2007) JaCVAM (2009)		OECD TG 437 (2009)
	Isolated chicken eye (ICE) test	*Ex-vivo*	ICCVAM (2007)	ESAC (2007) JaCVAM (2009)		OECD TG 438 (2009)
	Hen's egg test-chorioallantoic membrane (HET-CAM)	*In vitro/ Ex-vivo*			EU Competent Authorities for Dangerous Substances Directive	
	Isolated rabbit eye test	*Ex-vivo*			EU Competent Authorities for Dangerous Substances Directive	

With minor modification from ALTTOX

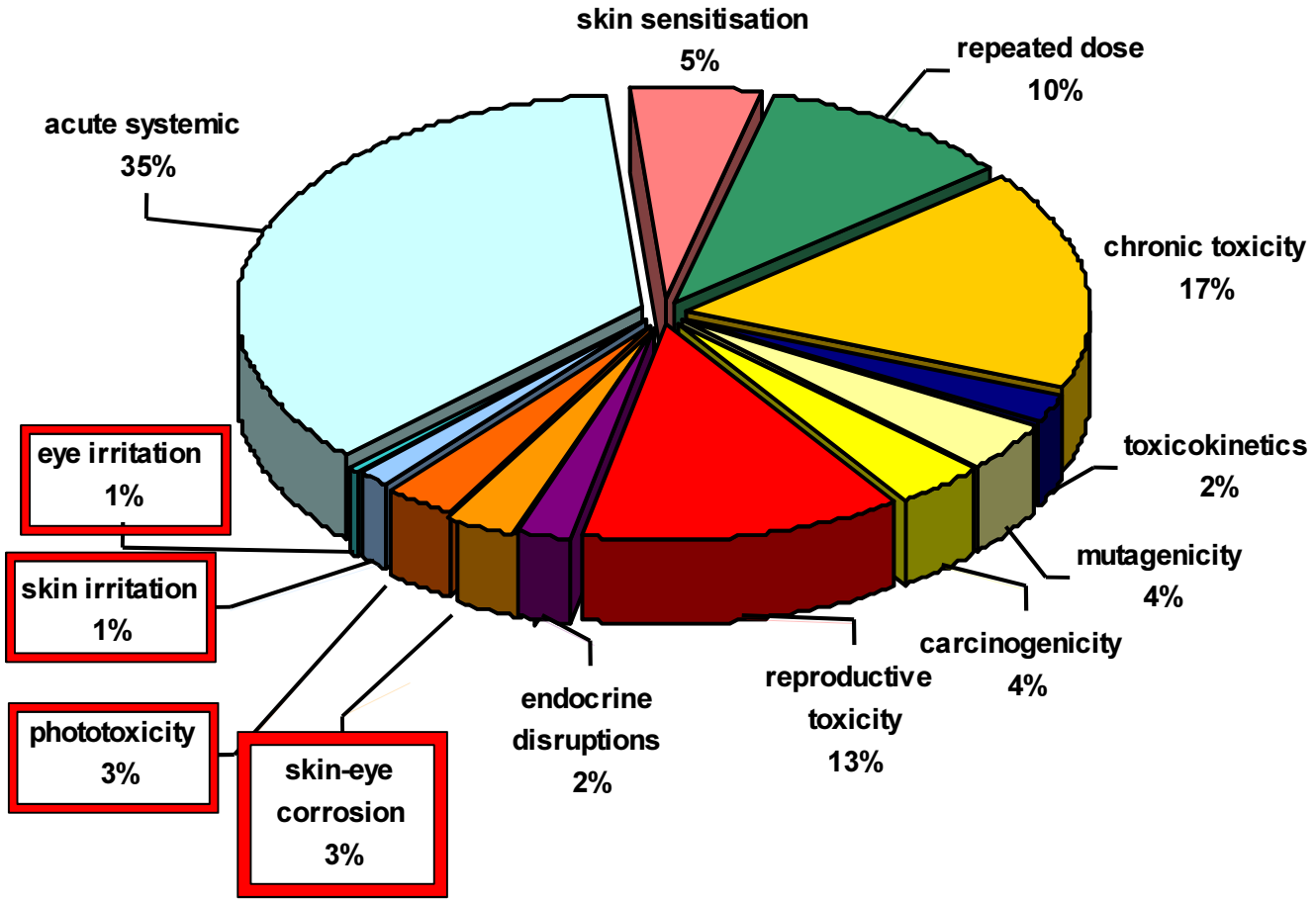
End-point and Method Name		Test Type[1]	Endorsement of Scientific Validity		Regulatory Acceptance National	International
Eye irritation						
	Cytosensor Microphysiometer modified (cytotoxicity/cell-function based *in vitro* assay)	*In vitro*	ESAC (2009)			
	Cytotoxicity/cell-function based *in vitro* assay: Fluorescein Leakage	*In vitro*	ESAC (2009)			
Genotoxicity						
	Bacterial reverse mutation (Ames) test	*In vitro*				OECD TG 471 (1997)
	In vitro cell gene mutation test	*In vitro*				OECD TG 476 (1997)
	In vitro chromosomal aberration test	*In vitro*				OECD TG 473 (1997)
	In vitro micronucleus test	*In vitro*	ESAC (2006)			Draft OECD TG 487
	In vitro sister chromatid exchange test	*In vitro*				OECD TG 479 (1986)
	In vitro unscheduled DNA synthesis test	*In vitro*				OECD TG 482 (1986)
	Saccharomyces cerevisiae gene mutation assay	*In vitro*				OECD TG 480 (1986)
	Saacharomyces cerevisiae mitotic recombination assay	*In vitro*				OECD TG 481 (1986)

With minor modification from ALTTOX

End-point and Method Name		Test Type[1]	Endorsement of Scientific Validity		Regulatory Acceptance National	International
Hematotoxicity: acute neutropenia						
	Colony forming unit granulocyte macrophage (CFU-GM) assay	*In vitro*	ESAC (2006)			
Phototoxicity						
	3T3 NRU Phototoxicity Test	*In vitro*	ESAC (1997)			OECD TG 432 (2004)
	3T3 NRU Phototoxicity Test: Application to UV filter chemicals	*In vitro*	ESAC (1998)			OECD TG 432 (2004)
Pyrogenicity						
	Human whole blood IL-1	*In vitro*	ESAC (2006)	ICCVAM (2008)[4]	European Pharmacopeia; US agencies	
	Human whole blood IL-6	*In vitro*	ESAC (2006)	ICCVAM (2008)[4]	European Pharmacopeia; US agencies	
	Human cryopreserved whole blood IL-1	*In vitro*	ESAC (2006)	ICCVAM (2008)[4]	European Pharmacopeia; US agencies	
	PBMC IL-6	*In vitro*	ESAC (2006)	ICCVAM (2008)[4]	European Pharmacopeia; US agencies	
	MM6 IL-6	*In vitro*	ESAC (2006)	ICCVAM (2008)[4]	European Pharmacopeia; US agencies	
	Limulus amebocyte lysate (LAL) test	*In vitro*			EDQM/European, Pharmacopeia;US Pharmacopeia	
Reproductive & developmental toxicity						
	Embryonic stem cell test	*In vitro*	ESAC (2002)			
	Micromass assay	*Ex vivo*	ESAC (2002)			
	Whole rat embryo assay	*Ex vivo*	ESAC (2002)			
Skin corrosion						
	Rat skin transcutaneous electrical resistance (TER)assay	*Ex vivo*	ESAC (1998)	ICCVAM (2002)		OECD TG 430 (2004)
	Corrositex® noncellular membrane	*In vitro*	ICCVAM (1999)	ESAC (2000)		OECD TG 435 (2006)
	EpiSkin® human skin model	*In vitro*	ESAC (1998)	ICCVAM (2002)		OECD TG 431 (2004)
	EpiDerm™ human skin model	*In vitro*	ESAC (1998)	ICCVAM (2002)		OECD TG 431 (2004)
	EST-1000 human reconstructed epidermis	*In vitro*	ESAC (2009)			OECD TG 431 (2004)
	SkinEthic™ human skin model	*In vitro*	ESAC (2006)			OECD TG 431 (2004)

End-point and Method Name		Test Type[4]	Endorsement of Scientific Validity		Regulatory Acceptance National	International
Skin irritation						
	EpiSkin® skin irritation test	*In vitro*	ESAC (2007)		EU test method B.46 in COM regulation 440/2008/EC	Draft OECD TG 439
	EpiDerm™ skin irritation test	*In vitro*	ESAC (2007)[5]		EU test method B.46 in COM regulation 440/2008/EC	Draft OECD TG 439
	EpiDerm™ Modified SIT	*In vitro*	ESAC (2008)		EU test method B.46 in COM regulation 440/2008/EC	Draft OECD TG 439
	SkinEthic RHE model	*In vitro*	ESAC (2008)		EU test method B.46 in COM regulation 440/2008/EC	Draft OECD TG 439
Skin sensitization						
	Reduced LLNA	*In vivo*	ESAC (2007)	ICCVAM (2009)		
	Local lymph node assay (LLNA)	*In vivo*	ICCVAM (1999)	ESAC (1999)		OECD TG 429 (2002)
	Nonradiolabelled LLNA: DA	*In vivo*	ICCVAM (2009)[6]	JaCVAM (2008)		Draft OECD TG
	LLNA: BrdU-ELISA	*In vivo*	ICCVAM (2009)[6]			Draft OECD TG
Vaccine potency						
	ELISA for erysipelas vaccines batch potency testing	*In vitro*	ESAC (2002)		EDQM/European Pharmacopeia	
	ELISA for human tetanus vaccines batch potency testing	*In vitro*	ESAC (2000)		EDQM/European Pharmacopeia	
	Toxin binding inhibition test for human tetanus vaccines batch potency testing	*In vitro*	ESAC (2000)		EDQM/European Pharmacopeia	

Animals used in Toxicology per Year



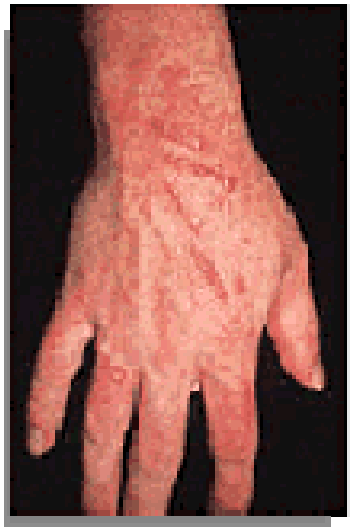
3rd Commission Report on the Number of Animal Used in the EU

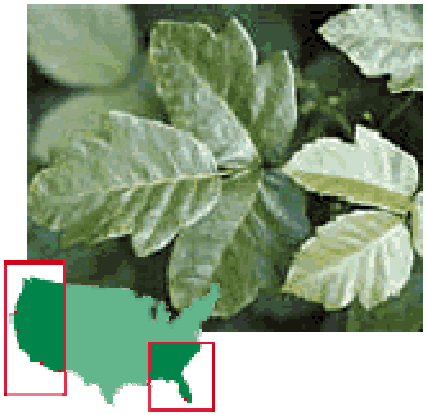
ESEMPIO

- ☒ Irritazione cutanea

Irritazione cutanea

Le dermatiti da contatto di tipo irritativo sono il risultato clinico di una risposta infiammatoria derivante principalmente dal rilascio da parte delle cellule epidermiche (cheratinociti) di citochine proinfiammatorie in risposta ad uno stimolo chimico.



