Use of *In Vitro* Skin Irritation/ Corrosion Test Methods for Toxicity Assessment of Pesticides

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Application of IATA

OECD Series on Testing and Assessment

Guidance Document on the Integrated Approach on Testing and Assessment for **Skin Corrosion and Irritation**

Examination of existing data

Epidemiological/clinical data

Experimental data (animal/*in vitro*/*ex vivo*/*in chemico*)

Non-testing strategies (read across/ bridging from structurally/biologically related substances)

Where are data insufficient ?

Conduct testing using a non-animal sequential testing strategy

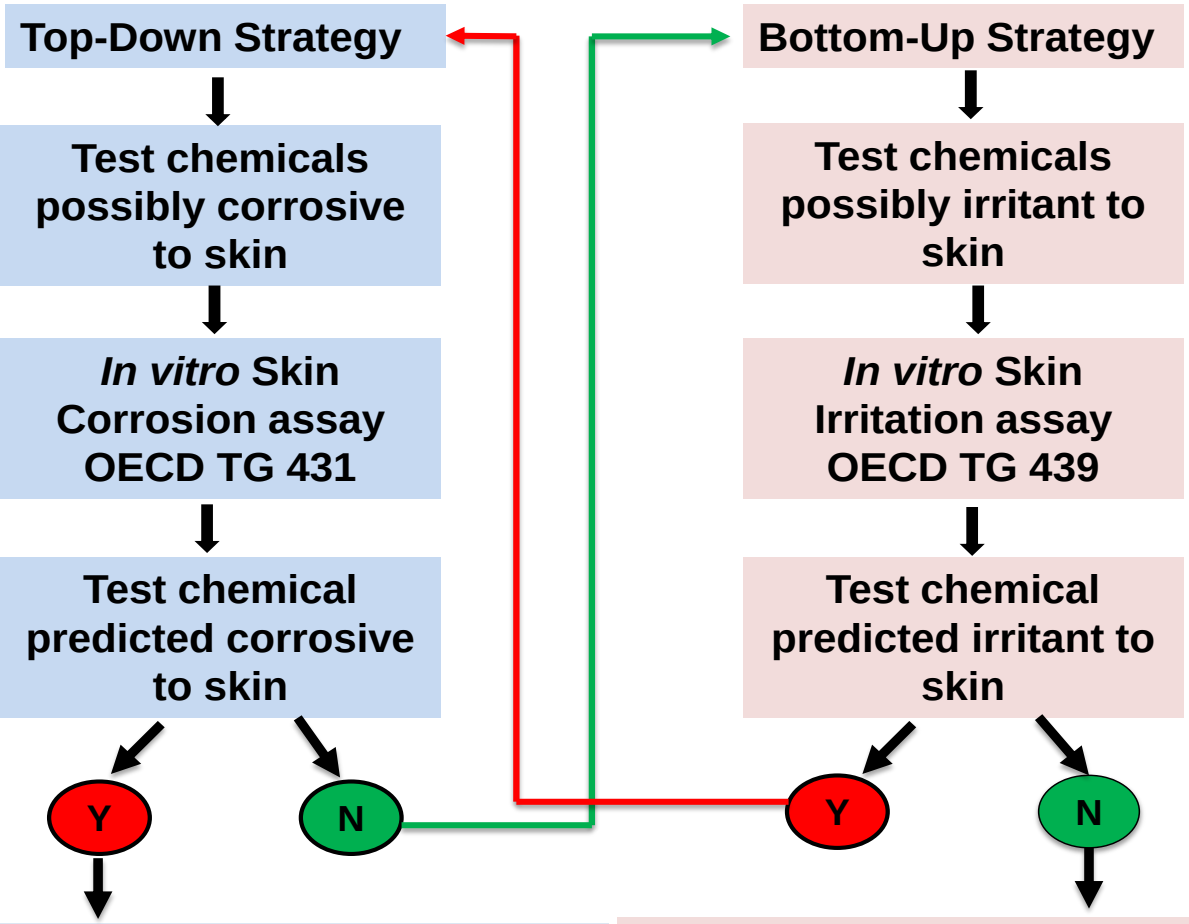
(OECD TG 430, 431, 435, 439)

Bottom-up approach: start with skin irritation test

Top down approach: start with skin corrosion test

Animal testing as last resort

Tiered Testing Strategies



- Test chemical may be labeled as corrosive to skin
- Test chemical may also be classified by the relevant packing group: Corrositex Assay (OECD TG 435)

- Test chemical may be labeled as non-irritant to skin
- Test chemical does not require any further skin irritation testing

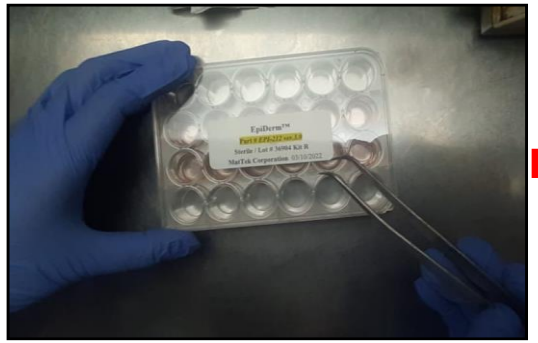
- **Top-down approach-** When test chemical is suspected to be corrosive (based on existing information)
- **Bottom-up approach-** When test chemical is not suspected to be corrosive

Wilt N. et al. The Toxicologist, 144, 89 (2015)

Reconstructed Human Epidermis (RhE) Test Method

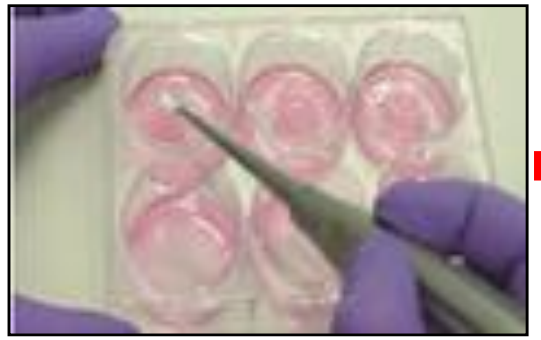
Test system:	RhE models: EpiDerm™ (EPI-200); EpiSkin™ (SM); SkinEthic™ RHE; epiCS®; LabCyte EPI-MODEL24
Assay endpoints:	Percent cell tissue viability by MTT assay
Assay control:	Negative control (sterile PBS or Normal saline; Positive control (KOH or glacial acetic acid exposure for 4 h; 5% SDS)
Applicability:	To classify corrosive and non-corrosive test substances Sub-categorization, i.e., 1A Vs. 1B, and 1C Vs. non-corrosive
Regulatory Status:	Validated and regulatory acceptance, OECD TG-431 (updated 2016)

