DB-ALM Protocol n° 183 : U937 Cell Line Activation Test for Skin Sensitization (U-SENS™)

Skin Sensitisation & Allergic Contact Dermatitis

The U-SENS™ is an *in vitro* test method designed to assess the skin sensitisation potential of substances by quantifying the change in the expression of the CD86 cell surface marker in the human cell line U937. The U-SENS™ assay is proposed to address the third key event (activation of dendritic cells) of the skin sensitisation Adverse Outcome Pathway (AOP).

Résumé

The U-SENS™ assay, an *in vitro* test method, has been developed and established within L'Oréal. This test method described in the following protocol is modelling the dendritic cell activation upon exposure to chemicals. Upon contact with sensitizers, U937 human histiocytic lymphoma cells are activated and increase the CD86 expression.

The U-SENS™ assay addressing one biological mechanism of the skin sensitisation pathway (formally described in an Adverse Outcome Pathway (AOP) for skin sensitization by the OECD, 2012) is foreseen to be combined with complementary information and evaluated in the context of Integrated Approaches to Testing and Assessment (IATA). In such context, the U-SENS™ assay is part of a decision strategy for skin sensitization hazard identification (contact skin sensitizer vs. non sensitizer). It is also foreseen to be a part of integrated test batteries which will be able to fully replace the *in vivo* test methods (i.e. Local Lymph Node Assay- LLNA, Buehler and Magnusson & Kligman) to predict the sensitizing potential of the chemicals in humans (risk and safety assessment).

On the basis of the data currently available, the U-SENS™ method was shown to be applicable to test chemicals (including cosmetics ingredients e.g. preservatives, surfactants, actives, dyes) covering a variety of organic functional groups, of physicochemical properties, skin sensitisation potencies (as determined in *in vivo* studies) (Piroird et *al*., 2015). The U-SENS™ assay is applicable to all mono-substances or mixtures (including pre- or pro-haptens) that are soluble in the aqueous testing conditions and compatible with flow cytometry analysis. The spectrum of reaction mechanisms known to be associated with skin sensitisation (i.e. Michael acceptor, Schiff base formation, acyl transfer agent, substitution nucleophilic bi-molecular (SN2), or nucleophilic aromatic substitution (SNAr)) are also covered. Besides, the theoretical limitations of the test method (high hydrophobic substances or color interference with the fluorescent flow cytometry measurement of coloring substances potentially leading to false negatives) have to be taken in account in the results' interpretation.

Experimental Description

Endpoint and Endpoint Measurement:

Since dendritic cell maturation upon exposure to a sensitizing agent is accompanied by changes in surface marker expression, these surface markers are primary biomarkers of a dendritic cell-based *in vitro* assay. The most frequently assessed markers are CD40, CD54, CD80, CD83, CD86, and HLA-DR. These surface markers are up-regulated upon dendritic cell maturation. The expression of the co-stimulatory molecule CD86 is adequate for *in vitro* testing since increases in CD86 expression are related upon *in vitro* exposure to contact sensitizers. In the current U-SENS™ assay, the following endpoints were measured and used to classify a chemical.

CD86 expression as a marker of cell activation is measured by flow cytometry following exposure to chemicals.

Cell viability is assessed using propidium iodide exclusion by flow cytometric analysis.

Endpoint Value:

For CD86 induction, the concentration of a chemical needed to reach a stimulation index (S.I) of 150% (EC150) is calculated.

For cytotoxicity, the concentration of a chemical needed to reach a cytotoxicity effect of 30% e.g. a remaining cell viability of 70% (CV70), is calculated.

Experimental System(s):

U937- Human histiocytic lymphoma cell line

Discussion

- Ethical issues: The test is based on a human cell line established more than 30 years ago. Thus no primary cells derived from cells or tissues sources are required to set-up the test methodology.
- Special equipment: The laboratories and study personnel involved should be skilled in cell cultures and flow cytometry. The flow cytometer should regularly undergo maintenance and daily/weekly cleaning procedures.The facility where the flow cytometer is located should keep a controlled temperature ~20°C (air conditioned) whenever the flow cytometer is on, in order to avoid drift in CD86 measurement. This is especially important if using a plate sampler.
- Amount of training: A training week was most of the time necessary to establish the test method in a naïve laboratory. It included a practical training in which (i) the main steps of the protocol was emphasized and (ii) then performed by the trainers. It also included depth discussions about the detailed protocol and a practical example-based workshop on concentration selection and conclusion.
- Obstacles pertaining to transferability: The U-SENS™ assay is based on measurements performed using a flow cytometer, generally sensible equipment. That is why the flow cytometer should regularly undergo maintenance and daily/weekly cleaning procedures and the involved study personnel being skilled in flow cytometry procedures to avoid any issue related to an external impact factor during the U-SENS experiment.
- Duration of the test: Three days are required to perform a run: cell treatment with test items is performed on day 1 then harvest, staining and data acquisition on day 3. A U-SENS experiment consisted of at least two independent runs in 2 different weeks. 2 to 3 weeks are required to overall define a call for a chemical. A trained experimenter can run at least 8 test items in a run and can perform two runs in one week (up to 16 different tests items per week).
- Possible adaptation of the protocol: it should be possible to run the test on current laboratory robots, as the method is performed in a 96-well plate format. By using robotics, the throughput might be enhanced.
- Costs: The consumables' cost per item excluding labor and fixed equipment has been estimated at approximately 250 euros. Contract Research Organization (CRO) testing costs are available upon request to the CRO.

Status

Participation in Validation Studies:

The U-SENS™ method has been evaluated in a validation study coordinated by L'Oréal (Alépée et *al*., 2015).
Subsequently it has been independently peer reviewed by the EURL ECVAM Scientific Advisory Committee (ESAC) (EURL ECVAM, 2016).

Considering all available evidence and input from regulators and stakeholders, the U-SENS™ was recommended by EURL ECVAM (EURL ECVAM, 2017) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling (UN, 2015). Examples of the use of U-SENS™ data in combination with other information, including historical data and eventually existing valid human data, are also reported elsewhere in the literature (OECD, 2016).

The U-SENS™ method proved to be transferable to laboratories experienced in cell culture techniques and flow cytometry analysis.
The level of reproducibility in predictions that can be expected from the test method is in the order of 90% and 84% within and between laboratories, respectively (Alépée et *al*., 2015).
Results generated in the validation study and other published studies (Piroird et *al*., 2015) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. United Nation Globally Harmonized System of Classification and Labelling of Chemicals- UN GHS Cat.1) from non-sensitisers is 86% (N=166) with a sensitivity of 91% (118/129) and a specificity of 65% (24/37).

Compared with human results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 77% (N=101) with a sensitivity of 100% (58/58) and a specificity of 47% (20/43).
False negative predictions compared to LLNA with the U-SENS™ are more likely to concern chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A).
The accuracy values given here for the U-SENS™ as a stand-alone test method are only indicative, since the test method should be considered in combination with other sources of information in the context of IATA.

Regulatory Acceptance:

The test method has been **adopted as OECD Test Guideline No. 442E** "In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome pathway for Skin Sensitisation" in October 2017. This key event based test guideline describes the U-SENS^{TM} method in Annex II "In Vitro Skin Sensitisation: U937 Cell Line Activation Test (U-SENS^{TM})". An updated version of the OECD TG 442E has been issued in June 2018 (OECD, 2018).

Proprietary and/or Confidentiality Issues

There are no Intellectual Property Rights on this test method.

There is no license fee with ATCC or Professor Nilsson (**kenneth.nilsson@igp.u.se**) for the use of the cell line (clone CRL1593.2) in the context of the OECD Test Guideline 442E.

Health and Safety Issues

General Precautions

- Store test chemicals in ventilated safety cup boards. Respect the special store conditions if necessary (e.g. special temperature, protected from light etc.)
- Non-coded test chemicals should be handled following chemical safety datasheet.
- Unknown and coded test chemicals with no or incomplete safety handling information should be considered as skin sensitizers and toxic and must be handled with maximum care.
- In accordance with chemical safety guidelines: use safety ventilated cabinet, wear gloves, and protections.
- After use, the material in contact with the sample and the assay medium should be decontaminated, prior to disposal.

MSDS Information

Trypan blue (Invitrogen, product code: 15250061) is classified as carcinogen category 2 (MSDS revised 25-Jun-2015).

Precautionary statements: do not handle until all safety precautions have been read and understood, use personal protective equipment as required. IF exposed or concerned: get medical advice/attention.

Abbreviations and Definitions

ATCC:	American Type Culture Collection
BLR:	Between-Laboratory Reproducibility
CD86 concentration response:	There is concentration-dependency (or concentration response) when a positive concentration (CD86 S.I. ≥ 150) is followed by a concentration with an increasing CD86 S.I.
CV70:	Concentration Value at which a chemical reach the cytotoxicity threshold (70%)
DMSO:	Dimethylsulphoxide
Drift:	A drift is defined by i) the corrected %CD86+ value of the untreated control replicate 3 is less than 50% of the mean of the corrected %CD86+ value of untreated control replicates 1 and 2; and ii) the corrected %CD86+ value of the negative control replicate 3 is less than 50% of mean of the corrected %CD86+ value of negative control replicates 1 and 2.
EC150:	Estimated Concentrations showing the 150% S.I. of CD86 expression
FCS:	Fetal Calf Serum
FL1:	FITC fluorescence
FL3:	Propidium iodide fluorescence
FSC:	Flow cytometry size parameter (Forward SCatter)
Invalidated run:	If one or more of the acceptance criteria is not met, the run is invalidated and should be repeated. The data should not be used for the U-SENS prediction.
LA:	Lactic acid CASRN 50-21-5
N:	Negative
NA:	Not applicable
NgC:	Negative control (Lactic acid)
Non concordant run:	Individual valid run conclusion not concordant with the majority of others individual valid runs conclusions. The data should not to be used for the mean final viability and CD86 SI determination.
P:	Positive
PBS:	Phosphate Buffer Saline
PI:	Propidium iodide
PC:	Positive control (TNBS)
RT:	Room Temperature
RPMI:	Complete culture medium (Roswell Park Memorial Institute)
SI:	Stimulation Index. Relative value of intensity in chemical-treated cells (% of IgG1 positive cells subtracted to the % of CD86 positive cells) compared to solvent/vehicle-treated cells (% of CD86+ control cells - % of IgG1+ control cells).
Solubility interference:	Crystals observed in the well under microscope following the 45±3 h exposure period.
SOP:	Standard Operating Procedure
SSC:	Flow cytometry granularity parameter (Side SCatter)
TNBS:	2,4,6-Trinitro-benzene-sulfonic acid (picrylsulfonic acid) CASRN 2508-19-2
WLR:	Within-Laboratory Reproducibility

Last update: 11 October 2017

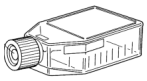
PROCEDURE DETAILS, Latest Version 18 May 2017

U937 Cell Line Activation Test for Skin Sensitization (U-SENS™)
DB-ALM Protocol n° 183

Quick flow chart U-SENS TM

A detailed description of the test method is available in the following sections of this protocol.

