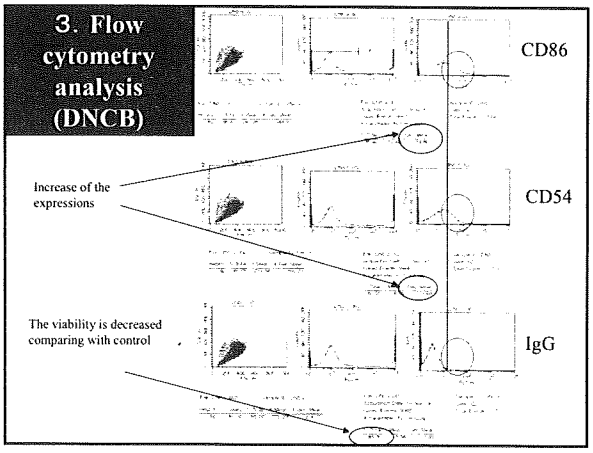
3. Flow cytometry analysis (DNCB)

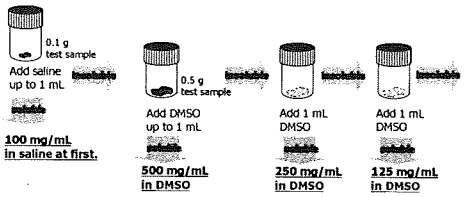


3. Setting of Flow cyotmetry

- You had better do the setting with using control and DNCB-treated cells.
- Set the voltage of FSC and SSC to appropriate levels. FSC and SSC are not needed for the analysis, but the FSC/SSC plot should be checked to make sure that a single population appears without contamination or excessive debris.
- Set and compensate the FL-1 and FL-3 voltage to appropriate position.
- Set R1 gate at the middle position between the peak of the negative fraction and the positive fraction in the FL-3 histogram using DNCB-treated cells. Please note that When self fluorescence chemicals tested, a peak of living cells could move to the right side.
- Check for leaks of fluorescence following the manufacturer's instructions. If necessary, the compensation FL-1 and FL-3 voltage should be conducted using FITC labeled-CD86 antibody and PI stained DNCB-treated cells.
- Keep the flow line in a flow cytometer clean, because we are to test many water insoluble chemicals. You should follow manufacturer's instruction.

4. Vehicle selection & Preparation of solutions

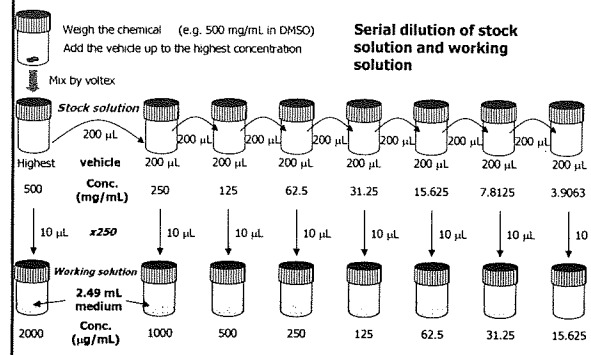
Determination of the highest concentration for dose finding assay.



First choice: Saline
Second choice: DMSO

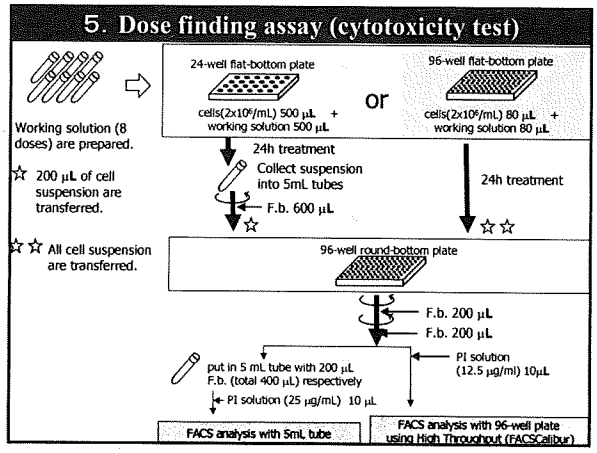
*: RPMI1640 can be used as an alternative of saline, if solubility is compatible.

4. Vehicle selection & Preparation of solutions



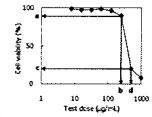
Dose finding assay: 1:2 serial dilution
Measurement CD86 &CD54: 1:1.2 serial dilution

5. Dose finding assay (cytotoxicity test)



5. Dose finding assay (cytotoxicity test)

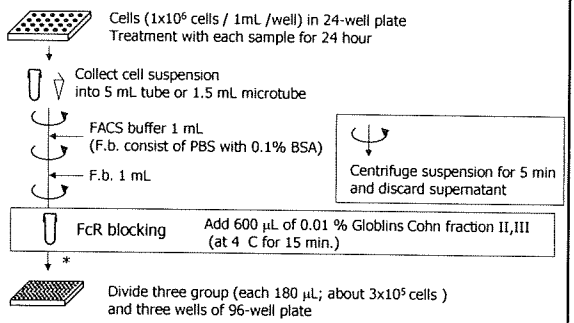
Calculation of CV75 value



The CV75 value is derived from the dose response curve as shown in the figure. CV75 is defined as the estimated concentration that is required to elicit 75% cell viability.