RhE Test Protocol



Tissue Receipt

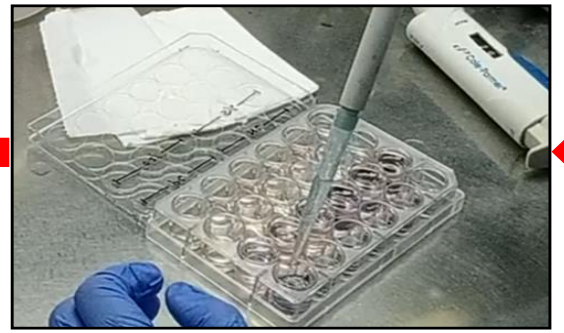
Pre-Incubation in assay medium (1 h)



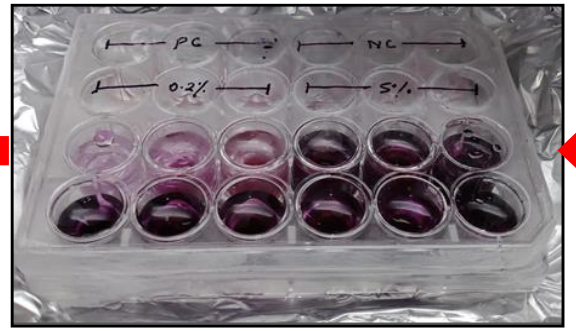
Tissue exposure to test item

Tissue rinsing

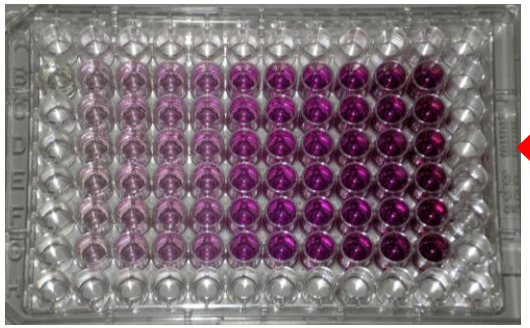
Blotting & drying the tissue surface



MTT Assay (3 h incubation)



Isopropanol Extraction (2 h)



OD at 570 nm

- MTT assay for the percent cell viability assessment
- OECD TG 431 (*In vitro* skin corrosion) and OECD TG 439 (*In vitro* skin irritation)

RhE-Corrosion: Prediction Models

For EpiSkin™ (SM) model

Viability measured following exposure time (3, 60 and 240 min)	Prediction to be considered UN GHS Category
< 35% after 3-min exposure	**Corrosive:** Optional Sub-category 1A
≥ 35% after 3-min exposure **AND** < 35% after 60-min exposure **OR** ≥ 35% after 60-min exposure **AND** < 35% after 240-min exposure	**Corrosive:** A combination of optional Sub-categories 1B and 1C
≥ 35% after 240-min exposure	Non-corrosive

Desprez B et al, Toxicology in Vitro, 29, 2055-2080 (2015)

RhE-Corrosion: Prediction Models

Prediction model for- EpiDerm™ (EPI-200), SkinEthic™ RHE, epiCS®

Viability measured following exposure time (3- and 60-min)	Prediction to be considered UN GHS Category
STEP 1	
< 50% after 3-min exposure	**Corrosive**
≥ 50% after 3-min exposure **AND** < 15% after 60-min exposure	**Corrosive**
≥ 50% after 3-min exposure **AND** ≥ 15% after 60-min exposure	**Non-corrosive**
STEP 2	
<25%; 18%; 15% after 3-min exposure	Optional Sub-category 1A
≥25%; 18%; 15% after 3-min exposure	A combination of optional Sub-categories 1B- and-1C

Desprez B et al, Toxicology in Vitro, 29, 2055-2080 (2015)

RhE Test Method-Skin Irritation Test

Test system:	**RhE models: EpiDermTM (EPI-200); EpiSkinTM (SM); SkinEthicTM RHE; LabCyte EPI-MODEL24; EpiCS®; Skin+®**
Assay endpoints:	Percent cell tissue viability by MTT assay
Assay control:	Negative control (sterile PBS or Normal saline; Positive control (1% SDS)
Applicability:	Determine skin irritancy of test substances either as a stand-alone replacement for *in vivo* skin irritation testing or as partial replacement test within a tiered testing strategy
Regulatory Status:	Validated and regulatory acceptance, OECD TG-439 (updated 2019)

OECD TG 439, (2019)

RhE-Irritation: Prediction Model

In vitro result	*In vivo* prediction	Prediction to be considered (GHS CATEGORY)
Mean Tissue Viability ≤50%	Irritant (I)	Category 2
Mean Tissue Viability > 50%	Non- irritant (NI)	No Category

Factors contributing to inaccurate MTT Assay

CASE-1: Subtoxic exposures may induce hormesis

Increased metabolism in response to cell damage-Incorrectly suggesting high viability

Solution:

- *Selecting test chemical exposures which result in a full range of cytotoxic responses*
- *Using adenosine triphosphate (ATP) endpoint assay- Measures cellular ATP content rather than metabolic rate*

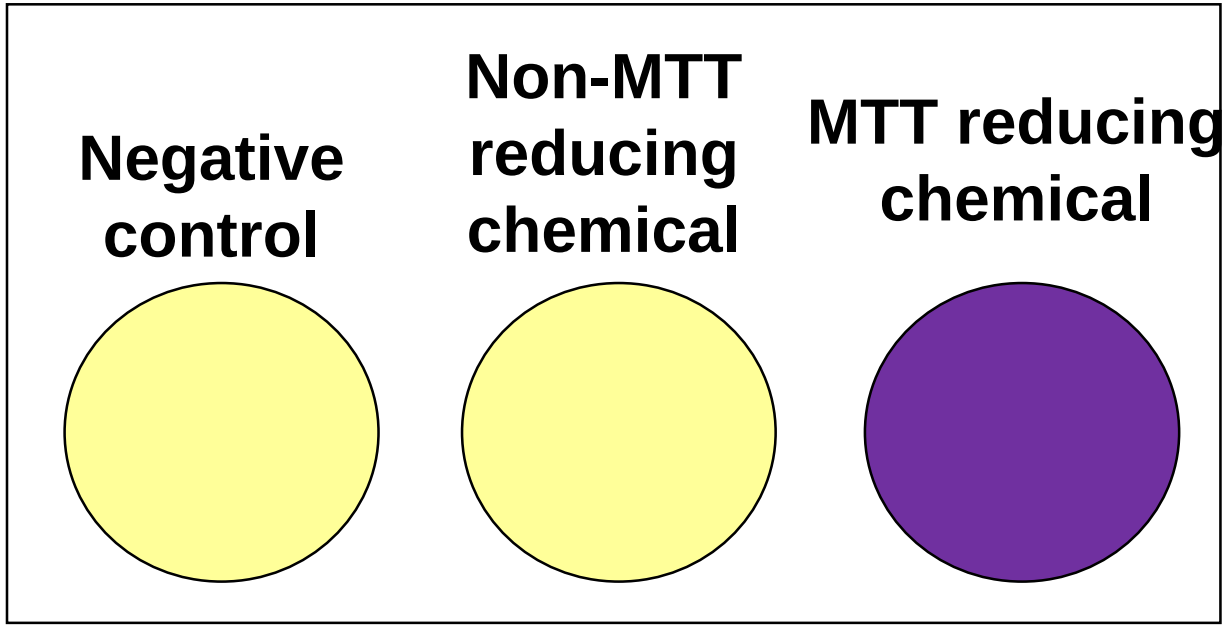
Factors contributing to inaccurate MTT Assay