CELL MAINTENANCE

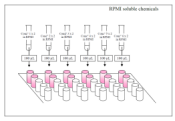


- For Day +1: Prepare a cell solution at 0.6 x 10^6 cell/mL
- For Day +2: Prepare a cell solution at 0.3 x 10^6 cell/mL
- For Day +3: Prepare a cell solution at 0.15 x 10^6 cell/mL

SOLUBILITY ASSESSMENT

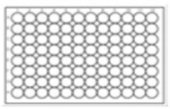
- Complete medium (50 mg/mL) preferentially or DMSO (50 mg/mL).
- Complete medium if no suitable vehicle found

PREPARATION OF TEST CHEMICALS



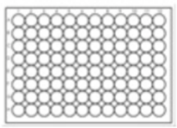
- Prepare a fresh stock solution at 0.4 mg/mL (complete medium) or 50 mg/mL (DMSO)
- PC (TNBS at 50 µg/mL), NgC (LA 200 µg/mL) and 0.4% DMSO final in complete medium if applicable
- First run concentrations : 1, 10, 20 ,50 ,100 and 200 µg/mL (final concentration)
- Next run concentrations : at least 4 usable concentrations between 0.1-200 µg/mL

CELL TREATMENT



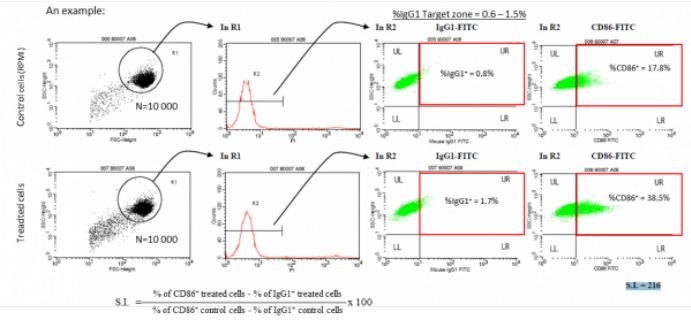
- In a sterile 96-well flat bottom plate (2 wells per conditions) add :
  - 100 µL/well of the test chemicals or control substances working solutions (2x concentrated)
  - 100 µL/well of suspended cells (0.5 x 10^6 cells/mL)
- Cover the plate(s) with a sealing tape
- Incubate 45±3 h at 37±2 °C, 5±1% CO_2, ≥ 95% humidity

CELL STAINING after 45±3 hours of exposure



- Check and report the solubility (interference if crystals or drops observed under the microscope)
- Wash once with 100 µL of ice-cold staining buffer and resuspend cells in 100 µL of staining buffer
- Stain the cells with FITC-labelled anti-CD86 or mouse IgG1 (5 µL/well, 30 min, 4°C, protected from light)
- Wash twice with 100 µL of ice-cold staining buffer
- Wash once with 100 µL of ice-cold PBS
- Resuspend cells in ice-cold PBS (50 µL in 96 wells-plate or 125 µL in microtubes)
- Add propidium iodide at 3 µg/mL final concentration just before acquisition

FLOW CYTOMETRY ACQUISITION /ANALYSIS



RUN PREDICTION

- **NOT CONCLUSIVE in the first run only**: CD86 S.I. higher than 150% at the highest non-cytotoxic concentration only.
- **NEGATIVE**: CD86 S.I. less than 150% at all non-cytotoxic concentrations and no interference (solubility, colour, cytotoxicity).
- **POSITIVE in all other cases**: CD86 S.I. higher than 150% with or without dose-response and/or interferences.
- **U-SENS™ PREDICTION:** at least two concordant runs are required

Prior to routine use of the U-SENS™ method for regulatory purposes, as recommended in OECD Test Guidelines 442E, laboratories should demonstrate technical proficiency by correctly predicting the proficiency chemicals listed in the following Table.

Proficiency substances	CAS RN	Physical state	*In vivo* prediction[1]	U-SENS™ Solvent/ Vehicle	U-SENS™ CV70 µg/mL	U-SENS™ EC150 g/mL[2]
4-Phenylenediamine	106-50-3	Solid	Sensitiser (strong)	Complete medium[3]	<30	Positive (≤10)
Picryl sulfonic acid	2508-19-2	Liquid	Sensitizer (strong)	Complete medium	>50	Positive (>50)
Diethyl maleate	141-05-9	Liquid	Sensitiser (moderate)	DMSO	10-100	Positive (≤20)
Resorcinol	108-46-3	Solid	Sensitiser (moderate)	Complete medium	>100	Positive (≤50)
Cinnamic alcohol	104-54-1	Solid	Sensitiser (weak)	DMSO	>100	Positive (10-100
4-Allylanisole	140-67-0	Liquid	Sensitiser (weak)	DMSO	>100	Positive (<200)
Saccharin	81-07-2	Solid	Non-sensitiser	DMSO	>200	Negative (>200)
Glycerol	56-81-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Lactic acid	50-21-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Salicylic acid	69-72-7	Solid	Non-sensitiser	DMSO	>200	Negative (>200)

CAS RN = Chemical Abstracts Service Registry Number

[1] The in vivo hazard and (potency) prediction is based on LLNA data.

[2] Reference Range based on historical observed values.

[3] Complete medium: RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin.

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Materials and Preparations

Cell or Test System

The human histiocytic lymphoma cell line U937 should be used for performing the U-SENS™ method. It is recommended that cells (CRL1593.2) are obtained from a well-qualified cell bank such as the American Type Culture Collection (**www.atcc.org**). This cell line is non adherent and is used as a cell suspension in a water-based culture medium.

Equipment

Fixed Equipment

- Sterile hood for cell culture work
- CO_2 incubator
- Centrifuge for tubes and 96-wells plates
- Flow Cytometer (optional: plate sampler): FACSCalibur (Becton Dickinson), FACSCanto II (Becton Dickinson) or similar
- Analytical Balance capable of accurately weighing up to 20 grams with 0.1 mg precision
- Water bath
- Roller plate
- 8 or 12 channel pipettes for volumes between 10 µL and 200 µL

Consumables

- Polypropylene non sterile V bottom shapped 96-well plates: ref. 738-001 VWR
- 96-well sterile flat bottom culture plates: ref. 734-2097 VWR
- 50mL/162cm^2 culture flasks: ref. 3151 Corning
- 25mL/75cm^2 culture flasks: ref. 430720 Corning
- 5mL sterile polystyrene hemolysis tubes + winged plugs: ref. 080064 Dutscher
- 12*75mm glass hemolysis tubes (6 mL): ref. 212-9861 VWR
- winged plugs for 6 mL glass hemolysis tubes: ref. 212-0131 VWR
- 16*100mm glass hemolysis tubes (15 mL): ref 734-4225 VWR
- winged plugs for 15 mL glass hemolysis tubes: ref. 0305502 Dutscher
- 0.22 microns filtration unit: ref. SCGVU05RE Milipore
- Sealing tape (sterile polyester): ref. 236366 NUNC
- Cytometer tubes 5mL (if no plate sampler): ref. 352235 BD Falcon
- 1,4mL Micronic U-tubes (if no plate sampler): ref. MP32022 Integra Biosciences
- Micronic 96 microtubes 1.4 mL rack (if no plate sampler): ref. MP22502 Integra Biosciences

Media, Reagents, Sera, others

- Trypan Blue: ref. 15250061 Invitrogen
- Apyrogenic distilled water
- DMSO: ref. D2650 Sigma-Aldrich
- Phosphate Buffer Saline w/o Ca^{2+} and Mg^{2+} (PBS) : ref 15-516F LANZA
- Fetal Calf Serum (FCS): ref. S1810 BioWest
- L-Glutamine, penicillin, streptomycin: ref. G1146 Sigma-Aldrich

- RPMI 1640: ref. 42001018 Gibco
- FITC Mouse IgG1 antibody: refs 555748 BD Pharmingen or GM4992 Caltag/Invitrogen
  FITC anti-human CD86 antibody: refs 555657 clone Fun-1 BD Pharmingen or MHCD8601 clone BU63 Caltag/Invitrogen.
  Other clones or supplier of the antibodies which passed the reactivity check may be used for the assay. Users may consider titrating the antibodies in their own laboratory's conditions to define the best concentration for use. Other detection system e.g., fluorochrome-tagged anti-CD86 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances listed in OECD Test Guidelines.
- Lactic Acid: CASRN 50-21-5, ≥ 85% purity, ref. L6661 Sigma-Aldrich
- Picrylsulfonic acid (2,4,6-Trinitro-benzene-sulfonic acid: TNBS): CASRN 2508-19-2, ≥ 99% purity, ref. 92822 Sigma-Aldrich
- 4% citrate tribasic buffer: ref. S5770 Sigma-Aldrich
- Propidium iodide: ref. P4170 Sigma-Aldrich

Nota bene. Alcohol-based disinfectants (like ethanol 70% or Meliseptol) should not be used in the U-SENS™ assay because these volatile products increase the CD86 basal level. The use of a surfactant rinsed with water is recommended.

Preparations

Media and Endpoint Assay Solutions

Fetal calf serum (FCS) inactivation

The fetal calf serum (FCS) is taken out of the freezer (-18 to -22°C). The FCS is allowed to thaw at room temperature, while a water bath is pre-warmed (temperature set to 56°C).
As soon as it is thawed, the FCS is placed in the 56°C water bath and the water temperature is allowed to equilibrate back to 56°C. The FCS is then incubated 30 min in the water bath at 56°C.
The inactivated FCS is taken out of the water bath and allowed to cool down to room temperature.
The inactivated FCS is aliquoted if and as required and frozen or stored in the fridge (2 to 8°C) for maximum one month (sterility required). As from now, unless otherwise mentioned, the FCS used is inactivated.

Complete medium

RPMI-1640 containing 10% heat inactivated FCS, 2 mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. After mixing all ingredients, the complete medium is filtered through a 0.22 µm filtration unit. The complete medium can be used during 15 days (sterility required). The non-complemented medium is called “RPMI-1640”.

Freezing medium

RPMI-1640 containing 20% heat inactivated FCS, 10% DMSO, 2mM L-Glutamine, 100U/mL penicillin and 100 µg/mL streptomycin. The required volume of fresh freezing medium is prepared extemporaneously before the freezing by adding 10% of FCS and 10% of DMSO to the complete medium prepared as indicated above. It is placed on ice until used (sterility required).

Staining buffer

PBS + 5% FCS (stored in the fridge, can be used during 1 month after its preparation, sterility not required).

Propidium iodide (PI) stock solution

50 µg/mL propidium iodide in 0.1% citrate buffer (can be used during 6 month stored in the fridge and protected from light, sterility not required).

Test Compounds

Solubilization

Chemicals are dissolved by adding the proper volume of vehicle (pre-heated at 37°C) into the glass vial/tube containing a pre-weighed amount of chemical. The closed vial/tube is placed for 15 min in a 37°C water bath and further agitated at room temperature on a roller plate for 15 min.

Nota bene. It has been reported that materials where the media comes into contact - tubes, stoppers etc. - may release chemicals into the media which might themselves influence the outcome of the test (e.g. tubes were shown to have deleterious effects on cells). Recommended tubes to use are:
- 6 or 15 mL glass hemolysis tubes + winged plug for weighing/stock solutions
- 5 mL sterile polystyrene hemolysis tubes + winged plug for dilutions.
For the same reasons, avoid caps containing latex/rubber or a joint of a similar chemical nature.

Solubility assessment

The solubility of the chemicals is assessed visually (particles, drops, cloudiness, non-miscible phases etc.). Complete medium or DMSO are the vehicles used in this assay. The vehicle has to be chosen from this list in this order of preference.

- Pre-heat the vehicles at 37°C.
- Prepare 2 benchmark tubes (complete medium and DMSO into 2 glasses vials/tubes), treat them like the stock solutions and use them as benchmark while visually assessing the solubility of the chemical by comparing control vs. stock solution in the same vehicle.
- Attempt to dissolve the chemical in the following vehicles, at the following concentrations:
    - Complete medium at 50 mg/mL
    - DMSO at 50 mg/mL
- If the chemical is soluble in complete medium (solubilised or stably dispersed), select the complete medium as solvent/vehicle independently of its solubility in DMSO. If the chemical is soluble only in DMSO (solubilised or stably dispersed), select this vehicle. If no suitable solvent/vehicle (neither complete medium nor DMSO) is found at this stage, select complete medium by default.

See procedures for *Solubility assessment* in **Annex 1** .

Nota bene*. Do not sonicate or heat > 37°C the stock solutions unless otherwise indicated by the supplier. Choose complete medium if DMSO is known to be incompatible with the chemical.*

Dilutions

- Pre-heat the solvent/vehicles at 37°C.
- Prepare a fresh stock solution of the chemical for each run, using the vehicle identified in the " *Solubility Assessment* " section on page 9.
- The concentration of the stock solution is 0.4 mg/mL in complete medium or 50 mg/mL in DMSO.
- The concentration of the stock solution of a chemical is the same for all runs.
- Weigh a precise mass and determine the proper volume to obtain the required stock solution concentration (e.g. for 0.4 mg/mL stock solution in complete medium weigh 2.4 mg and add 6 mL in 12 mL haemolysis glass tube).

Dilutions of chemicals and solvent/vehicle controls are performed to insure a constant percentage of the solvent/vehicle in the final volume of cell suspension in the well (0.4% for DMSO).

The final concentrations used vary between the runs according to the rules explained in paragraph " *Concentrations selection for the successive runs* " on page 13. However, in all the cases, the following guidelines should be followed (see illustrations for *dilutions procedures* in **Annex 2**):

- For complete medium soluble chemicals: prepare 2x concentrated solutions in complete medium (either by serial dilutions or by diluting the stock solution/intermediate solution, whichever is the most accurate)
- For DMSO soluble chemicals
    - Prepare 250x concentrated solutions in DMSO (either by serial dilutions or by diluting the stock solution/intermediate solution, whichever is the most accurate).
    - From these, prepare 2x concentrated solutions in complete medium by diluting each 1:125 (8 µL DMSO solution + 992 µL complete medium).
    - Prepare a DMSO vehicle control (8 µL DMSO + 992 µL complete medium).

