EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
In Vitro Methods Unit

ANNEX I TO THE CONTRACT N……..

TECHNICAL SPECIFICATIONS

Open call for tender on "Phase III – Prevalidation of three methods, subdivided into three lots:
Lot 1: Direct Peptide Reactivity Assay (DPRA)
Lot 2: Myeloid U937 Skin Sensitisation Test (MUSST)
Lot 3: human Cell Line Activation Test (hCLAT)

Open call for tender ref. OJ 2009/S 166-239612 of 29/08/2009.
Ref. IHCP/2009/I/03/53/OC

SUMMARY
This call is to invite laboratories to participate in the initial phase (prevalidation) of a project whose aim is to deliver validated alternative methods/strategies for skin sensitisation testing to be used by chemical and cosmetic industries for the safety assessment of products. The selected laboratories will work in close contact with multinational companies as well as the European Commission Joint Research Centre for the initial assessment of three of those tests involving flow-cytometry and HPLC methods.

1. INTRODUCTION

This proposal is addressing the requirement to make available alternative methods in the field of skin sensitisation to reduce or eliminate the need for animal testing
The objective of this proposal is to assess the reproducibility and preliminary predictive capacity of three partial replacement test methods for the identification of potential skin sensitising chemicals. The contractor(s) will participate to this study together with other laboratories.

1.1. Background to ECVAM

The European Centre for the Validation of Alternative Methods (ECVAM) within the Institute for Health and Consumer Protection (IHCP) of the European Commission Joint Research Centre is responsible for co-ordinating the validation of alternative test methods at the European Union level. ECVAM was established in accordance with a communication from the European Commission to the Council and the European Parliament in October 1991 and in response to Article 23 of Directive 86/609/EEC. ECVAM's goal, as defined in 1993 by its Scientific Advisory Committee (ESAC), is to promote the scientific and regulatory acceptance of in vitro methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals.

1.2. Regulatory framework

The field of alternatives is currently driven by the expectation from both Chemicals and Cosmetics policies. In particular, the 7th Amendment to the Cosmetics Directive foresees a testing ban on animals for cosmetics ingredients by 2009 and a marketing ban in the European Community for cosmetic ingredients tested on animals by 2009 for all human health effects with the exception of repeated-dose toxicity (including skin sensitisation), reproductive toxicity and toxicokinetics. For these endpoints the cut-off date for the marketing ban will be 2013. The new legislation for chemicals (REACH) foresees data requirements for more than 30.000 existing chemicals produced at levels above 1 ton/year. Within REACH the availability of validated in vitro methods has the potential to substantially reduce animal testing.

In view of the above, it has become of highest importance to identify predictive test methods tailored for *in vitro* investigation of chemical compounds for their potential to cause skin sensitisation to humans.

1.3. Scientific background

At the moment there are no validated alternative methods for the assessment of the skin sensitisation potential of chemicals. For complex endpoints such as skin sensitisation, the challenge of replacing whole animal studies with in vitro assays requires an integration of approaches, each reflecting one of the mechanistic steps occurring in vivo. There is a general consensus within the scientific community regarding the key steps which should be considered when designing in vitro methods for skin sensitisation. ECVAM has organised and hosted a number of workshops in which experts in the field convened to review the state-of-the art of methods specifically designed to cover such mechanisms. These include methods based on the use of dendritic cells and those based on the measurement of chemicals' reactivity towards proteins.

Three alternative methods developed by Colipa (the European Cosmetics Association) associated Industries and optimised within Colipa ring trials have been now elected as being ready to enter a formal Phase III Prevalidation study. This would allow for a more robust assessment of their reproducibility and preliminary predictive capacity as a basis for their incorporation into a testing strategy for achieving full replacement of the currently used animal tests.