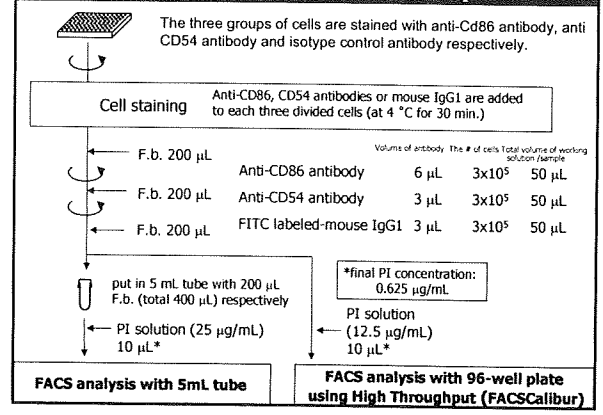
Note:
- At least two independent PI assays should be performed to derive a reliable CV75. The mean of at least two CV75 values is used for setting the dose-range for the main experiment.
- Eight final concentrations (mg/mL) are used for each chemical in the experiment (see section III). These are at 1.2×, 1×, 1/1.2×, 1/1.22×, 1/1.23×, 1/1.24×, 1/1.25×, and 1/1.26×CV75 the determined CV75 concentration.
- If CV75 can not be determined, stock solution (8 doses) will be prepared by serial 1:2 dilutions from the highest soluble dose. The final maximum concentration of test chemicals should not exceed 5000 mg/mL (if vehicle is saline) or 1000 mg/mL (if vehicle is DMSO).

6. Measuring of CD86 and CD54 expression



After 24-hour treatment, cells are washed and FcR of the cells are blocked, then cells are divided into 3 aliquots.

6. Measuring of CD86 and CD54 expression



6. Measuring of CD86 and CD54 expression

Example of 24well plate template

1. Control	2. DMSO	3. DNCB 4μg/mL	4. sampleA CV75/1.2⁴	5. CV75/1.2³	6. CV75/1.2⁴
7. Sample A CV75/1.2⁵	8. CV75/1.2²	9. CV75/1.2	10. CV75	11. CV75*1.2	12. Control Or DMSO
13. sampleB CV75/1.2⁵	14. CV75/1.2³	15. CV75/1.2⁴	16. CV75/1.2³	17. CV75/1.2²	18. CV75/1.2
19. sampleB CV75	20. CV75*1.2				

If more than one chemical is tested at the same time, a vehicle control for each vehicle (medium or DMSO) is set on each test group of chemicals.

7. Data analysis & interpretation

The **Relative Fluorescence Intensity (RFI)** is used as an indicator of CD86 and CD54 expression, and is calculated as follows for each concentration of every chemical:

$$RFI = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype control cells}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells}}$$

MFI = Geometric Mean fluorescence intensity

Prediction model: The chemicals must be labelled as a sensitizer,

If 2 of 3 independent run data at any dose exceed the MFI of CD86 ≥ 150, and/or
If 2 of 3 independent run data at any dose exceed the MFI of CD54 ≥ 200,

- Cell viability of medium and DMSO controls should be **more than 90 %.**
- In the positive control (DNCB), RFI values of both CD86 and CD54 should be over the positive criteria ("CD86 > 150 and CD54 > 200") and cell viability should be more than 50 %.
- Cell viability at the highest dose for each test chemical should be **less than 90%.**
- Cell viability of at least 4 doses in each experiment should be more than 50%.

7. Data analysis & interpretation

Table. Summary of the data from independent three experiment

sample	dose (μg/mL)	ratio	CD86 1	CD86 2	CD86 3	CD54 1	CD54 2	CD54 3	Cell viability(IgG) 1	Cell viability(IgG) 2	Cell viability(IgG) 3
Sample A (vehicle : DMSO)	8.2		114	113	133	87	82	68	95	98	97
	9.8		103	128	150	101	93	72	93	97	96
	11.8		113	**173**	**167**	105	112	82	92	96	96
	14.1		**181**	**219**	**175**	119	124	90	90	94	94
	16.9		122	**255**	**199**	157	136	118	88	92	92
	20.3		144	**286**	**224**	**238**	**221**	149	85	87	90
	24.4	CV75	97	**287**	**218**	**212**	**245**	166	76	81	83
	29.3		91	**176**	**189**	156	**205**	165	55	72	76
Sample B (vehicle : Saline)	409.1		75	92	71	82	78	58	95	98	98
	490.9		73	78	73	81	76	62	94	98	97
	589.1		67	75	74	75	72	58	95	97	97
	706.9		64	79	80	77	73	59	94	97	97
	848.3		60	80	78	89	75	61	94	97	96
	1018.0		59	71	67	90	75	60	91	97	96
	1221.6	CV75	63	81	67	100	78	64	88	94	94
	1465.9		73	93	75	129	102	84	81	86	89
Sample C (vehicle : DMSO)	170.3		69	92	76	91	88	69	95	98	97
	229.5		74	86	73	112	99	65	95	98	97
	275.4		71	87	78	107	99	68	95	98	97
	330.5		70	87	79	106	110	71	94	97	96
	396.6		74	80	77	108	114	73	93	97	97
	475.9		74	85	70	122	128	77	92	96	96
	571.1	CV75	74	96	75	134	142	91	83	95	94
	685.3		89	120	80	153	187	99	77	88	88

