DB-ALM Protocol n° 191 : SkinEthic™ HCE Eye Irritation Test Solid (EITS)

Eye Irritation

The SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test Solid (EITS) is an *in vitro* assay used to assess the acute ocular irritation potential of solid chemicals.

Résumé

The purpose of the SkinEthic™ HCE EIT method is to assess the eye irritation potential of chemicals using the Reconstructed Human Corneal Epithelium model.

The **SkinEthic™ HCE EIT method** is used to assess the serious eye damage/eye irritation potential of liquid (**EITL**: Eye Irritation Testing of Liquids) and solid test chemicals (**EITS**: Eye Irritation Testing of Solids).
Two protocols depending on the physical state of the testing chemicals have been developed and they are both available from DB-ALM: the current protocol is for solid chemicals (EITS), whereas the SkinEthic™ HCE EITL is for liquid/viscous chemicals (**DB-ALM Protocol No. 190**).

The **SkinEthic™ HCE EITS**, hereafter described, has been validated in a study coordinated by L'Oreal (Alépée et *al*., 2016a), and subsequently independent peer reviewed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Scientific Advisory Committee (ESAC opinion No. 2016-02).
This test method, accepted in the OECD Test Guideline No. 492 (OECD, 2017a), is recommended to identify **solid chemicals** that **do not require classification for eye irritation or serious eye damage** according to United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS).
However, **SkinEthic™ HCE EIT**, and this EITS protocol, is not intended to differentiate on its own between serious eye damage and eye irritation and this differentiation will need to be addressed by another tier of a test strategy in the context of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2017c).

On the basis of the data currently available, the SkinEthic™ HCE EITS protocol has been shown to be applicable to a wide range of solid chemicals, covering a large variety of chemical types, chemical classes, functional groups, molecular weights, LogPs, chemical structures, colored and/or direct MTT reducers, etc. (Alépée et *al*., 2016a). This test method allows the hazard identification of mono and multi-component test solid chemicals. Gasses and aerosols cannot be also evaluated with the current protocol.

Liquid/viscous chemicals cannot be evaluated with the current protocol but their assessment can be performed using SkinEthic™ HCE EITL assay (Alépée et *al*., 2016b) which is described in the **DB-ALM Protocol No. 190**.

Experimental Description

Endpoint and Endpoint Measurement:

Cell viability determination is based on cellular mitochondrial dehydrogenase activity, measured by tetrazolium salt MTT reduction [(3-4,5-dimethyl triazole 2-yl) 2,5-diphenyltetrazoliumbromide] (Mossman, 1983).
Viable cells of the tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent).

The extracted MTT formazan may be quantified using either a standard absorbance (Optical Density (OD)) measurement or a High/ Ultra-high Performance Liquid Chromatography (HPLC/UPLC) spectrophotometry procedure (Alépée et *al*., 2015; Alépée et *al*., 2016a).

Endpoint Value:

The reduction of cell viability in test chemicals treated tissues is compared to treated tissues with negative control (100% viability) and expressed as a %. The MTT- formazan reduction % in viability is used to predict the eye hazard potential of the test chemical.

Experimental System(s):

Human Corneal Epithelium model (SkinEthic™ HCE):
When cultured at the air-liquid interface in a chemically defined medium on a permeable synthetic membrane insert, the transformed human corneal epithelial cell line forms a corneal epithelial tissue. Obtained tissues are ultra-structurally (tissue morphology and thickness) similar to the corneal epithelium of the human eye (Nguyen et *al*., 2003).
As *in vivo* epithelium, the SkinEthic™ HCE model is characterized by the presence of intermediate filaments, mature hemi-desmosomes and desmosomes, and specific cytokeratins. The 0.5 cm² multilayered epithelium contains at least 4 cell layers, including columnar cells and wing cells.

Discussion

Ethical issues.The test is based on an *in vitro* system, no ethical issues are related.

Special equipment. No specific equipment is needed (only classical laboratory devices are required to perform the test method).

Amount of training required. Two training days are necessary to establish the test method in a naïve laboratory. It includes a practical training in which *(i)* the main steps of the protocol are emphasized *(ii)* a demonstration of the method are observed and *(iii)* then performed by the trainers. It also includes depth discussions about the detailed protocol.

Duration of the test. 3 days are required to perform a run: A run begins on Day 1 with tissue conditioning, on Day 2 with cell treatment with chemicals, and on Day 3 with data acquisition. A trained experimenter can perform at least 13 test chemicals in a run.

Costs.Testing costs are available upon request to the testing facilities (e.g. Contract Research Organization).

Status

Participation in Validation Studies:

The **SkinEthic™ HCE** method for testing solid chemicals (**EITS protocol**) has been evaluated in a multicentre validation study coordinated by L'Oréal (Alépée et *al*., 2016a).

The **SkinEthic™ HCE EITS protocol** was proved to be transferable to laboratories considered to be naïve in the conduct of the assays and also to be reproducible within- and between laboratories.
The level of reproducibility in terms of concordance of predictions that can be expected from SkinEthic™ HCE EITS from data on 60 solid chemicals is in the order of 95% within laboratories and 96.7% between laboratories, respectively (Alépée et *al*., 2016a).
Considering the data obtained in the validation study (Alépée et *al*., 2016a), the SkinEthic™ HCE EITS has an overall accuracy of 81% (based on 95 solid chemicals), sensitivity of 91% (based on 42 *in vivo* known classified chemicals), false negative rate of 9% (based on 42 chemicals), specificity of 74% (based on 53 *in vivo* known No category chemicals) and false positive rate of 26% (based on 53 chemicals), when compared to reference *in vivo* rabbit eye test data (OECD TG 405) classified according to the UN GHS classification system (OECD, 2017d).

Following the validation study, the EURL ECVAM Scientific Advisory Committee (ESAC) has independently peer reviewed the **Skin Ethic™ HCE EIT** method (ESAC Opinion, 2016) which can used for testing of liquid (see **DB-ALM Protocol No. 190**) and solid chemicals as described in this current protocol.

From the validation study (Alépée et *al*., 2016a) and the independent peer review (ESAC Opinion, 2016), it was concluded that the **SkinEthic™ HCE EIT method** and more specifically the **EITS protocol** is able to correctly identify **solid chemicals** (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS without further testing, within a testing strategy such as the Bottom-Up/Top-Down approach suggested by Scott et al. e.g., as an initial step in a Bottom-Up approach or as one of the last steps in a Top-Down approach (Scott et *al.* 2010; UN , 2015; OECD, 2017c).

However, the **SkinEthic™ HCE EIT method** is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation). This differentiation should be considered in combination with other sources of information in the context of an IATA (OECD, 2017c).

Regulatory Acceptance:

The **SkinEthic™ HCE EIT method** using solid (EITS protocol, e.g. the current DB-ALM Protocol No.191) and liquid (EITL protocol, DB-ALM Protocol No.190) testing chemicals is recommended as **scientifically valid according to OECD Test Guideline (TG) 492** which was adopted on 9th October 2017 (OECD, 2017a, OECD, 2017b).

Proprietary and/or Confidentiality Issues

The Reconstructed Human Tissue SkinEthic™ HCE technology, associated to production of model and media are proprietary to Episkin, France.

No intellectual property rights are associated with the present test method

Health and Safety Issues

General Precautions

Normal handling procedures for biological materials should be followed:

- It is recommended to wear glasses and gloves during handling.
- After use, the epithelium, the material, and all media in contact with the tissue should be decontaminated (for example, by using a 10% solution of bleach in appropriate containers), prior to elimination.

Safety instructions for working with test chemicals:

- Test chemicals should be handled following material safety datasheet. Store the test chemicals in ventilated safety cupboards. Respect specific storage conditions if necessary (special temperature, protected from light, etc.) according to the material safety datasheet guidelines.
- Unknown test chemicals with no or incomplete safety handling information should be considered as irritating and toxic and must be handled with maximum care in accordance with test chemical safety guidelines.

MSDS Information

Safety precautions:

MTT (R68, R36, R37, R38 / H315, H319, H335, H341)
Isopropanol (R11, R36, R67 / H225, H319, H336)
Methyl Acetate (H225, H319, H336)

Work in ventilated cabinets: to prevent accidental contact wear protective gloves, and if necessary safety glasses.