The three methods under evaluation are:

1. Direct Peptide Reactivity Assay (DPRA) **Lot I**
2. Myeloid U937 Skin Sensitisation Test (MUSST) **Lot II**
3. human Cell Line Activation Test (hCLAT) **Lot III**

1.4. Purpose and objectives of the study

Purpose

The purpose of a prevalidation study is to ensure that the protocol of a test method is sufficiently standardised and optimised for inclusion in a large-scale formal validation study. Specifically, the objective of this phase III prevalidation study is the assessment of the three methods protocol performance by testing, under blind conditions in at least three laboratories, an appropriate number of test materials, selected, coded and distributed independently, Prerequisite for entering this phase of prevalidation is the availability of a standardised protocol and of a preliminary defined prediction model.

Objectives

The objective of the Phase III Prevalidation study is the evaluation of the reproducibility and ability of the DPRA, the MUSST and hCLAT to reliably discriminate between potential skin sensitising substances and non-sensitisers.

The prevalidation/validation study will be conducted in accordance to:

1. ECVAM's validation principles (Balls *et al*., 1995)

2. principles and criteria documented in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34, OECD, 2005)

3. the Modular Approach to Validation (Hartung et al. 2004).

1.5. Adherence to quality principles (e.g. GLP or equivalent).

In agreement with OECD guideline Nr. 34 on the validation of Alternative Testing Methods, data should be preferably generated according to the principles of Good Laboratory Practice (GLP) or equivalent. Where this is not possible, the participating laboratories should observe general quality principles with respect to documentation, traceability and accountability.

1.6. Overall Study Structure

The DPRA and the MUSST will be evaluated in three laboratories: the lead laboratory, the In-Vitro Methods Unit (IVMU) of the IHCP laboratory plus an additional laboratory to be identified. The h-CLAT will be evaluated in four laboratories: two lead laboratories, the In-Vitro Methods Unit (IVMU) of the IHCP laboratory plus an additional laboratory to be identified.

The contractors might be responsible for evaluating more than one test depending on the Lot(s) for which they chose to apply and the award procedure regarding the tender as specified in the Administrative Annex.

The lead laboratories will be the laboratories where the tests have been developed:

1. Procter & Gamble (USA) for the DPRA
2. L'Oréal Research (France) for the MUSST
3. Kao Corporation (Japan) and Shiseido (Japan) for the hCLAT.

The participation of these three laboratories is necessary because of their unique know-how with the test methods under evaluation.

All responsibilities of the participating laboratories (in particular, work programme and data submission deadlines to be met) will be agreed upon by the European Commission represented here by ECVAM, the Study Management Team, and the contractors.

1.7. Role of the Management Team

The Study Management Team (SMT) is responsible for the overall management of the study. Specifically its role foresees:

- Ensuring that the purpose and objectives (goal) of the study are clearly defined
- Ensuring that a detailed project plan is available and that is clearly understood by all involved parties. (the project plan forms the basis of an agreement among the sponsor, the SMT, the lead and participating laboratories)
- Overseeing the conduct of the validation study and its progression including meeting the deadlines
- Approving of study design, protocols, time schedules, number and type of test chemicals, data management procedures
- Ensuring and monitoring over a proper information exchange
- Interpreting the outcome of the study and drawing conclusions
- Approving study reports and publications

The composition of the Study Management Team will be defined by ECVAM.

1.8. Data analysis

The statistical analysis of the data will be carried out independently by the IHCP biostatistics team.

1.9. Technical responsible

The technical responsible for this study is Silvia Casati - JRC - ECVAM

1.10. Project monitoring

The European Commission (ECVAM) and the Validation Management Team will monitor the work progress via e-mail exchange and teleconferences with the contractor and the personnel involved in the study.

1.11. Intellectual Property Agreement

The contracting party agrees that the experimental work commissioned and described in the present technical annex cannot be published or presented, even in part, without the official approval by ECVAM.

The name of the Commission has to be added on all publications resulting by this work.

1.12. Starting date of the project

The foreseen starting date of the project is February 2010.