Results of 3 runs Values of RFI

8 doses
Serial dilution 1.2

Let's judge each chemical; Negative or Positive?

Thank you for your attention!

and Question?

Direct Peptide Reactivity Assay (DPRA)
human Cell Line Activation Test (h-CLAT)
Myeloid U937 Skin Sensitisation Test (MUSST)

Phase III Pre-validation Study draft experimental design

Study Objective

The objective of the study is to pre-validate, in a formal inter-laboratory study, the direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitisation Test (MUSST) in view of their future incorporation into a testing strategy for replacing the currently used regulatory animal tests (Buehler Test and Guinea Pig Maximisation Test (OECD TG 406, TG B06 EU Regulation 440-2008) and the Local Lymph Node Assay (LLNA, OECD TG 429, TG B42 EU Regulation 440-2008).

The phase III prevalidation study will be conducted in accordance with the 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) and according to the Modular Approach to Validation (Hartung et al., 2004).

Study Goals

The primary goal of this Phase III Prevalidation Study is an evaluation of the transferability and reliability (reproducibility within and between laboratories) of the DPRA, h-CLAT and MUSST when challenged with a set of coded chemicals.

Secondary goals of the study are:

1. a preliminary evaluation of the ability of the three tests to reliably discriminate skin sensitising (S) from non-sensitising (NS) chemicals as defined by the Globally Harmonised System (GHS) for the classification and labelling of substances for skin sensitisation (category 1; no category) and as implemented in the European Commission Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixture.

2. a preliminary evaluation of the ability of the three tests to sub-categorise skin sensitising chemicals into Sub-category 1A and Sub-category 1B as adopted in the 3rd revised version of the GHS.

Sample Size

The parameters requested for the calculations are the following:

$\pi \rightarrow$ Expected proportion of concordant classifications among laboratories

$\pi - \delta \rightarrow$ Lower border of the Confidence Interval for the expected proportion π. The lower border ensures that the true proportion of concordant classifications will not be lower than this threshold.

$\alpha \rightarrow$ Type I Error: it's the probability to conclude that there is a significant difference in the mean performances of the three laboratories when in the truth there is not

$1 - \beta \rightarrow$ Statistical Power: it's the probability to correctly detect an existing difference in the performances of the three laboratories

$\beta \rightarrow$ Type II Error: it's the probability not to detect a significant difference among the mean performances of the three laboratories when in the truth there is.

Acceptable parameters for a significant statistical analysis of the Between Laboratory Reproducibility (BLR) are shown below:

π	0.9
$\pi - \delta$	0.65
α	0.05
$1 - \beta$	0.75
β	0.25

From these parameters, the number of chemicals required can be calculated to be at least 21

Since previous studies have shown that Within Laboratory Reproducibility (WLR) is systematically higher than the BLR, the parameters have been adapted to evaluate the size of the subset of chemicals which will be tested in triplicate within each laboratory:

π	0.95
$\pi - \delta$	0.65
α	0.05
$1 - \beta$	0.8
β	0.2

From these parameters, it can be calculated that the subset of chemicals used for the evaluation of the WLR should contain at least 13 chemicals

Experimental Design

Because sensitising chemicals (and in particular, weak or moderate sensitisers) are more informative for the evaluation of the reproducibility of these methods than negative chemicals, the ratio of sensitisers to non-sensitisers has been set to 2:1

On the basis of the above calculations, the following design has been selected:

- For evaluation of the BLR, 24 chemicals tested once in every laboratory, 16 sensitizers and 8 non-sensitizers

- For evaluation of the WLR, 15 chemicals tested two further times in each laboratory, the same subset being used at every site. 10 sensitizers and 5 non-sensitizers.

The 15 chemicals used to evaluate the WLR will be selected by stratified random sampling from the 24 chemicals used to evaluate the BLR.

This will provide the information needed to evaluate the within- and between- laboratory reproducibility of both the prediction results and the raw data, for the three tests evaluated.