Positive Control(s)

The positive control chemical is the **picrylsulfonic acid** (2,4,6-Trinitro-benzene-sulfonic acid, TNBS, CASRN 2508-19-2, ≥ 99% purity) at 50 µg/mL solubilised in complete medium.

TNBS is provided as a 1 M solution (i.e. 293 mg/mL). This solution is to be aliquoted as 10 µL aliquots to be stored in the freezer. On the treatment day, 283 µL complete medium is added to one frozen aliquot in order to obtain a 10 mg/mL solution.

- TNBS used as positive control (final concentration 50 µg/mL): on the treatment day, dilute it 1:100 (10 µL TNBS + 990 µL complete medium) in order to obtain a 0.1 mg/mL stock solution.
- If complete medium is also used as a test item (see QC run in section "*Acceptance criteria*" on page19, final concentration max 100 µg/mL): on the treatment day, dilute it 1:50 (20 µL TNBS + 980 µL complete medium) in order to obtain a 0.2 mg/mL stock solution.

The control stock solutions are placed for 15 min in a 37°C water bath and further agitated at room temperature on a roller plate for 15 min at the same time as the chemicals to evaluate.

Negative Control(s)

The negative control chemical is the **lactic acid** (LA, CASRN 50-21-5, ≥ 85% purity) at 200 µg/mL solubilised in complete medium (final concentration).
Lactic acid is provided as neat liquid lactic acid to be stored at room temperature. Based on the density, its concentration is considered to be 1209 mg/mL
On the treatment day, a 10 mg/mL solution of LA is prepared (e.g. 8.3 µL lactic acid are added to 992 µL complete medium). This solution is diluted 1:25 (40 µL lactic acid + 960 µL complete medium) in order to obtain a 0.4 mg/mL stock solution.

Method

Routine Culture Procedure

Cells thawing
Cells are thawed any day (but typically on Friday or Tuesday) that allows a passage of the cells to begin 3 days later and compatible with the maintenance of cell culture procedure described below (typically on Monday or Friday, respectively).
A vial is placed in a 37°C water bath for a few seconds until the content melts. Then, the content is transferred in a tube containing 25 mL of pre-warmed complete medium. The tube is centrifuged (5 min, 400 g), the supernatant removed and the cell pellet re-suspended in approximately 1 mL pre-warmed fresh complete medium. The cells are counted (using trypan blue exclusion in order to check that cell viability is ≥ 85%), diluted at 1.5 x 10^5 cells/mL and placed in a culture flask in the incubator for 3 days until next passage.

Cell banks
The human histiocytic lymphoma U937 cells (clone CRL1593.2) are recommended for performing the U-SENS™ method.
The cell bank vial (clone CRL1593.2) is thawed and cells are cultured for 1.5 to 2 weeks. Cells are then frozen as master cell bank vials.
Cells from a vial of the master cell bank are thawed and cultured for 1.5 to 2 weeks. Cells are frozen as working cell bank vials (~20 vials).
Cells from a vial of the working cell bank are thawed, cultured (minimum 1 week) and used for the runs. From this point, do not culture the cells more than 6 weeks (7 weeks after thawing, i.e. 21 passages).
Other U937 cellular clones may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results.

Cells freezing
All media and vials are to be kept on ice.
Cells pipetted from the flask are centrifuged (5 min, 400 g), re-suspended in complete medium and counted. The proper volume (corresponding to 10 vials maximum) of cell suspension is distributed in new tubes. These tubes are centrifuged and processed one by one: removing of the supernatant, adding cold freezing medium in order to have 5x10^6 cells/mL, quickly aliquoting (1 mL / vial) and placing in a properly designed freezing box (such as isopropanol box) pre-cooled in the freezer (> 45 min). The freezing box with the vials is then placed without delay in a -80°C freezer. After 24 h to 48 h, all vials are transferred to a proper liquid nitrogen container or similar.

Cells maintenance and culture
Cells are incubated at 37±2°C, 5±1% CO_2, ≥ 95% humidity.
Cells are passaged at 3x10^5 cells/mL when to be cultured for 2 days (typically on Monday and Wednesday) and at 1.5x10^5 cells/mL when to be cultured for 3 days (typically on Friday): cells are centrifuged (5 min, 400 g), the medium is discarded, the cells are re-suspended in pre-warmed complete medium and counted (using trypan blue exclusion in order to check that cell viability is ≥ 90%), and adjusted to the proper cell concentration.

	Day -3 (Friday)	Day 0 (Monday)	Day 1 (Tuesday)	Day 2 (Wednesday)	Day 3 (Thursday)	Day 4 (Friday)
Culture	Passage 1.5x10^5 c/mL	Passage 3x10^5 c/mL		Passage 3x10^5 c/mL		Passage 1.5x10^5 c/mL

Sizes of the culture flasks :

- 25 cm^2 culture flasks for 8 to 12 mL of cell suspension;
- 75 cm^2 culture flasks for 20 to 30 mL of cell suspension;
- 162 cm^2 culture flasks for 40 to 60 mL of cell suspension;
- 225 cm^2 culture flasks for 80 to 120 mL of cell suspension.

Cytometer maintenance
Each lab should maintain its flow cytometer as recommended by the manufacturer. Maintenance and cleaning (daily and weekly) procedures are to be performed strictly, by competent staff, and documented.

Test Material Exposure Procedures

Cell treatment with chemicals

- On day 0 (typically on Monday), the cells are seeded at $6x10^5$ cells/mL (for use on day 1, typically on Tuesday) or at $3x10^5$ cells/mL (for use on day 2, typically on Wednesday) in complete medium (RT).
- Treatment with the chemicals is performed on day 1 or day 2 (typically on Tuesday or Wednesday). Harvest, staining and reading are performed 45 h (± 3 h) later (on day 3 or 4, typically on Thursday or Friday, respectively).

The following Table summarizes the operations for the pre-culture and the assay:

	Day -3 (Friday)	**Day 0 (Monday)**	**Day 1 (Tuesday)**	**Day 2 (Wednesday)**	**Day 3 (Thursday)**	**Day 4 (Friday)**
Pre-Culture/ Assay	Passage $1.5x10^5$ c/mL	Passage $6x10^5$ c/mL	Treatment start		Harvest and Read	
	Passage $1.5x10^5$ c/mL	Passage $3x10^5$ c/mL		Treatment start		Harvest and Read

Other pre-cultured conditions than those described above may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results, for example by testing the proficiency substances recommended in OECD Test Guidelines.

Chemicals have to be evaluated in at least two independent runs, using cells from a different passage number. Runs performed the same week (as described in the Table above) are not independent because runs performed using cells from the same passage number.

- On the day when the treatment starts (Day 1 or Day 2), cells are centrifuged (5 min, 400 g) and re-suspended in complete medium (pre-heat RT) and counted assessing the viability (trypan blue exclusion). Adjust the cell concentration to $0.5x10^6$ cells/mL.

Nota bene. *If the cell viability is < 90% or if the cell concentration in the flask is more than 2 x 10^6 cells/mL, the cells are not to be used*.

- In a sterile 96-well flat-bottom plate, add:

  100 µL/well of the chemical (2x concentrated) or solvent/vehicle *AND*
  100 µL/well U937 (0.5×10^6 cells/mL)
  The final cell density is 0.25×10^6 cells/mL.

- For each concentration, 2 wells are prepared (one for IgG1 negative control and the other for CD86 staining).
- For the concentrations selection process for the first run and all further runs, please refer to paragraph "*Concentrations selection for the successive runs*" on page 13.
- If none of the chemicals in a single assay is soluble in complete medium, it is still required to include the complete medium controls as a general run control.
- Three pairs (6 wells) of complete medium untreated control, solvent/vehicle control, negative and positive controls are prepared for each plate of each run.
- Systematically cover each 96-well plate with a sealing tape. The sealing tape is used to prevent possible collateral effects of volatile chemicals.
- Cells ± chemicals at the appropriate concentrations are cultured for 45±3 h.

See suggested *plate template* in **Annex 3**.

Nota bene.
-It is required to prepare 3 pairs (6 wells) of vehicle control wells allowing for identification of an outlier if necessary.
-It is required to place the DMSO solvent/vehicle control wells just before and after the chemicals solubilised in DMSO.
-In cases where more than 1 plate is prepared for a run to accommodate the testing needs, do not split concentrations of the same chemical on 2 different plates, and do not separate a chemical from its solvent/vehicle control on 2 different plates.
-It is required to place a complete medium vehicle control well at the end of the plate to evaluate possible drift of CD86 signal (especially important when using a plate sampler equipped cytometer, see " *Flow cytometry acquisition settings*" section on page 16).
-Limiting the number of utilized wells to 80 wells/plate should prevent any drift in CD86 expression if using a plate auto-sampler. If the samples are going to be analyzed manually tube by tube, they are to be kept on ice throughout the flow cytometry acquisition before being put through the cytometer. They are therefore not subject to plate drift and it is not necessary to limit the number of wells used to 80. So, if required, the full 96-well plate can be used.
-In cases where a drift is observed (see "*Run acceptance criteria*" on page 18), the results of the run are to be discarded. For all further runs use half plates including all required controls, always refraining from splitting concentrations of a chemical on 2 different plates.
-Cooling the plate support of the plate auto-sampler using an ice pack might be considered in order to prevent the drift effect.

Concentrations selection for the first run

Because a dose finding assay is not conducted, for the first run, 6 final concentrations should be tested (1, 10, 20, 50, 100 and 200 µg/mL) into the corresponding solvent/vehicle either in complete medium or in 0.4% DMSO in complete medium.

Concentrations selection for the successive runs

For the subsequent runs, starting from the 0.4 mg/mL in complete medium or 50 mg/mL in DMSO, solutions of the test chemicals, at least 4 working solutions (at least 4 concentrations), are prepared using the corresponding solvent/vehicle.The concentrations for the second run (run 2) are chosen based on the results of the first run. The concentrations for any further run are chosen based on the individual results of all previous runs (See examples of *Concentrations selection* in **Annex 4**).

Objectives

- Show and/or confirm concentration-dependency of CD86 increase at non-toxic concentrations *OR*
- Show and/or confirm the absence of CD86 increase up to the maximum non-toxic concentration
- Confirm cytotoxicity, if any.

Authorized concentrations

Because too narrow steps between concentrations could jeopardize the concentration effect measurement, it is required to stick to concentrations among the following positive list.

The authorized usable final concentrations (µg/mL) are: 1 – 2 – 3 – 4 – 5 – 7.5 – 10 – 12.5 –15 –20 – 25 – 30 – 35 – 40 – 45 – 50 –60 – 70 – 80 – 90 – 100 – 120 – 140 – 160 – 180 – 200.

The maximum final concentration is 200 µg/mL.
In the case of a CD86 positive value at 1 µg/mL, then 0.1 µg/mL is evaluated in order to find the concentration of the test chemical that does not induce CD86 above the positive threshold.

Minimum requirements

- Bearing in mind the objectives stated above, the following rules should be applied
- As many concentrations as possible (at least 2) must be common with the previous test(s), for comparison purposes;
- Confirm (reproduce) the highest non cytotoxic CD86-negative concentration, if any;
- Confirm all non-cytotoxic CD86-positive concentrations, if any;
- Confirm the lowest cytotoxic concentration, if any;
- In order to investigate the concentration-response of CD86, any other required concentrations are to be chosen evenly spread between the EC150 (or the highest negative non cytotoxic concentration) and the CV70 (or the highest concentration allowed by the solubility assessment).
  See **Annex 7** for the calculation of the EC150 and CV70 values.
- If the first two runs have very different patterns (viability and/or CD86), it is recommended to reproduce as many common concentrations as possible from the first two runs and not focus too much on a specific area (e.g. in cases where there are solubility issues).
- In the case where a decreasing viability or color interference or solubility interference could prevent the concentration-response, it might be necessary to investigate new concentrations among the positive concentrations.
- One should not perform more than 5 independent runs per chemical (including 4 valid runs and maximum 1 invalid run).

Nota bene. If 2 consecutive independent runs and/or 2 independent runs for a given chemical are invalidated, the testing is stopped. An investigation has to be performed in order to understand and correct the underlying problem. Starting with newly thawed U937 cells is suggested. Moreover, performing a QC run (see section "*Acceptance criteria*" on page 18) might be one of the ways to gather informative data.

Endpoint Measurement

Cell staining

- *Solubility interference*: At 45±3 h, the aspect of the cells is checked and a precipitate evaluation is done under the microscope. If crystals are observed at this stage (see example in **Annex 5**), it will be documented in the study report.
- Then all cells are transferred with a multichannel pipette into V-shaped microtiter plates (keeping the same plate template, line by line, changing the tips, 2 up and down pipetting in tilted plate before transferring the 200 µL) and centrifuged (200 g, 5 min, 4°C*). Supernatants are discarded by inverting the plate. The plates are tapped gently on a paper towel, briefly vortexed (2 to 3 sec.) to break the pellet before adding any buffer and the cells washed once with 100 µL/well of ice-cold staining buffer (approximately 4°C). For the wash, the 100 µL of staining buffer are distributed line by line, without changing the tips if they don't touch the pellets.

  ** It is not required to pre-cool the centrifuge at 4°C. The temperature will go down during centrifugation.*

- During this last centrifugation, 5 µL (e.g. 0.25 µg) of the FITC-labelled mouse IgG1 (isotype) or CD86 antibodies / well are distributed on another plate. For each chemical treatment, 1 well is used for IgG1 staining and the other for CD86 staining (see suggested template in paragraph "*Cell treatment with chemicals*" section under "Test material exposure procedures" on page 12).

Nota bene. Users may consider titrating the antibodies in their own laboratory's conditions to define the best concentration for use. Other detection system e.g., fluorochrome-tagged anti-CD86 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances recommended in OECD Test Guidelines.

- Then, cells are re-suspended in 100 µL ice-cold staining buffer (distributed line by line, without changing the tips if they don't touch the pellets) and transferred (line by line, changing the tips, 1 up and down pipetting in laid flat plate before transferring the 100 µL) into the other V-shaped microtiter plate containing the antibodies.
- Cells are incubated on ice, protected from light for 30 min.
- Cells are then centrifuged (200 g, 5 min, 4°C), washed twice in 100 µL ice-cold staining buffer and once in 100 µL ice-cold PBS. For the washes, the 100 µL of staining buffer or PBS are distributed line by line, without changing the tips if they don't touch the pellets.
- If samples are to be analyzed manually tube by tube, resuspend the cells in a final volume of 125 µL of ice-cold PBS and transfer them into 1.4 mL microtubes* (keep the microtubes disposition in the rack exactly as the samples in the plate template, line by line, changing the tips, 1 up and down pipetting in laid flat plate before transferring the 200 µL).

OR

- If samples are to be analyzed automatically using a plate auto-sampler, re-suspend the cells in a final volume of 50 µL of ice-cold PBS (again keeping the same plate template, line by line, without changing the tips if they don't touch the pellets).
- Place the tube rack(s) or the plate(s) on ice, protected from light (e.g. using foil).

Nota bene.
o At each washing step, do not re-suspend cells by repeated pipetting but by briefly vortexing the plate containing the cell pellets after discarding the liquid.
o Make sure to keep all buffers and cells on ice during staining and washing steps.

Flow cytometry settings and analysis

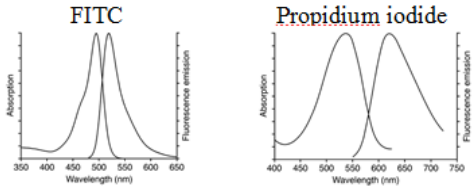
Nota bene. The cytometry template, settings and gates are to be prepared in advance with untreated U937 cells stained with IgG1 and propidium iodide (PI).The settings of this preparatory run should be used unchanged in all further runs of the study. During each plate acquisition, make sure to check the RPMI1 cells are in the right place.

In case of drastic changes on the cytometer (e.g. following maintenance operations), re-prepare the template, settings and gates described above.

- If samples are to be analyzed manually tube by tube:
    - Dilute the PI stock solution to 8 µg/mL in cold PBS. Add 75 µL of this PI solution (8 µg/mL) to each tube of only the first 2 columns of the microtubes rack.
    - Place each microtube in a proper 5 mL cytometer tube, vortex and insert it into the cytometer. Acquire successively each pair of IgG1 and CD86 tubes.
    - Once the first 2 columns have been acquired, proceed to the next 2 columns with the addition of PI and so forth until all samples have been processed.
    - Reminder: all tubes are to be kept on ice and protected from light all through the time between the end of the staining and their analysis in the cytometer.
- If samples are to be analyzed automatically using a plate auto-sampler:
    - Dilute the PI stock solution to 8 µg/mL in cold PBS. Add 30 µL of this PI solution (8 µg/mL) to each well of the plate and immediately insert the plate into the plate sampler.
    - In cases where additional plates have been prepared, process them the same way once the first one has been fully acquired on the cytometer.
    - Reminder: the plate(s) has(ve) to be kept on ice and protected from light until the PI is added.
- Whichever way is used to analyze the samples, the final PI concentration is 3 µg/mL.

Flow cytometry acquisition settings

- Cells are displayed within a size (FSC) and granularity (SSC) dot plot set to log scale in order to clearly identify the population in a first gate R1 and eliminate the debris.
- The cytometer is set so that a total of 10,000 cells in gate R1 are acquired for each well.
- When the cell viability is low, up to 20,000 cells including dead cells could be acquired. Alternatively, data can be acquired for one minute after the initiation of the analysis.
- If the samples are to be analyzed manually, the acquisition can be stopped manually after 40 sec for cytotoxic chemicals if the acquisition time for the majority (at least 3/4) of the first complete medium samples are less than 20 sec.
- The FITC acquisition channel (labeled from now on as FL1) should be set for the optimal detection of the FITC fluorescence signal, and the PI acquisition channel (labeled from now on as FL3) should be set for the optimal detection of DNA-bound PI fluorescence signal.



- If the samples are to be analyzed manually:
    - Do not use a tube loader auto-sampler. Automated tube samplers are slow enough to allow temperature of cells to increase and lead to drift in CD86 expression.
    - Select the fastest acquisition settings. In that case, it is foreseen that all 80 tubes of a plate are read in about 45 min.
- If using a plate sampler equipped flow cytometer: the small liquid volume in each well favors the warming up of the cells thus leading to CD86 signal loss (well-known recycling of surface antigens phenomenon), i.e. a drift in basal CD86 expression measurement.
    - Make sure the room is properly air conditioned to avoid heating up of the acquisition chamber. It has been measured that the temperature can rise above 30°C within the small space where the plate is set, when the surrounding room temperature stays at around 22°C.
    - Cooling the plate support of the plate auto-sampler using an ice pack before analyzing plates might be considered in order to prevent the drift effect.
    - Select fast acquisition settings so that a plate is read in about 15 min.

Flow cytometry analysis settings

- Cells from the same R1 gate are displayed within a FL3 / SSC dot plot (scale fixed for all the run). Viable cells are delineated by placing a second gate R2 selecting the population of PI-negative cells (FL3 channel). In case of population shift (coloring chemical), adjust the R2 gate as required for the concerned concentration(s).
- Percentage of FL1-positive cells is then measured among these viable cells gated on R2 (within R1).
- Cell surface expression of CD86 is analyzed in a FL1 / SSC dot plot gated on viable cells (R2). For the complete medium / IgG1 wells, the analysis marker is set close to the main population so that all three, if possible, or at least two of the complete medium controls have IgG1 within the target zone = 0.6 to 0.9%. In case this is not achievable, set the analysis marker so that one complete medium control has IgG1 within the target zone = 0.6 to 0.9% and another complete medium control has IgG1 within the target zone = 0.9% to 1.5%. In case this is still not achievable, the IgG1 data are too widely spread and the run has to be discarded. Results from any complete medium well with IgG1 < 0.6% or > 1.5% will be discarded at the data exploitation step.
- Then the percentage of IgG1+ in one well and of CD86+ cells in the second well is measuredfor each condition, without moving the analysis marker. See an *example of flow cytometer plots* in **Annex 6**.

Acquisition and storage of the raw data

- All data shall be archived.
- All calculations shall be done on exact values and not on visible values (on screen data such as rounded up values).
- All flow cytometry values are to be exported and archived as a file (e. g. stat text file for BD CellQuest software).
- The analysis plots/stats should be kept for each concentration, ideally including an electronic file.
- For each condition, if the "% viability mean" is < 70%, no IgG1 or CD86 percentage of positive cells is recorded (only the propidium iodide / viability data are recorded in that case).

Stimulation indexes calculation

For each viable condition ("% viability mean" ≥ 70%), the % of IgG1 positive cells is subtracted to the % of CD86 positive cells. And results are expressed as **stimulation indexes (S.I.)** calculated as follows:

$$\textbf{S.I.} = \frac{\%\ \text{of CD86}^+\ \textit{treated cells} - \%\ \text{of IgG1}^+\ \textit{treated cells}}{\%\ \text{of CD86}^+\ \textit{control cells} - \%\ \text{of IgG1}^+\ \textit{control cells}} \times 100$$

% of IgG1+ untreated control cells:
referred to as percentage of FL1-positive IgG1 cells defined with the analysis marker (accepted range of ≥ 0.6% and < 1.5%) among the viable untreated cells.
% of IgG1+/CD86+ control/treated cells:
referred to as percentage of FL1-positive IgG1/CD86 cells measured without moving the analysis marker among the viable control/treated cells.

The percentage of control cells (solvent/vehicle, i.e. complete medium or DMSO) is the mean of the 3 values obtained, unless one (outlier) is clearly out of the range of the other two (see below).

Outlier to discard

- If IgG1 level of a complete medium control is < 0.6% or > 1.5% positive cells, it is discarded.
- For each solvent/vehicle control (complete medium or DMSO), average the CD86-IgG1 % positive cells of the 3 wells. If one control is > 25% above or below the mean, it is an outlier to be discarded (if more than one outlier is identified, only discard the one furthest away from the mean).

Color interference

Color interference is defined as a shift of the FITC-labelled IgG1 dot-plot (IgG1 FL1 Geo Mean S.I. ≥ 150%). There is color interference if the *X* GeoMean fluorescence of the IgG1 well is 50 % higher than the *X* GeoMean fluorescence of the vehicle control IgG1 well (IgG1 X GeoMean S.I. ≥ 150).

For FL1 fluorescence:

If a color interference is preventing from identifying viable versus dead cells (propidium iodide channel), a comment should be added to state that from such concentration onward it was not possible to perform the evaluation due to color interference. No viability nor IgG1/CD86 data are to be recorded in that case.

$$\textbf{IgG1 FL1 x GeoMean S.I.} = \frac{\textit{treated cells}\ \text{IgG1 FL1 x}\ \textit{GeoMean}}{\textit{vehicle control cells}\ \text{IgG1 FL1 x}\ \textit{GeoMean}} \times 100$$

If a color interference is preventing from identifying viable versus dead cells (propidium iodide channel), a comment should be added to state that from such concentration onward it was not possible to perform the evaluation due to color interference. No viability nor IgG1/CD86 data are to be recorded in that case.

Acceptance Criteria

Cells acceptance criteria

- Cell viability at thawing: cell viability measured by trypan blue exclusion should be ≥ 85% (not be applied to the original cells bank vial). If not, the cells have to be discarded and another vial has to be thawed.
- Cell viability during maintenance: cell viability measured by trypan blue exclusion should be ≥ 90% (not to be applied at the first passage after thawing). If not, the cells have to be discarded and a new vial has to be thawed.
- Cell growth during maintenance: the cells should not grow more than 2 x 10^6 cells/mL. If this happens, start a new culture from the working cell bank.

Run quality control/acceptance criteria

If one or more of the following acceptance criteria is not met, the run is invalidated and should be repeated.

Nota bene. For the following criteria, the "corrected %$CD86^+$ value" is defined as the percentage of $CD86^+$ cells minus the percentage of $IgG1^+$ cells (%$CD86^+$-%$IgG1^+$).

- At least two complete medium controls have IgG1 within the target zone = 0.6% to 1.5% (preferred target zone = 0.6% to 0.9%).
- After discarding one complete medium control outlier if required, the corrected %CD86+ of the 2 remaining complete medium controls should be ≤ 25% above or below their mean.
- The average viability of the untreated cells (complete medium) should be > 90 %.
- There should be no drift in CD86 expression. Drift is defined as the following:
    - The corrected %CD86+ complete medium 3 control cells value is less than 50% of mean of the corrected %CD86+ complete medium 1 & complete medium 2 values.

    AND

    - The corrected %CD86+ LA3 value is less than 50% of mean of the corrected %CD86+ LA 1 & LA 2 values.
- The average of complete medium untreated cells corrected CD86+ values expression should be ≥ 2% and ≤ 25%.
- Positive control TNBS: at least 2 out of 3 TNBS wells should be positive (CD86 SI ≥ 150).
- Negative control LA: at least 2 out of 3 LA wells should be negative (CD86 SI < 150).

Chemicals quality control/acceptance criteria

If one or more of the following acceptance criteria is not met, all data on chemicals solubilized in DMSO are discarded and the run for these chemicals should be repeated.

- The average viability of the DMSO solvent/vehicle control should be > 90 %.
- After discarding one DMSO control outlier if required, the corrected %CD86+ of the 2 remaining DMSO controls should be ≤ 25% above or below their mean.
- After discarding outliers if required, the mean of DMSO solvent/vehicle control CD86 S.I. should be < 250 %.
- If solubility issues are observed on the FSC / SSC dot plots (see examples in **Annex 5** and **Downloads** section of **U-SENS™ protocol** for more information), it will be reported.

QC runs

Before their first use, all antibodies, cells and FCS must undergo a quality control (QC) process in each laboratory. Each new pair of antibodies (any new batch of either CD86 or the related IgG1 control), new batch of U937 cells or new batch of FCS is to be tested in 2 validated following QC runs (2 different weeks).
A QC run includes the vehicle controls complete medium and DMSO, the negative control LA and the positive control TNBS, the LA and TNBS used as test items (see *QC plate template* in **Annex 3**).
The QC run has to be analyzed and the data reported as any testing run.
The pair of antibodies, the new batch of cells or the new batch of FCS is validated if for 2 valid following runs:

- The average of all complete medium CD86-IgG1 % positive cells is ≥ 2% and ≤ 25%
- TNBS is considered POSITIVE by the U-SENS™ prediction model (see **Downloads** section of **U-SENS™ protocol** for more information). For the QC run only, the positive response of TNBS should be concentration dependent. There is concentration dependency (or concentration response) when a positive concentration (CD86-IgG1 S.I. ≥ 150) is followed by a concentration with a higher CD86-IgG1 S.I.
- LA is considered NEGATIVE by the U-SENS™ prediction model.

Data Analysis

Data should be reported in files: one separate file for each run (e.g. Excel, see **Annex 9** for further details) and one single general summary file (e.g. U-SENS™ predictor application- see **Downloads** section of **U-SENS™ protocol** for more information).

- *Run file:* should include the filled plate(s) template, the exported flow cytometry results, the codes, solvent/vehicle and concentrations for each test item and control, the required calculated values (S.I.) and means with identification of any outlier or invalidated value and any solubility issue if applicable.

- *Summary file*: should include the run reference of the individual runs performed for each test item, the proper individual run values, all comments related to the test items, the individual run conclusions, the U-SENS™ final classification, the calculated individual EC150 and CV70 values (whenever possible), the calculated viability and CD86 means, the overall EC150 and CV70 values (whenever possible) and the appropriates comments on the interferences if observed (color interference, solubility or reproducibility issues).

Prediction Model

For CD86 expression measurement, each test chemical is tested in at least four concentrations and in at least two independent runs (performed on a different day) to derive a single prediction (NEGATIVE or POSITIVE). See **Annex 8** for details regarding prediction model.

The individual conclusion of an U-SENS™ run is considered NEGATIVE (hereinafter also referred to as N) if the S.I. of CD86 is less than 150% at all non-cytotoxic concentrations (cell viability ≥ 70%) and if no interference is observed (cytotoxicity, solubility or colour regardless of the non-cytotoxic concentrations at which the interference is detected).
In all other cases: S.I. of CD86 higher or equal to 150% or interferences observed, the individual conclusion of an U-SENS™ run is considered POSITIVE (hereinafter also referred to as P).

- An U-SENS™ prediction is considered NEGATIVE if at least two independent runs are N.
  If the first two runs are both N, the U-SENS™ prediction is considered NEGATIVE and a third run does not need to be conducted.

- An U-SENS™ prediction is considered POSITIVE if at least two independent runs are P.
  If the first two runs are both P, the U-SENS™ prediction is considered POSITIVE and a third run does not need to be conducted.

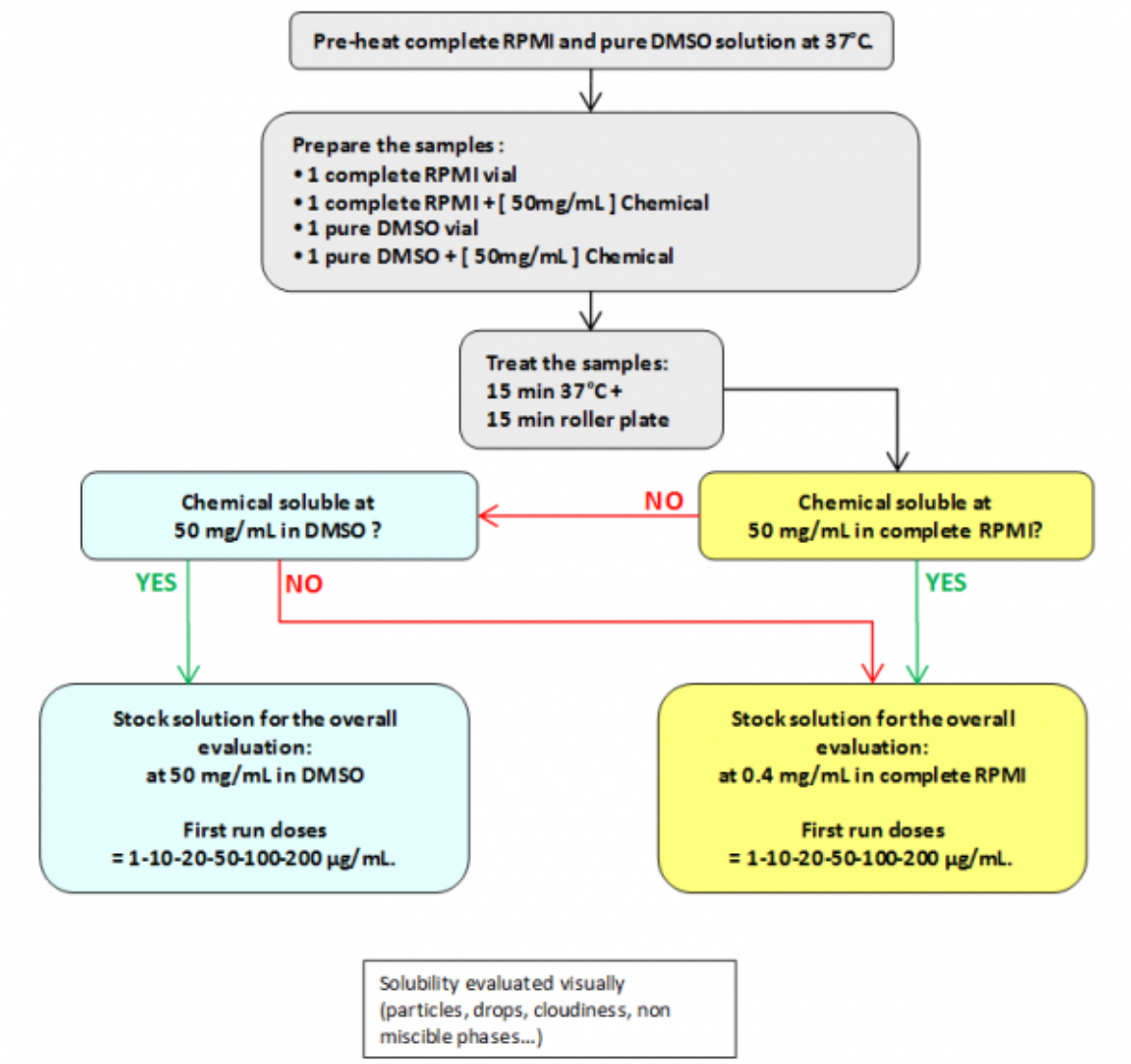
- Because a dose finding assay is not conducted, there is an exception if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only.
  The run is then considered to be concluded NOT CONCLUSIVE (NC), and additional concentrations (between the highest non cytotoxicity concentration and the lowest cytotoxicity concentration) should be tested in additional runs.
  In case a run is identified as NC, at least 2 additional runs should be conducted, and a fourth run in case runs 2 and 3 are not concordant (N and/or P independently).
  Follow up runs will be considered positive even if only one non cytotoxic concentration gives a CD86 equal or above 150%, since the concentration setting has been adjusted for the specific test chemical.
  The final prediction will be based on the majority result of the three or four individual runs (i.e. 2 out of 3 or 2 out of 4).

The U-SENS™ assay is applicable to all mono-substances or mixtures that are soluble in the aqueous testing conditions and compatible with flow cytometry analysis, except membrane disrupting substances (like surfactants). False negatives cannot be excluded with poor-water soluble substances (like some polymers or vegetal extracts).
Finally, results should be interpreted with care for substances that may interfere with CD86 induction pathways due to their own biological activity (like some topical drugs or some vegetal extracts). Besides, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available in the public domain on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the test method to a specific category of mixtures, the U-SENS™ assay should not be used for that specific category of mixtures.

The U-SENS™ test method is foreseen to be part of a battery or integrated testing approach for replacement of the existing *in vivo* assays for hazard identification (contact skin sensitizer vs. non sensitizer). It is also foreseen to be a part of an integrated approach which will be able to fully replace the *in vivo* test methods (i.e. LLNA, Buehler and Magnusson & Kligman) to ultimately make skin sensitization risk assessment decisions possible without animal testing.

Annexes

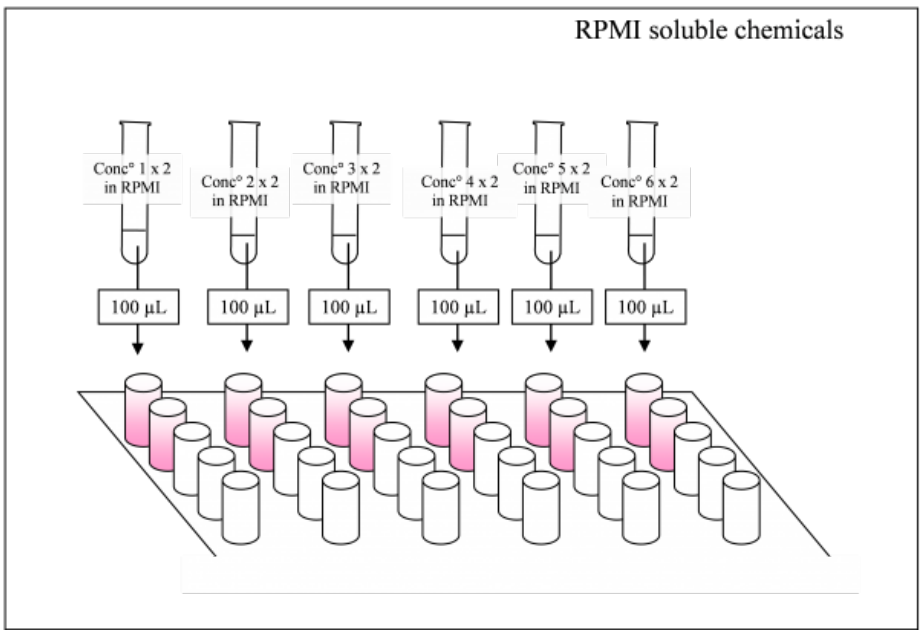
ANNEX 1: Solubility assessment

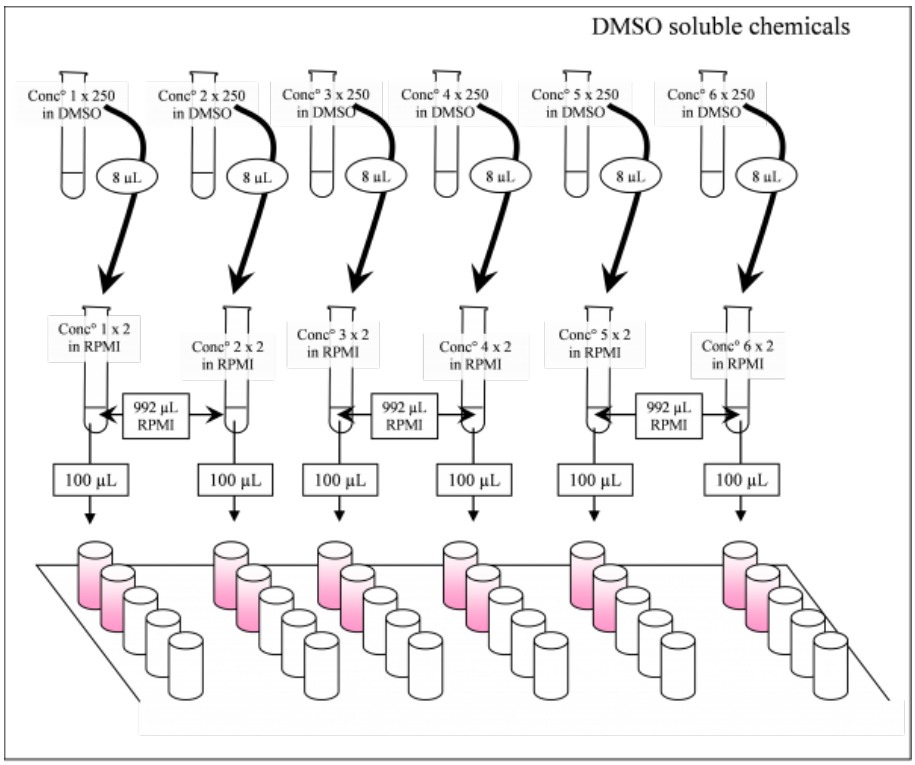


Nota Bene: The complete RPMI referred to the complete medium (RPMI-1640 containing 10% heat inactivated FCS, 2mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin).

ANNEX 2: Illustrations for the dilutions procedures

The Conc° 1 to Conc° 6 represent the final concentrations required in the 96 well plate.





Nota Bene: RPMI referred to the complete medium (RPMI-1640 containing 10% heat inactivated FCS, 2mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin).

ANNEX 3: Plate templates

Suggested plate template for cell treatment with chemicals :

		IgG1	CD86	IgG1	CD86	IgG1	CD86	IgG1	CD86	IgG1	CD86	
	1	2	3	4	5	6	7	8	9	10	11	12
A		*001* **RPMI 1**	*002* **RPMI 1**	*017* Chem 1 Conc 3	*018* Chem 1 Conc 3	*033* Chem 2 Conc 5	*034* Chem 2 Conc 5	*049*	*050* 0 0	*065*	*066* 0 0	
B		*003* **RPMI 2**	*004* **RPMI 2**	*019* Chem 1 Conc 4	*020* Chem 1 Conc 4	*035* Chem 2 Conc 6	*036* Chem 2 Conc 6	*051*	*052* 0 0	*067*	*068* 0 0	
C		*005* LA 1 (200)	*006* LA 1 (200)	*021* Chem 1 Conc 5	*022* Chem 1 Conc 5	*037* DMSO 1	*038* DMSO 1	*053* DMSO 3	*054* DMSO 3	*069*	*070* 0 0	
D		*007* LA 2 (200)	*008* LA 2 (200)	*023* Chem 1 Conc 6	*024* Chem 1 Conc 6	*039* DMSO 2	*040* DMSO 2	*055*	*056* 0 0	*071*	*072* 0 0	
E		*009* TNBS 1 (50)	*010* TNBS 1 (50)	*025* Chem 2 Conc 1	*026* Chem 2 Conc 1	*041*	*042* 0 0	*057*	*058* 0 0	*073*	*074* 0 0	
F		*011* TNBS 2 (50)	*012* TNBS 2 (50)	*027* Chem 2 Conc 2	*028* Chem 2 Conc 2	*043*	*044* 0 0	*059*	*060* 0 0	*075* TNBS 3 (50)	*076* TNBS 3 (50)	
G		*013* Chem 1 Conc 1	*014* Chem 1 Conc 1	*029* Chem 2 Conc 3	*030* Chem 2 Conc 3	*045*	*046* 0 0	*061*	*062* 0 0	*077* LA 3 (200)	*078* LA 3 (200)	
H		*015* Chem 1 Conc 2	*016* Chem 1 Conc 2	*031* Chem 2 Conc 4	*032* Chem 2 Conc 4	*047*	*048* 0 0	*063*	*064* 0 0	*079* **RPMI 3**	*080* **RPMI 3**	

Conc 1 is the lowest and Conc 6 is the highest concentration (µg/mL).

QC plate template:

		IgG1	CD86	IgG1	CD86	IgG1	CD86	IgG1	CD86	IgG1	CD86	
	1	2	3	4	5	6	7	8	9	10	11	12
A		*001* **RPMI 1**	*002* **RPMI 1**	*017* TNBS (1)	*018* TNBS (1)	*033* RPMI	*034* RPMI	*049* RPMI	*050* RPMI	*065* LA (20)	*066* LA (20)	
B		*003* **RPMI 2**	*004* **RPMI 2**	*019* TNBS (12.5)	*020* TNBS (12.5)	*035* RPMI	*036* RPMI	*051* RPMI	*052* RPMI	*067* LA (50)	*068* LA (50)	
C		*005* LA 1 (200)	*006* LA 1 (200)	*021* TNBS (25)	*022* TNBS (25)	*037* RPMI	*038* RPMI	*053* RPMI	*054* RPMI	*069* LA (100)	*070* LA (100)	
D		*007* LA 2 (200)	*008* LA 2 (200)	*023* TNBS (50)	*024* TNBS (50)	*039* RPMI	*040* RPMI	*055* RPMI	*056* RPMI	*071* LA (200)	*072* LA (200)	
E		*009* TNBS 1 (50)	*010* TNBS 1 (50)	*025* TNBS (75)	*026* TNBS (75)	*041* RPMI	*042* RPMI	*057* RPMI	*058* RPMI	*073* DMSO 3	*074* DMSO 3	
F		*011* TNBS 2 (50)	*012* TNBS 2 (50)	*027* TNBS (100)	*028* TNBS (100)	*043* RPMI	*044* RPMI	*059* RPMI	*060* RPMI	*075* TNBS 3 (50)	*076* TNBS 3 (50)	
G		*013* DMSO 1	*014* DMSO 1	*029* RPMI	*030* RPMI	*045* RPMI	*046* RPMI	*061* LA (1)	*062* LA (1)	*077* LA 3 (200)	*078* LA 3 (200)	
H		*015* DMSO 2	*016* DMSO 2	*031* RPMI	*032* RPMI	*047* RPMI	*048* RPMI	*063* LA (10)	*064* LA (10)	*079* **RPMI 3**	*080* **RPMI 3**	

All concentrations shown (in parenthesis on the third line) for LA and TNBS are final concentrations in µg/mL.

Nota Bene: RPMI referred to the complete medium (RPMI-1640 containing 10% heat inactivated FCS, 2mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin).

ANNEX 4: Examples of concentrations selection for run 2

The following examples show the application of the rules in order to determine the concentrations that should be used for run 2 (hereafter defined as test 2). Please note that EC150 and CV70 values indicated below are just illustrative values.

µg/ml	test 1 viab	test 1 CD86	test 2 choice
1	OK	neg	
2			
3			
4			
5			
7.5			
10	OK	neg	
12.5			
15			
20	OK	neg	x
25			
30			
35			
40			
45			
50	OK	neg	x
60			
70			
80			
90			
100	OK	neg	x
120			
140			
160			
180			
200	OK	neg	x

µg/ml	test 1 viab	test 1 CD86	test 2 choice
1	OK	neg	
2			
3			
4			
5			
7.5			
10	OK	neg	
12.5			
15			
20	OK	neg	
25			
30			
35			
40			
45			
50	OK	neg	x
60			
70			
80			
90			
100	OK	neg	x
120			x
140			x
160			x (CV70=173)
180			
200	tox		x

µg/ml	test 1 viab	test 1 CD86	test 2 choice
1	OK	neg	x
2			x
3			x
4			x (CV70=4.75)
5			
7.5			
10	tox		x
12.5			
15			
20	tox		
25			
30			
35			
40			
45			
50	tox		
60			
70			
80			
90			
100	tox		
120			
140			
160			
180			
200	tox		

µg/ml	test 1 viab	test 1 CD86	test 2 choice
1	OK	neg	
2			
3			
4			
5			
7.5			
10	OK	neg	
12.5			
15			
20	OK	neg	x
25			
30			
35			
40			
45			
50	OK	pos	x
60			
70			
80			x
90			
100	OK	pos	x
120			
140			x (CV70=156)
160			
180			
200	tox		x

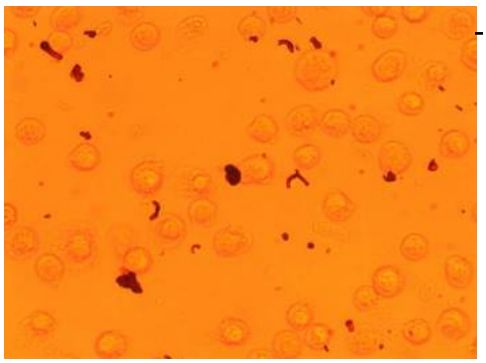
optional dose

µg/ml	test 1 viab	test 1 CD86	test 2 choice
1	OK	neg	
2			
3			
4			
5			
7.5			
10	OK	neg	
12.5			
15			
20	OK	neg	x
25			
30			x (EC150=23)
35			
40			x
45			
50	OK	pos	x
60			
70			x (CV70=88)
80			
90			
100	tox		x
120			
140			
160			
180			
200	tox		

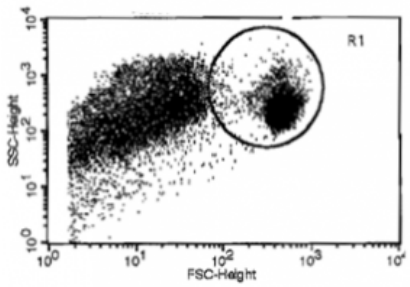
µg/ml	test 1 viab	test 1 CD86	test 2 choice
0.1			x
1	OK	pos	x
2			
3			
4			
5			
7.5			
10	OK	pos	x
12.5			
15			
20	OK	pos	x
25			
30			
35			
40			
45			
50	tox		x
60			
70			
80			
90			
100	tox		
120			
140			
160			
180			
200	tox		

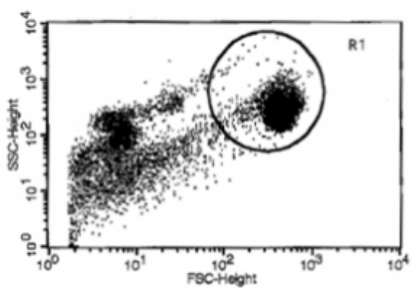
ANNEX 5: Examples of solubility issues observations

Crystals observed at 45±3 h under the microscope:

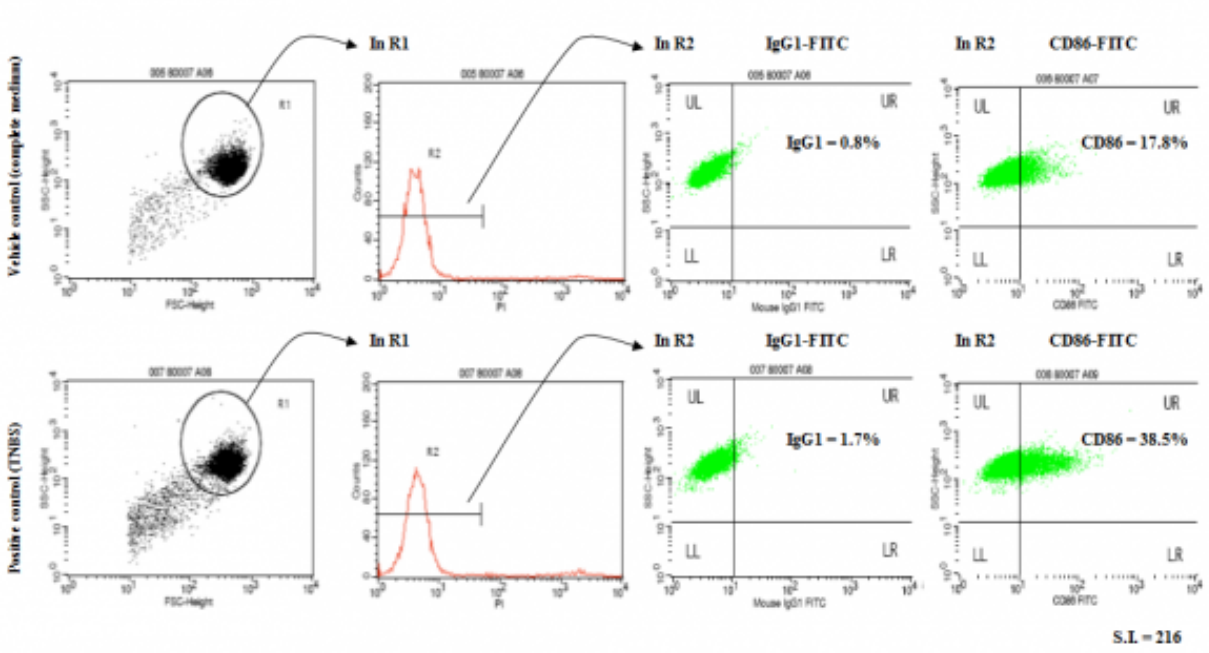


Solubility issues observed on the FSC / SSC dot plots:





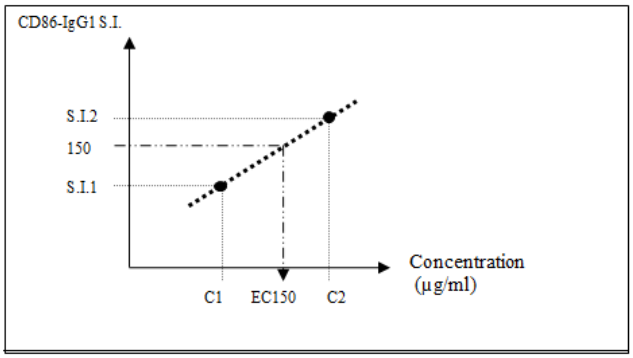
ANNEX 6: Simplified examples of flow cytometer plots



ANNEX 7: EC150 and CV70 values calculation

EC150

Theoretical concentration at which the chemical induces a S.I. of 150, calculated by log-linear interpolation:



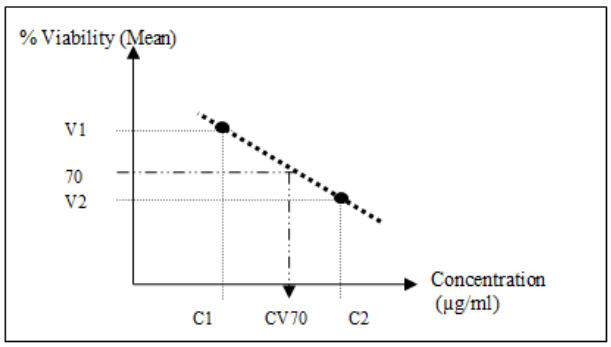
EC150 = C1 + [(150 – S.I.1) / (S.I.2 – S.I.1) * (C2 – C1))

C1 is the highest concentration in µg/mL with a CD86 S.I. < 150% (S.I. 1).

C2 is the lowest concentration in µg/mL with a CD86 S.I. ≥ 150% (S.I. 2).

CV70

Theoretical concentration at which the chemical induces 30% cytotoxicity i.e. a remaining viability of 70%, calculated by loglinear interpolation:



CV70 = C1 + [(V1 - 70) / (V1 – V2) * (C2 – C1)]

V1 is the minimum value of cell viability over 70%

V2 is the maximum value of cell viability below 70%

C1 and C2 are the concentrations showing the value of cell viability V1 and V2 respectively.

Overall EC150 and CV70

To be calculated based on the CD86-IgG1/viability mean values.

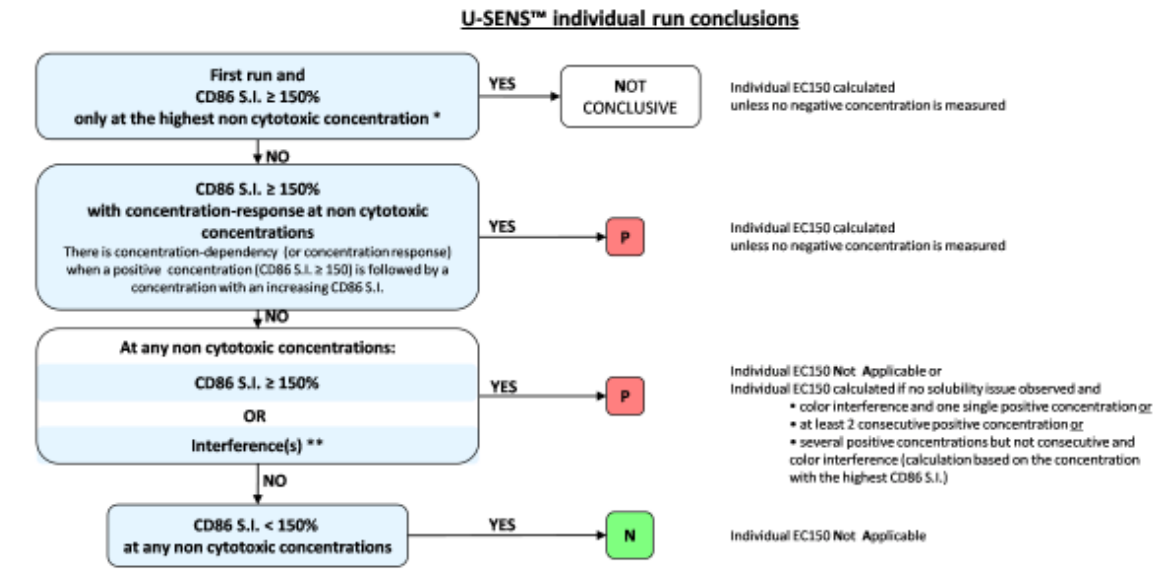
The EC150 and CV70 values (whenever possible) are calculated

- for each run : the individual EC150 and CV70 values are used as tools to investigate the concentration response effect of CD86 increase,

- based on the average viabilities, the overall CV70 is determined,

- based on the average S.I. of CD86 values, the overall EC150 is determined for the test chemical predicted as POSITIVE with the U-SENS™.

ANNEX 8: U-SENS™ Prediction Model



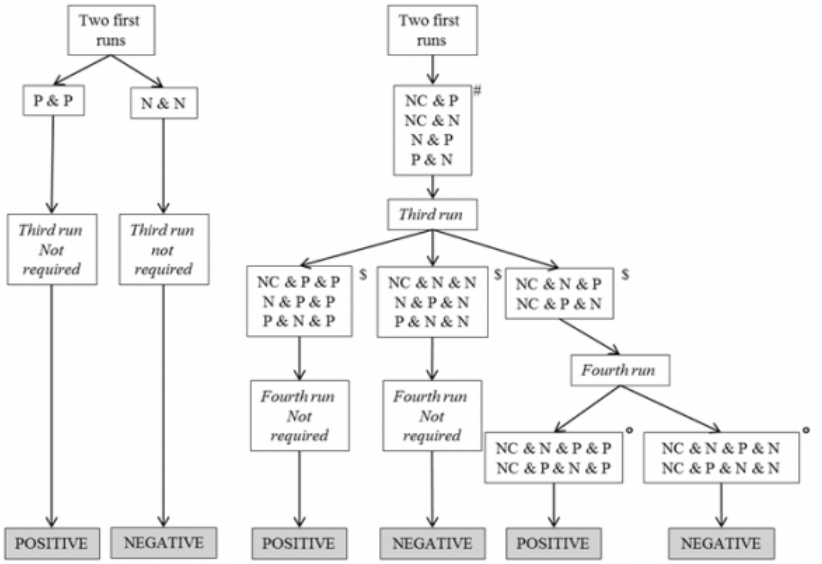
* Non cytotoxic concentration: cell viability ≥ 70%
** Interferences :

- **Solubility** : crystals, drops...observed at 45±3 h under the microscope
- **Color interference**: cell coloring properties observed (IgG1 FL1 Geo Mean S.I. ≥ 150)
- **Cytotoxicity** : highest non cytotoxic concentration < 100 µg/mL due to cytotoxicity

An U-SENS™ prediction is considered

- **NEGATIVE if the individual conclusions are Negative (N) in at least two independent runs,**
- **POSITIVE if the individual conclusions are Positive (P) in at least two independent runs.**

Prediction model used in the U-SENS™ test method.

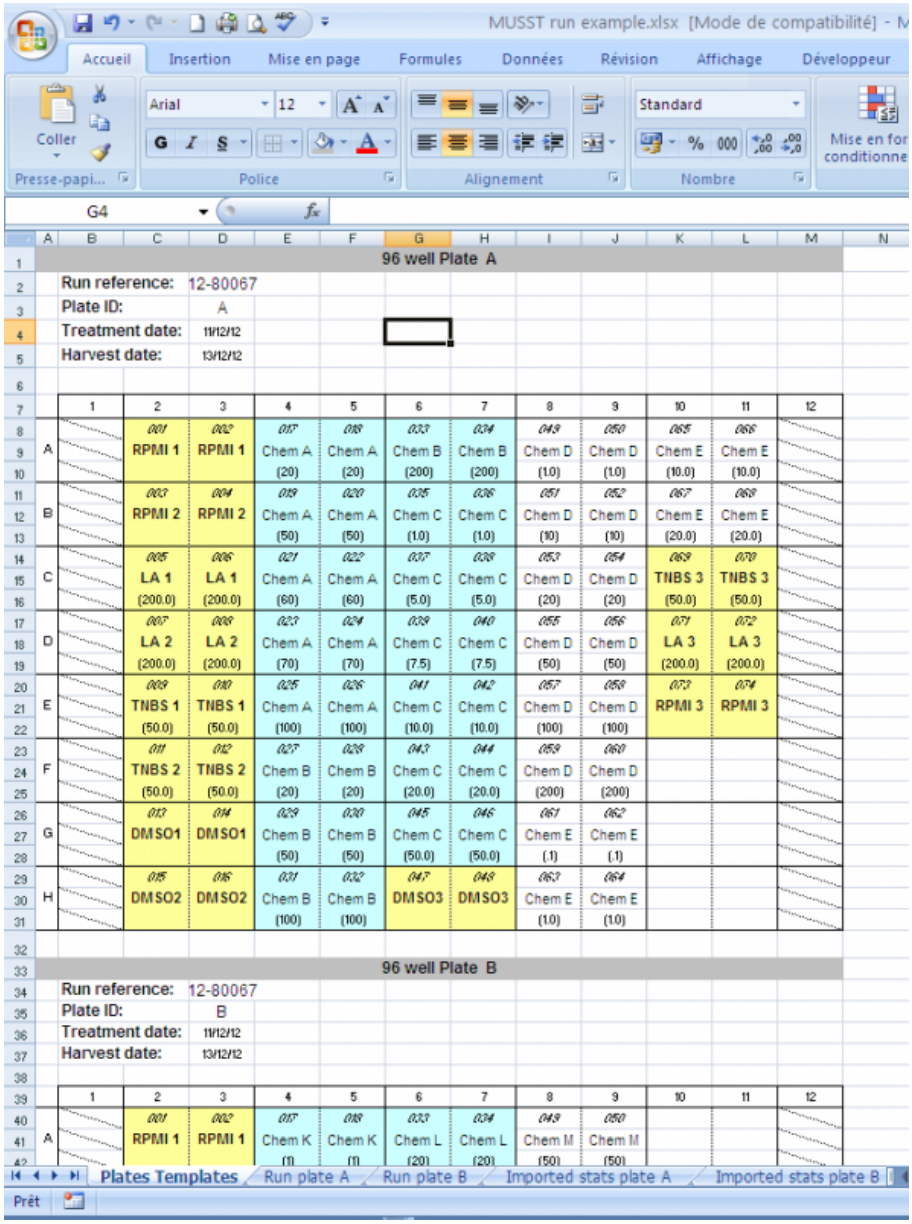


N: Run with no CD86 positive or interference observed;
P: Run with CD86 positive and/or interference(s) observed;
NC: Not Conclusive. First run with No Conclusion when CD86 is positive at the highest non-cytotoxic concentration only;
#: A Not Conclusive (NC) individual conclusion attributed only to the first run conducts automatically to the need of a third run to reach a majority of Positive (P) or Negative (N) conclusions in at least 2 of 3 independent runs.
$: The boxes show the relevant combinations of results from the three runs on the basis of the results obtained in the two first runs shown in the box above.
°: The boxes show the relevant combinations of results from the four runs on the basis of the results obtained in the three first runs shown in the box above.

ANNEX 9: Example of data treatment in Excel files

U-SENS run Excel file:

- Fill in the plate(s) template in the "Plates Templates" sheet



MUSST run example.xlsx [Mode de compatibilité] - M

Accueil | Insertion | Mise en page | Formules | Données | Révision | Affichage | Développeur

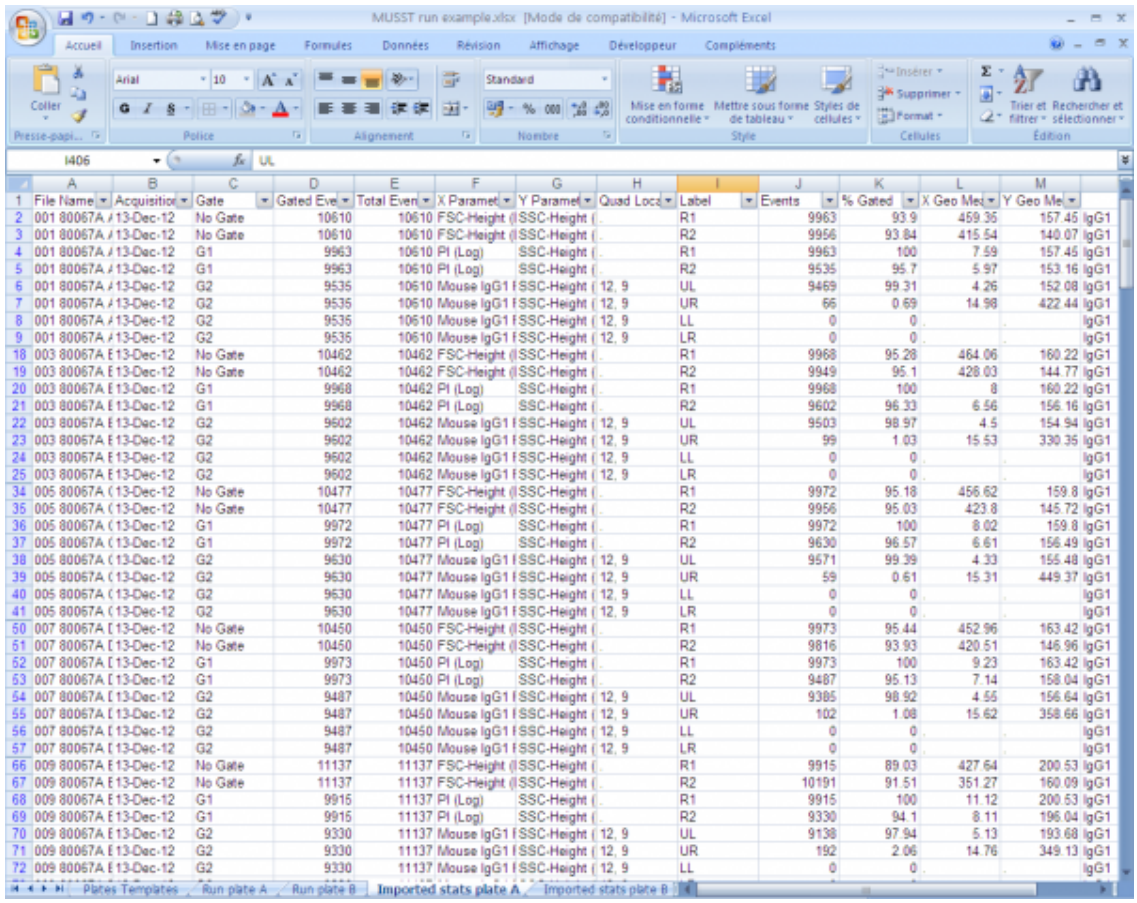
G4

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	96 well Plate A													
2		Run reference:	12-80067											
3		Plate ID:	A											
4		Treatment date:	11/12/12											
5		Harvest date:	13/12/12											
6														
7		1	2	3	4	5	6	7	8	9	10	11	12	
8			001	002	017	018	033	034	049	050	065	066		
9	A		RPMI 1	RPMI 1	Chem A (20)	Chem A (20)	Chem B (200)	Chem B (200)	Chem D (1.0)	Chem D (1.0)	Chem E (10.0)	Chem E (10.0)		
10														
11			003	004	019	020	035	036	051	052	067	068		
12	B		RPMI 2	RPMI 2	Chem A (50)	Chem A (50)	Chem C (1.0)	Chem C (1.0)	Chem D (10)	Chem D (10)	Chem E (20.0)	Chem E (20.0)		
13														
14			005	006	021	022	037	038	053	054	069	070		
15	C		LA 1 (200.0)	LA 1 (200.0)	Chem A (60)	Chem A (60)	Chem C (5.0)	Chem C (5.0)	Chem D (20)	Chem D (20)	TNBS 3 (50.0)	TNBS 3 (50.0)		
16														
17			007	008	023	024	039	040	055	056	071	072		
18	D		LA 2 (200.0)	LA 2 (200.0)	Chem A (70)	Chem A (70)	Chem C (7.5)	Chem C (7.5)	Chem D (50)	Chem D (50)	LA 3 (200.0)	LA 3 (200.0)		
19														
20			009	010	025	026	041	042	057	058	073	074		
21	E		TNBS 1 (50.0)	TNBS 1 (50.0)	Chem A (100)	Chem A (100)	Chem C (10.0)	Chem C (10.0)	Chem D (100)	Chem D (100)	RPMI 3	RPMI 3		
22														
23			011	012	027	028	043	044	059	060				
24	F		TNBS 2 (50.0)	TNBS 2 (50.0)	Chem B (20)	Chem B (20)	Chem C (20.0)	Chem C (20.0)	Chem D (200)	Chem D (200)				
25														
26			013	014	029	030	045	046	061	062				
27	G		DMSO1	DMSO1	Chem B (50)	Chem B (50)	Chem C (50.0)	Chem C (50.0)	Chem E (.1)	Chem E (.1)				
28														
29			015	016	031	032	047	048	063	064				
30	H		DMSO2	DMSO2	Chem B (100)	Chem B (100)	DMSO3	DMSO3	Chem E (1.0)	Chem E (1.0)				
31														
32														
33	96 well Plate B													
34		Run reference:	12-80067											
35		Plate ID:	B											
36		Treatment date:	11/12/12											
37		Harvest date:	13/12/12											
38														
39		1	2	3	4	5	6	7	8	9	10	11	12	
40			001	002	017	018	033	034	049	050				
41	A		RPMI 1	RPMI 1	Chem K (1)	Chem K (1)	Chem L (20)	Chem L (20)	Chem M (50)	Chem M (50)				
42														

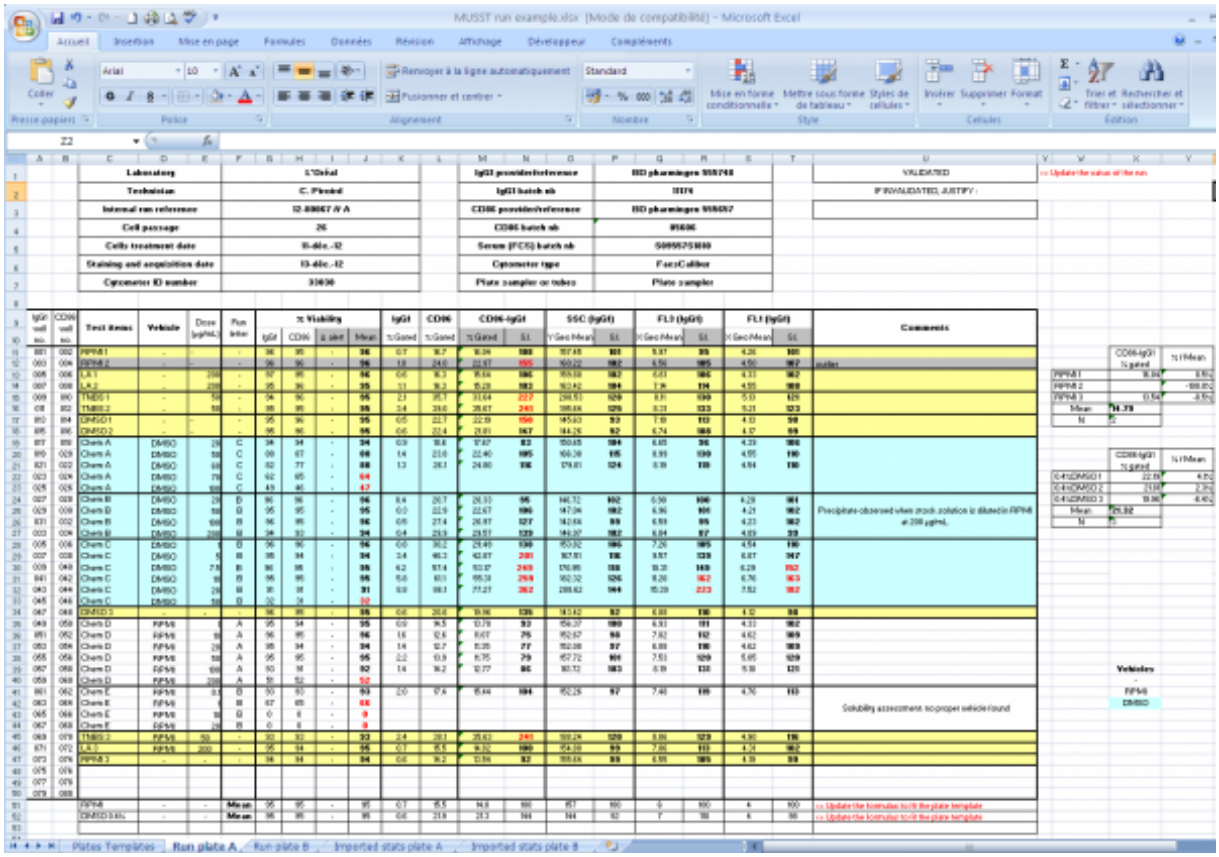
Plates Templates | Run plate A | Run plate B | Imported stats plate A | Imported stats plate B

Prêt

- Import the flow cytometry results (stats exportation txt file) in the "Imported stats" sheet:



- Select proper values using Excel filters and copy/paste them in the "run" sheet
- Apply data exploitation rules in order to generate the calculated values (S.I. and means)
- Format as grey background outlier's values and explain the reason of the discarding in the comments column
- Format as strikethrough and grey background invalidated values (if chemicals acceptance criteria not met or technical issue in some wells).
- For each chemical, report in the comments column: solubility issues if any (complete solubility of the stock solution not achieved, suspension, crystals/droplets in the well at 45±3 h, unusual dot-plots at doses) or unusual aspect of the cells at 45h (± 3 h).
- Only solubility issues observed under the microscope at 45±3 h may impact the individual conclusions.



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  Toxicology In Vitro 25;30(1 Pt B), 373-82
- EURL ECVAM (2017)
  EURL ECVAM Recommendation on the use of non-animal approaches for skin sensitisation testing **Link to document** (last access 11.10.2017)
  European Union Reference Laboratory for Alternatives to Animal Testing
- EURL ECVAM (2016)
  ESAC Opinion on the L'Oréal-coordinated study on the transferability and reliability of the U-SENS™ test method for skin sensitisation testing. ESAC Opinion No. 2016-03 of 24 June 2016; EUR 28178 EN; doi:10.2787/815737. **Link to document** (last access 15.06.2017)
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  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2016)
  OECD Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, ENV/JM/HA(2016)29. **Link to document** , **Link to Annex 1** and **Link to Annex 2** .
  OECD Environmental Health and Safety Publications
- OECD (2012)
  OECD Series on Testing and Assessment No. 168: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. **Link to Part 1** , **Link to Part 2** (last access 20.08.2013)
  OECD Environmental Health and Safety Publications
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  The Myeloid U937 Skin Sensitization Test (U-SENS) Addresses the Activation of Dendritic Cell Event in the Adverse Outcome Pathway for Skin Sensitization.
  Toxicology In Vitro 29 (5), 901–916
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  Globally Harmonised System of Classification and Labelling of Chemicals (GHS); New York and Geneva: United Nations.Sixth revised edition. **Link to document** (last access 15.06.2017)