Lot 1

Direct Peptide Reactivity Assay - DPRA

Test Method Summary

The DPRA is a chemistry-based assay exploiting the fact that chemical allergens have electrophilic properties and are therefore able to react with the nucleophilic side chains of amino acids to form covalent bonds. The underlying rationale of the DPRA is that if a chemical is capable of reacting with proteins then it has the potential to act as sensitisers. The endpoint measured in this method is the depletion of two synthetic peptides (containing respectively a cysteine and a lysine) from the reaction mixture following incubation with the test chemicals. The % of peptide depletion is calculated from the reaction mixture after 24 hours incubation with the test chemical by HPLC-UV. The DPRA allows for a yes/no answer in terms of sensitisation potential and furthermore allows quantifying chemicals reactivity in four classes Minimal, Low, Moderate, High on the basis of a classification three model based on the average of cysteine and lysine depletion values (Gerberick et al, 2007).

Draft Study Outline

The study design outlined below shall be agreed upon by the Study Management Team. The final study design will be released to the laboratories/contractors only once endorsed by the Study Management Team.

The study shall be structured in two phases:

1. Phase A, for the training of the participating laboratories, for test method transferability and for confirmation of the test method protocols.

2. Phase B, for the assessment of the protocol performance by testing, under blind conditions in three laboratories per test, about 30 chemicals (the exact number of chemicals will be proposed by the study biostatistician and agreed upon by the Study Management Team) that are selected, coded and distributed independently.

1) Phase A:

Phase A Stage I: Training of the Participating Laboratories

Stage 1 foresees the training of the selected laboratory at Procter & Gamble (Cincinnati, USA). The lead laboratory will be responsible for issuing a training SOP and for releasing a training report (*Phase A Stage I Report*) to the Study Management Team on the outcome of the training. The training duration should not exceed one week. Travel costs and accommodation costs will be in charge of the contractor.

Phase A Stage II: Test Method Transferability

This stage of the study is designed to assess the test method transferability. The contractor will have to test in house at least 5 chemicals. The Study Management Team will decide whether these chemicals will have to be tested uncoded, coded, or under both conditions. The

contractor will be responsible for producing a *Phase A Stage II* report to be submitted to the Study Management Team. Results of *Phase A Stage II* will be reviewed by the Study Management Team before progression.

2) Phase B: Test method reproducibility (within- and between-laboratory reproducibility)

Phase B Stage I:
During this phase the DPRA will be evaluated in 3 laboratories (P&G, the IVM laboratory and the contractor's laboratory) with a preliminary set of coded compounds (about 5, the exact number will be decided by the Study Management Team). It is the responsibility of the contractor to release a *Phase B Stage I* study report upon completion of testing. Progression to Phase B Stage II is dependent upon decision of the Study Management Team following review of the submitted data.

Phase B Stage II:
During this phase the DPRA will be evaluated in 3 laboratories (P&G, the IVM laboratory and the contractor's laboratory) with an additional number of coded chemicals (about 25, the exact number will be defined by the Study Management Team). It is responsibility of the contractor to release a Phase B Stage II study report upon completion of testing. The within- and between-laboratory reproducibility will be evaluated according to the objectives of the study agreed upon beforehand by the Study Management Team.

Data generated during Phase B will be used for the preliminary assessment of the predictive capacity of the test methods.

All the chemicals tested in Phases A Stage II and B (Stage I and Stage II) will be purchased, coded and distributed to the participating laboratories by the IVM unit of the IHCP.

Reports

A first interim report after phase A Stage II (4 months from the signature of the contract) and a second interim report after phase B Stage I (6 months from the signature of the contract) and a detailed final report after phase B Stage II (9 months from the signature of the contract) shall be required from the Contractor.

The reports should detail all the observations and results (including raw data) obtained during the planning and conduction of the project and should report any deviations from the established plan.

An electronic copy and four paper copies of the interim and final reports shall be delivered to ECVAM.

Time for completion of the study

The expected time-frame for the completion of the study is 9 months.

Proposed timescale:
Mo: 0 Signature of the contract and start of phase A

Mo: + 4	Interim report concerning phase A Stage II to be sent to the Commission (ECVAM)
Mo + 6	Interim report concerning phase B Stage I to be sent to the Commission (ECVAM) and start of phase B stage II.
Mo + 9	Final report to be sent to Commission (ECVAM)

Response to the tender

In their response applicants must include the following costs

- All costs foreseen for a complete evaluation of the transferability, reproducibility and preliminary predictive capacity of the DPRA as described in the draft study outline.