Statistical Analyses

All statistical analysis will be performed on the data deriving from valid runs and experiments only.

The following analysis plan can be amended if a scientific motivation appears and with the agreement of the VMT.

For all the assays, descriptive statistics on positive and negative controls values will be calculated.

For all the statistical tests included in the analysis, significance will be set at 0.05.

Although no specific criteria have been defined to evaluate the performance of the assays, the minimum requirements are implicitly stated in the parameters set for the sample size calculations.

According to these, the expected concordance for the Between Laboratory Reproducibility is around 90%, with a lower border for the 95% Confidence Interval equal to 65%, while the expected concordance for the Within Laboratory Reproducibility is around 95%, with the same lower border for the 95% Confidence Interval as for the BLR.

Please note that even though the predictive capacity analysis will include the calculation of Positive and Negative Predictive Values, these measures are dependent on prevalence and thus cannot be generalized.

1) DPRA

RELIABILITY

Since 3 full experiments will be performed at each site, the prediction model for a given chemical will be applied three times in each laboratory. Concordance in the sensitiser/non-sensitiser classification and in the assignment of a reactivity class will be evaluated within and between laboratories.

The analysis of reliability will also be performed on peptide depletion values obtained by the participating laboratories.

Reliability will be explored both as WLR and BLR.

Normality of data distributions will be checked using the Shapiro-Wilk test; in case of non-normality of the data, variance stabilizing transformations will be applied (e.g. logarithmic, square-root). If the transformation still does not normalize the data, non-parametric analyses will be performed (i.e. Kruskal-Wallis test).

WLR: The variability within each laboratory will be evaluated both as concordance among runs and as variability of each run. Differences in mean among the performance of the runs within each laboratory could be assessed also using an one-way analysis of variance (ANOVA).

BLR: Differences in the means between the peptide depletion values generated by the laboratories will be checked using a comparison by chemical, using a one-way analysis of variance (ANOVA). The ANOVA model will include only the Laboratory factor as an independent variable. The concordance of predictions obtained for each chemical by the participating laboratories will be compared as well.

PREDICTIVE CAPACITY

Replicate values within each run will be averaged for each peptide. A sensitiser/non-sensitiser classification will be derived from the average of the 2 peptides values according to the Prediction Model.

2x2 contingency tables, comparing the prediction results with the existing proposed classification, will be built in order to calculate sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test.

Moreover, as the prediction model allows a classification of the chemicals in four distinct sensitization reactivity classes, 4x4 contingency tables will be built in order to compare the prediction results obtained applying the 4-classes prediction model with the existing proposed classification (which is considered as the gold standard).

An estimate of the concordance between the two classifications reported in the 4x4 contingency tables will be given using Cohen's Kappa statistic. As Kappa statistic ranges between 0 and 1, agreement between classifications can be considered good if it's at least equal to 0.61 (Altman DG (1991) Practical statistics for medical research. London: Chapman and Hall according to Altman at al.).

No criteria have been established for the predictive capacity indices to be acceptable.

2) h-CLAT

RELIABILITY

Since 3 full experiments will be performed at each site, the prediction model for a given chemical will be applied three times in each laboratory. Concordance in the sensitiser/non-sensitiser classification will be evaluated within and between laboratories.

The analysis of reliability will also be performed on the RFI, EC150 and EC200 values as obtained by the participating laboratories.

Reliability will be explored both as WLR and BLR.

Normality of data distributions will be checked using the Shapiro-Wilk test; in case of non-normality of the data, variance stabilizing transformations will be applied (e.g. logarithmic, square-root). If the transformation still does not normalize the data, non-parametric analyses will be performed (i.e. Kruskal-Wallis test).

WLR: The variability within a single laboratory will be explored separately for CD86 and CD54 values. It will be evaluated using the concordance among runs and also a one-way analysis of variance including the run as unique independent factor.

The mean RFI, EC150 and EC200 values generated in each independent experiment will be compared for each chemical, using a one-way analysis of variance (ANOVA). The ANOVA model will include only the Experiment factor as independent variable.

BLR: The variability between laboratories will be explored separately for CD86 and CD54 values, as well as for the CV75 value obtained in the dose-finding experiments.

CV75 values will be tested for differences in mean before analyzing the variability of CD86 and CD54.

In case more than one measurement is produced by at least one laboratory, then CV75 will be compared using a one-way analysis of variance (ANOVA) including the Laboratory as unique independent factor.

Since the CV75 estimate is produced by each laboratory, then two estimates will be considered different if a relative difference of at least 20% (absolute value) is found.
Relative difference between two estimates is defined as follows:

$$\Delta = \frac{|Estimate1 - Estimate2|}{Estimate1}$$

If no significant difference is found between the CV75 estimates across laboratories, then all the doses tested for the assay will be taken into account for the Between Laboratory Reproducibility analysis; otherwise only the doses falling in the same range will be included in further analyses.