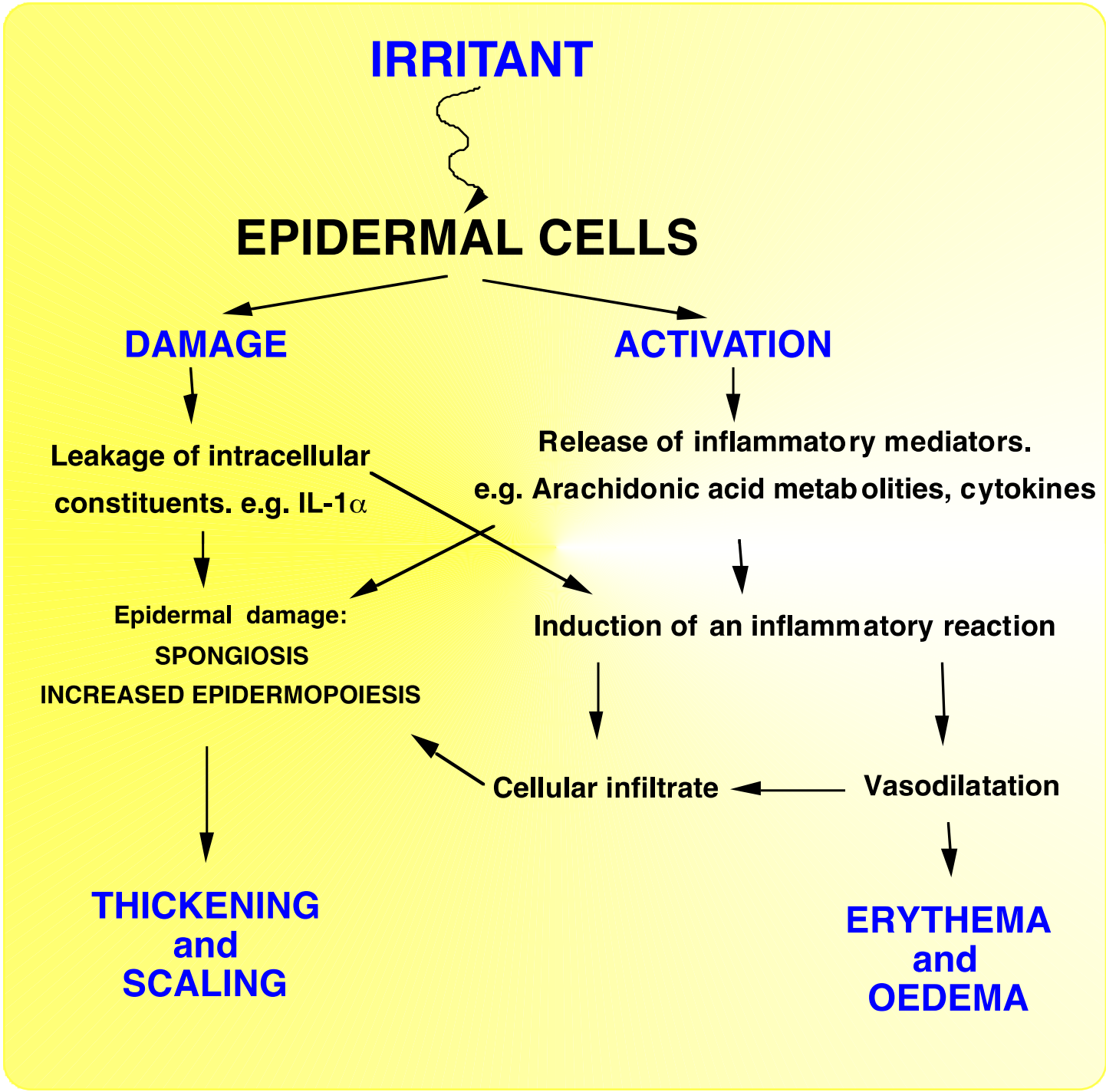


Contact Dermatitis: Inflammatory response to topical irritant caused by contact with Poison Oak

Irritazione cutanea

Cambiamenti patofisiologici

1. Distruzione della barriera cutanea
2. Modificazioni delle cellule epidermiche
3. Rilascio di citochine

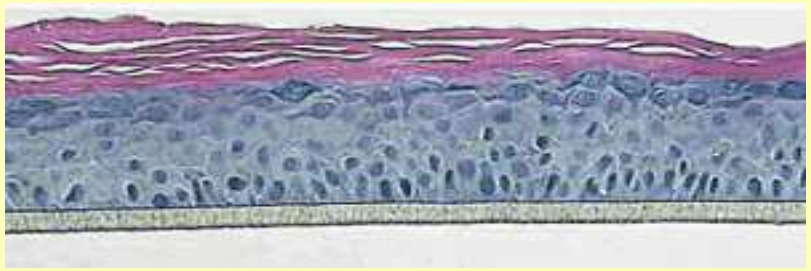

IRRITANT
EPIDERMAL CELLS
DAMAGE
ACTIVATION
Leakage of intracellular constituents. e.g. IL-1α
Release of inflammatory mediators. e.g. Arachidonic acid metabolities, cytokines
Epidermal damage:
SPONGIOSIS
INCREASED EPIDERMOPOIESIS
Induction of an inflammatory reaction
Cellular infiltrate
Vasodilatation
THICKENING and SCALING
ERYTHEMA and OEDEMA

EXPERIMENTAL MODEL

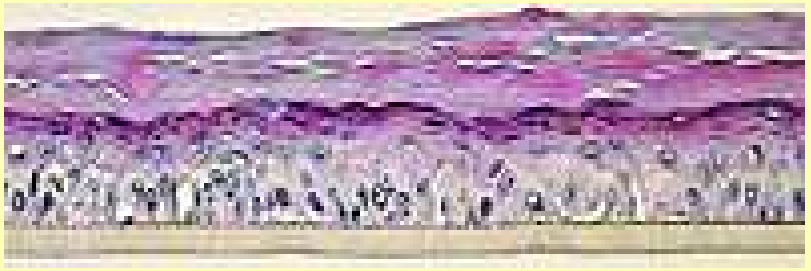
reconstituted human epidermis

EpiDerm™ **Episkin™**

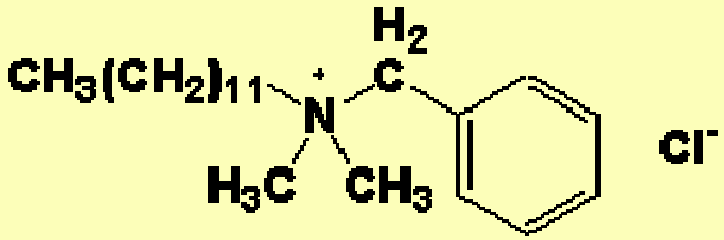
Cosmital™ **Skinethic™**



Control



Benzalkonium chloride



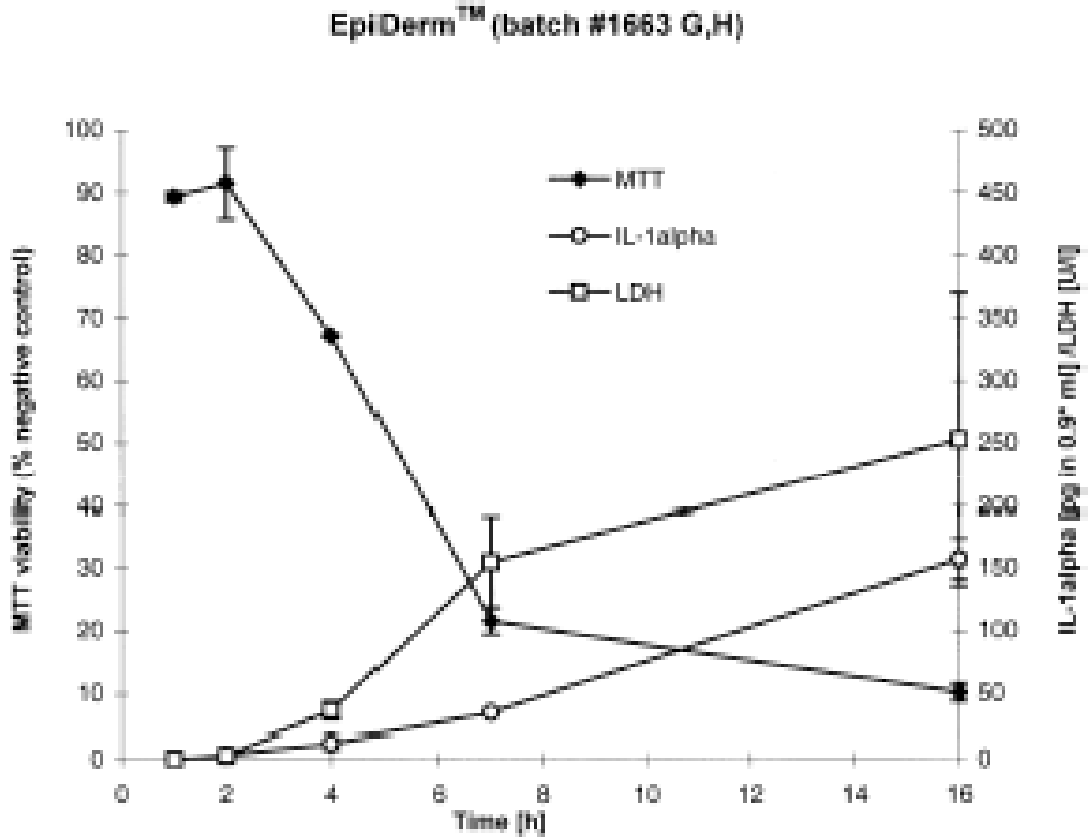


Fig. 1. Time-dependent response of EpiDerm™ tissue cultures (one batch as an example) after topical application of the test formulation 2B10: cell viability determined as MTT reduction capacity, release of the proinflammatory mediator IL-1α and leakage of the cytoplasmic enzyme LDH. Each value represents the mean±SD of two replicate cultures. *Volume of assay medium per culture.

Es. benzalkonium chloride 1%

In vitro tests for skin irritation (R38)

15 -60 MINUTES EXPOSURE

42 HOURS POST- INCUBATION

MTT TEST

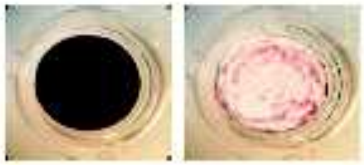
Endpoint 1

DOSE:

EpiDerm (0,63 cm²) : 25 mg or 30 µl- 60min

EPISKIN (0,38 cm²) : 10 mg or µl- 15 min

SkinEthic (0,5 cm2): 16 mg or µ- 42 min



MTT assay:

EpiDerm: 0,3 ml; 1 mg/ml MTT

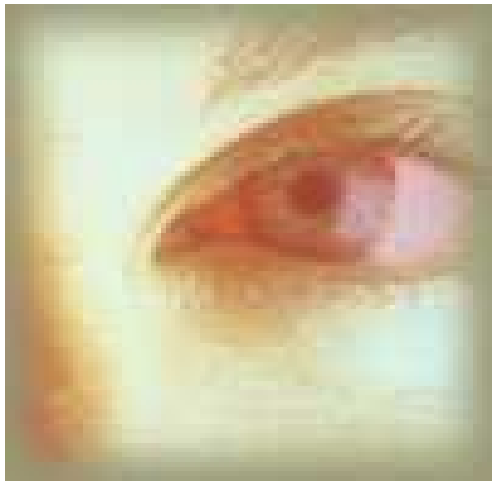
EPISKIN: 2 ml; 0,3 mg/ml MTT

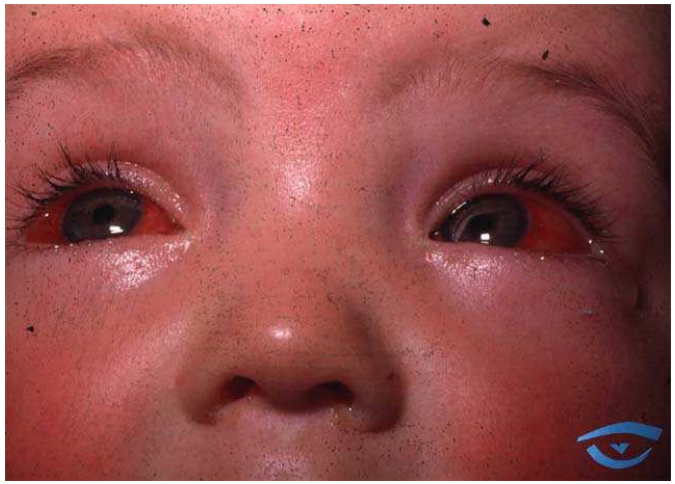
Viability > 50% of negative control: Non-irritant

Viability < 50% of negative control: Irritant

ESEMPI

☒ Irritazione oculare

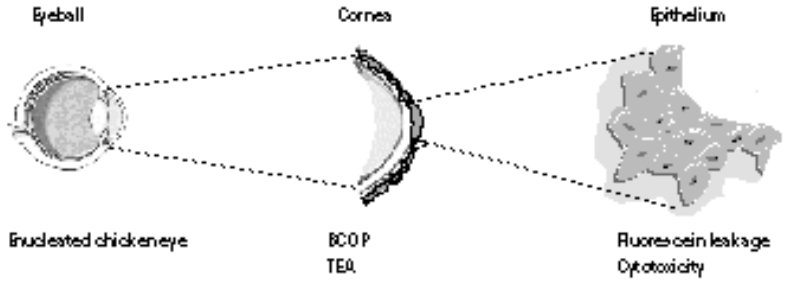




VALUTAZIONE DELLA TOSSICITA' OCULARE

3. Metodi alternativi:

- Occhio isolato,
- cornea isolata (BCOP);
- HET-CAM;
- modelli 3-D;
- colture cellulari (red blood cells).