- Travel costs in economy, including tickets, accommodation and any other related cost for the training of the personnel at the Procter & Gamble lead laboratory (Cincinnati, USA).

The evaluation of the tenders will be conducted as established in the Administrative Annex.

Estimated value: about 16.000,00 EUR

Direct Peptide Reactivity Assay Summary of the Procedure, Equipments and Materials

Principle and Scope

The reactivity of a test chemical versus a synthetic Cysteine or Lysine containing peptide is evaluated by combining the test material with a solution of the peptide and monitoring the remaining concentration of the peptide following 24 hours incubation time at room temperature. The peptide is a custom material containing phenylalanine to aid in detection and either Cysteine ("C") or Lysine ("K") as the reactive center. Relative concentrations of the peptide following the 24 hour reaction time are determined by high performance liquid chromatography with gradient elution and UV detection at 220nm. The method is applicable to test chemicals soluble in acetonitrile or other non-reactive, water-miscible solvent at a 100 mM concentration.

Reference

Gerberick,G.F.et al. "Development of a Peptide Reactivity Assay for Screening Contact Allergens" Toxicological Sciences 81, 332-343 (2004)

Equipment and Materials

APPARATUS	SUGGESTED TYPE (or Equivalent)

Liquid Chromatograph with light-excluding Autosampler capable of delivering 0.35 mL/min flow rate	Waters Alliance 2695, Waters Corp. Milford MA Column oven included *Note: autosampler needle depth must be adjusted to avoid the vial bottom*
UV Detector capable of measuring UV absorbance at 220 nm and 258 nm,	Waters 996 Photodiode Array (preferred)
Glass Autosampler Vials	Compatible with Autosampler
pH meter with electrode and calibration buffers	Capable of reading +/- 0.01 pH units
HPLC Column	Agilent Zorbax SB-C18 2.1 mm x 100 mm x 3.5 micron Part # 861753-902
Guard Column	Phenomenex Security Guard C18 4 mm x 2 mm Part # AJO-4286
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1mg readability
Dispensing Pipets capable of delivering 250 - 750 µL and 50 µL	Eppendorf Research Adjustable Pipets

Other common lab equipments, such as centrifuge or Vortex™ (or equivalent) and some generic glassware, are necessary.

Reagents

The main reagents required are:

Common buffers (ex. Phosphate Buffers, Ammonium Acetate Buffer)

Common HPLC buffers (ex. Trifluoroacetic acid, Acetonitrile)

Cysteine Peptide Ac-RFAACAA-COOH, purified > 90% by HPLC (Synbiosci, Livermore CA, USA)

Lysine Peptide Ac-RFAAKAA-COOH , purified >90% by HPLC (Synbiosci, Livermore CA, USA)

Other common lab reagents can be required.

Sample Analysis

Samples are prepared in triplicate and two peptide- test chemical ratios are evaluated in two different sequences.

The HPLC analysis is performed using a flow of 0.35ml/min and a linear gradient from 10% to 25% of a mobile phase. Total run time is 20’ including re-equilibration.

Each HPLC analysis sequence includes the following:

- 6 peptide concentration standards
- 1 Reference control (in triplicate) analysed regularly along the analysis sequence
- 1 Positive control (in triplicate)
- Each tested chemical (in triplicate)

Samples are prepared in batches of no more than 22 test materials per analysis sequence. Each chemical will have to be evaluated in triplicate in three independent experiments (days).

Two runs are needed for each experiment, one for the Cysteine peptide and one for the Lysine peptide.

Required analyses involve generation of chromatograms at two wavelengths, 220 nm and 258 nm, and determination of peak area.

The final detailed protocol shall be agreed upon by the Validation Management Team and will be provided to the selected laboratory by ECVAM.

For the purpose of the award of the contract, the tenderers are invited to submit a cost estimate for the testing of 30 chemicals.