The RFI, EC150 and EC200 values for CD86 and CD54 will be averaged across the three experiments for each concentration. Differences in means between the averaged RFI values generated by the laboratories will be checked using a comparison by chemical, using a one-way analysis of variance (ANOVA).

The ANOVA model will include only the Laboratory factor as independent variable.

PREDICTIVE CAPACITY

Classifications (sensitiser versus non-sensitiser) for each chemical will be derived from the RFI values obtained for CD86 and CD54, according to the predefined prediction model.

2x2 contingency tables, comparing the prediction results with the existing proposed classification, will be built in order to calculate sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test

3) MUSST

RELIABILITY

Since 3 full experiments will be performed at each site, the prediction model for a given chemical will be applied three times in each laboratory. Concordance in the sensitiser/non-sensitiser classification will be evaluated within and between laboratories.

The analysis of reliability will also be performed on SI and EC150 values as obtained by the participating laboratories.

Reliability will be explored both as WLR and BLR.

Normality of data distributions will be checked using the Shapiro-Wilk test; in case of non-normality of the data, variance stabilizing transformations will be applied (e.g. logarithmic, square-root). If the transformation still does not normalize the data, non-parametric analyses will be performed (i.e. Kruskal-Wallis test).

WLR: The reproducibility within each laboratory will be evaluated using the concordance of the predictions among the performed runs.

Moreover the mean SI ad EC150 values generated in each independent experiment will be compared for each chemical, using a one-way analysis of variance (ANOVA). The ANOVA model will include only the Experiment factor as the independent variable.

BLR: The predictions obtained for the same chemical in the different laboratories will be compared.
Moreover, Tthe SI and EC150 values will be averaged across the three experiments for each concentration. Differences in means between the averaged SI values generated by the laboratories will be checked using a comparison by chemical, using a one-way analysis of variance (ANOVA). The ANOVA model will include only the Laboratory factor as an independent variable.
The predictions obtained for the same chemical in the different laboratories will be compared as well.

PREDICTIVE CAPACITY

A single classification (sensitiser versus non-sensitiser) for each chemical will be derived from the SI values, according to the predefined prediction model.

2x2 contingency tables, comparing the prediction results with the existing proposed classification, will be built in order to calculate sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test

For all the statistical tests included in the analysis, significance will be set at 0.05.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
In-Vitro Methods Unit
European Centre for the Validation of Alternative Methods (ECVAM)

Direct Peptide Reactivity Assay (DPRA)
human Cell Line Activation Test (h-CLAT)
Myeloid U937 Skin Sensitisation Test (MUSST)

Phase III Pre-validation Study

Draft Project Plan

Content

Study Objective and Goals

Study Objective

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The Phase III Pre-validation study will be conducted in accordance with the principles and criteria documented in the OECD Guidance Document No 34, on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005) and according to the Modular Approach to Validation (Hartung et al., 2004).

Study Goals

The primary goal of this Phase III Pre-validation Study is an evaluation of the transferability and reliability (reproducibility within and between laboratories) of the DPRA, h-CLAT and MUSST when challenged with a set of coded chemicals.

Secondary goals of the study are:

1. A preliminary evaluation of the ability of the three tests to reliably discriminate skin sensitising (S) from non-sensitising (NS) chemicals as defined by the Globally Harmonised System (GHS) for the classification and labelling of substances for skin sensitisation (category 1; no category) and as implemented in the European Commission Regulation (EC) No 1272/2008 (EU, 2008b) on classification, labelling and packaging (CLP) of substances and mixture.

2. A preliminary evaluation of the ability of the three tests to sub-categorise skin sensitising chemicals into Sub-category 1A and Sub-category 1B as adopted in the 3rd revised version of the GHS.

Study Coordination and Sponsorship

Study Coordination

The overall study coordination will be conducted by ECVAM. This will include the organisation of all the different aspects of the pre-validation study including meetings and teleconferences. ECVAM will also be responsible for producing standard reporting templates.

Study Sponsorship

ECVAM will finance:

- The study coordination
- The management of the study (including support and management of the VMT meetings)
- The travel and accommodation costs for its relevant personnel to be trained at the lead laboratories
- The purchase, coding and distribution of chemicals to the laboratories
- The conduct of the DPRA and h-CLAT at the In Vitro Methods Unit's laboratories
- The costs of subcontracting external laboratories as additional sites contracted to carry out the three test methods as part of this study.
- The independent statistical support
- The independent QC audit of the data
- The publication of the study findings

JaCVAM, Kao, Shiseido will finance:

- The participation of the h-CLAT lead laboratories (Kao and Shiseido) representatives at the VMT meetings and other related activities.
- The conduct of the h-CLAT at the Shiseido and Kao laboratories
- The onsite training to the personnel of the other two laboratories involved in the prevalidation of the h-CLAT

Procter & Gamble will finance:

- The conduct of the DPRA at the P&G laboratories
- The onsite training to the personnel of the other two laboratories involved in the pre-validation of the DPRA

L'Oréal will finance:

- The conduct of the MUSST at the L'Oréal laboratories
- The onsite training to the personnel of the other two laboratories involved in the pre-validation of the MUSST.