CASE-2: MTT interaction

Test chemical may directly reduce MTT causing an overestimation of tissue viability

Solution:

- *Assessment of direct MTT reduction*
- *Testing of killed tissues in parallel*



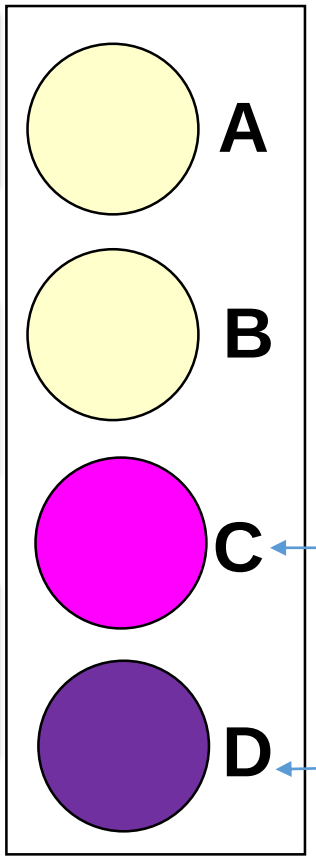
Assessment of direct MTT reduction

Fill tube with test chemical to be evaluated or water for control

Add 300 µL of MTT ready to use solution (1 mg/mL) and mix

Incubate mixture for 3 hours at 37°C protected from light

If MTT solution color turns blue/purple, test item is presumed to directly reduce MTT



Use killed tissue control if test chemical interference is less than 30% of negative control value

For more than 30%- Expert judgement

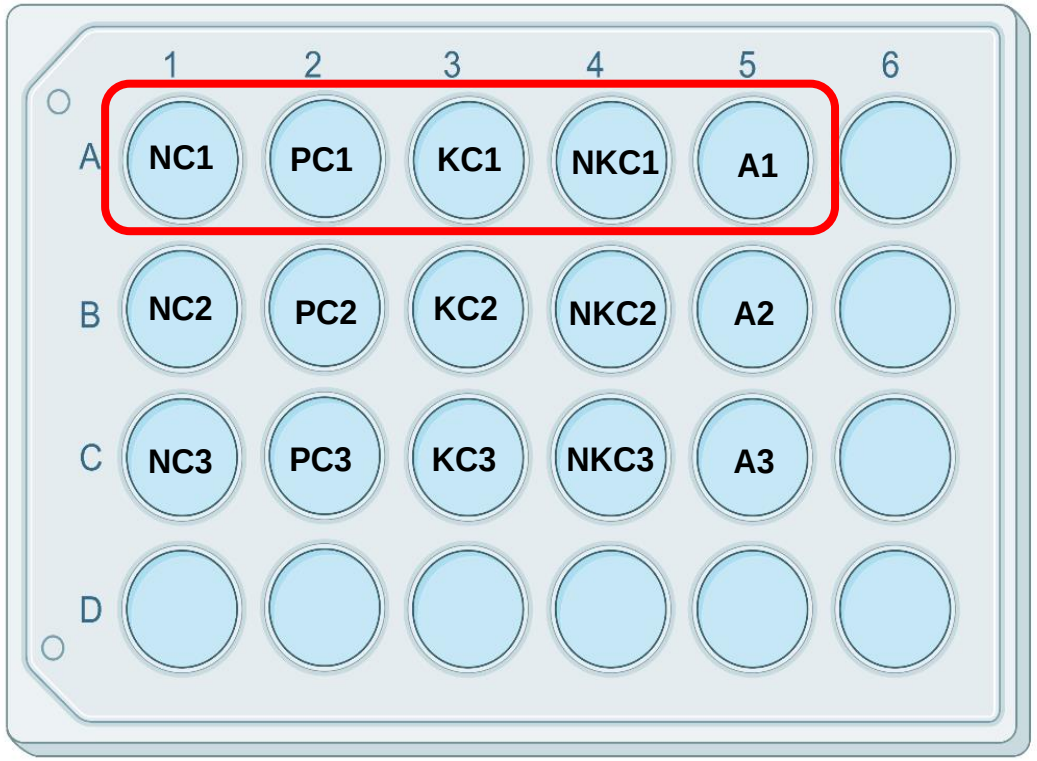
A: Control
B : Test chemical 1: no interaction
C : Test chemical 2: slight interaction
D: Test chemical 3: strong interaction

Testing killed tissues in parallel

Standard MTT assay	Killed Control (KC)+ Test Item	Negative Killed control
Living tissues + Negative control tissues + Positive control tissues	Living tissues placed in distilled water, incubated at 37°C, 5% CO_2, ≥ 90% RH for 24h Discard water and freeze tissue	Living tissues placed in distilled water, incubated at 37°C, 5% CO_2, ≥ 90% RH for 24h Discard water and freeze tissue
Exposure of living tissues to test chemical	Thaw at RT, killed tissues exposed to test chemical	Thaw at RT, killed tissues treated with water

MTT assay for percent viability evaluation

%Viability calculation when using killed control



A1= Test chemical A1

PC- Positive control

NC-Negative control

NKC- Negative killed control

KC-Killed control

% Viability PC= OD (PC)/OD (NC) x 100

Corrected killed control (CKC) = OD (KC) - OD (NKC)

% Viability CKC= OD (CKC)/ OD (NC) x 100

% Viability A= OD (A)/OD (NC) x 100

Corrected % Viability A =
% Viability A - % Viability CKC

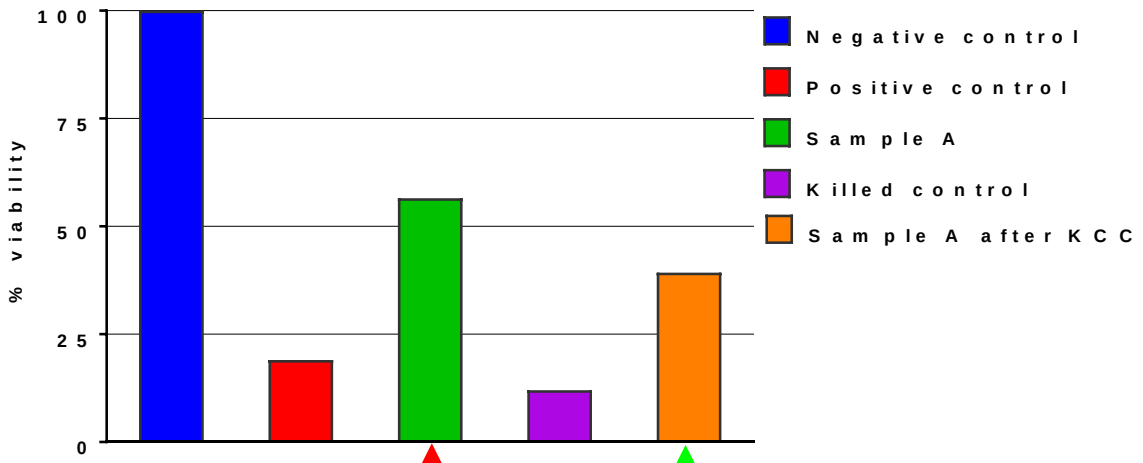
Mean Corrected % Viability A= (%CA1 +%CA2 + %CA3)/3

Calculate mean and SD for all groups

Effect of "Killed control" on % viability

Killed Control (KC) Correction

Test Item	KC Value (a)	Negative KC Value (b)	Corrected KC Value (a-b=c)
A	0.473	0.190	0.283



Sample A OD correction

Negative control OD	Non-corrected Sample A OD (d)	Corrected KC Value (c)	Corrected Sample A OD (d-c)
1.64	0.927	0.283	0.644

% Viability without KC correction= 0.927/1.64 x 100 =56.5%
(Non-irritant prediction)

% Viability with KCC = 0.644/1.64 x 100 = 39.3%
(Irritant prediction)

Factors contributing to inaccurate MTT Assay

CASE-3: Coloration interference

- Dyes and coloring test items able to stain tissues or interact with MTT
- Show interfere with viability assessment due to residual chemical color (unrelated to mitochondrial activity)

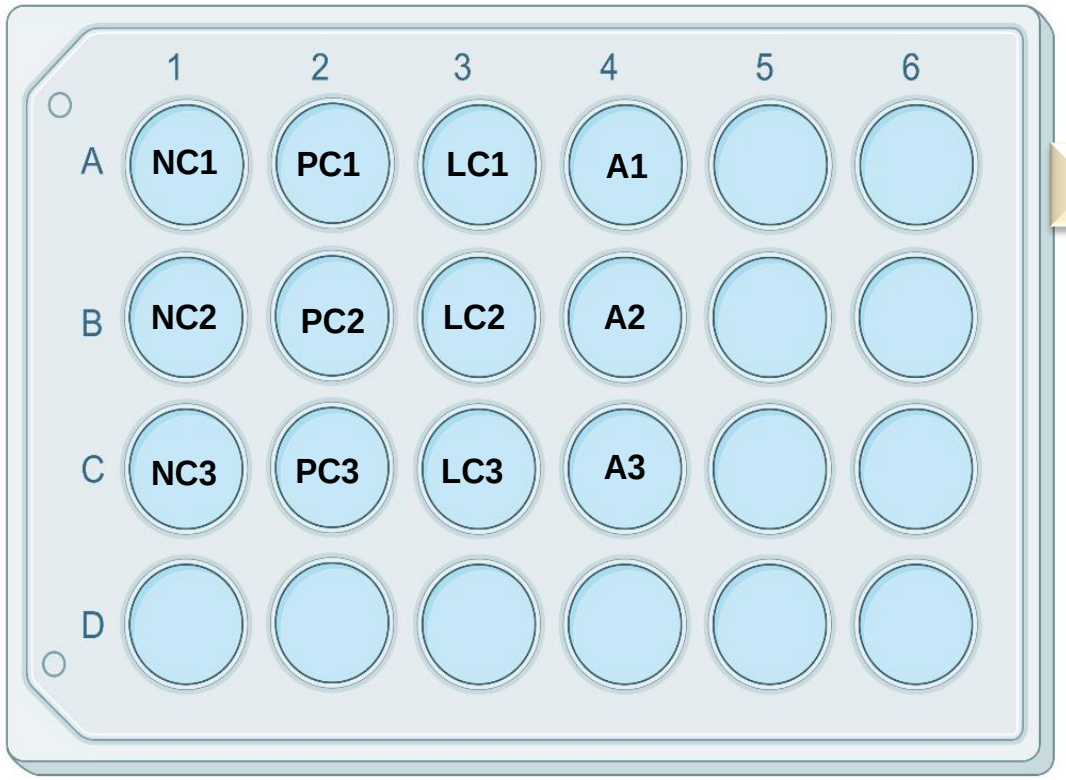
Solution:

- *Inclusion of 'Living-MTT' control*
- *Inclusion of both "Living-MTT' and 'Killed-MTT' controls*

Adapted controls for coloured test chemical

- **Living-MTT Control:** Living tissues exposed to test item, and processed identical experimental conditions without **MTT incubation** **(colored test items not interfering with MTT).**
- **Killed-MTT Controls:** Killed tissues exposed to test item, and processed identical experimental conditions with and without **MTT incubation** **(colored test items interfering with MTT).**

% viability calculation for colorant not interfering with MTT



% Viability PC= OD (PC)/OD (NC) x 100

% Viability Living control (LC)=OD(LC)/OD(NC)x 100

% Viability A= OD (A)/OD (NC) x 100

Corrected % Viability A =
% Viability A - % Viability LC

Mean Corrected % Viability A= (%CA1 +%CA2 + %CA3)/3

Calculate mean and SD for all groups

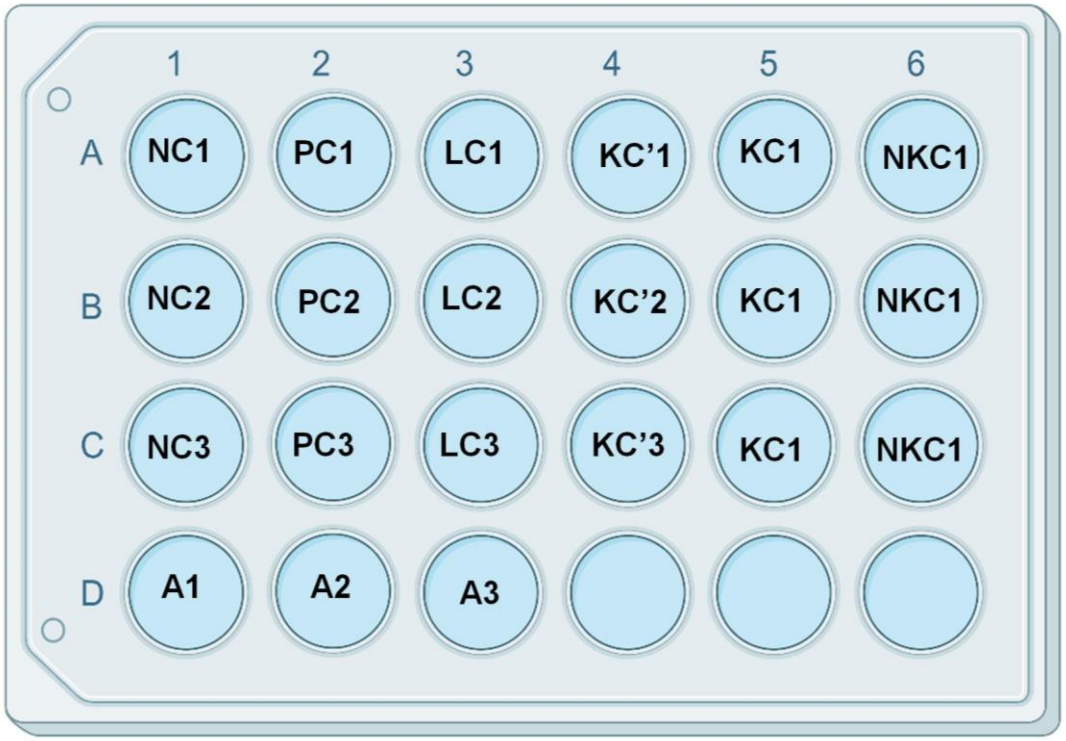
A1= Test chemical A1

PC- Positive control

NC-Negative control

LC-Living control without MTT incubation

% viability calculation for colorant interfering with MTT



A1= Test chemical A1
PC- Positive control
NC- Negative control
LC- Living control without MTT incubation
KC'- Killed control without MTT incubation
KC- Killed control with MTT
NKC- Negative killed control

% Viability PC= OD (PC)/OD (NC) x 100

% Living control (LC) = OD (LC)/ OD (NC)x 100

% Killed Control (KC) = OD (KC)/ OD (NC)x 100

Corrected Killed Control (CKC) = OD (KC)- OD (NKC)

% Corrected Killed Control (CKC) = OD (CKC)/ OD (NC)x 100

% Viability A= OD (A)/OD (NC) x 100

Corrected % Viability A =
(% Viability A - % Viability LC) – (% Viability CKC) + (% Viability KC')

Mean Corrected % Viability A= (%CA1 +%CA2 + %CA3)/3

Inclusion of adapted control: Changes in final viability

Condition	% Mean viability (%TS)	% Mean viability (% CKC)	% Mean viability (% LC)	% Mean viability (% KC)	Final corrected viability	Final viability
	Living+MTT	Killed+MTT	Living-MTT	Killed-MTT		
1	80.9	-	-	-	%TS	80.9
2	80.9	10.9	-	-	%TS - %CKC	70.0
3	80.9	-	19.0	-	%TS -%LC	61.9
4	80.9	10.9	19.0	5.0	%TS - % CKC - LC% + % KC	56.0

% Mean viability of Test Sample (%TS)

% Mean viability of Corrected killed control (% CKC)

% Mean viability of Living control without MTT incubation (% LC)

% Mean viability of Killed control without MTT incubation (% KC)

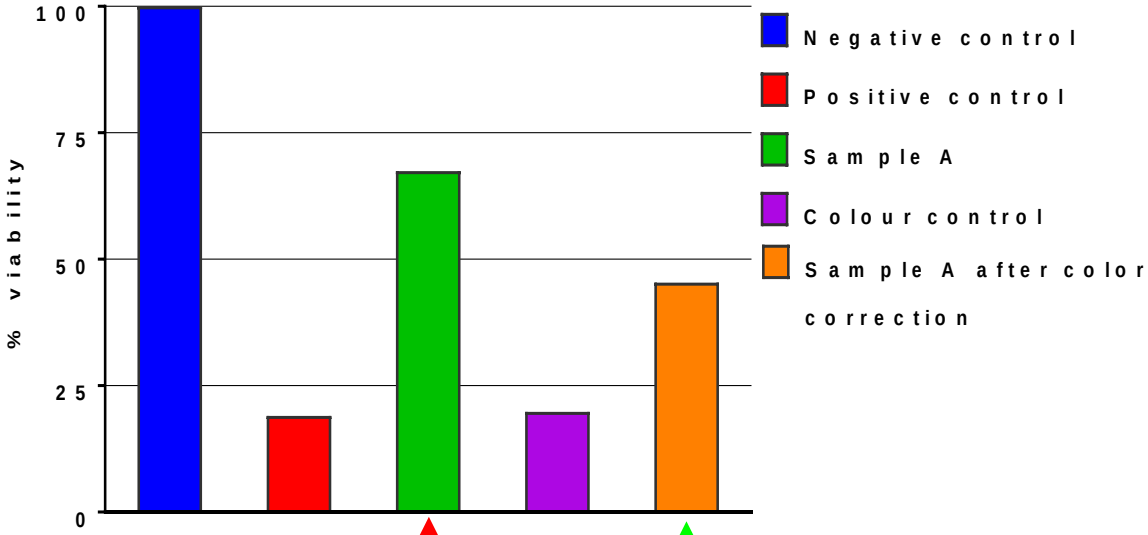
Effect of "Colour control" on percent viability

Color control Correction

OD of Sample A (a)	OD of Color control (b)	Corrected sample OD of A (a-b=c)
1.170	0.385	0.785

% viability correction

OD of Negative control (d)	% viability of non-corrected sample A (a/d)*100	% viability of corrected sample A (c/d)*100
1.73	67.4	45.4



% Viability without color correction= 1.170/1.730 x 100 =67.4% (Non-irritant prediction)

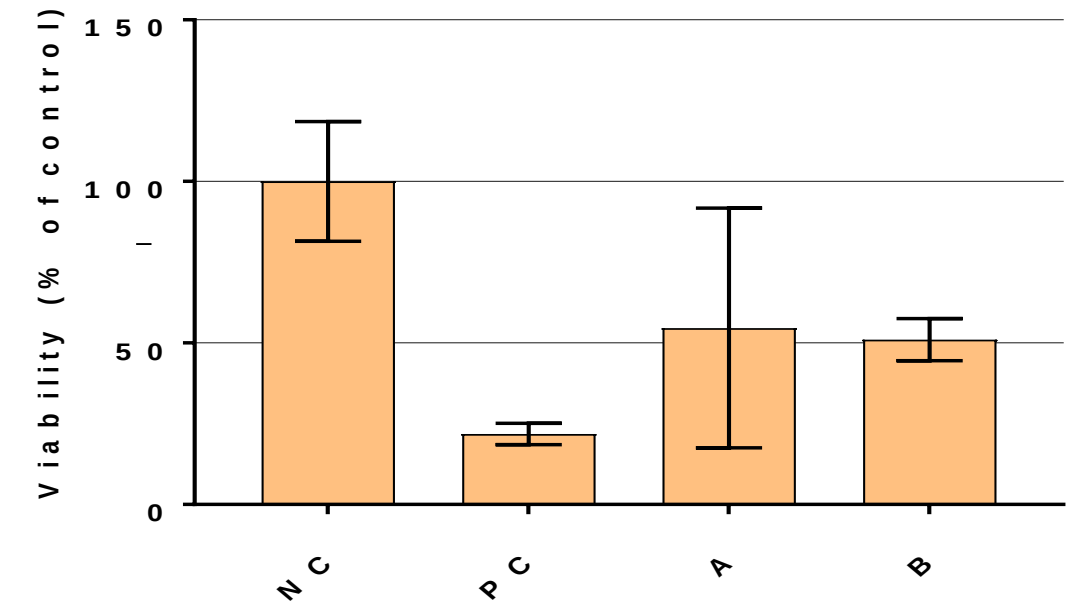
% Viability with color correction = 0.7/1.73 x 100 =45.4% (Irritant prediction)

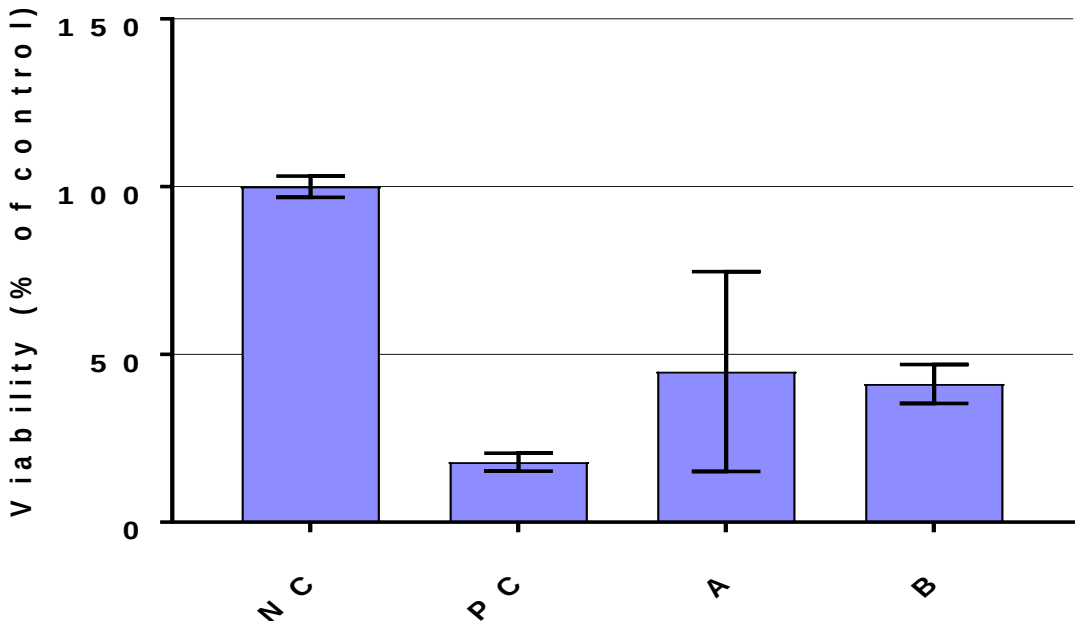
CASE-4: Adjusting values with valid controls

Test Item	Mean OD Value	Mean of viability (%)	SD of viability
NC	1.665	100.0	18.5
PC	0.363	21.8	3.3
A	0.910	54.6	37.1
B	0.850	51.0	6.5

Test Item	Mean OD Value	Mean of viability (%)	SD of viability
NC	2.023	100.0	3.2
PC	0.363	17.9	2.7
A	0.910	44.9	29.8
B	0.833	41.2	5.8

NC- Negative control; PC-Positive control

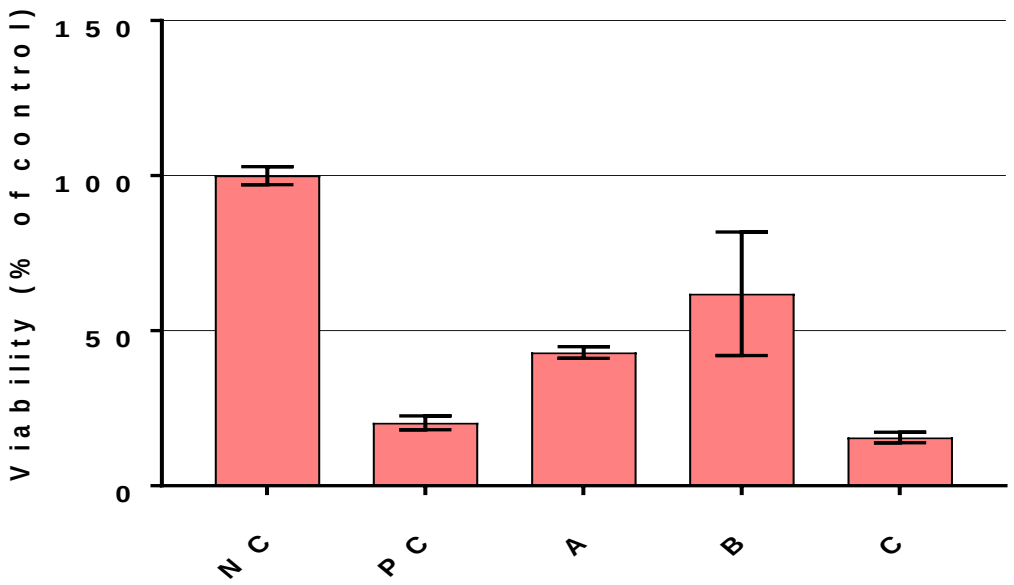




CASE 5: Variations in test item exposure

A	Viscous liquid, residual test item remains after rinsing	Residual test item may have increased toxicity **(Irritant?)**
B	Non-viscous liquid, tissue of one replicate was damaged while removing mesh	Damage tissue could have resulted in high SD **(Non-Irritant?)**
C	Solid white powder, completely removed from tissues	Clear irritant/corrosive **(Irritant?)**

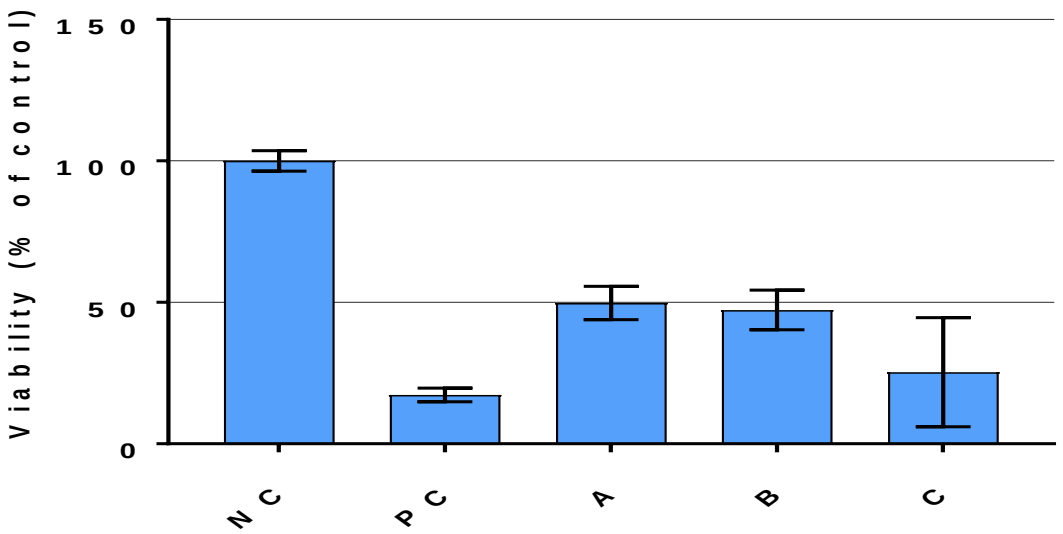
Test item	Mean OD Value	Mean of viability (%)	SD of viability
NC	2.020	100.0	2.9
PC	0.410	20.3	2.2
A	0.869	43.0	1.9
B	1.250	61.9	19.9
C	0.315	15.6	1.7



CASE-6: Analyzing conflicting results

Test item	Tissue	OD Value	%Viability
A	1	0.890	44.0
	2	1.120	55.4
	3	0.942	46.6
B	1	1.059	52.4
	2	0.799	39.5
	3	1.030	50.9
C	1	0.410	20.3
	2	0.859	42.5
	3	0.081	4.0

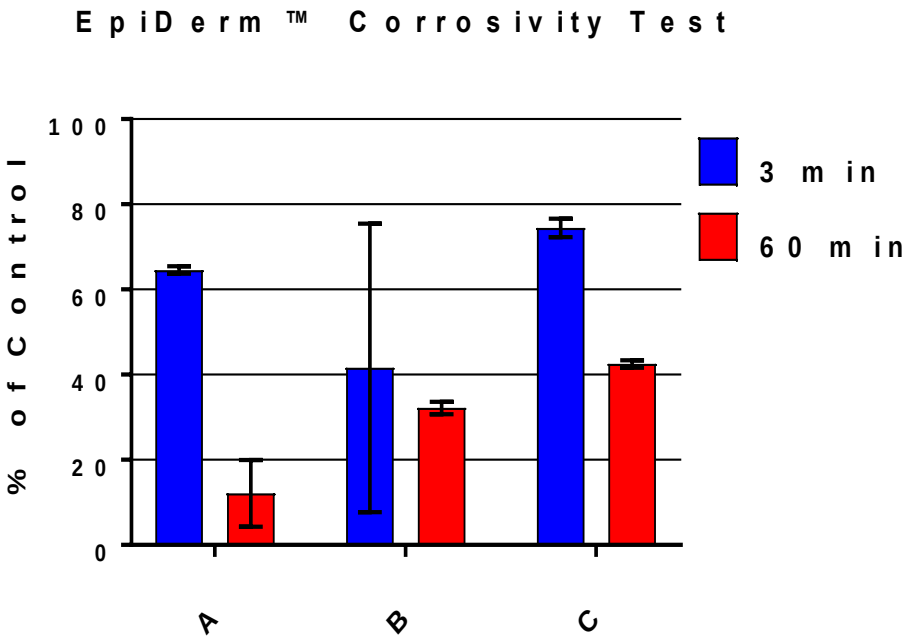
Test item	Mean OD	Mean of viability (%)	SD of viability
NC	2.022	100.0	3.6
PC	0.349	17.3	2.4
A	1.008	49.8	5.9
B	0.957	47.3	7.0
C	0.512	25.3	19.3



- Individual tissue values (A and B) showing different irritation rankings
- High standard deviation for test article C

CASE-7: Tissue loss during testing

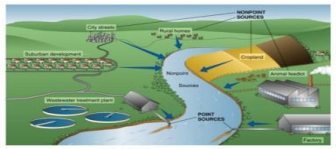
Test Article	Exposure Time	R- 1 % Viability	R-2 % Viability	R-3 % Viability	Mean % Viability
A	3	65.2	64.9	63.6	64.57
	60	**3.2**	18	15.2	12.13
B	3	62	**2.5**	60.3	41.6
	60	33.4	30.6	32.5	32.17
C	3	76.9	73.5	72.9	74.43
	60	43.2	42.8	41.5	42.50

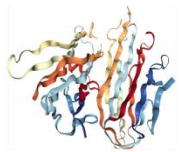
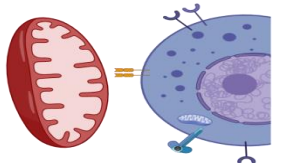
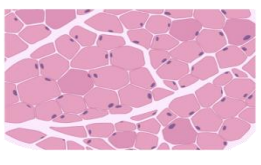
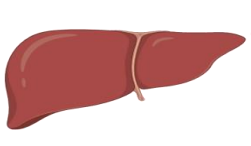
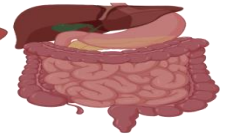


Tissue loss observed for test item-A at 60 min and for test item-B at 3 min

Perspectives, challenges and togetherness

Linking of cellular and molecular events to the events of regulatory interest




Sources: Environmental Containments | Exposure | Molecular initiating events | Organelle and cellular level effects | Tissue effects | Organ effects | Organ systems effects | Individual effects | Population effects | Community effects

Toxicity Pathway

Mode of Action

Adverse Outcome Pathway

Source to Outcome Pathway

High-throughput assays

2D cultures: cell lines/primary cells/ stem cells/ iPSCs

3D reconstructed tissues/ Organoids/ Organ on Chip

Computational Modeling

Thank You

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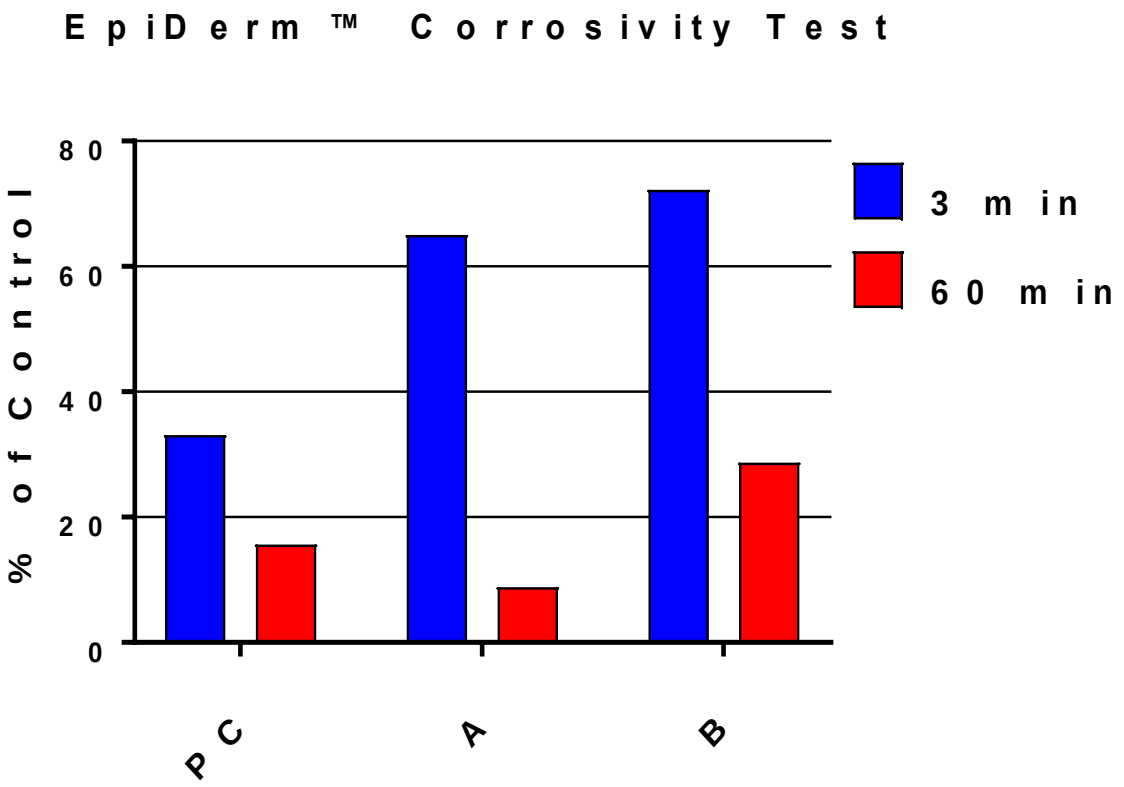
Evaluating corrosivity results

Test Item	Exposure Time	OD value	% Viability
PC	3	0.453	33.1%
	60	0.214	15.6%
A	3	0.889	65.0%
	60	0.121	8.8%
B	3	0.987	72.2%
	60	0.392	28.7%



- **Sample B- Non- corrosive**
- **Sample A- Corrosive; Consider optional sub-categorization for Sample A**

Evaluating corrosivity results

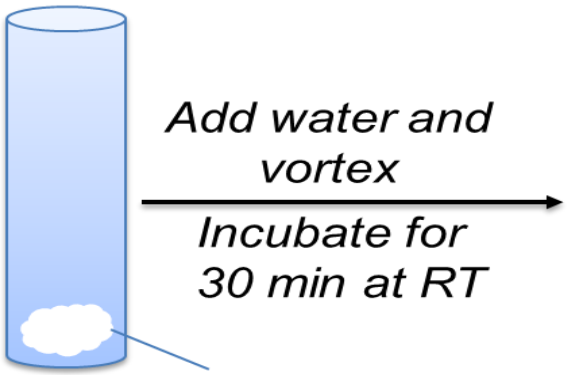
Applying Optional Subcategories

Test Item	Exposure Time	OD Value	Viability	Sub-Category
Positive Control	3	0.453	33.1	**1B/1C**
	60	0.214	15.6	
A	3	0.889	65.0	**1B/1C**
	60	0.121	8.8	

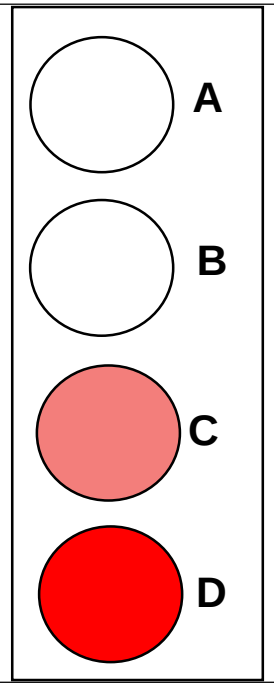
- Viability of 3 minute exposure <25% = **Subcategory 1A**
- Viability of 3 minute exposure ≥25% = **Subcategory 1B/1C**

Checking for colorant properties

- **For colored test chemicals-** Simply include adapted control/controls
- **For uncolored test chemicals-** Possible interference should be first checked



Direct visual observation



For colored solution:

Tissue staining ability of test chemical should be checked

- **YES-** Include adapted controls
- **NO-** Do not include

A: Control
B: Test chemical 1: no color
C: Slight coloration of a red Test chemical
D: Strong coloration of a red Test chemical