Lot 2

Myeloid U937 Skin Sensitisation Test - MUSST

Test Method Summary

The MUSST is a Dendritic Cells-like based assay which uses human myeloid U937 cells. The endpoint measured is the up-regulation of the co-stimulatory molecule CD86 as a marker of cell activation by flow cytometry analysis following 48h incubation with the test chemicals. The sensitising potential of a chemicals is determined when the marker expression exceed a certain threshold with respect to the vehicle control.

Draft Study Outline

The study design outlined below shall be agreed upon by the Study Management Team. The final study design will be released to the laboratories/contractors only once endorsed by the Study Management Team.

The study shall be structured in two phases:

3. Phase A, for the training of the participating laboratories, for test method transferability and for confirmation of the test method protocols.

4. Phase B, for the assessment of the protocol performance by testing, under blind conditions in three laboratories per test, about 30 chemicals (the exact number of chemicals will be proposed by the study biostatistician and agreed upon by the Validation Management Team) that are selected, coded and distributed independently.

1) Phase A:

Phase A Stage I: Training of the Participating Laboratories

Stage 1 foresees the training of the selected laboratory at l'Oréal Research (Aulnay-sous-Bois, France). The lead laboratory will be responsible for issuing a training SOP and for releasing a training report (*Phase A Stage I Report*) to the Study Management Team on the outcome of the training. The training duration should not exceed one week. Travel costs and accommodation costs will be in charge of the contractor.

Phase A Stage II: Test Method Transferability

This stage of the study is designed to assess the test method transferability. The participating laboratories will have to test in house at **least 3** chemicals. The Study Management Team will decide whether these chemicals will have to be tested uncoded, coded, or under both conditions. The participating laboratories will be responsible for producing a *Phase A Stage II* report to be submitted to the Study Management Team. Results of *Phase A Stage II* will be reviewed by the Study Management Team before progression.

2) Phase B: Test method reproducibility (within- and between-laboratory reproducibility)

Phase B Stage I:

During this phase the MUSST will be evaluated in 3 laboratories (l'Oréal Research, the IVM laboratory and the contractor's laboratory) with a preliminary set of coded compounds (about 5, the exact number will be decided by the Study Management Team). It is the responsibility of the participating laboratories to release a *Phase B Stage I* study report upon completion of testing. Progression to Phase B Stage II is dependent upon decision of the Study Management Team following review of the submitted data.

Phase B Stage II:

During this phase the MUSST will be evaluated in 3 laboratories (l'Oréal Research, the IVM laboratory and the contractor's laboratory) with an additional number of coded chemicals (about 25, the exact number will be defined by the Study Management Team). It is responsibility of the participating laboratories to release a Phase B Stage II study report upon completion of testing. The within- and between-laboratory reproducibility will be evaluated according to the objectives of the study agreed upon beforehand by the Study Management Team.

Data generated during Phase B will be used for the preliminary assessment of the predictive capacity of the test methods.

All the chemicals tested in Phases A Stage II and B (Stage I and Stage II) will be purchased, coded and distributed to the participating laboratories by the IVM if the IHCP.

Reports

A first interim report after phase A Stage II (4 months from the signature of the contract) and a second interim report after phase B Stage I (6 months from the signature of the contract) and a detailed final report after phase B Stage II (9 months from the signature of the contract) shall be required from the Contractor.

The reports should detail all the observations and results (including raw data) obtained during the planning and conduction of the project and should report any deviations from the established plan.

An electronic copy and four paper copies of the interim and final reports shall be delivered to ECVAM.

Time for completion of the study

The expected time-frame for the completion of the study is 9 months.

Proposed timescale:

Mo: 0	Signature of the contract and start of phase A
Mo: + 4	Interim report concerning phase A Stage II to be sent to the Commission (ECVAM)

Mo + 6	Interim report concerning phase B Stage I to be sent to the Commission (ECVAM) and start of phase B stage II.
Mo + 9	Final report to be sent to Commission (ECVAM)

Response to the tender

In their response applicants must include the following costs

- All costs foreseen for a complete evaluation of the transferability, reproducibility and preliminary predictive capacity of the MUSST as described in the draft study outline.
- Travel costs in economy, including tickets, accommodation and any other related cost for the training of the personnel at the L'Oréal Research lead laboratory (Aulnay-sous-Bois, France).

The evaluation of the tenders will be conducted as established in the Administrative Annex.

Estimated value: about 60.000,00 EUR.

Myeloid U937 Skin Sensitisation Test Summary of the Procedure, Equipments and Materials

Principle and Scope

The MUSST is based on the augmentation of CD86 in human myeloid U937 cells following exposure to chemicals. U937cells are exposed for 48h to a concentration range of the test chemical (from 4 to 6 concentrations) selected from preliminary range-finding studies and based on the CV70, the concentration that affords 70% cell viability. After the 48hr incubation period, cells are stained with FITC-labeled anti CD86 antibody and analysed by flow cytometry. Cell viability at each concentration tested is measured concurrently with propidium iodide (PI). The sensitising potential of a chemical is determined when the marker expression exceeds a certain threshold with respect to the vehicle control in at least two experiments.

Equipment and Materials

- The Contractor's laboratory should be **fully equipped for standard cell culture maintenance and experiments,** which include biosafety cabinets, CO_2 incubators, phase microscopes, cryostorage facilities, autoclave, waterbath, sonicator etc.

Additional equipment required include:

APPARATUS	**SUGGESTED TYPE (or Equivalent)**
Flow cytometer with automated sample acquisition from a 96-well plate, able to measure FSC, SSC, as	BD FACSCanto™ II High Throughput Sampler Option

well as the fluorescence signals of Propidium Iodide (PI) and FITC.	
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1mg readability
Dispensing Pipets capable of delivering 1-1000 • l.	Eppendorf Research Adjustable Pipets
5ml hemolysis tubes + winged plugs	212-9861, VWR International
5ml sterile polystyrene hemolysis tubes + winged plugs	L1773X, Fisher Bioblock Scientific.
V-shaped microtiter plates (96-wells)	

Other common lab equipments, such as a centrifuge able to accommodate microtiter plates, Vortexes™ (or equivalent) and some generic glassware, are necessary.

Reagents

The main reagents required are:

U937 cell line (ATCC # CRL-1593.2)
RPMI-1640 (Gibco # 42401-018)
Fetal Calf Serum (BioWest # S1810)
L-Glutamine, penicillin, strptomycin (Sigma # G1146)
DMSO (Sigma #D2650)
FITC labelled mouse IgG1 (BD Pharmingen #555748)
FITC labelled anti-human CD86 (BD Pharmingen #555657)
Propidium Iodide

Other common lab reagents can be required.

Sample Analysis

Every complete run includes testing 6 concentrations of each chemical, as well as a positive, negative, culture medium and vehicle controls.

On day 0, the cells are counted and passaged

On day 2, the cells are counted and treated.

On day 4, the cells are washed and stained for CD86 (or control IgG1), as well as with Propidium Iodide. The samples are then analysed by flow cytometry in the microtiter plate (96 wells).

Each chemical will need to be tested in at least two independent runs, the whole procedure will be repeated three times (3 full experiments).

The number of replicates per run will be decided upon by the Study Management Team and may vary from 1 to 3.

Before each run, the Flow Cytometer needs to be calibrated according the manufacturer's instructions:

- Verification of the optical alignment
- Monitoring of fluorescence resolution and sensitivity.
- Compensation for spectral overlap.

The final detailed protocol shall be agreed upon by the Validation Management Team will be provided to the selected laboratory by ECVAM.

For the purpose of the award of the contract, the tenderers are invited to submit a cost estimate for the testing of 30 chemicals.

Lot 3

Human Cell Line Activation Test – h-CLAT

Test Method Summary

The h-CLAT is based on the use of THP1 cells, a human pro-monocytic cell line as surrogate for Dendritic Cells. The readout of the test is the change in the expression of cell membrane markers (CD54 and CD86) measured by flow cytometry analysis following 24h incubation with the test chemical. The sensitising potential is identified when receptor expression exceed the thresholds in relation to vehicle control.

Draft Study Outline

The study design outlined below shall be agreed upon by the Study Management Team. The final study design will be released to the laboratories/contractors only once endorsed by the Study Management Team.

The study shall be structured in two phases:

5. Phase A, for the training of the participating laboratories, for test method transferability and for confirmation of the test method protocols.

6. Phase B, for the assessment of the protocol performance by testing, under blind conditions in three laboratories per test, about 30 chemicals (the exact number of chemicals will be proposed by the study biostatistician and agreed upon by the Study Management Team) that are selected, coded and distributed independently.

1) Phase A:

Phase A Stage I: Training of the Participating Laboratories

Stage 1 foresees the training of the selected laboratory at one of the two lead laboratories (Kao Corporation Safety Science Research Laboratories, Akabane Ichikaimachi, Haga, Tochigi 321-3497 Japan or Shiseido Co. Ltd Quality Assessment Center Fukuura, Kanazawa-ku, Yokohama, Kanagawa, Japan). The lead laboratory will be responsible for issuing a training SOP and for releasing a training report (*Phase A Stage I Report*) to the Study Management Team on the outcome of the training. The training duration should not exceed one week. Travel costs and accommodation costs will be in charge of the contractor.

Phase A Stage II: Test Method Transferability

This stage of the study is designed to assess the test method transferability. The participating laboratories will have to test in house at **least 3** chemicals. The Study Management Team will decide whether these chemicals will have to be tested uncoded, coded, or under both conditions. The participating laboratories will be responsible for producing a *Phase A Stage II*

report to be submitted to the Study Management Team. Results of *Phase A Stage II* will be reviewed by the Study Management Team before progression.

2) Phase B: Test method reproducibility (within- and between-laboratory reproducibility)

Phase B Stage I:

During this phase the hCLAT will be evaluated in 4 laboratories (Kao Corporation, Shiseido, the IVM laboratory and the contractor's laboratory) with a preliminary set of coded compounds (about 5, the exact number will be decided by the Study Management Team). It is responsibility of the participating laboratories to release a *Phase B Stage I* study report upon completion of testing. Progression to Phase B Stage II is dependent upon decision of the Study Management Team following review of the submitted data.

Phase B Stage II:

During this phase the hCLAT will be evaluated in 4 laboratories (Kao Corporation, Shiseido, the IVM laboratory and the contractor's laboratory) with an additional number of coded chemicals (about 25, the exact number will be defined by the Study Management Team). It is the responsibility of the participating laboratories to release a Phase B Stage II study report upon completion of testing. The within- and between-laboratory reproducibility will be evaluated according to the objectives of the study agreed upon beforehand by the Study Management Team.

Data generated during Phase B will be used for the preliminary assessment of the predictive capacity of the test methods.

All the chemicals tested in Phases A Stage II and B (Stage I and Stage II) will be purchased, coded and distributed to the participating laboratories by the IVM if the IHCP.

Reports

A first interim report after phase A Stage II (4 months from the signature of the contract) and a second interim report after phase B Stage I (6 months from the signature of the contract) and a detailed final report after phase B Stage II (9 months from the signature of the contract) shall be required from the Contractor.

The reports should detail all the observations and results (including raw data) obtained during the planning and conduction of the project and should report any deviations from the established plan.

An electronic copy and four paper copies of the interim and final reports shall be delivered to ECVAM.

Time for completion of study

The expected time-frame for the completion of the study is 9 months.

Proposed timescale:

Mo: 0	Signature of the contract and start of phase A
Mo: + 4	Interim report concerning phase A Stage II to be sent to the Commission (ECVAM)
Mo + 6	Interim report concerning phase B Stage I to be sent to the Commission (ECVAM) and start of phase B stage II.
Mo + 9	Final report to be sent to Commission (ECVAM)

Response to the tender

In their response applicants must include the following costs

- All costs foreseen for a complete evaluation of the transferability, reproducibility and preliminary predictive capacity of the hCLAT as described in the draft study outline.
- Travel costs in economy, including tickets, accommodation and any other related cost for the training of the personnel at one of the two lead laboratories (Kao Corporation, Shiseido, Japan)

The evaluation of the tenders will be conducted as established in the Administrative Annex.