Organisation

The management structure of this study and the responsibilities of the Validation Management Team are shown in Figure 1.

The Validation Management Team (VMT) is composed of:

Validation Management Group (VMG)

Chair (David Basketter)
Co-chair (Silvia Casati)
Representative of the coordinating organisation (Alexandre Angers)
Chair of the Chemical Selection Group (CSG) (Thomas Cole)
ECVAM biostatistician (André Kleensang; alternate Anna Compagnoni)
Industry representative (Pierre Aeby)
External expert (Sebastian Hoffmann)
External expert (Jon Richmond)

Lead laboratory Representatives

Procter & Gamble (G. Frank Gerberick)
L'Oréal (Jean Marc Ovigne)
Shiseido (Takao Ashikaga)
Kao Corporation (Hitoshi Sakaguchi)

Liaisons

JaCVAM (Hajime Kojima; alternate Yasuo Ohno)
NICEATM (William S. Stokes; alternate Eleni Salicru)
ICCVAM (Joanna M. Matheson; alternate Abigail Jacobs)
Health Canada (not identified)

The strategic decisions will be taken by the VMG only. Other members on the VMT will not have a voting right on such decisions. The lead laboratories representatives should only be consulted for technical issues and will not be involved on discussions regarding the chemical selection. The liaisons will be involved in all discussions but will not take part in strategic decision making.

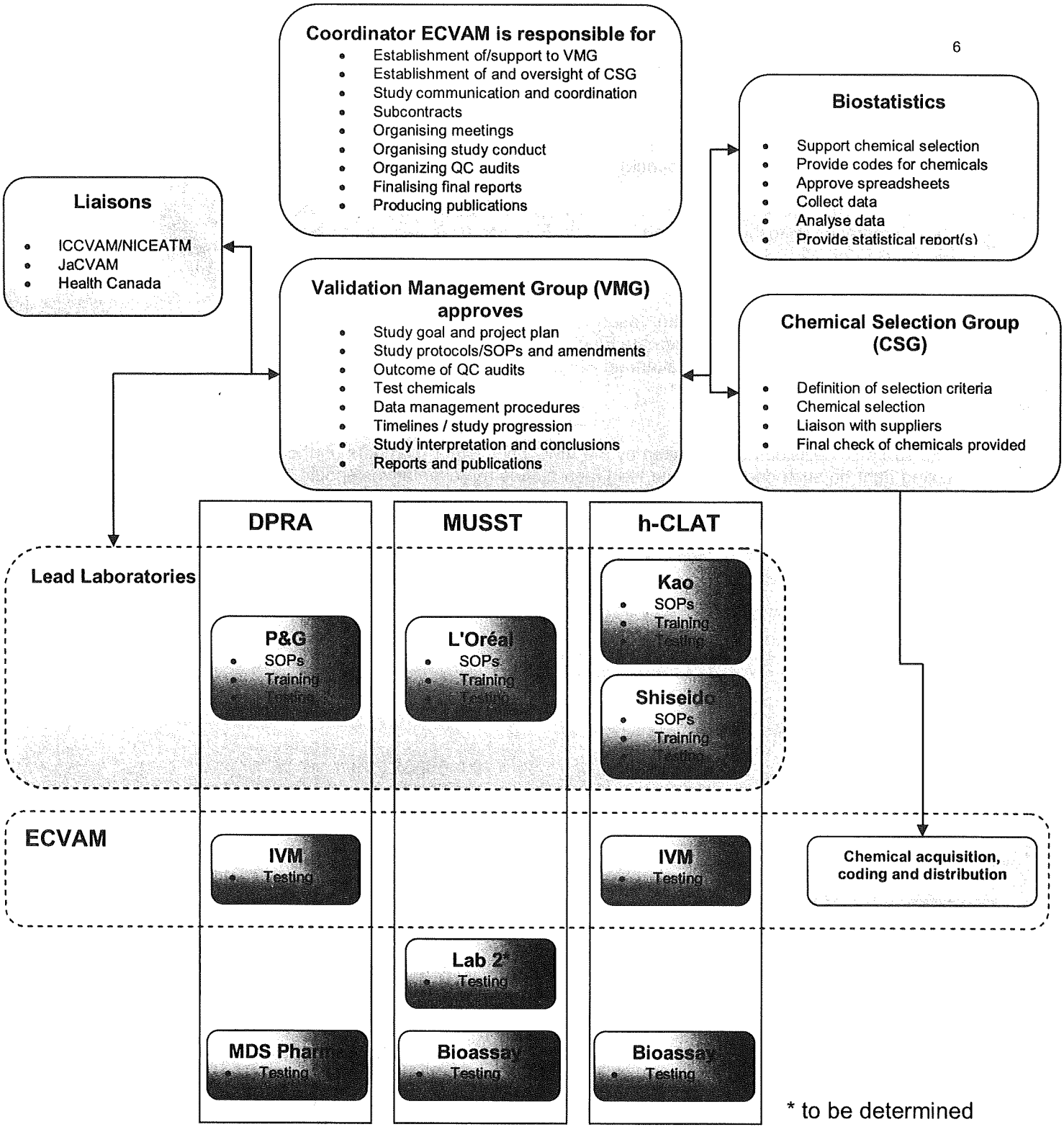

Figure 1: Management Structure of the study.