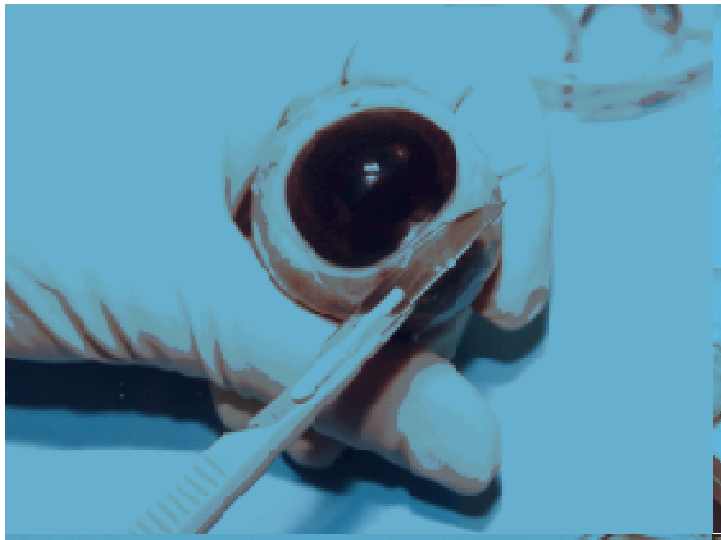
BCOP, bovine corneal opacity and permeability
HET-CAM, hen's egg test on chorioallantoic membrane

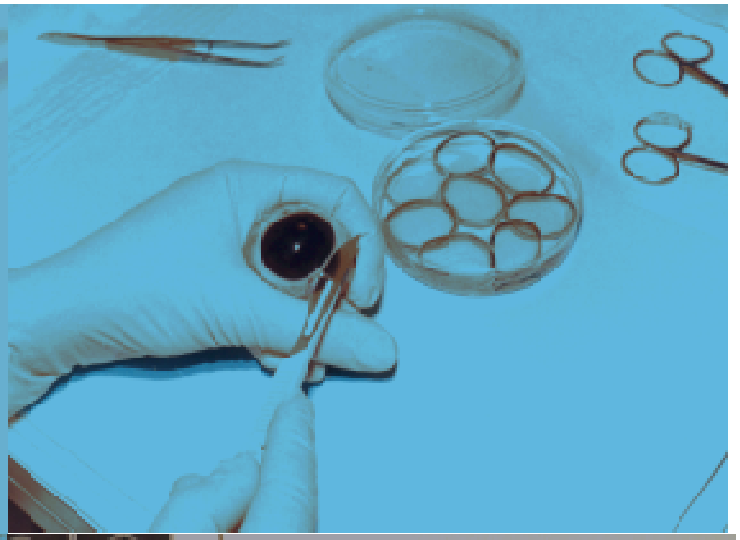
Metodi accettati o in convalida

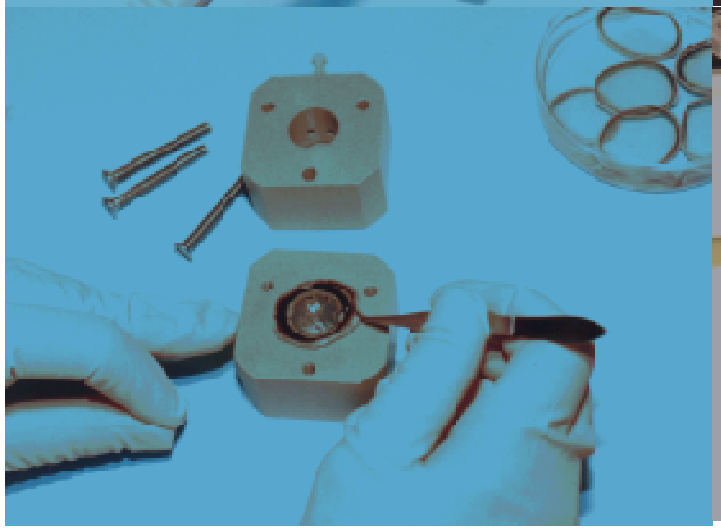
- Het-Cam (Francia e Germania)
- Fluoresceine Leakage test and the Cytosensor microphysiometer test to identify ocular corrosives and severe irritants. (ESAC)
- **The Bovine Corneal Opacity and Permeability (BCOP) and the Isolated Chicken Eye (ICE) test methods for eye irritation**
  Regulation: OECD Test Guidelines 437 and 438, adopted in September 2009.
  - Si accetta solo il dato positivo per la classificazione europea R41

ESAC= ECVAM Scientific Advisory Committee

CORNEA BOVINA ISOLATA



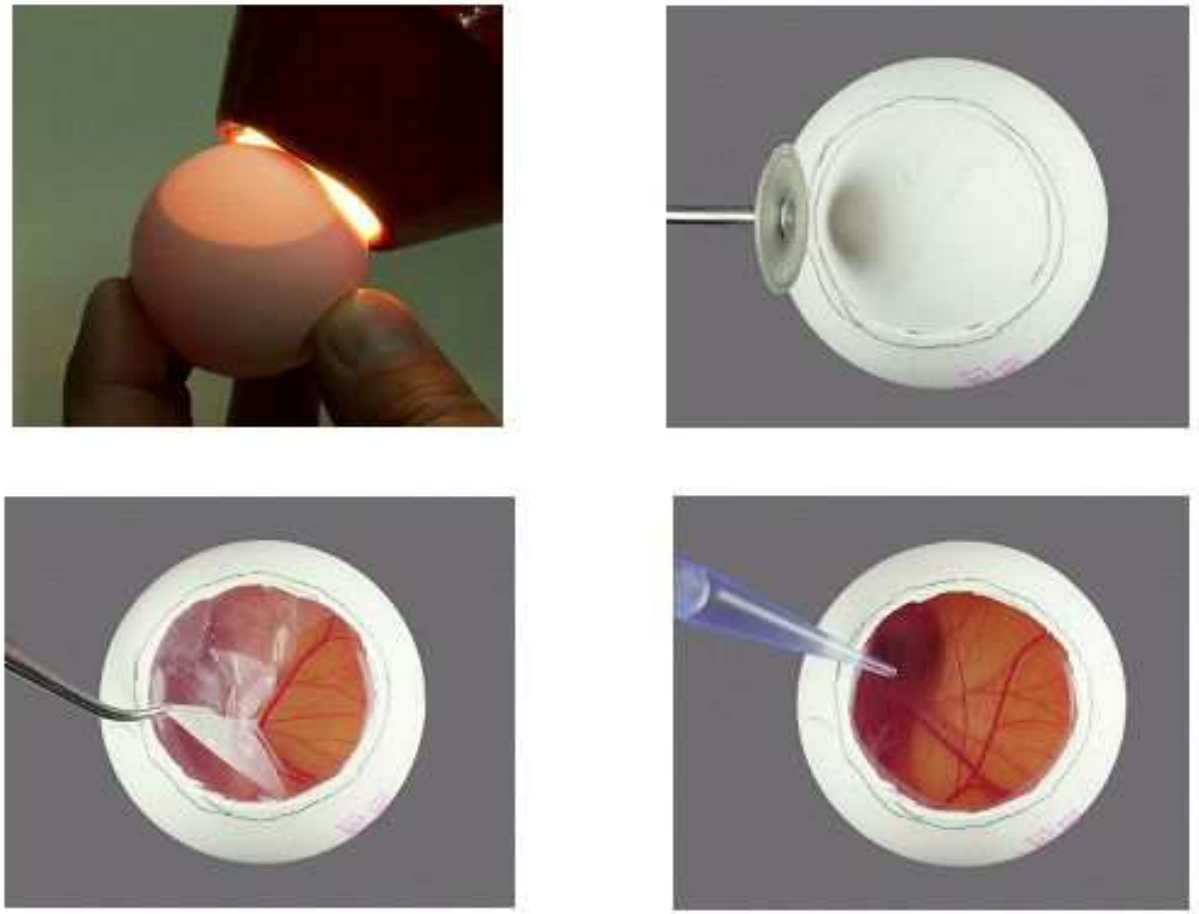




CORNEA BOVINA ISOLATA

- Opacizzazione: modificazione al passaggio della luce
- Modificazione nell'intergrità della barriera epiteliale: passaggio della fluoresceina
- Esame istologico
- Misurazione dell'idratazione della cornea

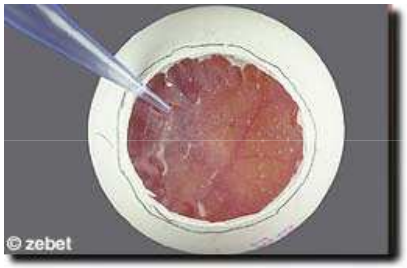
Het-Cam (Heng's test-chorioallantoic membrane)



Metodo approvato dalla Legislazione Francese

Uovo di gallina fecondato di 10 giorni

Applicazione di 0,3 ml di prodotto



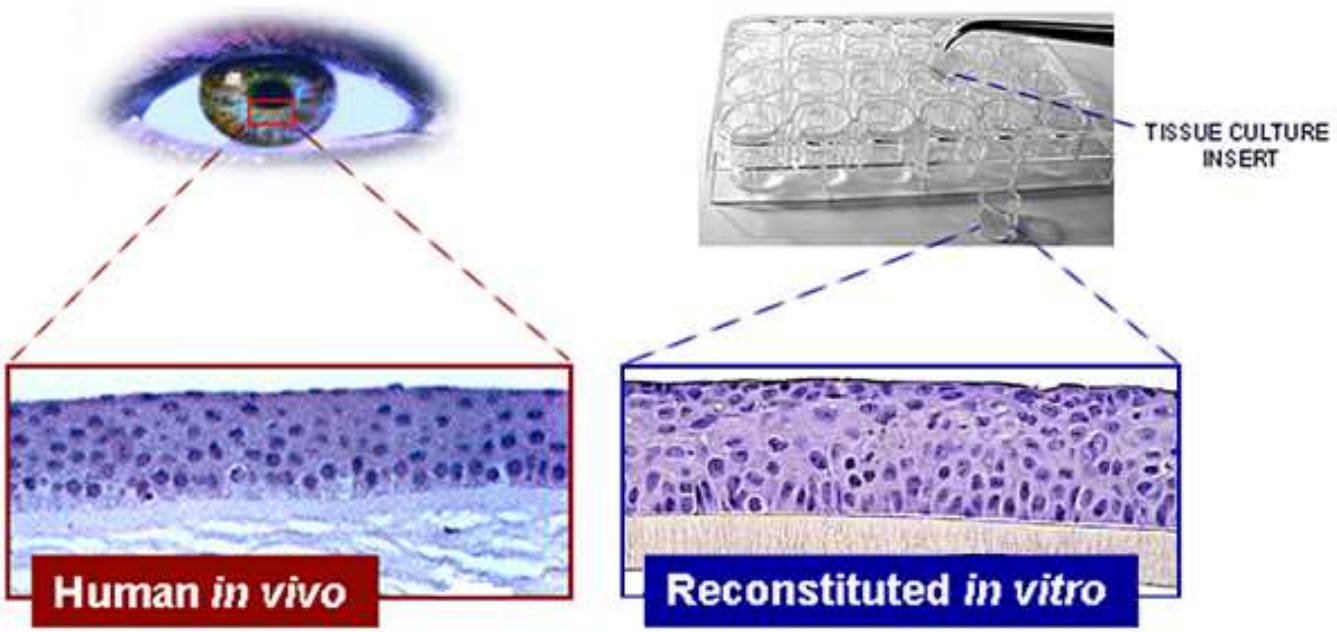
Osservazione a 5 min:

- Vascolarizzazione
- Emorragia
- Coagulazione

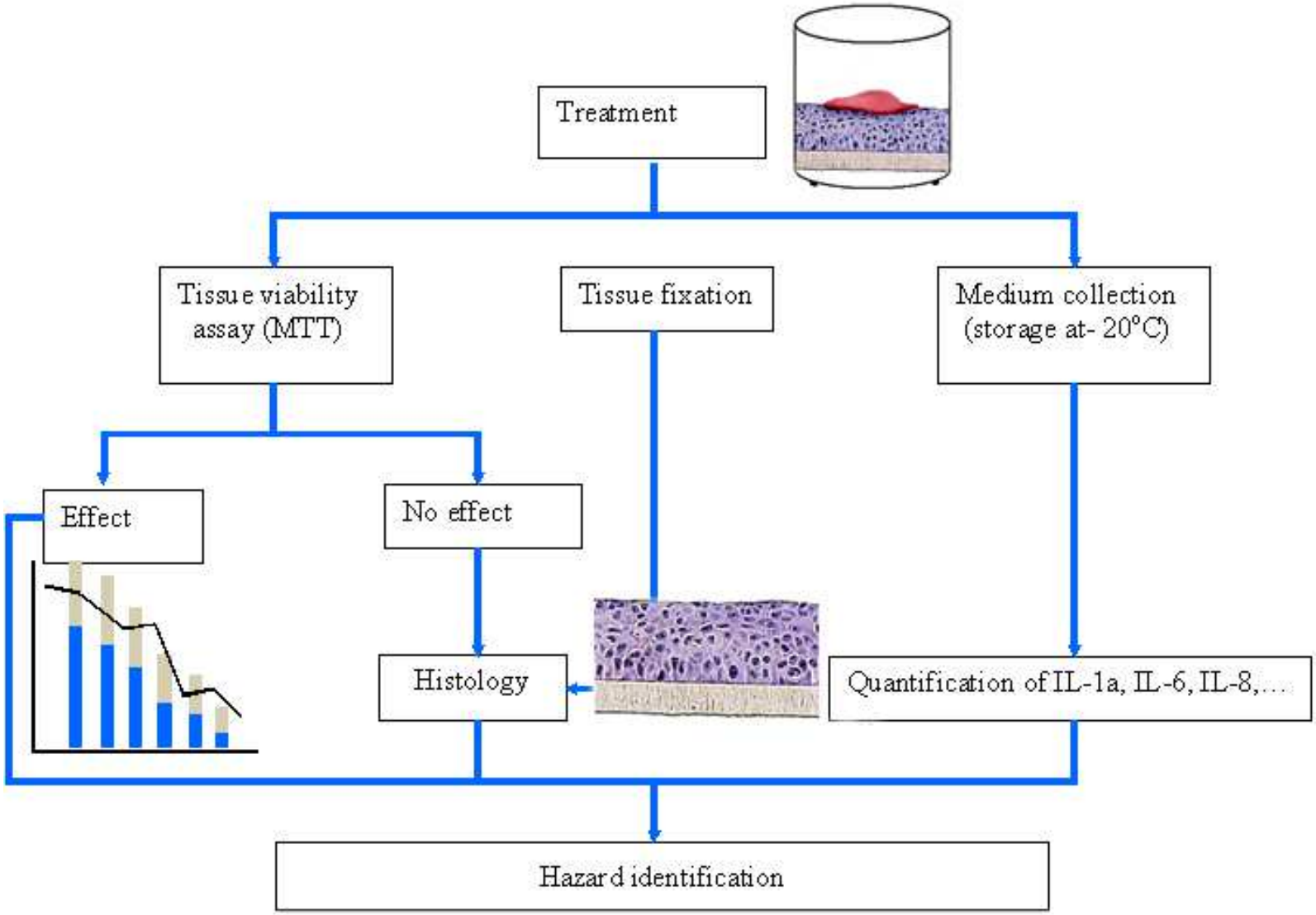
VALORACIÓN Fenómeno	Tiempo		
	T ≤ 30 s	**30 s ≥ T ≤ 2 min**	**2 min ≥ T ≤ 5 min**
Hiperemia	5	3	1
Hemorragia	7	5	3
Coagulación	9	7	5

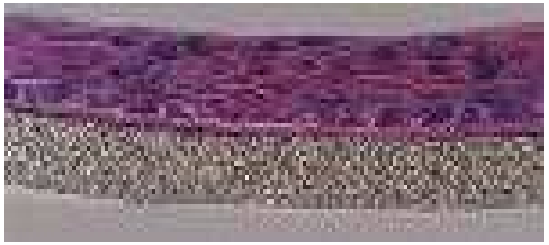
ÍNDICE HET-CAM	CATEGORÍAS
N < 1	PRACTICAMENTE NO IRRITANTE
1 ≤ N ≥ 5	LIGERAMENTE IRRITANTE
5 ≤ N ≥ 9	MODERADAMENTE IRRITANTE
N ≥ 9	IRRITANTE

Modelli 3D



Epiocular® MatTek, SkinEthic, HCE™, Gillette HCE-T


Treatment
Tissue viability assay (MTT)
Tissue fixation
Medium collection (storage at- 20°C)
Effect
No effect
Histology
Quantification of IL-1a, IL-6, IL-8,…
Hazard identification

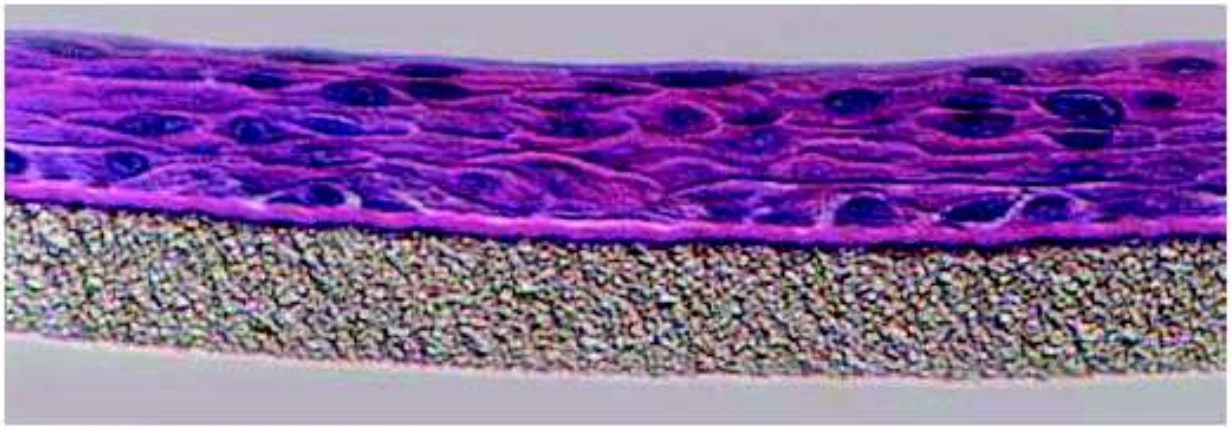


EpiOcular™

CLASSIFICAZIONE IN VIVO (Draize Test)	MAS score	EpiOcular CLASSIFICAZIONE	ET50 range (min)
Non irritante	0	Non irritante	>60 min
Minimo	0.1-25.0	Mild	31-60
Moderato	25.1-50.0	Moderato	3-30
Severo	50.1-110.0	Severo	<3

ET50 è il tempo necessario a ridurre del 50 % la vitalità del tessuto.

Reconstructed Human Tissue model EpiOcular – one of suggested alternatives



EpiOcular model by MatTek Corporation

Sensitivity: 94%
Specificity: 68%

(COLIPA pre-validated in 2007,
ECVAM validation running)

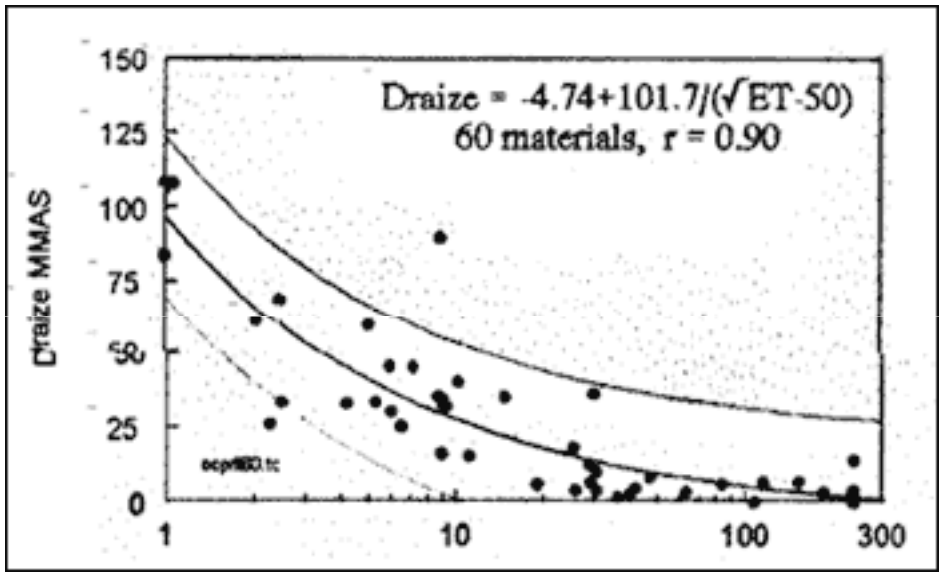
Assumption:

Eye Irritating chemicals are cytotoxic in specific test conditions of the in vitro eye irritation test

Selected in vitro design:

- exposure time: 30 min
- post-exposure time: 3 h for liquids, overnight for solids
- Irritating chemicals decrease the viability below 60%
- MTT viability assay

Correlazione tra il test di Draize e ET-50



Determination of EpiOcular prediction model -- Correlation of MTT ET-50 results to Draize rabbit eye data.

ET50 è il tempo necessario a ridurre del 50 % la vitalità del tessuto.

Modelli cellulari: Citotossicità

Culture cellulari primarie o stabilizzate di cheratinociti o fibroblasti

- **Neutral Red Uptake (NRU):** si valuta la capacità di inibizione della captazione del rosso neutro dopo un'esposizione di 24-48 h e si calcola la NRU50 o IC50
- **Neutral red release (NRR):** liberazione del colorante dopo esposizione per 1-3 min e si calcola la NRR50

Emolisi e denaturazione dell'emoglobina (RBC) Protocollo Invittox 37

- Determinazione della concentrazione che provoca la lisi del 50% dei globuli rossi (LD)
- Determinazione del rilascio di emoglobina rispetto a SDS (ID)
- Calcolo del rapporto LD/ID

L/D > 100 Non irritante

L/D > 10 Leggermente irritante

L/D > 1 Moderatamente irritante

L/D > 0,1 Irritante

L/D < 0,1 Molto irritante

Emolisi e denaturazione dell'emoglobina (RBC)
Protocolo Invittox 99

- Determinazione della CH50 (lisi del 50% degli eritrociti)
- Denaturazione dell'emoglobina a 541 nm
- Determinazione della Dlow (la concentrazione minima che provoca denaturazione dell'emoglobina) e della DMax (% massima di denaturazione a qualsiasi concentrazione)

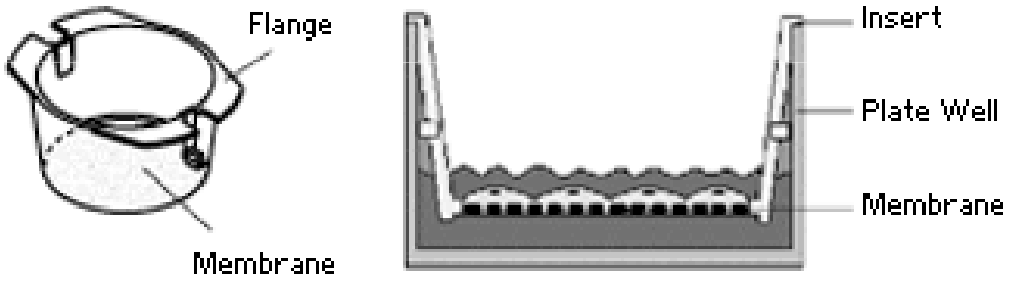
Emolisi e denaturazione dell'emoglobina (RBC)
Protocolo Invittox 99

Modello di predizione:

RBC-Parámetro	**Irritación ocular**
D_{max} ≥ 50% and D_{low} ≤ 10,000 mg/l	severo
H_{50} ≤ 500 mg/l	
D_{max} ≥ 50% and D_{low} = 100,000 mg/l	Moderado
D_{max} 20 – 50% and D_{low} ≤ 10,000 mg/l	
H_{50} > 500 – 10,000 mg/l	
no effects seen at 100,000 mg/l	no irritante

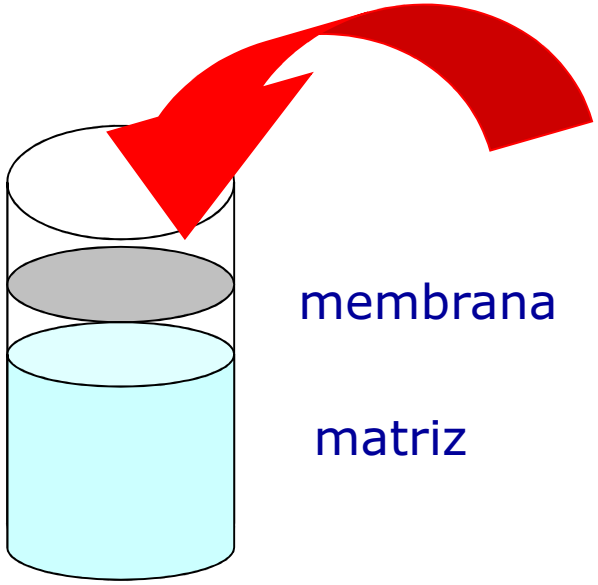
Alterazioni delle funzioni cellulare

- Fluorescein leakage, FL



- Microfisiometro: Alterazioni del metabolismo; produzione di H+

Eyetex ®
IRRITECTION ®



La membrana regola il rilascio del prodotto.

La matrice è formata da proteine,
glicoproteine, carboidrati, lipidi.

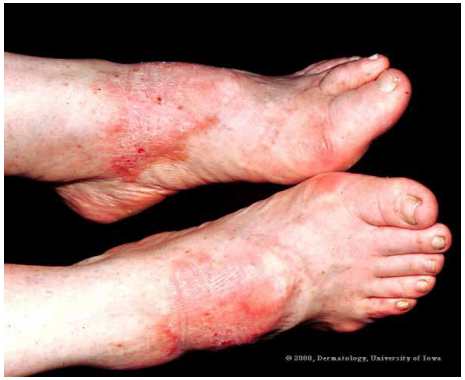
Si valuta la denaturazione della matrice.

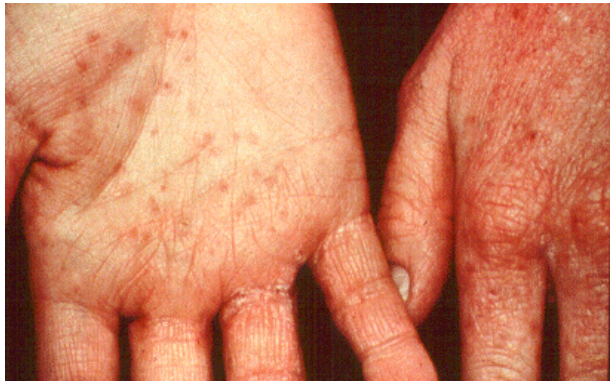
SCHEMA DELLA PRESENTAZIONE

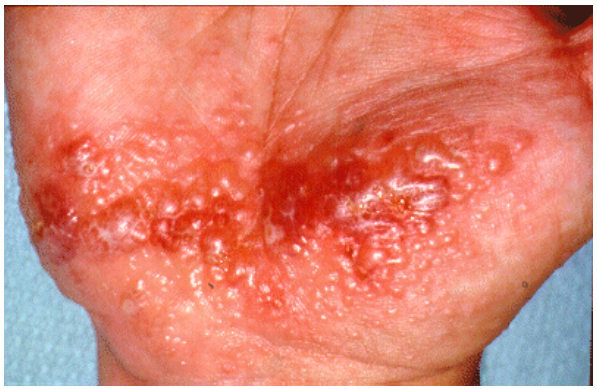
- Perchè sviluppare metodi alternativi
- Definizione di metodo alternativo
- Convalida di un metodo alternativo
- Metodi convalidati
- **Metodi in convalida**

ESEMPI

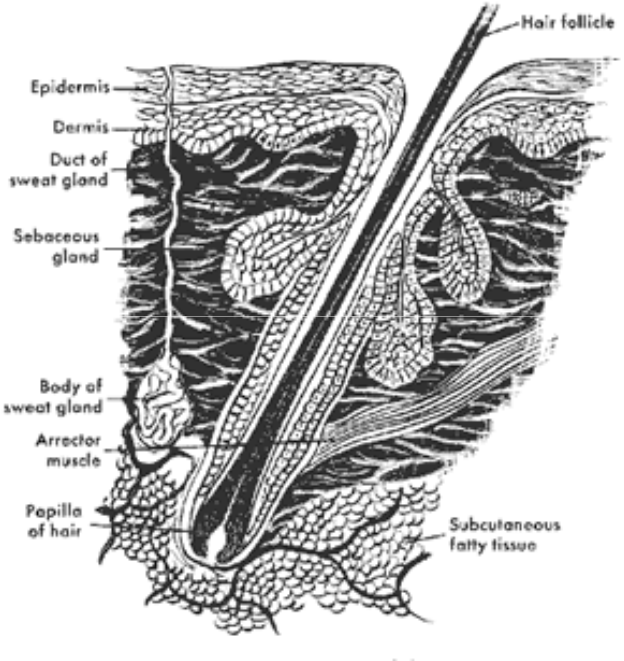
☒ Sensibilizzazione





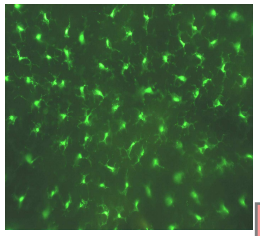


Strategy?



- To apply our **mechanistic understanding** of ACD to the design of predictive in vitro alternative test methods.
- **Different mechanistic approaches**:
  - Skin penetration/metabolization
  - Hapten binding (QSAR)
  - Antigen-specific immune response

Choice of experimental model to study contact dermatitis in vitro



Key passages

1. Absorption and local trauma – proinflammatory cytokine production (danger signals)

2. Protein binding

3. Antigen processing

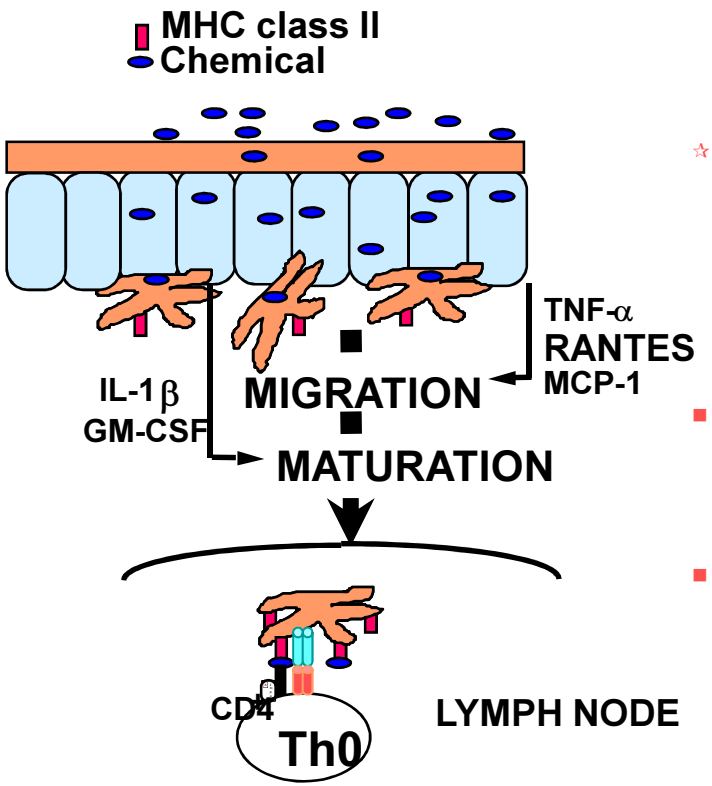
4. Langerhans cells/dermal DCs maturation and migration

5. Antigen presentation to Th cells and the generation of memory T cells (immunogenicity)

Key events in chemical-induced skin sensitization and *in vitro* opportunities

KEY EVENT	*IN VITRO* OPPORTUNITIES
1. Skin penetration	Human skin biopsis, pig skin; Reconstituted human epidermis
2. Binding to macro-molecules (i.e., proteins)	QSAR/Expert systems; Peptide binding assay
3. Local trauma and generation of danger signals	Keratinosens™; KC activation; NCTC2544 IL-18 assay; KC gene expression profile
4. Langerhans cells maturation and migration	DC-like up-regulation of class II antigens and costimulatory molecules, i.e. CD54, CD86; Cytokine release, i.e. IL-8; LC-like MUTZ-3 cells migration assay; DC-like gene expression profile (GARD)
5. Antigen presentation to T_H cells and memory T-cell generation	*In vitro* T-cell activation

KERATINOCYTES



- In principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen presenting capacity.
- **However, in addition to chemical processing, LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure.**
- Chemical must cause sufficient local trauma to induce/augment cutaneous cytokine production.
- The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than non-irritant ones (Grabbe *et al.*, 1996).
- **In this case, the potency of chemicals to induce cutaneous sensitization may be assessed as a function of KC cytokine expression.**

Toxicology and Applied Pharmacology 245 (2010) 281–290

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*

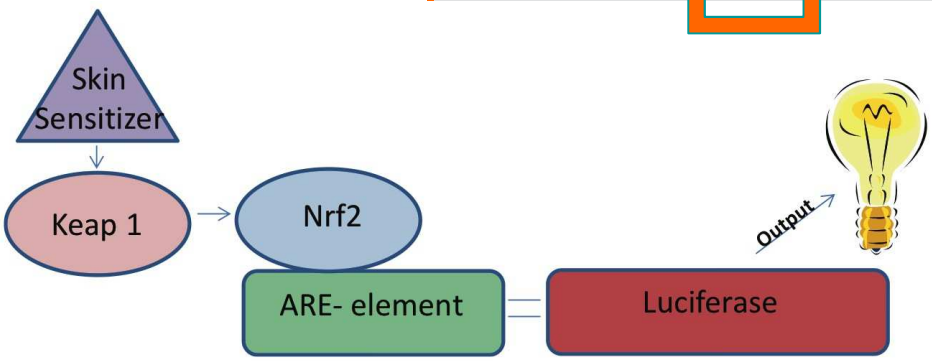
Roger Emter [a], Graham Ellis [b], Andreas Natsch [a,*]

[a] Givaudan Schweiz AG, Ueberlandstrasse 138, CH-8600 Duebendorf, Switzerland
[b] Givaudan Schweiz AG, 5 Rue de la Parfumerie, CH-1214 Vernier, Switzerland

Table 2
Cooper statistics for the different test lists.

	SILVER list	Sens-it-iv list	ECVAM list	ICCVAM list	SILVER list with reactivity data[a]
Correct positives	38	12	11	13	41
False negatives	5	2	1	2	2
Correct negatives	19	9	4	7	19
False positives	5	1	0	0	5
n test chemicals	[illegible]	24	16	22	[illegible]
Sensitivity	88.4	85.7	91.7	86.7	95.3
Specificity	79.2	90.0	100.0	100.0	79.2
Accuracy	85.1	87.5	93.8	90.9	89.6

[illegible]sitive in (i) KeratinoSens assay or if (ii) adduct [illegible]ity assay.

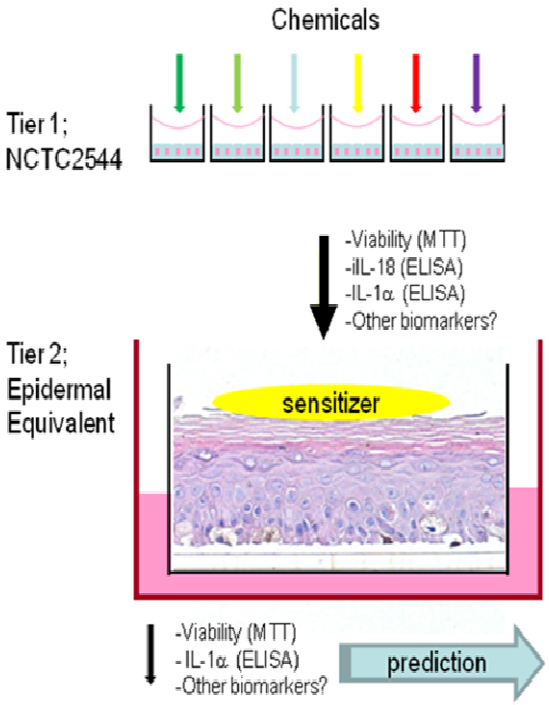


The Sens-it-iv toolbox (2011)

IL-18 detection and potency assessment: A 2-tiered approach

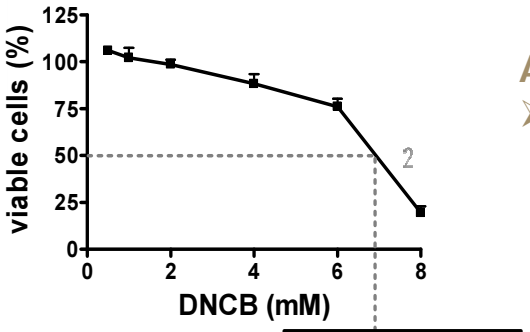
3Rs Management and Consultancy, Lyngby, Denmark
3RsMC.eu@gmail.com; +45 31538020

A 2-tiered approach for identification and classification of skin sensitizers



- Identification of contact sensitizers in Tier 1
  -IL-18 expression

- Identification of potency of sensitizers in Tier 2
  - EC50, MTT
  - IL-1α expression



NCTC2544/IL-18:

- 30 chemicals tested
- WLR: >95%
- Transferable
- BLR: >95%
- Accuracy: 97% (labeling)

RHE potency test:

- 16 chemicals tested
- WLR: >95%
- Transferable
- BLR: >95%
- Concordancy: 92% (classification)

Assessed by Cosmetics Europe

- 10 coded compounds

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NCTC 2544 AND IL-18

Toxicology in Vitro 23 (2009) 789–796

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Contingency table for the NCTC 2544 assay including COLIPA project

Sensitivity 16.5/19 = **86.8%**

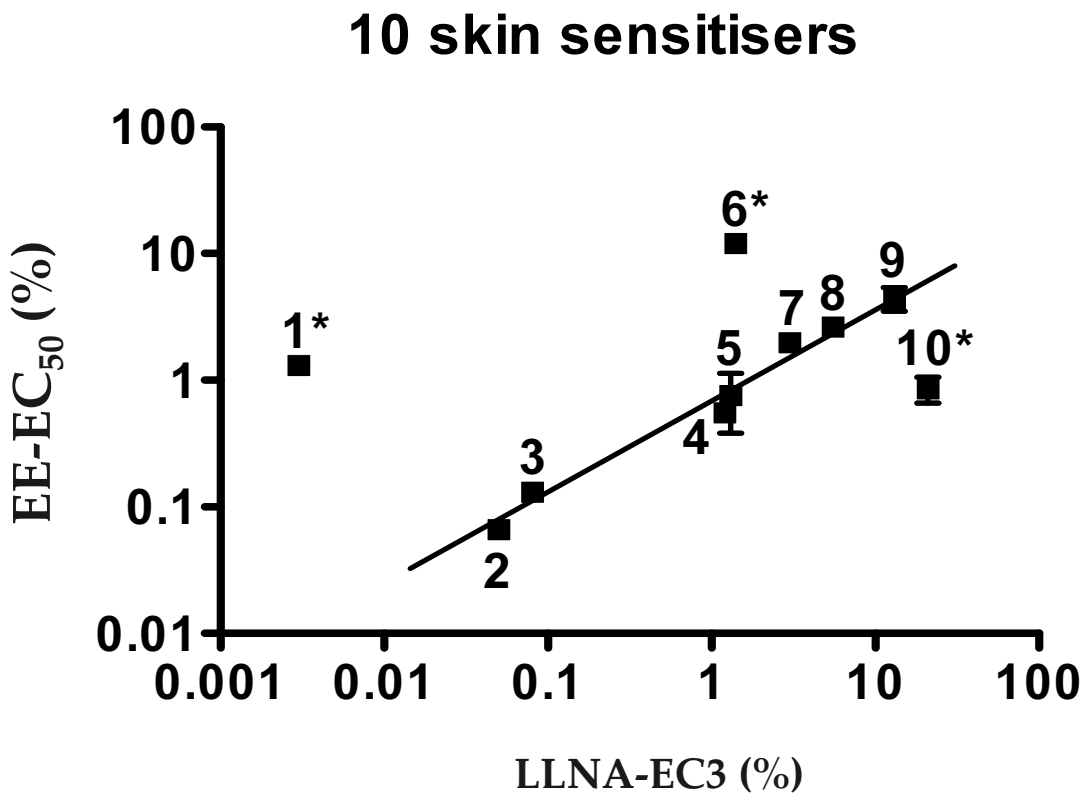
Specificity 9.5/10 = **95%**

Accuracy 26/29 = **89.7%**

- Similar results were also obtained using primary human KC and other keratinocyte cell lines, confirming the relevance of the proposed model and the possibility to use different source of KC

What about potency?

A RHE potency assay with potential applicability for classification *in vitro*



3Rs Management and Consultancy, Lyngby, Denmark
3RsMC.eu@gmail.com; +45 31538020

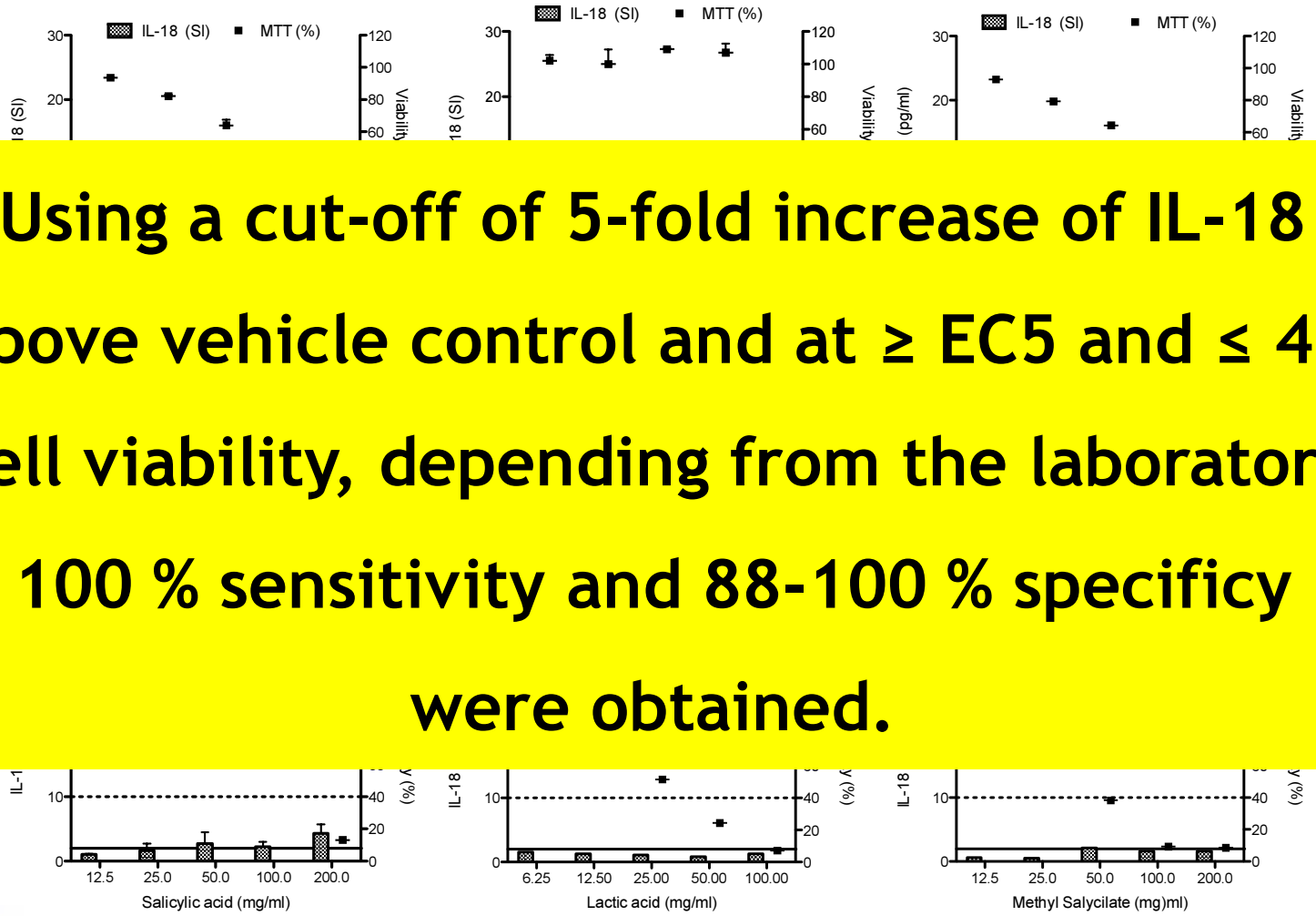
(Sue Gibbs, VUMC)

IL-18 SECRETION AS A MARKER FOR IDENTIFICATION OF CONTACT SENSITIZERS IN THE EPIDERM IN VITRO HUMAN SKIN

Using a cut-off of 2-fold increase of IL-18 above vehicle control, 10/11 contact sensitizers were correctly identified by the IL-18 assay (**90.9% sensitivity**) with no false positive results (**100% specificity**).

Chemical Tested: 2,4-Dinitrochlorobenzene (DNCB), 2-Mercaptobenzothiazole (2-MBT), 4-Nitrobenzylbromide (4-NBB), Cinnamaldehyde, Cinnamyl Alcohol, Eugenol, Glycerol, Glyoxal, Isoeugenol, Lactic Acid, Phenol, p-Phenylenediamine (ppd), Resorcinol, Salicylic Acid, Tetramethylthiurame disulfide (TMTD)

ZonMw



Using a cut-off of 5-fold increase of IL-18 above vehicle control and at ≥ EC5 and ≤ 40 cell viability, depending from the laboratory 100 % sensitivity and 88-100 % specificy were obtained.

SENS-IT-IV

TOXICOLOGICAL SCIENCES 117(1), 81–89 (2010)
doi:10.1093/toxsci/kfq182
Advance Access publication June 20, 2010

Keratinocyte Gene Expression Profiles Discriminate Sensitizing and Irritating Compounds

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[1]To whom correspondence should be addressed at Laboratory for Health Protection Research, National Institute for Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands. Fax: +31-30-2744446. E-mail: rob.vandebriel@rivm.nl.

Gene Set Enrichment Analysis showed upregulation of " Keap1 dependent" and "oxidative stress" gene lists. KC expression profiling can identify contact sensitizers. Moreover, our data suggest that contact sensitizers induce the oxidative stress pathway in KC.

Langerhans cells: restrictions

- *Difficult to isolate from human skin.*
- *Low viability.*
- *Shortage of available human skin*

ALTERNATIVE USE OF LC:

- *Human peripheral blood mononuclear cells*
- *CD34+ hematopoietic progenitor cells from cord blood*

ALTERNATIVE USE OF DC

- *Use of cell lines such as THP-1, KG-1, U937, MUTZ-3 (human monocytic leukemia cell lines)*

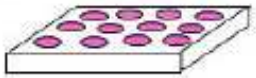
Toxicol Appl Pharmacol. 2009 May 1;236(3):372-82. Epub 2009 Feb 14.

Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound.

dos Santos GG, Reinders J, Ouwehand K, Rustemeyer T, Scheper RJ, Gibbs S.

Human Cell Line Activation Test (h-CLAT)*

Procedure



THP-1
1x10^6 cells /mL

24h

Culture with chemicals,
8 doses based on CV75

Flow cytometric analysis

Cell staining (CD86 & CD54)

FcR blocking

Relative Fluorescence Intensity (RFI)

$$\text{RFI} = \frac{\text{MFI of chemical treated cells - MFI of chemical treated Isotype control cells}}{\text{MFI of vehicle control cells - MFI of vehicle Isotype control cells}} \times 100$$

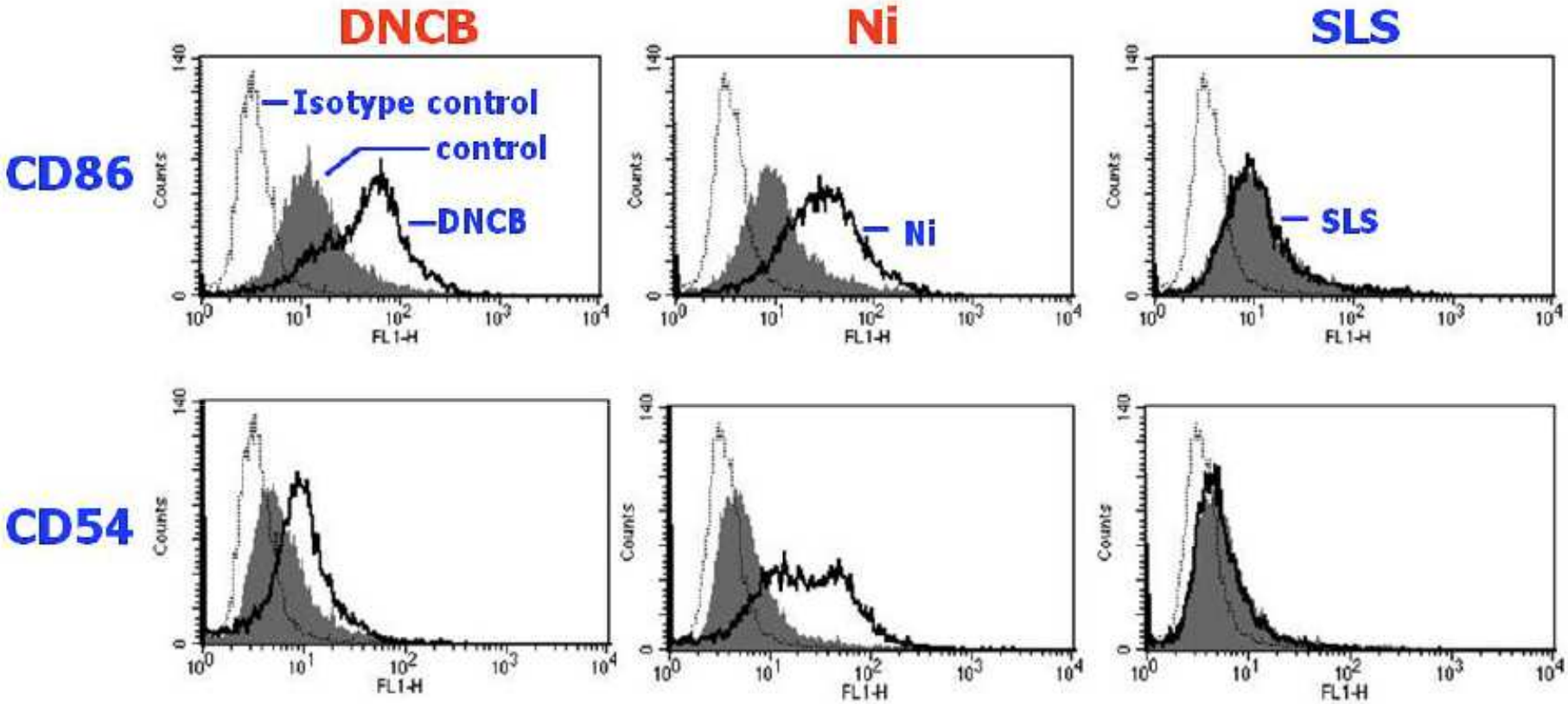
MFI = geometric mean fluorescence intensity

Prediction Model

- Viability ≥ 50% by Propidium Iodide
- Positive criteria: CD86 RFI ≥ 150% and/or CD54 RFI ≥ 200%
- Positive: 2 of 3 independent data at any dose should exceed the positive criteria

*: Ashikaga et al., 2006 Toxicol In Vitro 767-73., Sakaguchi et al., 2006 Toxicol In Vitro 774-84.

Histogram of CD86 / CD54 expression



DNCB and Ni (typical allergens) enhanced both CD86 and CD54 expressions but SLS (non-allergen) did not.

Miyazawa et al., Toxicology in Vitro 2007

Comparative evaluation with LLNA and human

h-CLAT vs LLNA

		h-CLAT	
		+(83)	-(34)
LLNA	+(85)	75	10
	-(32)	8	24

Sensitivity: 75/85 (88%)
Specificity: 24/32 (75%)
Positive predictivity: 75/83 (90%)
Negative predictivity: 24/34 (71%)
Accuracy: 99/117 (85%)

h-CLAT vs human

		h-CLAT	
		+(51)	-(20)
Human	+(55)	46	9
	-(16)	5	11

Sensitivity: 46/55 (84%)
Specificity: 11/16 (69%)
Positive predictivity: 44/51 (90%)
Negative predictivity: 11/20 (55%)
Accuracy: 57/71 (80%)

Good predictive capacity, but some false negative / positive

Nukada et al., WC7 2009, Ashikaga et al., ATLA 2010, Nukada et al., ESCD 2010

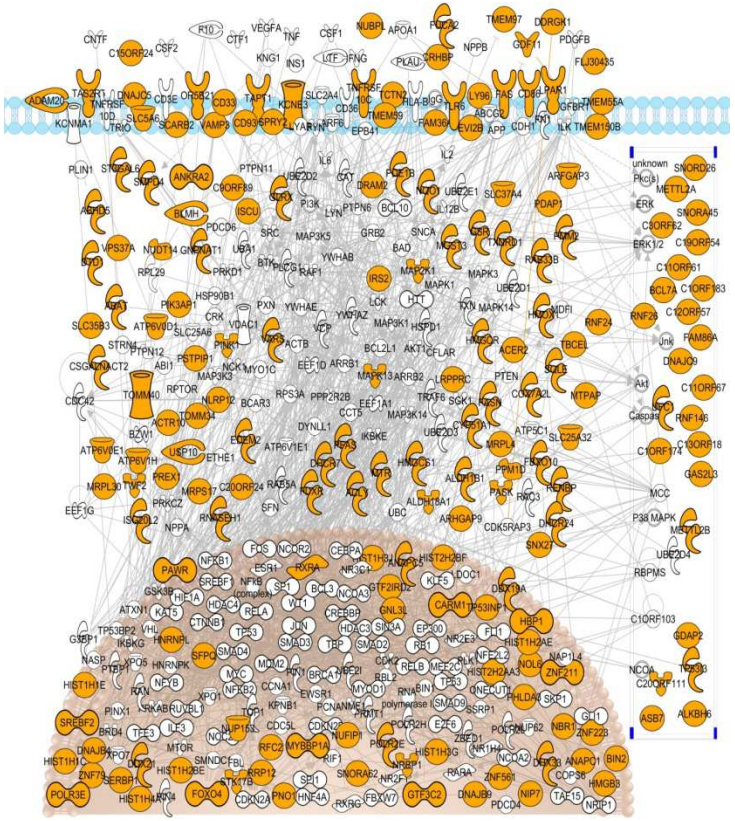
The Genomic Allergen Rapid Detection (GARD) test

SenzaGen AB

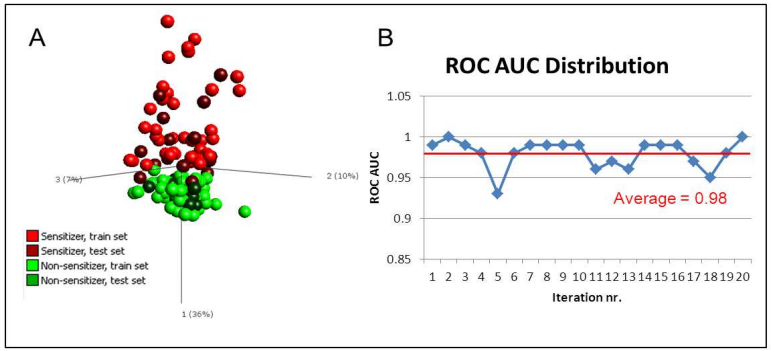
3Rs Management and Consultancy, Lyngby, Denmark
3RsMC.eu@gmail.com; +45 31538020

Genomic signatures for identification of skin and respiratory sensitizers

3RsMC
3Rs Managment and Consultancy
www.3RsMC.eu



- **Sensitization pathways**
  - MUTZ-3 cell line
    - 6 pathways/ 200 genes (*GARD*)
    - Accuracy: >97%

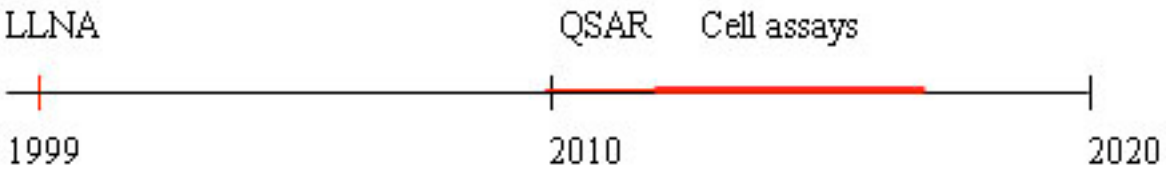


”SenzaGen AB”

Johansson et al., BMC Genomics. 2011 Aug 8;12:399.

Methods under validation at ECVAM

- THP-1: human Cell Line Activation Test (h-CLAT), chemical allergens are predicted by the up-regulation of CD86 and CD54 expression when cells are exposed to subtoxic concentrations of chemicals.
- U937: CD86 upregulation
- Peptide binding assay: the ability of known chemical allergens to bind with nucleophilic amino acids has been shown to correlate to the skin sensitization potential of a chemical.



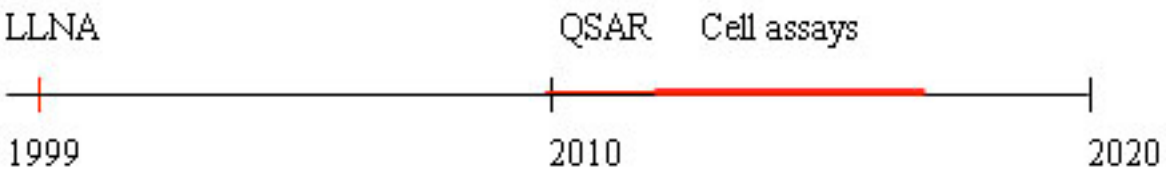
Methods under validation at JaCVAM

- THP-1: IL-8 Luc assay

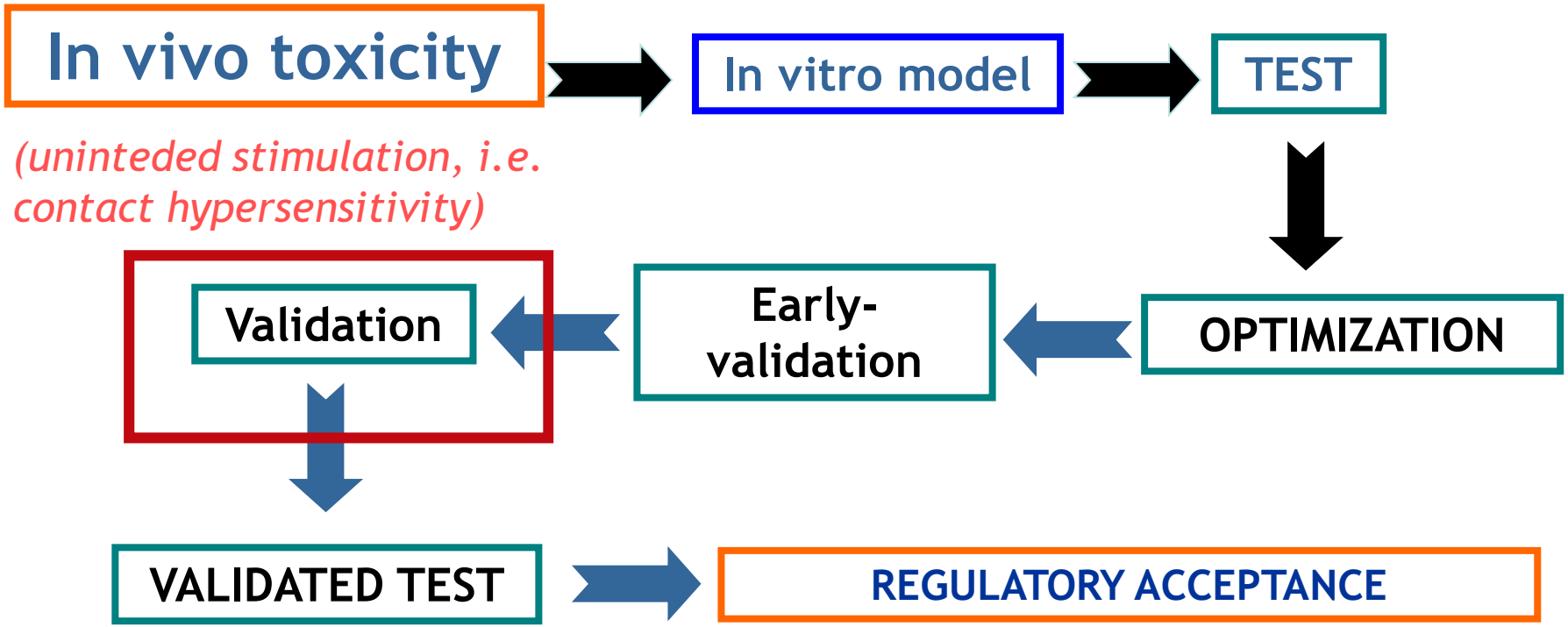
Takahashi T, Kimura Y, Saito R, Nakajima Y, Ohmiya Y, Yamasaki K, Aiba S.

An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8.

Toxicol Sci. 124(2):359-69, 2011 Sep 13.



Development of alternative in vitro test



Timetables for Phasing-Out Animal Experimentation

Chair - DG Entr / ECVAM

Stakeholders

- European Cosmetic Toiletry and Perfumery Association (COLIPA)
- European Federation for Cosmetic Ingredients (EFfCI)
- European Chemical Industry Council (CEFIC)
- Animal welfare groups (Eurogroup for Animal Welfare, ECEAE)
- European Organisation of Cosmetic Ingredients Industries and Services (UNITIS - natural origin ingredients)
- DG ENV, DG RES, DG SANCO (and SCCNFP)
- OECD

EC Timetables for phasing-out animal testing *

Time estimates assuming that optimal conditions are met

Toxicological Endpoints	ESAC endorsement	Full replacement of animal tests with methods adopted at EU level *	Testing and marketing cut-off dates
Skin Corrosion	Validated	**EU Annex V B.40** (OECD TG 430 and TG 431)	11 March 2009
Acute Phototoxicity	Validated	**EU Annex V B.41** (OECD TG 432)	
Skin Absorption / Penetration	2005	**2006** (OECD TG 428)	
Skin Irritation for hazard identification for risk assessment	2006 / 2007 > 2008	**2007 / 2008** **2010**	
Photogenotoxicity	2007	**2008**	
Eye Irritation	2008	**2009**	

*** substantial reduction of animal use could be achieved earlier**

Time estimates from stakeholders report on current status of alternative methods for cosmetics testing

Toxicological Endpoints	Full replacement of animal tests with methods adopted at EU level *	Marketing ban cut-off dates **
Acute Toxicity	**> 10 years**	11 March 2009
Genotoxicity / Mutagenicity	**> 12 years**	
Toxicokinetics / Metabolism	**> 12 years**	
Skin Sensitisation	**12-14 years**	11 March 2013
Photo-allergy (-sensitisation)	**> 15 years**	
Subacute & Subchronic Toxicity	**not estimated**	
Carcinogenicity	**not estimated**	
Reproductive & Developmental Toxicity	**not estimated**	

* Time estimates from 2004

** Testing ban cut-off date is 11 March 2009 for all toxicological endpoints

Table 1
Current status of available validated replacement alternative methods.

Existing alternative methods per endpoint[a]	Refine	Reduce[b]	Replace
Acute oral toxicity			
Fixed dose method [EC B.1 bis, OECD 420]	X	X	
Acute toxic class method [EC B.1tris, OECD 423]	X	X	
Up-and-down procedure [OECD 425]	X	X	
Acute inhalation toxicity			
Acute toxic class method [OECD 436]	X	X	
Acute dermal toxicity			
No validated alternative			
Skin corrosivity			
Rat skin TER[c] test [EC B.40, OECD 430]			X
Reconstructed human epidermal equivalents: EpiSkin™/EpiDerm™/SkinEthic™/EST[d]-1000 [EC B.40 bis, OECD 431]			X
Skin irritation			
EpiSkin™/Modified Epiderm™ SIT[e]/SkinEthic™ RHE[f] [EC B.46]			X
Eye irritation			
BCOP[g]/ICE[h] [OECD 437/438]		X	
Cytosensor microphysiometer test method/Fluorescein leakage test (ESAC, 2009)		X	
Skin sensitisation			
LLNA[g] LLNA[i] [EC B.42, OECD 429]	X	X	
rLLNA (ESAC, 2007b)	X	X	
Dermal absorption			
In vitro dermal absorption [EC B.45, OECD 428]			X
Mutagenicity/genotoxicity			
Bacterial Reverse Mutation Test [EC B.13/14, OECD 471]			X
In Vitro Mammalian Cell Gene Mutation Test [EC B.15, OECD 476]			X
In Vitro Micronucleus Test [EC B.12, OECD 474]			X
In Vitro Mammalian Chromosome Aberration test [EC B.10, OECD 473]			X
Reproductive Toxicity			
Whole Embryo Culture (WEC) test/MicroMass (MM) test/Embryotoxic Stem Cell Test (EST) (ESAC, 2001)		X	
Repeated dose toxicity/carcinogenicity/toxicokinetics			
No alternative methods available			
Photo-induced toxicity			
3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) [EC B.41, OECD 432]			X

[a] "EC B." methods are taken up in Regulation No. 440/2008 (EU, 2008b, 2009d) OECD methods are available through the OECD Guidelines for "Testing of Chemicals, Section 4: Health Effects" at http://www.oecdilibrary.org/oecd/content/books (consulted Sep 2009).

Table 2
Foreseeable data packages for European cosmetic ingredients that might be submitted to the SCCS according to the actual status of validated replacement alternatives.

EU cosmetic ingredients tested before 11 March 2009	EU cosmetic ingredients tested after 11 March 2009	EU cosmetic ingredients tested after 11 March 2013
Identification/physico-chemistry	Identification/physico-chemistry	Identification/physico-chemistry
Acute toxicity		
Skin corrosivity/irritation	Skin corrosivity/irritation	Skin corrosivity/irritation
Eye irritation	Screening for eye irritation	Screening for eye irritation
Skin sensitisation	Skin sensitisation[a]	
In vitro dermal absorption	*In vitro* dermal absorption	*In vitro* dermal absorption
Repeated dose toxicity	Repeated dose toxicity[a]	
In vitro and *in vivo* mutagenicity/genotoxicity	*In vitro* mutagenicity/genotoxicity	*In vitro* mutagenicity/genotoxicity
Reproductive toxicity	Reproductive toxicity[a]	
Carcinogenicity	Carcinogenicity[a]	
Chronic toxicity	Chronic toxicity[a]	
Toxicokinetic studies	Toxicokinetic studies[a]	
In vitro phototoxicity	*In vitro* phototoxicity	*In vitro* phototoxicity
Human data	Human data	Human data

[a] Animal test(s) performed outside the EU.

UTILIZZO PRATICO

- Nella valutazione della fototossicità, corrosione, irritazione cutanea ed oculare.
- Nella determinazione dell'assorbimento percutaneo.
- Nello sviluppo di nuove formulazioni contenenti sostanze potenzialmente irritanti i metodi in vitro consentono la selezione di quelle migliori.
- Nello screening di qualsiasi formulazione prima delle prove sull'uomo i metodi in vitro consentono con buona approssimazione di identificare il potenziale irritante.
- Nello studio del meccanismo d'azione.

CONCLUSIONI

- A tutt'oggi la maggior parte dei metodi alternativi validati sono metodi di '*reduction*' e '*refinement*', mentre per i metodi di '*replacement*'sono stati validati solo quelli relativi ad effetti topici, per i quali le interrelazioni tra organi e fenomeni cinetici hanno un peso minore.
- Il raggiungimento di una completa eliminazione dei modelli animali è un obiettivo purtoppo ancora lontano, *ma la ricerca in questa direzione è un fervido divenire*.

SITOX Informa XV n.1 Febbraio-Marzo 2012

GRAZIE PER L'ATTENZIONE

DOMANDE?