Abbreviations and Definitions

°C:	Degree Celsius
µL:	Microliter
%:	Percentage
No pred:	No prediction can be made
EC:	European Commission
EITL:	Eye Irritation Test Liquid
EITS:	Eye Irritation Test Solid
EU CLP:	European Classification Labelling and Packaging Regulation
HCE:	Human Corneal Epithelium
HPLC:	High Performance Liquid Chromatography

hr/hrs:	Hour/hours
IP :	Isopropanol
KU :	Negative control killed treated tissue
LLOQ:	Lower Limit Of Quantification
mg:	Milligram
Min:	Minute
mL:	Milliliter
MTT:	3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazoliumbromide
NC:	Not Classified
NgC:	Negative Control
nm:	Nanometer
NSC_{killed}:	Non Specific Color in killed tissues–killed tissue without MTT incubation
NSC_{living}:	Non Specific Color in living tissues–living tissue without MTT incubation
NSMTT:	Non Specific MTT reduction in killed tissue–killed tissue with MTT incubation
OD:	Optical Density
PBS- :	PBS without Ca^{2+} & Mg^{2+}
PC:	Positive Control
Qualified Test:	The test of a test chemical is qualified (qualified test) if it meets the acceptance criteriafor the test within a qualified run. Otherwise, the test is considered as Non-Qualified (invalid).
Run:	Set of up to 2 series by experimenter conducted with the same tissue batch within the same day.
RhT:	Reconstructed human Tissue
RT:	RoomTemperature
Series:	a set of up to 13 test chemicals plus Negative Control (NgC) and Positive Control (PC) all concurrently tested on at least 2 tissues replicates.
Test:	A test chemical tested on at least two tissue replicates when the cytotoxic effect is quantitatively measured by using the MTT assay. A reported technical issue before the viability measurement is not considered as a “Test” for the test chemical.
TT:	TestTreatment
ULOQ:	Upper Limit Of Quantification
UN GHS:	United Nations Globally Harmonized System
UPLC:	Ultra-high Performance Liquid Chromatography
V:	Volume

Last update: 16 October 2017

PROCEDURE DETAILS, Latest version 7 June 2017

SkinEthic™ HCE Eye Irritation Test Solid (EITS)
DB-ALM Protocol n° 191

Quick flow chart

The experimental procedures for the *in vitro* **SkinEthic™ HCE EITS** protocol are briefly outlined here below. A detailed description of the different steps is available in the following sections of this protocol.

Receipt: Transfer epithelium from agarose to maintenance medium in 6-well plate
(1 mL Tissue maintenance medium/well)
↓
Culture inserts equilibration period: Incubate at least overnight
(37±2°C , 5±1% CO_2, ≥ 90% humidity)
↓
Tissue conditioning :Transfer tissues to fresh maintenance medium in the 6-well plate
1 plate/test items (2 tissues/ plate)
(used the same plate for Receipt, Treatment, Post-incubation periods)
Incubate at least 30 min (37±2°C , 5±1% CO_2, ≥ 90% humidity)
↓
Treatment: 2 tissues each with 30±2 µL PBS without Ca^{2+} & Mg^{2+} (Negative Control)
or 30±2 µL methyl acetate (Positive Control)
or 30 µL PBS without Ca^{2+} & Mg^{2+} (PBS^-) + 30±2 mg test chemical
↓
Treatment Period: Incubate for 4 hours ± 5 min (37±2°C, 5±1% CO_2, ≥ 90% humidity)
↓
Rinse with PBS^- (25 mL: 2 mL/ jet)
↓
Post-soak Immersion: Immerse tissues in 4 mL fresh maintenance medium, in 12-well plates
↓
Post-soak Period: Incubate for 30±2 min at room temperature (RT)
↓
Transfer tissues to fresh maintenance medium in the same 6-well plates
↓
Post-incubation Period: Incubate for 18 hrs ± 30 min (37±2°C, 5±1% CO_2, ≥ 90% humidity)
↓
Viability: Transfer tissues into MTT solution (1 mg/mL) in 24-well plates (300µL MTT/well)
↓
Incubate tissues for 3 hours ± 15 min (37±2°C, 5±1% CO_2, ≥ 90% humidity)
↓
Extraction: Place the inserts on 1.5 mL isopropanol in 12-well plates
↓
Extract formazan at least 2 hours at RT
↓
Remove the insert and **homogenize** formazan extract
↓
Read OD with microplate spectrophotometer at 570±30 nm
and/or **analyse** the extract samples by **HPLC/UPLC spectrophotometry**

Contact Details

Dr. Nathalie Alépée
Research & Innovation
L'ORÉAL
1 Avenue Eugène Schueller
Aulnay sous Bois 93600
France
email: nalepee@rd.loreal.com
telephone: +33 (0)1 48 68 91 02

EPISKIN sales
Sales Department
4 rue Alexander Fleming
Lyon Cedex 07 69366
France
email: sales@episkin.com
telephone: +33 (0) 4 37 28 22 00

Materials and Preparations

Cell or Test System

The SkinEthic™ HCE tissues are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm²) with the necessary culture media (maintenance medium).
The SkinEthic™ medium and SkinEthic™ HCE tissue model are provided by Episkin (France) with related technical documentation (**www.episkin.com**).

Description of the kit	Comment	Storage conditions	Shelf life
SkinEthic™ HCE units (0.5 cm^2)	Tissues are shipped on semi solid agar's medium in order to maintain good shipment conditions for the tissues	RT	see technical data sheet
SkinEthic™ Maintenance Medium	Culture medium for incubations	Fridge	see technical data sheet

Store the SkinEthic™ HCE tissues at room temperature until their transfer into SkinEthic™ maintenance medium. Store the SkinEthic™ maintenance medium in the fridge and pre-warm only at room temperature (RT).

Equipment

Fixed Equipment

Item	Use
Microbiological safety cabinet (laminar flow hood)	*safe work under sterile conditions*
Non-sterile ventilated cabinet	*safe work with test chemicals, applications,washes*
Cell incubator 37±2°C , 5±1% CO_2, ≥ 90% humidity	*tissues incubations*
Plate reader (96 well) with a 570± 30 nm wavelength	*Optical Density readings (MTT formazan)*
Laboratory balance (accuracy 0.1 mg)	*test chemicals weighing*
Shaker plates	*shaking before reading (formazan extraction sample)*
HPLC/UPLC-UV/Visible spectrophotometry	*Performance Liquid Chromatography readings (MTT formazan)*

Consumables

Item	Use
1 sterile bottle	*reconstituting MTT reagent stock solution*
1 sterile bottle	*diluting MTT in assay medium*
1 glass funnel	*dropping wash fluids in the bottle*
Wash bottle (500 mL)	*collecting wash fluids*
Adjustable multi-step pipette, 25 mL	*distributing 1 mL maintenance medium and PBS*
Adjustable multi-step pipettes, 5 mL	*distributing maintenance medium, MTT, Isopropanol and PBS*
Adjustable micro-pipette – 0 to 200 µL	*pipetting 200 µL formazan extracts*
Adjustable positive displacement micro-pipette 0- 50 µl	*application of 30 µL*
Stop-watches/Timers	*controlling contact and step times*
Small clean blunt-edged forceps	*handling tissue inserts*
Spatula	*weighing powder*
Small glass weight boat	*weighing powder*
Mortar and pestle	*grinding granular*
96-well plates	*reading Optical Density*
12-well plates	*post soak and IP extraction*
24-well plates	*MTT incubation*
6-well sterile plates	*transfer tissue inserts upon receipt treatment, post incubation*
“Parafilm”	*covering plates during formazan extraction*
cotton tip swabs	*drying the tissue surface*

Nylon mesh Ø=7.5 mm (sefar Nitex 03-150/38 or equivalent)	*for sticky or powder difficult to apply*
HPLC/UPLC vial	*HPLC/UPLC measurement vial*
HPLC/UPLC reverse phase column	*HPLC/UPLC measurement*

Media, Reagents, Sera, others

Item	**Use**
Isopropanol (CASRN 67-63-0)	*formazan extraction*
MTT reagent (3-4,5-dimethyl thiazole 2-yl)2,5-diphenyltetrazolium bromide(CASRN 298-93-1, Sigma M2128 or equivalent)	*viability measurements, reagent*
Dulbecco's D-PBS without Ca^{2+} & Mg^{2+} GIBCO 14190-144 or equivalent (PBS^{-})	*rinsing tissues , Negative Control (NgC), MTT solubilisation*
Tissue maintenance medium (SkinEthic™)	*tissues culture, incubations and MTT dilution*
Sterile distilled water	*checking for color test chemical*
Solvents HPLC/UPLC grade	*HPLC/UPLC measurement*
Formazan (CASRN 57360-69-7); purity >97% (Sigma 88417 or equivalent)	*HPLC/UPLC validation system*
Methyl acetate (CASRN 79-20-9); purity >99% (Sigma 45999 or equivalent)	*Positive Control (PC)*

Preparations

Media and Endpoint Assay Solutions

MTT stock solution preparation

- Prepare a 5 mg/mL MTT solution in PBS^{-}
- Thoroughly mix this stock solution during 15±2 minutes at RT.
- Keep in the fridge (2 to 8°C) protected from light up to 16 days

MTT ready to use solution preparation

- Pre warm maintenance medium at RT
- Dilute MTT stock solution preparation 1/5 with maintenance medium (1v+4v, final concentration: 1 mg/mL)
- Keep at RT, protect from light until use (do not exceed 3 hours storage)
  Note: MTT solution is light sensitive. Protect it from light using foil.

The culture medium (maintenance medium) is delivered with the SkinEthic HCE tissues; it is store in the fridge. All these solutions and media are prepared or open under a safety cupboard.

Test Compounds

Preparation of test chemicals

Solid test chemicals (even granular powder) should be crushed to a very fine powder, if necessary, using a mortar and a pestle.

Application of test chemicals

The test chemical (± color, ± MTT reducer) is topically applied onto HCE tissues. For detailed experimental setup see the section "*Test Material Exposure Procedures*" on p.16.

Prior to routine use of the SkinEthic™ HCE EITS protocol **for regulatory purposes**, as recommended in OECD Test Guidelines 492, laboratories should demonstrate **technical proficiency** by correctly predicting the **seven proficiency solid chemicals** (see Table 1 in the following page).

Table 1. List of proficiency solid chemicals

Chemical Name	CAS RN	Organic Functional Group [1]	Physical State	Viability (%) [2]	Prediction	MTT Reducer	Colour Interfer.
***In Vivo* Category 1 [3]**							
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	0.2±0.1	No prediction can be made	N	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	5.3±4.1	No prediction can be made	N	N
***In Vivo* Category 2A [3]**							
Sodium benzoate	532-32-1	Aryl; Carboxylic acid	S	0.6±0.1	No prediction can be made	N	N
***In Vivo* Category 2B [3]**							
2,2-Dimethyl-3-methylene bicyclo [2.2.1] heptane	79-92-5	Alkane, branched with tertiary carbon; Alkene; Bicycloheptane; Bridged-ring carbocycles; Cycloalkane	S	15.8±1.1	No prediction can be made	N	N
***In Vivo* No Category [3]**							
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Aromatic heterocyclic halide; Aryl halide; Urea derivatives	S	101.9±6.6	No Cat	N	N
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl	S	97.7±5.6	No Cat	N	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	92.9±5.1	No Cat	N	N

Abbreviations:

CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; Colour interf. = colour interference with the standard absorbance (Optical Density (OD)) measurement of MTT formazan; S=solid.

*1 Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (**https://www.qsartoolbox.org/**).*

2 Based on results obtained with SkinEthic™ HCE EITS in the validation study (Alépée et al,2016a).

3 Based on results from the in vivo rabbit eye test (OECD TG 405) and using the UN GHS.

Positive Control(s)

Methyl acetate is used as Positive Control (PC).

Negative Control(s)

Phosphate Buffer solution without Ca^{2+} & Mg^{2+} (PBS^{-}) is used as Negative Control (NgC).

Note. *The negative and positive controls correspond to the quality control named viability in OECD TG 492 that must be performed for each run by the user. This data is not provided by the tissue supplier.*

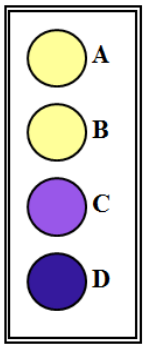
The following paragraphs describe the procedures to check **direct MTT reduction** and/ or **color interfering of testing chemicals prior to experiments**.
An **illustrative flowchart** providing guidance and summarising the steps to follow to identify and handle **direct MTT-reducers** and/or **color interfering chemicals** is provided as **Annex 4** on page 30.

Checking for direct MTT reduction of test chemicals (Annex 4)

Relative conversion of MTT by the tissue is the parameter evaluated in this assay, therefore it is necessary to assess the non-specific reduction of MTT by the test chemicals used.
Prior to experiments, **test chemicals** should be put in contact with the MTT solution as described below.

- ***When Optical Density (OD) is chosen as endpoint for viability assessment***

  This verification might be performed once before starting the experiment (ideally the week before the study).
  To identify this possible interference, each test chemical is checked for its ability to reduce MTT without tissue (**step 1**). In case of identified MTT interaction, proceed to **step 2**.



A: control
B: test chemical 1: no interaction
C: test chemical 2: slight interaction
D: test chemical 3: strong interaction

Step 1:
- Fill tubes or a 24-well plate with 30±2 mg of the test chemical to be evaluated or water for control.
- Add 300 µL of MTT solution (1 mg/mL) and mix.
- Incubate the mixture for 3 hours ± 15 minutes at 37±2°C, 5±1% CO_2, ≥90% humidity protected from light (test conditions).

If the MTT solution color turns blue or purple, the test chemical interacts with the MTT (see *picture on the left*).
It is then necessary to evaluate during the future studies the part of OD due to the non-specific reduction of the MTT (*i.e.* by using killed epithelium tissues) to define the %NSMTT (Non-Specific reduction of the MTT) value .

Step 2:
- Use killed tissues that possess no metabolic activity but can absorb and bind the test chemical like viable tissues (*see page 16 for more details*).
- Each MTT interacting test chemical is applied onto at least two killed tissues using this EITS protocol. In addition to that, at least two killed tissues are treated with PBS^{-} as control (negative control killed tissue, KU).
- For details see **Figure 1** (condition 2), p.13.

The evaluation of direct MTT reduction of test chemical (**steps 1** and **2 described above**) is performed only on one occasion (one single run even if additional runs are required to classify the test chemical).

- The Evaluation of Test chemical and MTT direct interaction has to be documented using the documentation sheet in **Annex 1**, p.27.
- Report systematically the part of OD due to the non-specific reduction of the MTT (to define the %NSMTT value for a MTT-reducing test chemical) for a test chemical before calculating the final viability (*see specific calculation under "Data Analysis" section, p.21*).

- ***When HPLC/UPLC-spectrophotometry is chosen as endpoint for viability assessment***

The same procedure as for OD measurement (*see paragraph above on this page*) is followed to evaluate the direct MTT reduction of test chemicals

Checking for color test chemicals only (Annex 4)

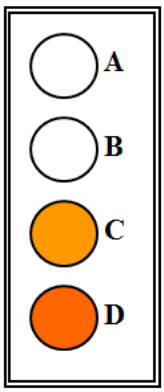
- ***When OD is chosen as endpoint***

Colored test chemicals or test chemicals able to develop a color after contact with the tissue can generate a remaining Non Specific Color ($\%NSC_{living}$). Therefore, each test chemical has to be checked for its colorant properties. Indeed, test chemicals that appear red, blue, black and green by absorbing light should be potentially considered as intrinsic colorants.

- Adapted controls should always be included for colored solid test chemicals.

- For uncolored solid test chemicals, this possible interference should first be checked (**step 1**) before deciding to include adapted controls (**step 2**).

Specific controls must be used in these cases consisting of test chemical-treated tissues that followed all the steps of the method except the MTT incubation.
%NSC$_{living}$ is determined after isopropanol extraction and OD reading in similar conditions (*see specific calculation under "Data Analysis" section, from p.21*)



A: Control
B: Test chemical 1:no color
C: Slight coloration of an orange Test chemical
D: Strong coloration of an orange Test chemical

***Note**: Orange is an example. A coloring test chemical can have of course another color.*

Step 1:
- Fill Eppendorf tubes with 10±2 mg of the test chemical to be evaluated.
- Add 90 µL of water.
- Vortex the solution for a few seconds.
- Incubate the solution for 30±2 min at RT.
- Perform a direct visual observation (*see picture on the left*).

When a colored solution is observed, the tissue staining ability of the test chemical should be checked (**step 2**), otherwise no adapted controls are required.

It is then necessary to evaluate during the study(ies) the part of OD due to the non-specific color (*i.e.* by using living epithelium tissues without MTT conversion test) to define the $\%NSC_{living}$ value (see *step 2 below*).

The visual possible interference should be checked once (**step 1**). In case the test chemical has a potential to color the tissue, possible interference (adapted controls, **step 2**) should be checked in parallel to the evaluation of a test chemical.

Step 2 :
- The Non-Specific Color ($\%NSC_{living}$) is quantified by using at least 2 living tissues per chemical. For details **Figure 1** (condition 3), p.13.
- Coloring test chemical controls are treated and handled like normal treated tissues except that they do not get into contact with the MTT solution as they are incubated in maintenance medium.

An independent %NSC_{living} control needs to be conducted for each test performed (concurrently to every testing: i.e. for each time in each run).

- The Evaluation of Test chemical and color direct interaction has to be documented using the documentation sheet in **Annex 2**, p.28.
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration (to define the %NSC_{living} value), for a test coloring chemical before calculating the final % viability (*see specific calculation under "Data Analysis" section, from p.21*).

- ***When HPLC/UPLC-spectrophotometry is chosen as endpoint:***

No pre-check or control are necessary.

Checking for color test chemicals with possible MTT direct interaction (Annex 4)

- ***When OD is chosen as endpoint:***

The test chemical intrinsic color can, in some cases, interfere with the MTT formazan extraction readings. Blue, dark purple and black test chemical may be directly tested on colorant controls without additional checking test due to their high probabilities to interfere with the blue MTT (formazan salt).

In that case, if the color of the test chemical interferes with the MTT pre-check, an additional adapted control is needed. Each coloring test chemical is applied onto at least two killed tissues and incubated in maintenance medium instead of MTT solution to determine the Non-Specific Color on killed tissues (% **NSC_{killed}**) (**see Figure 1**, condition 4 on p.13).

The evaluation of %NSC_{killed} is performed only on one occasion (one single run even if additional runs are required to classify the test chemical).

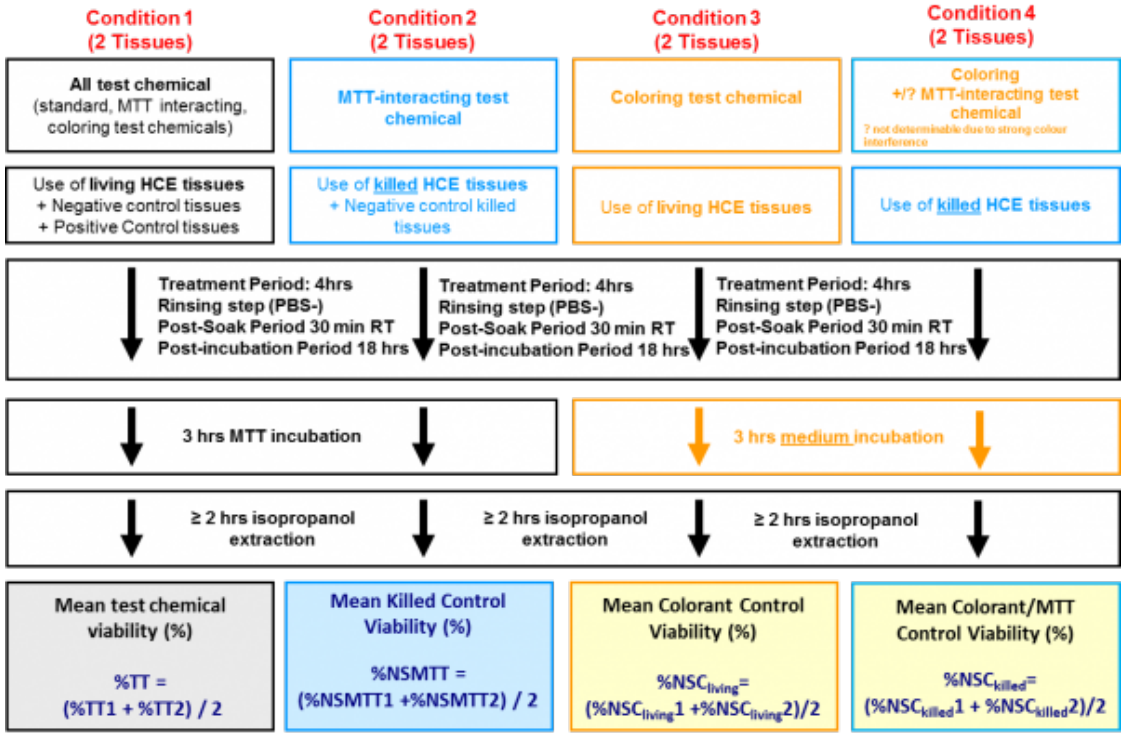
- Results are documented using the documentation sheets available as **Annex 1**, p.27 and **Annex 2**, p.28.
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration on killed tissues (to define the %NSC_{killed} value for a coloring MTT-reducer test chemical) before calculating the final % viability (*see specific calculation under "Data Analysis" section, from p.21*)

- ***When HPLC/UPLC-spectrophotometry is chosen as endpoint*:**

Colored test chemicals or test chemicals that become colored in contact with water or isopropanol that interfere too strongly with the MTT-reduction assay may still be assessed using HPLC/UPLC-spectrophotometry instead of standard absorbance (OD).
As this analytical method allows the separation between MTT formazan and test chemical, NSC controls (%NSC_{living} or %NSC_{killed}) are never required.

Based on this separation capacity of HPLC/UPLC system, two distinct peaks could be generated. In case of overlapping pattern, an alternative separation method should be considered. Evaluation of direct MTT reduction will be performed to define the %NSMTT (at least two killed tissues with MTT incubation). **See Figure 2**, p.14.

Figure 1. Summary of adapted controls choice depending of test chemical physical properties (when OD method is chosen)

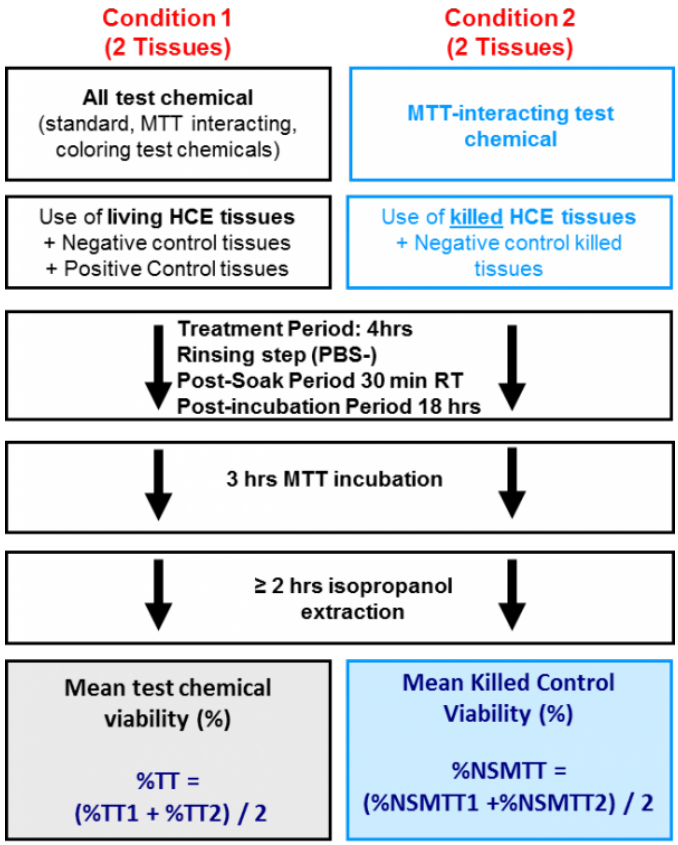


Case by case test conditions for OD reading

	MTT interaction	Coloration interference	Test conditions	Final Corrected Viability
Case 1	-	-	1	%TT
Case 2	+	-	1 + 2	%TT - %NSMTT
Case 3	-	+	1 + 3	%TT - $\%NSC_{living}$
Case 4	+ or ?	+	1 + 2 + 3+ 4	%TT - % NSMTT - $\%NSC_{living}$+ $\%NSC_{killed}$

Results for test chemicals producing %NSMTT and/or $\%NSC_{living}$ and/or $\%NSC_{killed}$ ≥ 50% of the negative control should be taken with caution.

Figure 2. Summary of adapted controls choice depending of test chemical physical properties (when HPLC/UPLC-spectrophotometry method is chosen)



Case by case test conditions for HPLC/UPLC-spectrophotometry endpoint

	MTT interaction	Coloration interference	Test conditions	Final Corrected Viability
Case 1	-	- or +	1	%TT
Case 2	+	- or +	1 + 2	%TT - %NSMTT

Results for test chemicals producing %NSMTT ≥ 50% of the negative control should be taken with caution.

Method

The Good Laboratory Practices with adherence to laboratory testing standards should be applied upon the need.

Test System Procurement

The SkinEthic™ HCE tissues are produced by Episkin (France) (**www.episkin.com**) and they are provided with the necessary maintenance medium. Each SkinEthic™ HCE tissue is controlled by the manufacturer.The human-derived HCE cells are free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the reconstructed tissue is checked by the supplier for absence of contamination by fungi and bacteria.
For the SkinEthic™ medium and SkinEthic™ HCE tissue model refer to the Technical Data and Certificate of Analysis provided by the supplier. Once received examine all kit components for integrity. If there is a question, a concern or something unusual, call + 33 (0) 4 37 28 22 00, **sales@episkin.com**.

Routine Culture Procedure

After reception and before proceeding to treatment steps (as described under the section "*Test material exposure procedures*" on p.16) SkinEthic™ HCE tissues are prepared as follows.

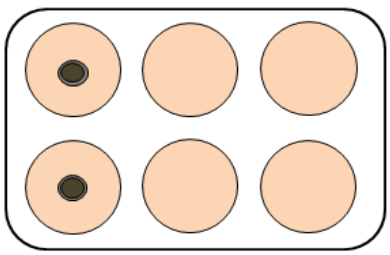
Reception

- Upon receipt of the epithelium kits, **pre-warm maintenance medium at RT** (if necessary) and **transfer each epithelium** from their transport packaging plate to 6-well plates containing 1 mL maintenance medium per well. ***Note:*** *The maintenance medium should be pre-warmed only at room temperature (and not at 37°C).*
- Verify the absence of air bubbles below tissues and incubate them at least overnight at 37±2 °C, 5±1% CO_2, ≥ 90% humidity until treatment steps. ***Note:*** *One plate is used for each test chemical, 2 wells per plate.*

Tissue conditioning

- Pre-warm the maintenance medium at RT.
- Before test chemical treatment, label the plate lid with the appropriate test chemical information.
- Dispense 1 mL of pre-warmed maintenance medium into the second and the third column of the 6-wells plate: the second column is for 4 hrs application' step and the third column for the 18 hrs post incubation step (see example of " *Application/post incubation plate design*" below).
- Transfer 2 SkinEthic™ HCE epithelia units into the 2 first wells filled with 1 mL maintenance medium (at least 2 replicate tissues per test chemical).
- Verify the absence of air bubbles below the tissues.
- Incubate the plates at 37±2°C, 5±1% CO_2, ≥ 90% humidity at least 30 min.

Application/post incubation plate design



1st column: *Tissue conditioning* -1 mL of maintenance medium
2nd column: *Application* - 1 mL of maintenance medium
3nd column: *Post incubation* - 1 mL of maintenance medium

- Two SkinEthic™ HCE tissues are used per chemical (Negative Control (NgC), Positive Control (PC), or test chemical).
- Only 1 chemical per plate
- The plate will be used for *tissue conditioning*, *application* and *post incubation* steps of the process.

***Remark**. In order to avoid cross contaminations between test chemicals leading to possible misclassifications in final results, only 1 chemical (NgC, PC, or test chemical) must be run per plate. The same plate will be used from tissue conditioning, application and post-incubation steps.*
Start with NgC, PC and then the test chemicals.

Water- killed epithelium preparation

Killed HCE tissues (also indicated as killed tissues) are used for MTT-interacting chemicals (p. 10) or MTT pre-check inconclusive chemicals due to color (p.12).
To prepare killed HCE tissues :

- Place the living epithelium in a 24-well plate pre-filled with 300 µL of distilled water.
- Incubate at 37±2°C, 5±1% CO_2, ≥ 90% humidity atmosphere for 24±1 hr.
- At the end of the incubation, discard the water.
- Keep killed epithelium frozen (dry) in freezer (-18 to -22°C) (*killed epithelium can be stored and used up to 6 months*).
- Tissues should be de-frozen before use at room temperature (at least 10 minutes) in 1 mL maintenance medium in 6-well plates).
- Further use of thawed killed HCE tissues is similar to living HCE tissues.
- Apply negative control and test chemical on killed tissues from the same batch.
- Proceed similarly to living tissues for application, rinsing, post-soak, etc.
- Perform this assay only once (on at least duplicate tissues) per test chemical when necessary.

Test Material Exposure Procedures

Tissue treatment

Negative control (PBS -), positive control (Methyl Acetate) and test chemicals applications

- For NgC, dispense **30±2 µL of PBS** - directly topically onto the tissue, always using a positive displacement pipette. Gently spread if necessary on the epithelium surface.
- For PC, dispense **30±2 µL of PC** directly topically onto the tissue, always using a positive displacement pipette. Gently spread if necessary on the epithelium surface.
- For test chemical, pre-moisten the tissues with 30±2 µL of PBS -
- Remove the tissue from the 6-well plate and place onto a dish of weighting to avoid any contamination of the maintenance medium by the test chemical during its disposal.
- Dispense **30 mg ± 2 mg** directly topically onto the tissue.
- Spread it on the epithelium surface without touching it and shake insert gently from side to side to ensure that the tissue is completely covered by the test chemical. Alternatively, pipette tip can be used to homogeneously cover the surface of the tissue with test chemicals.
- Treat tissues at adapted time intervals according to the necessary rinsing-off intervals, *i.e.* 60 seconds, to be adapted depending on the operator experience.
- Place the tissue, in the second column (see an example of *Plate design* on p.15), containing1 mL maintenance medium.
- Verify the absence of air bubbles below tissues and incubate treated tissues for **4±0.1 hours** at 37±2°C, 5±1% CO_2, ≥ 90% humidity with lids on.

Record time and details in the documentation sheet (**Annex 3** , p.29), as well as the weighting step.

End of treatment and rinsing procedure

- At the end of the exposure (4±0.1 hrs), the test chemical (and controls) will be removed by rinsing tissues with PBS $^{-}$. Tissues are rinsed by using a 25 mL adapted multi-pipette. Adjust the distribution to 2 mL per push.
- Process tissue per tissue.
- Maintain the insert over a glass funnel with forceps (to collect the wash fluids in the wash bottle).
- Rinse with **25 mL of PBS** $^{-}$ (at 5 to 8 cm distance from the tissue, directing to the wall of the insert in order to have a gentle action on the surface of the tissues) in order to remove the residual test chemical from the tissue surface. Use ever more the vortex movement to wash the tissue.
- Remove remaining PBS $^{-}$ onto the tissue by energised reversals. *Do not use cotton swab to dry the tissues at this step .*
- For tissues on which the test chemical has not been removed by standard washing procedure with PBS $^{-}$, an alternative option is proposed. Fulfil insert with PBS $^{-}$ onto the tissue and use cotton swabs to gently remove any residual test chemical avoiding contact with tissue.

Post-Soak Period

- After rinsing, transfer immediately the rinsed tissue to a 12-well plate containing **4 mL** of fresh pre-warmed maintenance medium.
- Immerge the tissue.
- Verify the absence of air bubbles below tissues and incubate the tissues for **30±2 minutes** at RT.
  ***Note** : This post-soak tissue immersion is intended to remove any test chemical inside the tissue.*
- At the end of the post-soak immersion, each tissue will be removed from the maintenance medium. The medium will be decanted off the tissue by returning the insert.
- Carefully dry the bottom of the insert by gently taping on a dry absorbent paper (***Figure A***) and the surface with a cotton swab (***Figure B***).

Document this step using documentation sheet in **Annex 3** , p.29.

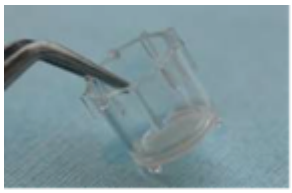


Figure A

Figure B

Post-Incubation Period

- Place the tissue, in the third column of a 6-well plate (see example of *Plate design* p.15), containing 1 mL maintenance medium.
- Verify the absence of air bubbles below tissues.
- incubate the tissues for **18 hours ± 30 min** at 37±2°C, 5±1% CO_2, ≥ 90% humidity.

MTT conversion test

The MTT conversion test is carried out at the end of the 18 hours ± 30 min post-incubation period . The results of this test are documented using the documentation sheet in **Annex 3** , p.29.

***Note** : Additional specific tissue controls for coloring test chemicals (%NSC $_{living}$ ± %NSC $_{killed}$) will be incubated with the **maintenance medium** (see **Figure 1** on p.13 , **conditions 3 and 4**).*

- Prepare MTT medium according to " *Preparation section* " on p.8.
- In a pre-labelled 24-well plate, dispense **300 µL** of MTT solution (**1 mg/mL** freshly prepared in maintenance medium) in 2 wells per test chemical (**Conditions 1 and 2**).
- For the **specific coloring controls,** dispense **300 µL** of maintenance medium instead of MTT medium (**Conditions 3 and 4**).
- Remove remaining maintenance medium (from post-incubation step) below the tissue from the

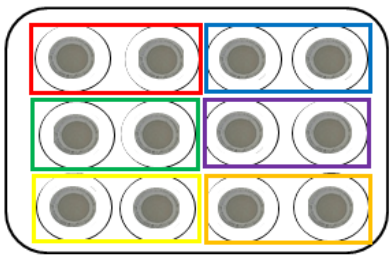
6-well plate, by gently blotted on absorbent material.

- Transfer the tissues in a 24-well pre-labelled plate containing the MTT-containing wells (or maintenance medium for coloring chemical).
- Verify the absence of air bubbles under the tissues.
- Incubate the tissues for **3 hours ± 15 minutes** 37±2°C, 5±1% CO_2, ≥ 90% humidity.
- Record starting time of MTT incubation (**Annex 3** , p.29).
- After the 3 hrs ± 15 min incubation, perform a quick contact of the tissue with dry absorbent paper.
- Place the inserts in **300 µL PBS** (in 24-well plates) to remove the excess of MTT solution (for **conditions 1 and 2**) or maintenance medium (for **conditions 3 and 4**).
- Record ending time of MTT/Maintenance medium incubation (**Annex 3** , p.29).

Formazan extraction

- Perform a quick contact of the tissue with dry absorbent paper.
- Pre-label 12-well plate containing **1.5 mL isopropanol.** To minimize any potential contamination of the isopropanol extraction solution with test chemical that may have remained on the tissue or with strongly colored test chemical, tissues should be extracted from the bottom only.
- During extraction, plates should be covered with “parafilm” to prevent evaporation. At least a layer of parafilm under the lid should be used (usually 3 layers: 2 layers on top of the wells under the lid. Put the lid on and cover with the third sheet of parafilm around the lid and plate; see **Figure C** and **D** below).
- Extract the MTT formazan crystals protected from light with gentle shaking on plate shaker (~ 120 rpm) for **2-3 hours at RT** .
- Remove the inserts from the 12-well plate.
- Homogenize the extraction solution vigorously up and down until a homogeneous solution is reached.

Example of Formazan extraction - plate design.



- Two SkinEthic ™ HCE tissues are used per chemical (NgC, PC, or test chemical).
- Up to 6 chemicals per plate.

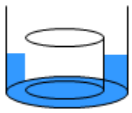


Figure C

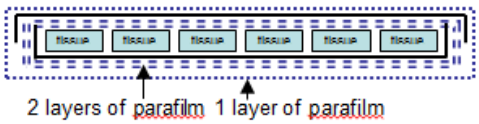


Figure D

Endpoint Measurement

Optical Density (OD) endpoint measurement

- For each tissue, transfer 200 µL / well of the formazan solution extract (i.e. 1.5 mL extraction solution) into two wells (2 x 200 µL) of a 96-well flat bottom microtiter plate (see examples below of plates' design).

For Conditions 1 and 3:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 1
B	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	
C	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 2
D	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	
E	TT11	TT12	TT13								BL	BL	Tissue 1
F	TT11	TT12	TT13								BL	BL	
G	TT11	TT12	TT13								BL	BL	Tissue 2
H	TT11	TT12	TT13								BL	BL	

NgC= negative control; PC= positive control; TT1...TT13= Test treatment 1...13; BL= blank (isopropanol 100%)

For Conditions 2 and 4:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	Killed Tissue 1
B	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	
C	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	Killed Tissue 2
D	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	
E	TT11	TT12	TT13								BL	BL	Killed Tissue 1
F	TT11	TT12	TT13								BL	BL	
G	TT11	TT12	TT13								BL	BL	Killed Tissue 2
H	TT11	TT12	TT13								BL	BL	

KU= negative control killed tissue; TT1...TT13= Test treatment 1...13 on killed tissues; BL= blank (isopropanol 100%)

Note. *Be careful of Isopropanol evaporation in 96-well plates: It is recommended to pool several test chemicals per plate but with a maximum of 60 wells filled per plate and to make the readings without delay in the same run (see example above). Moreover the filling time should not exceed 20 min.*

- Read Optical Density (OD) by using a spectrophotometer microtiter plate reader equipped with a 570±30 nm, without using a reference filter.
- Use isopropanol as blank (200 µL / well).
- Link OD values with the appropriate treatment conditions and replicate on the raw data documents (or files).
- Perform the Quality Control of the raw data and adapt archiving upon needs.

HPLC/UPLC-spectrophotometry endpoint measurement

- **For negative control only** : transfer 200 µL / well of the formazan solution extract (*i.e.* 1.5 mL extraction solution) into two wells (2 x 200 µL) of a 96-well flat bottom microtiter plate and read Optical Density (OD) at 570±30 nm wavelength.
- Use isopropanol as blank (200 µL / well).
- **For all conditions included negative control** : transfer at least 100 µL into an HPLC/UPLC vial (samples can also be frozen 2 months maximum).
- Use a validated analytical method on a qualified HPLC/UPLC-UV/Visible system see documentation on validation of analytical method available in the **Downloads** section of this protocol on **DB-ALM website**).
- Measure peak area at the retention time of the Formazan at the wavelength defined in the validated analytical method.
- Link Area values with the appropriate treatment conditions and replicate on the raw data documents (or files).
- Perform the Quality Control of the raw data and adapt archiving upon needs.

Acceptance Criteria

Common acceptance criteria

- The mean Optical Density (OD_{NgC}) at 570±30 nm of the two replicate tissues treated with negative control should be > 1.0 with an upper acceptance limit of ≤ 2.5.
- The acceptance criteria of the negative control by absorbance (optical density) should be met for both endpoints even if HPLC/UPLC-spectrophotometry is chosen as the endpoint measurement.
- The difference of viability between the two replicate tissues of a single test chemical should be ≤ 20% in the same run whatever the test item (for NgC, PC, test chemical and all adapted controls).
- The PC data meet the acceptance criteria if the mean viability, expressed as % of the NC, is ≤ 20% and the difference value is ≤ 20%.
- The acceptance criteria of the NgC and PC should be met for interpreting the test chemical data.
- For a given test chemical, a single testing run composed of 2 tissue replicates should be sufficient when the classification is unequivocal and if the difference value is ≤ 20 %. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50 ± 5%, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

Specific HPLC acceptance criteria

Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (US FDA, 2001; Alépée et *al*., 2015).
These key parameters and their acceptance criteria are shown in in the document "***Validation of an analytical method on a HPLC/UPLC-spectrophotometry system***" available in the **Downloads** section of this protocol on **DB-ALM website**.
Once the acceptance criteria defined in the document (see **Downloads**) have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this protocol.

A run is qualified if both the NgC and PC data fulfil the acceptance criteria requirements. Otherwise, the run will be considered as non-qualified. Non-qualified runs have to be documented and reported.

A single testing run composed of at least two tissue replicates should be sufficient for a test chemical when the resulting classification is unequivocal (independently of the endpoint: OD or HPLC/UPLC spectrophotometry).
However, in cases of borderline results, such as non-concordant replicate measurements, a second run may be considered, as well as a third one in case of discordant results between the first two runs.

Data Analysis

Main data calculation steps :

(a) Blanks : calculate the mean OD of isopropanol 100% from the 8 wells for each 96-well plate.

(b) Negative PBS - treated controls (NgC) : subtract blank mean value from individual tissues ODs (2 values from each of two tissues). Calculate the mean OD for each individual tissue.
Corrected mean OD for the 2 tissues corresponds to 100% viability.

(c) Positive control (PC) : subtract blank mean value from individual tissues ODs
(2 values from each of two tissues). Calculate the mean OD for each individual tissue.

(d) Test chemical: subtract blank mean value from individual tissues ODs
(2 values from each of two tissues). Calculate the mean OD for each individual tissue.

(e) Viability % : calculate for each treated epithelium the percentage of viability relative to the mean OD of negative control. Calculate viability mean values for each test chemical.

(f) Variability for each test : calculate the viability difference between the two tissues replicates (for NgC, PC and test chemical).

The **calculation procedures** to follow **under different conditions** and according the analytical **method chosen** (see **Figure 1**, p.13 and **Figure 2**, p.14 for summary) are described in details in the following paragraphs.

For viability Tests only – Normal calculation procedure (Condition 1)

A.Calculation for OD reading

- MEAN OD CALCULATION

*Negative Control (NgC)	$OD_{NgC1} = OD_{NgC1raw} - OD_{blank\ mean}$
Individual OD Negative Control (NgC)	$OD_{NgC2} = OD_{NgC2raw} - OD_{blank\ mean}$
Mean OD Negative Control	$\mathbf{OD_{NgC} = [OD_{NgC1} + OD_{NgC2}] / 2}$

The mean OD of the two Negative Control replicates (PBS - treated) corresponds to 100% reference viability.

*Positive Control (PC)	$OD_{PC1} = OD_{PC1\ raw} - OD_{blank\ mean}$
OD Positive Control (PC)	$OD_{PC2} = OD_{PC2\ raw} - OD_{blank\ mean}$
Mean OD Positive Control	$\mathbf{OD_{PC} = [OD_{PC1} + OD_{PC2}] / 2}$

*Test Treatment	$OD_{TT1} = OD_{TT1\ raw} - OD_{blank\ mean}$
OD Test Treatment (TT)	$OD_{TT2} = OD_{TT2\ raw} - OD_{blank\ mean}$
Mean OD Test Treatment	$\mathbf{OD_{TT} = [OD_{TT1} + OD_{TT2}] / 2}$

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control1	$\%NgC1 = [OD_{NgC1} / \text{mean } OD_{NgC}] \times 100$
% Negative Control2	$\%NgC2 = [OD_{NgC2} / \text{mean } OD_{NgC}] \times 100$
% mean Negative Control	**%NgC= (%NgC1 + %NgC2)/2**

% Positive Control1	$\%PC1 = [OD_{PC1} / \text{mean } OD_{NgC}] \times 100$
% Positive Control2	$\%PC2 = [OD_{PC2} / \text{mean } OD_{NgC}] \times 100$
% mean Positive Control	**%PC= (%PC1 + %PC2)/2**

% Test Treatment 1	$\%TT1 = [OD_{TT1} / \text{mean } OD_{NgC}] \times 100$
% Test Treatment 2	$\%TT2 = [OD_{TT2} / \text{mean } OD_{NgC}] \times 100$
% Mean Test Treatment	**%TT = (%TT1 + %TT2)/2**

B.Calculation for HPLC/UPLC - spectrophotometry endpoint

- MEAN OD CALCULATION

*Negative Control (NgC)

Mean Area Negative Control — **Area $_{NgC}$ = [Area $_{NgC1}$ + Area $_{NgC2}$] / 2**

The mean Area of the two Negative Control replicates (PBS - treated) corresponds to 100% reference viability.

*Positive Control (PC)

Mean Area Positive Control — **Area $_{PC}$ = [Area $_{PC1}$ + Area $_{PC2}$] / 2**

*Test Treatment

Mean Area Test Treatment — **Area $_{TT}$ = [Area $_{TT1}$ + Area $_{TT2}$] / 2**

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control1	%NgC1 = [Area $_{NgC1}$ / mean Area $_{NgC}$] x 100
% Negative Control2	%NgC2 = [Area $_{NgC2}$ / mean Area $_{NgC}$] x 100
% mean Negative Control	**%NgC= (%NgC1 + %NgC2)/2**
% Positive Control1	%PC1 = [Area $_{PC1}$ / mean Area $_{NgC}$] x 100
% Positive Control2	%PC2 = [Area $_{PC2}$ / mean Area $_{NgC}$] x 100
% mean Positive Control	**%PC= (%PC1 + %PC2)/2**
% Test Treatment 1	%TT1 = [Area $_{TT1}$ / mean Area $_{NgC}$] x 100
% Test Treatment 2	%TT2 = [Area $_{TT2}$ / mean Area $_{NgC}$] x 100
% Mean Test Treatment	**%TT = (%TT1 + %TT2)/2**

The mean relative viability is used for classification according to the **Prediction Model** *on page 26.*

MTT interacting test chemical calculation procedure (Condition 2)

Test chemicals that interfere with MTT can produce non-specific reduction of the MTT .
It is necessary to evaluate the OD or area due to the non-specific reduction **(%NSMTT)** and to subtract it before calculations of final viability.

OD *or* **Area $_{KU}$** : OD or Area untreated killed tissues + MTT incubation
OD *or* **Area $_{kt}$** : OD or Area test chemical treated killed tissues + MTT incubation
OD *or* **Area $_{NgC}$** : mean OD or Area negative control living tissues + MTT incubation

A.Calculation for OD reading

- Non-specific MTT reduction calculation (%NSMTT)

% Killed Test Treatment 1	%NSMTT1= [(OD $_{kt1}$ - OD $_{ku}$)/ OD $_{NgC}$]x100
% Killed Test Treatment 2	%NSMTT2= [(OD $_{kt2}$ - OD $_{ku}$)/ OD $_{NgC}$]x100
% Mean Non Specific MTT reduction	**%NSMTT = (%NSMTT1 + %NSMTT2)/2**

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment1	*%FV $_{C\ NSMTT1}$ = % TT1- %NSMTT*
%Final viability Test Treatment 2	*%FV $_{C\ NSMTT2}$ = % TT2- %NSMTT*
Mean Final Viability	**% *FV $_{C\ NSMTT}$* = (FV $_{C\ NSMTT1}$ +FV $_{C\ NSMTT2}$)/2**

B.Calculation for HPLC/UPLC - spectrophotometry endpoint

- Non-specific MTT reduction calculation (%NSMTT)

% Killed Test Treatment 1	%NSMTT1= [(Area $_{kt1}$ -Area $_{ku}$)/ Area $_{NgC}$]x100
% Killed Test Treatment 2	%NSMTT2= [(Area $_{kt2}$ -Area $_{ku}$)/ Area $_{NgC}$]x100
% Mean Non Specific MTT reduction	**%NSMTT = (%NSMTT1 + %NSMTT2)/2**

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1	*%FV $_{C\ NSMTT1}$ = % TT1- %NSMTT*
%Final viability Test Treatment 2	*%FV $_{C\ NSMTT2}$ = % TT2- %NSMTT*
Mean Final Viability	**% *FV $_{C\ NSMTT}$* = (FV $_{C\ NSMTT1}$ +FV $_{C\ NSMTT2}$)/2**

The mean final viability is used for classification according to the ***Prediction Model*** *on p.26.*

Coloring test chemicals calculation procedure (Condition 3)

For test chemicals detected as able to Color the tissues, it is necessary to evaluate the non-specific OD due to the residual chemical Color (unrelated to mitochondrial activity) and to subtract it before calculations of the “true” viability %.
Note. *This calculation procedure is not applicable to HPLC/UPLC-spectrophotometry.*

OD $_{TT\text{-}MTT}$: OD treated tissue without MTT incubation
OD $_{NgC}$: Mean OD Negative Control (living tissues + MTT incubation)

- Non-specific Color CALCULATION (%NSC $_{living}$)

% Non-Specific Color tissue 1	%NSC $_{living}$ 1= [(OD $_{TT1\text{-}MTT}$ / OD $_{NgC}$] x 100
% Non-Specific Color tissue 2	%NSC $_{living}$ 2= [(OD $_{TT2\text{-}MTT}$ / OD $_{NgC}$] x 100
Mean % Non-Specific Color	**%NSC $_{living}$ = (%NSC $_{living}$ 1+%NSC $_{living}$ 2)/2**

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1	*%FV $_{C\ NSCliving1}$ = % TT1-* %NSC $_{living}$
%Final viability Test Treatment 2	*%FV $_{C\ NSCliving2}$ = % TT2-* %NSC $_{living}$
Mean Final Viability	**% *FV $_{C\ NSCliving}$* = (FV $_{C\ NSCliving1}$ + FV $_{C\ NSCliving2}$)/2**

The mean final viability is used for classification according to the ***Prediction Model*** *on p.26.*

Coloring +/? MTT interacting test chemical calculation procedure (Condition 4)

Colored interfering test chemicals are usually identified in pre-checks as being also potential direct MTT reducers due to their intrinsic Color, thus leading to the use of %NSMTT controls together with %NSC $_{living}$ controls.

However, the Non-Specific MTT Reduction (%NSMTT) control also includes the binding of the test chemical to the killed tissues and thus binding is corrected twice leading to an overestimation of the toxic effect. This can be corrected with the use of a third set of control (%NSC $_{killed}$).
Note. *The %NSC $_{killed}$ control is not applicable to HPLC/UPLC-spectrophotometry .*

OD $_{kt\text{-}MTT}$: OD Killed test tissue treated tissues without MTT incubation
OD $_{NgC}$: mean OD negative control living tissues

- NON SPECIFIC Color WITHOUT MTT % CALCULATION (%NSC $_{killed}$)

% Non-Specific Color without MTT tissue 1	%NSC $_{killed1}$ = (OD $_{kt\text{-}MTT1}$ /OD $_{NgC}$)x100
% Non-Specific Color without MTT tissue 2	%NSC $_{killed2}$ = (OD $_{kt\text{-}MTT2}$ /OD $_{NgC}$)x100
Mean % Non-Specific Color without MTT	**%NSC $_{killed}$ = (%NSC $_{killed1}$ + %NSC $_{killed2}$)/2**

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1	*%FV $_{C\ TT1}$ = % TT1- %NSMTT-* %NSC $_{living}$ + %NSC $_{killed}$
%Final viability Test Treatment 2	*%FV $_{C\ TT2}$ = % TT2- %NSMTT-* %NSC $_{living}$ + %NSC $_{killed}$
% Mean Final Viability	***FV $_{C\ TT}$ = (FV $_{C\ TT1}$ + FV $_{C\ TT2}$)/2***

The mean final viability is used for classification according to the ***Prediction Model*** *on p.26.*

Remarks on data analysis

If the variability of the interfering test chemical is not significantly higher than normal, correction using adapted controls should be allowed as long as the interference is not extreme.
If variability is significantly higher than normal (above 140% of the negative control), it is assumed that the amount of test chemical retained by the tissue after exposure and post-treatment incubation varies significantly between different tests.
In this situation, the following rules are applied when **OD endpoint is chosen**:

- **IF** the mean of % Non-Specific Color on living tissues (%NSC $_{living}$) or % Non-Specific MTT reduction (%NSMTT) of the qualified run is less than or equal to (≤) 50%,
  THEN the test chemical is considered to be compatible with the test method.
- **IF** the mean of %NSC $_{living}$ or %NSMTT or [%NSC $_{living}$ + %NSMTT - %NSC $_{killed}$] of the qualified run is greater than (>) 50% AND the classification remains the same upon correction,
  THEN the test chemical is considered to be compatible with the test method.
- **IF** the mean of %NSC $_{living}$ or %NSMTT or [%NSC $_{living}$ + %NSMTT - %NSC $_{killed}$] of the qualified run is greater than (>) 50% AND the classification of the qualified run changes upon correction,
  THEN this test chemical is considered to be incompatible with the test method. In this case, use of another method or of a default classification as classified (category 1) should be considered.

Results for test chemical producing %NSMTT and/or %NSC $_{living}$ and/or %NSC $_{killed}$ ≥ 50% of the negative control should be taken with caution.

In the table below examples of calculations to be performed depending on the conditions.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Mean Viab %NSC $_{living}$	Mean viab %NSC $_{killed}$	Final Corrected Viability	Final Viability
	Living+MTT	*Killed+MTT*	*Living-MTT*	*Killed-MTT*		
1	81.2	-	-	-	%TT	81.2
2	101.2	11.2	-	-	%TT - %NSMTT	90.0
3	81.2	-	41.2	-	%TT - %NSC $_{living}$	40.0
4	101.2	11.2	20	11	%TT - % NSMTT - %NSC $_{living}$ + %NSC $_{killed}$	81.0
See specific section on calculation for OD reading	***p.21 (A)***	***p.22 (A)***	***p.23 (condition 3)***	***p.24 (condition 4)***		

For Colored test chemicals interfering too strongly with the MTT-reduction assay an alternative endpoint may be required (e.g. **HPLC-UPLC-spectrophotometry**). In this case, one single test should be sufficient independently of how strong the Color interference is, unless the test chemical is also a strong MTT reducer (*i.e.* , killed control values > 50% of the negative control) and correction from control tissues is required. In the table below examples of calculations.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Final Corrected Viability	Final Viability
	Living+MTT	*Killed+MTT*		
(H)1	81.2	-	%TT	81.2
(H)2	101.2	11.2	%TT - %NSMTT	90.0
See specific section on calculation for HPLC/UPLC-Spectrophotometry endpoint	***p.22 (B)***	***p.23 (B)***		

Prediction Model

The present test method is recommended to identify solid chemicals that do not require classification for eye irritation or serious eye damage according to UN GHS (UN GHS No Category) without further testing. However, the SkinEthic™ HCE EITS is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation).

The prediction model of the SkinEthic™ HCE EITS classifies chemicals into 2 groups:

-UN GHS No Category (NC), and

-“Category 1 / Category 2 (Cat. 1 / Cat. 2)” without further conclusion whether the test chemical belongs to the GHS Cat. 1 or Cat. 2.

Due to the high over-prediction rate shown in the SkinEthic™ HCE EITS validation (26.4%), part of the chemicals predicted as “Cat.1/ Cat.2” can actually be falsely positive. Therefore the OECD TG 492 (2017a) interprets the positive result of the SkinEthic™ HCE EITS as “no prediction can be made”.

The prediction model (PM) is described below:

Criteria for *in vitro* interpretation	**Classification**
Mean tissue viability > 50 %	No Category (NC)
Mean tissue viability ≤ 50 %	No prediction can be made

A single testing run should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run might be considered, as well as a third one in case of discordant results between the first two runs.

For a full evaluation of eye severe damage / eye irritation effects, the distinction will need to be addressed by another tier of a test strategy in the context of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2017c).

Annexes

Annex 1: Evaluation of Test chemicals - MTT direct interaction (3hrs±15min)

Laboratory: …… Study : ……

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Interaction Blue Color Yes / No

Date: …… ID and signature:……

Annex 2: Evaluation of test chemicals - Color interaction (30min±2min)

Laboratory: …… Study N°…....

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Ability to Color Yes / No

Date: …….. ID and signature:……

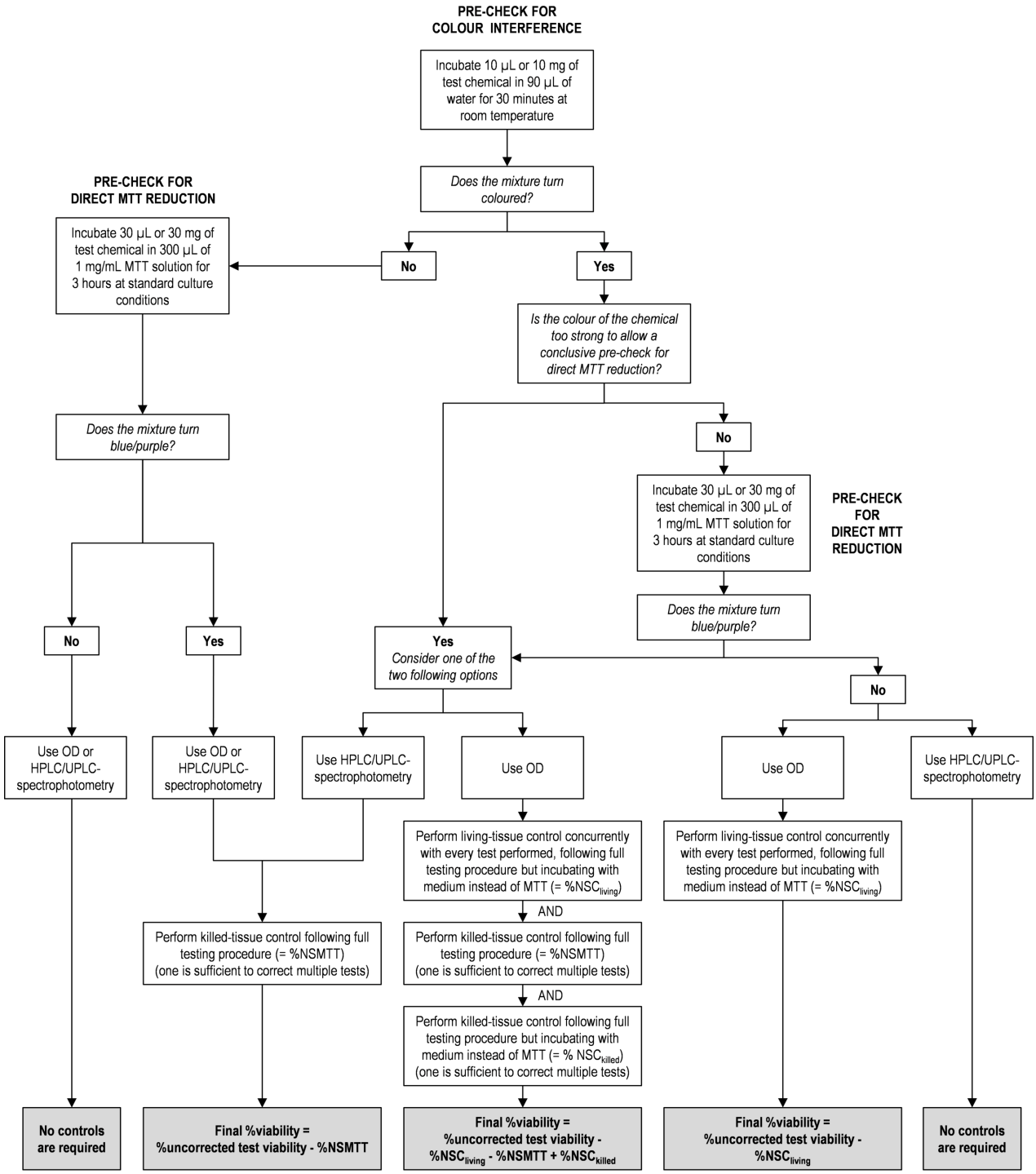
Annex 3: Incubation timings

Laboratory: …… Study N°…..... Batch N°:…….. Series N°:……..

Test chemical Name Or code	Treatment 4±0.1 hrs		Post-Soak Treatment 30±2 min	Post incubation 18 hrs ± 30 min	MTT incubation 3 hrs ± 15min	Formazan extraction
	Start time (hh:mm)	End time/Start time (hh:mm)	End time/Start time (hh:mm)	End time/Start time (hh:mm)	End time/Start time (hh:mm)	Date and End time (hh:mm)

Date: …….. ID and signature:…….

Annex 4: Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals



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