Chemical Selection Group

The roles and responsibilities of the chemical selection group are shown in figure 1

Chemicals Selection Group Members:
Thomas Cole (ECVAM)
Luca Tosti (ECVAM)
Anna Compagnoni (ECVAM)
William S. Stokes (NICEATM)
Dave Allen (NICEATM)
External expert TBD
External expert TBD

Testing Facilities Involved

Lead Laboratories

DPRA
Procter & Gamble Company
Miami Valley Innovation Center
P.O. Box 538707
Cincinnati, OH 45253-8707

h-CLAT
Global R&D, Safety and Microbial
Kao Corporation
2606 Akabane
Ichikai-Machi, Haga-Gun
Tochigi 321-3497 Japan

Shiseido Quality Assessment Center
2-12-1, Fukuura, Kanazawa-ku, Yokohama
236-8643, Japan

MUSST
L'Oréal
1 avenue Eugène Schueller BP 22
93601 Aulnay-sous-Bois CEDEX
France

2nd Laboratory DPRA, h-CLAT

In-House Validation and Training Laboratory
JRC.I.03 In-Vitro Methods Unit/ECVAM
European Commission
Joint Research Center
Institute for Health and Consumer Protection
Via E. Fermi, 2749
I-21027 Ispra, Italy

***2nd Laboratory MUSST,* TBD,**

3rd Laboratory DPRA

MDS Pharma Services
329 Impasse du Domaine Rozier
Les Oncins
69210 Saint Germain sur l'Arbresle
France

3rd laboratory h-CLAT, MUSST

Bioassay GmbH
Im Neuenheimer Feld 515
69120 Heidelberg
Germany

Testing Facilities and Key Personnel

General Capabilities

The laboratories (Testing Facilities) shall be capable of performing the following:

- The Lead Laboratories shall prepare Standard Operating Procedures (SOPs) for the DPRA, h-CLAT, MUSST and shall provide training to, and confirm the competence of, the technical staff of the other testing facilities.
- The Testing Facilities shall perform the assay according to the SOPs and the specified study phases.
- The Testing Facilities shall provide Study Phase Reports to ECVAM.
- Testing Facilities that are compliant with Good Laboratory Practices (GLPs) shall perform the study in accordance with GLPs.
- Testing Facilities that are not GLP compliant shall perform all aspects of the Study in the "spirit of GLP"following minimum quality requirements which is are defined in the section "Quality Assurance".
- All Testing Facilities shall adhere to this Study Plan and any authorised revisions or supplement, throughout the pre-validation study.

Testing Facility

The Testing Facility shall have competence in performing the DPRA, h-CLAT and MUSST and shall provide competent personnel, adequate facilities, equipment, supplies, proper health and safety guidelines policies and practices, and satisfactory quality assurance procedures.

Personnel

Study Directors

Each Testing Facility shall appoint a Study Director, a scientist of appropriate education, training, and experience in the assay performance. The Study Director represents the single point of study control with ultimate responsibility for the overall technical conduct of the study as well as for the interpretation, analysis, documentation and reporting of the results and GLP adherence or implementation in the spirit of GLP.

The Study Director must maintain records of the qualifications, training, experience, and a job description for each individual involved in the pre-validation study as well as for providing them with the SOPs for the study incorporating any pertinent information obtained from the Study Plan and Test Methods Protocol.

The Study Director is responsible for the collection and archiving of data generated by his/her laboratory and to send them to the Statistician contact person of the Management Team according to the deadlines established in the Study Plan.

The Study Directors are also responsible for sending timely Study Reports to the contact person of the Management Team that will monitor the progress of the Study. Such reports should include all relevant raw data as well as all deviations from the study plan and SOPs.