Estimated value: about 60.000,00 EUR.

Human Cell Line Activation Test

Summary of the Procedure, Equipments and Materials

Principle and Scope

The h-CLAT is based on the augmentation of CD86 and CD54 expression in THP-1 cells following exposure to sensitising chemicals. THP-1 cells are cultured with test chemicals for 24hr using 8 concentrations selected from preliminary range-finding studies and based on the CV75, the concentration that affords 75% cell viability. After the 24hr incubation period, cells are stained with anti human CD86 and CD54 antibodies and the mean fluorescence intensity (MFI) is measured by flow cytometry. Cell viability at each concentration tested is measured concurrently with propidium iodide (PI). The sensitising potential of a chemical is determined when the marker expression exceed a certain threshold with respect to the vehicle control at any dose in two of three independent experiments.

References

Ashikaga T, Yoshida Y, Hirota M, Yoneyama K, Itagaki H, Sakaguchi H, Miyazawa M, Ito Y, Suzuki H, Toyoda H. Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. Toxicol In Vitro. 2006 Aug;20(5):767-73.

Sakaguchi H, Miyazawa M, Yoshida Y, Ito Y, Suzuki H. Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1. Arch Dermatol Res. 2007 Feb;298(9):427-37.

Equipment and Materials

- The Contractor's laboratory should be **fully equipped for standard cell culture maintenance and experiments**, which include biosafety cabinets, CO_2 incubators, phase microscopes, cryostorage facilities, autoclave, waterbath, sonicator etc

Additional equipments required include:

APPARATUS	**SUGGESTED TYPE (or Equivalent)**
Flow cytometer able to measure the fluorescence signals of Propidium Iodide (PI) and FITC in a 96-well format	FACSCalibur™ (Becton Dickinson) or EPICS XL-MCL System II (Beckman Coulter)
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1mg readability
Dispensing Pipets capable of delivering 1-1000 • l.	Eppendorf Research Adjustable Pipets

Other common lab equipments, such as a centrifuge, Vortexes™ (or equivalent) and some generic glassware, are necessary.

Reagents

The main reagents required are:

TPH-1 cell line (ATCC # TIB-202)
RPMI-1640 (Gibco # 42401-018)
Fetal Calf Serum (BioWest # S1810)
L-Glutamine, penicillin, strptomycin (Sigma # G1146)
DMSO (Sigma #D2650)
b-Mercaptoethanol (GIBCO #21985-023)
Globulins Cohn Fraction II,III (Sigma #G2388-10G)
FITC labelled mouse IgG1 (DAKO #X0927)
FITC labelled anti-human CD54 (DAKO #F7143)
FITC labelled anti-human CD86 (BD Pharmingen #555657)
Propidium Iodide (Sigma #P4170)

Other common lab reagents can be required.

Sample Analysis

Before each run a dose-finding study for each chemicals has to be performed.

Every complete run consists of 8 concentrations of each chemical, as well as a positive, negative, and vehicle controls.

On day 0, the cells are counted and passaged

On day 2, the cells are counted, and treated.

On day 3, the cells are washed and stained for CD86, CD54 or control IgG1, as well as with Propidium Iodide. The samples are then analysed by flow cytometry in a microtiter plate (96 well plate) or in individual tubes.

Each chemical will need to be tested in at least three independent runs, the whole procedure will be repeated three times (3 full experiments).

The number of replicates per run will be decided upon by the Study Management Team and may vary from 1 to 3.

Before each run, the Flow Cytometer needs to be calibrated according the manufacturer's instructions:

- Verification of the optical alignment
- Monitoring of fluorescence resolution and sensitivity.
- Compensation for spectral overlap.

The final detailed protocol shall be agreed upon by the Validation Management Team will be provided to the selected laboratory by ECVAM.

For the purpose of the award of the contract, the tenderers are invited to submit a cost estimate for the testing of 30 chemicals.

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