Giornata INT.E.G.RA. :
test di corrosività cutanea *in vitro*
OECD 435 «Corrositex®»

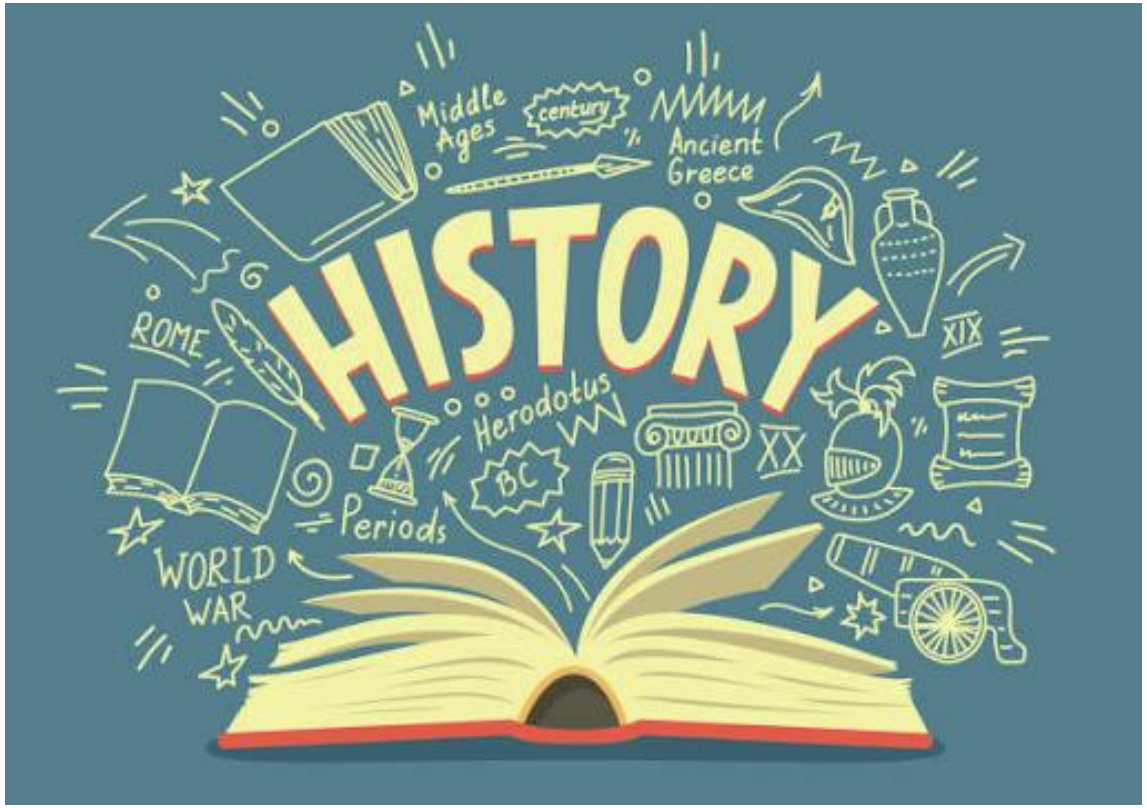
Leo Salvi, 2024

Introduzione ai Test In Vitro

- I test in vitro sono esperimenti eseguiti su cellule o tessuti isolati in ambienti controllati, come provette o piastre Petri.
- Questi metodi sono cruciali per valutare la sicurezza e l'efficacia di sostanze chimiche, cosmetici e prodotti farmaceutici senza l'uso di animali.
- L'obiettivo è aderire al principio delle 3R (Replacement, Reduction, Refinement), migliorando al contempo l'accuratezza scientifica e l'efficienza dei test.

Storia dei Test In Vitro

- - Anni '70: Inizio della consapevolezza sui limiti etici e scientifici dei test sugli animali.
- - Anni '80: Primi sviluppi significativi dei metodi in vitro per la valutazione della citotossicità e della mutagenicità.
- - 1991: Fondazione dell'ECVAM (European Centre for the Validation of Alternative Methods), che ha promosso la validazione dei metodi in vitro.
- - 1997: Prima linea guida OECD per un metodo in vitro (Test di Ames per la mutagenicità).
- - Anni 2000: Adozione globale crescente di test in vitro con linee guida e regolamenti specifici istituiti da FDA, ECHA, e altre agenzie regolatorie.



Importanza dei Test In Vitro

- - Etica: Riduzione significativa dell'uso di animali nei test, rispondendo alle crescenti preoccupazioni etiche.
- - Efficienza: Processi più rapidi e costi ridotti rispetto ai test sugli animali, con tempi di risposta test che possono essere ridotti da mesi a giorni.
- - Precisione: Valutazioni mirate e dettagliate sui meccanismi cellulari e molecolari, che migliorano l'affidabilità e la riproducibilità dei risultati.

Impatto sui Cosmetici

- - 1986: Iniziativa di sviluppo di modelli in vitro per test sui cosmetici nei laboratori dell'industria.
- - 2003: Unione Europea introduce il divieto parziale di test sugli animali per i cosmetici.
- - 2009: Direttiva UE sui Cosmetici (Regolamento CE 1223/2009) completa il divieto di sperimentazione animale per i prodotti cosmetici finiti e gli ingredienti.

- Test in vitro vengono spesso utilizzati per valutare il potenziale di irritazione cutaneo e oculare

Applicazioni nei Prodotti Chimici

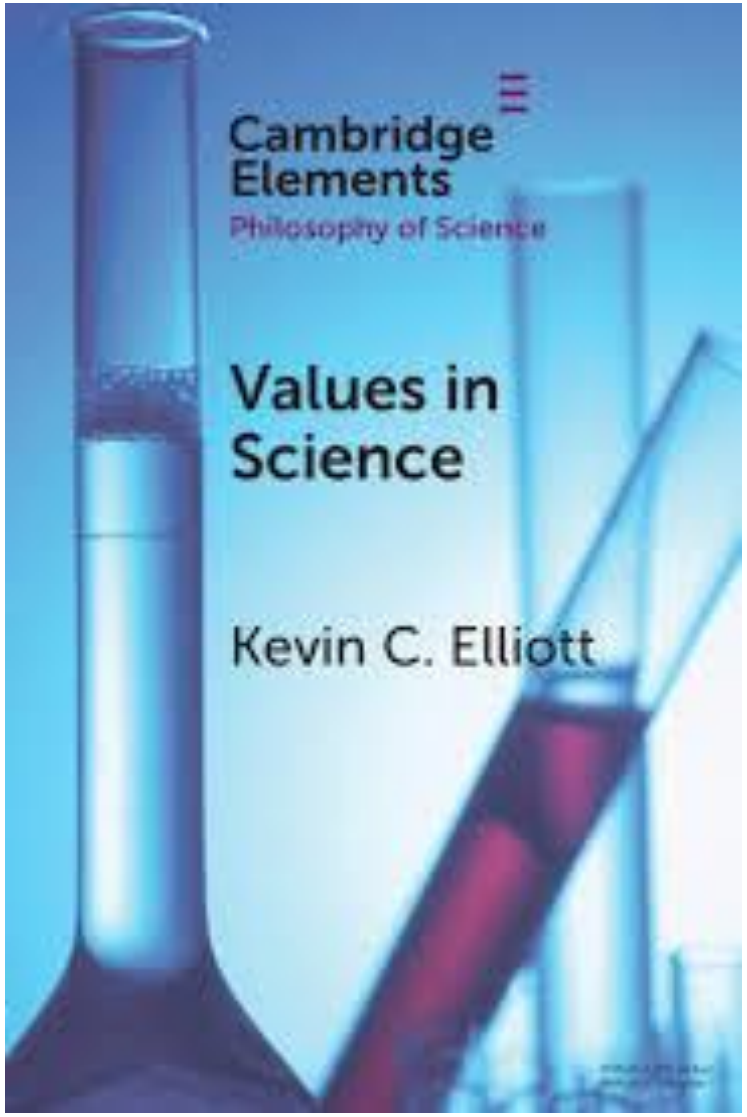
- - 2007: Implementazione del regolamento REACH nell'Unione Europea per la registrazione, valutazione, autorizzazione e restrizione delle sostanze chimiche, che promuove l'uso di test in vitro.
- - Metodi In Vitro:
- - Test di Ames (OECD TG 471): Per valutare la mutagenicità di sostanze chimiche usando batteri.
- - Test di citotossicità (LDH, MTT): Per misurare la capacità di una sostanza di uccidere cellule in coltura.
- - Corrositex: Per la valutazione della corrosività, importante nelle linee guida per la manipolazione e lo stoccaggio di sostanze chimiche.

Test In Vitro nei Farmaceutici

- - Pre-Screening Preclinico:
- - Anni '90: Inizio dell'uso estensivo di test in vitro per lo screening iniziale dei farmaci.
- - Screening ADME: Test per assorbimento, distribuzione, metabolismo ed eliminazione delle sostanze.
- - Modelli Avanzati:
- - 1997: Sviluppo dei primi modelli di epitelio umano ricostituito utilizzati nei test di assorbimento e irritazione.
- - Innovazioni Recenti:
- - Test Microfluidici:"Organo-su-chip" per modellare più accuratamente la fisiologia umana, migliorando la previsione degli effetti dei farmaci.

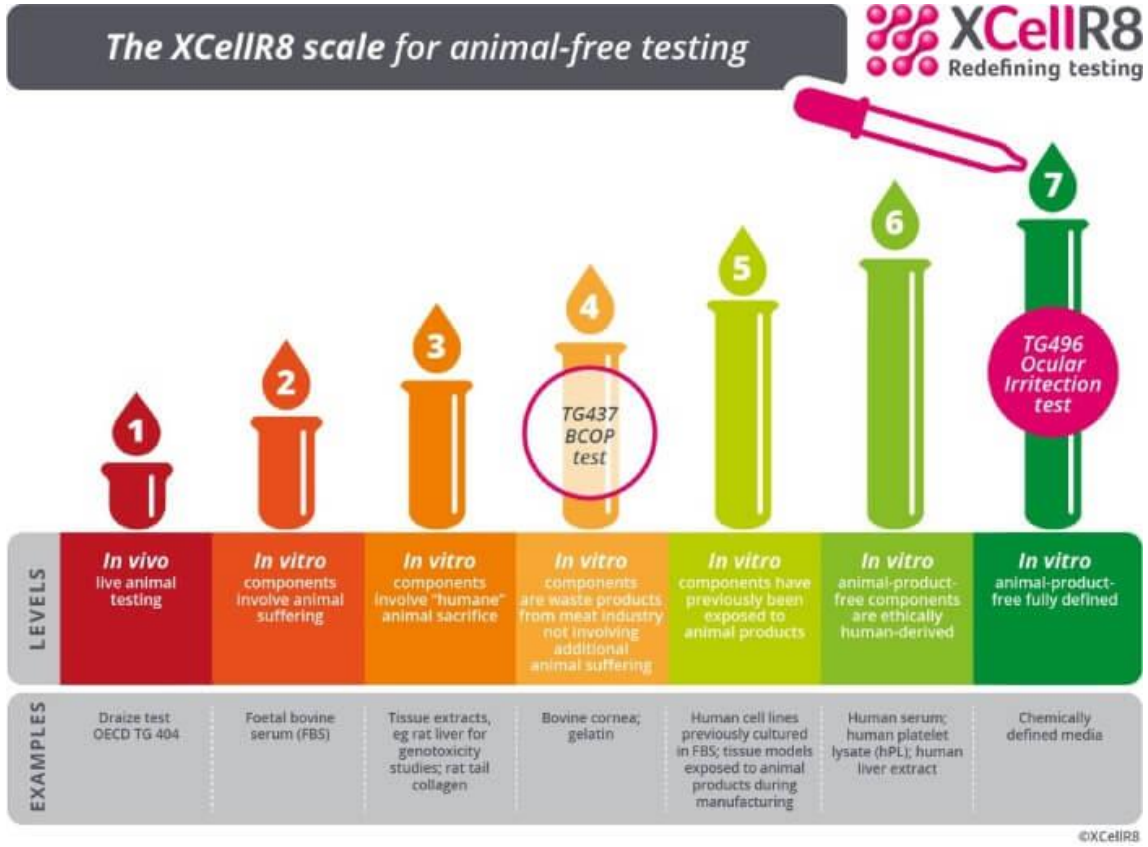
Valore Scientifico dei Test In Vitro

- - **Riproducibilità**:
- - Anni '90: Prime dimostrazioni della riproducibilità dei test in vitro nei contesti accademici e industriali.
- - **Standardizzazione**: Linee guida OECD e altri organismi regolatori che promuovono test validati e standardizzati.
- - **Controllo Sperimentale**:
- - Capacità di isolare variabili specifiche per studi mirati su meccanismi fisiopatologici e tossicologici.



Conclusione

- - I test in vitro rappresentano una pietra miliare per l’evoluzione delle pratiche di test nell'industria chimica, cosmetica e farmaceutica.
- - Con le continue innovazioni, questi test offrono un futuro promettente per prodotti più sicuri ed efficaci con un minore impatto etico.
- - Il miglioramento continuo delle metodologie in vitro garantisce una maggiore protezione per i consumatori e una migliore comprensione scientifica dei rischi associati alle nuove sostanze.



Irritection Assay System (IAS) Ocular and Dermal Irritection

Test Execution and Experimental Protocols

Contents of the Kits

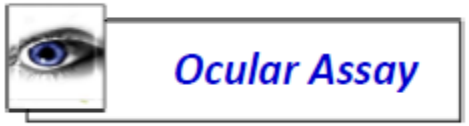
Scientific Background

- The Irritection Assay Systems are in vitro 100% animal free tests that: **Detect**, **Predict** and **Rank** the ocular and dermal irritation potential of several type of materials and substances.

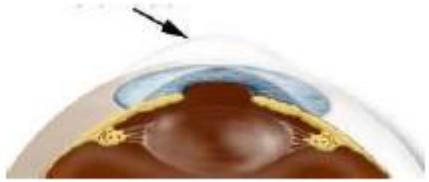
  - Cosmetic products
  - Household products
  - Pharmaceuticals
  - Chemical Substances
  - Raw Materials

Scientific Background

- The IAS mimic the biochemical phenomena of the ocular and dermal irritation as they would occur in vivo



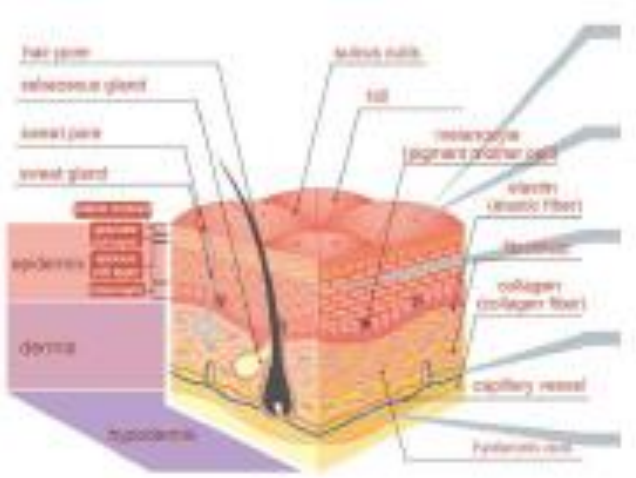
- The corneal irritancy of a chemical is known to be related to the propensity of said chemical to promote denaturation and disruption of corneal proteins



Scientific Background

Dermal Assay

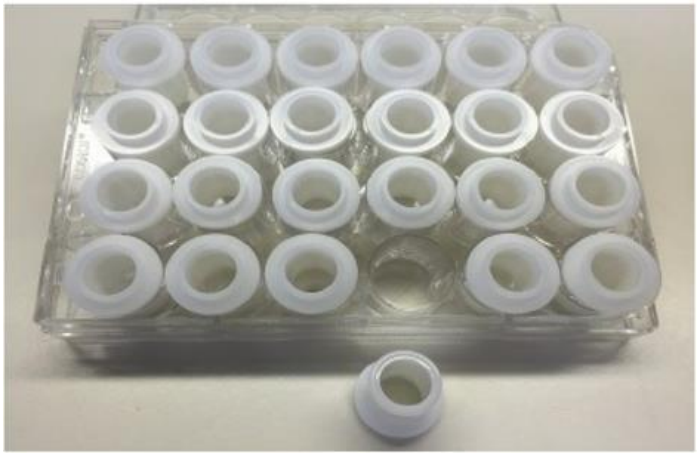
- Chemicals that cause dermal irritation are known to induce alterations in the structure of ketarin, collagen and other dermal proteins.



Scientific Background

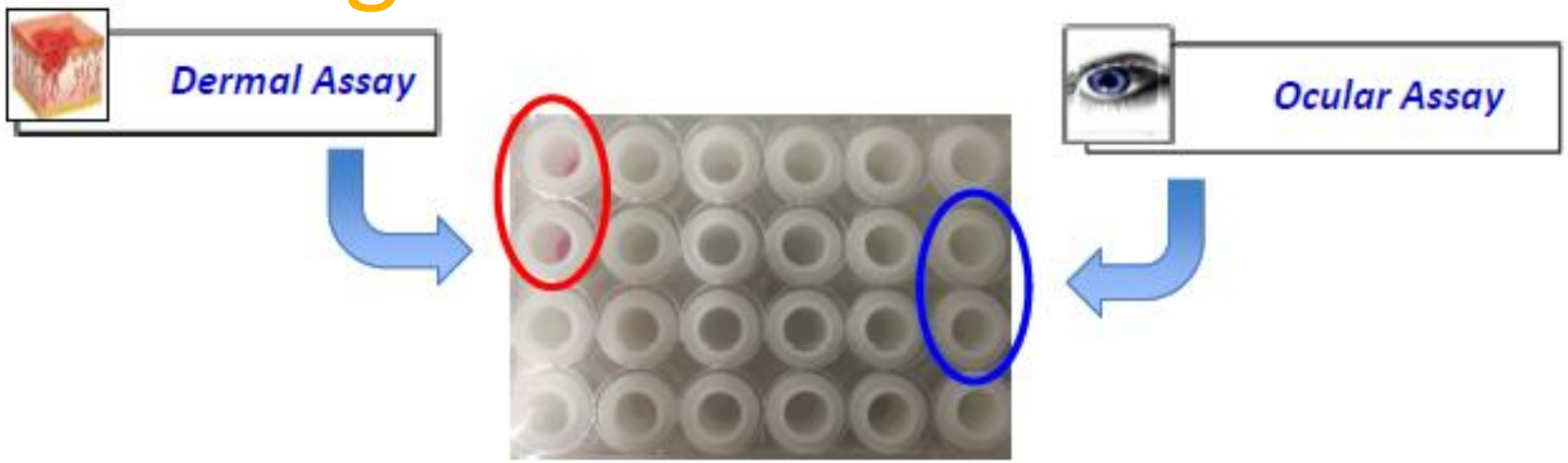
- In order to mimic these biochemical phenomena the methods developed make use of 2 important components

Membrane Discs



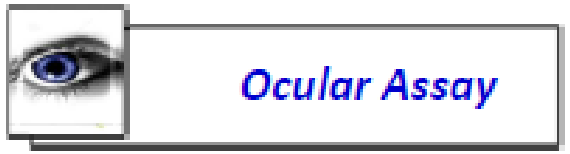
Propriatory Protein Reagent

Scientific Background – Membrane Discs



- The main difference in the membrane discs between dermal and ocular kits is that the dermal membrane discs have been coated with keratin and collagen in order to more closely mimic the barrier properties of the dermis.
- Additionally a red dye is bound to these proteins during manufacturing, to indicate disruption of the keratin/collagen biobarrier when testing is performed

Scientific Background – Proprietary Protein Reagent

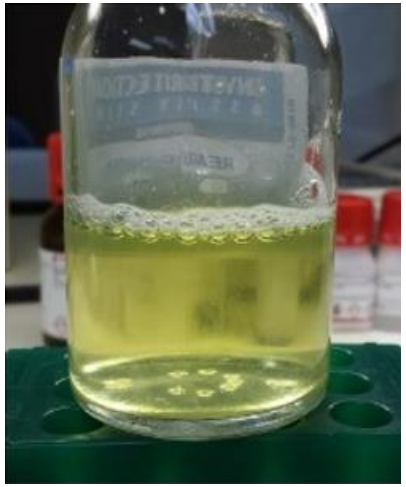


- The Dermal proprietary protein reagent contains additional globulins with respect to the Ocular one.

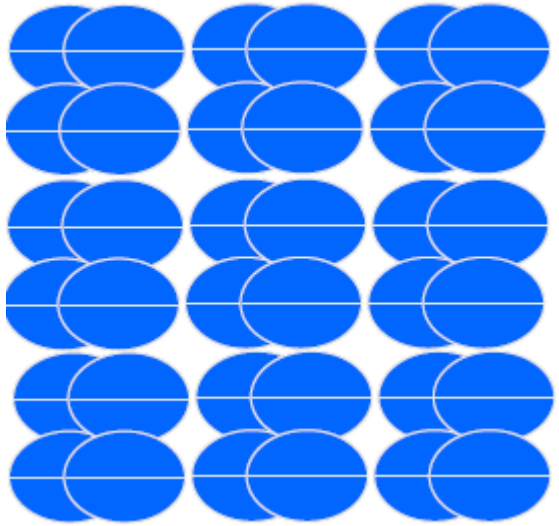
- The major constituent is an oligomeric protein cosisting of 12 subunits. With a moleculae weight of about 320kD, lipids and low molecular weight components

Scientific Background

- When the proprietary protein reagent is rehydrated with a buffering salt solution, proteins, glycoproteins, lipids and other low molecular weight components combine with additional reagent constituents and form an ordered macromolecular matrix.
- Mimicking this way the structure of the Dermis or the Cornea



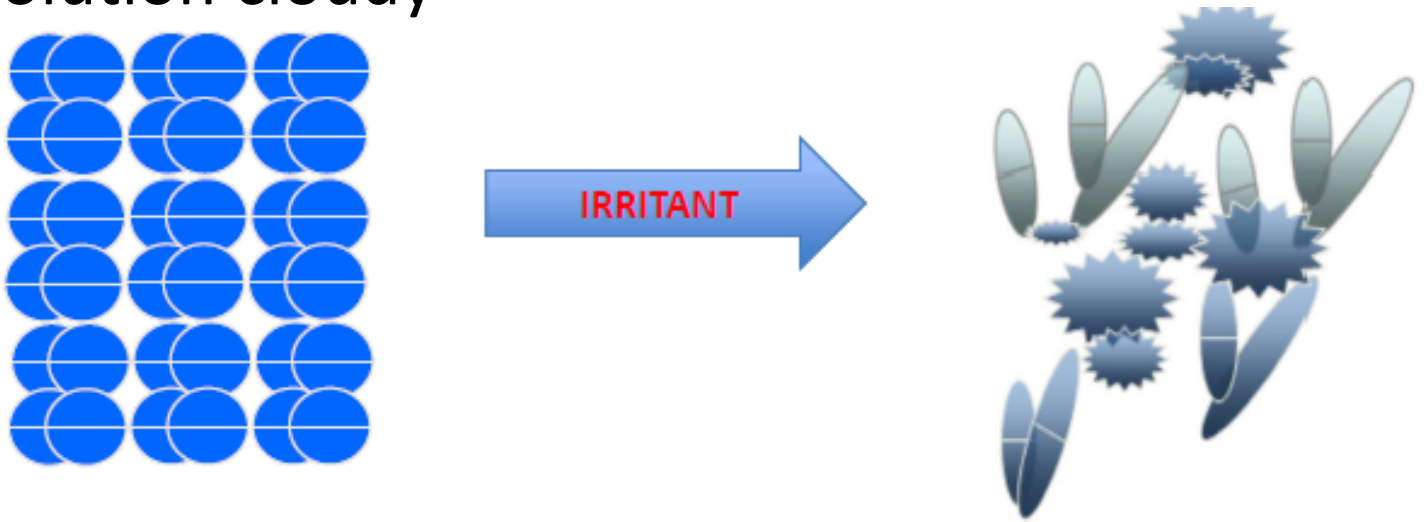
Reconstituted Protein Solution



Ordered Macromolecular Matrix

Scientific Background

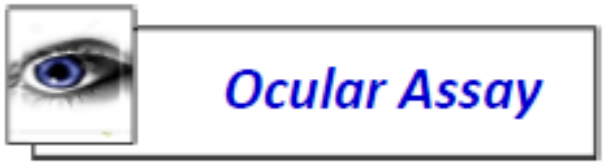
- The application of chemical irritants the protein reagent solution will lead to protein denaturation. This change in conformation will disrupt the highly ordered macromolecular matrix which will gradually form minute insoluble particles making the clear protein solution cloudy

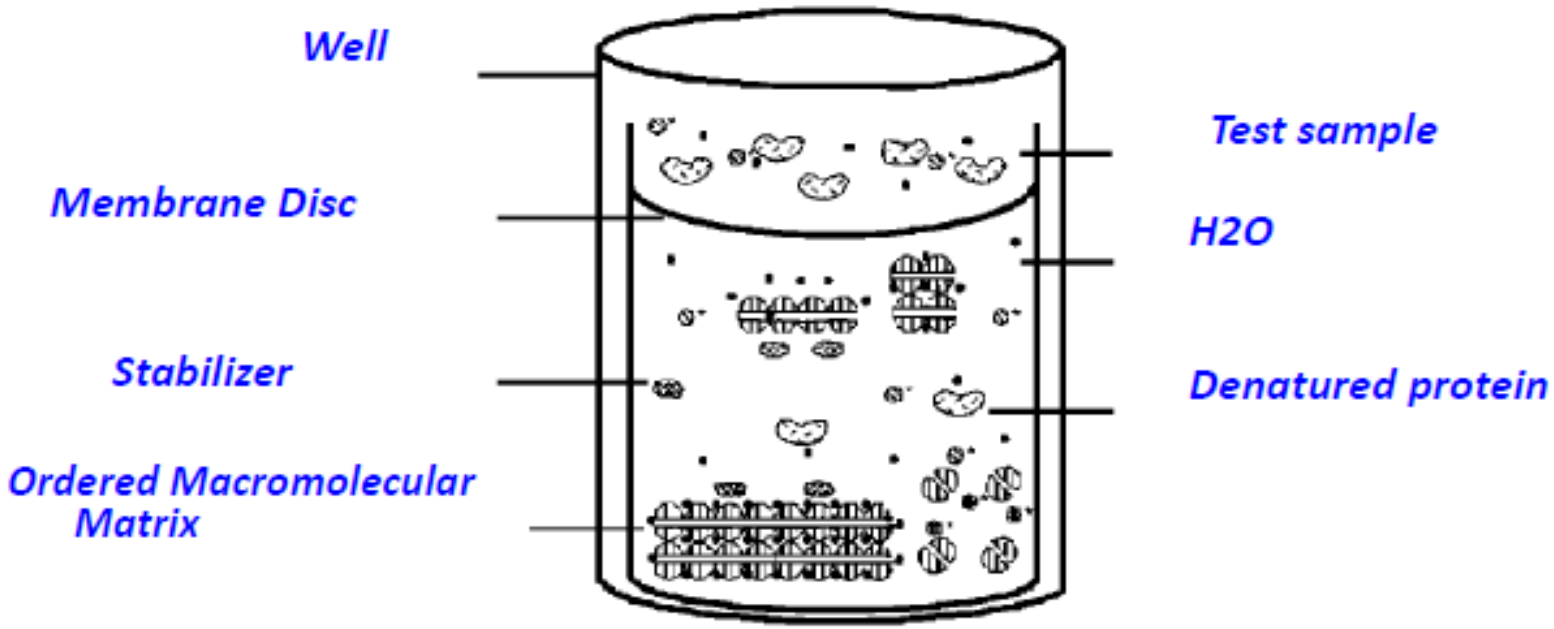


Ordered Macromolecular Matrix
Clear Solution

Disordered Macromolecular Matrix
Cloudy Solution

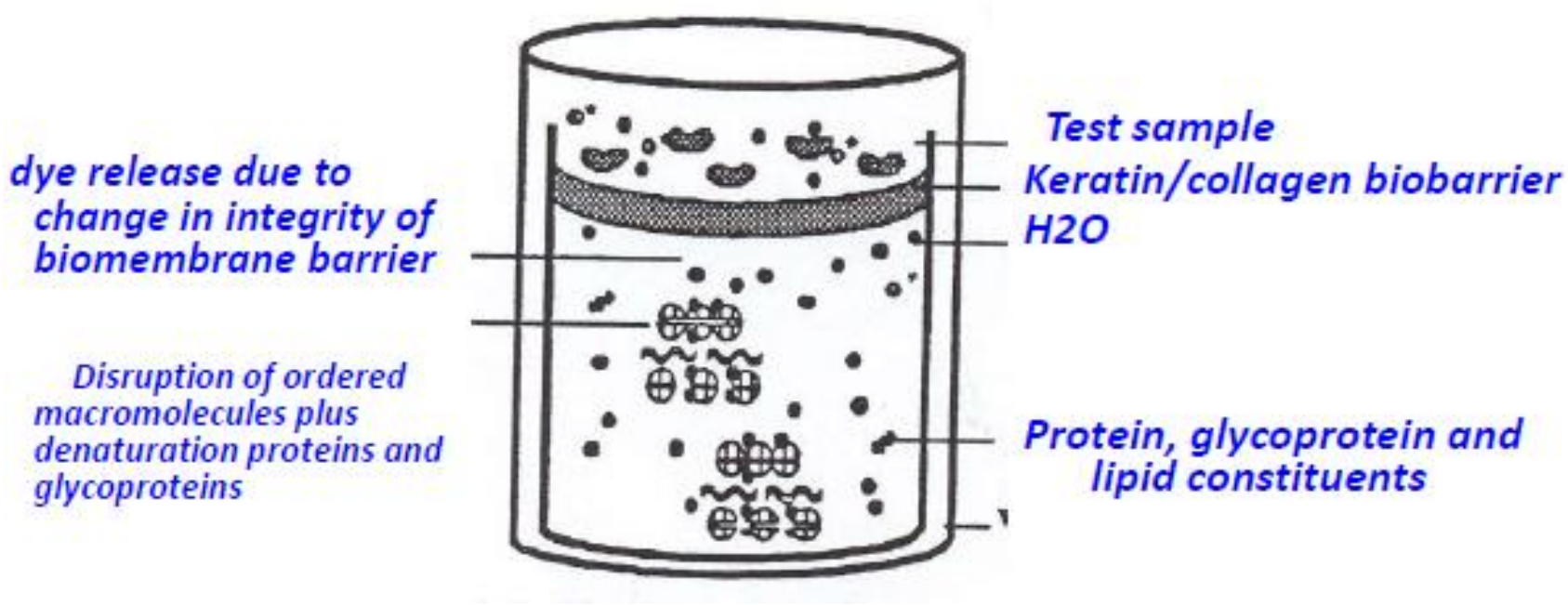
Scientific Background



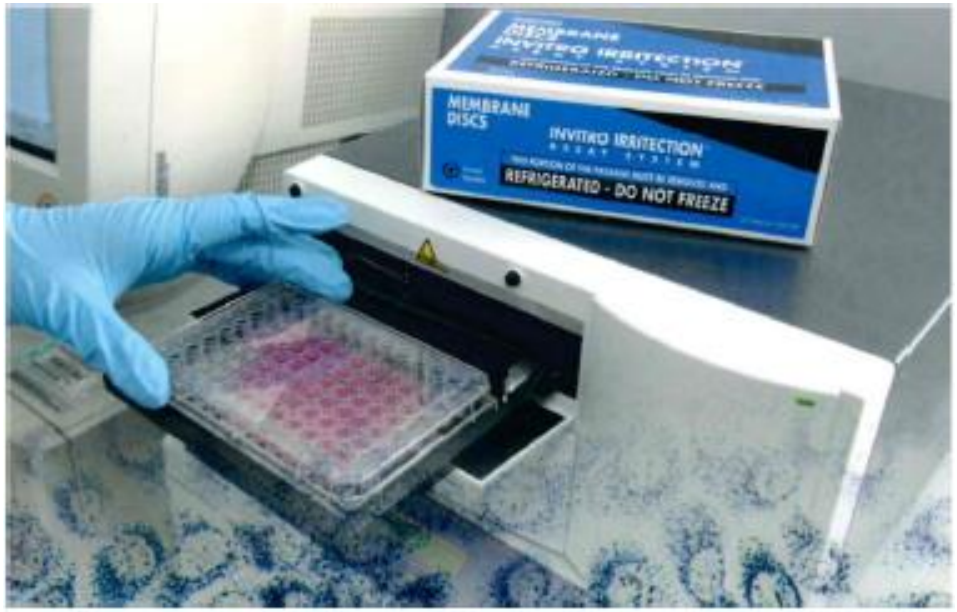


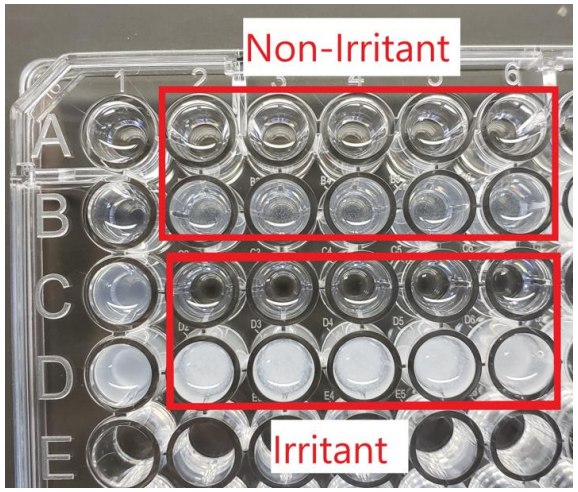
Scientific Background

Dermal Assay



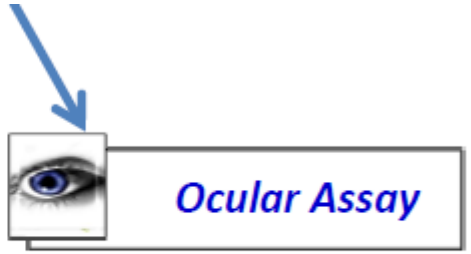
Scientific Background / Quantification





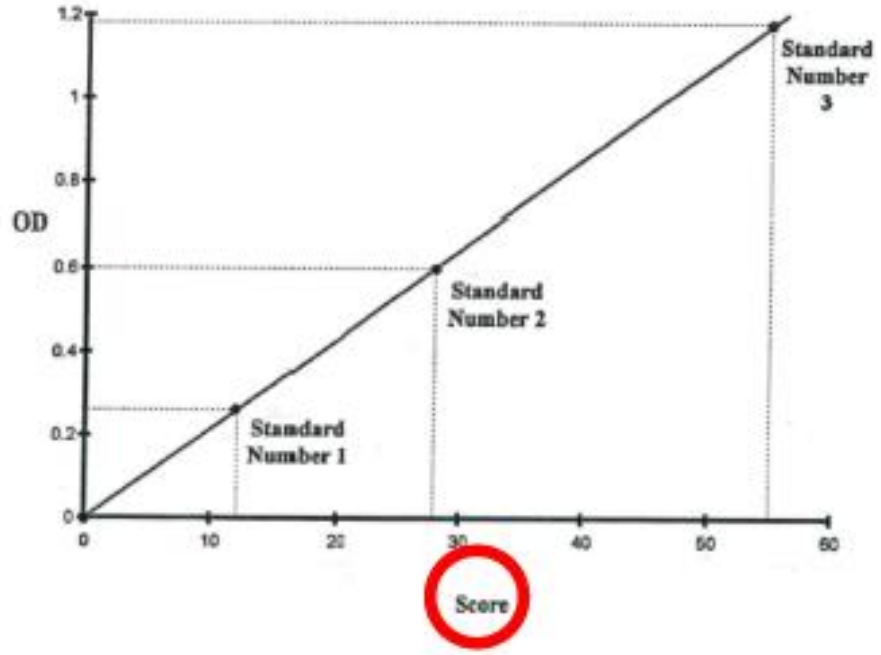
- These phenomena can be quantified by measuring the change in light scattering that occurs as the protein matrix becomes disrupted and the turbidity of the solution increases.
- The light scattering or optical density can be measured by using a plate reader, fitted with the Irritection Assay System software

450nm



405nm

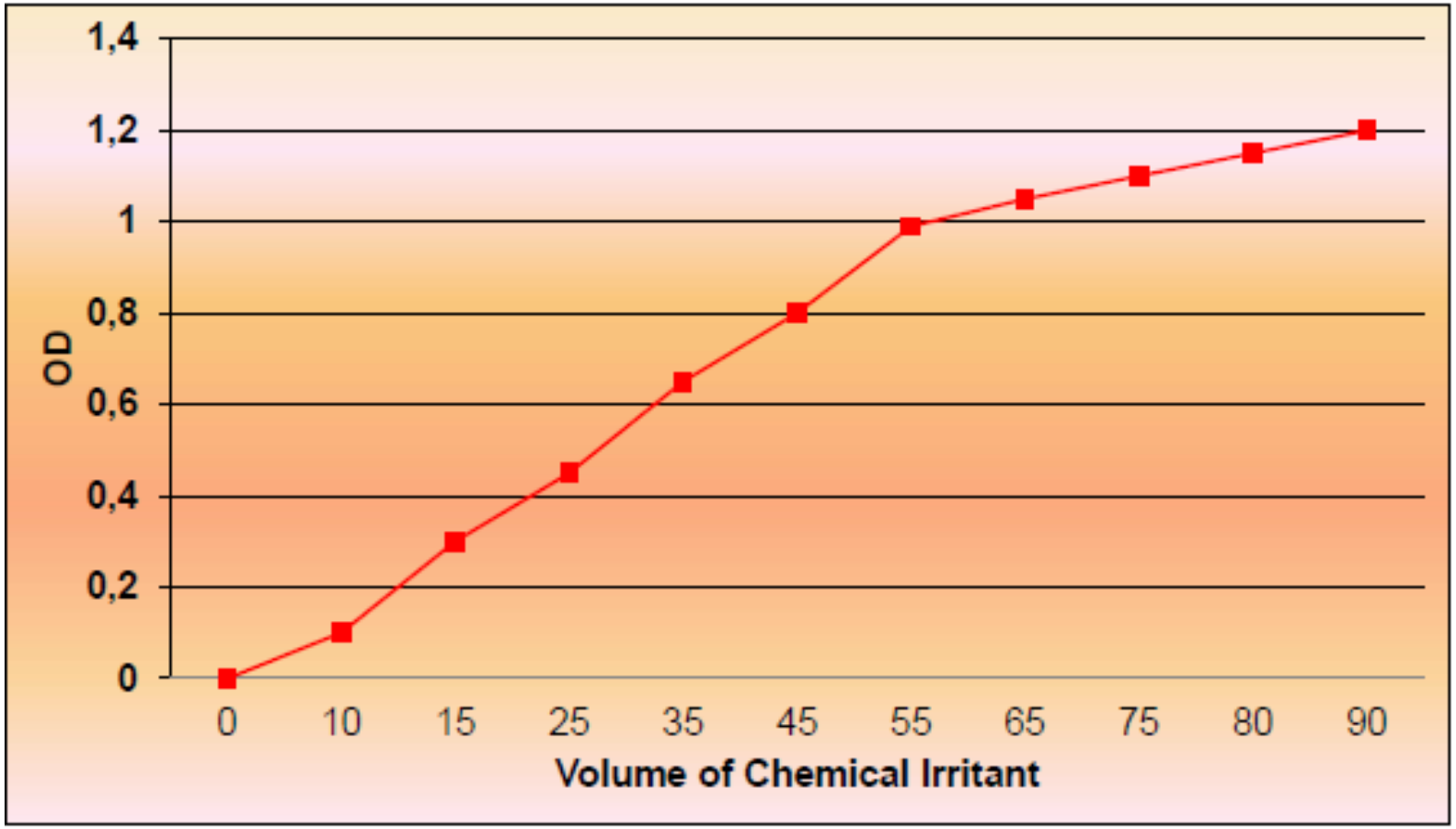
Scientific Background



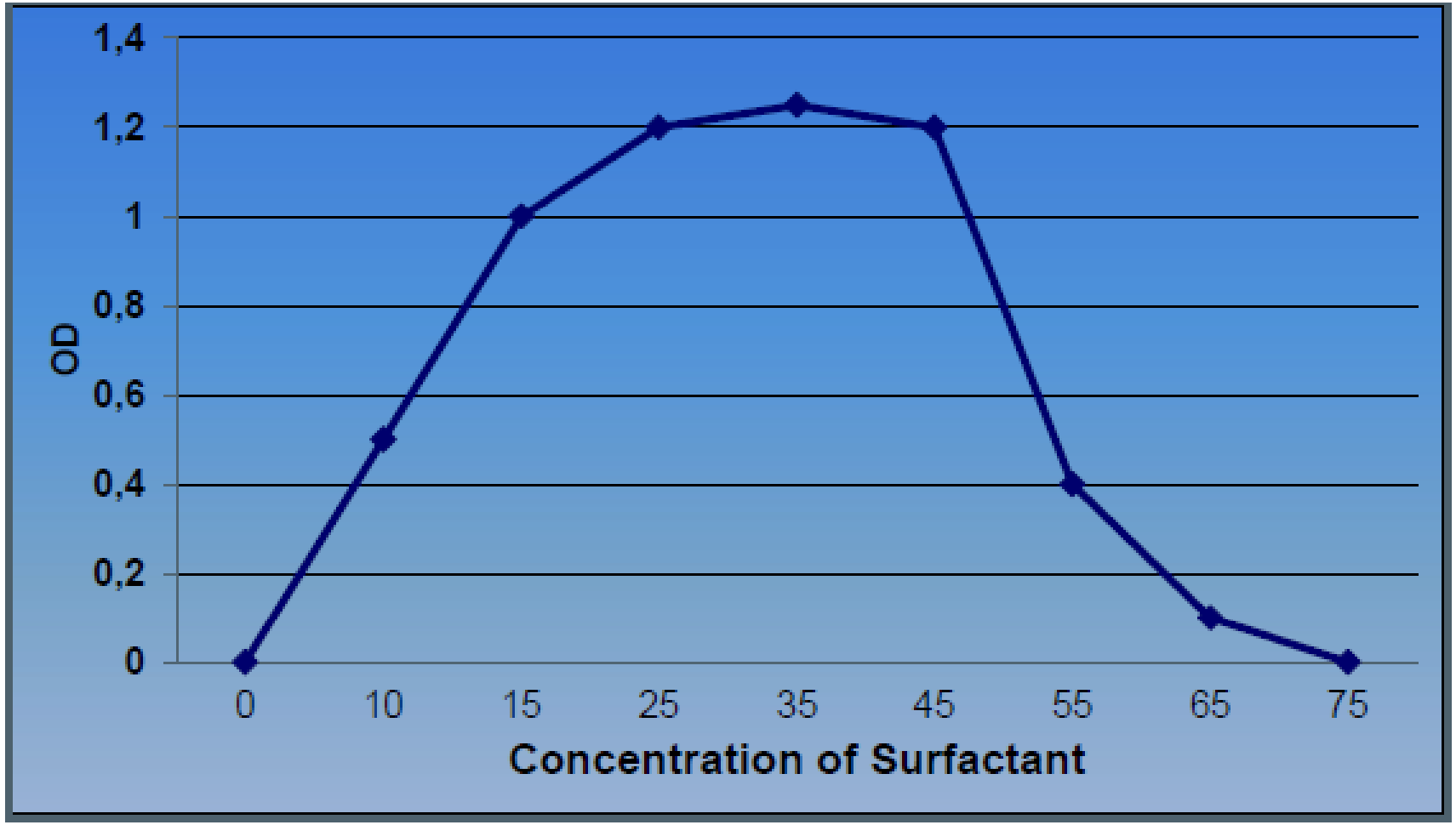
- Calibrator substances of known irritancy potential are measured together with the test samples.
- This allows the construction of a standard curve that directly relates the Optical Density measurements to an Irritection test score determined by in vivo studies

- The comparison of OD produced by the test sample to the standard curve permits the calculation of an irritancy score that has been shown to be directly related to the potential irritancy of the test material

Scientific Background / Typical Chemical Irritants

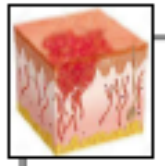


Scientific Background / Surfactant Materials



Scientific Background / Irritancy Score

Human Irritancy Equivalent (HIE) Score	Predicted Dermal Irritancy Classification
0.00 - 0.90	Non-Irritant
0.90 - 1.20	Non-Irritant/ Irritant
1.20 - 5.00	Irritant



Dermal Assay

Ocular Assay

Irritection Draize Equivalent (IDE)	Predicted Ocular Irritancy Classification
0.0 – 12.5	Minimal Irritant
>12.5 – 30	Mild Irritant
>30 – 51	Moderate Irritant
> 51	Severe Irritant

Test Execution and Experimental Protocols

Contents of the Kits

Test Execution and Experimental Protocols

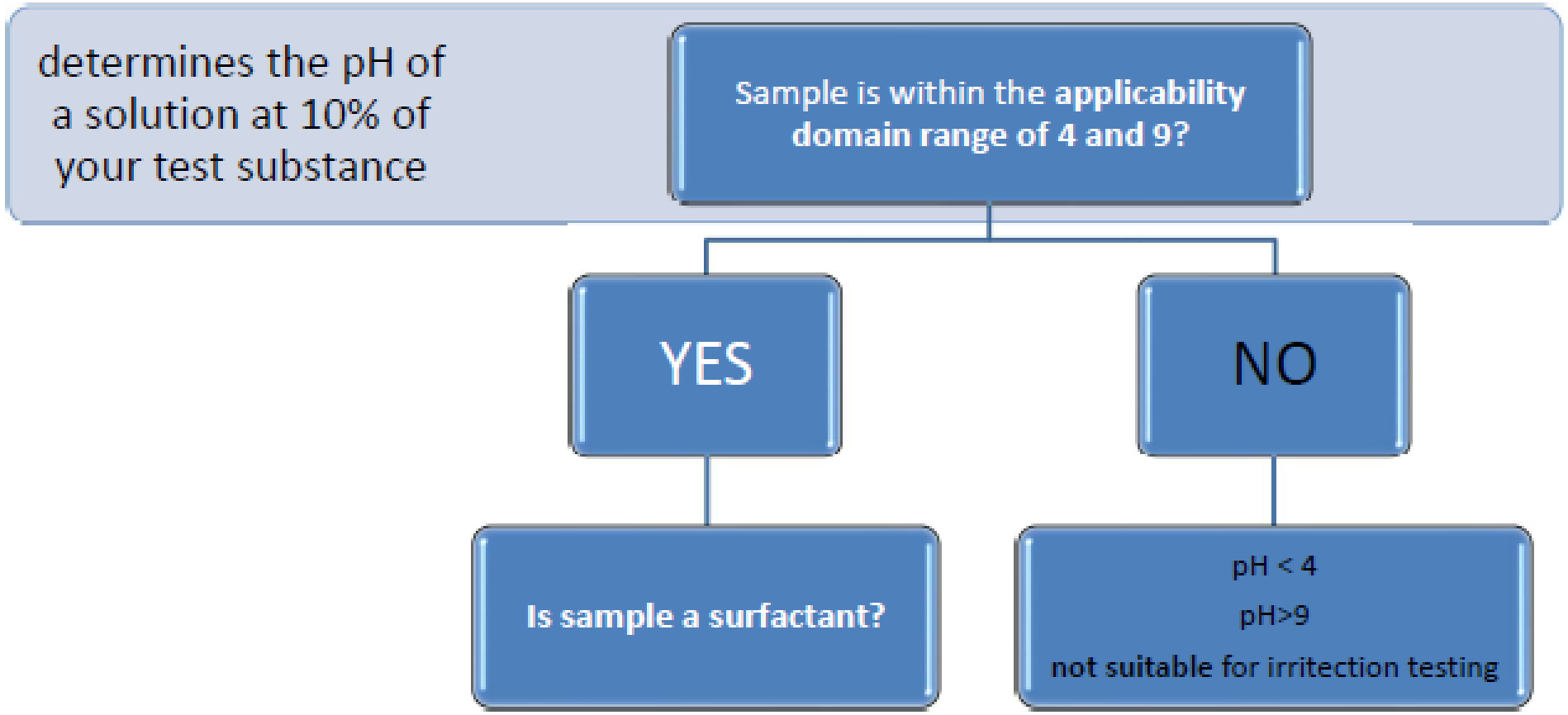
Contents of the Kit	
Proprietary Reagent Powder	When hydrated forms a solution containing an ordered macromolecular matrix
Hydrating solution	Employed to rehydrate the Reagent Powder and facilitate formation of the ordered protein matrix
Blanking Buffer	Employed as control solution which accounts for the test sample background contribution to the assay
Activator_A	Lowers the pH of the reagent solution the appropriate level to initiate formation of the ordered macromolecular matrix
Calibrators: Cal0, Cal1, Cal2, Cal 3	Known irritants that are employed in each assay to provide standardization and determination of irritancy scores
Quality Controls: QC1 and QC2	Known irritants that are employed in each assay as quality assurance controls to ensure proper performance of the assay
Inhibition Check	Strong irritant substance that is employed as a positive control to check for false negative results at completion of assay
Membrane Discs	Semi permeable membranes that facilitate controlled delivery of the test sample to protein reagent

Kit Formats

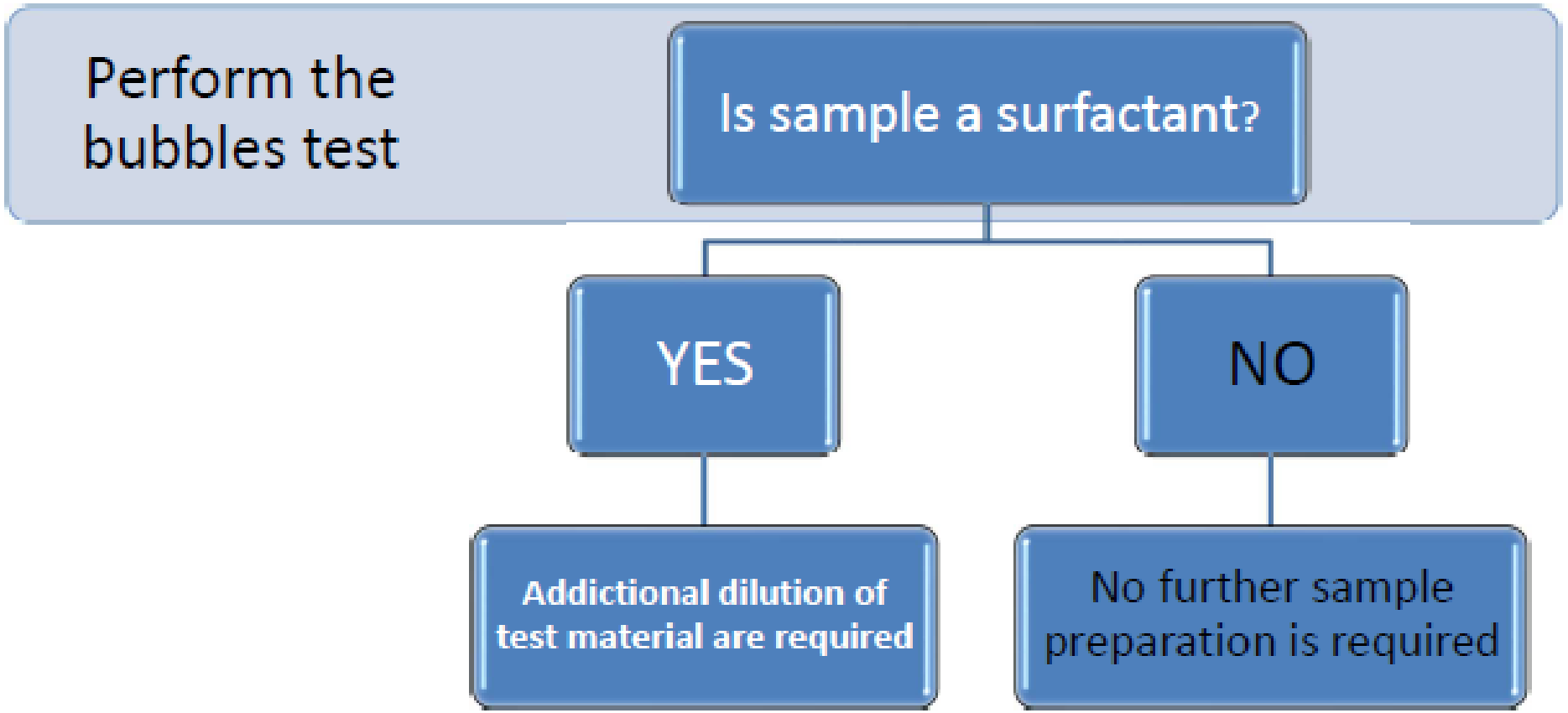
Both Ocular and Dermal Irritection kits come in a variety of sizes that can accommodate testing of multiple substances

- 1 sample kit
- 2 samples kit
- 3 samples kit
- 4 samples kit

Test Execution / 1. Preparation of Test Substance



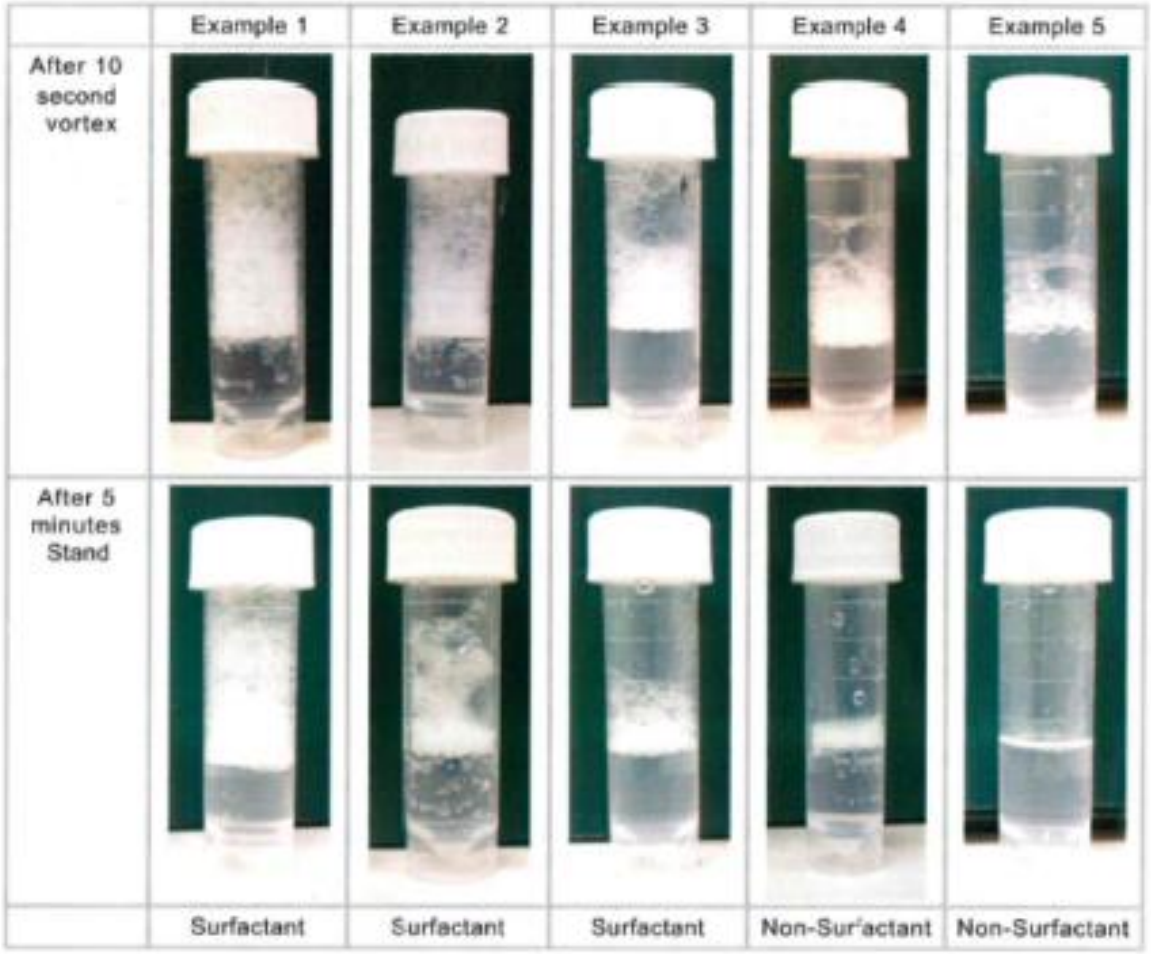
Test Execution / 1. Preparation of Test Substance



Test Execution / 1. Preparation of Test Substance

Bubbles Test

- Solution at 10% of your test substance
- Vortex for 10 seconds
- Allow it to stand for 5 minute
- Examine the sample to see if there is persistent layer of bubbles



Test Execution / 1. Preparation of Test Substance

Non Surfactant Chemical → Volume Dependent dose response protocol

Surfactant Chemical → Concentration Dependent dose response protocol

Test Execution / 1. Preparation of Test Substance

IAS	Protocol	Doses applied
Dermal	Volume Dependent	25, 50, 75, 100, 125 µL or mg*
Dermal	Concentration Dependent	1, 5, 10, 25, 50%
Ocular	Volume Dependent	25, 50, 75, 100, 125 µL or mg*
Ocular	Concentration Dependent	0,3125, 0,625, 1,25, 2,5, 5%

*In cases of solids or waxy liquids weigh in mg rather than µL

Ocular Irritection / OECD 496 / DB/ALM-157

- On the 24th of October 2019, Ocular Irritection received OECD approval with the protocol number OECD 496

OECD/OCDE **496**

Adopted:
24 October 2019

OECD GUIDELINE FOR TESTING OF CHEMICALS

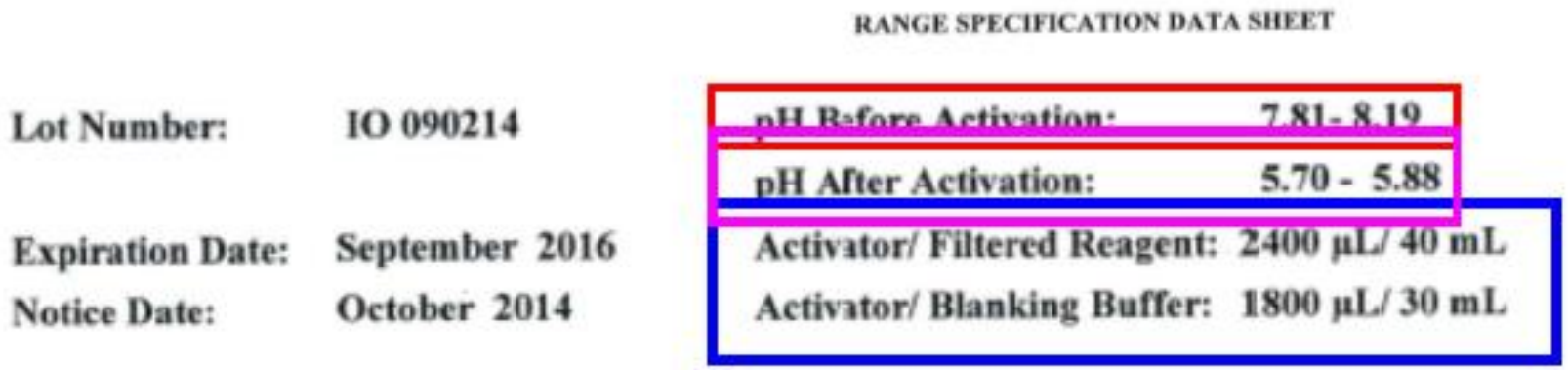
In vitro Macromolecular Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

Test Execution / 2. Reagent Preparation

- Place Hydrating Solution in 25°C incubator and remove membrane discs plate from refrigerator. Allow them to come to room temperature. Do this procedure 2 hours before commencing the test.
- **Rehydration:** Pour all of the hydrating solution into the reagent powder and gently swirl. Let the dissolved reagent stand at RT for about 10 minutes
- **Filtration:** Pour all of the dissolved reagent into a funnel using the filter paper provided within the kits.

Test Execution / 2. Reagent Preparation

- Record the pH of the dissolved and filtered reagent verifying that it falls within the specifications provided in the Range Specification Data Sheet



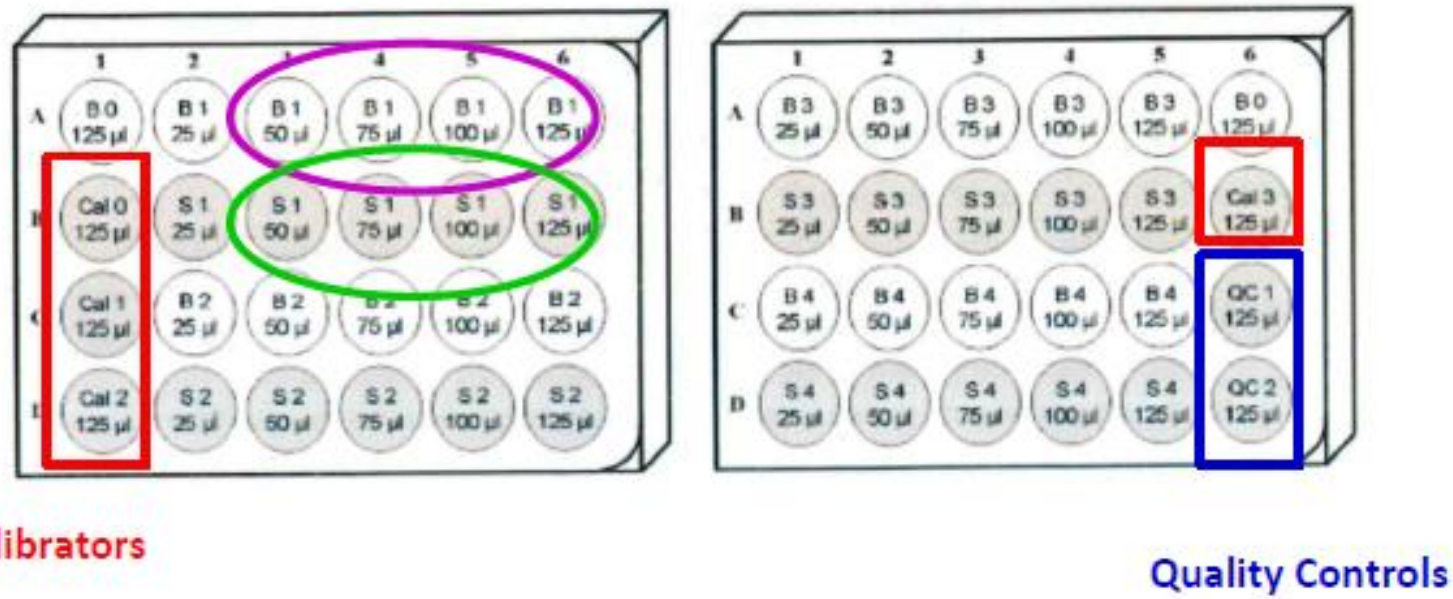
RANGE SPECIFICATION DATA SHEET

Lot Number:	IO 090214	pH Before Activation:	7.81 - 8.19
		pH After Activation:	5.70 - 5.88
Expiration Date:	September 2016	Activator/ Filtered Reagent:	2400 µL/ 40 mL
Notice Date:	October 2014	Activator/ Blanking Buffer:	1800 µL/ 30 mL

- Activation: add the activator reagent to the dissolved and filtered reagent and record the pH once more. Make sure it falls within the specifications of the Range Specification Data Sheet. Add the activator to the Blanking Buffer as well

Test Execution / 3. Test Material Exposure Procedure

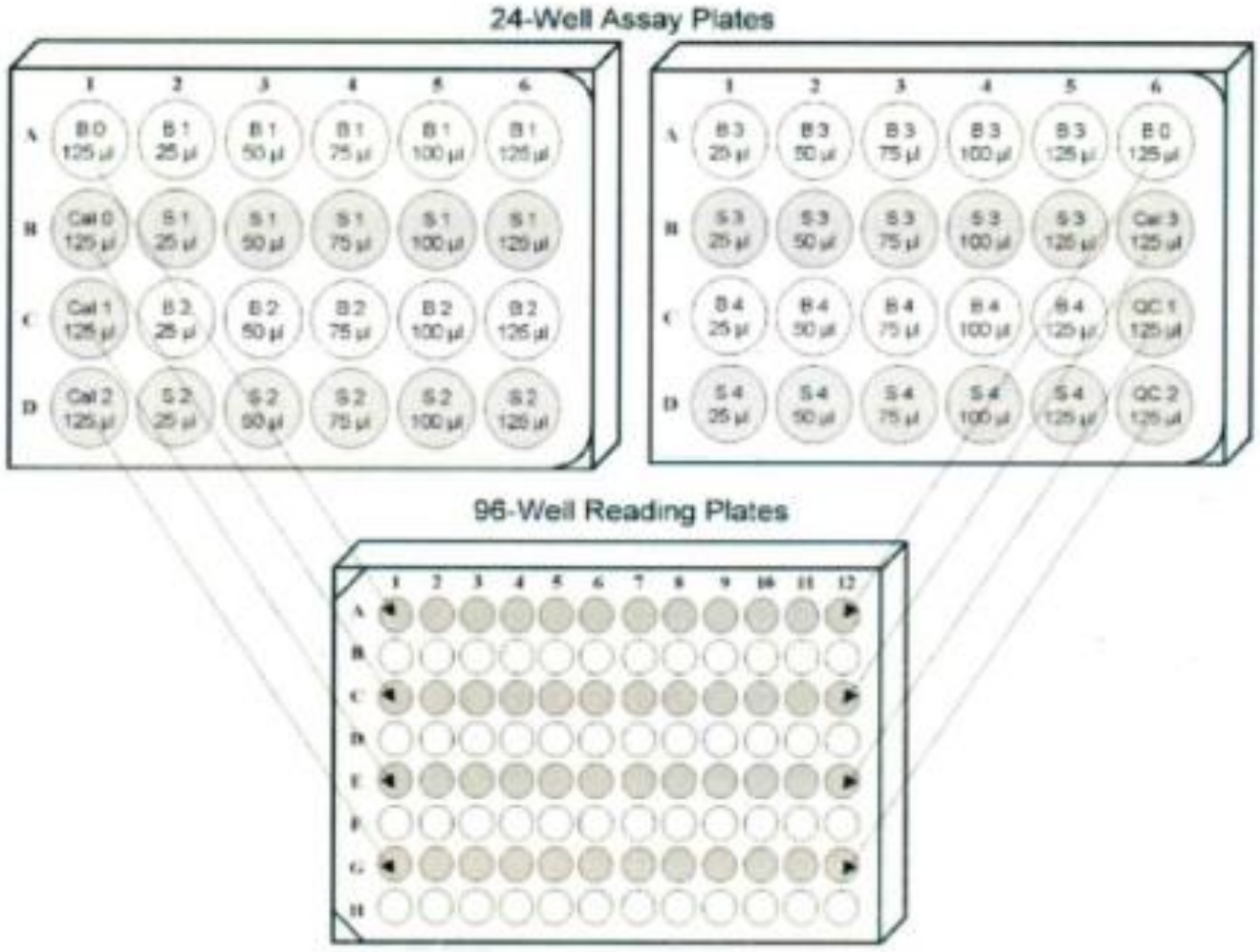
- Follow the schemes provided in the instructions manual about how to layout samples, calibrators and quality controls on the 24 well plate.



Note that for DB-ALM protocol surfactant, the samples are directly pipetted directly in the wells below the membrane discs

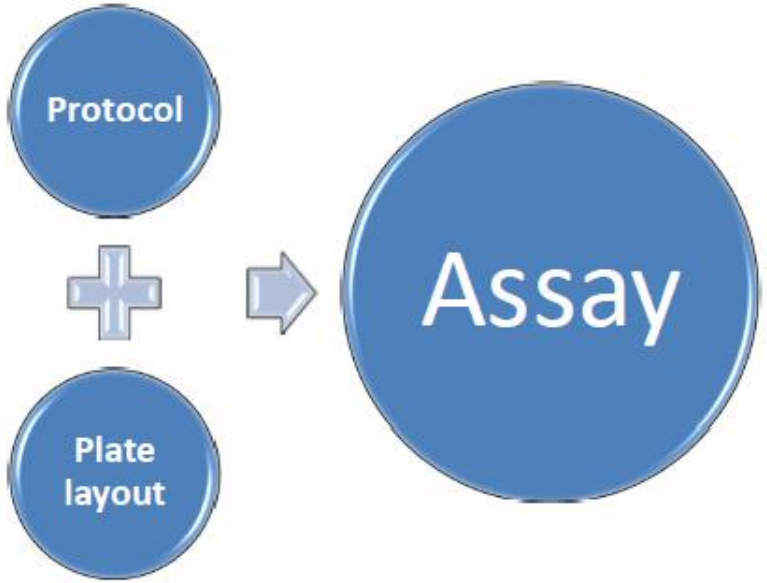
Test Execution / 4. Incubation and After

- Cover the 24 well plate with parafilm and incubate for 24 hours.
- After incubation transfer the 24 well assay plate to a 96 well reading plate according to the diagram of the manual
- Place into the plate reader and set up the software for reading.



Irritection Software

- Serves as a user interface to the plate reader
- Processing of spectrophotometric readings
- Compares the increase in OD produced by the samples to the standard curve
- Calculates the ‘’irritancy score’’ for each tested sample
- Provides graphical representation of the results



Irritection Software / Qualified Assay

ASSAY REPORT - ORIGINAL

Sample Description	:		Date	:	06/04/15
Sample Number	:	15_IT021	Time	:	10:53:56
Product Type	:	COSMETICO	Technician Name	:	LOSINI
Assay Method	:	Dermal	Kit Lot Number	:	ID120213
Protocol	:	Cosmetic_ID120213_04_6_	Reagent temperature	:	0.0
Incubation Time	:	24.0 hours	Reagent pH Before Activation	:	10.01
Plate Layout	:	5 Samples*4 Volumes	Reagent pH After Activation	:	8.30
Instrument Type	:	Tecan (auto select)	Sample pH	:	
Wavelength	:	450nm	Assay Number	:	3
Comment	:		Assay Qualification	:	Qualified

Sample Results:

Dose	Sample OD	Blank OD	Net OD	Irritancy Score	Irritancy Classification	Qualification
50 ul	471	9	462	2.03	Irritant	Qualified
75 ul	565	6	559	2.40	Irritant	Qualified
100 ul	628	6	622	2.64	Irritant	Qualified
125 ul	719	9	710	2.98	Irritant	Qualified

Calibrator Values:

Designation	OD	Irritancy Score	Range Limit (OD)	Qualification
Cal 0	170	0.00	0 - 200	Range qualified
Cal 1	196	1.00	104 - 260	Range qualified
Cal 2	454	2.00	330 - 630	Range qualified
Cal 3	977	4.00	810 - 1430	Range qualified

Quality Control Values:

Designation	OD	Irritancy Score	Range Limit (Score)	Qualification
QC 1	147	0.75	0.11 - 0.95	Range qualified
QC 2	723	3.03	0.94 - 3.60	Range qualified

* Mean value from assay data history
** Mean value from protocol defaults or adjusted value due to calibrator zero substitution
[ ] Value before substitution

Results Interpretation and Data Analysis

Indicates that values obey expected behaviour of typical dose response curve

Dose	Sample OD	Blank OD	Net OD	Irritancy Score	Irritancy Classification	Qualification
25 mg	124	-4	128	7.4	Minimal	Qualified
50 mg	114	-1	115	6.7	Minimal	Qualified
75 mg	140	-2	142	8.2	Minimal	Qualified
100 mg	151	-4	155	9.0	Minimal	Qualified
125 mg	134	-5	139	8.0	Minimal	Qualified

The highest irritancy score calculated by the software is the irritancy of the test sample and it is called Maximum Qualified Score (MQS)
It is the value that correlates the most with the in vivo irritancy property of the test material

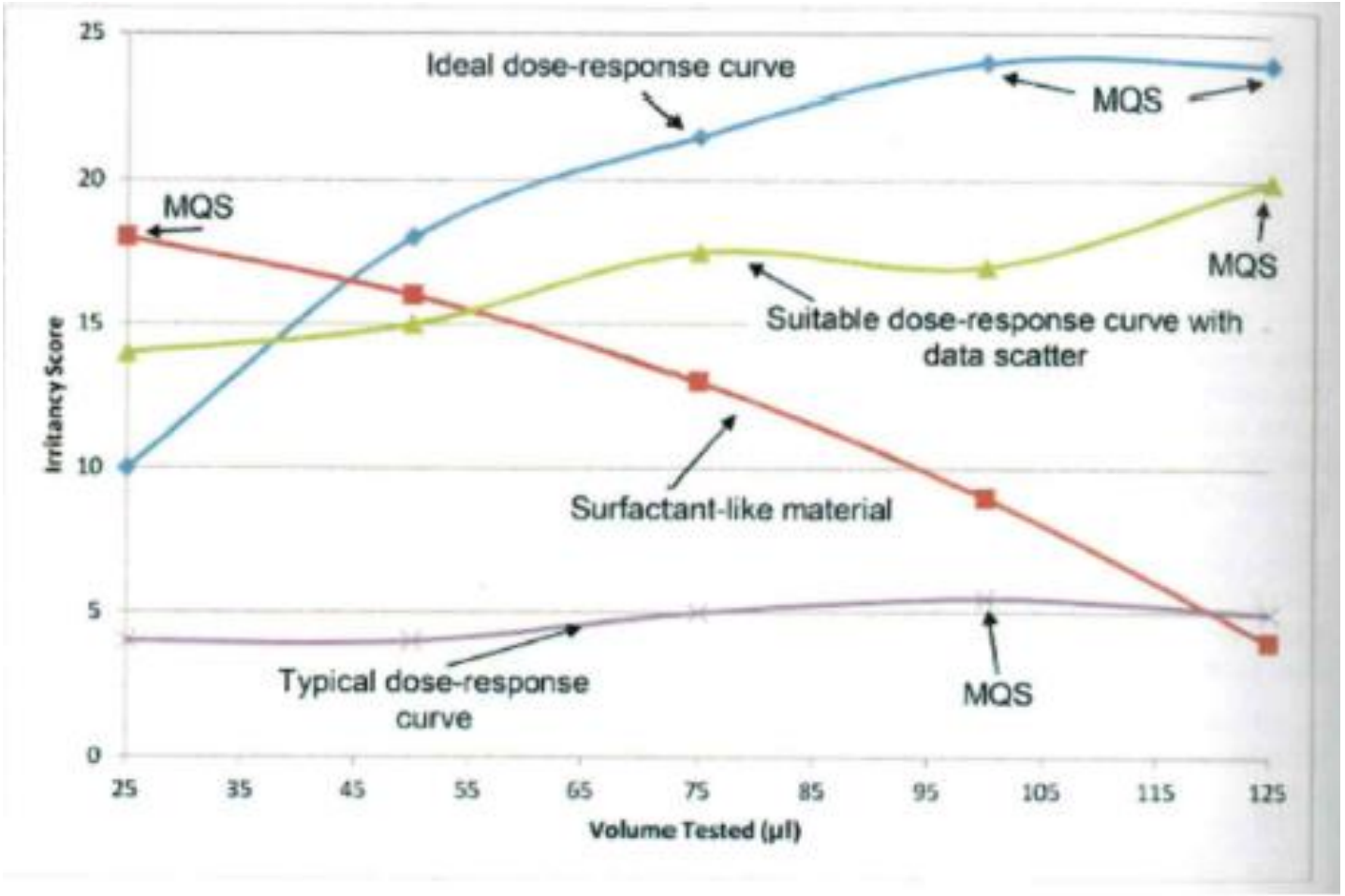
Results Interpretation and Data Analysis / Reporting

Sample	Protocol	Method	Max IDE Score	Predicted Dermal Irritancy Classification
Campione 1	Cosmetic / Volume dependent dose response curve	Ocular Irritection	9.0	Minimal Irritant

Interpretation of Ocular Irritection® Results According to Protocol

Protocol / DIE	DB-ALM 157	OECD 496	GHS Classification
0 – 12,5	Minimal	No Category	Non-Irritant
> 12,5 – 30	Mild	No Prediction	Irritant
> 30 – 51	Moderate	Category 1	
> 51	Severe		

Various Dose Response Curves



Grazie per l'attenzione

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