DPRA:

- TBA Leslie Foertsch (Procter & Gamble)
- Siegfried Morath (In Vitro Methods Unit)
- Fabien Marguerite (MDS Pharma)

hCLAT:

- Hitoshi Sakaguchi (Kao)
- Takao Ashikaga (Shiseido)
- Ingrid Langezaal (In Vitro Methods Unit)
- Axel Hohenstein Sven-Michael Cords (Bioassay)

MUSST:

- Cécile Piroird (L'Oréal)
- TBA (Lab #2)
- Sven-Michael CordsAxel Hohenstein (Bioassay)

Quality Assurance (QA) DirectorOfficer

For Testing Facilities that are GLP compliant the Quality Assurance Director Officer shall monitor the pre-validation study and assure conformity with GLP requirements, and document and report compliance failures for all aspects of the study (facilities, equipment, personnel, methods, practices, records, controls, SOPs, final reports (for data integrity), and archives). The Quality Assurance Director is entirely separate from and independent of the personnel engaged in the direction and conduct of that study.

Testing Facilities which are not GLP-compliantperforming the study in the spirit of GLP, shall appoint an individual to assure that all records, documents, raw data and reports are available to the Validation Management Team if an inspection is requested, and ensure that the quality assurance provisions detailed in the section "Quality Assurance" (see below) have been implemented.

- Gerard Bowe (In Vitro Methods Unit)
- John Troutman TBA (Procter & Gamble)
- Minehiro Okuda (Kao)
- Tuyoshi Yoshida (Shiseido)
- Cécile PiroirdJean Marc Ovigne (L'Oréal) (to be discussed)

- Natascha Hach (Bioassay)
- Catherine Liang (MDS Pharma)

Safety Officers

A designated **Safety Officer** (not otherwise involved in the actual conduct of the pre-validation study) at each participating laboratory will receive the blinded (coded) test chemicals and shall transfer the test chemicals to those responsible for safe storage of the materials or performance of the test of the Laboratory without revealing the details of the contents of the test chemical containers. Sealed material safety data sheets will accompany the test chemicals and the Safety Officer shall retain the package until the completion of the pre-validation study. At the end of the pre-validation study, the Safety Officer shall return the unopened package to the Management Team. If any Testing Facility personnel should open the package at any time during the pre-validation study, the Safety Officer shall promptly notify the Management Team through the designated contacts.

- Salvador Fortaner (In Vitro Methods Unit)
- Cindy RyanTBA (Procter & Gamble)
- Yukio Fukuda (Kao)
- Shigenobu Hagino (Shiseido)
- Alexandre Staropoliy (L'Oréal)
- Natascha Hach (Bioassay)
- Cédric Faura (MDS Pharma)

Experimental Team of at the Testing Facilities

The conduct of the DPRA, h-CLAT and MUSST require personnel trained and competent in the specific techniques and general laboratory procedures. Each individual engaged in the conduct of or responsible for the supervision of a pre-validation study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties.

When the same site is responsible for more than one study, it is important required that different individuals perform the methods.

Experimental Team:

DPRA:

- Leslie Foertsch (Procter & Gamble)
- Nicholaos Parissis (In Vitro Methods Unit)
- Sylvie Borget (MDS Pharma)
- Raphaëlle VoutaTBA (Lab #3MDS Pharma)

hCLAT:

- Takao AshikagaTBA (Shiseido)
- Yuko NukadaTBA (Kao)
- Charlotte Bostroem (In Vitro Methods Unit)
- Kirstin Young (Bioassay)

MUSST:

- TBA (L'Oréal)
- TBA (Lab #2)
- Ursula Vettermann (Bioassay)

Note: Tobias Nehrbaß will supervise the flow cytometry analyses for both the hCLAT and MUSST experiments at the Bioassay site

Quality Assurance

GLP compliant laboratories:

GLP-compliant laboratories shall conduct this pre-validation study in compliance with Good Laboratory Practice Standards (OECD, 1999).

Non GLP compliant laboratories

For the laboratories participating in the pre-validation study which do not have formally implemented GLP, it is considered that the following requirements (Balls, et al., 1995) are essential for the mutual acceptance of information produced in the pre-validation process:

- Qualified personnel, and appropriate facilities, equipment and materials shall be available
- Records of the qualifications, training and experience, and a job description for each professional and technical individual, shall be maintained.
- SOPs shall be established and followed.
- A study plan shall be provided, and any amendments to this shall be documented.
- For each study, an individual with appropriate qualifications, training and experience shall be appointed to be responsible for its overall conduct and for any report issued.
- Apparatus used for the generation of data shall be inspected regularly, cleaned, maintained and calibrated according to SOPs and manufacturers' instructions. Records of these processes shall be kept, and made available for inspection on request.
- Reagents shall be labelled, as appropriate, to indicate their source, identity, concentration and stability. The labelling shall include the preparation and expiry dates, and specific storage conditions.
- The origin of the biological system shall be well defined, and its homogeneity and stability shall be assured.
- All data generated during a study shall be recorded directly, promptly and legibly by the individual(s) responsible. These entries shall be attributable and dated.
- Data generated as direct computer input shall be identified at the time of input by the individual(s) responsible.
- All changes to data shall be identified with the date and the identity of the individual responsible, and a reason for the change shall be documented at the time.

Study Phases and Schedule

The study shall be undertaken in two structured and sequential phases: