Appendix 1

Project Plan

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test
Phase III Prevalidation
Project Plan

Version	Author	Reviewer	Approver	Date of approval
4	Silvia Casati	Alexandre Angers	VMG	19/11/2010

Document history

Version	Date	Drafted by	Comments
2	03/06/2010	Alexandre Angers	Updated on the basis of the comments received from the laboratories
3	24/06/2010	Alexandre Angers	Removed obsolete Annex II Added Annex II: Amended Timelines following the review of the study progress at the 4th VMT meeting of June 23rd-24th, 2010
4	19/11/2010	Alexandre Angers	Added Annex III Clarified secondary study goal
5	5/05/2011	Alexandre Angers	Added Ricerca as a 4th laboratory for the MUSST Added Annex IV

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

Phase III Prevalidation Study

Project Plan

Study Objective and Goals

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) with a view to 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 (OECD, 1992),and Method B06 of EU Regulation 440-2008 (EU 2008a), and the Local Lymph Node Assay, OECD TG 429 (OECD, 2002, and Method B42 of EU Regulation 440-2008 (EU 2008a).Achieving this ultimate goal will require data integration and further validation activities which are outside the objective of this study..

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 mixtures.

2. Where possible, a preliminary consideration of the ability of the three tests to contribute to sub-categorisation of skin sensitising chemicals, e.g. 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 necessary Validation Management Group (VMG)/Validation Management Team (VMT) meetings and teleconferences, preparation of meeting minutes, management of a web-site dedicated to the study for sharing documents with the VMG/VMT and coordination of communications between all parties.

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 and for the personnel of the Finnish Centre for Alternative Methods (FICAM) to be trained at the lead laboratories
- The purchase, coding and distribution of chemicals to the laboratories
- The purchase and supply of the necessary antibodies to FICAM for the conduct of the MUSST assay.
- 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 if appropriate
- 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 of 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 of 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 of the personnel of the other two laboratories involved in the pre-validation of the MUSST.

FICAM will finance:

- The conduct of the MUSST at the FICAM laboratories

Organisation

The management structure of this study and the responsibilities of the VMT are shown in Figure 1.

The 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 (Anna Compagnoni (up to January 2011), André Kleensang (up to September 2010))
Industry representative (Pierre Aeby)
External expert (Sebastian Hoffmann)
External expert (Jon Richmond)
JaCVAM representative (Aiba Setsuya)

Lead laboratory Representatives

Procter & Gamble (G. Frank Gerberick)
L'Oréal (Jean Marc Ovigne, Nathalie Alépée)
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 voting rights on such decisions. The lead laboratories representatives should only be consulted for technical issues and will not be involved in discussions regarding the chemical selection. The liaisons will be involved in all discussions but will not take part in strategic decision making.

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)
William S. Stokes (NICEATM/ICCVAM)

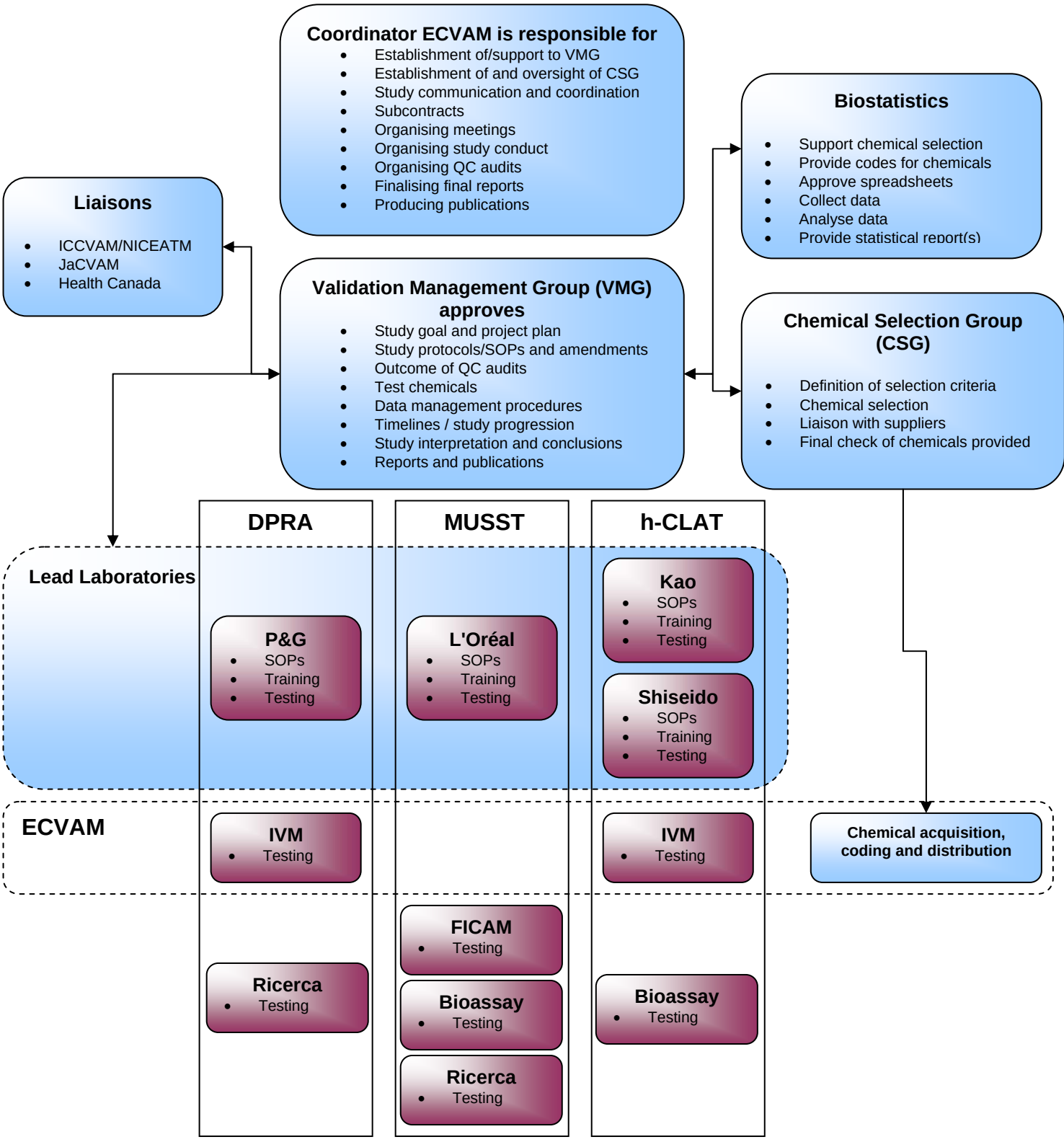

Figure 1: Management Structure of the study.

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
In-Vitro Methods Unit/ECVAM
Institute for Health and Consumer Protection
Joint Research Centre
European Commission
Via E. Fermi, 2749
I-21027 Ispra, Italy

2nd Laboratory MUSST

Finnish Centre for Alternative Methods (FICAM)
University of Tampere
Medical School
Medisiinarinkatu 3
33014 Tampere
Finland

3rd Laboratory DPRA, 4th Laboratory MUSST

Ricerca Biosciences SAS
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 Study Personnel

General Capabilities

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

- The Lead Laboratories shall prepare finalised Test Method Protocols 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 in adherence to the finalised Test Method Protocols and to the study phases described in the Project Plan.
- The Testing Facilities shall provide Study Phase Reports to ECVAM.
- Testing Facilities that are compliant with Good Laboratory Practices (GLP) shall perform the study in accordance with GLP.
- Testing Facilities that are not GLP compliant shall demonstrably perform all aspects of the Study adhering to the minimum quality requirements which are defined in the section "Quality Assurance".
- All Testing Facilities shall adhere to this Project 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.

Study 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, the documentation and reporting of the results, as well as GLP adherence or adherence to the minimum quality requirements.

The Study Director is responsible for the collection and archiving of data generated by his/her laboratory and to send them to the contact persons (Alexandre Angers, Silvia Casati) of the VMG according to the deadlines established in the Project Plan.

The Study Directors are also responsible for sending timely Study Reports to the contact persons of the VMG that will monitor the progress of the Study. Such reports should include all relevant experimental data as well as details of commentary on all deviations from the Project Plan and Test Method Protocols.

The study directors will be the primary contact point for the communications between the VMG and the testing facilities unless otherwise requested.

Quality Assurance (QA) Officer

For Testing Facilities that are GLP compliant the Quality Assurance Officer shall 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 Officer is entirely separate from and independent of the personnel engaged in the direction and conduct of that study.

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

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 the responsible personnel of the laboratory. Sealed Material Safety Data Sheets (MSDS) 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 VMG contact persons. If any Testing Facility personnel should open the package at any time during the pre-validation study, the Safety Officer shall promptly notify the VMG through the designated contacts.

Experimental Team

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 required that different individuals perform the experimental work involved in each study.

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.
- 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.
- Instruments used for the generation of experimental data shall be inspected regularly, cleaned, maintained and calibrated according to established SOPs, if available, or to 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.
- 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.
- 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 and explained at the time.

Study Phases and Schedule

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

1. Phase A, for the training of the participating laboratories, for test method transfer and for confirmation of the Test Method Protocols.
2. Phase B, for the assessment of the protocol performance by testing, under blind conditions in all the laboratories, the 24 chemicals that are selected, coded and distributed independently (15 of which being tested three times at each site).

Phase A

Phase A covers the training of the laboratory personnel at the lead laboratories, Procter & Gamble for the DPRA, L'Oréal for the MUSST, Shiseido and Kao for the h-CLAT (Stage I), and the testing of a defined number of chemicals in their own laboratory (Stage II).

Stage I: The lead laboratories will be responsible for issuing a definitive Test Method Protocol and a Training Plan, for training personnel from the other testing sites, and for releasing a Training Report (Phase A Stage I Training Report) on the outcome of the training.

Stage II: The trained personnel will transfer the test method to their own laboratories. The laboratories will have to successfully perform the method procedures, and test in house a number of chemicals defined by the lead laboratories in their transfer study plans. The lead laboratories will be responsible for issuing a Transfer Plan, acceptable to the VMG, as well as a statement on the outcome of the transfer. The laboratories to which the method is transferred will be responsible for submitting a Transfer Report (Phase A Stage II Transfer Report) to the VMG.

Chemicals to be used for Phase A Stage II will not be tested coded and will have to be purchased by the laboratories to which the method is being transferred.

Results of Phase A Stage II will be reviewed by the VMT before a decision is taken to proceed to Phase B. If the Phase A Stage II results do not meet the transfer acceptance criteria, the VMG will work with the testing facility and the lead laboratory to identify the problems and determine what further action is to be taken. Each laboratory will advance to phase B once they have submitted Transfer Reports acceptable to the VMG.

Phase B

During Phase B, the laboratories will generate data to evaluate the test methods' reproducibility and for the preliminary assessment of their predictive capacity. 24 coded chemicals will be tested by each laboratory. The test items will include sensitisers and non-sensitisers. For the evaluation of the between-laboratory reproducibility the 24 chemicals will be tested once in each laboratory. For the evaluation of the within laboratory reproducibility a subset of 15 chemicals will be tested two further times in each laboratory.

Stage I: During this stage, the test methods will be evaluated with a set of 9 coded chemicals tested once (1 experiment involves performing a number of qualified runs required to apply once the prediction model as defined in the Test Method Protocol of each method). It is the responsibility of the laboratories to prepare and submit a Phase B Stage I Report upon completion of testing. Progression to Phase B Stage II is dependent upon decision of the VMG following review of the submitted data.

Results of Phase B Stage I will be reviewed by the VMG before progression. If the preliminary analysis of Stage I data raises concerns regarding the proper implementation/conduct of the tests at any of the testing facilities, the VMG will then work with the testing facility and the lead laboratory to identify the problems to determine what further action shall be taken. Each laboratory will advance to Stage II once they have submitted Stage I report acceptable to the VMG.

Stage II: During this stage, the test methods will be evaluated with an additional set of 15 coded chemicals tested 3 times (3 independent experiments, see definition above). It is the responsibility of the laboratories to prepare and submit a Phase B Stage II Report acceptable to the VMG upon completion of testing..

The within- and between-laboratory reproducibility will be evaluated in light of the objectives of the study determined beforehand by the VMG. Data generated during Phase B will also be used for the preliminary assessment of the predictive capacity of the test methods.

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

Communication between laboratories

The participating laboratories are allowed to freely communicate and meet during the training and transfer phases of the validation study. Such meetings will be organized by the lead laboratories and can occur without a formal approval by the VMG. However, during the testing phase (Phase B), the participating laboratories will no longer contact each other without the previous knowledge and approval by the VMG. All VMG approved meetings or other forms of communication between the participating laboratories during the testing phase will be organized by the Study Coordinator ECVAM in collaboration with the lead laboratories and summary details of the information exchanged made known to the VMG.

Reports

All reports shall be provided to the designated contacts of the VMG in electronic format (i.e. e-mails with attachments)

Monthly reports

For Phase B I and Phase B II each testing facility will provide a Monthly Progress Report, on the first Monday of each calendar month. These reports will include interim information of the study progress, and should follow the template shown in Annex I. In these Monthly Progress Reports, all the experimental data generated during the previous month should be sent to the VMG designated contacts using the supplied data reporting templates.

Phase A Stage I Report

The Training Report shall document all the experimental procedure steps that have been performed and the critical points that are discussed during the training session including conclusions regarding the outcome of the training.

Phase A Stage II Report

The Transfer Report shall describe the transfer, plan and the transfer acceptance criteria relevant to the report , as well as the transfer results.

Phase B Stage I Report

At the conclusion of Phase B Stage I a report shall be provided by each Study Directors to the designated contacts of the VMG and shall be submitted in electronic format unless otherwise specified in the contract signed by the contracting laboratories with the European Commission.

Information/data contained in the report should have undergone the appropriate quality checks.

Phase B Stage II Report
At the conclusion of Phase B Stage II a report shall be provided by each Study Director to the designated contacts of the VMG. The report shall be submitted in electronic format unless otherwise specified in the contract signed by the contracting laboratories with the European Commission.

Information/data contained in the report should have undergone the appropriate quality checks.

Templates for reporting study Phases B Stage I and B Stage II will be provided by ECVAM in due time.

Deliverables

The following table summarises the reports to be compiled for the purpose of this study and the estimated completion timelines. Timelines might need to be reviewed during the study.

PHASE	**REPORT**	**Estimated due Dates**		
End of Phase A Stage I (lead laboratories)	Training Report	March 31st, 2010		
End of Phase A Stage II (trained laboratories)	Transfer Report	June 15th, 2010		
		DPRA	**h-CLAT**	**MUSST**
End of Phase B Stage I (all laboratories)	Phase B Stage I Report	July 15th, 2010	August 31st, 2010	August 31st, 2010
End of Phase B Stage II (all laboratories)	Phase B Stage II Report	September 15th, 2010	March 15th, 2011	March 15th, 2011

Pre-validation Report

The pre-validation final report will be drafted by ECVAM with the support of the VMG and will include the results of the study and the VMG conclusions/recommendations on the outcome of the study. The draft study report shall be circulated to the lead laboratories for review and comments prior to finalisation. The VMG shall review all comments received and make revisions if deemed appropriate. The VMG is also responsible for approving the final version of the document.

Note: All the documentation sent to the VMT (text documents, spreadsheets, presentations etc.) should be compatible with Microsoft Office 2003 programs.

Test chemicals

Chemical Distribution Organisation

The In-House Validation and Training Laboratory of the In-Vitro Methods Unit/ECVAM (IHCP, JRC, European Commission) will be responsible for the purchase, independent coding and shipping of the chemicals tested in Phase B to the participating laboratories. The personnel involved in the chemical coding and distribution shall be independent from the personnel involved in the conduct of the pre-validation study.

Responsible: Thomas Cole (IVMU-IHCP- European Commission)

Chemical Coding

All test chemicals will be randomly coded. Each chemical will have a code that is unique for each Testing Facility and for each experiment. The chemicals codes will be generated and provided by ECVAM.

Health and Safety

Each Testing Facility shall conform to all local, state, and federal statutes in effect at the time of this pre-validation study. The designated Safety Officer shall be the point of contact for health and safety issues.

Receipt of Chemicals

Test chemicals will be packaged so as to minimize damage and risk to handlers during transit and will be shipped to the Testing Facility in accordance with relevant regulatory procedures. Chemicals will be packaged and shipped so as to conceal their identities. The Testing Facility shall be notified by the shipping organisation when the test chemicals are shipped, shall make proper provision for their receipt, and promptly acknowledge that they have been received.

The coded test chemicals together with the MSDS will be shipped to the Safety Officer. Upon receipt at the facility, the test chemicals shall be stored in appropriate storage conditions as indicated in the unsealed accompanying documentation **and stored up to six month following the submission of Phase B Stage II report to ECVAM.**

Each MSDS related to a specific chemical will be sealed in a single envelope, labelled with the corresponding chemical code, for use only in an emergency. At the end of the pre-validation study, the Safety Officer shall return the unopened MSDSs to the VMG contact persons. If any of the sealed envelopes containing the MSDS is opened by the laboratory, the Safety Officer shall immediately notify the VMG designated contacts.

The Study Director of each Testing Facility shall receive essential information about test chemical to allow performing the test method protocol. Upon receipt, each testing facility must complete and return the Test Chemical Receipt Report.

Handling of Test Chemicals

Appropriate routine safety procedures shall be followed in handling the test chemicals unless otherwise specified in the unsealed documentation supplied at the time of chemical distribution.

Test Facility personnel shall be instructed to treat all coded test chemicals as ***potential sensitisers*** and to dispose of laboratory waste as toxic waste. The health and safety information package provided to the Testing Facility Safety Officer shall be opened at the Testing Facility only in an emergency/need-to-know situation.

Preparation and Solubilisation of Test Chemicals

Laboratories participating in the study will **not** be instructed which specific solvent to use to solubilise the chemicals. Each laboratory shall determine the most appropriate solvent for a particular chemical following the solubilisation procedures described in the relevant Test Method Protocol.

Data Collection, handling and analysis

Experimental data will be collected using the data reporting templates and will be sent by the laboratories to the designated contacts of the VMG. A quality check of the reporting templates will be performed by the VMG designated contacts before transferring them to the ECVAM biostatistician in charge of their analysing them. ECVAM will be responsible for the quality control of the processes of data collection, handling and analysis, as well as of the final biostatistical report. The data management procedure is to be approved by the VMT.

Data will be analysed according to the statistical approaches approved by the VMG.

Records and Archives

At the end of the Pre-validation study, the original raw (if applicable) and processed data shall be submitted to ECVAM for storing and archiving. In addition, other records relevant to this Pre-validation study (instrument logs, calibration records, facility logs, etc.) should be made available for inspection upon request by the VMG.

Copies of all raw and derived data shall be stored and archived at the participating Testing Facility for at least five years after completion of the pre-validation study. The data which are stored electronically shall be periodically copied, and backup files shall be produced and maintained.

Alterations of the Project Plan

No changes in the Project Plan shall be made without the knowledge and consent of the VMG.

Study Timeline

The following table summarises the critical phases of the study and the tentative completion timelines. The reported timelines have been defined on the basis of the feedback received from all laboratories to the draft version of the Project Plan. Timelines may be reviewed and revised by the VMG during the study.

TASK	Estimated completion timelines		
Chemical Selection	January 13th, 2010		
Experimental Design	January 13th, 2010		
Finalised SOPs	February 15th, 2010		
Formal start of the study	March 1st, 2010		
End Phase A Stage I (training)- Lead Labs	March 31st, 2010		
Project Plan	April 30th 2010		
Chemicals coding and distribution	May 31tst, 2010		
End Phase A Stage II (transferability) -Trained Labs	June15th, 2010		
Updated SOPs (if necessary)	June 15th, 2010		
	DPRA	**h-CLAT**	**MUSST**
End of Phase B Stage I (testing) – All Labs	July 31st, 2010	August 31st, 2010	August 31st, 2010
End of Phase B Stage II (testing) – All Labs	September 15th, 2010	March 15th, 2011	March 15th, 2011

Documents and Data

1. ECVAM after consultation with the VMT supplies prevalidation study documentation 'in confidence' to participating laboratories. Unless and until ECVAM places these documents in the public domain, they may not be published or communicated/distributed to other third parties without the knowledge and consent of ECVAM after consultation with the VMT.

2. All study data generated by the contracted laboratories is the property of the European Commission/ECVAM. It may not be published communicated/distributed to other third parties without the knowledge and consent of the European Commission/ECVAM, and the knowledge of the VMT.

3. Study data generated by the lead laboratory are co-owned by the European Commission/ECVAM and the concerned laboratory. For the h-CLAT, data generated by the lead laboratories will be co-owned by the European Commission/ECVAM, JaCVAM, and the lead laboratories. Until the validation study has been concluded and the outcomes published by ECVAM and the VMT, this data may not be published or circulated/distributed to third parties without the knowledge and consent of ECVAM after consultation with the VMT.

4. ECVAM reserves the right to be the first to promptly publish and communicate the outcomes of the validation process.

References

OECD (1992). *OECD Guideline for the Testing of Chemicals No. 406*: *Skin Sensitisation,* 9pp. Paris, France: Organisation for Economic Cooperation and Development.

EU (2008a) Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). *Official Journal No **L 142**, 31/05/2008, p. 0001-0739.*

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OECD (2005). OECD Series on Testing and Assessment *No. 34*. *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, 96pp. Paris, France: Organisation for Economic Cooperation and Development.

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Anon. (2003). *Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Part 3: Health and Environmental Hazards*, pp. 151-158. New York, NY, USA, and Geneva, Switzerland: United Nations Organisation.

EU (2008b) Regulation (EC) No 1272/2008 (16 December 2008) of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. *Official Journal of the European Union **L 353**, (31/12/2008) p. 1-1355*.

OECD (1999) *OECD series on principles of good laboratory practice and compliance monitoring No 5, Compliance of Laboratory Suppliers with GLP Principles.* Paris, France: Organisation for Economic Cooperation and Development.

Balls, M., Blaauboer, B.J., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski, D., Spielmann, H. & Zucco, F. (1995). Practical aspects of the validation of toxicity test procedures. ECVAM Workshop Report 5. *ATLA* 23, 129–147

Annex I - Monthly Report Template

Testing Facility:

Author of Report:

Date:

Status of Activities:

Problems Encountered/Resolutions:

Projected Testing Schedule:

Annex II: Amended timelines following the review of the study progress at the 4th VMT meeting of June 23rd-24th, 2010

In view of the progress made, the following, updated study timelines were agreed upon. Timelines might need to be further reviewed during the study.

TASK	Estimated completion timelines		
	DPRA	**h-CLAT**	**MUSST**
End Phase A Stage II	August 31st, 2010	July 31st, 2010	August 31st, 2010
Updated SOPs	August 31st, 2010	August 31st, 2010	August 31st, 2010
End of Phase B Stage I	September 30th, 2010	November 30th, 2010	October 31st, 2010
End of Phase B Stage II	November 30th, 2010	November 30th, 2011	May 31st, 2011

Annex III: Amended timelines following the review of the study progress at the 5th VMT meeting of November 18th-19th, 2010

In view of the progress made, the following, updated study timelines were agreed upon. Timelines might need to be further reviewed during the study.

TASK	Estimated completion timelines		
	DPRA	**h-CLAT**	**MUSST**
End Phase A Stage II	February 28th, 2011	February 28th, 2011	February 28th, 2011
Updated SOPs	February 28th, 2011	February 28th, 2011	February 28th, 2011
End of Phase B Stage I	TBD	TBD	TBD
End of Phase B Stage II	May 31st, 2011	November 30th, 2011	November 30th , 2011

Annex IV: Amended timelines following the review of the study progress at the 6th VMT meeting of March 24th-25th, 2011

In view of the progress made, the following, updated study timelines were agreed upon. Timelines might need to be further reviewed during the study.

TASK	Estimated completion timelines		
	DPRA	**h-CLAT**	**MUSST**
End of Phase B Stage I	done	June 30th, 2011	September 30th, 2011
End of Phase B Stage II	August 2011	September 2012	May 2012

Appendix 2

Experimental Design

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
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
Experimental design

Version	Author	Reviewer	Approver	Date of approval
1	Anna Compagnoni Andre Kleensang Silvia Casati Alexandre Angers	VMG	VMG	19/11/2010

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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 pre-validation 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 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) (United Nations 2009) 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 used for the calculations are the following:

$\boldsymbol{\pi}$ → Expected proportion of concordant classifications among laboratories

$\boldsymbol{\pi - \delta}$ → 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.

α → 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 – β → Statistical Power: it's the probability to correctly detect an existing difference in the performances of the three laboratories.

β → 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. The expected proportion of concordant classifications is consistent with the data from the test submissions to ECVAM (see appendix I):

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 needed for the evaluation of the WLR should consist of 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 sensitisers and 8 non-sensitisers.
- For evaluation of the WLR, 15 chemicals tested two further times in each laboratory, the same subset being used at every site. 10 sensitisers and 5 non-sensitisers.

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 WLR and BLR of both the prediction results and the raw data, for the three tests evaluated.

Definitions:

For the purpose of the pre-validation study, the testing of a single test chemical within a single run will be defined as “test”.

A set of x number of test chemicals plus the relevant controls as defined in the corresponding SOP, all concurrently tested constitute a “run”.

The total number of tests, as defined in the corresponding SOP, needed to classify the chemical as sensitiser or non-sensitiser is called “experiment” (1 test for the DPRA, three tests for the h-CLAT, at least two tests for the MUSST).

Acceptance criteria for the run and the results for each of the chemicals tested within a single run (test) are defined in the corresponding SOP of each test method.

For the analysis of the BLR 3 independent qualified experiments (MUSST, h-CLAT), are needed.

Provisions for retesting in case the run or the results for a single chemical do not meet the acceptance criteria are described in the test method SOPs.

Statistical Analyses

Note: The following analysis plan can be amended if scientifically justified and with the agreement of the VMT.

Statistical analysis will be applied to the data from valid runs and experiments only.

The statistical analyses on the test method's reliability will mainly focus on the concordance of the predictions obtained. Reliability will be explored both as WLR and BLR.

Additionally, descriptive and inferential statistical analyses will be performed on the raw data obtained, as described in the following sections; because of the limited sample sizes available, the results of the inferential tests applied will not be used to draw any conclusion, but will only be given as additional descriptive information.

In particular, descriptive statistics such as mean, standard deviation and coefficient of variation (CV) will be generated for each repetition of the experiment; moreover, repetitions will be compared using t-test or Analysis of Variance (ANOVA) as appropriate.

In case significant differences are found, further exploratory analyses will be carried out in order to find the most likely source of variation (e.g. different CV75 values generated for the same chemical in the same laboratory).

For all the assays, descriptive statistics on positive and negative controls values will be calculated including frequency of invalid runs/experiments per chemical and laboratory.

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

Evaluation of the reproducibility will be performed in terms of the concordance of the prediction obtained in the different laboratories (BLR) or within the three experiments in the same laboratory (WLR). Other indexes for the evaluation of concordance, such as the Cohen's Kappa coefficient, will also be calculated, when applicable.

In order to combine the results from the 9 chemicals tested once per laboratory (Stage I), and the 15 chemicals tested three times in each laboratory (Stage II), approaches such as weighted pair wise comparisons of the results from the Stage II chemicals will be considered.

The expected performance of the test methods are implicit in the parameters used in the calculations of the sample size required (see above). These are consistent with the preliminary values for the BLR calculated from the test submissions (see Appendix I).

	DPRA	h-CLAT	MUSST
Concordance from submitted data:	0.91	0.87	0.88

However, based on the performance of methods previously evaluated at ECVAM, the target performance for this study will be set at 80% for the Between Laboratory Reproducibility and 85% for the Within Laboratory Reproducibility.

Please note that even though the predictive capacity analysis will include the calculation of Positive and Negative Predictive Values, these measures are dependent on the proportion of sensitisers and non-sensitisers in the chemicals selected for this specific study, and thus cannot be generalised

1) DPRA

RELIABILITY

Concordance in the sensitiser/non-sensitiser classification will be evaluated between laboratories and, for the subset of 15 chemicals, within laboratories.

Additionally, descriptive statistical analyses will be performed on the peptide depletion values obtained by the participating laboratories.

PREDICTIVE CAPACITY

Replicate depletion 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 constructed in order to obtain a preliminary evaluation of sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test. In addition 95% confidence intervals will be calculated for all parameters.

Moreover, as the prediction model allows a classification of the chemicals into four distinct 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 reference standard).

A preliminary estimate of the concordance between the "obtained" and "true" 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).

2) h-CLAT

RELIABILITY

Unlike the other methods which are tested in three laboratories, including one lead laboratory, the h-CLAT will be tested in four laboratories, including two lead laboratories. In order to compensate for this difference, two BLR calculations will be performed comparing the results of the two naïve laboratories to each lead laboratory, in turn (i.e. one comparing Lead Lab 1, Naïve lab 1 and Naïve lab 2, the other comparing Lead Lab 2, Naïve lab 1 and Naïve lab 2).

Concordance in the sensitiser/non-sensitiser classification will be evaluated between laboratories and, for the subset of 15 chemicals, within laboratories.

Additionally, descriptive and inferential statistical analyses will be performed on the CV75, RFI, EC150 and EC200 values as obtained by the participating laboratories.

The mean EC150 and EC200 values, applicable, will be compared between laboratories and among experiments for each chemical, using ANOVA.

CV75 values will be tested for differences in mean between laboratories, and between experiments within the same laboratory.

The variability within a single laboratory will be explored separately for CD86 and CD54 values. The RFI values for CD86 and CD54 will be averaged across the three runs and across all concentrations for each experiment. Differences in means between the averaged RFI values will be checked across experiments and across laboratories using a comparison by chemical, using ANOVA.

PREDICTIVE CAPACITY

A classification (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 obtain a preliminary evaluation of sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test. In addition 95% confidence intervals will be calculated for all parameters.

3) MUSST

RELIABILITY

Concordance in the sensitiser/non-sensitiser classification will be evaluated between laboratories and, for the subset of 15 chemicals, within laboratories.

Additionally, descriptive and inferential statistical analyses will be performed on the SI and EC150 values as obtained by the participating laboratories. The mean EC150 values, when available, will be compared for each chemical, using ANOVA. The ANOVA model will include Experiment and Laboratory factor as the independent variables.

The SI values will be averaged across the runs and across all concentrations for each experiment. Differences in means between the averaged SI values will be checked across experiments and across laboratories using a comparison by chemical, using ANOVA.

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 obtain a preliminary evaluation of sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test. In addition 95% confidence intervals will be calculated for all parameters.

Appendix I: Evaluation of concordance of prediction from the submitted data:

MUSST

sample	L'OREAL	BEIERSDORF	SHISEIDO	
Ethylene Diamine	S	S	S	Conc
PPD	S	S	S (?)	Conc
TNBS	S	S	S	Conc
Eugenol	S	S	S (?)	Conc
Isoeugenol	S	S	S (?)	Conc
Lactic Acid	NS	NS	NS	Conc
SDS	S (?)	S (?)	NS	Disc
Salycilic Acid	NS	NS	NS	Conc

Overall Concordance: 7 / 8 = 0.88

hCLAT

sample	LAB A	LAB B	LAB C	LAB D	LAB E	
Methylchloroisothiazolinone/ methylisothiazolinone (act. 1.5%)	NA	+	+	+	+	Conc
DNCB	+	+	+	+	+	Conc
1,4-Dihydroquinone	+	+	+	+	+	Conc
1,4-Phenylenediamine	NA	+	+	+	+	Conc
Methyldibromo glutaronitrile	NA	+	+	+	+	Conc
2-Mercaptobenzothiazole	NA	+	+	+	+	Conc
Cinnamic aldehyde	NA	-	+	+	+	Disc
Tetramethylthiuram disulfide	NA	+	+	+	+	Conc
Hexyl cinnamic aldehyde	-	-	-	-	-	Conc
Eugenol	+	+	+	+	+	Conc
Benzalkonium chloride	+	-	-	-	-	Disc
Sodium lauryl sulfate	-	-	-	-	-	Conc
Vanillin	-	-	-	-	-	Conc
Glycerol	NA	-	-	-	-	Conc
Salicylic acid	NA	+	+	+	+	Conc

Overall Concordance: 13 / 15 = 0.87

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DPRA

Chemicals	Prediction Model Results				
	P&G	Givaudan	L'Oreal	Kao	
5-Chloro-2-methyl-4-isothiazolin-3-one	+	+	+	+	Conc
4-Nitrobenzylbromide	+	+	+	+	Conc
Glutaraldehyde	+	+	+	+	Conc
pPhenylenediamine	+	+	+	+	Conc
Benzyl bromide	+	+	+	+	Conc
Propyl gallate	+	+	+	+	Conc
Methyldibromo glutaronitrile	+	+	+	+	Conc
Isoeugenol	+	+	+	+	Conc
Glyoxal	+	+	+	+	Conc
2-Hydroxyethyl acrylate	+	+	+	+	Conc
2-Mercaptobenzothiazole	+	+	+	+	Conc
Ethylenediamine	-	+	-	-	Disc
Cinnamaldehyde	+	+	+	+	Conc
Trimellitic anhydride	-	+	-	-	Disc
Citral	+	+	+	+	Conc
1-Chlorooctadecane	-	-	-	-	Conc
Cyclamen aldehyde	+	+	+	+	Conc
Imidazolidinyl urea	+	+	+	+	Conc
5-Methyl-2,3-hexadione	+	+	+	+	Conc
Penicillin G	+	+	+	-	Disc
Butyl glycidyl ether	+	+	+	+	Conc

Glycerol	-	-	-	-	Conc
Isopropanol	-	-	-	-	Conc
Methyl salicylate	-	+	+	-	Disc
Diethyl Phthalate	-	-	-	-	Conc
Vanillin	+	+	+	+	Conc
Octanoic acid	-	-	-	-	Conc
Propylene glycol	-	-	-	-	Conc
p-Benzoquinone	+	+	+	+	Conc
2,4-Dinitrochlorobenzene	+	+	+	+	Conc
Oxazolone	+	+	+	+	Conc
Formaldehyde	+	+	+	+	Conc
2-Phenylpropionaldehyde	+	+	+	+	Conc
Diethyl maleate	+	+	+	+	Conc
Benzylideneacetone	+	+	+	+	Conc
Farnesal	+	+	+	+	Conc
2,3-Butanedione	+	+	+	+	Conc
4-Allylanisole	+	+	+	+	Conc
Hydroxycitronellal	+	+	+	+	Conc
Butanol	-	-	-	-	Conc
6-Methylcoumarin	-	-	-	-	Conc
Lactic acid	-	-	-	-	Conc
4-Methoxyacetophenone	-	-	-	-	Conc

Overall Concordance = 39 / 43 = 0.91

Appendix 3

Chemical Selection report

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Validation of Alternative Methods Unit

Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test Validation Study

Chemical Selection Report

Version	Author	Reviewer	Approver	Date of approval
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1. Introduction

Regulatory hazard assessment of substances includes evaluation of their skin sensitisation potential. Due to the unavailability of validated alternative non-animal methods skin sensitisation hazard assessment is performed according to standard *in vivo* test methods: Guinea Pig Maximisation Test (GPMT) and Buehler Test (BT) [OECD TG 406 (OECD, 1992); Method B06, EU Regulation 440/2008 (EU, 2008a)]. In 2002 the Local Lymph Node Assay (LLNA) was validated as an alternative reduction (generally requiring fewer animals)/refinement method and adopted as an official test guideline [OECD TG 429 (OECD, 2002, 2010); Method B42, EU Regulation 440/2008 (EU, 2008a)].

Three *in vitro* test systems for skin sensitisation, developed as alternatives to the traditional *in vivo* procedures, were submitted to ECVAM for formal validation: 1) Direct Peptide Reactivity Assay (DPRA); 2) human Cell Line Activation Test (h-CLAT); 3) Myeloid U937 Skin Sensitisation Test (MUSST). The primary objective of the study was an evaluation of transferability and reliability (within- and between laboratory reproducibility).

Based on statistical estimation of worthy sample size and feasibility of experimental testing compatible with available resources and reasonable project timeframe, the study Validation Management Group opted for selection of 24 chemicals to be tested under blind conditions by the participating laboratories

The test chemicals for the validation study were selected by an independent Chemicals Selection Group (CSG) appointed by ECVAM and chaired by Dr. Thomas Cole (ECVAM). In addition to the chair, the CSG was composed of Dr. Luca Tosti (ECVAM); Dr. David Basketter (chair of the study) and Dr. Bill Stokes (NICEATM/ICCVAM). The chemical selection strategy was presented and approved by the VMG at its 1st meeting. The final list of chemicals was presented and endorsed by the VMG at its 4th meeting before the initiation of the blind testing phase.

This document describes the chemical selection process.

2. LLNA sensitisation potency, GHS classification, and reference chemicals

The standard local lymph node assay (LLNA) identifies contact allergens as a function of induced T lymphocyte proliferation in draining lymph nodes following topical exposure of mice to test chemicals. Proliferation is quantified as a stimulation index (SI) compared to vehicle controls where, for regulatory purposes, SI ≥ 3 defines a positive allergen. SI provides a parameter for dose-response measurement, allowing determination of relative potency (EC3) by linear interpolation. EC3 is an indicator of the relative amount of chemical required to induce SI = 3. Lower equivalent quantity (EC3) therefore corresponds to higher sensitisation potency. By convention, order of magnitude EC3 thresholds define potency categories, *i.e.* extreme (EC3 < 0.1); strong (0.1 < EC3 < 1); moderate (1 < EC3 < 10); weak (10 < EC3 < 100); no category (EC3 > 100).

Regulatory classification and labelling of chemicals implements the globally harmonised system (GHS) (UN, 2009), subordinate to EU Regulation 1272/2008 (EU, 2008b) on classification, labelling and packaging (CLP) of substances and mixtures. Under GHS, positive skin sensitisation is allocated category 1, with sub-categories 1A and 1B differentiating more severe from less severe potency. GHS assigns 'no category' to negatives (non-sensitisers). Criteria for classification from animal experiments depend on the particular test method (GPMT, BT, LLNA). For LLNA, where EC3 is determined, category 1A corresponds to EC3 ≤ 2, and category 1B corresponds to EC3 > 2. A secondary objective of

the validation study was a preliminary evaluation of the ability of the three tests to discriminate skin sensitisers from non-sensitisers (category 1 versus no category) with additional refinement to discrimination of sub-categories, 1A and 1B.

LLNA has become established as a benchmark for comparative evaluation of analogous methods that are based on the same or similar principle and that measure or predict the same endpoint. To facilitate consistency and smoother evaluation of similar method, OECD has defined three elements of performance standards: 1) essential test method components; 2) accuracy (predictive capacity) and reliability values; 3) a minimum list of reference chemicals (OECD, 2005). Specifically for skin sensitisation, a set of internationally harmonised LLNA performance standards (PS) have been elaborated (ECVAM, 2008; ICCVAM) including 22 reference chemicals. Significance of these PS reference chemicals was recognised in the chemical selection procedure for this *in vitro* validation study, with inclusion of relevant eligible chemicals.

3. Objective for *in vitro* testing

Considering essential relevance of sensitizing chemicals (and those with weak or moderate potency as more challenging to the *in vitro* systems) compared to non-sensitisers, for evaluation of reproducibility, the proportion of sensitisers to non-sensitisers was set at 2:1. Statistical estimation then yielded a testing programme for the 24 selected chemicals, as follows:

1) BLR (between laboratory reproducibility): The full complement of 24 chemicals (16 sensitisers and 8 non-sensitisers) to be tested once in all participating laboratories.
2) WLR (within laboratory reproducibility): A sub-set of 15 from the 24 chemicals (10 sensitisers and 5 non-sensitisers) to be tested twice again in each laboratory.

The validation schedule was then arranged in two phases:

Phase A: training of the transfer laboratories in relevant method, where selection and supply of chemicals would be the independent responsibility of the respective lead laboratory.

Phase B: testing of the 24 coded chemicals by the 10 laboratories participating in the ring trial validation study. In practice, this Phase B was arranged as two sequential stages. In Stage I, a randomly sampled sub-set of 9 chemicals would be tested by each laboratory in a single experiment, allowing the VMG an opportunity to monitorthe implementation of the testing programme and verify that no serious issues were arising before the majority of the testing was performed Assuming satisfactory pilot review, the study would then progress to Stage II, testing the remaining set of 15 chemicals in three independent experiments. Effectively, the full complement of 24 chemicals would have been tested at least once, for analysis of BLR, and 15 would have been tested in two additional independent experiments for evaluation of WLR.

4. Strategy for chemical selection

Combination of two recognised databases provided a convenient master source for review of eligible substances:

1) ICCVAM database of 103 chemicals, subsequently supplemented with some unpublished additions, provided by NICEATM ((ICCVAM database (NICEATM).

2) LLNA database of 341 chemicals from compilations published in the scientific literature (Gerberick et al., 2005; Kern et al., 2010).

Taking into account occurrence of duplication between the two databases, their integration resulted in an effective combined database of 369 chemicals. The database compiles the available skin sensitisation classifications from BT, GPMT and/or LLNA references, including relative potency (EC3 value) and GHS classifications. In addition, the database compiles physical-chemical properties and miscellaneous information such as commercial availability and purity quality.

The basis of chemical selection was to provide a range of sensitisation potency (*i.e.* extreme, strong, moderate, weak) ranked according to LLNA EC3 values. Inclusion of chemicals of moderate and weak potencies would challenge the *in vitro* test method sensitivity (ability to detect positives). Similarly, inclusion of non-sensitisers would test *in vitro* test method specificity (ability to detect negatives,). Furthermore, the chemical selection would aim for a balance of physical state (solid versus liquid) and would avoid association of structural analogues, unless contrasting skin sensitisation potential was evident.

During their respective development and optimisation, the three *in vitro* methods had been used to evaluate certain chemicals listed in these databases, as described in the original submissions of the methods to ECVAM. Acknowledging this, the chemical selection for this study was designed to include:

- A small quota of "tested" substances (i.e. substances reported as being previously tested by the method in the original submission to ECVAM
- A majority of "untested" substances (i.e. substances not found as part of the being reported as previously tested chemicals in the original submission to ECVAM)

To ensure parity between the three *in vitro* methods object of the validation study, the only "tested" chemicals that were considered were those already tested by all three methods and that have been correctly predicted by each method with respect to the *in vivo* classifications. The only exception to this criterion was the inclusion in the final list of 2-Mercaptobenzothiazole, a chemical previously tested in the DPRA and in the h-CLAT but not in the MUSST.

Inclusion in the final list of a proportion of chemicals already successfully tested provided an opportunity to confirm the reproducibility of the test method with these chemicals when tested under blind conditions and by other laboratories.

The ratio of tested to untested chemicals was set in advance at 1:2 by the VMG (i.e., 8 tested, 16 untested).

5. Criteria for chemical selection

A primary eligibility criterion for the chemical selection was the availability of quality assured *in vivo* data for comparative evaluation of *in vitro* results. In particular, availability of both LLNA and GPMT *in vivo* data, with concordance of corresponding skin sensitisation classification as an assurance of quality, formed the basis for short-listing candidate chemicals. Availability of accepted human data was adopted as a secondary criterion, in case of insufficient eligibility under the primary criterion.

The source database yielded only 11 eligible chemicals reported as previously tested in all three methods, qualified unequivocally as consistently and correctly classified by the three methods. From the same source database, 215 untested chemicals were found. However, 191 were registered with LLNA *in vivo* data alone, leaving only another 24 eligible for selection by the primary criterion.

Although 35 chemicals (11 tested, 24 untested) might have been sufficient for reduction to a definitive set of 24, final confirmation of the chemicals would be subject to review of individual suitability and commercial availability. Evidently, this would lead to an eventual shortfall unless supplemented by alternatives. Therefore, in collaboration with NICEATM, 8 additional untested chemicals were identified from an unpublished updated version of the ICCVAM database, increasing the total number of candidates to 43, and now providing both an adequate and practical shortlist.

Chemicals were eliminated from the shortlist for specific reasons, i.e., unknown potency category, structural analogy, incompatible physical properties, etc. The final selection admitted 9 LLNA performance standards (PS) reference chemicals, present among the candidate shortlist. In particular, nickel chloride and xylene (both with ambiguous *in vivo* classification from LLNA and GPMT, but with known human response) were considered eligible under the secondary selection criterion. Nickel chloride (human positive, GPMT positive, LLNA negative) is accepted as a PS true positive reference chemical (i.e., LLNA false negative). Xylene (human negative, LLNA positive) is accepted as a PS true negative reference chemical (i.e., LLNA false positive). In addition, Kathon CG, a commercial aqueous mixture including 1.2% CMI (5-chloro-2-methyl-4-isothiazolin-3-one) was selected, making exception to a general preference for pure substances with discrete properties. CMI is an LLNA PS reference chemical of extreme potency, and the commercial preparation is a recognised source.

The review of individual suitability reduced the candidate list to 26 fully eligible chemicals. Subsequently however, 3 of these (all moderate potency) were found to be not readily available commercially, effectively leaving only 23 selected. Therefore, expediency justified inclusion of 2-mercaptobenzothiazole, an LLNA PS reference chemical, also with moderate potency. This substance had been successfully tested in DPRA and h-CLAT, but not tested in MUSST. Rationale for inclusion was the assumption that MUSST would also yield a correct prediction, concurrent with h-CLAT (both cell-based assays).

Following acquisition of the chemicals, laboratory solubility experiments were conducted (at ECVAM) according to each *in vitro* test method SOP, to confirm unbiased compatibility with the three systems to avoid loosing information from the study because of solubilisation problems.

Table 1 lists the 43 candidate chemicals, with summary note on selection/rejection. Table 2 consolidates the 24 definitively selected set, covering a range of skin sensitisation potential and balance of physical state. The 24 chemicals fulfil the strategic objective of 8 tested versus 16 untested, with balanced representation of potency and physical state. Table 3 compiles the full official classification and labelling of the 24 selected chemicals, according to Annex VI of Regulation 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures.

Included in Annex are references to *in vivo* studies for the selected set of 24 chemicals.

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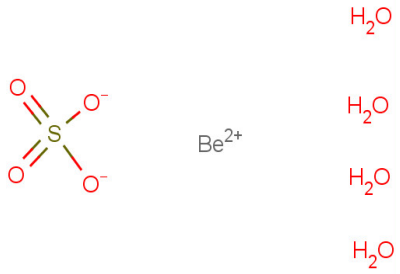
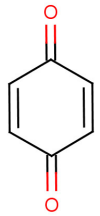
SSPS_Chemical selection report.pdf

Table 1. 43 eligible chemicals: 1st 24 selected

	Name	CAS #	Structure	Remarks
1	beryllium sulphate	7787-56-6		**Selected (*in vitro* untested)**
2	Kathon CG (1.2% CMI)	26172-55-4 (CMI)		**Selected (*in vitro* tested)**: aqueous solution containing 1.2% CMI (5-chloro-2-methyl-4-isothiazolin-3-one); LLNA PS reference chemical
3	benzoquinone	106-51-4		**Selected (*in vitro* tested)**
4	4-phenylene diamine	106-50-3		**Selected (*in vitro* tested)**: LLNA PS reference chemical

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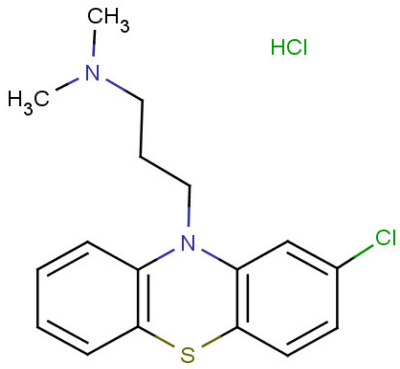
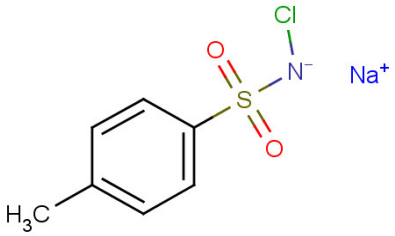
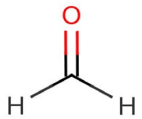
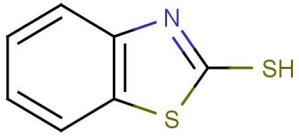
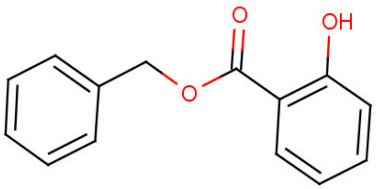
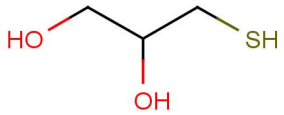
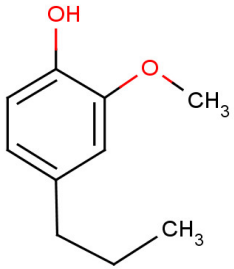
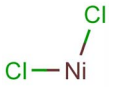
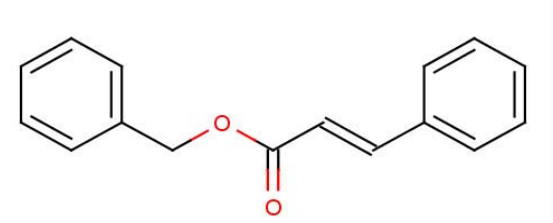
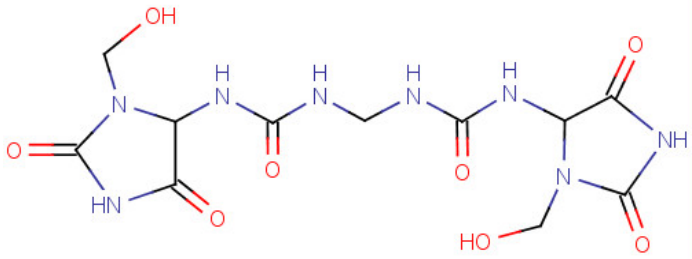
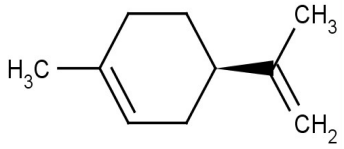
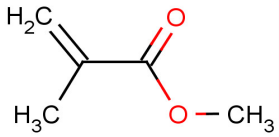
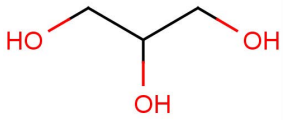
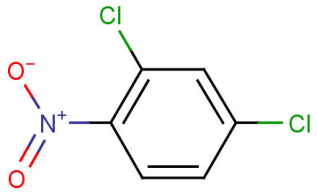
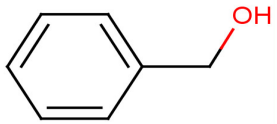
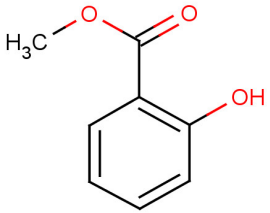
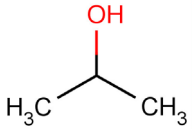
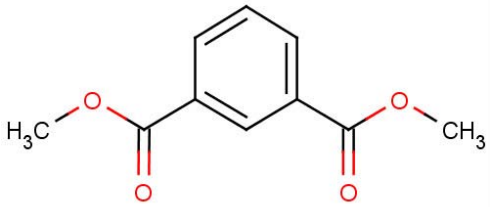
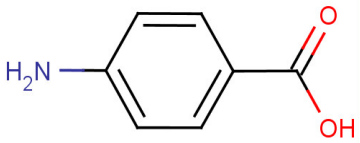
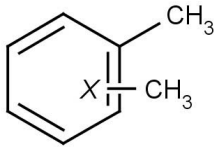
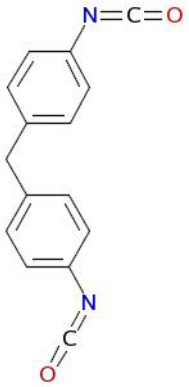
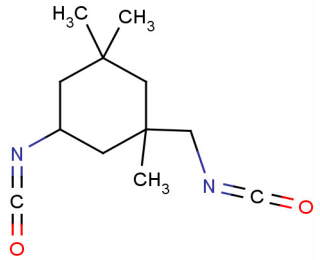
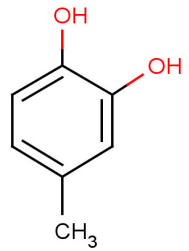
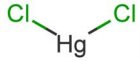
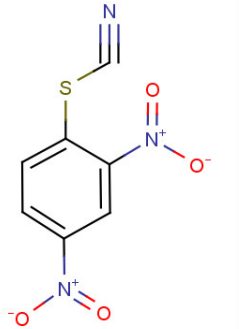
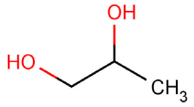
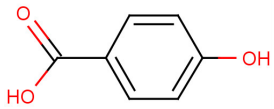
5	chlorpromazine HCl	69-09-0		**Selected (*in vitro* untested)**
6	chloramine T	149358-73-6		**Selected (*in vitro* untested)**
7	formaldehyde	50-00-0		**Selected (*in vitro* tested)**
8	2-mercapto benzothiazole	149-30-4		**Selected**: replacement alternative (available); LLNA PS reference chemical

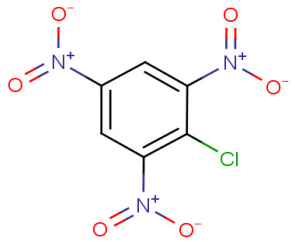
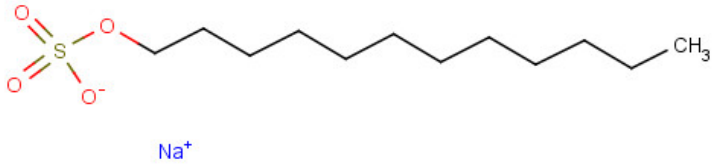
9	benzyl salicylate	118-58-1		**Selected (*in vitro* untested)**
10	1-thioglycerol	96-27-5		**Selected (*in vitro* untested)**: positive: structurally similar to glycerol (negative)
11	dihydro eugenol	2785-87-7		**Selected (*in vitro* untested)**
12	nickel chloride	7718-54-9		**Selected (*in vitro* untested)**: LLNA PS reference chemical (human positive, LLNA false negative)

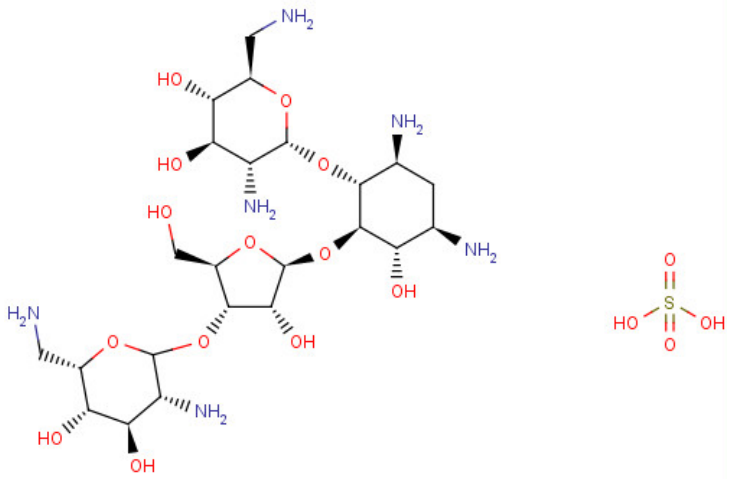
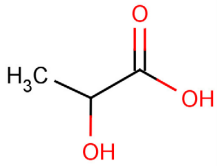
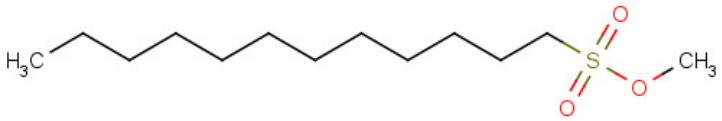
13	benzyl cinnamate	103-41-3		**Selected (*in vitro* untested)**: ICCVAM database (unpublished update)
14	imidazolidinyl urea	39236-46-9		**Selected (*in vitro* tested)**: LLNA PS reference chemical
15	R(+)-limonene	5989-27-5		**Selected (*in vitro* untested)**: ICCVAM database (unpublished update)
16	methyl methacrylate	80-62-6		**Selected (*in vitro* untested)**: LLNA PS reference chemical
17	glycerol	56-81-5		**Selected (*in vitro* tested)**

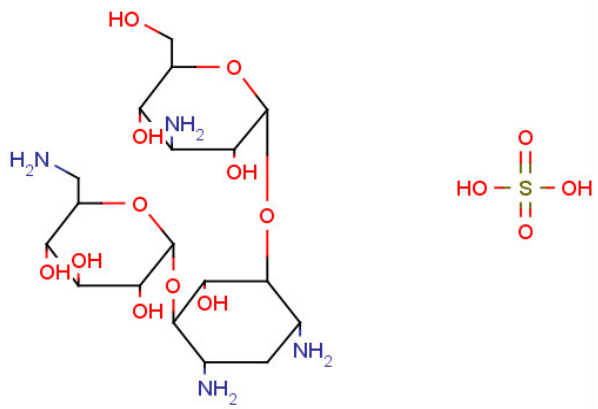
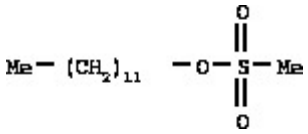
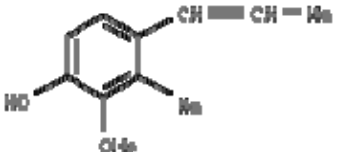
18	2,4- dichloro nitrobenzene (DCNB)	611-06-3		**Selected (*in vitro* untested)**: negative: structurally similar to positive LLNA PS reference chemical (2,4-dinitrochlorobenzene) (DNCB)
19	benzyl alcohol	100-51-6		**Selected (*in vitro* untested)**: ICCVAM database (unpublished update)
20	methyl salicylate	119-36-8		**Selected (*in vitro* tested)**: LLNA PS reference chemical
21	isopropanol	67-63-0		**Selected (*in vitro* tested)**
22	dimethyl isophthalate	1459-93-4		**Selected (*in vitro* tested)**

23	4-amino benzoic acid	150-13-0		**Selected (*in vitro* tested)**
24	xylene	1330-20-7		**Selected (*in vitro* untested)**: LLNA PS reference chemical (human negative, LLNA false positive)
25	diphenyl methane 4,4-diisocyanate	**101-68-8**		**Rejected**: potency not available
26	isophorone diisocyanate	**4098-71-9**		**Rejected**: potency not available

27	4-methyl catechol	**452-86-8**		**Rejected**: potency not available
28	mercuric chloride	**7487-94-7**		**Rejected**: potency not available
29	2,4-dinitro thiocyano benzene	**1594-56-5**		**Rejected**: potency not available
30	propylene glycol	**57-55-6**		**Rejected (*in vitro* tested)**: negative: structurally similar to glycerol (also negative, selected)
31	4-hydroxy benzoic acid	**99-96-7**		**Rejected (*in vitro* tested)**: negative: structurally similar to 4-amino benzoic acid (also negative, selected)

32	lanolin	**8006-54-0**	Fat-like substance derived from sheep wool. Contains a complex combination of esters and polyesters, consisting chiefly of cholesteryl and isocholesteryl esters of the higher fatty acids.	**Rejected**: fat: insoluble in aqueous media (incompatible *in vitro*)
33	picryl chloride	**88-88-0**		**Rejected**: hazardous (explosive)
34	cocoamido propyl betaine	**61789-40-0**	Systematic name: 1-Propanaminium, 3-amino-N-(carboxymethyl)-N,N-dimethyl-, N-coco acyl derivs., hydroxides, inner salts	**Rejected**: uncertain composition / purity
35	sodium laurel sulphate (SLS)	**151-21-3**		**Rejected**: uncertain compatibility *in vitro*; LLNA PS reference chemical (false positive)

36	neomycin sulphate	**1405-10-3**		**Rejected**: animal negative, human positive
37	lactic acid	**50-21-5**		**Rejected (*in vitro* tested)**: MUSST negative control; LLNA PS reference chemical
38	methyl dodecane sulphonate	2374-65-4		**Rejected**: uncertain availability

39	kanamycin sulphate	**25389-94-0**		**Rejected**: uncertain availability
40	dodecyl methane sulphonate	51323-71-8		**Rejected**: uncertain availability; ICCVAM database (unpublished update)
41	3-methyl isoeugenol	186743-29-3		**Rejected**: uncertain availability; ICCVAM database (unpublished update)

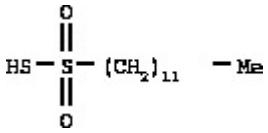
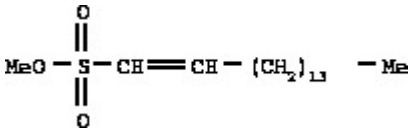
42	dodecyl thiosulphonate	127089-67-2		**Rejected**: reserve selection (not required); ICCVAM database (unpublished update)
43	methyl hexadecane sulphonate	26452-48-2		**Rejected**: reserve selection (not required); ICCVAM database (unpublished update)

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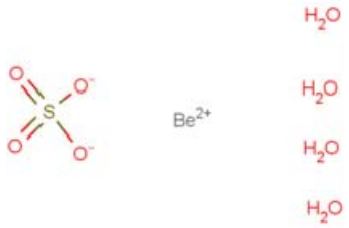
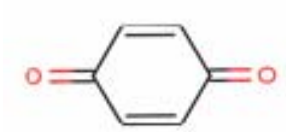
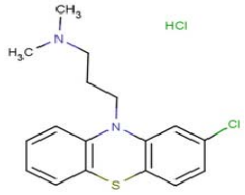
Table 2. 24 selected chemicals :

Seq. No. (notes)	Chemical Name	Structure	CAS#	State	Purity	LLNA potency category	LLNA	GP	HMT	MEST	HPTA	EC3	GHS potency category	Official CLP according to EC No. 1272/2008	DPRA R&D result	h-CLAT R&D result	MUSST R&D result	References (see Annex I)
1	Beryllium sulfate		7787-56-6	S	≥99%	extreme	+	+	+			0.001	1A	Not listed				1,2
2 (1)	Kathon CG (1.2% CMI)		26172-55-4 (CMI)	L	~1.2%	extreme	+	+			+	0.009	1A	Not listed	+	+	+	3,4,5,6
3	Benzoquinone		106-51-4	S	≥99%	extreme	+	+				0.0099	1A	Not Classified	+	+	+	7,4,5
4 (1)	4-Phenylene diamine		106-50-3	S	≥99%	strong	+	+	+	+	+	0.11	1A	Not Classified	+	+	+	8,7,4,1,2,9, 10,5,11,12
5 (2)	Chlor promazine HCl		69-09-0	S	≥98%	strong	+	+	+			0.14	1A	Not listed				1,10,2

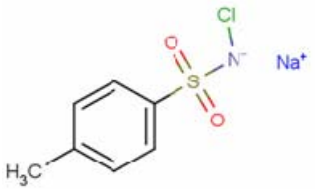
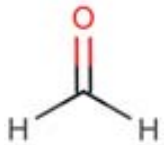
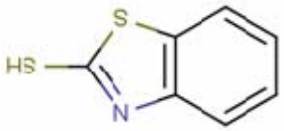
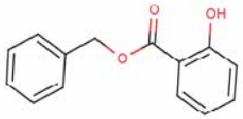
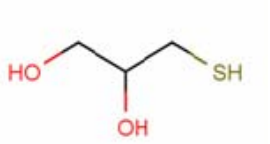
6	Chloramine T		149358-73-6	S	≥98%	strong	+	+			+	0.4	1A	Not Classified				7

Table 2. (continued)

Seq. No. (notes)	**Chemical Name**	**Structure**	**CAS#**	**State**	**Purity**	**LLNA potency category**	**LLNA**	**GP**	**HMT**	**MEST**	**HPTA**	**EC3**	**GHS potency category**	**Official CLP according to EC No. 1272/2008**	**DPRA R&D result**	**h-CLAT R&D result**	**MUSST R&D result**	**References (see Annex I)**
7 (8)	Formaldehyde		50-00-0	L	37%	strong	+	+	+	+	+	0.61	1A	H317 (Cat.1) may cause skin sensitisation	+	+	+	13,8,14,7,1,15,10
8 (1)	2-Mercapto benzothiazole		149-30-4	S	97%	moderate	+	+	+		+	1.7	1A	H317 (Cat.1) may cause skin sensitisation	+	+		7,4,1,2,9,10,12,5
9	Benzyl salicylate		118-58-1	L	≥99%	moderate	+	+				2.9	1B	Not listed				16,17,18
10 (3)	1-Thioglycerol		96-27-5	L	≥97%	moderate	+	+	+			3.6	1B	Not listed				2,1,10

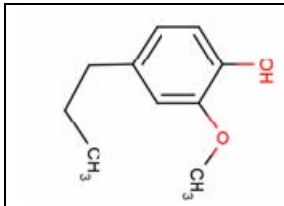
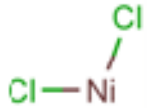
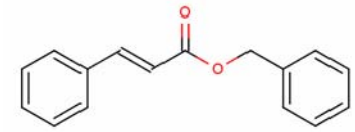
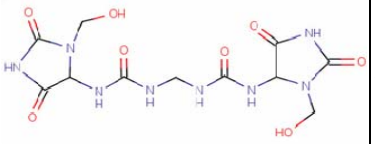
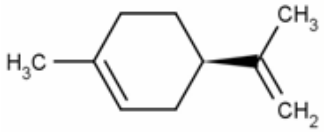
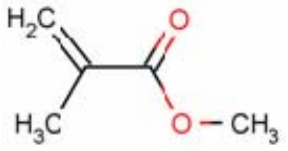
11	Dihydro eugenol		2785-87-7	L	≥99%	moderate	+	+				6.8	1B	Not listed				Unpublished Uniliver data
12 (1) (8)	Nickel chloride		7718-54-9	S	100%	no category (false negative) (4)	-	+					NC (false negative)	H317 (Cat.1) may cause skin sensitisation				7,19,20,12

Table 2. (continued)

Seq. No. (notes)	Chemical Name	Structure	CAS#	State	Purity	LLNA potency category	LLNA	GP	HMT	MEST	HPTA	EC3	GHS potency category	Official CLP according to EC No. 1272/2008	DPRA R&D result	h-CLAT R&D result	MUSST R&D result	References (see Annex I)
13 (9)	Benzyl cinnamate		103-41-3	S	≥98%	weak	+	+				18.4	1B	Not listed				17,18
14 (1)	Imidazolidinyl urea		39236-46-9	S	~95%	weak	+	+			+	24	1B	Not listed	+	+	+	7,11,10,21,22
15	R(+)-Limonene		5989-27-5	L	≥99%	weak	+	+				69	1B	H317 (Cat.1) may cause skin sensitisation				17,18
16 (1)(5)(10)	Methyl methacrylate		80-62-6	L	99%	weak	+	+				90	1B	H317 (Cat.1) may cause skin sensitisation				23

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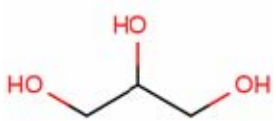
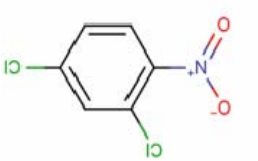
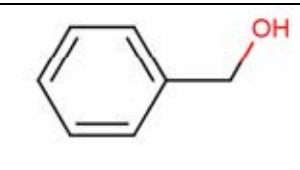
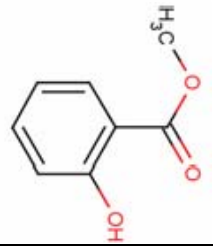
Seq. No. (notes)	Chemical Name	Structure	CAS#	State	Purity	LLNA potency category	LLNA	GP	HMT	MEST	HPTA	EC3	GHS potency category	Official CLP according to EC No. 1272/2008	DPRA R&D result	h-CLAT R&D result	MUSST R&D result	References (see Annex I)
17 (3)	Glycerol		56-81-5	L	99%	no category	-	-					NC	No listed	-	-	-	9
18 (6)(11)	2,4-Dichloro nitrobenzene		611-06-3	S	≥98%	no category	-	-					NC	Not listed				2,24,19
19	Benzyl alcohol		100-51-6	L	99.8%	no category	-	-					NC	Not classified				17,18

Table 2. (continued)

Seq. No. (notes)	Chemical Name	Structure	CAS#	State	Purity	LLNA potency category	LLNA	GP	HMT	MEST	HPTA	EC3	GHS potency category	Official CLP according to EC No. 1272/2008	DPRA R&D result	h-CLAT R&D result	MUSST R&D result	References (see Annex I)
20 (1)	Methyl salicylate		119-36-8	L	≥99%	no category	-	-	-				NC	Not listed	-	-	-	1,2,19,10,25,5

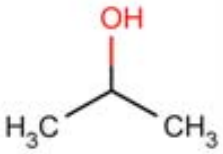
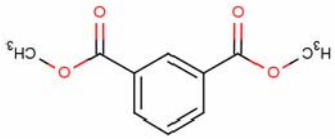
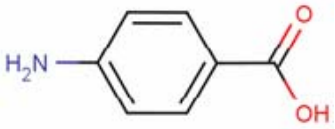
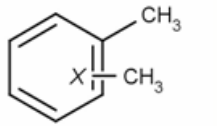
21 (1)	Isopropanol		67-63-0	L	≥99%	no category	-	-					NC	Not classified	-	-	-	2,25,9,26
22	Dimethyl isophthalate		1459-93-4	S	99%	no category	-	-					NC	Not listed				7,5
23	4-Amino benzoic acid		150-13-0	S	≥99%	no category	-	-	-	+	+		NC	Not listed				4,27,2,5
24 (1)	Xylene		1330-20-7	L	98.5%	weak (false positive (7)	+		-			95.8	1B (false positive)	Not classified				2,28,12

Table 2 general notes:
24 chemicals: 3 extreme; 4 strong; 5 moderate; 4 weak; 8 negative
24 chemicals: 12 liquids + 12 solids
22 chemicals (15 +ve, 7 -ve): concordant LLNA / GP *in vivo* result
8 chemicals: tested by all 3 *in vitro* methods (all with concordant results)
9 chemicals: LLNA PS reference chemicals
Table 2 specific notes:

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(1) LLNA performance standard (PS) reference chemicals
(2) *in vivo* test conducted according to obsolete guideline (valid at time of study)
(3) thioglycerol (positive) structurally similar to glycerol (negative); also irritant to eyes, respiratory system, and skin; glycerol (negative) structurally similar to thioglycerol (positive)
(4) positive, *human* (false negative in the LLNA): assigned 'moderate' by expert opinion
(5) positive, *human*, based on clinical experience
(6) DCNB (negative) structurally similar to DNCB (PS positive 'extreme', tested *in vitro*)
(7) false positive, in the LLNA: assigned 'no category' by expert opinion
(8) official CMR classification: hazard indicated on vial label
(9) low mp (34-37ºC) forming solid mass at RT: requires melting (water bath ~35ºC) for aliquot manipulation
(10) observed tendency to dissolve polystyrene (e.g., pipettes): glassware recommended for aliquot manipulation
(11) low mp (29-32ºC) forming solid mass at RT: requires melting (water bath ~30ºC) for aliquot manipulation

Table 3. Official full classification and labelling (C&L): 24 chemicals definitive selection set

Chemical	CAS#	Official C&L (Reg 1272/2008, Annex VI)
Beryllium sulfate	7787-56-6 (13510-49-1)	not listed
Kathon CG (75% aq.) components:		components not listed
5-chloro-2-methyl-4-isothiazolin-3-one (~1.2%)	26172-55-4 (CMI)	
2-methyl-4-isothiazolin-3-one (~0.4%)	2682-20-4 (MI)	
magnesium nitrate (~22%)	10377-60-3	
Benzoquinone	106-51-4	**Acute toxicity, Inhalation (Category 3)** H331 Toxic if inhaled. **Acute toxicity, Oral (Category 3)** H301 Toxic if swallowed. **Eye irritation (Category 2)** H319 Causes serious eye irritation. **Specific target organ toxicity - single exposure (Category 3)**

		H335 May cause respiratory irritation. **Skin irritation (Category 2)** H315 Causes skin irritation. **Acute aquatic toxicity (Category 1)** H400 Very toxic to aquatic life.
4-Phenylenediamine	106-50-3	**Acute toxicity, Inhalation (Category 3)** H331 Toxic if inhaled. **Acute toxicity, Dermal (Category 3)** H311 Toxic in contact with skin. **Acute toxicity, Oral (Category 3)** H301 Toxic if swallowed. **Eye irritation (Category 2)** H319 Causes serious eye irritation. **Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Acute aquatic toxicity (Category 1)** H400 Very toxic to aquatic life. **Chronic aquatic toxicity (Category 1)** H410 Very toxic to aquatic life with long lasting effects.
Chlorpromazine HCl	69-09-0	not listed
Chloramine T	127-65-1 (149358-73-6)	**Acute toxicity (Category 4)** H302 Harmful if swallowed. **Skin corrosion (Category 1B)** H314 Causes severe skin burns and eye damage. **Respiratory sensitization (Category 1)** H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.

Table 3 (continued)

Formaldehyde	50-00-0	**Acute toxicity, Inhalation (Category 3)** H331 Toxic if inhaled. **Acute toxicity, Dermal (Category 3)** H311 Toxic in contact with skin. **Acute toxicity, Oral (Category 3)** H301 Toxic if swallowed. **Skin corrosion (Category 1B)** H314 Causes severe skin burns and eye damage. **Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Carcinogenicity (Category 2)** H351 Suspected of causing cancer. **Specific target organ toxicity - single exposure (Category 3)** H335 May cause respiratory irritation.
2-Mercaptobenzothiazole	149-30-4	**Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Acute aquatic toxicity (Category 1)** H400 Very toxic to aquatic life. **Chronic aquatic toxicity (Category 1)** H410 Very toxic to aquatic life with long lasting effects.

Benzyl salicylate	118-58-1	not listed
1-Thioglycerol	96-27-5	not listed
Dihydroeugenol	2785-87-7	not listed
Nickel chloride	7718-54-9	**Carcinogenicity, Inhalation (Category 1A)** H350i May cause cancer by inhalation. **Germ cell mutagenicity (Category 2)** H341 Suspected of causing genetic defects. **Reproductive toxicity (Category 1B)** H360 May damage fertility or the unborn child. **Acute toxicity, Inhalation (Category 3)** H331 Toxic if inhaled. **Acute toxicity, Oral (Category 3)** H301 Toxic if swallowed. **Specific target organ toxicity - repeated exposure (Category 1)** H372 Causes damage to organs through prolonged or repeated exposure. **Skin irritation (Category 2)** H315 Causes skin irritation. **Respiratory sensitization (Category 1)** H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled. **Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Acute aquatic toxicity (Category 1)** H400 Very toxic to aquatic life. **Chronic aquatic toxicity (Category 1)** H410 Very toxic to aquatic life with long lasting effects.

Table 3 (continued)

Benzyl cinnamate	103-41-3	not listed
Imidazolidinyl urea	39236-46-9	not listed
R(+)- Limonene	5989-27-5	**Flammable liquids (Category 3)** H226 Flammable liquid and vapour. **Skin irritation (Category 2)** H315 Causes skin irritation. **Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Acute aquatic toxicity (Category 1)** H400 Very toxic to aquatic life. **Chronic aquatic toxicity (Category 1)** H410 Very toxic to aquatic life with long lasting effects.
Methyl methacrylate	80-62-6	**Flammable liquids (Category 2)** H225 Highly flammable liquid and vapour. **Skin irritation (Category 2)** H315 Causes skin irritation. **Skin sensitization (Category 1)** H317 May cause an allergic skin reaction. **Specific target organ toxicity - single exposure (Category 3)** H335 May cause respiratory irritation.

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Glycerol	56-81-5	not listed
2,4-Dichloronitrobenzene	611-06-3	not listed
Benzyl alcohol	100-51-6	**Acute toxicity (Category 4)** H332 Harmful if inhaled. **Acute toxicity (Category 4)** H302 Harmful if swallowed.
Methyl salicylate	119-36-8	not listed
Isopropanol	67-63-0	**Flammable liquids (Category 2)** H225 Highly flammable liquid and vapour. **Eye irritation (Category 2)** H319 Causes serious eye irritation. **Specific target organ toxicity - single exposure (Category 3)** H336 May cause drowsiness or dizziness.
Dimethyl isophthalate	1459-93-4	not listed
4-Aminobenzoic acid	150-13-0	not listed
Xylene	1330-20-7	**Flammable liquids (Category 3)** H226 Flammable liquid and vapour. **Acute toxicity, Inhalation (Category 4)** H332 Harmful if inhaled. **Acute toxicity, Dermal (Category 4)** H312 Harmful in contact with skin. **Skin irritation (Category 2)** H315 Causes skin irritation.

Annex I.: References to *in vivo* studies for the selected set of 24 chemicals

1. Basketter, D.A., Scholes, E.W. and Kimber, I. (1994). The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test, Food and Chemical Toxicology. **32(6)**, pp. 543–547
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5. Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A. and Aptula, A.O. (2007) Mechanistic Applicability Domain Classification of a Local Lymph Node Assay Dataset for Skin Sensitization. Chemical Research in Toxicology. **20 (7)**, pp 1019–1030
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12. Kligman, A. (1966). The identification of contact allergens by human assay (III). The maximization test: a procedure for screening and rating contact sensitizers. Journal of Investigative Dermatology. **47**, pp. 393–409
13. Kimber, I. and Weisenberger, C. (1989). A murine local lymph node assay for the identification of contact allergens. Archives of Toxicology. **63, pp.** 274-282
14. Maurer, T. and Kimber, I. (1991) Draining lymph node cell activation in guinea pigs: Comparisons with the murine local lymph node assay. Toxicology. **69(2)**, pp. 209-218

15. Basketter, D.A., Gerberick, F.G., Kimber, I. (2001). Measurement of allergenic potency using the local lymph node assay. Trends in Pharmacological Sciences. **22(6)** pp. 264-265

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Available documents on request: List of check lists for solubility, sampling and shipping of the test items

- SSPS_chemicals solubility check
- SSPS_chemicals sampling check
- SSPS_chemicals shipping check

Appendix 4

Stratified random sampling for the selection of 9 Chemicals

STRATIFIED RANDOM SAMPLING FOR THE SELECTION OF 9 CHEMICALS

INTRODUCTION

A probability sampling method is any method of sampling that uses some form of random selection, in order to guarantee that all the units in the population have the same probability of being selected.

Stratified random sampling is a method of sampling which is convenient when the total population to be sampled can be naturally divided into classes which are internally homogeneous according to the outcome of interest.

When such a case arises, a simple random sampling is performed within each sub-class, in order to guarantee the representativeness of the final sample.

For the purpose of this exercise, we have selected a proportional allocation, where the number of sample items to be selected from each stratum is proportional to the numerousness of the stratum itself.

METHODS

For the case of this pre-validation study, a list of 24 chemicals has been provided.
These chemicals are divided into 5 classes according to their potency as sensitisers. For the majority of the chemicals, the potency classes were assigned according to the ECETOC classifications (ECETOC technical report #87). The two exceptions were Xylene and Nickel Chloride, which are respectively a false positive and a false negative in the in vivo assays. Xylene was assigned to the "Negatives", while Nickel Chloride was assigned to the "Moderates" based on expert judgment (David Basketter).

The list of chemicals, together with their potency class, is shown in the following table.

Potency category	Chemicals
3 Extreme	5-Chloro-2-methyl-4-isothiazolin-3-one
	Benzoquinone
	Beryllium sulfate
4 Strong	Formaldehyde
	Chloramine T
	Chlorpromazine hydrochloride
	4-Phenylenediamine
5 Moderate	Dihydroeugenol
	Nickel chloride
	1-Thioglycerol
	2-Mercaptobenzothiazole
	Benzyl salicylate
4 Weak	Imidazolidinyl urea
	Methyl methacrylate
	Benzyl cinnamate
	R(+)- Limonene
8 Negatives	Methyl salicylate

	Glycerol
	Isopropanol
	Dimethyl isophthalate
	4-Aminobenzoic acid
	2,4-Dichloronitrobenzene
	Xylene
	Benzyl alcohol

The number of chemicals to be sampled in each class was determined as follows:

Potency Class	**Population Numerousness**	**Proportion**	**Sample Numerousness**
EXTREME	3	3/24 = 0.125	0.125*9 = 1.125 ≈ 1
STRONG	4	4/24 = 0.166	0.166*9 = 1.5
MODERATE	5	5/24 = 0.208	0.208*9 = 1.875 ≈ 2
WEAK	4	4/24 = 0.166	0.166*9 = 1.5
NEGATIVES	8	8/24 = 0.333	0.333*9 = 3
Total	***24***		***9***

As the number of chemicals to be sampled in classes STRONG and WEAK resulted to be equal to 1.5, 1 chemical was selected in each class, while the 3rd additional chemical was randomly selected among the remaining chemicals belonging to the two classes pooled together.

Random numbers in the relevant range were generated using SAS System, version 9.0; SAS code is shown in the next section.

As a different number of chemicals had to be chosen in each class (and then random numbers in different ranges had to be generated), the sampling procedure was divided in subsequent steps, in order to correctly sample the desired number of items from each class. Before performing the sampling, a numerical sequential code was given to each chemical in each class, as an identifier. The sampling scheme is shown below (please note that the codes for Step 5 were added in the table after performing Step 3).

Potency category	**Chemicals**	**Step1**	**Step2**	**Step3**	**Step4**	**Step5**
3 Extreme	5-Chloro-2-methyl-4-isothiazolin-3-one	1				
	Benzoquinone	2				
	Beryllium sulfate	3				
4 Strong	Formaldehyde			1		1
	Chloramine T			2		2
	Chlorpromazine hydrochloride			3		3
	4-Phenylenediamine			4		*
5 Moderate	Dihydroeugenol		1			
	Nickel chloride		2			
	1-Thioglycerol		3			
	2-Mercaptobenzothiazole		4			
	Benzyl salicylate		5			
4 Weak	Imidazolidinyl urea			1		*
	Methyl methacrylate			2		4
	Benzyl cinnamate			3		5
	R(+)- Limonene			4		6

8 Negatives	Methyl salicylate				1	
	Glycerol				2	
	Isopropanol				3	
	Dimethyl isophthalate				4	
	4-Aminobenzoic acid				5	
	2,4-Dichloronitrobenzene				6	
	Xylene				7	
	Benzyl alcohol				8	

STEP 1 : 1 EXTREME SENSITISER

In Step 1, 1 Extreme Sensitiser was sampled out of the 3 in the entire population.

```
** STEP 1: 1 Extreme out of 3;
data step1;
 chemical=int(3*ranuni(0))+1;
 output;
run;
```

The outcome of the procedure was the following:

chemical
2

corresponding to Benzoquinone.

STEP 2 : 2 MODERATE SENSITISERS

In Step 2, 2 Extreme Sensitisers were sampled out of the 5 in the entire population.

```
** STEP 2: 2 Moderate out of 5;
data step2;
 chemical1=int(5*ranuni(0))+1;
 chemical2=int(5*ranuni(0))+1;
 output;
run;
```

The outcome of the procedure was the following:

chemical1	chemical2
3	1

corresponding to 1-Thioglycerol and Dihydroeugenol.

STEP 3: 1 STRONG AND 1 WEAK SENSITISER

In Step 3, 1 Strong Sensitiser out of the 4 in the population, and also 1 Weak Sensitiser out of the 4 in the population were sampled.

```
** STEP 3: 1 Strong out of 4 and 1 Weak out of 4;
data step3;
do i=1 to 2;
 chemical=int(4*ranuni(0))+1;
 output;
end;
run;
```

The outcome of the procedure was the following:

i	chemical
1	4
2	1

corresponding to 4-Phenylenediamine and Imidazolidinyl urea.

STEP 4: 3 NON SENSITISERS

In Step 4, 3 Negative chemicals were sampled out of the 8 in the entire population.

```
** STEP 4: 3 Negatives out of 8;
data step4;
 chemical1=int(8*ranuni(0))+1;
 chemical2=int(8*ranuni(0))+1;
 chemical3=int(8*ranuni(0))+1;
 output;
run;
```

The outcome of the procedure was the following:

chemical1	chemical2	chemical3
8	2	6

corresponding to Benzyl alcohol, Glycerol and 2,4-Dichloronitrobenzene.

STEP 5: 1 STRONG OR WEAK SENSITISER

In Step 5, the last chemical belonging either to the Strong or to the Weak Sensitisers was sampled out of the 6 remained after removing the two chemicals (one Strong and one Weak Sensitiser) sampled during Step 3.

```
** STEP 5: 1 Strong/Weak out of remaining 6;
data step5;
 chemical=int(6*ranuni(0))+1;
 output;
run;
```

The outcome of the procedure was the following:

chemical

4

corresponding to Methyl methacrylate.

CONCLUSION: FINAL LIST

Potency category	Chemicals
1 Extreme	Benzoquinone
1 Strong	4-Phenylenediamine
2 Moderate	Dihydroeugenol
	1-Thioglycerol
2 Weak	Imidazolidinyl urea
	Methyl methacrylate
3 Negative	Glycerol
	2,4-Dichloronitrobenzene
	Benzyl alcohol

Appendix 5

Chemicals Coding, Aliquoting and Shipping Guidelines

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
In-Vitro Methods Unit

Direct Peptide Reactivity Assay, human Cell Line Activation Test, Myeloid U937 Skin Sensitisation Test Phase III Prevalidation

Chemicals Coding, Aliquoting and Shipping Guidelines

Version	Authors	Reviewer	Approval	Date of approval
1	Luca Tosti Alexandre Angers	Thomas Cole	Thomas Cole	28/04/2011

Document history			
Version	Date	Drafted by	Comments

Chemicals coding, aliquoting and shipping guidelines

Table of contents:

This document describes procedures for coding, aliquoting and shipping of chemicals, relevant to laboratories participating in the Skin Sensitisation Prevalidation Study.

Test item code format

Codes generated for labelling of chemical aliquots comprise four characters: an initial letter, designated per laboratory (Table 1) followed by a number corresponding to Stage (Phase B) and experiment (Table 2). A unique letter pair is then allocated to each aliquot, resulting in the format: X0XX

Table 1: Code letter assignment to test facility laboratory

P&G	**IVMU (DPRA)**	**RICERCA**	**Kao**	**Shiseido**	**IVMU (hCLAT)**	**Bioassay (hCLAT)**	**L'Oréal**	**FICAM**	**Bioassay (MUSST)**
A	B	C	D	E	F	G	H	J	K

Table 2: Code number assignment to stage / experiment

Stage I	Stage II (first replicate)	Stage II (second replicate)	Stage II (third replicate)
0	1	2	3

In practice, unique random text strings comprising two letters are generated from True Random Number Service (internet: Random.org). The maximum combinations are 676 (=26x26).

Combinations including the letter I or O are eliminated, to avoid possible confusion with the numbers 1 or 0.

Each chemical is then allocated a sequential number, 1 – 24, for internal reference of the ECVAM chemical selection group only:
1 – 9: those to be tested only once (1 aliquot)
10 – 24: the remaining 15 chemicals for triplicate testing (3 aliquots)

Following the order of the randomly generated sequence, a two letter combination (code) is assigned to each aliquot, unique for each method, laboratory and replicate, illustrated for one chemical only (Table 3a & 3b). Codes for all chemical aliquots are recorded in an Excel spreadsheet format kept by the Chemical Selection Group. The identity of the chemicals to which the codes are assigned remain confidential from the VMT and the biostatisticians. Copies of the tables, indicating only codes for the respective aliquots but not identifying the chemical names, are prepared for un-biased biostatistical analysis of reproducibility.

Table 3a: Example of codes assigned to chemical aliquots for Phase B Stage I (tested once)

method	**DPRA**			**h-CLAT**				**MUSST**		
lab	A	B	C	D	E	F	G	H	J	K
lab/expt	**A0**	**B0**	**C0**	**D0**	**E0**	**F0**	**G0**	**H0**	**J0**	**K0**
code	**AR**	**KS**	**QM**	**SE**	**YZ**	**RL**	**GW**	**EP**	**CQ**	**YS**

Table 3b: Example of codes assigned to chemical aliquots for Phase B Stage II (tested three times)

method	DPRA			h-CLAT				MUSST		
lab	A	B	C	D	E	F	G	H	J	K
lab/expt	**A1**	**B1**	**C1**	**D1**	**E1**	**F1**	**G1**	**H1**	**J1**	**K1**
code	**VE**	**CK**	**SB**	**CZ**	**MP**	**MD**	**LE**	**KT**	**UP**	**ZZ**
lab/expt	**A2**	**B2**	**C2**	**D2**	**E2**	**F2**	**G2**	**H2**	**J2**	**K2**
code	**NK**	**HY**	**MK**	**GL**	**PT**	**VS**	**LG**	**WC**	**SN**	**HF**
lab/expt	**A3**	**B3**	**C3**	**D3**	**E3**	**F3**	**G3**	**H3**	**J3**	**K3**
code	**EE**	**TW**	**HE**	**BG**	**EU**	**WY**	**VC**	**GG**	**BW**	**TL**

Introducing a further element of differentiation, aiming to preclude parallel systematic testing of the chemicals in a uniform sequence between the laboratories, an arbitrary running order is assigned to each set of aliquots supplied to individual participants. In practice, an integer is allocated randomly to each aliquot, respective of recipient laboratory, illustrated for four laboratories (Table 4a & 4b). Each consignment check-list (described later) is then prepared in sequential order of the integer, resulting in a random order of chemical number for each laboratory, to be followed as test item assay order.

Table 4a: Examples of random integer assignment to chemical aliquots for Phase B Stage I (tested once)

	LAB # 1	LAB # 2	LAB # 3	LAB # 4
CHEMICAL # 1	6	2	4	5
CHEMICAL # 2	9	4	5	8
CHEMICAL # 3	2	1	3	3
CHEMICAL # 4	5	3	6	4
CHEMICAL # 5	3	7	8	7
CHEMICAL # 6	8	9	2	9
CHEMICAL # 7	1	5	9	2
CHEMICAL # 8	4	6	7	6
CHEMICAL # 9	7	8	1	1

Table 4b: Examples of random integer assignment to chemical aliquots for Phase B Stage II (tested three times)

	LAB # 1	LAB # 2	LAB # 3	LAB # 4
CHEMICAL # 10 expt 1	11	24	21	14
CHEMICAL # 10 expt 2	24	17	23	11
CHEMICAL # 10 expt 3	10	15	12	22
CHEMICAL # 11 expt 1	15	13	17	12
CHEMICAL # 11 expt 2	20	12	10	20
CHEMICAL # 11 expt 3	23	14	15	16

CHEMICAL # 12 expt1	17	21	16	15
CHEMICAL # 12 expt 2	14	18	20	21
CHEMICAL # 12 expt 3	22	22	18	18
CHEMICAL # 13 expt 1	13	19	13	19
CHEMICAL # 13 expt 2	16	11	14	23
CHEMICAL # 13 expt 3	19	20	22	24
CHEMICAL # 14 expt 1	18	10	24	17
CHEMICAL # 14 expt 2	12	23	11	13
CHEMICAL # 14 expt 3	21	16	19	10
CHEMICAL # …	…	…	…	…

Aliquot preparation

All test item chemicals are purchased from Sigma-Aldrich, with delivery to ECVAM. On arrival, the chemicals are stored in a repository at the appropriate temperature (ambient or 4ºC). All chemicals are handled in a standard fume hood with protective gloves.

In line with method SOPs, aliquots (solid and liquid) are prepared by weighing (~4.0 g) into 15 ml amber glass vials with melamine resin caps (Sigma cat no. 27003) using an analytical balance (accuracy 0.0001g). Solid materials are sampled from the supply containers with a spatula, liquids via pipette. Vials are additionally sealed with parafilm.

To avoid cross-contamination between test items, aliquots are prepared one chemical at a time, preparing the aliquots for all laboratories, cleaning instruments and removing disposable materials after each chemical.

Vial labels

As a general precaution for test item handling by recipient laboratories, and considering the coded nature of the chemicals, all vial labels include the hazard indication: *Very Toxic*. Official hazard classifications (if applicable, according to Annex I, Directive 67/548) are compiled for each chemical. In addition, the MSDS for each chemical is reviewed, in particular noting any indication of CMR toxicity, where special handling precautions may be relevant. In cases of either official or significant CMR potential, the respective vial label includes indication of this additional hazard.

Specifically for the DPRA method, the vial label displays a rounded molecular weight and purity, enabling calculation of test solution concentration by the relevant laboratories.

The molecular weights of the chemicals are rounded to the first decimal place (% rounding < 0.1%) (Table 5).

Table 5: Purity and MW indicated for DPRA test item preparation

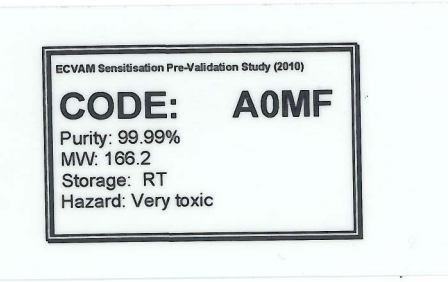
ID		Purity %	rounded MW
	Phase B1		
1	Benzoquinone	99.80	108.1
2	4-phenylenediamine	99.90	108.1
3	dihydroeugenol	99.99	166.2
4	1-thioglycerol	98.00	108.2
5	Imidazolidinyl urea	95.00*	388.3
6	Methyl methacrylate	99.90	100.1
7	Glycerol	100.00	92.1
8	2-4 dichloronitrobezene	99.10	192.0
9	benzyl alcohol	99.94	108.1
	Phase B2		
10	Kathon CG	**100.00**	**8849.1****
11	Beryllium sulphate	99.99	177.1
12	Formaldehyde	**100.00**	**80.1****
13	chloramine T	98.00	227.6
14	Chlorpromazine hydrochloride	99.00	355.3
15	2-Mercaptoebenzothiazole	96.5	167.3
16	Benzyl salicylate	99.90	228.2
17	Benzyl cinnamate	98.10	238.3
18	R(+) Limonene	99.1	136.2
19	Methyl salicylate	100.00	152.1
20	Isopropanol	99.90	60.1
21	Dimethyl isophthalate	99.90	194.2
22	4 Aminobenzoic-acid	100.00	137.1
23	Nickel Chloride	99.99	129.6
24	Xylene	98.6	106.2

* Catalogue purity, lot purity not found on the Certificate of Analysis.
** note Appendix III

Information indicated on the vial labels, with examples, is as follows:

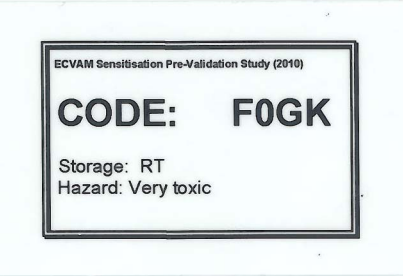
DPRA

Study title
CODE
Purity
Molecular weight
Storage temperature
Hazard: Very toxic
CMR (if applicable)



h-CLAT & MUSST

Study title
CODE
Storage temperature
Hazard: Very toxic
CMR (if applicable)



Aliquot weighing (sampling) and vial labelling is conducted in the presence of a second person, monitoring and verifying individual weights and labels, recoded in a checklist as follows:

Sampling check Date:_____

Method		Lab	Run	Code	Substance ID	temp	MW	Weight (g)	Check	
DPRA	P&G	**A**	**0**	**GC**	(4)	+4ºC				
DPRA	IVMU	**B**	**0**	**QU**	(4)	+4ºC				
DPRA	RICERCA	**C**	**0**	**LC**	(4)	+4ºC				
h-CLAT	Kao	**D**	**0**	**TN**	(4)	+4ºC				
h-CLAT	Shiseido	**E**	**0**	**DJ**	(4)	+4ºC				
h-CLAT	IVMU	**F**	**0**	**KG**	(4)	+4ºC				
h-CLAT	Bioassay	**G**	**0**	**JN**	(4)	+4ºC				
MUSST	L'Oreal	**H**	**0**	**DL**	(4)	+4ºC				
MUSST	FICAM	**J**	**0**	**LM**	(4)	+4ºC				
MUSST	Bioassay	**K**	**0**	**KE**	(4)	+4ºC				

Balance I.D.:1128450765

Performed By:_____ Date:_____

Verified By:_____ Date:_____

The weighing and labelling records are kept in a separate file.

Aliquot shipment

Transportation of dangerous goods is regulated by the International Air Transport Association (IATA) which requires package labelling with a standard UN number, assigned according to toxicity class. Limited quantities, such as sample material for diagnostic analyses, are exempt from specific transportation requirements. The test item chemicals are considered as potentially hazardous goods, with UN number, if applicable, indicated in section 14 of the Material Safety Data Sheet (MSDS).

The courier for the JRC is Noe' Spedizioni SRL, which provides a shipping note/letter template for completion with the following:

United Nations (UN) number
Full sender's address
Full receiver's address
Box dimension and weight
Description of content as "Samples for Analysis"

For samples shipped outside the EU, the note includes a declaration of "Dangerous Goods in Expected quantities" and a "proforma invoice".

For the first batches of nine chemicals, two boxes are prepared, one for room temperature (RT) storage chemicals, another for chemicals that should be stored at +4°C. The box with chemicals stored a $+4^{o}$C is packed with cold blocks and some dry ice, to preserve the temperature for the duration of the shipment. Packing of the boxes is conducted in the presence of a second person, monitoring and verifying individual labels, recorded as a checklist as follows:

Shipping check Date:_____

Method		Lab	Run	Code	Substance ID	Temp	Check	
DPRA	P&G	**A**	**0**	**PR**	(1)	+4ºC		
DPRA	P&G	**A**	**0**	**AF**	(2)	RT		
DPRA	P&G	**A**	**0**	**MF**	(3)	RT		
DPRA	P&G	**A**	**0**	**GC**	(4)	+4ºC		
DPRA	P&G	**A**	**0**	**YY**	(5)	+4ºC		
DPRA	P&G	**A**	**0**	**FU**	(6)	+4ºC		
DPRA	P&G	**A**	**0**	**XL**	(7)	RT		
DPRA	P&G	**A**	**0**	**QG**	(8)	RT		
DPRA	P&G	**A**	**0**	**AR**	(9)	RT		

Performed By:_____ Date:_____

Verified By:_____ Date:_____

According to the IATA regulation all chemicals should travel accompanied by their MSDS. A decoding list and corresponding MSDSs are enclosed in an envelope, labeled "For customs use only". Additionally, respective MSDSs are sealed in individual envelopes, labelled only with the corresponding code, to be kept by the safety officer at the testing site for the whole duration of the study, and to be unsealed only in the case of an emergency. All envelopes are sealed with tape and signed across the seal, allowing subsequent inspection of integrity.

The shipment is addressed to the Safety Officer of the participating laboratory, as disclosed by each testing site at the beginning of the study. Shipments are made on Monday morning, to avoid delays in delivery caused by week-ends. Availability of a safety officer for goods receipt is confirmed by e-mail prior to shipment, avoiding delivery during staff absence due to holidays etc.

The participant laboratories are advised of the shipment by e-mail (see Appendix II), which includes a consignment check-list indicating the content of the boxes. This allows the recipient to confirm the complete and intact delivery of the test items, and the integrity of the sealed MSDS and decoding list envelopes. The recipients are instructed to return the completed check-list, with the envelope containing the "for customs only" decoding list, to ECVAM. The following is an example of the consignment check-list sent to the laboratories:

Consignment check-list Date:_____

Please check and complete the following:
(*) vial received? (Yes/No)
(**) MSDS envelope sealed? (Yes/No)

Method		Lab	Run	Code	Substance	MW	Purity	Temp	ECVAM note	Vial received?(*)	MSDS sealed?(**)	Test Lab Comments
DPRA	IVMU	B	0	BY	chem#1	108.1	99.8	+4ºC				
DPRA	IVMU	B	0	QU	chem#2	108.2	98	+4ºC				
DPRA	IVMU	B	0	RB	chem#3	100.1	99.9	+4ºC	Use glassware for sampling.			
DPRA	IVMU	B	0	YR	chem#4	192	99.1	RT	Melt in water bath (~ 35° C) for weighing			
DPRA	IVMU	B	0	JA	chem#5	108.1	99.9	RT				
DPRA	IVMU	B	0	PE	chem#6	388.3	95.00*	+4ºC				
DPRA	IVMU	B	0	KS	chem#7	108.1	99.94	RT				
DPRA	IVMU	B	0	FR	chem#8	92.1	100	RT				
DPRA	IVMU	B	0	KU	chem#9	166.2	99.99	RT				

Checked By:_____ Date:_____

Expiration date and vial quantity

Participant laboratories which are GLP (Good Laboratory Practice) compliant are provided with the expiration date and quantity of the test items for each vial that is sent. The following is an example of the expiration date and vial quantity list:

Method		Lab	Run	Code	Running order	Expiration date	Vial quantity (g)
DPRA	IVMU	B	0	BY	chem#1	Apr-12	4.11
DPRA	IVMU	B	0	QU	chem#2	Feb-14	4.10
DPRA	IVMU	B	0	RB	chem#3	Aug-12	4.10
DPRA	IVMU	B	0	YR	chem#4	Apr-12	4.00
DPRA	IVMU	B	0	JA	chem#5	Dec-12	4.10
DPRA	IVMU	B	0	PE	chem#6	Apr-12	4.00
DPRA	IVMU	B	0	KS	chem#7	Jul-12	4.00
DPRA	IVMU	B	0	FR	chem#8	Sep-12	4.20
DPRA	IVMU	B	0	KU	chem#9	Apr-12	4.10

Appendix I – Kathon CG and Formaldehyde information for the DPRA

On April 30th, 2010, the following message was sent to all VMG members.

The responses received showed that option 2 is preferred by the majority of the VMG.

From: ANGERS Alexandre (JRC-ISPRA)
Sent: Friday, April 30, 2010 10:26 AM
To: CASATI Silvia (JRC-ISPRA); COLE Thomas (JRC-ISPRA); COMPAGNONI Anna (JRC-ISPRA); 'David Basketter'; 'Jon Richmond'; KLEENSANG Andre (JRC-ISPRA); 'Pierre Aeby'; 'Sebastian Hoffmann'
Subject: ECVAM Skin Sensitisation Prevalidation Study - Question concerning the DPRA molecular weights

Dear VMG members,

We have been working on the information to provide to the laboratories involved in the DPRA study, and we would like to consult you on a specific issue.

As was discussed during the last Teleconference, due to the fact that the DPRA SOP requests the chemical to solubilised as a 100mM solution, we will need to supply them with the molecular weight of the compounds. We agreed that, although this will affect the independence of the experiments used to evaluate the within-laboratory reproducibility, there was no way to avoid it.

Further analysis of the formula showed that there is additional information needed, namely the purity. At this point, we have two options, which are described in the document attached to this mail. In summary, either we supply the molecular weight AND the purity, or we correct for the purity in the molecular weight we supply.

For most chemicals, there are little practical differences between the two. Neither option solves the independence of the WLR repeats. However, for two for the chemicals, formaldehyde and Kathon CG, giving the purity might reveal the identity of the chemical, at least to the lead laboratory, as they would become easy to recognize for those who have experience with manipulating them.

We would like to ask you for your opinion on the subject, and if there is an option you would think is more appropriate, if any.

Thank you in advance,

Best regards,

Alexandre

Attached document:

The formula to calculate the target weight in the DPRA SOP is

$TW = \left(\frac{MW}{\%Purity} \right) \times 30$, dissolved in 3 ml solvent for a final 100mM solution

For mixtures there are two options:
1) We provide both the Molecular Weight and the % Purity
2) We correct the Molecular Weight for 100% purity and tell them to consider this material as 100% pure.

Formaldehyde:

For option 1, we need to provide both the MW (30.03 g/mol) and the %Purity (37.5)

$$TW = \left(\frac{30.03}{37.5} \right) \times 30 = 24.02mg$$

For option 2, we can correct by multiplying both the numerator and denominator by $\frac{100}{37.5}$:

$$TW = \left(\frac{30.03 \times \frac{100}{37.5}}{37.5 \times \frac{100}{37.5}} \right) \times 30$$

$$TW = \left(\frac{80.1}{100} \right) \times 30 = 24.02mg$$

So a 30.03 g/mol compound which is 37.5% pure is equivalent to an 80.1 g/mol compound which is 100% pure, in the equation used in the SOP.

Kathon CG:

Kathon CG is 1.59% active ingredient, composed of 0.4% of MI (114.14 g/mol) and 1.19% of MCI (149.6 g/mol).

The weighted average molecular weight of the active compound is then

$$\left(114.14 \times \frac{0.4}{1.59} \right) + \left(149.6 \times \frac{1.19}{1.59} \right) = 140.7 g / mol$$

For option 1, we need to provide a MW of 140.7 g/mol and a purity of 1.59%

For option 2, we can correct for 100% purity:

$$TW = \left(\frac{140.7 \times \frac{100}{1.59}}{1.59 \times \frac{100}{1.59}} \right) \times 30$$

$$TW = \left(\frac{8849.1}{100} \right) \times 30$$

So a 140.7 g/mol compound which is 1.59% pure is equivalent to an 8849.1 g/mol compound which is 100% pure, in the equation used in the SOP.

Note:

Since both mixtures are solutions, there is an option 3) which would be to give them the molarity of the solution, so that they can calculate how to dilute it to 100mM

Kathon CG, using the weighted average, is 113 mM

Formaldehyde is 12.2M

However, there are no such instructions or formula in the current SOP to dilute a chemical solution to the correct concentration, and it would need to be modified.

Note 2:

We could also do this for all the chemicals which are not 100% pure.

At the same time, the following e-mail was sent to the DPRA lead laboratory representatives:

From: ANGERS Alexandre (JRC-ISPRA)
Sent: Wednesday, May 05, 2010 9:36 AM
To: 'Foertsch, Leslie'
Cc: CASATI Silvia (JRC-ISPRA); Gerberick, Frank
Subject: ECVAM Skin Sensitisation Prevalidation Study - Question concerning the DPRA molecular weights

Dear Leslie,

We are in the process of gathering the information to be included for testing the chemicals with the DPRA. We realized that, in addition to the molecular weights, we would need to provide the purity. This has led to some concerns in our discussions that this would make some chemicals easy to recognize despite the coding.

One suggestion was made, which we would like to run by you. I will choose, as an example, the case of 40% Glyoxal which has been used in the training and transfer experiments.

To calculate the required weight, you would use the formula

(mw / purity) x 30, which is (58.04 / 40) x 30

We would like, for the chemicals with low (and potentially recognizable) purities to supply a corrected molecular weight, that would include the purity calculation. In this example, the molecular weight would be multiplied by 100/40, and become 145.1. This chemical would then be labeled as having a mw of 145.1 and a purity of 100%.

The calculation

(145.1 / 100) x 30 would then give the exact same target weight.

Before we proceed, we would like to ask you if there is any problem or concern with this method that you would see, based on your experience with the test method.

Thank you in advance,

Alexandre

And the response received was:

From: Foertsch, Leslie [mailto:foertsch.lm@pg.com]
Sent: Wednesday, May 05, 2010 1:18 PM
To: ANGERS Alexandre (JRC-ISPRA)
Cc: CASATI Silvia (JRC-ISPRA); Gerberick, Frank
Subject: RE: ECVAM Skin Sensitisation Prevalidation Study - Question concerning the DPRA molecular weights

Dear Alexandre,

You're right, there are a handful of chemicals that would be easy to identify by their purity. I don't see that this would be a problem for the DPRA. Please go ahead with the proposed plan.

Regards,

Leslie

Appendix II: E-mail template for the shipment of the chemicals

This is to inform you that chemicals for phase B stage 1 items have been shipped

You will receive two boxes, one with 5 chemicals at room temperature and one with 4 chemicals at 4oC.

The tracking numbers for the two boxes are XXX and XXX (include also the company)

Each box will contain:

- The coded vials with chemical samples.
- The chemical's MSDS in sealed envelopes labeled with the corresponding codes.
- A sealed envelope containing a decoding list and an extra copy of the MSDSs. This envelope is for customs use only, please do not open it. This enveloped will be clearly labeled "For customs only".

Note that the box may also be accompanied by shipment documents containing information that can reveal the chemicals identity.

Please read carefully the actions before opening the boxes:

Actions

- If the shipment documents are delivered together with the boxes, please destroy those documents before handing the box to the technical team.
- Open the boxes and return to ECVAM the sealed envelopes containing the decoding list "for customs only". If these envelopes have been unsealed by customs, please report this information, and return them nonetheless.
- Hand over the box to the technical team. They will be responsible for compiling the consignment check list (attached in the e-mail) to confirm receipt of vials and integrity of the sealed MSDS.
- The completed check list should be scanned and sent to ECVAM by e-mail as early as possible.

In addition, please find attached to this e-mail a file describing the coding scheme designed for the study, for your information.

If you have any question, do not hesitate to ask us.

Appendix 6

DPRA results template

Vehicle	Code	Conc/ replicate	Cysteine																		Lysine																		Mean Depletion	Reactivity Class (CYS + LYS)	Reactivity Class (CYS only)	Criterion	Criterion met?		
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Lowers at	Absorb.s at 220 nm	Retent. Time similar to prepr.	Co-elution	If available: Peak Area at 258 nm	220/258 ratio	Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Lowers at	Absorb.s at 220 nm	Retent. Time similar to prepr.	Co-elution	If available: Peak Area at 258 nm	220/258 ratio					
	STANDARD																																											
		STD1	xx	xx																			xx	xx																					
		STD2	xx	xx																			xx	xx																					
		STD3	xx	xx																			xx	xx																					
		STD4	xx	xx																			xx	xx																					
		STD5	xx	xx																			xx	xx																					
		STD6	xx	xx																			xx	xx																					
		Dil Buff	xx	xx																			xx	xx																					
	REF CTRL A																																											
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		r2	xx	#VALUE!																			xx	#VALUE!																					
		r3	xx	#VALUE!																			xx	#VALUE!																			Range Mean Conc Lys [0.45 to 0.55]		#VALUE!	
	CO-ELUTION CTRL																	Y/N	Y/N	Y/N																	Y/N	Y/N	Y/N					Tick the row where there is a "Y" at co-elution.		
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Acetonitrile		Chem 2																																										
Acetonitrile		Chem 3																																										
Acetonitrile		Chem 4																																										
Acetonitrile		Chem 5																																										
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Acetonitrile	REF CTRL C																																											
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		r3	xx	#VALUE!																			xx	#VALUE!																			Range Mean Conc Cys [0.45 to 0.55]		#VALUE!	
water	REF CTRL C (if needed)																																										Range Mean Conc Lys [0.45 to 0.55]		#VALUE!	
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		r2	xx	#VALUE!																			xx	#VALUE!																			Peak Area CV ctrl C < 15% - Lys		#DIV/0!	
		r3	xx	#VALUE!																			xx	#VALUE!																			Range Mean Conc Cys [0.45 to 0.55]		#VALUE!	
Water:Aceto	REF CTRL C (if needed)																																										Range Mean Conc Lys [0.45 to 0.55]		#VALUE!	
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		r2	xx	#VALUE!																			xx	#VALUE!																			Peak Area CV ctrl C < 15% - Lys		#DIV/0!	
		r3	xx	#VALUE!																			xx	#VALUE!																			Range Mean Conc Cys [0.45 to 0.55]		#VALUE!	
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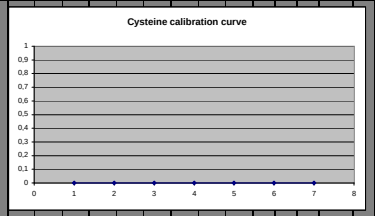

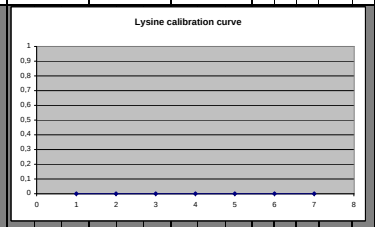

Vehicle	Code	Conc/ replicate	Cysteine																		Lysine																		Mean Depletion	Reactivity Class (CYS + LYS)	Reactivity Class (CYS only)	Criterion	Criterion met?
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Cleared	Absorbs at 220 nm	Retent. Time similar to ampyl.	Co-elution	# available Peak Area at 220 nm	220/258 ratio	Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Cleared	Absorbs at 220 nm	Retent. Time similar to ampyl.	Co-elution	# available Peak Area at 220 nm	220/258 ratio					
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
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		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 16																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 17																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 18																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 19																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 20																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 21																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 22																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 23																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 24																																															
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		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 25																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					
Acetonitrile	CHEMICAL 26																																															
	code:	r1	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	#VALUE!	xx	#VALUE!	xx	xx	#DIV/0!	#DIV/0!	#DIV/0!	#####	#####	#####	#DIV/0!	#DIV/0!	#DIV/0!	Y/N	Y/N	Y/N	Y/N	Y/N	xx	########	#DIV/0!	#DIV/0!	#DIV/0!	SD % Depl Cys < 14,9	#DIV/0!
		r2	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########				SD % Depl Lys < 11,6	#DIV/0!
		r3	xx	#VALUE!	xx	xx															xx	#VALUE!	xx	#VALUE!	xx	xx															xx	########					

Appendix 7

DPRA data QC form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
In-Vitro Methods Unit

SSPA_DPRA_VMG_OTH_Data_QC_V2.pdf

Data Quality Checks Procedures - DPRA

Author: Alexandre Angers

Version 1: 4/11/2010
Version 2: 4/11/2010 Small modifications after using it the first time

Description:

Upon receipt by the contact points of ECVAM, all the filled DPRA reporting templates will undergo quality checks according to the procedures described below.

One form (checklist) should be printed (Page 2 onwards, depending on the number of chemicals) for every received template, and filled up by hand. Once checked, the template can be saved in the official data folder, and the checklist should be scanned and saved as a PDF file in the same folder, with the same name as the original template (adding CHECKLIST at the end of the file name). The template is locked against editing with the following password: ssps

The check should include confirmation that the values in the checked cells are within range, and, if possible, that the formula in the cell has not been modified.

File name:

Received:

Checked by:

Part A – System Suitability

Cell	Description	To check	OK? (Initials)
BI5	r2 acceptance criteria (Lysine)	> 0.99	
BI9	r2 acceptance criteria (Cysteine)	> 0.99	
Q12	Ref Control A (Cysteine)	0.5 +- 0.05	
AQ12	Ref Control A (Lysine)	0.5 +- 0.05	

Part B – Co-Elution

Cell	Description	To check	OK? (Initials)
AA17-AA42	Co-Elution (Cysteine)	"Yes" if Y and Z are "Yes" Co-eluting chemicals:	
BA17-BA42	Co-Elution (Lysine)	"Yes" if AY and AZ are "Yes" Co-eluting chemicals:	

Part C- Suitability over time

Cell	Description	To check	OK? (Initials)
P43	Ref Control B (Cysteine)	< 0.15	
AP43	Ref Control B (Lysine)	< 0.15	

Part D – Solvent Controls

Cell	Description	To check	OK? (Initials)
Q50,P50	Ref Control C (Cysteine) (Solvent 1)	Q50: 0.5 +- 0.05 P50: < 0.15	
AQ50, AP50	Ref Control A (Lysine) (Solvent 1)	AQ50: 0.5 +- 0.05 AP50: < 0.15	
Q54, P54	Ref Control A (Cysteine) (Solvent 2)	Q54: 0.5 +- 0.05 P54: < 0.15	
AQ54, AP54	Ref Control A (Lysine) (Solvent 2)	AQ54: 0.5 +- 0.05 AP54: < 0.15	
Q58, P58	Ref Control A (Cysteine) (Solvent 3)	Q58: 0.5 +- 0.05 P58: < 0.15	
AQ58, AP50	Ref Control A (Lysine) (Solvent 3)	AQ58: 0.5 +- 0.05 AP58: < 0.15	

Part E – Positive Control

Cell	Description	To check	OK? (Initials)
T62, U62	CA depletion values (Cysteine)	T62 > 60.8 U62 < 14.9	
AT62, AU62	CA depletion values (Lysine)	69.4 > AT62 > 40.2 AU62 < 11.6	

Part F – Chemicals

Chemical 1:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 2:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 3:
a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)
b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)
c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 4:
a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)
b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)
c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 5:
a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)
b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)
c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 6:
a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)
b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)
c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 7:
a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)
b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)
c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 8:
a. Solvent. Same indicated in Column A as on the General Information worksheet?

Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 9:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 10:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 11:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 12:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 13:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?

(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 14:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 15:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 16:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 17:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 18:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 19:

a. Solvent. Same indicated in Column A as on the General Information worksheet?

Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 20:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 21:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 22:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 23:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 24:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?

(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 25:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Chemical 26:

a. Solvent. Same indicated in Column A as on the General Information worksheet?
Random result from Column K: Proper solvent control used?
(Cysteine?) (Lysine?)

b. Co-elution Same indicated as in Part B?
(Column AA?) (Column BA?)

c. %SD criteria met?
(Cysteine? U, < 14.9) (Lysine? AU, < 11.6)

Appendix 8

DPRA SOP version 2

P&G	**Direct Peptide Reactivity Assay (DPRA) Standard Operating Procedure**	**Version 2** Pages: 23 Annexes: 0
European Centre for the Validation of Alternative Methods (ECVAM) **Skin Sensitisation Prevalidation Study**		

Issued by	Procter & Gamble	*Date:10/12/2009(Internal version 9)*
Approved by	Validation Management Group	*Date:12/01/2010*
Distributed by	ECVAM	*Date:*

Revision History:

Revision	*Date:*	*Description of change:*
Version 2	September 2010	Addition of an analysis sequence example and additional changes based on comments from the training/transfer phase. The main changes are: - Note about Acetonitrile(page 4) - Area ratio of the peptide peak at 220/258 (page 18) - Co-elution with reactivity (page 19) - Example HPLC Analysis (page 21)

Direct Peptide Reactivity Assay

PRINCIPLE AND SCOPE

The reactivity of a test chemical and synthetic Cysteine or Lysine containing peptides is evaluated by combining the test chemical with a solution of the peptide and monitoring the remaining concentration of the peptide following 24 hours of interaction 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. Samples are prepared and analyzed in triplicate in batches of up to 26 chemicals (including controls) to keep the total HPLC analysis time less than 30 hours. The method is applicable to test chemicals soluble in acetonitrile or other non-reactive, water-miscible solvent at a 100 mM concentration.

REFERENCES

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

Gerberick, G.F. et al. "Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach" Tox. Sci. **97**, 417-427 (2007)

APPARATUS	SUGGESTED TYPE (or Equivalent)
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1mg readability
Glass Vials with Teflon or polyethylene-lined closure, nominal 4 mL capacity	Qorpak 2502T, Supplier VWR Scientific Catalog #66009-557
Dispensing Pipets capable of delivering 250 - 750 µL and 50 µL	Eppendorf Research Adjustable Pipets *verify accuracy at time of use*
Liquid Chromatograph with light-excluding Autosampler capable of delivering 0.35 mL/min flow rate	Waters Alliance 2695, Waters Corp. Milford MA *Note: avoid Waters 2795 design with bottom-draw autosampler needles or adjust needle depth to avoid bottom*
UV Detector capable of measuring UV absorbance at 220 nm	Waters 996 Photodiode Array (preferred) Or Waters 2487 Fixed Wavelength Absorbance detector
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 *Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3 micron particle Part #*

	00D-4251-B0
Guard Column	Phenomenex Security Guard C18 4 mm x 2 mm Part # AJO-4286
Optional: Laboratory Automated Pipetting System with appropriate tools to deliver 50 µL – 750 µL volumes	Beckman Biomek 2000

CHEMICALS AND SPECIAL MATERIALS	**SUGGESTED TYPE (or Equivalent)**
Trifluoroacetic Acid	Sigma-Aldrich 299537 99+%, redistilled, for protein sequencing
Sodium Phosphate, Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$, FW=138.0) CASNo 10049-21-5	ACS Reagent Grade, Aldrich S9638
Sodium Phosphate, Dibasic Heptahydrate ($Na_2HPO_4 \cdot 7H_2O$ FW=268.0) CASNo 7782-85-6	ACS Reagent Grade, Aldrich S9390
Ammonium Acetate $NH_4C_2H_3O_2$ FW=77.08	ACS Reagent Grade, Sigma Aldrich 238074
Ammonium Hydroxide NH_4OH, 28 - 30%	ACS Reagent Grade, Sigma Aldrich 320145
Acetonitrile, HPLC Grade ****	HPLC Grade, Sigma Aldrich 439134 Or HPLC Grade, Fisher Scientific A/0626/17
Purified Water	HPLC Grade or Millipore Milli-Q grade
Cysteine Peptide (store refrigerated) Ac-RFAACAA-COOH, MW=751.9 90-95% purity	RS Synthesis, Louisville KY, USA or Synbiosci, Livermore CA, USA *Note: material contains a mixture including the peptide with one less alanine ("A") unit which may co-elute.*
Lysine Peptide (store refrigerated) Ac-RFAAKAA-COOH MW= 776.2 90-95% purity	RS Synthesis, Louisville KY, USA or Synbiosci, Livermore CA, USA
Cinnamic Aldehyde, 93% purity (Positive Control) **CAS [104-55-2]**	Sigma Aldrich Catalog # W22361-3

REAGENT	**PREPARATION**
100 mM Sodium Phosphate, Monobasic	Using a 1 Liter volumetric flask, dissolve 13.8 g of Sodium Phosphate Monobasic Monohydrate in purified water and dilute to final volume of 1

	Liter. Store refrigerated.
100 mM Sodium Phosphate, Dibasic	Using a 1 Liter volumetric flask, dissolve 26.8 g of Sodium Phosphate Dibasic Heptahydrate in purified water and dilute to final volume of 1 Liter. Store refrigerated.
100 mM Phosphate Buffer, pH=7.5	Combine 18 mL of 0.1 M Sodium Phosphate Monobasic with 82 mL of 0.1M Sodium Phosphate Dibasic. Mix well and measure pH using a calibrated pH meter. Adjust pH to 7.5 +/- 0.05 with either the monobasic (to acidify)or dibasic (basify) solution.
100 mM Ammonium Acetate Buffer, pH=10.2:	Dissolve 1.542 g of Ammonium Acetate in 200 mL purified water. Adjust the pH to 10.2 by dropwise addition of Ammonium Hydroxide using a pH meter calibrated at pH 7 & 10.
HPLC Mobile Phase A: 0.1% (v/v) Trifluoroacetic Acid in Water	Add 1.0 mL of Trifluoroacetic acid to 1 Liter of HPLC grade Water.
HPLC Mobile Phase B 0.085% (v/v) trifluoroacetic acid in acetonitrile	Add 850 microliters of Trifluoroacetic Acid to 1 liter of HPLC grade acetonitrile.

****** A Note about acetonitrile:**

Some supplies of acetonitrile have had a negative impact on peptide stability (particularly cysteine). This can be assessed when starting a new batch of acetonitrile by performing the following test prior to running the assay.

1. Prepare a small amount of 0.501 mg/mL cysteine peptide solution in phosphate buffer.
2. Prepare an autosampler vial containing 750uL peptide solution and 250uL acetontrile.
3. Incubate for 24 hours.
4. Visually inspect the vial for precipitation.
5. Set up an HPLC run using the conditions defined in this SOP. Inject this sample every 2-3 hours for approximately 48 hours.
6. Compare the peak areas for each injection. The CV should be <15%

PROCEDURE

The procedure is divided into six main categories:

- **PRE WORK:** Prepare HPLC system, Pre-weigh test chemicals and peptide, determine the appropriate solvent for each test chemical.
- **SOLUTION PREPARATION:** Dissolve test chemicals and peptide immediately before the assay.
- **ASSAY PROCEDURE:** Combine solutions to start peptide reaction.
- **HPLC ANALYSIS:** Analysis of the final reaction mixture.
- **DATA ANALYSIS & CALCULATIONS:** Analysis of the data obtained.

- **DATA REPORTING (FOR CYSTEINE AND LYSINE):** Data reporting.
- **ACCEPTANCE CRITERIA:** Acceptance criteria for the run, test chemical and data.

PRE-WORK

Prepare HPLC System

Prepare an HPLC system with a UV detector (220 nm) and mobile phase A and B described in the reagent section.

1. Column Equilibration: Install a new guard column cartridge for each set of chemicals. Install the HPLC column (see Apparatus section) and equilibrate the column at 30 °C with 50% A, 50% B for at least 2 hours before use. Condition the column by running the gradient at least twice before using the column.
2. Column Storage Conditions: If the column will be stored for more than a week, fill the column with acetonitrile (without Trifluoroacetic Acid) and cap tightly. Store at room temperature.
3. System Shutdown: Following analysis, maintain a low flow (typically 0.05 mL/min) of 50% A: 50% B through the system and decrease column temperature to approximately 25 °C. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid), remove the column from the HPLC system and cap tightly and purge acid containing mobile phases from the system using a mixture of either 1:1 (v/v) acetonitrile:water or 1:1 (v/v) methanol:water.

Solubility Assessment and Pre-weigh Test Chemicals

Solubility of the test chemicals in a suitable solvent should be assessed before performing the actual assay. An appropriate solvent will dissolve the test chemical completely, i.e. by visual inspection the solution must not be cloudy nor have noticeable precipitate. Acetonitrile is the preferred solvent for test chemicals, however not all chemicals are soluble in acetonitrile. The following solubilization procedure for the selection of the appropriate solvent should be followed.

1. Evaluate solubility by preparing an approximately 100mM solution in **acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
2. If the test chemical is not soluble in acetonitrile, attempt to prepare a 100mM solution in **water**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
3. If the test chemical is not soluble in acetonitrile or water alone, attempt to prepare a 100mM solution in a 1:1 mixture of **water:acetonitrile** (this works well for many organic salts). Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
4. If the test chemical is not soluble in either acetonitrile or water, attempt to prepare a 100mM solution in **isopropanol.** Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
5. If the test chemical is not soluble in either acetonitrile, water or isopropanol, attempt to prepare a 100mM solution in **acetone or a 1:1 acetone:acetonitrile mix.** Vortex to mix. If the test chemical does not

solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).

6. As a last option, if the chemical is not soluble in any of these solvents, attempt to dissolve the same amount of test chemical in **300 microliters of dimethyl sulfoxide** and dilute the resulting solution with 2700 **microliters of acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
7. If the test chemical is not soluble in this mixture, dissolve the same amount of test chemical in **1500 microliters of dimethyl sulfoxide** and dilute the resulting solution with **1500 microliters of acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).

Note: Water is not a good solvent choice for anhydrides due to their reactivity with water.

Test chemicals are pre-weighed into clean, dry 4mL glass vials. Test chemicals will be dissolved in 3.0 mL of the appropriate solvent determined in the "Solubility Assessment" Pre-Work (see above) to prepare a 100 mM solution immediately before use. The weight of test chemical to be added to the vial is determined based on the molecular weight ("MW") and purity. If no purity information is available, assume 100% purity.

1. Calculate the target weight of test chemical needed to prepare 3.0mL of a 100mM solution of test chemical using the formula:

$$3\text{mL} \times \frac{1\text{ L}}{1000\text{mL}} \times \frac{100\text{ mmoles}}{\text{L}} \times \text{MW}\left(\frac{\text{mg}}{\text{mmole}}\right) \times \frac{100}{\%\text{Purity}} = \frac{\mathbf{MW}}{\mathbf{\%\,Purity}} \times \mathbf{30} = \mathbf{Target\ Weight\ (mg)}$$

2. Weigh the target amount (+/- 10% of target) of test chemical directly into a glass vial and record the actual weight, identity, molecular weight and purity.
3. Tightly close each vial and store under appropriate conditions until ready to perform testing. Appropriate storage conditions for each test chemical are determined based on supplier information.
4. Control Samples: Cinnamic aldehyde is used as the Positive Control for the assay and is included in every assay run. "Reference Controls" are also included with each study. A Reference Control is a peptide solution where the test chemical is replaced by the solvent used to dissolve it. Reference Controls for each solvent used to solubilize the test chemicals should be included in every assay run together with the samples (see Reference Controls in the scheme on pages 8 and 9) and are used to verify that the solvent does not impact the Percent Peptide Depletion. The appropriate Reference Controls for each chemical are used to calculate Percent Peptide Depletion.

Pre-weigh Cysteine or Lysine peptide for stock solutions (0.667 mM)

Note: Do not add buffer to the peptide solid until ready to begin the assay

Cysteine Peptide Ac-RFAACAA-COOH, 0.667 mM, 0.501 mg/mL: The assay will require approximately 800 µL/sample replicate All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock needed, weigh an appropriate amount of peptide into a large vial or test tube. For example, to prepare 25 mL of solution, weigh 0.01215 g of Cysteine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.501 mg/mL. Record the exact weight of peptide added to the vial.

Lysine Peptide Ac-RFAAKAA-COOH, 0.667 mM, 0.518 mg/mL: The assay will require approximately 800µL/sample replicate. All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock solution needed, weigh an appropriate amount of peptide into a large vial of test tube. For example, to prepare 25 mL of solution, weigh 0.0129 g of Lysine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.518 mg/mL. Record the exact weight of peptide added to the vial.

Note:

- When starting a new lot of peptide, a small amount should be dissolved in the appropriate buffer at ~0.5 mg/mL and injected through the HPLC to verify that the chromatogram is similar to previous batches.

SOLUTION PREPARATION

1. Suitable run sequence sizes are 1-26 test chemicals, in addition to the Positive Control and Reference Controls. If additional solvents are required, additional Reference Controls need to be prepared and the number of test chemicals will need to be reduced. This run sequence size permits the first HPLC injection to occur 24 hours after mixing the test chemical and peptide and the last HPLC injection to occur no more than 30 hours later. Appropriate controls must be included in each run sequence.
2. Label three autosampler vials for each test chemical and control corresponding to the triplicate preparations.
3. Pre-weigh all test chemicals as described above. Do not dissolve until ready to use.
4. Pre-weigh Cysteine or Lysine peptide as described above. Do not dissolve until ready to use.

Test Chemical Solution Preparation

Solubility of the test chemical in the appropriate solvent is evaluated in the pre-work section. 100mM solutions of test chemicals in the appropriate solvents are prepared fresh, immediately before use.

1. When ready to perform the assay, calculate and weigh out the appropriate amount of test chemical needed to prepare a 100mM solution. Dissolve the test chemical by adding 3.0mL of the appropriate solvent. The resulting solution should have a test chemical concentration of 100 mM.
2. Mix vial to dissolve the test chemical. Slight sonication (less than 1 minute) may be used if needed. If the test chemical is not completely dissolved, do not

proceed with that specific test chemical in the selected solvent. Re-evaluate alternative solvents (see pre-work section) to find a suitable choice.

3. Record and report the final solvent choice for each chemical.

Positive Control Solution Preparation

The Positive Control (cinnamic aldehyde) is soluble in acetonitrile.

1. Calculate the target weight of cinnamic aldehyde needed to prepare 3.0mL of a 100mM solution of test chemical using the formula:

$$3\text{mL} \times \frac{1\text{ L}}{1000\text{mL}} \times \frac{100\text{ mmoles}}{\text{L}} \times \text{MW}\left(\frac{\text{mg}}{\text{mmole}}\right) \times \frac{100}{\%\text{Purity}} = \frac{\mathbf{MW}}{\mathbf{\%\,Purity}} \times \mathbf{30} = \mathbf{Target\ Weight\ (mg)}$$

2. Weigh the target amount (+/- 10% of target) of test chemical directly into a glass vial and record the actual weight, identity, molecular weight and purity.

3. Tightly close each vial and store under appropriate conditions until ready to perform testing. Appropriate storage conditions for each test chemical are determined based on supplier information.

4. Dissolve in 3mL of acetonitrile.

Peptide Stock Solution Preparation

1. **Cysteine Peptide:** Add the appropriate amount of pH 7. 5 phosphate buffer to make a 0.667 mM solution of Cysteine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\mathbf{mL\ pH\ 7.5\ Buffer} = \frac{\mathbf{mg\ Peptide}}{\mathbf{0.501\ mg/mL}}$$

2. **Lysine Peptide:** Add the appropriate amount of pH 10.2 Ammonium Acetate buffer to make a 0.667 mM solution of Lysine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\mathbf{mL\ pH\ 10.2\ Buffer} = \frac{\mathbf{mg\ Peptide}}{\mathbf{0.518\ mg/mL}}$$

ASSAY PROCEDURE

Reference Control, Positive Control, Co-elution Control and Sample Preparation

Samples are prepared in triplicate for both peptides. Each assay (Cys and Lys) may be prepared concurrently (if two HPLC systems are available) or on separate days (if only one HPLC is available).

1. Assemble the following previously prepared reagents, solvents and solutions:
    a. Peptide stock solution,
    b. Appropriate buffer (pH 7.5 for Cysteine Peptide, pH 10.2 for Lysine peptide),
    c. Acetonitrile
    d. Test chemical solution (or solvent for Reference Controls)

2. Using 1 mL autosampler vials as containers, prepare the sample by adding the reagents in the quantity and order listed below, with gentle mixing during addition. Record the time of addition of the test chemical to the peptide solution.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM test chemical	**1:50 Ratio, Lysine Peptide** 0.5 mM Peptide, 25 mM test chemical
750 µL Cysteine peptide solution (or pH 7.5 phosphate buffer for Co-elution Controls) **200 µL Acetonitrile** **50 µL Test chemical solution (or solvent for Reference Controls)**	**750 µL Lysine peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution Controls)** **250 µL Test chemical solution (or solvent for Reference Controls)**

3. Cap the vials, vortex to mix and place in the HPLC autosampler (dark) at 25 °C for 24 hours. HPLC analysis of the batch of samples should start 24 hours after the test chemical was added to the peptide solution.

Standard Preparation

Standards are prepared in a solution of 20% Acetonitrile:Buffer while samples will have a mixture of 25% solvent:buffer. This difference does not adversely impact the chromatography or stability of the samples and standards.

Using serial dilution, prepare standards of the peptide stock solution covering the range from 1 - 0.0167mM.

1. **Prepare approximately 10 mL of <u>dilution buffer</u>** by mixing 8 mL of buffer (pH 7.5 for Cysteine peptide, pH 10.2 for Lysine peptide) with 2 mL of acetonitrile.
2. **Prepare the initial standard, "STD1"** at 0.534 mM by diluting 1600 µL of the peptide stock solution (at 0.667 mM) with 400 µL acetonitrile.
3. **Dilute 1.0 mL of standard STD1 with an equal volume of dilution buffer and continue** in a serial manner to give standards with nominal concentrations noted below. Include a blank of dilution buffer as STD 7.

	STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**Dilution Buffer**
mM Peptide	**0.534**	**0.267**	**0.1335**	**0.0667**	**0.0334**	**0.0167**	**0.000**

Serial Dilution Procedural Details

1) Label 5 glass vials (nominal 2-5 mL volume) with codes STD2 – STD6.
2) Add 1.00 mL of dilution buffer to vials STD2 through STD6
3) Transfer 1.00 mL of Standard STD1 to vial STD2. Mix with minimal air entrainment
4) Transfer 1.00 mL from vial STD2 to vial STD3. Mix with minimal air entrainment
5) Continue in a similar manner for standards STD4 through STD6.
6) Transfer standards to autosampler vials for analysis

HPLC ANALYSIS

Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the weak and strong solvents and a column temperature of 30ºC. The HPLC analysis is performed using a flow of 0.35 mL/min and a linear gradient from 10% to 25% Acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Inject equal volumes of each standard, sample and control. The injection volume may vary according to the system used (typically in the range from 3-10 µL). On some systems, 10µL injection volumes lead to unacceptably broad peaks and smaller injection volumes need to be used. Absorbance is monitored at 220 nm. If using a Photodiode Array detector, absorbance at 258 nm should also be recorded. Re-equilibrate the column under initial conditions for at least 7 minutes. *Note: The 7 minute re-equilibration time was determined using a Waters 2695 HPLC system. Other systems may require more or less re-equilibration time due to system mixing volume. Shorter equilibration times will be acceptable if peak retention times are stable.*

HPLC Conditions

Column	Preferred Column: Zorbax SB-C18 2.1mm x 100 mm x 3.5 micron Agilent Part Number 861753-902 Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3 micron particle (Part # 00D-4251-B0 may require flowrates of 0.3mL/min) or any other C18 column that demonstrates acceptable peak resolution. *Note: Both columns are semi-micro scale and require careful connections to minimize extracolumn peak broadening. Waters 2695 Alliance HPLC systems provide suitable peak shapes. Semi-micro HPLC systems may improve peak resolution and it may be possible to decrease analysis time on other systems.*			
Column Temperature	30 ºC			
Sample temperature	25 ºC			
Detector	Photodiode Array detector or Fixed Wavelength Absorbance detector with 220 nm signal for quantitation			
Injection Volume	~7 µL (Volume varies according to the HPLC system. If peaks are too broad, the volume should be decreased) Set the autosampler needle depth to avoid drawing sample from the bottom of the vial.			
Run Time	20 minutes			
Flow Conditions	**Time**	**Flow**	**%A**	**%B**
	0 min	0.35 mL/min	90	10
	10 min	0.35 mL/min	75	25
	11 min	0.35 mL/min	10	90
	13 min	0.35 mL/min	10	90
	13.5 min	0.35 mL/min	90	10
	20 min	end run		

Note: Visually inspect samples prior to HPLC analysis. Generally, precipitation is not a problem. However, if a precipitate is observed, this should be noted in the data reporting template. Samples may be centrifuged at low speed (100-400 xg) in the vial to force precipitate to the bottom of the vial as a precaution, since large amounts of precipitate may clog the HPLC tubing or columns. **Precipitate formation and removal must be recorded and reported.**

Filtering samples or use of high speed polypropylene centrifuge tubes to remove precipitate has not been evaluated and may lead to loss of peptide through adsorption, therefore this is not recommended.

Prepare two separate analysis sequences, based on the example below: 1. Calibration standards, Reference Controls A and Co-elution Controls and, 2. Stability of Reference Controls over analysis time (Reference Controls B) and sets of replicates (Reference Controls C, Positive Control and test chemicals). The first analysis sequence can be timed to complete prior to the end of the 24 hour incubation and the second sequence should be timed to assure that the injection of the first sample starts 24 (+/-2) hours after the test chemical was mixed with the peptide solution. Alternatively, since there is no chemical reaction occurring in the calibration standards, Reference Controls and Co-elution controls, the first analysis sequence can be timed to run shortly after assay setup is complete rather than directly before the second analysis sequence.

Example HPLC Sample Analysis Sequences
(A more specific analysis sequence can be found at the end of the SOP.)

STD1 **STD2** **STD3** **STD4** **STD5** **STD6** **Dilution Buffer** **Reference Control A, rep 1** **Reference Control A, rep 2** **Reference Control A, rep 3**	***Calibration Standards and Reference Controls*** *Verify linearity of response* *Verify precision and accuracy of pipetting* *System Suitability:* *r^2 >0.990* *Mean peptide concentration of Reference Controls A =* *0.50 +/- 0.05 mM*
Co-elution Control 1 **Co-elution Control 2** **Co-elution Control 3** . . .	***Co-elution Controls*** *Verify co-elution of test chemicals with peptide*
Reference Control B, rep 1 **Reference Control B, rep 2** **Reference Control B, rep 3**	***Reference Controls*** *Verify stability of Reference Controls over analysis time* *(see also below)*
Reference Control C, rep 1 §, † **Cinnamic aldehyde, rep 1** **Sample 1, rep 1** **Sample 2, rep 1**	***First set of replicates*** *Note: Start first set of replicates 24 +/- 2 hours after peptide:test chemical mixing.*

Sample 3, rep 1 . . .	
Reference Control C, rep 2 [§] **Cinnamic aldehyde, rep 2** **Sample 1, rep 2** **Sample 2, rep 2** **Sample 3, rep 2** . . .	***Second set of replicates***
Reference Control C, rep 3 [§] **Cinnamic aldehyde, rep 3** **Sample 1, rep 3** **Sample 2, rep 3** **Sample 3, rep 3** . . . [†]	***Third set of replicates*** *For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM*
Reference Control B, rep 4 **Reference Control B, rep 5** **Reference Control B, rep 6**	***Reference Controls*** *Verify stability of Reference Controls over analysis time:* *CV of peptide peak areas of the nine Reference Controls B and C in acetonitrile must be < 15.0%*

[§] Three replicates for Reference Controls C should be included in the analysis sequence for each solvent that is used to dissolve test chemicals. These should be run with the Samples and are used to separately calculate the Percent Peptide Depletion in each solvent and verify that they do not impact the Percent Peptide Depletion.

[†] The difference in time between the first injection of the first replicate and the last injection of the last replicate should not exceed 30 hours.

DATA ANALYSIS & CALCULATIONS

The concentration of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area of the appropriate peaks and calculating the concentration of peptide using the linear calibration curves derived from the standards. The Cysteine peptide includes other peaks that elute near the peptide of interest. Refer to the example chromatogram for appropriate integration of the peak.

The percent depletion of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area and dividing that by mean peak area of the reference controls.

1. Integrate the appropriate peaks and determine peak area for standards, samples and controls. The peak area of each integrated peak must be reported. The peaks should be consistently integrated "valley to valley". There may be some instances when this is not practical, but should be appropriate for most chromatograms.
2. Generate a linear calibration curve with forced coordinate intercept at y = 0 based on the concentration of standards (equal weighting) and the peak area. Suitable calibration curves will have an r^2>0.990. Calculate the mean peptide concentration in Reference Controls A, SD and CV. The mean should be 0.50 +/- 0.05 mM. Values outside of this range may indicate a pipetting or sample preparation error. The peptide concentration of Reference Controls A and C (see analysis sequence above) must be reported.
3. Calculate the mean peptide peak area at 220 nm for the nine Reference Controls B and C in acetonitrile, SD and CV. The CV must be < 15.0%
4. Calculate the mean peptide peak area at 220 nm for the three Reference Controls C for each solvent used.
5. Calculate the mean peptide concentration (mM) for the three Reference Controls C for each solvent used, SD and CV. The mean should be 0.50 +/- 0.05 mM.
6. UV absorbance is a general detection method and interfering peaks may occur. If there is uncertainty regarding the identity of the peak, verify the UV absorbance spectrum and retention time are consistent with the Reference Control C injections.
7. Some test chemicals will co-elute with the cysteine or lysine peptides. In order to detect possible co-elution of the test chemicals with a peptide, the test chemicals included in the run must be injected alone ("Co-elution Controls") at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent. If a chemical absorbs at 220 nm and has a similar retention time as a peptide (overlap of "valley to valley" integration periods), then co-elution of the test chemical with that peptide should be reported. In order to assure that baseline noise is not being called interference, the "interfering" chemical peak should have a peak area that is >10% of the mean peptide peak area in the appropriate Reference Control. The chromatograms of the reaction mixtures should also be inspected in case of possible co-elution to verify if the peaks of the chemical and the peptide are indeed not baseline separated. If co-elution occurs, proper integration and calculation of Percent Peptide Depletion (see below) is not possible. The data should be recorded as "interference" for the peptide the chemical co-elutes with. When a Photodiode Array detector is used, co-elution of chemical and peptide

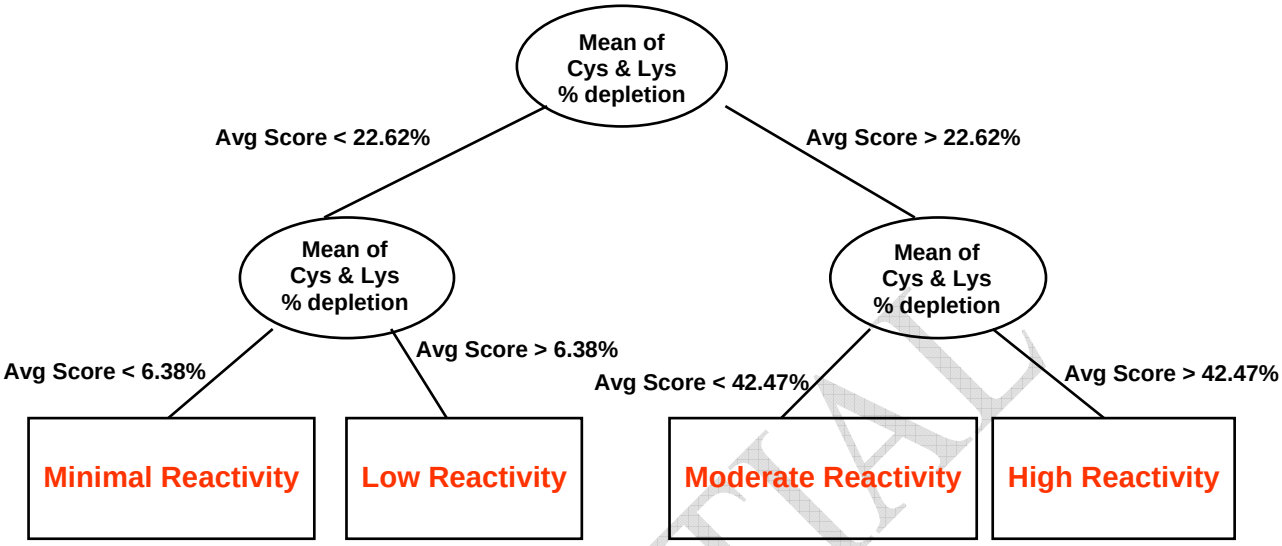
may also be verified by looking at the UV spectrum at 258nm in addition to 220nm and calculating the area ratio of 220/258. This value should be consistent over all samples and standards for a pure peptide peak and thus gives a measure of peak purity. Calculation of peak purity (area ratio of 220/258) might not be possible if the chemical is highly reactive with the peptide leading to very small peaks.

8. For the Positive Control and for each test chemical, calculate the Percent Peptide Depletion in each replicate from the peptide peak area of the replicate injection and the mean peptide peak area in the three relevant Reference Controls C (in the appropriate solvent), by using the following formula. The Percent Peptide Depletion of every injected Positive Control and test chemical replicate must be reported. Moreover, the mean Percent Peptide Depletion of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

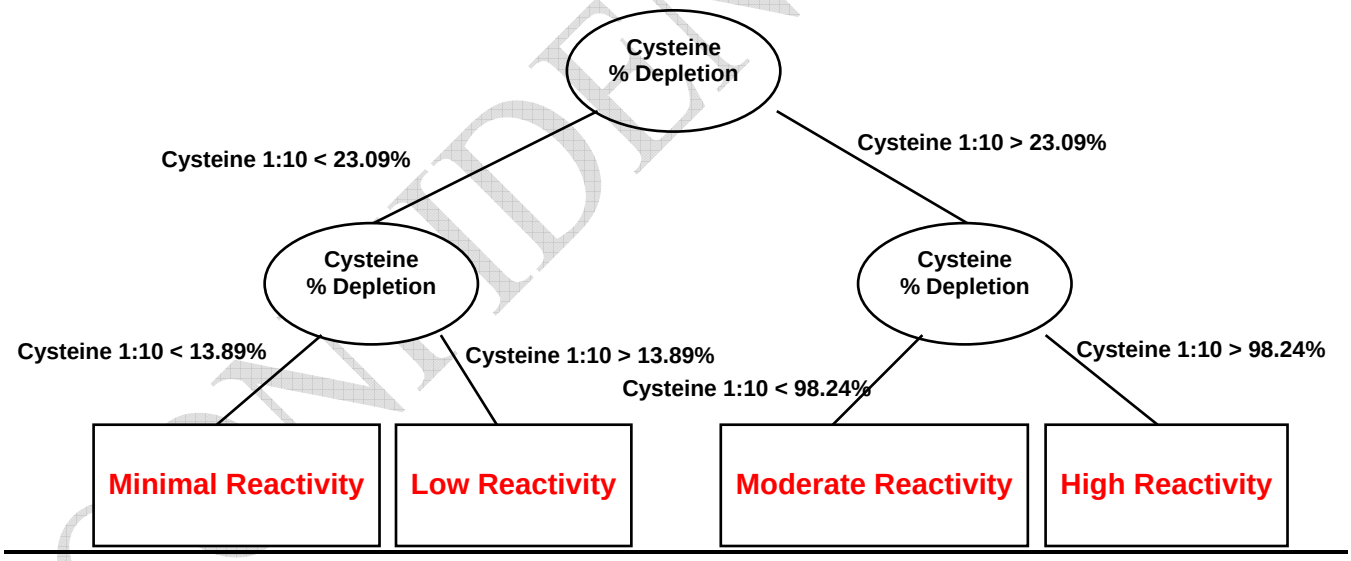
$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls } C}\right)\right] \times 100$$

9. If the Percent Peptide Depletion is < - 10.0%, it should be considered unacceptable as this may be a situation of co-elution or inaccurate peptide addition to the reaction mixture. If this happens, proper integration and calculation of Percent Peptide Depletion is not possible. The data should be recorded as “interference” if co-elution was observed for that peptide. If co-elution was not observed the run should be repeated for that chemical..
10. Calculate the mean of the Percent Cysteine and Percent Lysine Depletions for the Positive Control and for each test chemical. Negative depletion values should be considered as “Zero” when calculating the mean. Assign a reactivity category to each test chemical by using the Cysteine 1:10/Lysine 1:50 prediction model below. In cases where a test chemical co-elutes with the lysine peptide, the Cysteine 1:10-only prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone, and the data should be reported as “inconclusive”. The reason for this is that the lysine reactivity does not carry enough weight to drive the prediction model.

Cysteine 1:10/Lysine 1:50 Prediction Model



Cysteine 1:10-only Prediction Model



DATA REPORTING (FOR CYSTEINE AND LYSINE)

SYSTEM SUITABILITY

- Peptide peak area at 220 nm of each Standard and Reference Control A replicate.
- The linear calibration curve should be graphically represented and the R^2 reported.
- Peptide concentration (mM) of each Reference Control A replicate.
- Mean peptide concentration (mM) of the three Reference Controls A, SD and CV.

ANALYSIS SEQUENCE

Reference Controls:

- Peptide peak area at 220 nm of each B and C replicate.
- Mean peptide peak area at 220 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean peptide peak area at 220 nm of the three appropriate Reference Controls C (for calculation of Percent Peptide Depletion).
- For each solvent used, the peptide concentration (mM) of the three appropriate Reference Controls C.
- For each solvent used, the mean peptide concentration (mM) of the three appropriate Reference Controls C, SD and CV.

Positive Control (cinnamic aldehyde)

- Peptide peak area at 220 nm of each replicate.
- Percent Peptide Depletion of each replicate.
- Mean Percent Peptide Depletion of the three replicates, SD and CV.

For each test chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time. If precipitate was re-solubilised or centrifuged.
- Peptide peak area at 220 nm of each replicate (for systems equipped with a PDA detector the peak area at 258 nm should also be reported).
- Percent Peptide Depletion of each replicate.
- Mean of Percent Peptide Depletion of the three replicates, SD and CV
- Mean of Percent Cysteine and Percent Lysine Depletion values.
- Reactivity class.

ACCEPTANCE CRITERIA

PEPTIDE REACTIVITY ASSAY RUN ACCEPTANCE CRITERIA

All criteria must be met for the whole run to be considered valid. If these criteria are not met, the run must be repeated for all test chemicals.

System Suitability:
Calibration Linearity $r^2 > 0.990$
Mean peptide concentration of Reference Controls A = 0.50 +/- 0.05 mM

Positive Control:
The mean Percent Peptide Depletion value of the three replicates for cinnamic aldehyde must fall within the ranges reported in the following table (based on 95% Tolerance Intervals):

	Percent Cysteine Depletion		Percent Lysine Depletion	
Positive Control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Cinnamic aldehyde	60.8	100.0	40.2	69.4

Maximum Standard Deviations for Positive Control replicates:
Standard Deviation for Percent Cysteine Depletion must be < 14.9%
Standard Deviation for Percent Lysine Depletion must be < 11.6%

Stability of Reference Controls over analysis time:
CV of peptide peak areas for the nine Reference Controls B and C in acetonitrile must be < 15.0%.

ACCEPTANCE CRITERIA FOR EACH TEST CHEMICAL

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of sample replicates:
Standard Deviation for Percent Cysteine Depletion must be < 14.9%
Standard Deviation for Percent Lysine Depletion must be < 11.6%

Reference Controls in the analysis sequence:
For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM

PEPTIDE REACTIVITY ASSAY DATA ACCEPTANCE CRITERIA

Presence of precipitate

If precipitate occurs at the end of the 24 hrs incubation period, This should be recorded and the sample analyzed.

Co-elution of test chemical with peptide

In cases where a test chemical co-elutes with the lysine peptide, the Cysteine 1:10-only prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone, and the data should be reported as "inconclusive".

1. Negative depletion values

If a negative Percent Peptide Depletion value is observed, it can be accepted if it is > -10.0%. This value should be considered "Zero" when calculating the mean Percent Depletion of Cysteine and Lysine for the prediction model. If the Percent Peptide Depletion is < - 10.0%, it should be considered unacceptable as this may be a situation of co-elution or inaccurate peptide addition to the reaction mixture. The data should be reported as "interference" If this happens, proper integration and calculation of Percent Peptide Depletion is not possible. The data should be recorded as "interference" if co-elution was observed for that peptide. If co-elution was not observed the run should be repeated for that chemical.

2. Co-elution Controls

If a chemical (Co-elution Control) absorbs at 220 nm and has a similar retention time as a peptide (Reference Control) (overlap of "valley to valley" integration periods), then co-elution of the test chemical with the peptide should be reported. In order to assure that baseline noise is not being called interference, the "interfering" chemical peak should have a peak area the is >10% of the mean peptide peak area in the appropriate Reference Control. The chromatograms of the reaction mixtures should also be inspected in case of possible co-elution to verify if the peaks of the chemical and the peptide are indeed not baseline separated. If co-elution occurs, proper integration and calculation of Percent Peptide Depletion (see below) is not possible. The data should be recorded as "interference" for that peptide.

3. Area ratio of the peptide peak at 220/258

When a Photodiode Array detector is used, co-elution of chemical and peptide may also be verified by looking at the UV spectrum at 258nm in addition to 220nm and calculating the area ratio of 220/258. This value should be consistent over all samples and standards for a pure peptide peak and thus gives a measure of peak purity. For each sample. a ratio in the following range would give a good indication that co-elution has not occurred: **90%<Mean Area ratio of control samples <110%.** However, calculation of peak purity (area ratio of 220/258) might not always be possible, particularly if the test chemical is highly reactive with the peptide leading to very small peaks.

4. Co-elution with reactivity

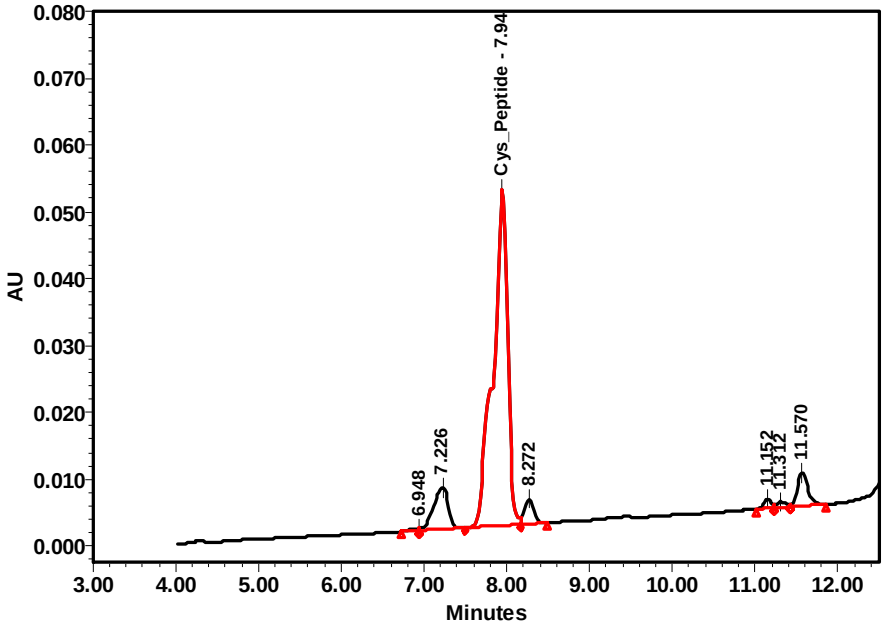
In some instances, a test chemical may have an overlapping retention time with either of the peptides and still be reactive with that peptide. If this is the case, percent depletion can still be calculated with a notation of "**co-elution – percent depletion estimated**". Co-elution will make the area of the peptide peak appear to be larger than it really is, therefore the calculated percent depletion may be **lower** than the true value. This estimated value can still be used in the prediction model with some additional notation. When using an estimated percent depletion for one peptide and the model predicts "High reactivity", the result is fine but should still be noted that the percent depletion is estimated. If the estimated percent depletion leads to a "Moderate reactivity" or "Low reactivity," the result should be noted as "≥ Moderate reactivity" or "≥ Low Reactivity" respectively. If the estimated percent depletion leads to "Minimal reactivity," the result should be reported as "Inconclusive." However, unless cysteine is the co-eluting peptide, the Cysteine-only prediction model should be used before using this estimated method.

The following table illustrates the different scenarios:

Mean depletion values	No co-elution	Co-elution with Cysteine alone or Cysteine and Lysine	Co-elution with Lysine only
Less than 6.38%	Minimal Reactivity	Inconclusive	Apply Cysteine-only prediction model
Between 6.38% and 22.62%	Low Reactivity	≥ Low Reactivity	Apply Cysteine-only prediction model
Between 22.62% and 42.47%	Moderate Reactivity	≥ Moderate Reactivity	Apply Cysteine-only prediction model
More than 42.47%	High Reactivity	High Reactivity	Apply Cysteine-only prediction model

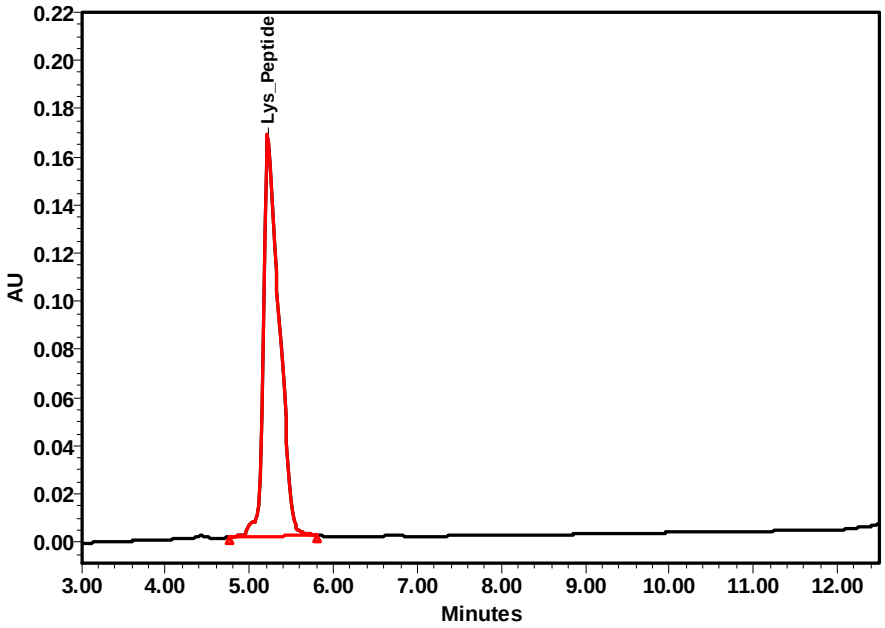
Example Chromatograms

Cysteine Peptide (Retention Time approximately 8-9 minutes)



Note: The Cysteine peptide shown contains a mixture including the peptide with one less alanine ("A") unit which causes the leading edge shoulder on the peak. Both peptides react in a comparable manner and are integrated together.

Lysine Peptide (Retention Time approximately 5-6 minutes)



Example HPLC Analysis

Example DPRA run:

You have 5 test chemicals. Chemical 1, 2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in isopropanol

You need to set up the following vials:

Analysis Sequence 1:

STD 1
STD 2
STD 3
STD 4
STD 5
STD 6
Dilution buffer blank
Reference Control A, rep 1 (made with acetonitrile)
Reference Control A, rep 2 (made with acetonitrile)
Reference Control A, rep 3 (made with acetonitrile)

Coelution Control for Chemical 1
Coelution Control for Chemical 2
Coelution Control for Chemical 3
Coelution Control for Chemical 4
Coelution Control for Chemical 5

Analysis Sequence 2:

Reference Control B, rep 1 (made with acetonitrile)
Reference Control B, rep 2 (made with acetonitrile)
Reference Control B, rep 3 (made with acetonitrile)

Reference Control C, rep 1 (made with acetontrile)
Reference Control C, rep 1 (made with isopropanol)
Cinnamic aldehyde, rep 1
Chemical 1, rep 1
Chemical 2, rep 1
Chemical 3, rep 1
Chemical 4, rep 1
Chemical 5, rep 1

Reference Control C, rep 2 (made with acetontrile)
Reference Control C, rep 2 (made with isopropanol)
Cinnamic aldehyde, rep 2
Chemical 1, rep 2
Chemical 2, rep 2

Chemical 3, rep 2
Chemical 4, rep 2
Chemical 5, rep 2

Reference Control C, rep 3 (made with acetontrile)
Reference Control C, rep 3 (made with isopropanol)
Cinnamic aldehyde, rep 3
Chemical 1, rep 3
Chemical 2, rep 3
Chemical 3, rep 3
Chemical 4, rep 3
Chemical 5, rep 3

Reference Control B, rep 4 (made with acetonitrile)
Reference Control B, rep 5 (made with acetonitrile)
Reference Control B, rep 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean peptide peak area of the Reference Control C made with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean peptide peak area of the Reference Control C made with isopropanol.

Appendix 9

DPRA SOP Version 3

P&G	**Direct Peptide Reactivity Assay (DPRA) Standard Operating Procedure**	**Version 3** Pages: 27 Annexes: 0
European Centre for the Validation of Alternative Methods (ECVAM) **Skin Sensitisation Validation Study**		

Issued by	Procter & Gamble	*Date: 05/01/2012*
Approved by	Validation Management Group	*Date: 12/01/2012*
Distributed by	ECVAM	*N/A*

Revision History:

Revision	*Date:*	*Description of change:*
Version 2	September 2010	Addition of an analysis sequence example and additional changes based on comments from the training/transfer phase. The main changes are: - Note about Acetonitrile(page 4) - Area ratio of the peptide peak at 220/258 (page 18) - Co-elution with reactivity (page 19) - Example HPLC Analysis (page 21)
Version 3	January 2012	-Remove Synbiosci as suggested peptide supplier -Include expiration date for ammonium acetate buffer -Clarification for estimating peptide depletion -Change the wording of “curve with forced coordinate intercept at y = 0” for the standard curve graph Additional clarifications in the sample preparation procedure

Direct Peptide Reactivity Assay

PRINCIPLE AND SCOPE

The reactivity of a test chemical and synthetic Cysteine or Lysine containing peptides is evaluated by combining the test chemical with a solution of the peptide and monitoring the remaining concentration of the peptide following 24 hours of interaction 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. Samples are prepared and analyzed in triplicate in batches of up to 26 chemicals (including controls) to keep the total HPLC analysis time less than 30 hours. The method is applicable to test chemicals soluble in acetonitrile or other non-reactive, water-miscible solvent at a 100 mM concentration.

REFERENCES

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

Gerberick, G.F. et al. "Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach" Tox. Sci. **97**, 417-427 (2007)

APPARATUS	SUGGESTED TYPE (or Equivalent)
Analytical Balance	Capable of accurately weighing up to 20 grams with 0.1mg readability
Glass Vials with Teflon or polyethylene-lined closure, nominal 4 mL capacity	Qorpak 2502T, Supplier VWR Scientific Catalog #66009-557
Dispensing Pipets capable of delivering 250 - 750 µL and 50 µL	Eppendorf Research Adjustable Pipets *verify accuracy at time of use*
Liquid Chromatograph with light-excluding Autosampler capable of delivering 0.35 mL/min flow rate	Waters Alliance 2695, Waters Corp. Milford MA *Note: avoid Waters 2795 design with bottom-draw autosampler needles or adjust needle depth to avoid bottom*
UV Detector capable of measuring UV absorbance at 220 nm	Waters 996 Photodiode Array (preferred) Or Waters 2487 Fixed Wavelength Absorbance detector
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 *Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3 micron particle Part #*

	00D-4251-B0
Guard Column	Phenomenex Security Guard C18 4 mm x 2 mm Part # AJO-4286
Optional: Laboratory Automated Pipetting System with appropriate tools to deliver 50 µL – 750 µL volumes	Beckman Biomek 2000

CHEMICALS AND SPECIAL MATERIALS	**SUGGESTED TYPE (or Equivalent)**
Trifluoroacetic Acid	Sigma-Aldrich 299537 99+%, redistilled, for protein sequencing
Sodium Phosphate, Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$, FW=138.0) CASNo 10049-21-5	ACS Reagent Grade, Aldrich S9638
Sodium Phosphate, Dibasic Heptahydrate ($Na_2HPO_4 \cdot 7H_2O$ FW=268.0) CASNo 7782-85-6	ACS Reagent Grade, Aldrich S9390
Ammonium Acetate $NH_4C_2H_3O_2$ FW=77.08	ACS Reagent Grade, Sigma Aldrich 238074
Ammonium Hydroxide NH_4OH, 28 - 30%	ACS Reagent Grade, Sigma Aldrich 320145
Acetonitrile, HPLC Grade ****	HPLC Grade, Sigma Aldrich 439134 Or HPLC Grade, Fisher Scientific A/0626/17
Purified Water	HPLC Grade or Millipore Milli-Q grade
Cysteine Peptide (store refrigerated) Ac-RFAACAA-COOH, MW=751.9 90-95% purity	RS Synthesis, Louisville KY, USA or JPT Peptide, Germany *Note: material contains a mixture including the peptide with one less alanine ("A") unit which may co-elute.*
Lysine Peptide (store refrigerated) Ac-RFAAKAA-COOH MW= 776.2 90-95% purity	RS Synthesis, Louisville KY, USA or JPT Peptide, Germany
Cinnamic Aldehyde, 93% purity (Positive Control) **CAS [104-55-2]**	Sigma Aldrich Catalog # W22361-3

REAGENT	**PREPARATION**
100 mM Sodium Phosphate, Monobasic	Using a 1 Liter volumetric flask, dissolve 13.8 g of Sodium Phosphate Monobasic Monohydrate in purified water and dilute to final volume of 1

	Liter. Store refrigerated.
100 mM Sodium Phosphate, Dibasic	Using a 1 Liter volumetric flask, dissolve 26.8 g of Sodium Phosphate Dibasic Heptahydrate in purified water and dilute to final volume of 1 Liter. Store refrigerated.
100 mM Phosphate Buffer, pH=7.5	Combine 18 mL of 0.1 M Sodium Phosphate Monobasic with 82 mL of 0.1M Sodium Phosphate Dibasic. Mix well and measure pH using a calibrated pH meter. Adjust pH to 7.5 +/- 0.05 with either the monobasic (to acidify)or dibasic (basify) solution.
100 mM Ammonium Acetate Buffer, pH=10.2:	Dissolve 1.542 g of Ammonium Acetate in 200 mL purified water. Adjust the pH to 10.2 by dropwise addition of Ammonium Hydroxide using a pH meter calibrated at pH 7 & 10. **Prepare fresh or use within 2 weeks**
HPLC Mobile Phase A: 0.1% (v/v) Trifluoroacetic Acid in Water	Add 1.0 mL of Trifluoroacetic acid to 1 Liter of HPLC grade Water.
HPLC Mobile Phase B 0.085% (v/v) trifluoroacetic acid in acetonitrile	Add 850 microliters of Trifluoroacetic Acid to 1 liter of HPLC grade acetonitrile.

****** A Note about acetonitrile:**

Some supplies of acetonitrile have had a negative impact on peptide stability (particularly cysteine). This can be assessed when starting a new batch of acetonitrile by performing the following test prior to running the assay.

1. Prepare a small amount of 0.501 mg/mL cysteine peptide solution in phosphate buffer.
2. Prepare an autosampler vial containing 750uL peptide solution and 250uL acetontrile.
3. Incubate for 24 hours.
4. Visually inspect the vial for precipitation.
5. Set up an HPLC run using the conditions defined in this SOP. Inject this sample every 2-3 hours for approximately 48 hours.
6. Compare the peak areas for each injection. The CV should be <15%

PROCEDURE

The procedure is divided into six main categories:

- **PRE WORK:** Prepare HPLC system, Pre-weigh test chemicals and peptide, determine the appropriate solvent for each test chemical.
- **SOLUTION PREPARATION:** Dissolve test chemicals and peptide immediately before the assay.
- **ASSAY PROCEDURE:** Combine solutions to start peptide reaction.
- **HPLC ANALYSIS:** Analysis of the final reaction mixture.
- **DATA ANALYSIS & CALCULATIONS:** Analysis of the data obtained.

- **DATA REPORTING (FOR CYSTEINE AND LYSINE):** Data reporting.
- **ACCEPTANCE CRITERIA:** Acceptance criteria for the run, test chemical and data.

PRE-WORK

Prepare HPLC System

Prepare an HPLC system with a UV detector (220 nm) and mobile phase A and B described in the reagent section.

1. Column Equilibration: Install a new guard column cartridge for each set of chemicals. Install the HPLC column (see Apparatus section) and equilibrate the column at 30 °C with 50% A, 50% B for at least 2 hours before use. Condition the column by running the gradient at least twice before using the column.
2. Column Storage Conditions: If the column will be stored for more than a week, fill the column with acetonitrile (without Trifluoroacetic Acid) and cap tightly. Store at room temperature.
3. System Shutdown: Following analysis, maintain a low flow (typically 0.05 mL/min) of 50% A: 50% B through the system and decrease column temperature to approximately 25 °C. If the system is to be idle for more than a week, fill the column with acetonitrile (without Trifluoroacetic acid), remove the column from the HPLC system and cap tightly and purge acid containing mobile phases from the system using a mixture of either 1:1 (v/v) acetonitrile:water or 1:1 (v/v) methanol:water.

Solubility Assessment and Pre-weigh Test Chemicals

Solubility of the test chemicals in a suitable solvent should be assessed before performing the actual assay. An appropriate solvent will dissolve the test chemical completely, i.e. by visual inspection the solution must not be cloudy nor have noticeable precipitate. Acetonitrile is the preferred solvent for test chemicals, however not all chemicals are soluble in acetonitrile. The following solubilization procedure for the selection of the appropriate solvent should be followed.

1. Evaluate solubility by preparing an approximately 100mM solution in **acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
2. If the test chemical is not soluble in acetonitrile, attempt to prepare a 100mM solution in **water**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
3. If the test chemical is not soluble in acetonitrile or water alone, attempt to prepare a 100mM solution in a 1:1 mixture of **water:acetonitrile** (this works well for many organic salts). Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
4. If the test chemical is not soluble in either acetonitrile or water, attempt to prepare a 100mM solution in **isopropanol.** Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
5. If the test chemical is not soluble in either acetonitrile, water or isopropanol, attempt to prepare a 100mM solution in **acetone or a 1:1 acetone:acetonitrile mix.** Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).

6. As a last option, if the chemical is not soluble in any of these solvents, attempt to dissolve the same amount of test chemical in **300 microliters of dimethyl sulfoxide** and dilute the resulting solution with 2700 **microliters of acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).
7. If the test chemical is not soluble in this mixture, dissolve the same amount of test chemical in **1500 microliters of dimethyl sulfoxide** and dilute the resulting solution with **1500 microliters of acetonitrile**. Vortex to mix. If the test chemical does not solubilize completely, the vial may be placed in a sonicating bath (1 minute or less).

Note: Water is not a good solvent choice for anhydrides due to their reactivity with water.

Test chemicals are pre-weighed into clean, dry 4mL glass vials. Test chemicals will be dissolved in 3.0 mL of the appropriate solvent determined in the "Solubility Assessment" Pre-Work (see above) to prepare a 100 mM solution immediately before use. The weight of test chemical to be added to the vial is determined based on the molecular weight ("MW") and purity. If no purity information is available, assume 100% purity.

1. Calculate the target weight of test chemical needed to prepare 3.0mL of a 100mM solution of test chemical using the formula:

$$3\text{mL} \times \frac{1\text{ L}}{1000\text{mL}} \times \frac{100\text{ mmoles}}{\text{L}} \times \text{MW}\left(\frac{\text{mg}}{\text{mmole}}\right) \times \frac{100}{\%\text{Purity}} = \frac{\textbf{MW}}{\textbf{\%Purity}} \times \textbf{30} = \textbf{Target Weight (mg)}$$

2. Weigh the target amount (+/- 10% of target) of test chemical directly into a glass vial and record the actual weight, identity, molecular weight and purity.
3. Tightly close each vial and store under appropriate conditions until ready to perform testing. Appropriate storage conditions for each test chemical are determined based on supplier information.
4. Control Samples: Cinnamic aldehyde is used as the Positive Control for the assay and is included in every assay run. "Reference Controls" are also included with each study. A Reference Control is a peptide solution where the test chemical is replaced by the solvent used to dissolve it. Reference Controls for each solvent used to solubilize the test chemicals should be included in every assay run together with the samples (see Reference Controls in the scheme on pages 8 and 9) and are used to verify that the solvent does not impact the Percent Peptide Depletion. The appropriate Reference Controls for each chemical are used to calculate Percent Peptide Depletion.

Pre-weigh Cysteine or Lysine peptide for stock solutions (0.667 mM)

Note: Do not add buffer to the peptide solid until ready to begin the assay

Cysteine Peptide Ac-RFAACAA-COOH, 0.667 mM, 0.501 mg/mL: The assay will require approximately 800 µL/sample replicate All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock

needed, weigh an appropriate amount of peptide into a large vial or test tube. For example, to prepare 25 mL of solution, weigh 0.01215 g of Cysteine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.501 mg/mL. Record the exact weight of peptide added to the vial.

Lysine Peptide Ac-RFAAKAA-COOH, 0.667 mM, 0.518 mg/mL: The assay will require approximately 800µL/sample replicate. All samples in a batch should use the identical peptide stock solution. Based on the amount of peptide stock solution needed, weigh an appropriate amount of peptide into a large vial of test tube. For example, to prepare 25 mL of solution, weigh 0.0129 g of Lysine peptide. Smaller or larger quantities may be prepared as appropriate, but the final concentration should always be 0.518 mg/mL. Record the exact weight of peptide added to the vial.

Note:

- When starting a new lot of peptide, a small amount should be dissolved in the appropriate buffer at ~0.5 mg/mL and injected through the HPLC to verify that the chromatogram is similar to previous batches.

SOLUTION PREPARATION

1. Suitable run sequence sizes are 1-26 test chemicals, in addition to the Positive Control and Reference Controls. If additional solvents are required, additional Reference Controls need to be prepared and the number of test chemicals will need to be reduced. This run sequence size permits the first HPLC injection to occur 24 hours after mixing the test chemical and peptide and the last HPLC injection to occur no more than 30 hours later. Appropriate controls must be included in each run sequence.
2. Label three autosampler vials for each test chemical and control corresponding to the triplicate preparations.
3. Pre-weigh all test chemicals as described above. Do not dissolve until ready to use.
4. Pre-weigh Cysteine or Lysine peptide as described above. Do not dissolve until ready to use.

Test Chemical Solution Preparation

Solubility of the test chemical in the appropriate solvent is evaluated in the pre-work section. 100mM solutions of test chemicals in the appropriate solvents are prepared fresh, immediately before use.

1. When ready to perform the assay, calculate and weigh out the appropriate amount of test chemical needed to prepare a 100mM solution. Dissolve the test chemical by adding 3.0mL of the appropriate solvent. The resulting solution should have a test chemical concentration of 100 mM. Note: For test chemicals that are expensive or in short supply, it is possible to prepare smaller volumes of solutions (i.e. 1 ml), as long as the actual weight of test chemical used to prepare the 100mM stock solutions can be measured within 10% of the calculated target.
2. Mix vial to dissolve the test chemical. Slight sonication (less than 1 minute) may be used if needed. If the test chemical is not completely dissolved, do not

proceed with that specific test chemical in the selected solvent. Re-evaluate alternative solvents (see pre-work section) to find a suitable choice.

3. Record and report the final solvent choice for each chemical.

Positive Control Solution Preparation

The Positive Control (cinnamic aldehyde) is soluble in acetonitrile.

1. Calculate the target weight of cinnamic aldehyde needed to prepare 3.0mL of a 100mM solution of test chemical using the formula:

$$3\text{mL} \times \frac{1\text{ L}}{1000\text{mL}} \times \frac{100\text{ mmoles}}{\text{L}} \times \text{MW}\left(\frac{\text{mg}}{\text{mmole}}\right) \times \frac{100}{\%\text{Purity}} = \frac{\mathbf{MW}}{\mathbf{\%\,Purity}} \times \mathbf{30} = \mathbf{Target\ Weight\ (mg)}$$

2. Weigh the target amount (+/- 10% of target) of test chemical directly into a glass vial and record the actual weight, identity, molecular weight and purity.

3. Tightly close each vial and store under appropriate conditions until ready to perform testing. Appropriate storage conditions for each test chemical are determined based on supplier information.

4. Dissolve in 3mL of acetonitrile.

Peptide Stock Solution Preparation

1. **Cysteine Peptide:** Add the appropriate amount of pH 7. 5 phosphate buffer to make a 0.667 mM solution of Cysteine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\mathbf{mL\ pH\,7.5\ Buffer} = \frac{\mathbf{mg\ Peptide}}{\mathbf{0.501\ mg/mL}}$$

2. **Lysine Peptide:** Add the appropriate amount of pH 10.2 Ammonium Acetate buffer to make a 0.667 mM solution of Lysine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial (from above) using the equation:

$$\mathbf{mL\ pH\,10.2\ Buffer} = \frac{\mathbf{mg\ Peptide}}{\mathbf{0.518\ mg/mL}}$$

ASSAY PROCEDURE

Reference Control, Positive Control, Co-elution Control and Sample Preparation

Samples are prepared in triplicate for both peptides. Each assay (Cys and Lys) may be prepared concurrently (if two HPLC systems are available) or on separate days (if only one HPLC is available).

1. Assemble the following previously prepared reagents, solvents and solutions:
    a. Peptide stock solution,
    b. Appropriate buffer (pH 7.5 for Cysteine Peptide, pH 10.2 for Lysine peptide),
    c. Acetonitrile
    d. Test chemical solution (or solvent for Reference Controls)

2. Using 1 mL autosampler vials as containers, prepare the sample by adding the reagents in the quantity and order listed below, with gentle mixing during addition. Record the time of addition of the test chemical to the peptide solution.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM test chemical	**1:50 Ratio, Lysine Peptide** 0.5 mM Peptide, 25 mM test chemical
750 µL Cysteine peptide solution (or pH 7.5 phosphate buffer for Co-elution Controls) **200 µL Acetonitrile** **50 µL Test chemical solution (or solvent for Reference Controls)**	**750 µL Lysine peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution Controls)** **250 µL Test chemical solution (or solvent for Reference Controls)**

3. Cap the vials, vortex to mix and place in the HPLC autosampler (dark) at 25 °C for 24 hours. HPLC analysis of the batch of samples should start 24 hours after the test chemical was added to the peptide solution.

Note: For each set of control/sample triplicates, the replicate vials should be prepared individually, using the same solutions.

Standard Preparation

Standards are prepared in a solution of 20% Acetonitrile:Buffer while samples will have a mixture of 25% solvent:buffer. This difference does not adversely impact the chromatography or stability of the samples and standards.

Using serial dilution, prepare standards of the peptide stock solution covering the range from 1 - 0.0167mM.

1. **Prepare approximately 10 mL of <u>dilution buffer</u>** by mixing 8 mL of buffer (pH 7.5 for Cysteine peptide, pH 10.2 for Lysine peptide) with 2 mL of acetonitrile.
2. **Prepare the initial standard, "STD1"** at 0.534 mM by diluting 1600 µL of the peptide stock solution (at 0.667 mM) with 400 µL acetonitrile.
3. **Dilute 1.0 mL of standard STD1 with an equal volume of dilution buffer and continue** in a serial manner to give standards with nominal concentrations noted below. Include a blank of dilution buffer as STD 7.

	STD1	STD2	STD3	STD4	STD5	STD6	Dilution Buffer
mM Peptide	**0.534**	**0.267**	**0.1335**	**0.0667**	**0.0334**	**0.0167**	**0.000**

Serial Dilution Procedural Details

1) Label 5 glass vials (nominal 2-5 mL volume) with codes STD2 – STD6.
2) Add 1.00 mL of dilution buffer to vials STD2 through STD6
3) Transfer 1.00 mL of Standard STD1 to vial STD2. Mix with minimal air entrainment
4) Transfer 1.00 mL from vial STD2 to vial STD3. Mix with minimal air entrainment
5) Continue in a similar manner for standards STD4 through STD6.

6) Transfer standards to autosampler vials for analysis

HPLC ANALYSIS

Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the weak and strong solvents and a column temperature of 30ºC. The HPLC analysis is performed using a flow of 0.35 mL/min and a linear gradient from 10% to 25% Acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Inject equal volumes of each standard, sample and control. The injection volume may vary according to the system used (typically in the range from 3-10 µL). On some systems, 10µL injection volumes lead to unacceptably broad peaks and smaller injection volumes need to be used. Absorbance is monitored at 220 nm. If using a Photodiode Array detector, absorbance at 258 nm should also be recorded. Re-equilibrate the column under initial conditions for at least 7 minutes. *Note: The 7 minute re-equilibration time was determined using a Waters 2695 HPLC system. Other systems may require more or less re-equilibration time due to system mixing volume. Shorter equilibration times will be acceptable if peak retention times are stable.*

HPLC Conditions

Column	Preferred Column: Zorbax SB-C18 2.1mm x 100 mm x 3.5 micron Agilent Part Number 861753-902 Alternate Column: Phenomenex Luna C18(2) 2.0 mm x 100mm x 3 micron particle (Part # 00D-4251-B0 may require flowrates of 0.3mL/min) or any other C18 column that demonstrates acceptable peak resolution. *Note: Both columns are semi-micro scale and require careful connections to minimize extracolumn peak broadening. Waters 2695 Alliance HPLC systems provide suitable peak shapes. Semi-micro HPLC systems may improve peak resolution and it may be possible to decrease analysis time on other systems.*			
Column Temperature	30 ºC			
Sample temperature	25 ºC			
Detector	Photodiode Array detector or Fixed Wavelength Absorbance detector with 220 nm signal for quantitation			
Injection Volume	~7 µL (Volume varies according to the HPLC system. If peaks are too broad, the volume should be decreased) Set the autosampler needle depth to avoid drawing sample from the bottom of the vial.			
Run Time	20 minutes			
Flow Conditions	**Time**	**Flow**	**%A**	**%B**
	0 min	0.35 mL/min	90	10
	10 min	0.35 mL/min	75	25
	11 min	0.35 mL/min	10	90
	13 min	0.35 mL/min	10	90
	13.5 min	0.35 mL/min	90	10
	20 min	end run		

Note: Visually inspect samples prior to HPLC analysis. Generally, precipitation is not a problem. However, if a precipitate is observed, this should be noted in the data reporting template. Samples may be centrifuged at low speed (100-400 xg) in the vial to force precipitate to the bottom of the vial as a precaution, since large amounts of precipitate may clog the HPLC tubing or columns. **Precipitate formation and removal must be recorded and reported.**

Filtering samples or use of high speed polypropylene centrifuge tubes to remove precipitate has not been evaluated and may lead to loss of peptide through adsorption, therefore this is not recommended.

Prepare two separate analysis sequences, based on the example below: 1. Calibration standards, Reference Controls A and Co-elution Controls and, 2. Stability of Reference Controls over analysis time (Reference Controls B) and sets of replicates (Reference Controls C, Positive Control and test chemicals). The first analysis sequence can be timed to complete prior to the end of the 24 hour incubation and the second sequence should be timed to assure that the injection of the first sample starts 24 (+/-2) hours after the test chemical was mixed with the peptide solution. Alternatively, since there is no chemical reaction occurring in the calibration standards, Reference Controls and Co-elution controls, the first analysis sequence can be timed to run shortly after assay setup is complete rather than directly before the second analysis sequence.

Example HPLC Sample Analysis Sequences
(A more specific analysis sequence can be found at the end of the SOP.)

STD1 **STD2** **STD3** **STD4** **STD5** **STD6** **Dilution Buffer** **Reference Control A, rep 1** **Reference Control A, rep 2** **Reference Control A, rep 3**	***Calibration Standards and Reference Controls*** *Verify linearity of response* *Verify precision and accuracy of pipetting* *System Suitability:* *r^2 >0.990* *Mean peptide concentration of Reference Controls A =* *0.50 +/- 0.05 mM*
Co-elution Control 1 **Co-elution Control 2** **Co-elution Control 3** . . .	***Co-elution Controls*** *Verify co-elution of test chemicals with peptide*
Reference Control B, rep 1 **Reference Control B, rep 2** **Reference Control B, rep 3**	***Reference Controls*** *Verify stability of Reference Controls over analysis time* *(see also below)*
Reference Control C, rep 1 §, † **Cinnamic aldehyde, rep 1** **Sample 1, rep 1** **Sample 2, rep 1**	***First set of replicates*** *Note: Start first set of replicates 24 +/- 2 hours after peptide:test chemical mixing.*

Sample 3, rep 1 . . .	
Reference Control C, rep 2 [§] **Cinnamic aldehyde, rep 2** **Sample 1, rep 2** **Sample 2, rep 2** **Sample 3, rep 2** . . .	***Second set of replicates***
Reference Control C, rep 3 [§] **Cinnamic aldehyde, rep 3** **Sample 1, rep 3** **Sample 2, rep 3** **Sample 3, rep 3** . . . [†]	***Third set of replicates*** *For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM*
Reference Control B, rep 4 **Reference Control B, rep 5** **Reference Control B, rep 6**	***Reference Controls*** *Verify stability of Reference Controls over analysis time:* *CV of peptide peak areas of the nine Reference Controls B and C in acetonitrile must be < 15.0%*

[§] Three replicates for Reference Controls C should be included in the analysis sequence for each solvent that is used to dissolve test chemicals. These should be run with the Samples and are used to separately calculate the Percent Peptide Depletion in each solvent and verify that they do not impact the Percent Peptide Depletion.

[†] The difference in time between the first injection of the first replicate and the last injection of the last replicate should not exceed 30 hours.

DATA ANALYSIS & CALCULATIONS

The concentration of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area of the appropriate peaks and calculating the concentration of peptide using the linear calibration curves derived from the standards. The Cysteine peptide includes other peaks that elute near the peptide of interest. Refer to the example chromatogram for appropriate integration of the peak.

The percent depletion of peptide is determined in each sample from absorbance at 220 nm, measuring the peak area and dividing that by mean peak area of the reference controls.

1. Integrate the appropriate peaks and determine peak area for standards, samples and controls. The peak area of each integrated peak must be reported. The peaks should be consistently integrated "valley to valley". There may be some instances when this is not practical, but should be appropriate for most chromatograms.
2. Generate a linear calibration curve based on the concentration of standards (equal weighting) and the peak area. Suitable calibration curves will have an r^2>0.990. Calculate the mean peptide concentration in Reference Controls A, SD and CV. The mean should be 0.50 +/- 0.05 mM. Values outside of this range may indicate a pipetting or sample preparation error. The peptide concentration of Reference Controls A and C (see analysis sequence above) must be reported.
3. Calculate the mean peptide peak area at 220 nm for the nine Reference Controls B and C in acetonitrile, SD and CV. The CV must be < 15.0%
4. Calculate the mean peptide peak area at 220 nm for the three Reference Controls C for each solvent used.
5. Calculate the mean peptide concentration (mM) for the three Reference Controls C for each solvent used, SD and CV. The mean should be 0.50 +/- 0.05 mM.
6. UV absorbance is a general detection method and interfering peaks may occur. If there is uncertainty regarding the identity of the peak, verify the UV absorbance spectrum and retention time are consistent with the Reference Control C injections.
7. Some test chemicals will co-elute with the cysteine or lysine peptides. In order to detect possible co-elution of the test chemicals with a peptide, the test chemicals included in the run must be injected alone ("Co-elution Controls") at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent. If a chemical absorbs at 220 nm and has a similar retention time as a peptide (overlap of "valley to valley" integration periods), then co-elution of the test chemical with that peptide should be reported. In order to assure that baseline noise is not being called interference, the "interfering" chemical peak should have a peak area that is >10% of the mean peptide peak area in the appropriate Reference Control. The chromatograms of the reaction mixtures should also be inspected in case of possible co-elution to verify if the peaks of the chemical and the peptide are indeed not baseline separated. If co-elution occurs and proper integration and calculation of Percent Peptide Depletion (see below) is not possible, the data should be recorded as "interference" for the peptide the chemical co-elutes with. When a Photodiode Array detector is used, co-elution of chemical and peptide may also be verified by looking at the UV spectrum at 258nm in addition to

220nm and calculating the area ratio of 220/258. This value should be consistent over all samples and standards for a pure peptide peak and thus gives a measure of peak purity. Calculation of peak purity (area ratio of 220/258) might not be possible if the chemical is highly reactive with the peptide leading to very small peaks.

8. For the Positive Control and for each test chemical, calculate the Percent Peptide Depletion in each replicate from the peptide peak area of the replicate injection and the mean peptide peak area in the three relevant Reference Controls C (in the appropriate solvent), by using the following formula. The Percent Peptide Depletion of every injected Positive Control and test chemical replicate must be reported. Moreover, the mean Percent Peptide Depletion of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

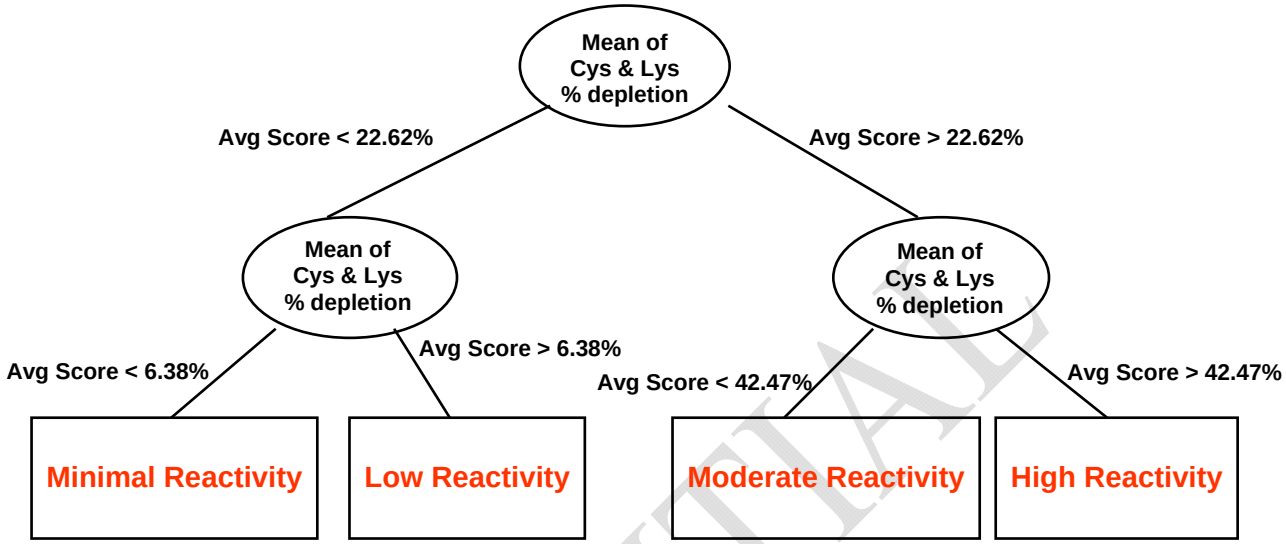
$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls } C}\right)\right] \times 100$$

9. If the Percent Peptide Depletion is < - 10.0%, it should be considered that this may be a situation of co-elution, inaccurate peptide addition to the reaction mixture or just baseline "noise." If this happens, the coelution controls (test chemical alone chromatograms and 220/258 ratio) should be carefully analyzed. If the peptide peak appears at the proper retention time and has the appropriate peak shape (see examples on page 22), the peak can be integrated. In this case, there may just be baseline noise causing the peptide peak to be bigger or their may be some co-elution/overlap in retention time of the peptide and test chemical. The calculated %-depletion should be reported as an "estimate." If this was only an issue for lysine, use the "cysteine-only" prediction model. If this is an issue with cysteine or both cysteine and lysine, use the table on page 20 of the SOP.

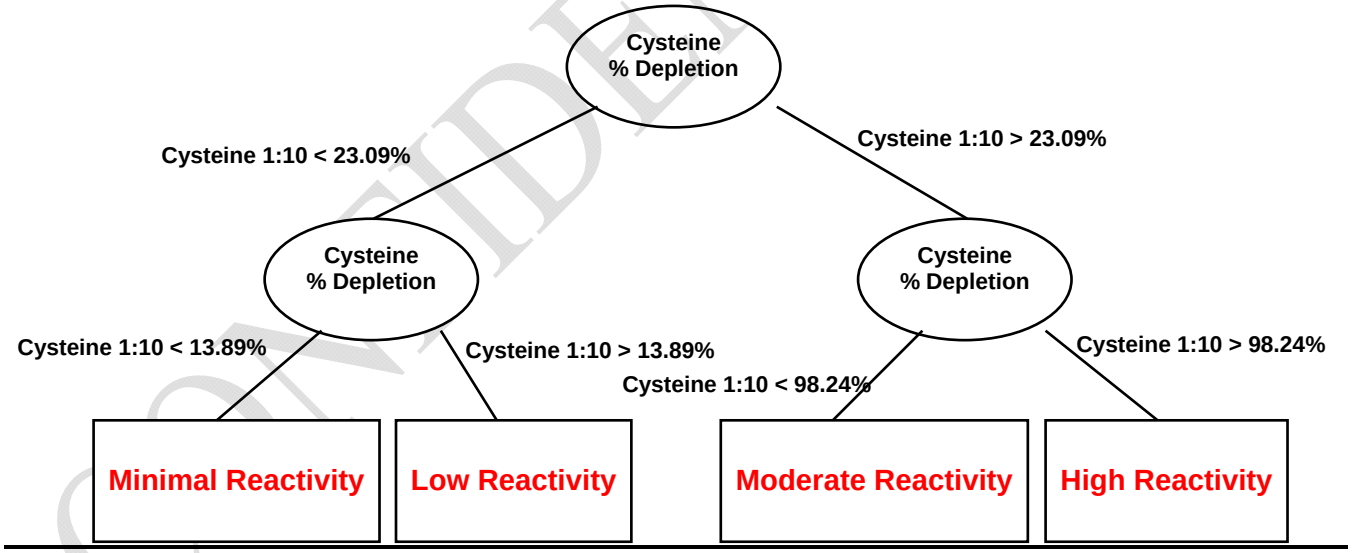
   If the peak does not have the proper shape due to complete overlap in retention time of the test chemical and peptide and can not be integrated, calculation of Percent Peptide Depletion is not possible. If this is an issue for lysine, use the "cysteine-only" model. If this is an issue for cysteine or both cysteine and lysine, the data must be reported as "inconclusive".

10. Calculate the mean of the Percent Cysteine and Percent Lysine Depletions for the Positive Control and for each test chemical. Negative depletion values should be considered as "Zero" when calculating the mean. Assign a reactivity category to each test chemical by using the Cysteine 1:10/Lysine 1:50 prediction model below. In cases where a test chemical co-elutes with the lysine peptide, the Cysteine 1:10-only prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide and percent depletion can not be estimated, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone, and the data should be reported as "inconclusive". The reason for this is that the lysine reactivity does not carry enough weight to drive a lysine-only prediction model.

Cysteine 1:10/Lysine 1:50 Prediction Model



Cysteine 1:10-only Prediction Model



DATA REPORTING (FOR CYSTEINE AND LYSINE)

SYSTEM SUITABILITY

- Peptide peak area at 220 nm of each Standard and Reference Control A replicate.
- The linear calibration curve should be graphically represented and the R^2 reported.
- Peptide concentration (mM) of each Reference Control A replicate.
- Mean peptide concentration (mM) of the three Reference Controls A, SD and CV.

ANALYSIS SEQUENCE

Reference Controls:

- Peptide peak area at 220 nm of each B and C replicate.
- Mean peptide peak area at 220 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean peptide peak area at 220 nm of the three appropriate Reference Controls C (for calculation of Percent Peptide Depletion).
- For each solvent used, the peptide concentration (mM) of the three appropriate Reference Controls C.
- For each solvent used, the mean peptide concentration (mM) of the three appropriate Reference Controls C, SD and CV.

Positive Control (cinnamic aldehyde)

- Peptide peak area at 220 nm of each replicate.
- Percent Peptide Depletion of each replicate.
- Mean Percent Peptide Depletion of the three replicates, SD and CV.

For each test chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time. If precipitate was re-solubilised or centrifuged.
- Peptide peak area at 220 nm of each replicate (for systems equipped with a PDA detector the peak area at 258 nm should also be reported).
- Percent Peptide Depletion of each replicate.
- Mean of Percent Peptide Depletion of the three replicates, SD and CV
- Mean of Percent Cysteine and Percent Lysine Depletion values.
- Reactivity class.

ACCEPTANCE CRITERIA

PEPTIDE REACTIVITY ASSAY RUN ACCEPTANCE CRITERIA

All criteria must be met for the whole run to be considered valid. If these criteria are not met, the run must be repeated for all test chemicals.

System Suitability:
Calibration Linearity $r^2 > 0.990$
Mean peptide concentration of Reference Controls A = 0.50 +/- 0.05 mM

Positive Control:
The mean Percent Peptide Depletion value of the three replicates for cinnamic aldehyde must fall within the ranges reported in the following table (based on 95% Tolerance Intervals):

	Percent Cysteine Depletion		Percent Lysine Depletion	
Positive Control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Cinnamic aldehyde	60.8	100.0	40.2	69.4

Maximum Standard Deviations for Positive Control replicates:
Standard Deviation for Percent Cysteine Depletion must be < 14.9%
Standard Deviation for Percent Lysine Depletion must be < 11.6%

Stability of Reference Controls over analysis time:
CV of peptide peak areas for the nine Reference Controls B and C in acetonitrile must be < 15.0%.

ACCEPTANCE CRITERIA FOR EACH TEST CHEMICAL

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

Maximum Standard Deviation of sample replicates:
Standard Deviation for Percent Cysteine Depletion must be < 14.9%
Standard Deviation for Percent Lysine Depletion must be < 11.6%

Reference Controls in the analysis sequence:
For each solvent used, the mean of the peptide concentrations of the three appropriate Reference Controls C = 0.50 +/- 0.05 mM

PEPTIDE REACTIVITY ASSAY DATA ACCEPTANCE CRITERIA

Presence of precipitate

If precipitate occurs at the end of the 24 hrs incubation period, This should be recorded and the sample analyzed.

Co-elution of test chemical with peptide

In cases where a test chemical co-elutes with the lysine peptide, the Cysteine 1:10-only prediction model can be used. In cases where the test chemical co-elutes with the cysteine peptide and the peptide peak can not be integrated, a determination of reactivity can not be made based on the Percent Depletion data from the lysine reaction alone, and the data should be reported as "inconclusive". If the peak for the cysteine peak can be integrated, follow the instructions below to determine an estimated Percent Depletion .

1. Negative depletion values

If the Percent Peptide Depletion is < - 10.0%, it should be considered that this may be a situation of co-elution, inaccurate peptide addition to the reaction mixture or just baseline "noise." If this happens, the coelution controls (test chemical alone chromatograms and 220/258 ratio) should be carefully analyzed. If the peptide peak appears at the proper retention time and has the appropriate peak shape (see examples on page 22), the peak can be integrated. In this case, there may just be baseline noise causing the peptide peak to be bigger or their may be some co-elution/overlap in retention time of the peptide and test chemical. The peptide peak is visible and can be integrated. The calculated %-depletion should be reported as an "estimate." If this was only an issue for lysine, use the "cysteine-only" prediction model. If this is an issue with cysteine or both cysteine and lysine, use the table on page 20 of the SOP.

If the peak does not have the proper shape due to complete overlap in retention time of the test chemical and peptide and can not be integrated, calculation of Percent Peptide Depletion is not possible. If this is an issue for lysine, use the "cysteine-only" model. If this is an issue for cysteine or both cysteine and lysine, the data must be reported as "inconclusive".

2. Co-elution Controls

If a chemical (Co-elution Control) absorbs at 220 nm and has a similar retention time as a peptide (Reference Control) (overlap of "valley to valley" integration periods), then co-elution of the test chemical with the peptide should be reported. In order to assure that baseline noise is not being called interference, the "interfering" chemical peak should have a peak area the is >10% of the mean peptide peak area in the appropriate Reference Control. The chromatograms of the reaction mixtures should also be inspected in case of possible co-elution to verify if the peaks of the chemical and the peptide are indeed not baseline separated. If co-elution occurs, proper integration and calculation of Percent Peptide Depletion (see below) is not possible. The data should be recorded as "interference" for that peptide.

3. Area ratio of the peptide peak at 220/258

When a Photodiode Array detector is used, co-elution of chemical and peptide may also be verified by looking at the UV spectrum at 258nm in addition to 220nm and calculating the area ratio of 220/258. This value should be consistent over all samples and standards for a pure peptide peak and thus gives a

measure of peak purity. For each sample. a ratio in the following range would give a good indication that co-elution has not occurred: **90%<Mean Area ratio of control samples <110%**. However, calculation of peak purity (area ratio of 220/258) might not always be possible, particularly if the test chemical is highly reactive with the peptide leading to very small peaks.

4. Co-elution with reactivity and estimated depletion values

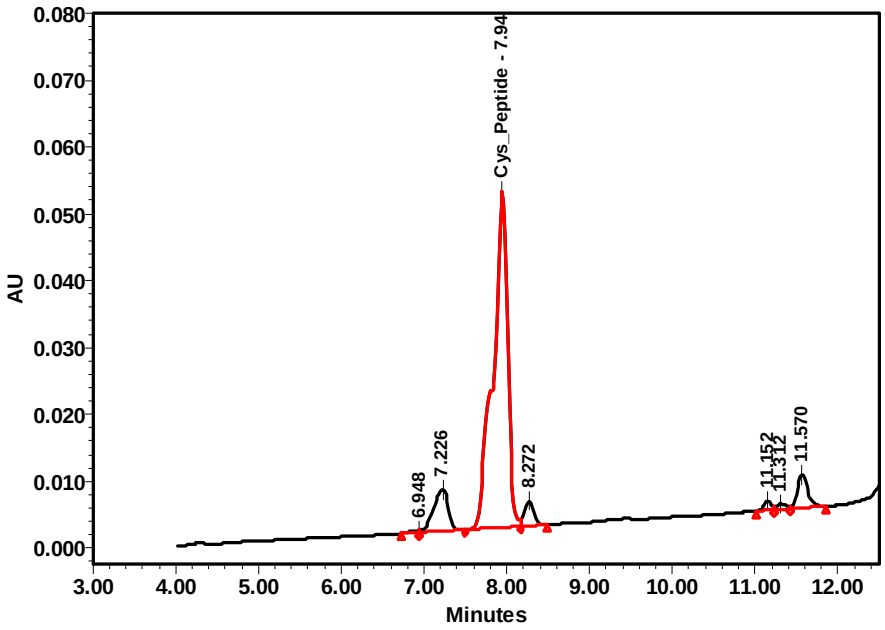
In some instances, a test chemical may have an overlapping retention time with either of the peptides and still be reactive with that peptide. If this is the case and the overlap in retention time between the test chemical and peptide is incomplete, percent depletion can still be calculated with a notation of "**co-elution – percent depletion estimated**". Co-elution will make the area of the peptide peak appear to be larger than it really is, therefore the calculated percent depletion may be **lower** than the true value. This estimated value can still be used in the prediction model with some additional notation. When using an estimated percent depletion for one peptide and the model predicts "High reactivity", the result is fine but should still be noted that the percent depletion is estimated. If the estimated percent depletion leads to a "Moderate reactivity" or "Low reactivity," the result should be noted as "≥ Moderate reactivity" or "≥ Low Reactivity" respectively. If the estimated percent depletion leads to "Minimal reactivity," the result should be reported as "Inconclusive." However, unless cysteine is the co-eluting peptide, the Cysteine-only prediction model should be used before using this estimated method.

The following table illustrates the different scenarios:

Mean depletion values	No co-elution	Co-elution with Cysteine alone or Cysteine and Lysine	Co-elution with Lysine only
Less than 6.38%	Minimal Reactivity	Inconclusive	Apply Cysteine-only prediction model
Between 6.38% and 22.62%	Low Reactivity	≥ Low Reactivity	Apply Cysteine-only prediction model
Between 22.62% and 42.47%	Moderate Reactivity	≥ Moderate Reactivity	Apply Cysteine-only prediction model
More than 42.47%	High Reactivity	High Reactivity	Apply Cysteine-only prediction model

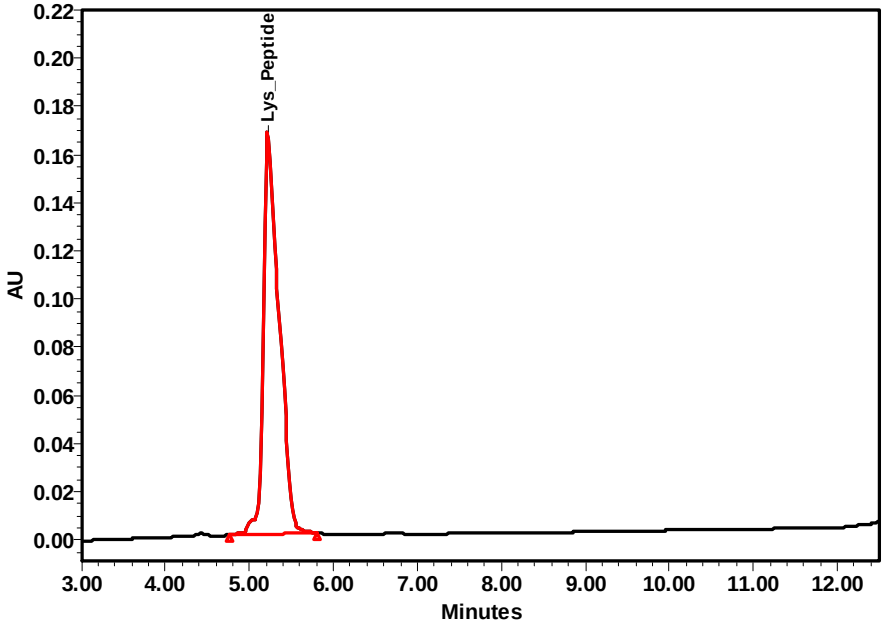
Example Chromatograms

Cysteine Peptide (Retention Time approximately 8-9 minutes)



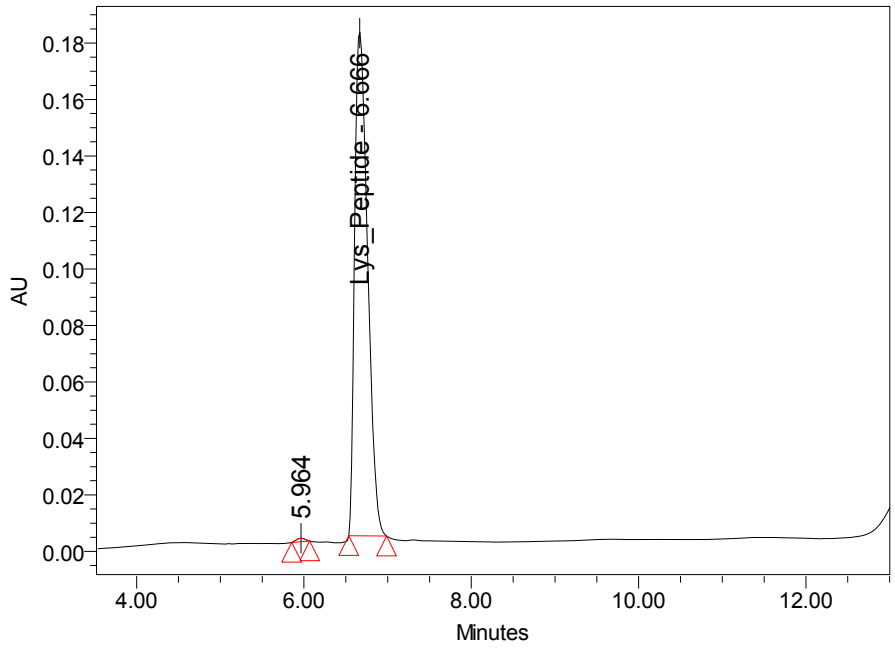
Note: The Cysteine peptide shown contains a mixture including the peptide with one less alanine ("A") unit which causes the leading edge shoulder on the peak. Both peptides react in a comparable manner and are integrated together.

Lysine Peptide (Retention Time approximately 5-6 minutes)

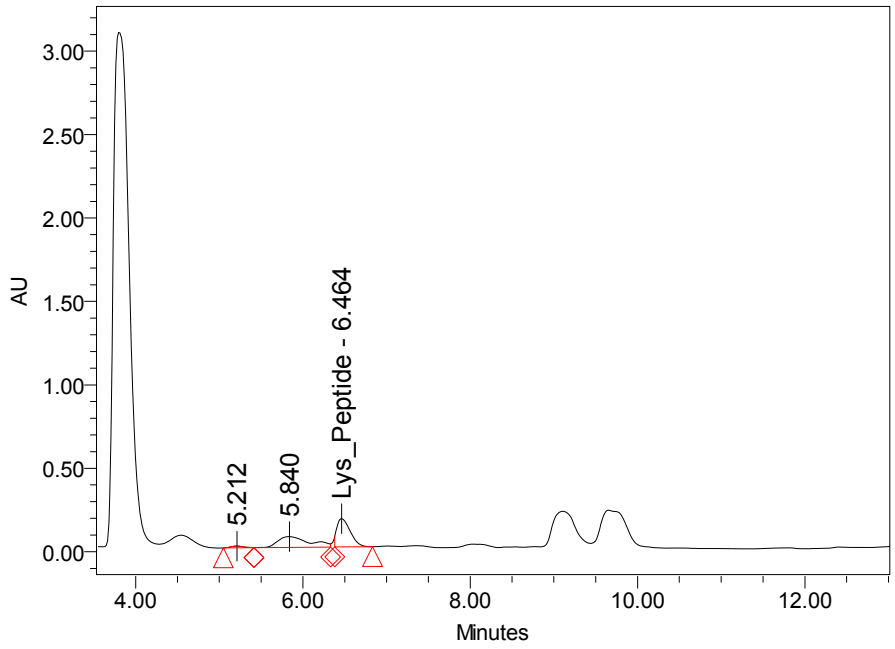


Examples of co-elution:

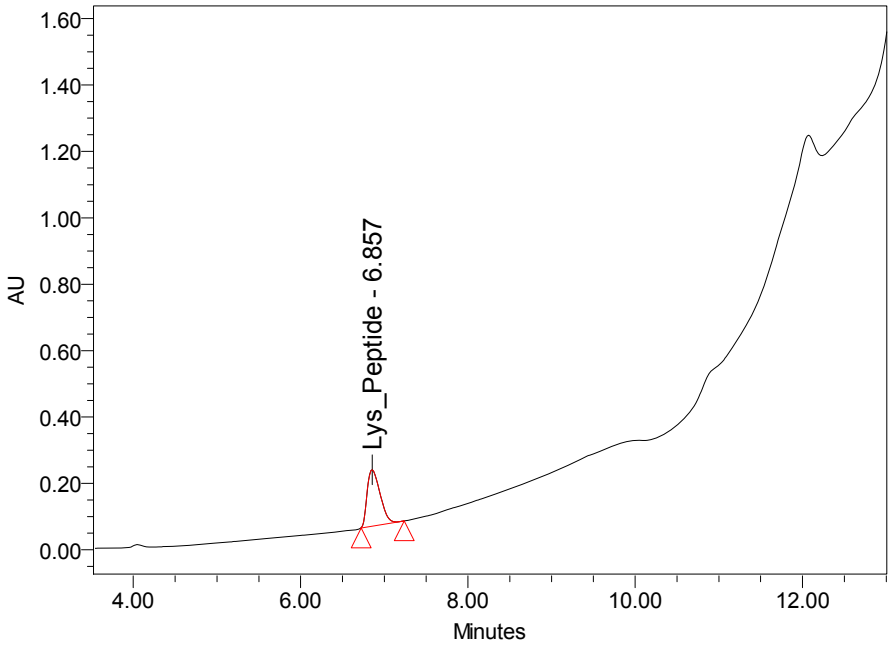
Situation 1: Possible co-elution or baseline noise/strange looking baseline. The peptide peak can be integrated, but should be considered an estimate.



The peptide peak is clearly visible and can be integrated. Perhaps there is some small test chemical peak with the same retention time which would make the peptide peak appear bigger and give a <-10% depletion.

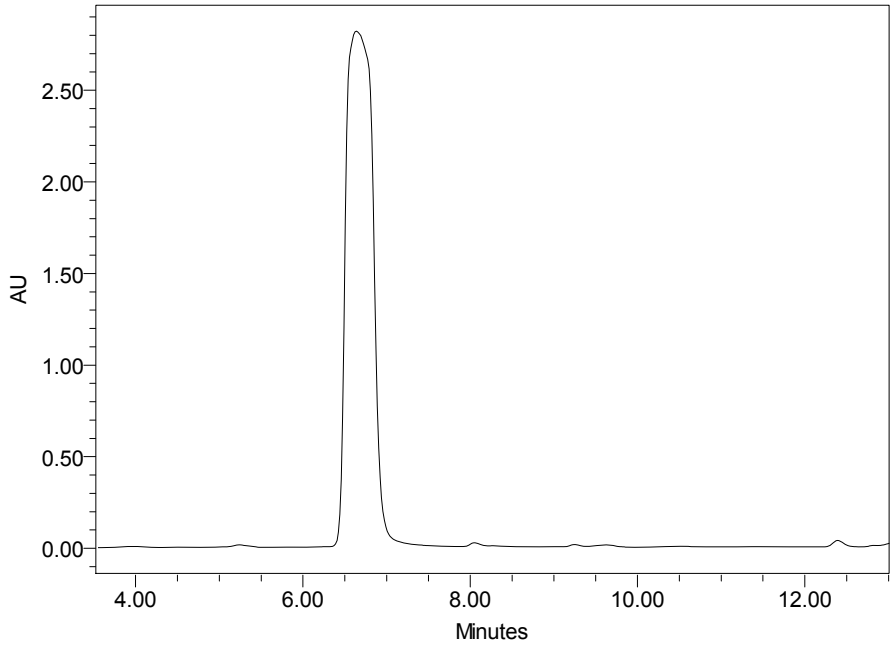


The lysine peak overlaps slightly with a test chemical peak. This would be reported as possible co-elution/estimated % depletion



The peptide peak can be integrated but the baseline is not flat so this should be considered an estimate because the "area under the curve" can not be determined with complete certainty.

Situation 2: The peptide peak can not be integrated



The test chemical peak has the same retention time as the peptide. This peak is not the peptide. In this case, there is no way of knowing if the peptide is fully depleted, partially depleted or not depleted at all. Therefore no estimate can be made and it must be reported as coelution

Example HPLC Analysis

Example DPRA run:

There are 5 test chemicals. Chemical 1, 2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in isopropanol

The following vials should be set up:

Analysis Sequence 1:

STD 1
STD 2
STD 3
STD 4
STD 5
STD 6
Dilution buffer blank
Reference Control A, rep 1 (made with acetonitrile)
Reference Control A, rep 2 (made with acetonitrile)
Reference Control A, rep 3 (made with acetonitrile)
Coelution Control for Chemical 1
Coelution Control for Chemical 2
Coelution Control for Chemical 3
Coelution Control for Chemical 4
Coelution Control for Chemical 5

Analysis Sequence 2:

Reference Control B, rep 1 (made with acetonitrile)
Reference Control B, rep 2 (made with acetonitrile)
Reference Control B, rep 3 (made with acetonitrile)
Reference Control C, rep 1 (made with acetontrile)
Reference Control C, rep 1 (made with isopropanol)
Cinnamic aldehyde, rep 1
Chemical 1, rep 1
Chemical 2, rep 1
Chemical 3, rep 1
Chemical 4, rep 1
Chemical 5, rep 1
Reference Control C, rep 2 (made with acetontrile)
Reference Control C, rep 2 (made with isopropanol)
Cinnamic aldehyde, rep 2
Chemical 1, rep 2
Chemical 2, rep 2

Chemical 3, rep 2
Chemical 4, rep 2
Chemical 5, rep 2

Reference Control C, rep 3 (made with acetontrile)
Reference Control C, rep 3 (made with isopropanol)
Cinnamic aldehyde, rep 3
Chemical 1, rep 3
Chemical 2, rep 3
Chemical 3, rep 3
Chemical 4, rep 3
Chemical 5, rep 3

Reference Control B, rep 4 (made with acetonitrile)
Reference Control B, rep 5 (made with acetonitrile)
Reference Control B, rep 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean peptide peak area of the Reference Control C made with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean peptide peak area of the Reference Control C made with isopropanol.

Appendix 10

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Development of a Peptide Reactivity Assay for Screening Contact Allergens

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Allergic contact dermatitis resulting from skin sensitization is a common occupational and environmental health problem. In recent years, the local lymph node assay (LLNA) has emerged as a practical option for assessing the skin sensitization potential of chemicals. In addition to accurate identification of skin sensitizers, the LLNA can also provide a reliable measure of relative sensitization potency; information that is pivotal in successful management of human health risks. However, even with the significant animal welfare benefits provided by the LLNA, there is still interest in the development of nonanimal test methods for skin sensitization testing. One characteristic of a chemical allergen is its ability to react with proteins prior to the induction of skin sensitization. The majority of chemical allergens is electrophilic and as such reacts with nucleophilic amino acids like cysteine or lysine. In order to determine if reactivity correlates with sensitization potential, 38 chemicals representing allergens of different potencies (weak to extreme) and nonsensitizers were evaluated for their ability to react with glutathione or three synthetic peptides containing either cysteine, lysine, or histidine. Following a 15-min reaction time for glutathione or a 24 h reaction period for the three synthetic peptides, the samples were analyzed by HPLC. UV detection was used to monitor the depletion of glutathione or the peptide following reaction. The results demonstrate that a significant correlation (Spearman correlation) exists between allergen potency and the depletion of glutathione ($p = 0.001$), lysine ($p = 0.025$), and cysteine ($p = 0.020$), but not histidine. The peptide with the highest sensitivity was cysteine (80.8%) whereas histidine was the least sensitive (11.5%). The data presented show that measuring peptide reactivity has utility for screening chemicals for their skin sensitization potency and thus potential for reducing our reliance on animal test methods.

Key Words: **allergens; alternatives; skin sensitization; peptide reactivity.**

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health problem, and the most common manifestation of immunotoxicity in humans. The acquisition of skin sensitization, and the subsequent elicitation of an allergic hypersensitivity reaction in the skin, are processes dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses (Kimber *et al*., 2000, 2002). For many years guinea pigs were the species of choice for the hazard identification of skin sensitizing chemicals. More recently, however, the local lymph node assay (LLNA) has been developed as an alternative approach based upon characterization of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals (Basketter *et al*., 2002; Dearman *et al*., 1999; Gerberick *et al*., 2000; Kimber *et al*., 1994, 2002). The LLNA has been adopted recently, as Testing Guideline 429, by the Organization for Economic Cooperation and Development (OECD, 2002) as a stand-alone test method for skin sensitization testing. However, one challenge facing investigators is the need to develop nonanimal based methods for the evaluation of new chemicals that will reduce significantly or eliminate the need for animals in skin sensitization testing in the future (Ryan *et al*., 2001).

There are a variety of characteristics that determine whether a chemical can function as a contact sensitizer (or allergen) including the ability to penetrate into the skin, react with protein, and be recognized as antigenic by immune cells. The correlation of protein reactivity with skin sensitization potential is well established (Dupuis and Benezra, 1982; Lepoittevin *et al*., 1998). In fact, Landsteiner and Jabcobs (1936) presented the origin of the reactivity hypothesis in their landmark paper looking at the underlying mechanisms of contact allergy. Thus, if a chemical is capable of reacting with protein either directly or after appropriate biotransformation, then it has the potential to act as a contact allergen.

The majority of chemical allergens (or their metabolites) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups, nucleophiles, capable of reacting with electrophilic allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine can also react with electrophiles (Ahlfors *et al*.,

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2003; Dupuis and Benezra, 1982; Lepoittevin *et al*., 1998). Thus, electrophilic allergens have the capability to react with nucleophilic amino acids in proteins, forming extremely stable covalent bonds, and therefore are involved in the triggering of skin sensitization resposnes.

Since protein reactivity is a key step in the induction of skin sensitization it was hypothesized that reactivity could be used to screen for the sensitization potential of chemicals. Therefore, a peptide-based assay was developed and chemicals of different allergenic potencies (weak to extreme) along with nonsensitizers were evaluated to determine if reactivity could be used as a potential skin sensitization screening tool. All chemicals tested have been evaluated in the LLNA and for each assigned a skin sensitization potency category: extreme, strong, moderate, weak, and nonsensitizers. These data demonstrate that a significant correlation exists between a chemical's skin sensitization potency and its ability to react with peptides containing nucleophilic amino acids such as cysteine and lysine.

MATERIALS AND METHODS

Test chemicals. The following chemicals with accompanying CAS numbers were purchased from the Sigma Chemical Company (St. Louis, MO): 2,4-dinitrochlorobenzene [97-00-7], *p*-benzoquinone [106–51-4], octanoic acid [124-07-2], 1,4-hydroquinone [123-31-9], glutaraldehyde [111-30-8], 4-(N-ethyl-N-2-methansulphonamido-ethyl)-2-methyl-1,4-phenylenediamine [CD3; 25646-71-3], 1-(4-methoxyphenyl)-1-penten-3-one [104-27-8], and lactic acid [50-21-5].

The following chemicals with accompanying CAS numbers were purchased from the Aldrich chemical company (Milwaukee, WI): diphenylcyclopropenone [97-00-7], phthalic anhydride [85-44-9], 2-hydroxyethylacrylate [818-61-1], 3-dimethylaminopropylamine [109-55-7], cinnamic aldehyde [104-55-2], 3-aminophenol [591-27-5], 3,4-dihydrocoumarin [119-84-6], α-hexylcinnamaldehyde [101-86-0], ethleneglycol dimethacrylate [97-90-5], diethyl phthalate [84-66-2], 2-hydroxypropylmethacrylate [923-26-2], oxazolone [5646-46-5], 1,2-benzisothiazolin-3-one [2634-33-5], phenylacetaldehyde [122-78-1], squaric acid [2892-51-5], citral [5392-40-5], diethyl maleate [141-05-9], α-amyl cinnamaldehyde [122-40-7], benzyl benzoate [120-40-7], hydroxycitronellal [107-75-5], lilial [80-54-6], 1-butanol [71-36-3], 4-hydroxybenzoic acid [99-96-7], 6-methylcoumarin [92-48-8], methyl salicylate [119-36-8], chlorobenzene [108-90-7].

Lauryl gallate [1166-52-5] was purchased from Alfa Aesar (Ward Hill, MA). Glycerol [56-81-5] was purchased from J.T. Baker (Phillipsburg, NJ). Hexane [110-54-3] was purchased from EM Science (Gibbstown, NJ). 5-Methy-2,3-hexanedione [13706-86-0] was purchased from Penta MFG (Livingston, NJ).

The purity of these chemicals was equal to or greater than 95% except for the following: α-hexylcinnamaldehyde (85%), oxazolone (90%), glutaraldehyde (70%), phenylacetaldehyde (90%), and lactic acid (85%). Stock solutions that were prepared from chemicals with less than 95% purity were adjusted for purity.

LLNA protocol and chemicals tested. The LLNA was conducted as described elsewhere (Basketter *et al*., 1996, 2002; Dearman *et al*., 1999; Gerberick *et al*., 2000; Kimber *et al*., 1994, 2002). Briefly, groups of CBA female mice (7–12 weeks of age) were exposed topically on the dorsum of both ears to 25 μl of test material, or to an equal volume of the relevant vehicle alone. Treatment was performed daily for three consecutive days. Five days following the initiation of exposure, all mice were injected via the tail vein with 250 μl of phosphate buffered saline (PBS) containing 20 μCi of tritiated thymidine. Mice were sacrificed 5 h later and the draining lymph nodes excised for each experimental group. The incorporation of tritiated thymidine measured by β-scintillation counting was reported in disintegrations per minute (dpm). A stimulation index (SI) was calculated for each allergen-treated group as the ratio of the dpm of the treated group over the dpm of the concurrent vehicle control. A substance was classified as a skin sensitizer if at one or more test concentrations it induced a three-fold or greater increase in local lymph node proliferative activity compared with concurrent vehicle-treated controls. The data reported in this article are derived from previously conducted studies. References for the sources of LLNA data for each of the chemicals are provided in Table 2.

Potency estimation in the LLNA. The approach to the estimation of relative skin sensitization potency of chemicals in the LLNA has been described previously in detail (Basketter *et al*., 1999). It is based upon the mathematical estimation of the concentration of chemical necessary to obtain a three-fold increase in proliferative activity in draining lymph nodes compared with concurrent vehicle-treated controls. It is termed the estimated concentration that yields a three-fold stimulation value (EC3). In these present investigations, existing dose response data for 38 chemicals evaluated in the LLNA have been used to derive EC3 values. In most cases calculation of the EC3 values was conducted by linear interpolation according to the equation:

$$EC3 = c + [(3 - d)/(b - d)] \times (a - c)$$

where the data points lying immediately above and below the SI value of 3 on the LLNA dose response plot have the coordinates (a,b) and (c,d), respectively.

For the remainder of the chemicals for which the lowest concentration tested resulted in a stimulation index of greater than 3, an EC3 value was extrapolated from the two lowest doses utilized (Gerberick *et al*., in press). The extrapolated EC3 value is calculated by log-linear interpolation between these two points on a plane where the x-axis represents the dose level and the y-axis represents the SI. The point with the higher SI is denoted (a,b) and the point with the lower SI is denoted (c,d). The formula for the extrapolated EC3 value is as follows:

$$EC3 = 2\ (\log2(c) + (3 - d)/(b - d) * (\log2(a) - \log2(c)))$$

The relative sensitizing potencies of the chemical allergens were categorized using an arbitrary classification scheme that has recently been proposed (Kimber *et al*., 2003). The system, shown in Table 1, is comprised of four sensitization potency categories based on EC3 values. Compounds that did not induce a three-fold increase at any concentration tested are categorized as nonsensitizing.

GSH reactivity assay. Glutathione (GSH), glutathione disulfide (GSSG), iodoacetic acid, potassium bicarbonate, dimethyl sulfoxide (DMSO), bathophenanthrolinedisulfonic acid (BPDS), ethanol, and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma. Perchloric acid (70%) and *m*-cresol purple were purchased from Aldrich. Glacial acetic acid, sodium acetate, methanol, and potassium hydroxide were purchased from J.T. Baker (Phillispsburg, NJ). A 2 mM stock solution of GSH and GSSG were prepared fresh in nitrogen purged water. A 200 mM stock solution of each test chemical was prepared in DMSO. Iodoacetic acid and *m*-cresol purple were prepared separately in water and combined by transferring 5 ml of 200 mM iodoacetic acid to 5 ml of 0.4 mM *m*-cresol purple. DNFB was prepared at 1% in ethanol. A working solution of 2 M potassium hydroxide/2.4 M potassium bicarbonate was prepared by adding 20 ml of 10 M potassium hydroxide to 80 ml of 3 M potassium bicarbonate. A 2.6 M sodium acetate solution was prepared by transferring 695 ml of glacial

TABLE 1
Classification of Relative Skin Sensitization Potency Using LLNA EC3 Values

EC3 value (%)	Potency classification
≥10–≤100	Weak
≥1–<10	Moderate
<1	Strong
<0.1	Extreme

acetic acid to 500 g of sodium acetate in 224 ml of water. All other reagents were prepared in water.

Conjugation of GSH to several different chemical classes has been examined under various conditions and these data were used to design the reaction conditions employed in this work (Boyland and Chasseaud, 1967; Esterbauer *et al*., 1975; Fry *et al*., 1992; Garle and Fry, 1989; Portoghese *et al*., 1989). GSH reactivity of a test chemical was evaluated in a 15 ml plastic Corning conical tube (Corning, NY) by transferring 50 µl of the GSH stock solution to 400 µl of sodium phosphate buffer, 100 mM (pH 7.4), followed by the addition of 50 µl of the test chemical stock solution. The final reaction contained 0.2 mM GSH and 20 mM of the test chemical. The reaction was incubated in a 25°C shaking water bath for 15 min. Additional samples containing GSH (0.05 to 200 mM) or GSSG (0.025 to 100 mM) were prepared without test chemical and these samples were used to define the calibration curve for each analysis. Immediately following incubation, GSH and GSSG were derivatized with DNFB according to the method of Farriss and Reed (1987). To each sample, 50 µl of BPDS, 100 µl of concentrated perchloric acid, 50 µl of 100 mM iodoacetic acid/0.2 mM *m*-cresol purple, and 480 µl of the working solution of 2 M potassium hydroxide/2.4 M potassium bicarbonate were added. The sample was mixed on a Vortex (Bohemia, NY) and placed in the dark at room temperature for 10 min. Unreacted GSH was derivatized by adding 1 ml of 1% DNFB. The sample was mixed on a Vortex and placed in the dark at room temperature for 60 min. The sample was spun in a centrifuge at 2000 rpm for 10 min and the supernatant was analyzed by HPLC.

Derivatized GSH and GSSG were separated and quantitated by reversed-phase (RP)-HPLC according to the method of Farriss and Reed (1987) on a Waters Alliance 2695 system (Waters Corporation, Milford, MA) using a Waters 2487 dual channel UV detector (365 nm) and a Waters Spherisorb analytical column (5 µm, NH_2, 4.6 mm × 250 mm). A gradient mobile phase was employed with a flow-rate of 1.5 ml/min, an injection volume of 25 µl, and a column temperature of 35°C. An initial mobile phase composition of 80% A (80% methanol) and 20% B (500 mM sodium acetate/64% methanol) was held for 5 min, followed by a 25 min linear gradient to a final composition of 20% A and 80% B. The column was allowed to equilibrate for 10 min between injections. GSH and GSSG had retention times of approximately 8 and 11 min, respectively.

All test chemicals were prepared and analyzed in duplicate on two different days. The mass of GSH and GSSG on column was calculated with the respective calibration curves. GSH depletion was determined by dividing the GSH in a sample containing a test chemical by the GSH in a sample with no test chemical, and multiplying by 100. GSSG was measured to determine if GSH depletion was a result of GSH conjugation to the test chemical or due to an oxido-reduction reaction with the test chemical.

Lysine, cysteine, and histidine reactivity assay. Peptides, Ac-RFAAKAA-COOH (lysine peptide, Fig. 1), Ac-RFAACAA-COOH (cysteine peptide), and Ac-RFAAHAA-COOH (histidine peptide), were made by the SynPep Corp. (Dublin, CA), and purified >90% by HPLC (Keough *et al*., 1997). Molecular weight confirmation was done by flow injection mass spectrometry with electrospray ionization in the positive mode. Ammonium acetate, ammonium hydroxide, sodium phosphate monobasic and sodium phosphate dibasic for the preparation of buffers were purchased from J.T. Baker. DMSO was obtained from Sigma. Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA, 99%) for the preparation of the HPLC mobile phase were purchased from EMD (Gibbstown, NJ) and Aldrich, respectively.

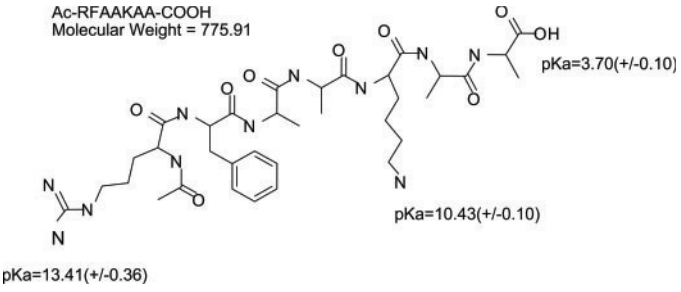


FIG. 1. Structure of synthetic peptide, Ac-RFAAKAA-COOH, showing the lysine side chain. The pKa of the amines is shown. The other synthetic peptides were similarly structured, except a cysteine or histidine residue was substituted for the lysine.

Peptide stock solutions were prepared to a final concentration of 1.25 mM in either 100 mM ammonium acetate buffer, pH 10.2 (lysine peptide), or 100 mM phosphate buffer, pH 7.5 (histidine and cysteine peptides). Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, or solubilized in DMSO and then diluted with an equal part acetonitrile. Triplicate reactivity samples were prepared containing 0.5 mM peptide, and either 5 mM or 25 mM test chemical for a peptide:test chemical ratio of 1:10 or 1:50. A Biomek 2000 automated workstation (Beckman Coulter, Fullerton, CA) was used to make additions of the peptide stock solution (400 µl), the appropriate buffer (350 µl), and the test chemical solutions (50 or 250 µl) into autosampler vials. In the case of the 1:10 ratio reactivity samples, 200 µl of acetonitrile was added to each vial. Samples without the test chemicals were also prepared in triplicate to function as controls. The autosampler vials were capped, gently vortexed, and incubated for 24 h at room temperature in the autosampler (dark) prior to HPLC analysis.

Calibration standards were prepared manually from the peptide stock solution, diluted into the appropriate buffer for the peptide, and contained either 5 or 25% acetonitrile. The peptide concentrations were 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.50, 1.0 mM.

A Waters Alliance 2695 and 996 PDA detector comprised the chromatographic system. A 10 µl injection of the reactivity samples was made onto the column. The peptides were separated from the test chemicals and products on a Zorbax SB-C18 (2.1 × 100 mm) stationary phase (Agilent Technologies, Wilmington, DE) which was preceded by a SecurityGuard cartridge guard system (Phenomenex, Torrance, CA) containing a C18 cartridge (2.0 × 4.0 mm). The column temperature was 30°C. The mobile phase consisted of 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B). A gradient of 90% (A) to 60% (A) over 25 min at a flow rate of 0.3 ml/min was used for the separation. The diode array detector scanned the wavelengths 210–400 nm. Chromatograms were extracted at 220 nm. Quantitation was performed using either Millenium32 or Empower software packages. Peptide reactivity with the test chemicals was reported as percent peptide depletion, which was determined as the reduction of the peptide concentration in the samples relative to the average concentration of the controls.

Data analysis. Sensitivity, specificity, positive predictivity, negative predictivity, and accuracy were calculated for each peptide based on an arbitrary depletion cut-off value used to determine whether the chemical had reacted with the peptide.

For the sensitizers, LLNA EC3 values and the peptide depletion percentages were compared using a Spearman correlation analysis (Hollander and Wolfe, 1973). Spearman correlations were calculated since both the raw and log-transformed EC3 values are not normally distributed and since Spearman correlations are based on the ranks of the data points rather than the actual values (i.e., no distributional assumptions are made). Correlation analyses and graphs were generated using S-Plus 6.2 (Insightful Corporation, Seattle, WA). A level of significance (alpha) of 0.05 was used for the correlation analyses.

RESULTS

Test Chemical Dataset: Chemical Information and Biological Data

Table 2 lists 38 chemicals along with their respective molecular weights and chemical structures. It is clear from reviewing the structures themselves that the dataset embraces the wide chemical diversity known to exist among skin allergens. For

TABLE 2
Chemical Structures and Their Potency Category Based on LLNA Data

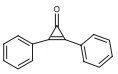
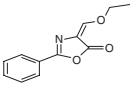
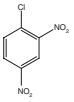
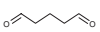
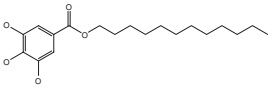
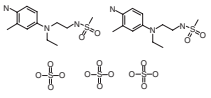
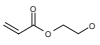
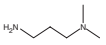
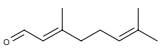
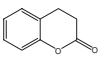
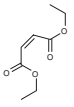
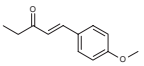
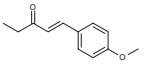
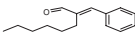
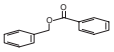
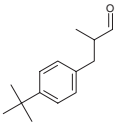
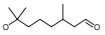
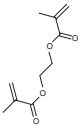
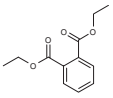
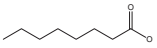
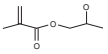
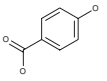
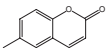
Chemical name[a]	MW	Chemical structure	LLNA EC3 (%)[b]	Potency category[c]	Reference
Diphenylcyclopropenone	206.25		*0.003**	*Extreme*	Ryan *et al*., 2000
Oxazolone	217.24		0.003	Extreme	Loveless *et al*., 1996
p-Benzoquinone	108.10		*0.01**	*Extreme*	Basketter and Scholes, 1992
2,4-Dinitrochlorobenzene	202.55		0.04	Extreme	Loveless *et al*., 1996
1,4-Hydroquinone	110.12		0.1	Strong	Kimber *et al*., 1998
Glutaraldehyde	100.10		0.1	Strong	Hilton *et al*., 1998
Phthalic anhydride	148.12		0.16	Strong	Dearman *et al*., 2000
Lauryl gallate	338.45		*0.3**	*Strong*	Unpublished
CD3	271.3		0.6	Strong	Unpublished
2-Hydroxyethyl acrylate	116.13		*1.4**	*Moderate*	Scholes *et al*., 1992
3-Dimethylaminopropylamine	102.20		2.2	Moderate	Wright *et al*., 2001
1,2-Benzisothiazolin-3-one	151.19		*2.3**	*Moderate*	Ashby *et al*., 1995
Phenylacetaldehyde	120.15		3.0	Moderate	Basketter *et al*., 2001
Cinnamic aldehyde	132.16		3.1	Moderate	Basketter *et al*., 2001
3-Aminophenol	109.10		3.2	Moderate	Ashby *et al*., 1995
Squaric acid	114.06		4.3	Moderate	Ryan *et al*., 2000
Citral	152.26		5.1	Moderate	Ashby *et al*., 1995
3,4-Dihydrocoumarin	148.10		5.6	Moderate	Ashby *et al*., 1995
Diethyl maleate	172.20		*5.8**	Moderate	Ryan *et al*., 2000

TABLE 2—*Continued*

Chemical name[a]	MW	Chemical structure	LLNA EC3 (%)[b]	Potency category[c]	Reference
1-(4-methoxyphenyl)-1-penten-3-one	190.26		*9.3**	Moderate	Ryan *et al*., 2000
α-Amyl cinnamaldehyde	202.31		10.6	Weak	Patlewicz *et al*., 2001
α-Hexylcinnamic aldehyde	216.33		11.7	Weak	Dearman *et al*., 2001
Benzyl benzoate	212.25		16.7	Weak	Smith and Hotchkiss, 2001
Lilial	204.30		18.7	Weak	Basketter *et al*., 2001
Hydroxycitronellal	172.3		23.0	Weak	Basketter *et al*., 1994
5-Methyl-2,3-hexanedione	128.17		25.8	Weak	Ryan *et al*., 2000
Ethyleneglycol dimethacrylate	198.24		36.5	Weak	Unpublished
Glycerol	92.10		NC	NS	Ryan *et al*., 2000
Hexane	86.18		NC	NS	Basketter *et al*., 1998
Diethyl phthalate	222.26		NC	NS	Ryan *et al*., 2000
Octanoic acid	144.20		NC	NS	Basketter *et al*., 1998
2-Hydroxypropyl methacrylate	144.19		NC	NS	Basketter and Scholes, 1992
1-Butanol	74.14		NC	NS	Ryan *et al*., 2000
4-Hydroxybenzoic acid	138.13		NC	NS	Ashby *et al*., 1995
6-Methylcoumarin	160.10		NC	NS	Ashby *et al*., 1995
Methyl salicylate	153.15		NC	NS	Kimber *et al*., 1998
Chlorobenzene	112.56		NC	NS	Ashby *et al*., 1995
Lactic acid	90.08		NC	NS	Basketter *et al*., 1998

Note. NC, not calculated; NS, non-sensitizing in LLNA.

[a]Chemical name—Each chemical listed in the table is associated with representative LLNA data and its specific literature citation.

[b]EC3 values calculated using the log-linear extrapolation are indicated by an asterisk (*).

[c]Potency category was determined by the following EC3 cutoff values: Extreme, <0.1%; Strong, ≥0.1% – <1%; Moderate, ≥1% – <10%; and Weak, ≥10% – ≤100%. Potency categories derived from extrapolated EC3 values are given in italics.

example, aldehydes, ketones, aromatic amines, quinones, and acrylates are represented in the dataset. In addition, the LLNA EC3 values and each chemical's potency category are displayed in Table 2. The dataset includes weak, moderate, strong, and extreme skin sensitizers, as well as nonsensitizers. For each chemical, the data shown in Table 2 derive from one representative experiment that we feel reflects accurately the results obtained with the chemical as the chemical might have been tested multiple times in LLNA studies. The specific reference for the source of the LLNA data for each chemical is indicated in Table 2. The dataset comprises 11 nonsensitizers; 7 weak sensitizers; 11 moderate sensitizers; 5 strong sensitizers; and 4 extreme sensitizers; a total of 38 compounds. For the skin sensitizers, the range of EC3 values spans from 0.003% for the extreme sensitizers, oxazolone and diphenylcyclopropenone, to 36.5% for the weak sensitizer, ethyleneglycol dimethacrylate. EC3 values estimated by the log-linear extrapolation method are marked with an asterisk (*) and the potency class for the chemical is shown in italics.

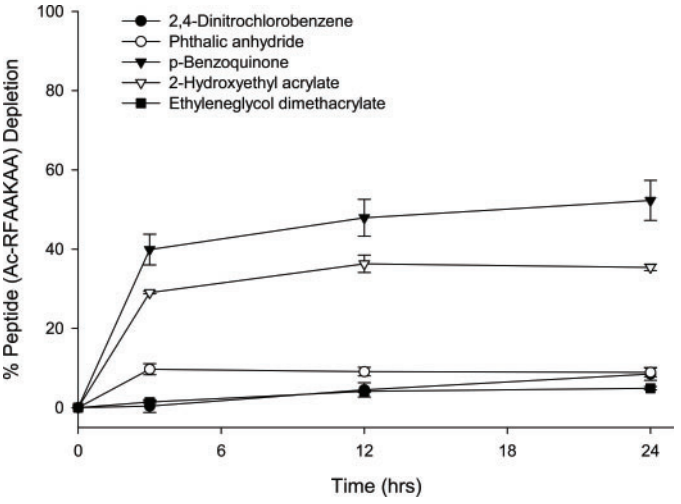


FIG. 2. Kinetics of lysine reactivity with allergens. The lysine peptide (0.5 mM) was examined with various test chemicals (5 mM) at 3, 12, and 24 h.

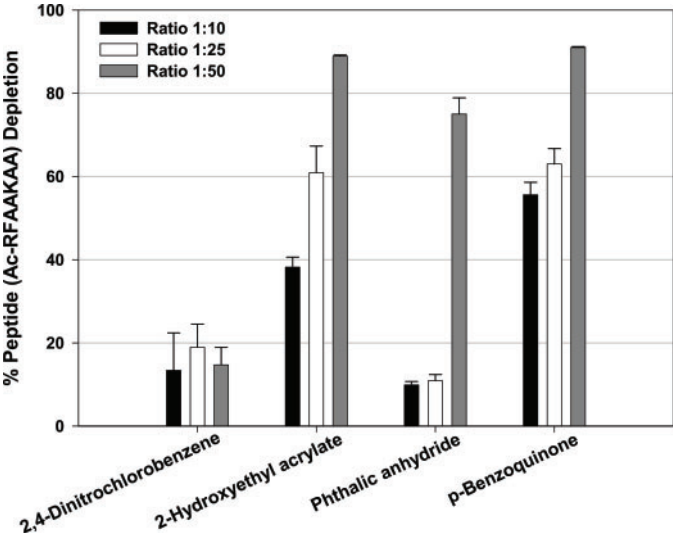


FIG. 3. Optimization of peptide to test chemical ratio for reactivity. The lysine peptide (0.5 mM) was tested with chemicals 5 mM (gray), 12.5 mM (black), and 25 mM (white) with a 24 h reaction time.

Optimization of Peptide Reactivity Assays

Conjugation of GSH to chemical allergens was evaluated using reaction conditions previously described for other chemicals (Boyland and Chasseaud, 1967; Esterbauer *et al*., 1975; Fry *et al*., 1992; Garle and Fry, 1989; Portoghese *et al*., 1989). The GSH and test chemicals were mixed at a ratio of 1:100 for 15 min at pH 7.4. The reactions were stopped at 15 min and the percent depletion of free, unreacted GSH was measured. Thus, the reactions conditions previously published for evaluating GSH reactivity with chemicals were used for evaluating our test chemical dataset.

For the synthetic peptides (containing lysine, cysteine, or histidine), a number of optimization experiments were conducted to determine the reaction conditions that would be most optimal for the majority of chemicals tested. Initially, the kinetics of the reaction using the lysine peptide and a peptide to test chemical ratio of 1:10 was evaluated. Figure 2 shows that reaction times of 3, 12, or 24 h gave similar results with the chemical allergens tested. For example, maximum reactivity (percent depletion of lysine peptide) was still evident at 24 h for both 1,4-benzoquinone and hydroxyethyl acrylate. For logistic reasons, the 24 h time point was chosen for analysis of the other chemicals in the dataset. The pH of the lysine peptide reaction was set at 10.5 based on its pKa and the need to deprotonate the primary amine of the lysine side chain to make it available for reactivity. Prior to analysis of the chemicals, the optimal test chemical to peptide ratio was determined for each peptide. Figure 3 shows clearly that the use of a peptide (lysine) to test chemical ratio of 1:50 was optimal for three of the four allergens tested. Thus, for the lysine peptide we used a 1:50 peptide to test chemical ratio with a 24 h reaction period. In preliminary experiments, the optimal pH for evaluating the reactivity of the lysine peptide was found to be 10.2 (data not shown). For the cysteine and histidine peptides, similar reaction conditions were used except that the reaction was conducted at pH 7.5 and a 1:10 peptide to test chemical ratio was used for the cysteine peptide.

Relationship of Peptide Reactivity and Potency Data

There are multiple hurdles a chemical must pass in order to initiate a sensitization response, including skin absorption, peptide reactivity, and immune recognition by T cells. Thus, it would not be expected that measuring peptide reactivity would predict the total biological response that occurs in animals or humans. However, this peptide reactivity

TABLE 3
Reactivity of Chemical Substances to Glutathione or Synthetic Peptides with Results Expressed as Percent Depletion

LLNA category	*(Conc. peptide:Conc. test substance)*	Glutathione (0.2 mM:20 mM)		Lysine (0.5 mM:25 mM)		Cysteine (0.5 mM: 5 mM)		Histidine (0.5 mM:25 mM)	
		Average	SD	Average	SD	Average	SD	Average	SD
Extreme	2,4 Dinitrochlorobenzene	43.6	2.6	14.7	4.2	100.0	0.0	0.3	4.9
	Diphenylcyclopropenone	22.0	7.5	0.7	3.8	98.8	2.0	−1.0	3.1
	Oxazolone	22.6	9.5	49.6	1.8	75.5	1.4	−4.9	11.6
	p-Benzoquinone	100.0	0.0	91.0	0.2	99.0	1.8	94.0	3.8
Strong	Phthalic anhydride	100.0	0.0	75.0	3.9	−1.9	1.0	−4.6	5.8
	1,4-Hydroquinone	76.5	6.1	51.1	6.5	83.3	0.9	30.0	0.9
	Glutaraldehyde	20.8	4.0	85.4	3.5	30.2	0.5	2.7	1.0
	Lauryl gallate	42.2	13.6	8.7	4.2	90.9	13.1	−0.1	1.0
	CD3	63.6	13.6	13.6	0.5	90.1	1.1	−8.9	4.0
Moderate	2-Hydroxyethyl acrylate	98.1	1.8	88.9	0.3	92.6	0.5	8.2	4.3
	3-Dimethylaminopropylamine	2.8	5.2	−1.8	1.9	10.2	3.4	1.9	2.5
	Cinnamic aldehyde	46.7	5.2	43.2	4.1	70.6	1.0	−3.8	7.8
	3-Aminophenol	2.0	2.4	1.2	1.7	6.6	1.5	2.0	2.4
	3,4-Dihydrocoumarin	2.3	2.6	7.5	1.0	ND	—	−1.8	2.8
	1,2-Benzisothiazolin-3-one	14.5	1.3	ND	—	97.7	0.1	ND	—
	Phenylacetaldehyde	−4.7	0.7	22.6	1.9	60.7	13.3	−3.1	0.8
	Squaric acid	16.5	4.0	4.8	4.9	46.9	8.7	3.1	0.4
	Citral	37.5	14.4	16.9	0.3	85.7	3.2	−7.9	1.0
	1-(4-Methoxyphenyl)-1-penten-3-one	−0.2	1.5	14.3	3.2	29.9	5.6	1.7	2.1
	Diethyl maleate	83.3	4.5	85.5	1.6	100.0	0.0	0.5	0.8
Weak	α-Hexylcinnamaldehyde	−2.6	3.2	−1.6	2.9	−0.3	1.2	−0.4	1.5
	5-Methyl-2,3-Hexandione	−2.6	9.9	7.5	1.1	25.8	4.0	23.1	3.9
	Hydroxycitronellal	−1.8	3.9	6.5	2.0	17.5	1.7	5.6	6.2
	Ethyleneglycol dimethacrylate	3.6	5.6	12.4	3.0	87.3	5.0	−1.2	1.8
	α-Amyl cinnamaldehyde	0.2	10.1	3.9	1.5	0.6	0.2	−1.1	1.7
	Benzyl benzoate	0.7	5.5	3.0	5.3	0.2	1.1	−2.5	0.8
	Lilial	7.7	0.8	0.7	0.2	14.0	6.4	−0.4	0.3
Non Sensitizers	Glycerol	1.2	4.2	2.1	0.9	−3.8	5.2	0.2	0.6
	Hexane	−0.8	4.1	−5.1	0.6	−0.4	0.8	−1.8	3.5
	Diethyl phthalate	10.9	13.3	−0.7	0.9	0.8	1.7	0.7	2.8
	Octanoic acid	−1.6	3.1	0.9	0.1	−1.0	0.7	0.7	0.3
	2-Hydroxypropyl methacrylate	5.5	4.8	ND	—	58.4	5.9	ND	—
	1-Butanol	6.1	7.5	1.2	0.8	−0.4	1.4	0.5	0.4
	4-Hydroxybenzoic acid	−1.0	5.8	2.2	2.1	−0.3	0.8	−0.4	0.2
	6-Methylcoumarin	−1.6	8.6	4.0	5.6	1.4	0.3	−1.6	2.5
	Methyl salicylate	4.2	3.5	1.6	0.3	0.3	0.8	0.5	1.1
	Chlorobenzene	3.2	2.3	1.3	0.2	0.4	0.2	−1.8	2.0
	Lactic acid	−1.1	11.1	0.8	0.5	−0.9	0.3	−0.8	1.3

information might be useful in helping to screen chemicals for their potential to cause a skin sensitization response. Table 3 summarizes our reactivity data for GSH and the three synthetic peptides that contained lysine, cysteine, or histidine. The chemicals in Table 3 are listed according to the four potency categories described previously (Table 1). For discussion purposes, we chose a peptide depletion of greater than 10% to indicate that the chemical had reacted with the starting peptide.

For GSH, all of the strong and extreme sensitizers caused depletion of greater than 10% with a range of 20.8 to 100% (Table 3). For the moderate sensitizers, 6 of 11 tested gave depletions of greater than 10% with a range of 14.5 to 98.1%. The only weak or nonsensitizer that caused GSH depletion of greater than 10% was the nonsensitizer diethyl phthalate which yielded 10.9% depletion.

The peptide depletion results for the three synthetic peptides were quite varied. The majority of chemical allergens tested with the cysteine peptide at a 1:10 ratio (21/26) caused greater than 10% depletion. All of the extreme sensitizers and four of five strong sensitizers caused depletion of greater than 20% with most of them with values greater than 75%. Interestingly, no depletion of the cysteine peptide was observed with the strong allergen phthalic anhydride. For the moderate sensitizers tested, all but one gave depletion values greater than 10% with a range of 10.2 to 100%. No data was obtained with 3,4-dihydrocoumarin because it co-eluted with the cysteine peptide. Four of seven weak allergens gave depletion of cysteine peptide at levels

greater than 10% with a range of 14.0 to 87.3%. The only nonsensitizer to give a response of greater than 10% was 2-hydroxypropyl methacrylate with a value of 58.4%. Using a peptide to test chemical ratio of 1:50 for the cysteine peptide increased the sensitivity of the assay as indicated by an increase in percent depletion values for weak and moderate allergens, but also increased the number of nonsensitizers detected (data not shown). Thus, the 1:10 peptide to test chemical ratio gave a better discrimination of sensitizers and nonsensitizers when using the cysteine peptide.

Depletion of the lysine peptide was generally seen more often with moderate to extreme sensitizers than with weak sensitizers. Three of four extreme and four of five of the strong sensitizers demonstrated depletion values of greater than 10%. For the moderate sensitizers, 6 of the 10 tested had values greater than 10%, whereas, only one of weak sensitizers, ethyleneglycol dimethacrylate, had a value of greater than 10%. Thus, 14 of 26 allergens tested gave lysine depletion results of 10% or greater. None of the nonsensitizers tested gave lysine depletion levels of greater than 10%.

For the histidine peptide that was run at a 1:50 ratio at pH of 7.4, only 3 of 26 sensitizers tested gave depletion results of greater than 10%. None of the nonsensitizers gave values of greater than 10%.

To summarize the potential usefulness of a 10% depletion cut-off for categorizing chemicals as sensitizers or nonsensitizers, the sensitivity, specificity, positive predictivity, and negative predictivity were calculated for each peptide. These statistics are shown in Table 4. Based on the specificity, most nonsensitizers (90.9% to 100.0%) are called negative, regardless of peptide. In terms of sensitivity, cysteine is the most promising peptide (80.8%) and histidine is not sensitive at all (11.5%). Most chemical substances called sensitizers are true sensitizers (93.8 to 100.0%) regardless of peptide, but many chemicals called nonsensitizers based on a 10% depletion cut-off are actually sensitizers (i.e., the negative predictivity range is 30.3 to 66.7%). The accuracy values for the glutathione, lysine, cysteine, and histidine were 65.8, 66.7, 83.8, and 36.1%, respectively.

The LLNA EC3 values for each sensitizer and the peptide depletion percentages were compared using a Spearman correlation analysis. Spearman correlations are based on the ranks of the data points rather than the actual values (Hollander and Wolfe, 1973). By computing Spearman correlations rather than Pearson correlations, weak sensitizers have the same amount of influence as stronger sensitizers on the results. In addition, potential outliers do not have a strong influence on the results. Scatter plots of EC3 values and the depletion percentage of each peptide, along with correlation analysis results, are shown in Figure 4. The results indicate that a moderately strong correlation exists between LLNA EC3 values and glutathione depletion ($r = -0.645$, $p = 0.0010$), lysine depletion ($r = -0.445$, $p = 0.0259$), and cysteine depletion ($r = -0.463$, $p = 0.0205$). LLNA EC3 values and histidine depletion are not significantly correlated ($r = 0.005$, $p = 0.9795$).

TABLE 4
Contingency Probability Statistics

	Peptide			
	Glutathione	Lysine	Cysteine	Histidine
Sensitivity	55.6%	53.8%	80.8%	11.5%
Specificity	90.9%	100.0%	90.9%	100.0%
Positive predictivity	93.8%	100.0%	95.5%	100.0%
Negative predictivity	45.5%	45.5%	66.7%	30.3%
Accuracy	65.8%	66.7%	83.8%	36.1%

DISCUSSION

Over the past 25 years there have been significant advances in our understanding of the chemical and biological processes associated with skin sensitization and allergic contact dermatitis. For example, there is an increased understanding of how chemical reactions play an important role in the initiation of skin sensitization (reviewed in Lepoittevin *et al*., 1998). Specifically, they are involved in the formation of hapten-protein complexes which are processed by antigen-presenting Langerhans cells in the skin for presentation to antigen-specific T cells.

Chemical allergens (haptens) or their metabolites are small molecular weight compounds (generally less than 500 Da) with electrophilic properties. In this regard, they are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine can react with electrophiles (Ahlfors *et al*., 2003; Dupuis and Benezra, 1982; Lepoittevin *et al*., 1998). Thus, electrophilic allergens react with nucleophilic amino acids to form extremely stable covalent bonds, which is critical to the initiation of a skin sensitization response. It is the understanding of these reaction types that led to the development of DEREK, an expert system designed to identify skin sensitization alerts (Barratt *et al*., 1994). Protein reactivity was also an important consideration in the development of several QSARs (e.g., Patlewicz *et al*., 2001; Roberts and Patlewicz, 2002). Since reactivity is one key step in the induction of skin sensitization it was our intention to investigate whether reactivity could be used to develop a quantitative peptide-based reactivity assay that would have utility for screening a chemical's skin sensitization potency as defined in the LLNA. The conditions of the peptide reactivity assays were set up to optimize our ability to screen for allergens, not to necessarily reproduce all of the varied physiological conditions on and in the skin where these reactions might take place. What we try to compare is the intrinsic reactivity of

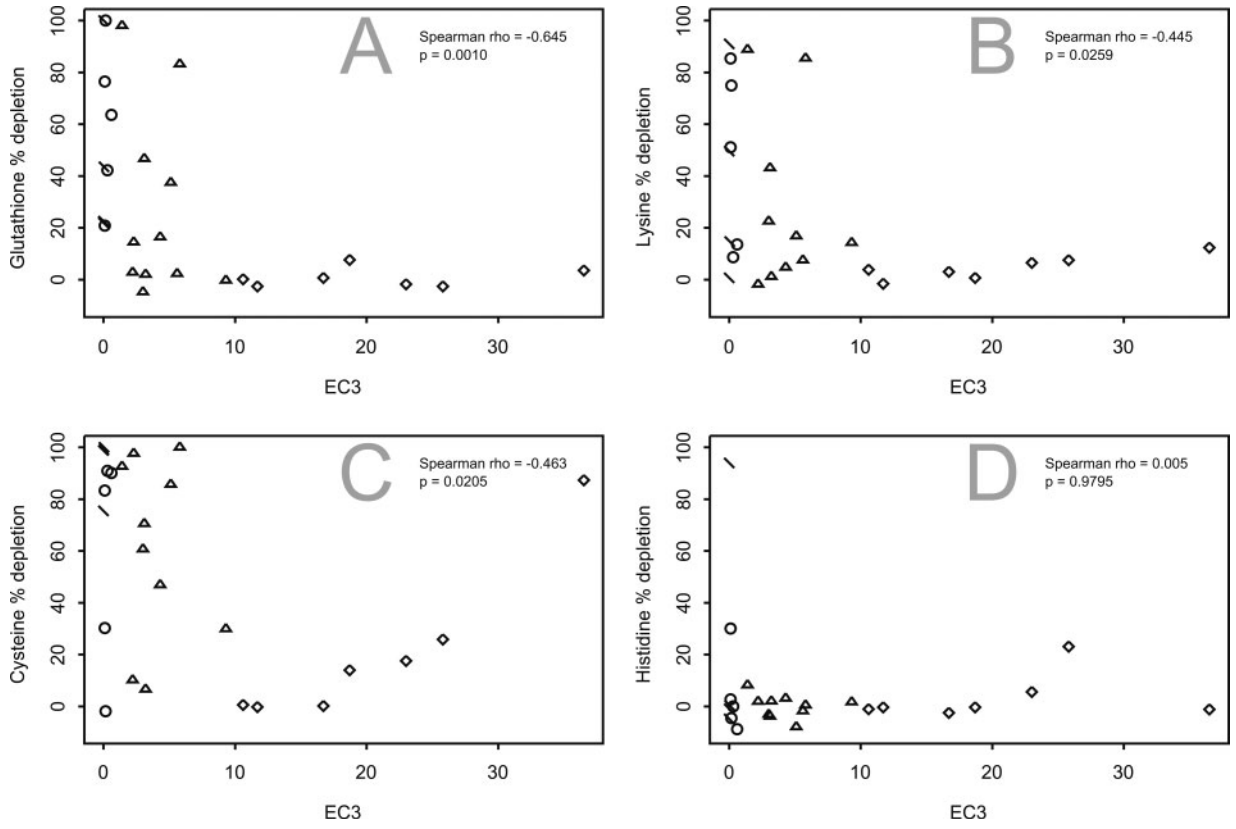


FIG. 4. Spearman correlation of LLNA EC3 data and peptide reactivity. LLNA potency category is denoted as Extreme (□), Strong (○), Moderate (△), or Weak (◇). Peptides include glutathione (A), lysine (B), cysteine (C), and histidine (D).

different haptens in water towards specific nucleophiles such as lysine. Even if the physiological pH is around 7.4 (this is an average value that does not reflect specific compartments and/or situations) the protonation status of amino groups in proteins is very diverse. Thus it has been shown for some lysine residues that they have very low pKa values compared to 'normal' primary amino groups. In human serum albumin for example, Lysine 166 has a low pKa value and is therefore able to react with electrophiles such as acetylsalicylic acid while other residues are not. As we are considering small peptides for which a matrix effect does not exist, the only way to mimic such reactive amino groups is to increase the pH to get a significant reactivity within 24 h time period used for the lysine peptide.

The LLNA EC3 values listed in Table 2 show a range of potency from 0.003% for the extreme allergen, oxazolone, to 36.5% for the weak allergen, ethyleneglycol dimethacrylate. The chemicals selected for the dataset are known skin allergens which have been reported to induce sensitization in animals and/or man. For each chemical listed in Table 2, a specific reference is given for the representative LLNA data shown, since most of these compounds have been tested several times in different laboratories (e.g., Dearman *et al*., 1998, 2001; Kimber *et al*., 1998; Loveless *et al*., 1996) and are well known allergens for the purpose of using them for evaluation of new methodologies (Gerberick *et al*., in press). The chemicals represented in the database comprise weak, moderate, strong, and extreme sensitizers, as well as nonsensitizing materials, as based on potency categorization criteria that have been developed recently by a European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Task Force (Kimber *et al*., 2003).

The results, as summarized in Table 3, demonstrate clearly that an association between the degree of peptide reactivity (as measured by peptide depletion) and sensitization potency is evident. For ease of discussion, we chose 10% depletion as a cutoff for classifying whether a test chemical had been detected or not with the different peptides. Additional studies are underway to increase the number of chemicals tested so that we can more formally analyze the data to determine more appropriate cutoffs to employ for the peptide reactivity assays. With the current dataset, a greater number of the extreme, strong, and moderate allergens reacted with GSH, cysteine, and lysine than did the weak and nonsensitizers. Based on the number of compounds with greater than 10% depletion, the cysteine peptide seems to be best at discriminating between the different potency categories followed by the GSH and lysine peptides (Table 3). The histidine peptide was limited in its ability to react with the known allergens. It is important to note that conditions used for each of the peptides were optimized in a

general way and are not specific for any chemical on an individual basis. Thus, some chemicals were not detected because they either co-eluted with the peptide (e.g., 3,4-dihydrocoumarin with cysteine peptide) or were incompatible with the solvent system (e.g., diphenylcyclopropenone in buffer with 25% acetonitrile). Even with these limitations, the results show that analysis of peptide reactivity demonstrates a significant correlation with sensitization potency (Fig. 4). Of course, one would not expect an extremely high correlation between reactivity and potency since other factors, such as skin penetration and immune recognition by T cells, are critical for the acquisition of skin sensitization.

Glutathione (GSH), a cysteine containing peptide, is the most prevalent cellular thiol and most abundant low molecular weight peptide present in cells (Deneke and Fanburg, 1989). Cellular GSH protects cells by detoxifying electrophilic compounds through conjugation of its nucleophilic sulfur, and by acting as an antioxidant (Deneke and Fanburg, 1989). It is unknown whether GSH is directly involved in ACD, however a clear correlation exists between sensitizing activity and ability to perturb GSH status in mouse skin (Schmidt and Chung, 1992, 1993). The perturbation of the GSH status is likely driven by direct GSH conjugation or by oxidative stress via free radical formation (Schmidt *et al*., 1990; Schmidt and Chung, 1992, 1993). Regardless of the mechanism, *in vivo* application of electrophilic chemicals to the skin depletes GSH and this is driven by the ubiquitous nature of GSH and its reactivity to soft electrophiles, particularly α,β-unsaturated carbonyl compounds. Our results show that all of the extreme and strong sensitizers demonstrated GSH depletion, whereas only 6 of 11 moderate sensitizers showed values greater than 10% (Table 3). None of weak sensitizers caused GSH depletion greater than 10%. In contrast, the cysteine peptide demonstrated greater sensitivity with most of the moderate sensitizers giving depletion values greater than 10%. In fact, the only two compounds that didn't react with cysteine were 3-aminophenol and 3,4-dihydrocoumarin which are prohaptens and are thought to require biotransformation for their ability to react with nucleophilic amino acids such as cysteine.

Only one compound, phthalic anhydride, reacted with GSH but not the cysteine peptide. The differences between the GSH and the cysteine peptide results might be due to the different test conditions used for the two peptide assays or to a difference in the peptide sequence. Thus, while in the cysteine peptide the terminal α-NH_2 group is blocked by an acetyl function, this terminal α-NH_2 group is free in the GSH peptide. It has been shown in previous studies on the covalent binding of 5-chloro-2-methylisothiazol-3-one (MCI) to model peptides that terminal α-NH_2 function can play a role in protein modifications (Alvarez-Sanchez *et al*., 2004). Not all sensitizers are expected to show the same reactivity towards SH/NH_2. This is well described by the Hard and Soft Acid and Base (HSAB) theory (Pearson, 1963) which reflects qualitatively interaction mechanisms between a nucleophile and an electrophile. Thus, GSH, which is a good model for a soft nucleophile, is expected to react more selectively with soft electrophiles such as α,β-unsaturated aldehydes, ketones, or esters while lysine which is a good example of a hard nucleophile should react more selectively with hard electrophiles such as phthalic anhydride.

In recent studies, lysine has been demonstrated to be an important nucleophile for allergens such as sultone and methylisothiazolone derivaties (Alvarez-Sanchez *et al*., 2003; Meschkat *et al*., 2001). Thus, we thought lysine would be an important nucleophile to use in our peptide reactivity studies. Overall, the lysine peptide worked well with extreme, strong, and some moderate sensitizers. However, only one weak allergen, ethyleneglycol dimethacrylate, was detected with the lysine peptide. The only compound detected with the lysine peptide but not the cysteine peptide was phthalic anhydride due to a chemical selectivity of phthalic anhydride for amino groups.

From a specificity standpoint, the GSH, lysine and cysteine peptides performed extremely well. Only two nonsensitizers gave depletion results greater than 10%. Diethyl phthalate gave 10.9% depletion of GSH and 2-hydroxypropyl methacrylate gave 58.4% depletion with the cysteine peptide. Although 2-hydroxypropyl methacrylate is reactive with cysteine it might be that its limited ability to penetrate the skin or be recognized by the immune system accounts for it being a nonsensitizer in the LLNA. As mentioned previously, the use of a 1:50 ratio for the cysteine peptide yielded additional nonsensitizers demonstrating reactivity (data not shown). Although these compounds have the ability to react with peptides in our artificial systems they might lack that ability to do so *in vivo*. Moreover, there could be other reasons such as a lack of immune recognition or materials are appropriately detoxified before they have the ability to initiate a sensitization response.

One potential challenge for developing *in vitro* methods for skin sensitization testing is that it is well known that some chemical allergens are prohaptens, and as such require biotransformation prior to initiating a skin sensitization response *in vivo* (Smith and Hotchkiss, 2001; Smith-Pease *et al*., 2003), The need for biotransformation has been demonstrated with many chemicals, such as the formation of benzoquinonediimine from azo hair dyes (Basketter and Goodwin, 1988), or orthoquinone from isoeugenol (Bertrand *et al*., 1997; Hotchkiss, 1998). Since prohaptens are an important class of sensitizers a few of these chemicals (aminophenol and 3,4-dihydrocoumarin) were also included and evaluated in this assay (Bertrand *et al*., 1997; Smith and Hotchkiss, 2001). The observation that neither of these chemicals reacted with the three peptides is consistent with the fact that they are prohaptens. However, 1,4-hydroquinone, which requires oxidation for reactivity, reacted with all of the peptides used, including histidine. It is believed that the 1,4-hydroquinone was air oxidized under the conditions of testing to benzoquinone. Based on the knowledge that some chemical allergens need to be biotransformed prior to reacting with proteins/peptides, it will be critical to incorporate a metabolism component to address these types of molecules. We are

evaluating currently a number of metabolic and oxidizing systems for use in our peptide reactivity work.

The goal of this work was to evaluate the use of peptide reactivity as a means for screening the skin sensitization potential of chemicals. The results presented show clearly that each of the peptides evaluated, except histidine, was useful in quantifying reactivity as measured by the depletion of the peptide. Measurement of peptide reactivity will be helpful in reducing our need for animal testing by allowing us to deselect chemicals with high reactivity and thus, the potential to be moderate to strong allergens. Thus, by performing a peptide reactivity assay one can reduce the number of compounds that would require testing in an animal model. It is hoped that with additional modification of the peptide reactivity assays (e.g., addition of metabolism component) and use of other nonanimal methods (e.g., dendritic cell based assay, skin penetration models), we will be even more successful in reducing our reliance on animals for skin sensitization testing in the future.

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Appendix 11

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Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach

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In the interest of reducing animal use, *in vitro* alternatives for skin sensitization testing are under development. One unifying characteristic of chemical allergens is the requirement that they react with proteins for the effective induction of skin sensitization. The majority of chemical allergens are electrophilic and react with nucleophilic amino acids. To determine whether and to what extent reactivity correlates with skin sensitization potential, 82 chemicals comprising allergens of different potencies and non-allergenic chemicals were evaluated for their ability to react with reduced glutathione (GSH) or with two synthetic peptides containing either a single cysteine or lysine. Following a 15-min reaction time with GSH, or a 24-h reaction time with the two synthetic peptides, the samples were analyzed by high-performance liquid chromatography. UV detection was used to monitor the depletion of GSH or the peptides. The peptide reactivity data were compared with existing local lymph node assay data using recursive partitioning methodology to build a classification tree that allowed a ranking of reactivity as minimal, low, moderate, and high. Generally, nonallergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity. Classifying minimal reactivity as nonsensitizers and low, moderate, and high reactivity as sensitizers, it was determined that a model based on cysteine and lysine gave a prediction accuracy of 89%. The results of these investigations reveal that measurement of peptide reactivity has considerable potential utility as a screening approach for skin sensitization testing, and thereby for reducing reliance on animal-based test methods.

Key Words: **allergens; alternatives; skin sensitization; peptide reactivity; prediction model.**

Allergic contact dermatitis (ACD) resulting from skin sensitization is a critical toxicological endpoint evaluated for all new chemicals developed for consumer and/or occupational use. The acquisition of skin sensitization and the subsequent elicitation of an ACD reaction in the skin are processes dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses (Kimber *et al.*, 2000). The local lymph node assay (LLNA) is viewed as the most appropriate skin sensitization test method for the evaluation of chemicals that have potential to come in contact with the skin (Cockshott *et al.*, 2006). The LLNA is based upon characterization of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals (Gerberick *et al.*, 2000; Kimber *et al.*, 2002). However, there is a critical need to develop non-animal–based methods for the evaluation of new chemicals that will reduce significantly or eliminate the need for animals in skin sensitization testing in the future (Jowsey *et al.*, 2006; Ryan *et al.*, 2001, 2005). This is of particular importance in view of the forthcoming European Union ban on *in vivo* testing of cosmetic and toiletry ingredients following the publication of the Seventh Amendment to the Cosmetic Directive (European Union Seventh Amendment to Cosmetic Directive) and for Registration, Evaluation, and Authorization of Chemicals that requires evaluation of a large number of chemicals.

Fortunately, the underlying chemical and cellular mechanisms of ACD are relatively well understood to aid scientists in the development of alternative methods for skin sensitization testing. It is believed that for a chemical to function as a contact sensitizer (or allergen), it must be capable of penetrating into the viable epidermis, react with protein, induce local trauma, and be recognized by the immune system. Thus, characterization of skin sensitization must integrate various sources of information from a battery of assays representing the key steps of skin allergy (Jowsey *et al.*, 2006). For example, investigators have undertaken recently the development of chemical reactivity screening methods for aiding in the assessment of a chemical's skin sensitization potential (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004; Kato *et al.*, 2003; Natsch *et al.*, in press).

The correlation of skin protein reactivity and skin sensitization is well established and has been known for many years (Dupuis and Benezra, 1982; Landsteiner and Jacobs, 1936;

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Lepoittevin *et al.*, 1998). It is accepted that if a chemical is capable of reacting with protein directly or after appropriate biotransformation, then it has the potential to act as an allergen. While a variety of mechanisms contribute to protein reactivity, it is generally recognized that this process involves the reaction of a small molecule, having electrophilic properties, with a nucleophilic amino acid on a protein. The majority of chemical allergens (or their metabolites) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups, nucleophiles, capable of reacting with electrophilic allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can also react with electrophiles (Ahlfors *et al.*, 2003; Dupuis and Benezra, 1982; Lepoittevin *et al.*, 1998).

Measuring chemical reactivity on nucleophile-containing peptides has potential utility for evaluating the skin sensitization potential of chemicals (Gerberick *et al.*, 2004). Specifically, it was demonstrated that peptides containing either cysteine or lysine along with glutathione (GSH) served as surrogate nucleophiles to quantitatively measure chemical reactivity. The purpose of this work was to examine the reactivity of a large set of test chemicals (38 from original study and 44 new chemicals for a total of 82) using lysine, cysteine, and GSH peptides at different peptide to chemical molar ratios to determine whether the degree of reactivity correlated with the compound's sensitization potency. The data were analyzed using a classification tree model approach to develop a pragmatic prediction model for assessing and interpreting the peptide reactivity assay data.

MATERIALS AND METHODS

Test chemicals. The rationale for selecting chemicals for evaluation was based on chemical diversity and on the availability of robust LLNA data representing a good distribution of weak, moderate, strong, and extreme allergens along with nonallergens. The following chemicals with accompanying purity and CAS numbers were purchased from Aldrich Chemical Company (Milwaukee, WI): 2-acetylcyclohexanone, 97% (874-23-7); a-amylcinnamaldehyde, 85% (122-40-7); benzaldehyde, 95% (100-52-7); 1,2-benzisothiazolin-3-one, 97% (2634-33-5); benzyl benzoate, 99% (120-51-4); benzylideneacetone, 99% (122-57-6); 1-butanol, 99.5% (71-36-3); chlorobenzene, 99% (108-90-7); cinnamaldehyde, 99% (14371-10-9); coumarin (91-64-5); cyclamen aldehyde, 90% (103-95-7); diethyl maleate, 97% (141-05-9); diethyl phthalate, 99.5% (84-66-2); diphenylcyclopropenone, 98% (886-38-4); ethyl acrylate, 99% (140-88-5); ethyl vanillin, 99% (121-32-4); ethyleneglycol dimethacrylate, 98% (97-90-5); farnesal, 85% (19317-11-4); formaldehyde (50-00-0); 2,4-heptadienal, 90% (5910-85-0); hexenal, 98% (6728-26-3); a-hexylcinnamaldehyde, 85% (101-86-0); 4-hydroxybenzoic acid, 99% (99-96-7); hydroxycitronellal, 95% (107-75-5); 2-hydroxyethyl acrylate, 96% (818-61-1); 2-hydroxypropyl methacrylate, 97% (923-26-2); 2-mercaptobenzothiazole, 98% (149-30-4); 6-methyl coumarin, 99% (92-48-8); methyl salicylate, 99% (119-36-8); methyl-2-nonynoate, 99% (111-80-8); methylparaben, 99% (99-76-3); metol, 99% (55-55-0); nonanoyl chloride, 96% (764-85-2); oxalic acid, 99% (144-62-7); oxazolone, 90% (15646-46-5); perillaldehyde, 92% (2111-75-3); phenylacetaldehyde, 90% (122-78-1); 2-phenylpropionaldehyde, 98% (93-53-8); phthalic anhydride, 99% (85-44-9); propyl gallate, 98% (121-79-9); propyl paraben, 99% (94-13-3); resorcinol, 99.5% (108-46-3); salicylic acid, 99% (69-72-7); squaric acid, 99% (2892-51-5); vanillin, 99% (121-33-5); vinylidene dichloride, 99% (75-35-4); vinyl pyridine (1337-81-1).

The following chemicals with accompanying purity and CAS numbers were purchased from Sigma Chemical Company (St Louis, MO): *p*-benzoquinone, 98% (106-51-4); CD3 (25646-71-3); 2,4-dinitrochlorobenzene, 99% (97-00-7); glutaraldehyde, 70% (111-30-8); imidazolidinyl urea, 95% (39236-46-9); isopropanol, 99% (67-63-0); isopropyl myristate, 98% (110-27-0); lactic acid, 85% (50-21-5); 1-(4-methoxyphenyl)-1-penten-3-one (104-27-8); nonanoic acid, 97% (112-05-0); octanoic acid, 98% (124-07-2); sulfanilamide, 99% (63-74-1); sulphanilic acid, 99% (121-57-3); trimellitic anhydride, 97% (552-30-7).

The following chemicals with accompanying purity and CAS numbers were purchased from Fluka Chemical Company (Milwaukee, WI): 4-allylanisole, 98% (140-67-0); benzoyl peroxide, 97% (94-36-0); 1-bromobutane, 99% (109-65-9); 2,3-butanedione, 99% (431-03-8); 5-chloro-2-methyl-4-isothiazolin-3-one (26172-55-4); ethylbenzoylacetate, 97% (94-02-0); fluorescein isothiocynate, 98% (3326-32-7); glyoxal (107-22-2); lilial, 95% (80-54-6); 4-methoxyacetophenone, 99% (100-06-1); palmitoyl chloride, 98% (112-67-4); propylene glycol, 99.7% (57-55-6); 2,2,6,6-tetramethyl-3,5-heptanedione, 98% (1118-71-4).

Hexane (110-54-3) was purchased from EM Science (Gibbstown, NJ). Bandrowski's base was purchased from ICN (Costa Mesa, CA). Glycerol, 99% (56-81-5) was purchased from J.T Baker (Phillipsburg, NJ).Tetracholorosalicylanilide (1154-59-2) was purchased from Eastman Kodak Company (Rochester, NY). Lauryl gallate, 98% (1166-52-5) was purchased from Alfa Aesar (Ward Hill, MA). 5-Methyl-2,3-hexandione (13706-86-0) was purchased from Penta MFG (Livingston, NJ). Kathon CG (55965-84-9) was purchased from Rohm & Haas (Philadelphia, PA). 2-Methyl-2H-isothiazol-3-one (2682-20-4) was supplied by J.-P. L.

LLNA protocol and chemicals tested. The LLNA data reported in this manuscript are derived from previously conducted studies. The LLNA studies were conducted as described elsewhere (Gerberick *et al.*, 2000; Kimber *et al.*, 2002). Briefly, groups of CBA female mice (7–12 weeks of age) were exposed topically on the dorsum of both ears to 25 μl of test material or to an equal volume of the relevant vehicle alone. Treatment was performed daily for three consecutive days. Five days following the initiation of exposure, all mice were injected via the tail vein with 250 μl of phosphate-buffered saline containing 20 μCi of tritiated thymidine. Mice were sacrificed 5 h later, and the draining lymph nodes were excised for each experimental group. The incorporation of tritiated thymidine measured by β-scintillation counting was reported in disintegrations per minute (dpm). A stimulation index (SI) was calculated for each allergen-treated group as the ratio of the dpm of the treated group over the dpm of the concurrent vehicle control. A substance was classified as a skin sensitizer if at one or more test concentrations it induced a threefold or greater increase in local lymph node proliferative activity compared with concurrent vehicle-treated controls.

Potency estimation in the LLNA. The method used to determine the relative skin sensitization potency of a chemical has been previously described and is based upon the mathematical estimation of the concentration necessary to induce a threefold increase in the proliferative activity in the draining lymph nodes relative to vehicle-treated mice (Basketter *et al.*, 1999). This estimated concentration, known as the EC3 value, is calculated by conducting a linear interpolation of coordinates above and below the value of three on the LLNA dose-response plot. The EC3 value for chemicals which had an SI greater than three for the lowest concentration tested was extrapolated from the two lowest doses evaluated (Ryan *et al.*, in press). EC3 values extrapolated by this method were calculated by log-linear interpolation between these two points on a plane in which the dose level and SI are represented on the x-axis and y-axis, respectively. Existing dose-response data from previously conducted LLNA experiments have been used to calculate the EC3 values for the chemicals used

in this manuscript. An arbitrary classification scheme based on EC3 values was used for categorizing the relative skin sensitization potency of chemicals evaluated in this study (Kimber *et al.*, 2003). This system classifies the sensitization potency of a chemical as extreme (EC3 < 0.1), strong (EC3 $\geq$ 0.1 to < 1), moderate (EC3 $\geq$ 1 to < 10), weak (EC3 $\geq$ 10 to $\leq$ 100), and nonsensitizing are not calculated.

The specific EC3 potency data used in this paper for the majority of chemicals are found in a recently published LLNA database paper (Gerberick *et al.*, 2005). References for the other chemicals used are as follows: diphenylcyclopropenone (Ryan *et al.*, 2000), phthalic anhydride (Dearman *et al.*, 1992), oxazolone (Loveless *et al.*, 1996), propyl gallate (Ashby *et al.*, 1995; Basketter and Scholes, 1992), metol (Ashby *et al.*, 1995; Basketter and Scholes, 1992), benzoyl peroxide (Kimber *et al.*, 1998), squaric acid (Ryan *et al.*, 2000), 2-methyl-2H-isothiazol-3-one (Estrada *et al.*, 2003), lilial (Basketter *et al.*, 2001), and nonanoic acid (P&G, unpublished data).

GSH, cysteine, and lysine peptide depletion assays. A method to measure reactivity of a test chemical with reduced GSH was recently developed (Gerberick *et al.*, 2004) which is based on a previously described method (Farriss and Reed, 1987). Briefly, 50 µl of a 2mM GSH stock solution prepared in oxygen-free 100mM sodium phosphate buffer (pH 7.4) and 50 µl of a 200mM test chemical prepared in dimethyl sulfoxide (DMSO) were added to 400 µl of oxygen-free 100mM sodium phosphate buffer (pH 7.4). The final reaction, containing 0.2mM GSH and 20mM of the test chemical, representing 1:100 molar ratio, was mixed and incubated for 15 min at 25°C with agitation. Control samples and standards used for defining the calibration curve for each analysis were prepared without test chemical for GSH (0.05–200mM) and glutathione disulfide (GSSG) (0.025–100mM). All samples were prepared in triplicate. Following incubation, GSH and GSSG in the samples and standards were derivatized with iodoacetic acid and 2,4 dinitrofluorobenzene. Derivatized GSH and GSSG were separated and quantitated by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Alliance 2695 system (Waters Corporation, Milford, MA) using a Waters UV detector (365 nm) and a Waters Spherisorb NH_2 analytical column (3 µm, 2.0 $\times$ 100 mm) under gradient conditions. Total GSH (GSH equivalents as GSH or GSSG) in each sample was determined from the calibration curve and used to calculate the percent peptide depletion relative to the mean concentration of total GSH in the control sample (no test chemical).

A method to measure reactivity of a test chemical with model heptapeptides containing lysine (Ac-RFAAKAA-COOH) or cysteine (Ac-RFAACAA-COOH) was recently developed (Gerberick *et al.*, 2004). Peptides were prepared and purified by the SynPep Corporation (Dublin CA, USA) to > 90% purity as measured by HPLC, and molecular weight confirmation was determined by flow injection positive-ion electrospray mass spectrometry. Briefly, 400 µl of a 1.25mM peptide stock solution prepared in buffer and a 100mM test chemical stock solution prepared in either acetonitrile or DMSO/acetonitrile were added to 100mM ammonium acetate buffer (pH 10.2) for the lysine peptide or 100mM sodium phosphate buffer (pH 7.5) for the cysteine peptide. The final reaction, containing 0.5mM of the peptide and 5 or 25mM of the test chemical, representing 1:10 and 1:50 molar ratios, was mixed and incubated in the dark for 24 h at 25°C. Control samples and standards used for defining the calibration curve for each analysis were prepared without test chemical for each peptide and ranged from 0.0156 to 1.0mM. All samples were prepared in triplicate. Following incubation, the peptide was quantified by reverse-phase HPLC (Waters 2695 Alliance) on a Zorbax SB-C18 column (3.5 µm, 100 $\times$ 2.1 mm) with UV detection at 220 nm (Waters 996 PDA detector) using an external standard linear calibration curve. The UV spectrum was collected from 210 to 400 nm to permit verification of the peptide peak identity. Peptide reactivity was reported as percent depletion based on the decrease in nonreacted peptide concentration in the sample relative to the average concentration measured in the control.

Classification tree model development. The goal was to develop a prediction model that would quantify in some way peptide depletion as related to level of reactivity. Various models were developed using classification tree methodology (Brieman *et al.*, 1983) and the recursive partitioning routines implemented in S-Plus 7.0 statistical software (2003, Insightful Corp., Seattle, WA). During model development, each peptide at each concentration (GSH 1:100, cysteine 1:10, cysteine 1:50, lysine 1:10, and lysine 1:50) was considered as a potential predictor. For each of the 82 chemicals examined, three measurements were taken as the percentage of peptide depletion for each peptide/concentration, and the average depletion was determined. Some potential models were developed using these peptide depletion averages as predictors, while other potential models were developed using peptide depletion percentages averaged further across various peptides/concentrations (e.g., the average of the cysteine 1:10 and lysine 1:50 percentages). Specifically, models were developed using the following peptide depletion values as potential predictors in each model: model #1, average of all five peptides as the only predictor; model #2, cysteine 1:10, cysteine 1:50, GSH 1:100, lysine 1:10, lysine 1:50; model #3, average of cysteine 1:10, GSH 1:100, and lysine 1:10 as the only predictor; model #4, average of cysteine 1:10, cysteine 1:50, lysine 1:10, and lysine 1:50 as the only predictor; model #5, average of cysteine 1:10 and lysine 1:50 as the only predictor; model #6, cysteine 1:10, cysteine 1:50, lysine 1:10, lysine 1:50.

Classification tree building begins with the root node, which includes all of the chemical compounds in the learning data set (a total of 56). Beginning with this node, if more than one variable is considered, S-Plus software finds the best possible variable (peptide and concentration) to split the node into two child nodes. In order to find the best peptide/concentration, the software checks all possible peptides/concentrations as well as all possible values of the peptide/concentration used to split the node. For example, suppose that an attempt is made to build a tree using cysteine 1:10 and lysine 1:10. For each of these peptides, the individual chemicals are rank ordered based on their depletion values as potential predictors. The root node is then split into two child nodes using the average of two adjacent values of one of the peptides. The rank-ordered cysteine 1:10 values are $-10, -3.8, -3.7, -1.9, -1.3, \ldots, 100$, so child nodes are created by splitting the root node based on cysteine 1:10 at -6.9 (the average of -10 and -3.8), -3.75 (the average of -3.8 and -3.7), etc. One child node represents all chemicals with peptide depletion less than the specified value, and the other node represents all chemicals with peptide depletion greater than the specified value. The software then seeks to maximize the average ''purity'' of the two child nodes. In other words, a pair of child nodes in which one node contains all nonsensitizers and weak sensitizers and the other node contains all moderate and strong sensitizers would be superior to a pair of child nodes in which each node contains a mix of chemicals from each sensitization category. Once the best pair of child nodes is determined, the process that was used on the root node is repeated on each child node. The splitting of nodes into child nodes continues in an iterative manner until the level of purity in the child nodes reaches a reasonable level or until a minimum sample size per node is reached.

In the various models fit to peptide reactivity data, splits were made until there were a total of four child nodes. Once these nodes were determined, each node was named based on the sensitization category most often represented in each node. The names assigned to each node include ''minimal reactivity,'' ''low reactivity,'' ''moderate reactivity,'' or ''high reactivity'' (corresponding to the prediction of non-, weak, moderate, and strong/extreme sensitizers, respectively). Chemicals of a different sensitization category than the category of the node in which they are included are considered to be misclassified.

This modeling procedure was conducted six times (once for each potential model previously mentioned) based on all 56 chemicals in the learning data set. Once the six models were determined, they were tested on 26 additional chemicals. Some advantages to using classification trees for prediction include ease of variable selection and model interpretation, no assumptions regarding the distribution of the data, predictor interaction effects are taken into account, models can discriminate on one or more variables, differing costs can be taken into account for different types of misclassification, and more than two response levels are easily handled.

The final classification tree model was assessed via scatterplots and by calculating Cooper statistics (Cooper *et al.*, 1979). Cooper statistics were used to determine how well the model distinguished sensitizers (of any strength) and

TABLE 1
Reactivity of Chemical Substances to GSH or Synthetic Peptides with Results Expressed as Percent Depletion of Nonreacted Peptide

Concentration of peptide:concentration of test substance	GSH 1:100 (0.2mM:20mM)		Lysine 1:10 (0.5mM:5mM)		Lysine 1:50 (0.5mM:25mM)		Cysteine 1:10 (0.5mM:5mM)		Cysteine 1;50 (0.5mM:25mM)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Strong/extreme										
Diphenylcyclopropenone	22.0	7.5	0.3	4.1	− 0.7	3.8	98.8	2.0	100.0	0.0
Oxazolone	22.6	9.5	42.9	3.2	49.6	1.8	75.5	1.4	89.3	2.6
Benzoyl peroxide	100.0	0.0	28.6	8.1	81.3	2.9	100.0	0.0	80.6	3.7
Kathon CG	46.7	9.3	4.5	1.0	3.9	1.0	99.1	1.6	99.5	0.9
Bandrowski's base	30.0	9.3	11.6	2.5	4.2	17.0	87.5	0.3	96.3	0.1
5-Chloro-2-methyl-4-isothiazolin-3-one	74.7	8.5	3.9	3.2	35.1	14.0	96.3	2.8	87.8	6.0
p-Benzoquinone	100.0	0.0	55.6	3.0	91.0	0.2	99.0	1.8	97.1	2.8
Tetrachlorosalicylanilide	0.7	2.3	− 0.2	0.7	9.0	24.0	36.8	20.0	96.5	0.7
2,4 Dinitrochlorobenzene	43.6	2.6	13.4	9.0	14.7	4.2	100.0	0.0	100.0	0.0
Glutaraldehyde	20.8	4.0	66.0	2.2	85.4	3.5	30.2	0.5	70.0	4.7
Fluorescein isothiocynate	92.6	1.5	15.5	0.3	61.1	1.5	100.0	0.0	100.0	0.0
Phthalic anhydride	100.0	0.0	9.9	0.8	75.0	3.9	− 1.9	1.0	− 5.5	2.0
Lauryl gallate	42.2	13.6	6.8	0.6	8.7	4.2	90.9	13.1	100.0	0.0
Propyl gallate	19.7	4.3	13.5	11.7	26.6	10.7	59.9	35.2	97.7	2.4
CD3	63.6	13.6	18.9	2.5	13.6	0.5	90.1	1.1	83.0	1.1
Trimellitic anhydride	97.6	4.0	6.5	0.7	43.7	4.9	− 1.1	5.7	− 14.8	5.7
Formaldehyde	37.5	3.5	0.7	0.6	11.2	3.5	60.4	4.1	75.0	3.0
Metol	86.1	3.4	34.2	3.8	44.7	3.8	100.0	0.0	38.3	3.1
Moderate										
2-Hydroxyethyl acrylate	98.1	1.8	38.2	2.4	88.9	0.3	92.6	0.5	92.2	0.1
Glyoxal	33.0	6.3	29.7	6.2	67.8	1.9	56.5	1.7	94.0	8.5
Vinyl pyridine	38.0	0.7	0.1	11.3	− 16.9	16.2	92.1	0.4	90.3	0.1
2-Mercaptobenzothiazole	24.0	5.9	− 1.9	1.2	− 3.0	0.6	97.5	4.2	99.2	0.7
Nonanoyl chloride	79.0	13.0	− 1.1	9.3	− 6.3	1.8	18.2	3.0	23.0	11.0
2-Methyl-2H-isothiazol-3-one	73.0	5.8	2.6	9.4	− 5.6	5.2	97.9	0.3	100.0	0.0
1,2-Benzisothiazolin-3-one	14.5	1.3	—	—	9.7	2.5	97.7	0.1	83.5	1.6
Methyl-2-nonynoate	92.7	4.1	2.5	2.9	3.2	4.0	100.0	0.0	100.0	0.0
Cinnamaldehyde	46.7	5.2	27.5	1.7	43.2	4.1	70.6	1.0	88.6	1.4
Phenylacetaldehyde	− 4.7	0.7	12.9	0.5	22.6	1.9	60.7	13.3	81.1	3.7
Benzylideneacetone	58.5	3.9	− 2.2	0.5	1.5	0.9	94.7	2.3	96.5	3.0
2,4-Heptadienal	93.0	2.5	19.8	3.5	23.9	5.0	97.3	0.1	93.4	2.7
Squaric acid	16.5	4.0	3.2	1.3	4.8	4.9	46.9	8.7	94.3	4.2
Trans-2-hexenal	68.0	3.9	2.8	1.8	3.6	2.6	97.9	0.3	93.0	1.0
Diethyl maleate	83.3	4.5	33.4	0.6	85.5	1.6	100.0	0.0	100.0	0.0
2-Phenylpropionaldehyde	3.7	3.9	8.8	2.1	21.2	1.6	48.2	7.1	100.0	0.0
Perillaldehyde	10.2	4.7	13.3	0.5	13.8	0.5	31.9	3.3	85.0	0.7
Palmitoyl chloride	77.0	14.1	0.2	0.4	26.6	1.3	25.5	6.6	60.1	5.2
1-(4-Methoxyphenyl)-1-penten-3-one	− 0.2	1.5	8.3	2.3	14.3	3.2	29.9	5.6	75.8	12.6
Weak										
α-Hexylcinnamaldehyde	− 2.6	3.2	1.0	1.5	− 1.6	2.9	− 0.3	1.2	1.0	2.4
α-Amyl cinnamaldehyde	0.2	10.1	2.2	1.2	3.9	1.5	0.6	0.2	0.7	10.6
2,3-Butanedione	0.5	4.1	23.7	1.3	27.0	3.9	79.0	20.8	75.5	16.8
Farnesal	10.0	2.6	5.9	0.6	8.5	13.6	16.4	3.5	71.1	6.7
Oxalic acid	− 2.9	3.1	0.0	1.4	− 0.9	0.7	0.9	5.8	− 5.8	7.7
Benzyl benzoate	0.7	5.5	2.9	0.9	3.0	5.3	0.2	1.1	− 2.2	5.5
4-Allylanisole	17.8	3.1	− 0.9	1.3	− 0.8	1.8	20.6	5.6	61.5	5.4
Lilial	7.7	0.8	0.8	0.8	0.7	0.2	14.0	6.4	71.6	15.5
Cyclamen aldehyde	10.4	5.5	0.3	0.4	1.0	0.4	18.9	8.1	46.1	9.7
Imidazolidinyl urea	30.7	3.0	0.2	1.0	1.3	1.9	52.3	6.0	74.7	2.3
5-methyl-2,3-hexandione	− 2.6	9.9	5.0	1.1	7.5	1.1	25.8	4.0	69.6	7.3
2,2,6,6-Tetramethyl-3,5-heptanedione	5.4	8.2	0.6	1.9	0.0	0.2	1.4	13.6	− 3.7	0.6
Ethyleneglycol dimethacrylate	3.6	5.6	4.5	1.6	12.4	3.0	87.3	5.0	100.0	0.0

TABLE 1—*Continued*

Concentration of peptide:concentration of test substance	GSH 1:100 (0.2mM:20mM)		Lysine 1:10 (0.5mM:5mM)		Lysine 1:50 (0.5mM:25mM)		Cysteine 1:10 (0.5mM:5mM)		Cysteine 1;50 (0.5mM:25mM)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Ethyl acrylate	89.8	− 4.5	24.0	20.7	93.7	1.3	96.4	0.3	97.6	2.1
Hydroxycitronellal	− 1.8	3.9	10.6	1.2	6.5	2.0	17.5	1.7	55.8	3.6
Nonsensitizers										
Glycerol	1.2	4.2	− 0.6	1.2	2.1	0.9	− 3.8	5.2	0.9	1.9
Hexane	− 0.8	4.1	− 0.7	0.3	− 5.1	0.6	− 0.4	0.8	0.3	2.3
Diethyl phthalate	10.9	13.3	0.7	1.0	− 0.7	0.9	0.8	1.7	3.3	4.6
Octanoic acid	− 1.6	3.1	− 0.3	0.7	0.9	0.1	− 1.0	0.7	2.7	3.7
2-Hydroxypropyl methacrylate	5.5	4.8	—	—	− 13.6	7.8	58.4	5.9	96.5	1.5
1-Butanol	6.1	7.5	1.2	2.5	1.2	0.8	− 0.4	1.4	− 4.1	4.3
4-Hydroxybenzoic acid	− 1.0	5.8	2.2	1.2	2.2	2.1	− 0.3	0.8	14.0	14.0
6-Methyl coumarin	− 1.6	8.6	0.2	2.5	4.0	5.6	1.4	0.3	− 0.3	3.9
Methyl salicylate	4.2	3.5	2.4	0.8	1.6	0.3	0.3	0.8	0.8	7.7
Chlorobenzene	3.2	2.3	1.4	0.8	1.3	0.2	0.4	0.2	− 2.7	2.2
Lactic acid	− 1.1	11.1	3.2	0.4	0.8	0.5	− 0.9	0.3	11.5	21.0
1-Bromobutane	4.0	3.3	0.2	1.2	1.2	0.4	13.8	3.6	47.6	24.1
2-Acetylcyclohexanone	4.3	4.1	− 4.6	2.2	12.5	0.5	18.2	4.4	40.8	8.5
4-Methoxyacetophenone	2.5	3.2	− 0.8	0.7	0.1	0.3	4.7	5.0	− 3.3	1.4
Ethylbenzoylacetate	3.9	3.0	− 0.6	0.6	1.9	0.4	2.3	5.5	0.5	0.5
Ethyl vanillin	− 0.7	3.1	—	—	9.7	5.5	1.1	17.0	—	—
Isopropanol	1.4	6.8	− 1.3	0.1	0.5	0.5	− 10.0	17.0	− 3.1	0.3
Propylene glycol	4.2	2.5	0.2	2.0	0.6	0.7	− 0.9	17.5	− 3.0	0.6
Sulfanilamide	12.8	4.5	0.3	1.6	0.8	0.5	− 1.3	17.3	− 2.1	0.2
Isopropyl myristate	4.9	− 8.7	3.5	2.5	− 4.0	17.3	0.8	1.7	− 2.2	2.9
Benzaldehyde	6.8	2.6	− 1.5	1.2	− 1.7	1.4	7.2	8.8	− 2.2	2.6
Methylparaben	3.4	4.2	− 0.6	2.3	− 0.4	0.8	3.6	6.8	− 5.4	6.3
Nonanoic acid	4.0	6.5	− 4.1	3.9	− 9.6	2.9	− 3.7	6.1	5.2	4.6
Propyl paraben	− 1.0	6.0	− 0.7	0.2	− 0.2	1.3	8.2	2.3	21.8	6.3
Resorcinol	3.6	6.2	− 0.9	1.7	− 0.8	1.9	1.6	5.6	2.3	2.0
Salicylic acid	− 8.2	− 5.2	− 6.9	2.7	—	—	3.5	4.2	9.3	5.6
Sulphanilic acid	− 6.0	2.3	− 0.3	1.6	0.5	1.0	5.3	5.5	1.4	4.1
Vanillin	1.5	4.7	0.2	2.0	− 6.6	3.6	3.2	5.5	34.2	5.1
Coumarin	1.0	3.8	− 9.9	2.9	− 14.9	22.0	1.0	4.6	− 14.5	10.1
Vinylidene dichloride	.0	5.3	− 0.8	7.8	− 4.3	18.2	2.4	1.7	4.0	1.7

nonsensitizers. The Cooper statistics calculated include sensitivity (the proportion of true sensitizers predicted as having low, moderate, or high reactivity), specificity (the proportion of true nonsensitizers predicted as having minimal reactivity), positive predictivity (the proportion of chemicals classified as having low, moderate, or high reactivity that are true sensitizers), negative predictivity (the proportion of chemicals classified as having minimal reactivity that are true nonsensitizers), and accuracy (the overall proportion of correct predictions). Cooper statistics were computed on the entire set of chemicals (up to 56 training set chemicals and 26 validation set chemicals).

RESULTS

Peptide Reactivity Data with GSH, Lysine, and Cysteine

Peptide depletion results on 38 chemicals using GSH, cysteine, and lysine peptides were previously published (Gerberick *et al.*, 2004). The ratios of peptide to chemical used were 1:100 for GSH, 1:50 for lysine, and 1:10 for cysteine. The results indicated a strong correlation between allergen potency and depletion of the nonreacted peptide. In this study, we have expanded the number of chemicals evaluated to 82 and added two experimental conditions: cysteine at 1:50 and lysine at 1:10. The results for the 82 test chemicals are presented in Table 1. The chemicals are listed in the order of lowest EC3 values (i.e., the most potent allergens) through nonsensitizers and include 18 extreme/strong sensitizers; 19 moderate sensitizers; 15 weak sensitizers; and 30 nonsensitizers based on an existing LLNA categorization scheme (Kimber *et al.*, 2003). The LLNA EC3 data reported in this manuscript are derived from previously conducted studies (Ashby *et al.*, 1995; Basketter and Scholes, 1992;

Basketter *et al.*, 2001; Dearman *et al.*, 1992; Estrada *et al.*, 2003; Gerberick *et al.*, 2005; Kimber *et al.*, 1998; Loveless *et al.*, 1996; Ryan *et al.*, 2000). Generally, it is evident that the more potent the allergen, the more peptide depletion that is observed (Table 1), specifically for the GSH and cysteine peptides. For the majority of the extreme and strong allergens, greater than 75% depletion was observed for the 1:50 cysteine peptide. Less peptide depletion was noted for the GSH and 1:10 cysteine peptides but again generally more depletion was observed with the more potent allergens. With 1:10 and 1:50 lysine peptides, peptide depletion was greater also with the more potent allergens but not to levels of the cysteine-containing peptides. Interestingly, phthalic anhydride and trimellitic anhydride demonstrated significant depletion with GSH and lysine 1:50 peptides but not with the others. Finally, only a few of the nonsensitizers (e.g., 2-hydroxypropyl methacrylate) demonstrated peptide depletion values similar to those observed with the allergens suggesting good specificity for peptide reactivity assays. It is important to note that for a few test compounds (1,2-benzisothiazolin-3-one; 2-hydroxypropyl methacrylate; ethyl vanillin; and salicylic acid), one or two peptide depletion values are missing due to either an incompatibility with the solvent system or the test compounds coeluted with peptide in the HPLC analysis.

Prediction Models Based on Classification Tree Model Analysis

One requirement for using the peptide reactivity assay data for screening the skin sensitization potential of chemicals was to find a robust method to analyze and categorize the data. Another need was to determine if each of the five peptides were necessary for screening since the possibility of reducing the amount of work necessary to analyze each chemical would increase throughput and reduce the cost and amount of test material required for testing. To address these two needs, we chose to use a classification tree model approach which is a form of binary recursive partitioning that is used when observations need to be assigned to a category based on a number of predictor variables (Brieman *et al.*, 1983). Specifically, the classification tree approach used an algorithm to evaluate all of the peptide reactivity depletion data for each chemical in the context of its known LLNA potency category. Table 2 lists six prediction models that were developed based on use of all of the peptide data or limited to the use of specific peptide data (e.g., exclusion of GSH data). For each model generated, the model predictors used for evaluation of the chemical data set is given along with the model's accuracy and number of misclassifications. Cooper statistics were used to determine how well the model distinguished sensitizers (of any strength) and nonsensitizers. The Cooper statistics calculated accuracy based on chemicals predicted as sensitizers if they were categorized as having low, moderate, or high reactivity versus chemicals predicted as nonsensitizers if they were categorized as having minimal reactivity. It is clear from use of Cooper statistics analysis that model #1, which incorporates all of the peptides and their ratios, delivers the highest accuracy and fewest number of misclassifications. However, it is important to note that the delivered accuracy for the other models (#2– #6), which incorporate fewer peptides (e.g., model #5), is not that dissimilar to the more peptide comprehensive model #1. The similarity between the models suggest that for screening purposes it might be adequate to go with a model that uses fewer peptides and thus requires less material and less time for analysis. Comparison shows that the model #1 (sum of GSH; cysteine 1:10 and 1:50; and lysine 1:10 and 1:50) yields an accuracy of 94% and five misclassifications, whereas a model based only on cysteine 1:10 and lysine 1:50 yields a reasonable accuracy of 89% and nine misclassifications. Thus, we chose model #5 which includes only cysteine 1:10 and lysine 1:50 as predictors for analyzing further our peptide reactivity data.

Cysteine 1:10 and Lysine 1:50 Classification Decision Tree Model (Model #5)

The decision tree model that incorporates cysteine 1:10 and lysine 1:50 as predictors is presented in Figure 1. The model is based on making decisions on the average of peptide depletion data for cysteine 1:10 and lysine 1:50. As indicated in the methods, the classification decision tree model was developed using the peptide depletion data along with the LLNA potency data. By incorporating different cutoffs generated by the model, we have chosen to name the peptide depletion reactivity categories as minimal, low, moderate, and high reactivity. Generally, chemicals with moderate to high reactivity are associated with moderate to strong allergenicity, while those categorized as having minimal to low reactivity include weak and nonsensitizers (Table 3). However, it would be inappropriate to consider that a simple peptide reactivity assay would have the capability to predict a chemical's sensitization potency. It is believed that to accomplish this task, additional assay data will be needed to make an accurate prediction of a chemical's skin sensitization potential (Jowsey *et al.*, 2006). As far as the capability of using this model for classifying a chemical as a sensitizer or nonsensitizer, the Cooper statistics show that this model performs very well with an accuracy of 89% (Table 2 and Fig. 2). The high values for the sensitivity (88%), specificity (90%), positive predictivity (94%), and negative predictivity (81%) suggest that this peptide reactivity model would perform well as a screening assay, especially if used along with other physiochemical or biological data (Fig. 2). The nine chemicals that are misclassified include six sensitizers (α-hexylcinnamaldehyde, α-amylcinnamaldehyde, benzyl benzoate, 2,2,6,6-tetramethyl-3,5-heptanedione, oxalic acid, and nonanoyl chloride) and three nonsensitizers (2-acetylcyclohexanone, 2-hydroxypropyl methacrylate, and 1-bromobutane).

TABLE 2
Classification Tree Models Based on GSH, Cysteine, and Lysine Peptide Depletion Data

Model name	Model predictors used	Number of chemicals	Accuracy (%)	Number of misclassifications
Model #1	GSH, Cys (1:10 and 1:50), Lys (1:10 and 1:50)	78	94	5
Model #2	GSH, Cys (1:10 and 1:50)	82	88	10
Model #3	GSH, Cys (1:10), Lys (1:10)	78	91	7
Model #4	Cys (1:10 and 1:50), Lys (1:10 and 1:50)	78	91	8
Model #5	Cys (1:10), Lys (1:50)	81	89	9
Model #6	Cys (1:10)	82	89	9

DISCUSSION

Our understanding of the chemical and biological processes associated with skin sensitization and ACD has advanced significantly in recent years. This knowledge is providing the foundation for the development of numerous alternative methods for skin sensitization testing. One particular area of development has been to apply our knowledge of how chemical reactivity plays an important role in the initiation of skin sensitization response (reviewed in Lepoittevin *et al.*, 1998). Specifically, we (Gerberick *et al.*, 2004) as well as others (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004a; Kato *et al.*, 2003; Natsch *et al.*, in press) have addressed the development of chemical reactivity screening methods for assessing the skin sensitization potential of chemicals.

It is believed that the formation of hapten-protein complexes is a prerequisite for the initiation of skin sensitization and which occurs prior to the processing of the complexes by antigen-presenting LC in the skin for the eventual presentation of the chemical to antigen-specific T cells. Chemical allergens (haptens) or their metabolites are small molecular weight compounds (generally less than 500 Da) with electrophilic properties. They are able to react with nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens. Lysine and cysteine are those most often cited but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Ahlfors *et al.*, 2003; Dupuis and Benezra, 1982; Lepoittevin *et al.*, 1998). Thus, electrophilic allergens are believed to react with nucleophilic amino acids to form a stable covalent bond which is critical to the initiation of a skin sensitization response. However, it must be realized that other mechanisms for hapten interaction need to be considered as well (Divkovic, 2006).

Since reactivity is one key step in the induction of skin sensitization, we have been interested in pursuing whether measuring a chemical's reactivity could be used to develop a quantitative peptide-based reactivity assay that would have utility for screening a chemical's skin sensitization potency as defined in the LLNA. We evaluated 38 chemicals representing allergens of different potencies (weak to extreme) and non-sensitizers for their ability to react with GSH or three synthetic peptides containing either cysteine, lysine, or histidine (Gerberick *et al.*, 2004). The results demonstrated that a significant correlation exists between allergen potency and the depletion of GSH, lysine, and cysteine but not histidine. It is important to note that our intent in developing a peptide reactivity approach was not for the purpose of reproducing the physiological conditions of reactivity. For example, the lysine peptide assay must be run at pH 10.5 for optimal reactivity of the amine group. Moreover, we have chosen to focus on only two nucleophiles, lysine and cysteine, for use in developing a screening assay for determining a chemical's reactivity potential. Although lysine has been demonstrated to be an

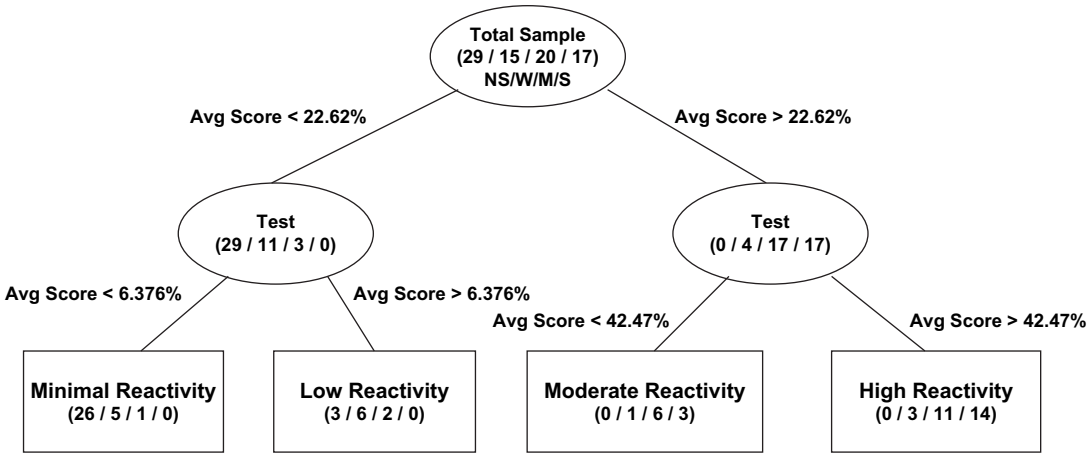

FIG. 1. Classification tree model based on the average of cysteine (1:10) and lysine (1:50) data.

TABLE 3
Comparison of Peptide Reactivity and Potency Data

Chemical name	EC3 value	LLNA category	Reactivity based on Cys (1:10) and Lys (1:50) data
Diphenylcyclopropenone	0.00030	Extreme	High
Oxazolone	0.0030	Extreme	High
Benzoyl peroxide	0.0040	Extreme	High
Kathon CG	0.0080	Extreme	High
Bandrowski's base	0.0080	Extreme	High
5-Chloro-2-methyl-4-isothiazolin-3-one	0.0090	Extreme	High
p-Benzoquinone	0.0099	Extreme	High
Tetrachlorosalicylanilide	0.040	Extreme	Moderate
2,4-Dinitrochlorobenzene	0.050	Extreme	High
Glutaraldehyde	0.10	Strong	High
Fluorescein isothiocynate	0.14	Strong	High
Phthalic anhydride	0.16	Strong	Moderate
Lauryl gallate	0.30	Strong	High
Propyl gallate	0.32	Strong	High
CD3	0.60	Strong	High
Trimellitic anhydride	0.60	Strong	Low
Formaldehyde	0.61	Strong	Moderate
Metol	0.80	Strong	High
2-Hydroxyethyl acrylate	1.4	Moderate	High
Glyoxal	1.4	Moderate	High
Vinyl pyridine	1.6	Moderate	Moderate
2-Mercaptobenzothiazole	1.7	Moderate	High
Nonanoyl chloride	1.8	Moderate	Minimal
2-Methyl-2H-isothiazol-3-one	1.9	Moderate	High
1,2-Benzisothiazolin-3-one	2.3	Moderate	High
Methyl-2-nonynoate	2.5	Moderate	High
Cinnamaldehyde	3.0	Moderate	High
Phenylacetaldehyde	3.0	Moderate	Moderate
Benzylideneacetone	3.7	Moderate	High
2,4-Heptadienal	4.0	Moderate	High
Squaric acid	4.3	Moderate	Moderate
Trans-2-hexanal	5.5	Moderate	High
Diethyl maleate	5.8	Moderate	High
2-Phenylpropionaldehyde	6.3	Moderate	Moderate
Perillaldehyde	8.1	Moderate	Moderate
Palmitoyl chloride	8.8	Moderate	Moderate
1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	Low
a-Hexylcinnamaldehyde	11	Weak	Minimal
a-Amylcinnamaldehyde	11	Weak	Minimal
2,3-Butanedione	11	Weak	High
Farnesal	12	Weak	Low
Oxalic acid	15	Weak	Minimal
Benzyl benzoate	17	Weak	Minimal
4-Allylanisole	18	Weak	Low
Lilial	19	Weak	Low
Cyclamen aldehyde	22	Weak	Low
Imidazolidinyl urea	24	Weak	Moderate
5-Methyl-2,3-hexanedione	26	Weak	Low
2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	Minimal
Ethyleneglycol dimethacrylate	28	Weak	High
Ethyl acrylate	28	Weak	High
Hydroxycitronellal	33	Weak	Low
Glycerol	NC[a]	NS[b]	Minimal
Hexane	NC	NS	Minimal
Diethyl phthalate	NC	NS	Minimal
Octanoic acid	NC	NS	Minimal
2-Hydroxypropyl methacrylate	NC	NS	Low
1-Butanol	NC	NS	Minimal
4-Hydroxybenzoic acid	NC	NS	Minimal
6-Methyl coumarin	NC	NS	Minimal
Methyl salicylate	NC	NS	Minimal
Chlorobenzene	NC	NS	Minimal
Lactic acid	NC	NS	Minimal
1-Bromobutane	NC	NS	Low
2-Acetylcyclohexanone	NC	NS	Low
4-Methoxyacetophenone	NC	NS	Minimal
Ethylbenzoylacetate	NC	NS	Minimal
Ethyl vanillin	NC	NS	Minimal
Isopropanol	NC	NS	Minimal
Propylene glycol	NC	NS	Minimal
Sulfanilamide	NC	NS	Minimal
Isopropyl myristate	NC	NS	Minimal
Benzaldehyde	NC	NS	Minimal
Methylparaben	NC	NS	Minimal
Nonanoic acid	21 (False +)	NS	Minimal
Propyl paraben	NC	NS	Minimal
Rsorcinol	NC	NS	Minimal
Salicylic acid	NC	NS	—
Sulphanilic acid	NC	NS	Minimal
Vanillin	NC	NS	Minimal
Coumarin	NC	NS	Minimal
Vinylidene dichloride	NC	NS	Minimal

[a]Not calculated.
[b]Nonsensitizer.

important nucleophile for allergens such as sultones and methylisothiazolone derivaties (Alvarez-Sanchez *et al.*, 2003; Meschkat *et al.*, 2001) and cysteine for α,β-unsaturated allergens (Ahlfors *et al.*, 2003), it is probable that nucleophiles other than lysine and cysteine are critical for the initiation of a sensitization response (Divkovic, 2006; Divkovic *et al.*, 2005). Thus, our approach will yield minimal information on how a specific chemical reacts with protein *in vivo* but does provide a means of quantifying reactivity for the purpose of screening skin sensitization potential.

Using GSH as a cysteine-containing peptide and two synthetic hepapeptides, one with lysine and other with cysteine, we expanded our analysis of chemical reactivity from 38 to 82 chemicals. The chemicals represented in the data set comprise weak ($n = 15$), moderate ($n = 19$), strong and extreme sensitizers ($n = 18$), as well as nonsensitizing materials ($n = 30$), as based on potency categorization criteria that have been developed by a European Centre for Ecotoxicology and Toxicology of Chemicals Task Force (Kimber *et al.*, 2003).

		Predicted Classification (based on classification tree model)		
		Non-Sensitizer	Sensitizer	total
Chemical Classification[a]	Non-Sensitizer	**26**	**3**	**29**
	Sensitizer	**6**	**46**	**52**
	total	**32**	**49**	**81**

table statistics for the shadowed 2 x 2 table

sensitivity:	88%
specificity:	90%
positive predictivity:	94%
negative predictivity:	81%
accuracy:	**89%**

[a]Based primarily on LLNA data

FIG. 2. Cooper statistics (nonsensitizers vs. sensitizers) for cysteine (1:10) and lysine (1:50) prediction model.

The LLNA EC3 values listed in Table 3 show a range of potency from 0.0003% for the extreme allergen, diphenylcyclopropenone, to 33% for the weak allergen, hydroxycitronellal.

The results, as summarized in Table 3, demonstrate that an association between the degree of peptide reactivity (as measured by nonreacted peptide depletion) and sensitization potency is evident. To help with interpretation of the peptide depletion data, we examined the utility of using classification tree methodology for development of a prediction model. Classification tree methodology involves an algorithm to group data based on one or more predictors. In this particular case, we used the peptide depletion data for each of the peptides (predictors) to see which ones would be used to subgroup the data. Although we used LLNA potency data for each of the 82 compounds to build the model, we chose to use high, moderate, low, and minimal reactivity as the category names for groups determined by the model. To evaluate each of the models for their hazard identification ability, we considered any compound that was categorized as high, moderate, or low as a skin sensitizer and those categorized as minimal as nonsensitizers. Cooper statistics were used to determine how well the different models distinguished sensitizers from nonsensitizers. All of the models generated are listed in Table 2. Model #1, that incorporated all of the peptide depletion data for each of the peptides, demonstrated a prediction accuracy of 94%. In addition, this model yielded only five misclassifications. Although the performance of this model is outstanding, it has the limitation of requiring the use of five different peptides. Moreover, model #1 involves the use of the GSH assay which involves a multiple-step procedure which can be challenging to transfer to other laboratories (data not shown). Thus, we were interested to see if a robust model could be developed that did not incorporate GSH into the decision tree. Table 2 summarizes the classification models and lists for each model the accuracy and number of misclassifications obtained with the model. Although the accuracy values are lower for the ''simpler'' models, they still show a very good ability to distinguish sensitizers from nonsensitizers. The one model we think demonstrates a good compromise between requiring fewer peptide ratios for analysis and no GSH is model #5, which includes use of cysteine at 1:10 and lysine at 1:50. Model #5 has a prediction accuracy of 89% with nine misclassifications. Of the six sensitizers classified as nonsensitizers, five of them are weak sensitizers (e.g., a-hexylcinnamaldehyde, benzyl benzoate). Nonanoyl chloride, a moderate sensitizer, was classified as a nonsensitizer. For chemicals that are misclassified, it is important to consider the chemical's water solubility as related to compatibility with the assay conditions as well as the possibility that the chemical is a prohapten and might require bioactivation prior to it reacting with nucleophile-containing peptides. Moreover, in some instances it might be prudent to review the LLNA data used to categorize a chemical as a sensitizer or nonsensitizer. For example, oxalic acid is categorized as weak sensitizer in the LLNA but the chemical does not contain apparent alerts or does human data exist to classify it as a sensitizer. Interestingly, a few of the nonsensitizers identified as sensitizers are compounds believed to have reactive properties (e.g., 2-hydroxypropyl methacrylate, 1-bromobutane). Moreover, two anhydride compounds are identified with the use of the lysine 1:50 peptide which support the use of using both cysteine and lysine for screening unknown chemicals. In addition to providing good assistance for hazard identification, the cysteine 1:10 and lysine 1:50 model provides quantifying data on reactivity that has potential for use, along with other data, for predicting the skin sensitization potency of an unknown chemical. Consistent with what we have observed in the past (Gerberick *et al.*, 2004), the amount of peptide depletion corresponds closely with the allergenic potency of the compound. Generally, moderate, strong, and extreme sensitizers show moderate to high reactivity, while weak and nonsensitizers show minimal to low reactivity (Table 3).

Of course, one would not expect an extremely high correlation between reactivity and potency since other factors, such as skin penetration and immune recognition by T cells, are critical for the acquisition of skin sensitization. It is not possible to say which event is most critical and it is likely unique for each chemical (e.g., reactivity for one chemical versus bioavailability for another chemical). Thus, it is very important to point out that it is not expected that the peptide reactivity assay alone should have the ability to predict a compound's sensitization potential. It is believed that to replace the LLNA, a battery of assays will be needed to reproduce the complex chemistry and biology that are involved in the induction of skin sensitization. Jowsey *et al.* (2006) have described this need in a very informative way by showing how different assays could provide quantitative information on different aspects known about the mechanism of ACD. For example, the peptide reactivity assay could serve as a first tier screening assay and also provide in time information needed to complete a holistic assessment of a chemical's skin sensitization potential.

One potential challenge for developing alternative methods for skin sensitization testing is that it is well known that some chemical allergens are prohaptens and as such require biotransformation prior to initiating a skin sensitization response *in vivo* (Smith and Hotchkiss, 2001). The need for biotransformation has been demonstrated with many chemicals, such as the formation of benzoquinonediimine from azo hair dyes (Basketter and Goodwin, 1988), or orthoquinone from isoeugenol (Bertrand *et al.*, 1997). Based on the knowledge that some chemical allergens need to be biotransformed prior to reacting with proteins/peptides, it will be critical to incorporate a metabolism component to address these types of molecules. We are currently evaluating a peroxidase/peroxide oxidizing system for use in a modified peptide reactivity assay.

The goal of this work was to evaluate the use of chemical reactivity as a means for screening the skin sensitization potential of chemicals. A prediction model was developed using a classification tree approach which allowed ranking the reactivity as minimal, low, moderate, or high as well as for assessing skin sensitization hazard. The results presented show clearly that using a cysteine- and lysine-based peptide depletion, assay demonstrates a good, but not perfect, association between chemical reactivity and allergenic potency. Generally, moderate to extreme allergens demonstrate high peptide depletion whereas weak and nonsensitizers demonstrate significantly less peptide depletion. It is hoped that with additional information from other *in vitro* assays and modification of existing peptide reactivity assays (e.g., addition of metabolism component), this methodology will be even more helpful in reducing our reliance on animals for skin sensitization testing in the future.

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Appendix 12

DPRA Training and Transfer plan

P&G	**Direct Peptide Reactivity Assay (DPRA) Training and Transfer Plan**	Version 2 Pages: 4 N° of Annexes: 0
European Centre for the Validation of Alternative Methods (ECVAM) Skin Sensitisation Prevalidation Study		

Issued by	Procter & Gamble	*Date:2/02/2010*
Approved by	Validation Management Group	*Date:17/02/2010*
Distributed by	ECVAM	*Date:18/02/2010*

Revision History:

Revision	*Date:*	*Description of change:*
Version 2	31/03/2010	Corrections in the catalog numbers of Glyoxal and 2,3-Butanedione, 97%

Representative of Validation Management Group	Dr. Alexandre Angers	*Date: 31/03/2010*

DPRA Training and Transfer

The laboratories and study personnel should be skilled in basic HPLC operation. The detailed SOP and training agenda will be provided to all training participates in advance of the training session.

Length of training session:

Participating laboratories should expect to spend 3 days in Cincinnati for training. Day 1 will be a full day. Days 2 and 3 will most likely be less than full days.

Training Agenda (Day 1)

Morning:

- P&G will present background information on the development of the DPRA and its prediction model
- The details of the SOP, instrumentation and assay setup will be discussed. Participants will have the opportunity to ask questions.
- Laboratory tour

Break for Lunch

Afternoon:

- Each participant will set up their own assay with a small number of test chemicals (approximately 5-6 test chemicals with the appropriate controls).
- All samples will be placed in the HPLC autosampler for their 24 hour incubation and the HPLC will be timed to begin at the appropriate time.
- Time for Questions & Answers regarding the material covered on Day 1.

Chemical	CAS	Potency	Sigma Aldrich catalog number
p-phenylenediamine	106-50-3	Strong	P6001
3,3,5-trimethylhexanoyl chloride, 98%	36727-29-4	Moderate	422959
Glyoxal, 40%	107-22-2	Moderate	50660
Citral, 95%	5392-40-5	Weak	C83007
Imidazolidinyl urea	39236-46-9	Weak	I5133
Glycerol, 99%	56-81-5	Non-sensitizer	G9012

Training Agenda (Day 2)

Morning:

- Data analysis and reporting will be discussed.

- The trainer will demonstrate proper integration techniques using "example" chromatograms.
- The discussion and demonstration will include simple/one-peak chromatograms, more complex chromatograms and situations of co-elution.
- Time for Questions and Answers

Afternoon:

- There is no scheduled training activity.
- The trainer will verify that the HPLC systems begin injecting samples at the appropriate time.

Training Agenda (Day 3)

Morning:

- Participants will analyze the data from the samples that they prepared on Day 1.
- Acceptance criteria and proper data reporting will be discussed.
- Time for Questions & Answers.
- Participants will be instructed to "practice" with the 5-6 training chemicals when they return to their "home" laboratories. Each laboratory can determine the number of practice runs they need to feel comfortable with the assay.

Transfer

- Each laboratory will complete 3 successful runs with the 5-6 chemicals used in training. All data will be reported to the Lead laboratory. The Lead Laboratory will determine each laboratory's readiness for the transfer phase.
- The transfer phase is not blinded.
- Test chemicals, peptides and all other reagents will be purchased by the individual laboratories.
- The following chemicals will be used for assessing transferability:

Chemicals	CAS	Potency	Sigma Aldrich catalog number
p-Benzoquinone, 98%	106-51-4	Strong	B10358
2,4-Dinitrochlorobenzene, 99%	97-00-7	Strong	237329
Oxazolone, >90%	15646-46-5	Strong	E0753
Formaldehyde, 37%	50-00-0	Strong	F15587
2-Phenylpropionaldehyde, 98%	93-53-8	Moderate	241369
Diethyl maleate, 97%	141-05-9	Moderate	D97703
Benzylideneacetone, 99%	122-57-6	Moderate	147885
Farnesal, >85%	19317-11-4	Weak	W401900-SAMPLE
2,3-Butanedione, 97%	431-03-8	Weak	31530

4-Allylanisol, 98%	140-67-0	Weak	A29208
Hydroxycitronellal, 95%	107-75-5	Weak	W258318-SAMPLE
Butanol, 99.4%	71-36-3	Non-sensitizer	360465
6-Methylcoumarin, 99%	92-48-8	Non-sensitizer	M36203
Lactic acid, 85%	50-21-5	Non-sensitizer	252476
4-Methoxyacetophenone, 99%	100-06-1	Non-sensitizer	117374

- Each test chemical will be tested in 2 independent runs with each peptide.
- Data will be submitted to the Lead Laboratory using the "data reporting template" provided during the training session.
- Each assay run must meet all acceptance criteria as described in the SOP in order to be considered a valid run.
- The Lead laboratory will consider the SOP transfer to be successful if the naïve laboratories assign (in each run) at least 14/15 chemicals correctly as "sensitizer" or "non-sensitizer" and at least 13/15 chemicals correctly to a reactivity category that is the same or one off from P&G's historical data. Each independent run must meet these criteria, and misclassifications may occur in any potency category.
- The Lead laboratory will then determine qualification based on this data.

Appendix 13

DPRA Training report (IVMU)

Product Safety and Regulatory Affairs
Central Product Safety

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DPRA Training Report

Training Location	**Procter & Gamble** **Miami Valley Innovation Center** **Cincinnati, OH**
Date	**May 25, 2010**
Number of Pages	**16**
Status	**Draft**
Training Laboratory	**Frank Gerberick Laboratory** **Procter & Gamble**
Trainer	**Leslie M Foertsch** **PO Box 538707** **Cincinnati, OH 45253** **Telephone: +1 513 627-0421** **Fax: +1 513 627-0400**
Trained Laboratory	**In Vitro Methods**
Trainee	**Siegfried Morath** **Building 58** **Via Enrico Fermi, 2749** **I-21027, Ispra (VA)** **Italy** **Telephone : +39 0332 789483** **Fax : +39 0332 785336** **Nicholaos Parissis** **Building 58** **Via Enrico Fermi, 2749** **I-21027, Ispra (VA)** **Italy** **Telephone : +39 0332 786224** **Fax : +39 0332 785336**

GLP Statement

Work conducted in the Procter & Gamble Laboratory was not intended to be conducted according to strict Good Laboratory Practices (GLP). The training activities were completed in the "spirit" of GLP and do meet the basic principles of good laboratory practice. They follow a sound protocol, are conducted by qualified personnel, are controlled by written and understood Standard Operating Procedures, are conducted in proper and adequate facilities, are conducted using calibrated and maintained equipment, are well documented and have fully retrievable raw data.

Abbreviations

DPRA	Direct Peptide Reactivity Assay
GLP	Good Laboratory Practices
HPLC	High-Performance Liquid Chromatography
LLNA	Local Lymph Node Assay
SD	Standard Deviation
SOP	Standard Operating Procedure
RSD	Relative Standard Deviation
UV	Ultraviolet

Background of the test method

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health problem, and the most common manifestation of immunotoxicity in humans. The acquisition of skin sensitization, and the subsequent elicitation of an allergic hypersensitivity reaction in the skin, is dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses. For many years guinea pigs were the species of choice for the hazard identification of skin sensitizing chemicals. More recently, however, the local lymph node assay (LLNA) has been developed as an alternative approach based upon characterization of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals (Kimber et al, 1994; Dearman et al, 1999; Gerberick et al, 2000; Kimber et al, 2002; Basketter et al, 2002). The LLNA has been adopted recently, as Test Guideline 429, by the Organization for Economic Cooperation and Development (OECD, 2002) as a stand-alone test method for skin sensitization testing. However, one challenge facing investigators is the need to develop non-animal based methods for the evaluation of new chemicals that will significantly reduce or eliminate the need for animals in skin sensitization testing in the future (Ryan et al, 2001; Casati et al, 2005; Ryan et al, 2005)**.**

There are a variety of characteristics that determine whether a chemical can function as a contact sensitizer (or allergen) including the ability to penetrate into the skin, react with protein, and be recognized as antigenic by immune cells. The correlation of protein reactivity with skin sensitization potential is well established (Dupuis and Benezra, 1982; Lepoittevin et al, 1998). In fact, Landsteiner and Jacobs (1936) presented the origin of the reactivity hypothesis in their landmark paper looking at the underlying mechanisms of contact allergy. Thus, if a chemical is capable of reacting with protein either directly or after appropriate biotransformation, then it has the potential to act as a contact allergen. The majority of chemical allergens (or their metabolites/oxidation products) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups, nucleophiles, capable of reacting with electrophilic allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine have been reported to react with electrophiles (Dupuis and Benezra, 1982; Lepoittevin et al, 1998; Ahlfors et al, 2003). Since protein reactivity is a key step in the induction of ACD it was hypothesized that an *in vitro* method could be developed to screen the sensitization potential of new chemicals based on reactivity.

Using nucleophile-containing synthetic peptides, we (Gerberick et al, 2004, 2007) have evaluated the utility of these peptides to screen for skin sensitization potential by measuring peptide depletion following incubation with allergens and non-allergens. For the synthetic heptapeptides that contain either cysteine or lysine, the ratio of peptide to chemical ratio used was 1:10 and 1:50, respectively. Following a 24 hour reaction period for the two synthetic peptides, the samples were analyzed by HPLC using UV detection to monitor the depletion of peptide following reaction.

As a test chemical may be a hapten, prehapten or a prohapten (Lepoittevin, 2006; Gerberick et al, 2008), it is critical to incorporate methods that allow for either spontaneous air-oxidation (simulating hapten formation by product aging) or metabolic activation (simulating the activation process of the prohapten in the skin). The DPRA is designed to primarily detect haptens that have the inherent reactivity to interact with peptides. However, we have been able to detect some prehaptens with the DPRA and are in the process of developing a next generation assay to evaluate even a greater number of prohaptens.

It is important to remember that skin sensitization is a complex, multi-step physiological process. Therefore, the DPRA is designed to be part of a battery or integrated testing approach for assessing the skin sensitization potential of chemicals.

Purpose of the training

The purpose of this training session was to train members of the In vitro Methods laboratory on the DPRA SOP. This training session included a discussion of the SOP, "hands on" setup of the assay and data analysis.

Timing

March 15 - 17, 2010

SOP covered by the training

Direct Peptide Reactivity Assay (DPRA) Standard Operating Procedure Version 1 for the European Centre for the Validation of Alternative Methods (ECVAM) Skin Sensitization Prevalidation Study.

Items used for the training

The following reagents and test materials were used for the training:

Phosphate Buffer pH 7.5 (prepared in advance February 23, 2010)
Ammonium acetate Buffer pH 10.2 (prepared in advance February 23, 2010)
Cysteine Peptide
Lysine Peptide
Acetonitrile, HPLC grade
Purified Water

Chemical	CAS	Potency	Sigma Aldrich catalog number
p-phenylenediamine	106-50-3	Strong	P6001

3,3,5-trimethylhexanoyl chloride, 98%	36727-29-4	Moderate	422959
Glyoxal, 99%	107-22-2	Moderate	128465
Citral, 95%	5392-40-5	Weak	C83007
Imidazolidinyl urea	39236-46-9	Weak	I5133
Glycerol, 99%	56-81-5	Non-sensitizer	G9012

Training Program:

Theoretical aspects

The training session began with an in depth discussion of the background to the development of the DPRA and the current SOP that is being used for the prevalidation studies. The trainer covered the SOP section by section.

Specific aspects of the procedure that were discussed in detail:

- The use of a photodiode array detector and/or the co-elution controls to help determine peptide peak identity and the presence of co-elution.
- Importance of peptide purity not exceeding 90-95%
- Solubility assessments and the order of solvents used in the "Solubility Assessment" section of the SOP.
- Description of the Reference Controls A, B, and C and how they are prepared and used. The generalized HPLC Sample Analysis Sequence in the SOP was found to be confusing. The trainer prepared a more specific Analysis Sequence.
- The changes/additions that have been made to the Data Analysis & Calculations were discussed. This includes the calculation of CV, use of the Reference Controls and additional calculations of peptide concentration.
- Use of the prediction models and when to use the Cysteine 1:10-only Prediction Model.
- The use of Acceptance Criteria is a new addition to the SOP. Specific acceptance criteria were discussed as well as when an entire assay needs to be repeated and when a single test chemical needs to be repeated.

Practical aspects

The equipment used at Procter & Gamble undergoes yearly preventative maintenance checks as well as calibrations. However, if there appears to be a problem with any instrument, service calls with the appropriate vendors and technicians are scheduled. Trainees were instructed to follow the equipment maintenance and calibration standards that are currently in place in their laboratory.

During the "hands on" portion of the training, the trainees were given the opportunity to setup an actual DPRA test run for cysteine and lysine. In the interest of time, the training chemicals described above and peptides were pre-weighed by the trainer the afternoon

before the training session and stored at 4°C. The buffers for peptide dissolution were also prepared in advance. All other aspects of assay setup described in the SOP were performed on site by the trainee.

There were no deviations to the test method SOP used during sample setup. There was a power outage during the night that the samples were being analyzed on the HPLC's. This caused the HPLC runs to shut down. The trainer restarted the runs early the following morning.

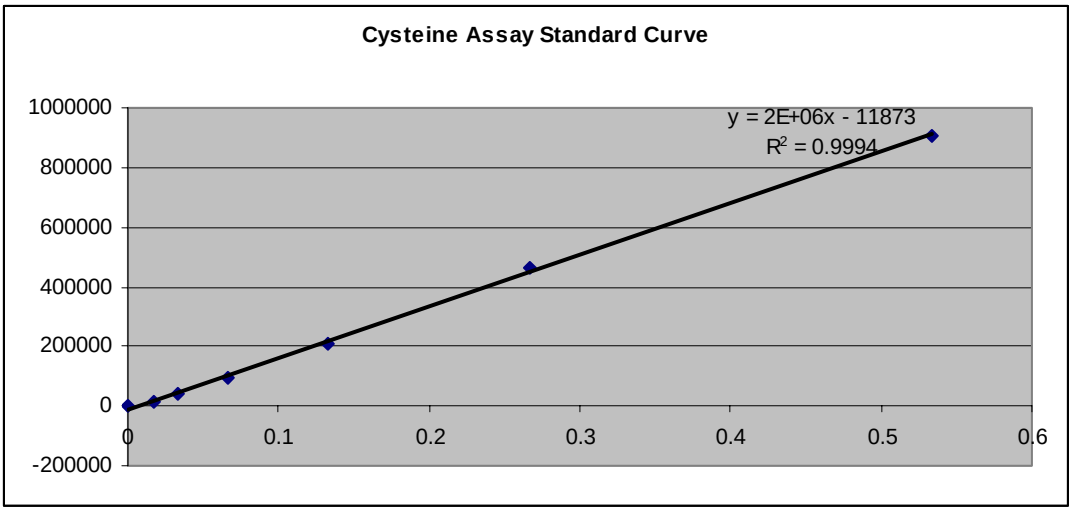
In order to discuss data analysis and reporting, the trainer used several historical DPRA assay runs to demonstrate data analysis along with the data from the run set up during the training session. Retention times and peptide peak appearance (page 21 of the SOP) were discussed as well as proper integration techniques. Calibration Standards graphed to verify linearity of response and system suitability was determined. The historical runs chosen by the trainer included test chemicals that exhibited single peak chromatograms and test chemicals that co-eluted with the peptides. The use of the 220/258 ratio was also discussed and demonstrated.

Results generated during the training

Cysteine Assay

System Suitability

	Concentration	Peak Area
Standard 1	0.533	908620
Standard 2	0.267	460680
Standard 3	0.133	212060
Standard 4	0.0667	96692
Standard 5	0.0333	40664
Standard 6	0.0166	15432
Standard 7	0	752
Ref Control A	0.512	874607
Ref Control A	0.488	833115
Ref Control A	0.507	865925
Mean	0.502	857882.333
Standard Dev.	0.013	21884.010
CV	0.025	0.026



The system suitability criteria have been met for this assay preparation.

Stability of Reference Control B over time

	Peak Area	Concentration
Reference Control B	858902	0.503
Reference Control B	880713	0.516
Reference Control B	865955	0.507
Reference Control B	869804	0.509
Reference Control B	846119	0.496
Reference Control B	830289	0.486
Mean	858630.333	0.503
Standard Dev.	18033.215	0.011
CV	0.021	0.021

Reference Control C

Reference Control C (acetonitrile)	876140	0.513
Reference Control C (acetonitrile)	853850	0.500
Reference Control C (acetonitrile)	867650	0.508
Mean	865880.000	0.507
Standard Dev.	11249.920	0.007
CV	0.013	0.013

Reference Control C (water)	852278	0.499
Reference Control C (water)	854734	0.501
Reference Control C (water)	854150	0.500
Mean	853720.667	0.500
Standard Dev.	1283.055	0.001
CV	0.002	0.002

Acceptance criteria have been met.

Cinnamic Aldehyde Positive Control

	Peak Area	% Depletion
Cinnamic Aldehyde	233553	73.0
Cinnamic Aldehyde	207263	76.1
Cinnamic Aldehyde	229631	73.5
Mean	223482.333	74.2
Standard Dev.	14182.581	1.638
CV	0.063	0.022

Acceptance criteria have been met.

Test Chemical Data

	Peak Area	% Depletion
p-phenylendiamine	1553	99.8
p-phenylendiamine	2748	99.7
p-phenylendiamine	964	99.9
Mean	1755.000	99.8
Standard Dev.	908.992	0.105
CV	0.518	0.001
3,3,5-trimethylhexanoyl chloride	694043	19.8
3,3,5-trimethylhexanoyl chloride	647387	25.2
3,3,5-trimethylhexanoyl chloride	620701	28.3
Mean	654043.667	24.5
Standard Dev.	37121.364	4.287
CV	0.057	0.175
glyoxal	35772	95.8

glyoxal	41261	95.2
glyoxal	11408	98.7
Mean	29480.333	96.5
Standard Dev.	15889.909	1.861
CV	0.539	0.019
citral	332104	61.6
citral	322336	62.8
citral	358600	58.6
Mean	337680.000	61.0
Standard Dev.	18764.015	2.167
CV	0.056	0.036
imidazolidinyl urea	619294	28.5
imidazolidinyl urea	603107	30.3
imidazolidinyl urea	665241	23.2
Mean	629214.000	27.3
Standard Dev.	32232.953	3.723
CV	0.051	0.136
glycerol	863249	0.3
glycerol	878721	-1.5
glycerol	846085	2.3
Mean	862685.000	0.4
Standard Dev.	16325.308	1.885
CV	0.019	5.110

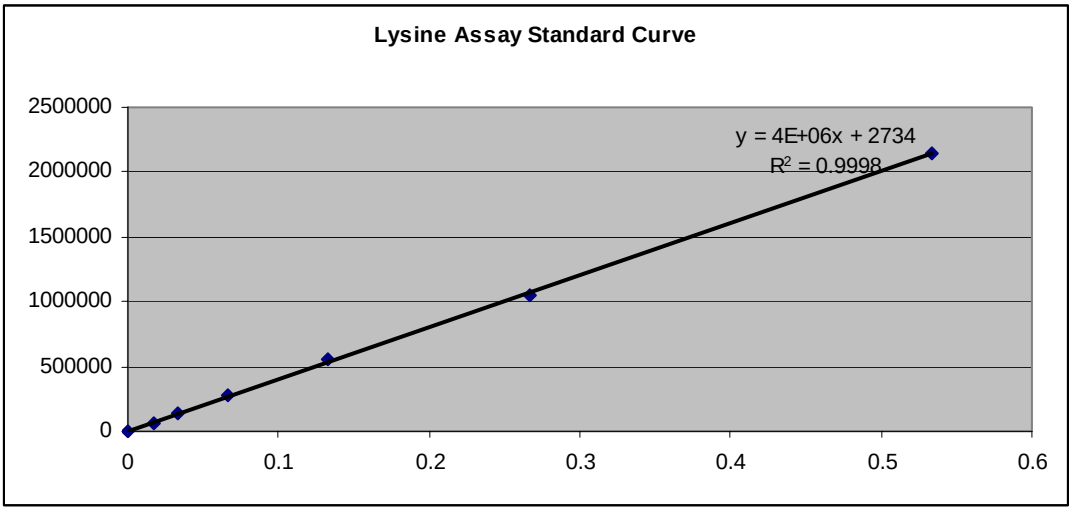
Acceptance criteria have been met.

Lysine assay

System Suitability

	Concentration	Peak Area
Standard 1	0.533	2152690
Standard 2	0.267	1054232
Standard 3	0.133	549252
Standard 4	0.0667	274179
Standard 5	0.0333	137916
Standard 6	0.0166	68713
Standard 7	0	139
Ref Control A	0.503	2028522
Ref Control A	0.507	2040873
Ref Control A	0.504	2029304
Mean	0.505	2032899.667
Standard Dev.	0.002	6916.170

CV	0.004	0.003



The system suitability criteria have been met for this assay preparation.

Stability of Reference Control B over time

	Peak Area	Concentration
Reference Control B	1985961	0.493
Reference Control B	1999694	0.497
Reference Control B	1985485	0.493
Reference Control B	1996551	0.496
Reference Control B	1993770	0.496
Reference Control B	1962211	0.495
Mean	1987278.667	0.495
Standard Dev.	13532.150	0.002
CV	0.007	0.003

Reference Control C

Reference Control C (acetonitrile)	1978034	0.491
Reference Control C (acetonitrile)	2007705	0.498
Reference Control C (acetonitrile)	1962211	0.487
Mean	1982650.000	0.492
Standard Dev.	23095.597	0.006
CV	0.012	0.011

Reference Control C (water)	1959583	0.487
Reference Control C (water)	1957888	0.486
Reference Control C (water)	1920983	0.477
Mean	1946151.333	0.483
Standard Dev.	21812.886	0.006
CV	0.011	0.011

Acceptance criteria have been met.

Cinnamic Aldehyde Positive Control

	Peak Area	% Depletion
Cinnamic Aldehyde	857837	56.7
Cinnamic Aldehyde	881915	55.5
Cinnamic Aldehyde	919528	53.6
Mean	886426.667	55.3
Standard Dev.	31091.980	1.568
CV	0.035	0.028

Acceptance criteria have been met.

Test Chemical Data

	Peak Area	% Depletion
p-phenylendiamine	1585669	20.0
p-phenylendiamine	1618441	18.4
p-phenylendiamine	1607777	18.9
Mean	1603962.333	19.1
Standard Dev.	16715.704	0.843
CV	0.010	0.044
3,3,5-trimethylhexanoyl chloride	1654666	16.5
3,3,5-trimethylhexanoyl chloride	1639963	17.3
3,3,5-trimethylhexanoyl chloride	1662623	16.1
Mean	1652417.333	16.7
Standard Dev.	11496.142	0.580
CV	0.007	0.035

glyoxal	525498	73.0
glyoxal	515712	73.5
glyoxal	534098	72.6
Mean	525102.667	73.0
Standard Dev.	9199.373	0.473
CV	0.018	0.006
citral	1768496	10.8
citral	1731776	12.7
citral	1729288	12.8
Mean	1743186.667	12.1
Standard Dev.	21953.799	1.107
CV	0.013	0.092
imidazolidinyl urea	1465551	26.1
imidazolidinyl urea	1521018	23.3
imidazolidinyl urea	1569455	20.8
Mean	1518674.667	23.4
Standard Dev.	51991.622	2.622
CV	0.034	0.112
glycerol	1996119	-0.7
glycerol	1998443	-0.8
glycerol	1993993	-0.6
Mean	1996185.000	-0.7
Standard Dev.	2225.734	0.112
CV	0.001	-0.164

Acceptance criteria have been met.

Co-elution control and 220/258 analysis were discussed and demonstrated (data not shown here).

Prediction Model Outcome

IVM Training	% Cysteine Depletion	% Lysine Depletion	Average	Reactivity Category
p-phenylendiamine	99.8	19.1	59.5	high
3,3,5-trimethylhexanoyl chloride	24.5	16.7	20.6	moderate
glyoxal	96.5	73.0	84.8	high
citral	61.0	12.1	36.6	moderate
imidazolidinyl urea	27.3	23.4	25.4	moderate
glycerol	0.4	-0.7	0.2	minimal

P&G Historical	% Cysteine Depletion	% Lysine Depletion	Average	Reactivity Category
p-phenylendiamine	93.0	23.5	58.3	high
3,3,5-trimethylhexanoyl chloride	29.3	19.3	24.3	moderate
glyoxal	56.5	67.8	62.2	high
citral	85.7	16.9	51.3	high
imidazolidinyl urea	52.3	1.3	26.8	moderate
glycerol	-3.8	2.1	1.1	minimal

The data obtained during the training session correlates well with P&G's historical data. All six chemicals were classified correctly as "sensitizer" or "non-sensitizer." Five out of the six chemicals were predicted into the same reactivity category as compared to historical data. Generally, we do not have a great concern when the predicted reactivity category differs by one. In this case, Citral was predicted as moderate in the training run and high in P&G's historical database.

Aside from this, it is important to note that technically the training run looked very clean. The acceptance criteria described in the SOP was met and the triplicate values for each chemical were very reproducible.

Statement on training outcome

The trainer and trainees felt that this training session was successful. The trainees came to the training with a strong background in the analytical chemisty and felt comfortable that they will be able to adopt this assay into their laboratory.

As part of the Training and Transfer plan, P&G requested that trainees use the training chemicals to help establish the assay in their laboratories. They were asked to report their data back to the trainer prior to beginning the official Transfer Phase and testing the transfer chemicals. P&G has not yet received this data from In vitro Methods.

Annex 1:

List of documents provided to the training participants

- Reprints of Peptide Reactivity Assay manuscripts published by the Gerberick laboratory, two papers covering the DPRA (Gerberick, 2004 and 2007) and one paper on the 2nd generation Peroxidase Peptide Reactivity Assay (Gerberick, 2009)

- An Excel document that is used by P&G to calculate the target amount of test chemical and peptide that needs to be weighed out. This workbook also contains a one-page assay set-up schematic.


DPRA assay set-up workbook

- An example HPLC Analysis Sequence demonstrating the vials that need to be prepared for the assay.


Example DPRA run

Annex 2:

Comments and questions from the trainees that arose during the training session:

- **Is it critical to use the suggested supplier for vials, columns and reagents for mobile phases and buffers?** No, it is not critical to use the suggested vials and mobile phase/buffer reagents. The suggested HPLC columns are the two columns that we have used. Similar columns from other vendors may work equally well, but we have never tried them.
- **Can other sources of peptide be used?** Synbiosci is the vendor that we have always used. We have recently found an additional vendor for the peptides. A European vendor is also an option as long as peptide performance is similar to Synbiosci peptides. The most important thing to remember when ordering peptide is to request 90-95% purity, not only is it less costly but it also improves solubility of the peptides.
- **Does %-purity of the peptides need to be accounted for when calculating the amount of peptide needed for the assay?** No, %-purity of peptide does not need to be accounted for.
- **Reference Controls A, B and C are confusing.** The controls and how they are used was discussed and the example analysis sequence was provided to help explain.
- **Example HPLC Sample Analysis Sequence in SOP is confusing**. The example analysis sequence was provided to help clarify.

Annex 3:

Additional remarks from trainer:

Due to the confusion regarding the analysis sequence and Reference controls, it may be helpful to modify that section of the SOP. Replacing the current text with the example analysis sequence that was provided during the training session may be helpful.

Annex 4:

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Appendix 14

DPRA Training report (Ricerca)

Product Safety and Regulatory Affairs
Central Product Safety

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DPRA Training Report

Training Location	**Procter & Gamble** **Miami Valley Innovation Center** **Cincinnati, OH**
Date	**May 15, 2010**
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Status	**Draft**
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Trained Laboratory	**MDS Pharma / Ricerca**
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UV	Ultraviolet

Background of the test method

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health problem, and the most common manifestation of immunotoxicity in humans. The acquisition of skin sensitization, and the subsequent elicitation of an allergic hypersensitivity reaction in the skin, is dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses. For many years guinea pigs were the species of choice for the hazard identification of skin sensitizing chemicals. More recently, however, the local lymph node assay (LLNA) has been developed as an alternative approach based upon characterization of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals (Kimber et al, 1994; Dearman et al, 1999; Gerberick et al, 2000; Kimber et al, 2002; Basketter et al, 2002). The LLNA has been adopted recently, as Test Guideline 429, by the Organization for Economic Cooperation and Development (OECD, 2002) as a stand-alone test method for skin sensitization testing. However, one challenge facing investigators is the need to develop non-animal based methods for the evaluation of new chemicals that will significantly reduce or eliminate the need for animals in skin sensitization testing in the future (Ryan et al, 2001; Casati et al, 2005; Ryan et al, 2005)**.**

There are a variety of characteristics that determine whether a chemical can function as a contact sensitizer (or allergen) including the ability to penetrate into the skin, react with protein, and be recognized as antigenic by immune cells. The correlation of protein reactivity with skin sensitization potential is well established (Dupuis and Benezra, 1982; Lepoittevin et al, 1998). In fact, Landsteiner and Jacobs (1936) presented the origin of the reactivity hypothesis in their landmark paper looking at the underlying mechanisms of contact allergy. Thus, if a chemical is capable of reacting with protein either directly or after appropriate biotransformation, then it has the potential to act as a contact allergen. The majority of chemical allergens (or their metabolites/oxidation products) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups, nucleophiles, capable of reacting with electrophilic allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine have been reported to react with electrophiles (Dupuis and Benezra, 1982; Lepoittevin et al, 1998; Ahlfors et al, 2003). Since protein reactivity is a key step in the induction of ACD it was hypothesized that an *in vitro* method could be developed to screen the sensitization potential of new chemicals based on reactivity.

Using nucleophile-containing synthetic peptides, we (Gerberick et al, 2004, 2007) have evaluated the utility of these peptides to screen for skin sensitization potential by measuring peptide depletion following incubation with allergens and non-allergens. For the synthetic heptapeptides that contain either cysteine or lysine, the ratio of peptide to chemical ratio used was 1:10 and 1:50, respectively. Following a 24 hour reaction period for the two synthetic peptides, the samples were analyzed by HPLC using UV detection to monitor the depletion of peptide following reaction.

As a test chemical may be a hapten, prehapten or a prohapten (Lepoittevin, 2006; Gerberick et al, 2008), it is critical to incorporate methods that allow for either spontaneous air-oxidation (simulating hapten formation by product aging) or metabolic activation (simulating the activation process of the prohapten in the skin). The DPRA is designed to primarily detect haptens that have the inherent reactivity to interact with peptides. However, we have been able to detect some prehaptens with the DPRA and are in the process of developing a next generation assay to evaluate even a greater number of prohaptens.

It is important to remember that skin sensitization is a complex, multi-step physiological process. Therefore, the DPRA is designed to be part of a battery or integrated testing approach for assessing the skin sensitization potential of chemicals.

Purpose of the training

The purpose of this training session was to train members of the Ricerca (previously MDS Pharma) laboratory on the DPRA SOP. This training session included a discussion of the SOP, "hands on" setup of the assay and data analysis.

Timing

March 2 – 4, 2010

SOP covered by the training

Direct Peptide Reactivity Assay (DPRA) Standard Operating Procedure Version 1 for the European Centre for the Validation of Alternative Methods (ECVAM) Skin Sensitization Prevalidation Study.

Items used for the training

The following reagents and test materials were used for the training:

Phosphate Buffer pH 7.5 (prepared in advance February 23, 2010)
Ammonium acetate Buffer pH 10.2 (prepared in advance February 23, 2010)
Cysteine Peptide
Lysine Peptide
Acetonitrile, HPLC grade
Purified Water

Chemical	CAS	Potency	Sigma Aldrich catalog number
p-phenylenediamine	106-50-3	Strong	P6001

3,3,5-trimethylhexanoyl chloride, 98%	36727-29-4	Moderate	422959
Glyoxal, 99%	107-22-2	Moderate	128465
Citral, 95%	5392-40-5	Weak	C83007
Imidazolidinyl urea	39236-46-9	Weak	I5133
Glycerol, 99%	56-81-5	Non-sensitizer	G9012

Training Program:

Theoretical aspects

The training session began with an in depth discussion of the background to the development of the DPRA and the current SOP that is being used for the prevalidation studies. The trainer covered the SOP section by section. Since the Ricerca researchers had used the DPRA in the past for work they had done with L'Oreal, they had a very good understanding of the assay. Therefore, we focused on changes that have been made to the SOP within the past year.

Specific aspects of the procedure that were discussed in detail:

- The use of a photodiode array detector and/or the co-elution controls to help determine peptide peak identity and the presence of co-elution.
- Importance of peptide purity not exceeding 90-95%
- Solubility assessments and the order of solvents used in the "Solubility Assessment" section of the SOP.
- Description of the Reference Controls A, B, and C and how they are prepared and used. The generalized HPLC Sample Analysis Sequence in the SOP was found to be confusing. The trainer prepared a more specific Analysis Sequence.
- The changes/additions that have been made to the Data Analysis & Calculations were discussed. This includes the calculation of CV, use of the Reference Controls and additional calculations of peptide concentration.
- Use of the prediction models and when to use the Cysteine 1:10-only Prediction Model.
- The use of Acceptance Criteria is a new addition to the SOP. Specific acceptance criteria were discussed as well as when an entire assay needs to be repeated and when a single test chemical needs to be repeated.

Practical aspects

The equipment used at Procter & Gamble undergoes yearly preventative maintenance checks as well as calibrations. However, if there appears to be a problem with any instrument, service calls with the appropriate vendors and technicians are scheduled. Ricerca is a GLP-certified laboratory and was instructed to follow the equipment maintenance and calibration standards that are currently in place in their laboratory.

During the "hands on" portion of the training, the trainees were given the opportunity to setup an actual DPRA test run for cysteine and lysine. In the interest of time, the training chemicals described above and peptides were pre-weighed by the trainer the afternoon before the training session and stored at 4°C. The buffers for peptide dissolution were also prepared in advance. All other aspects of assay setup described in the SOP were performed on site by the trainee.

There were no deviations to the test method SOP used. At the time of the training, P&G did have some problems with the HPLC systems. A recent software/server upgrade caused an unforeseen problem with the software that controls the equipment. The trainer did not feel this would be a problem for the trainees because they have experience running the DPRA assay.

In order to discuss data analysis and reporting, the trainer used several historical DPRA assay runs to demonstrate data analysis. Retention times and peptide peak appearance (page 21 of the SOP) were discussed as well as proper integration techniques. Calibration Standards graphed to verify linearity of response and system suitability was determined. The historical runs chosen by the trainer included test chemicals that exhibited single peak chromatograms and test chemicals that co-eluted with the peptides. The use of the 220/258 ratio was also discussed and demonstrated.

Results generated during the training

Due to software problems, the HPLC's were unable to be run. The training chemicals were also purchased by Ricerca and were used to help them set the assay up in their laboratory. Data from Ricerca's training experiments can be found in the following Excel tables.


Ricerca Training Data


Ricerca Training Data

Statement on training outcome

The trainer and trainees felt that this training session was successful. The trainees came to the training with a strong background in the DPRA assay and felt comfortable with the changes and additions that have been made to the SOP.

As part of the Training and Transfer plan, P&G requested that trainees use the training chemicals to help establish the assay in their laboratories. They were asked to report their data back to the trainer prior to beginning the official Transfer Phase and testing the transfer chemicals. Ricerca reported their data back to P&G on April 15, 2010. The only concern for the data was that the cysteine depletion for cinnamic aldehyde was slightly above the acceptable range (60.8% - 96.6%). This is consistently observed by Ricerca and could be due to lot to lot variability of cinnamic aldehyde. This criterion is a new part of the SOP and may need to be adjusted during the transfer phase.

Annex 1:

List of documents provided to the training participants

- Reprints of Peptide Reactivity Assay manuscripts published by the Gerberick laboratory, two papers covering the DPRA (Gerberick, 2004 and 2007) and one paper on the 2nd generation Peroxidase Peptide Reactivity Assay (Gerberick, 2009)
- An Excel document that is used by P&G to calculate the target amount of test chemical and peptide that needs to be weighed out. This workbook also contains a one-page assay set-up schematic.

DPRA assay set-up workbook

- An example HPLC Analysis Sequence demonstrating the vials that need to be prepared for the assay.

Example DPRA run

Annex 2:

Comments and questions from the trainees that arose during the training session:

- **Is it critical to use the suggested supplier for vials, columns and reagents for mobile phases and buffers?** No, it is not critical to use the suggested vials and mobile phase/buffer reagents. The suggested HPLC columns are the two columns that we have used. Similar columns from other vendors may work equally well, but we have never tried them.
- **Can other sources of peptide be used?** Synbiosci is the vendor that we have always used. We have recently found an additional vendor for the peptides. A European vendor is also an option as long as peptide performance is similar to Synbiosci peptides. The most important thing to remember when ordering peptide is to request 90-95% purity, not only is it less costly but it also improves solubility of the peptides.
- **Does %-purity of the peptides need to be accounted for when calculating the amount of peptide needed for the assay?** No, %-purity of peptide does not need to be accounted for.

- **Reference Controls A, B and C are confusing.** The controls and how they are used was discussed and the example analysis sequence was provided to help explain.
- **Example HPLC Sample Analysis Sequence in SOP is confusing**. The example analysis sequence was provided to help clarify.

Annex 3:

Additional remarks from trainer:

Due to the confusion regarding the analysis sequence and Reference controls, it may be helpful to modify that section of the SOP. Replacing the current text with the example analysis sequence that was provided during the training session may be helpful.

The trainer also suggests that Ricera purchase a new sample of Cinnamic Aldehyde to be used as the positive control in future studies. If the depletion values for cysteine are still above the acceptable range, the Acceptance Criteria may need to be modified slightly. This will be reassessed at the end of the transfer phase.

Annex 4:

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Appendix 15

Statistical Report

Appendix 15

STATISTICAL DATA ANALYSIS FOR THE ECVAM SKIN SENSITISATION PREVALIDATION STUDY

Direct Peptide Reactivity Assay (DPRA)

DRAFT REPORT

Prepared for:
European Commission - Joint Research Center
Collaboration Agreement between European Commission and Adriaens Consulting

Service Contract CCR.IHCP.C438312.X0

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December 2011

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1 BACKGROUND

With the entry into force of the 7th Amendment to the Cosmetics Directive (EU 2003) and the new European chemicals regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) there is a stronger need for having alternative non-animal methods available for skin sensitisation. In the first quarter of 2009 three partial replacement methods for skin sensitization were formally submitted to ECVAM. Such methods, namely the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitisation Test (MUSST) were developed by the European Cosmetics Association (Colipa) associated industries and optimized within Colipa ring trials. Based on the information provided on these methods ECVAM concluded that they were sufficiently developed and standardized to be included in the ECVAM validation process.

The DPRA, which is a partial replacement in chemico test method, is the subject of this report. Nucleophile-containing synthetic peptides are used to screen for skin sensitization potential by measuring peptide depletion following incubation with allergens and non-allergens. For the synthetic heptapeptides that contain either cysteine or lysine, the ratio of peptide to chemical ratio used is 1:10 and 1:50, respectively. Following a 24 hour reaction period for the two synthetic peptides, the samples are analyzed by HPLC using UV detection to monitor the depletion of peptide following reaction. Average peptide depletion data for cysteine and lysine are then used in a classification tree model in which chemicals are classified as having minimal, low, moderate or high reactivity. When co-elution with the lysine peptide prevents a determination of an accurate depletion value with a sufficient level of confidence, a prediction model is available to classify the chemical into reactivity classes based on the depletion value for the Cysteine peptide alone.

The primary goal of the ECVAM skin sensitization prevalidation study is an evaluation of the transferability and reliability (reproducibility within and between laboratories) of the test method in view to their future use in an integrated non-animal approach for replacing the currently used regulatory animal tests.

As secondary goals of the study, the experimental data were used to perform:

a) a preliminary evaluation of the ability of the test 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 mixtures.

b) Where possible, a preliminary consideration of the ability of the test to contribute to sub-categorisation of skin sensitising chemicals, e.g. into Sub-category 1A and Sub-category 1B as adopted in the 3rd revised version of the GHS.

The current report, presents the outcome of the statistical analysis of the DPRA where the transferability and reliability were evaluated in three independent laboratories. The statistical analysis were performed according to the study goals and in compliance with the study experimental design agreed by the Validation Management Team and were applied to the data from valid runs and experiments only. Note that the VMG requested in a specific case an exception to this rule.

2 METHODS

2.1 Design of the study

The within and between laboratory reproducibility (WLR and BLR, respectively) of the DPRA assay was assessed in 3 laboratories. Procter & Gamble (P&G) acted as lead laboratory, the In-Vitro Methods Unit of ECVAM (IVMU) acted as naïve laboratory, and Ricerca as partly naïve laboratory. Based on a statistical evaluation (for more details see Phase III Pre-validation Study experimental design) the following design was approved by the VMG:

- For evaluation of the BLR, 24 chemicals tested once in every laboratory (16 sensitisers and 8 non-sensitisers).
- For evaluation of the WLR, a subset of 15 chemicals from those used for the evaluation of the BLR, tested two additional times in each laboratory, the same subset being used at every site (10 sensitisers and 5 non-sensitisers).

The study was structured in, conducted, and evaluated as, two sequential phases:

- Phase A: training of the participating laboratories (phase A1), test method transfer and confirmation of the Test Method Protocols (phase A2). The results or not part of the current statistical data analysis.
- Phase B: assessment of the protocol performance by testing under blind conditions in all the laboratories. The results or the subject of the current statistical data analysis.
    - Phase B1: 9 chemicals were tested once in each laboratory (evaluation BLR)
    - Phase B2: 15 chemicals were tested 3 times in each laboratory (evaluation WLR)

As described in the SOP each experiment is composed of one run to evaluate the cysteine depletion and another run to evaluate the lysine depletion. The SOP states that up to 25 chemicals can be accommodated within a single run. Therefore, the 9 chemicals or the three required independent assessments of the 15 chemicals were always tested within the same run. The data are presented laboratory by laboratory, and are referred to as experiment 1 (9 chemicals), experiment 2, experiment 3, and experiment 4.

2.2 Data management

For the statistical analyses, a summary template was designed by the statistician, and the results were transferred to this template by ECVAM. This summary template contained internal checks that ensured that no mistakes were made in the transfer of the results. The final conclusions for each chemical were then compared to the conclusions of the reports sent by the laboratories as an additional check. The details of the study results generated at the different laboratories are described in the DPRA study report.

2.3 Statistical data analysis

2.3.1 Reproducibility of the control values

Descriptive statistics (mean and standard deviation) of the reference controls and of the peptide depletion values were calculated for each of the 4 runs in the 3 laboratories. The frequency of invalid runs/experiments was reported.

2.3.2 Within laboratory reproducibility

The WLR was assessed with data generated with a subset of 15 chemicals tested in three independent experiments in each laboratory (Study Phase B2).

The main determinant of the test method's reliability assessment was on the concordance of classification, as sensitiser (S) or non-sensitiser (NS), which were determined from the peptide depletion values (average of the 2 peptide values according to the prediction model (PM) presented in Figure 1 or in case of co-elution with lysine according to the PM presented in Figure 2). The concordance of classification with regard to the 4 reactivity classes was also considered.

Additionally, descriptive and inferential statistical analyses (ANOVA F-test) were performed on the raw peptide depletion data. The mean cysteine and lysine depletion for each chemical was compared between the 3 independent experiments by one-way ANOVA. A critical α-level = 0.027 was used to corrected for the number m (m=15) of hypothesis tested (rough False Discovery Rate (RFDR): α(m+1)/2m). In case the null hypothesis of equal means was rejected a post-hoc analysis was performed based on Tuckey's procedure. The results of the inferential tests applied will only be considered as additional descriptive information because of the sample sizes (three replicates). In case of small sample size violations against ANOVA assumptions (normal distribution of the errors and homogeneity of variances) are difficult to assess and confirm. In case of heterogeneity of variances, the standard F-test (ANOVA) is not appropriate because its type I error (false positive, reject a true null-hypothesis) rates exceed the intended level 5%. All analyses were performed with R version 2.14.0.

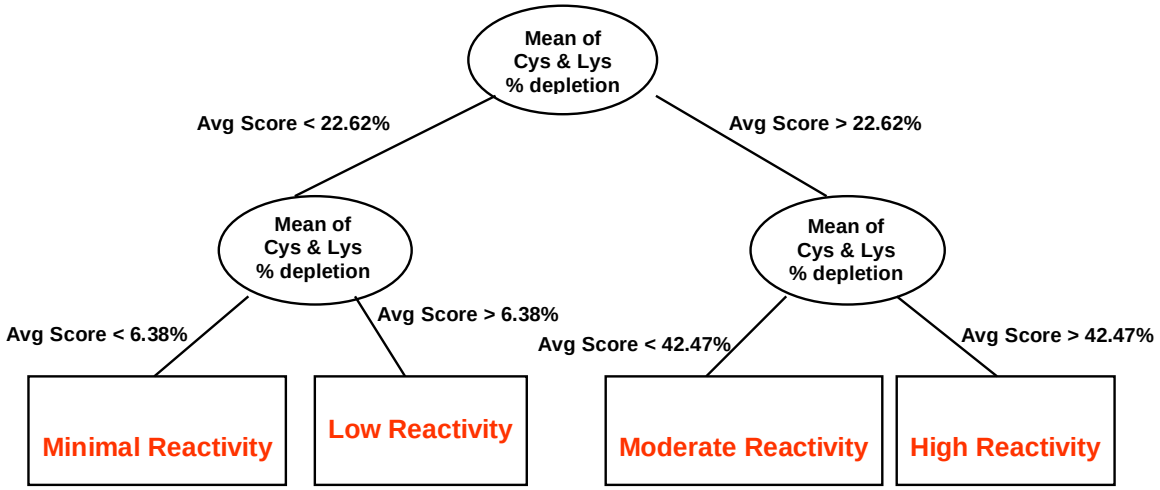

Figure 1 Cysteine 1:10/Lysine 1:50 prediction model

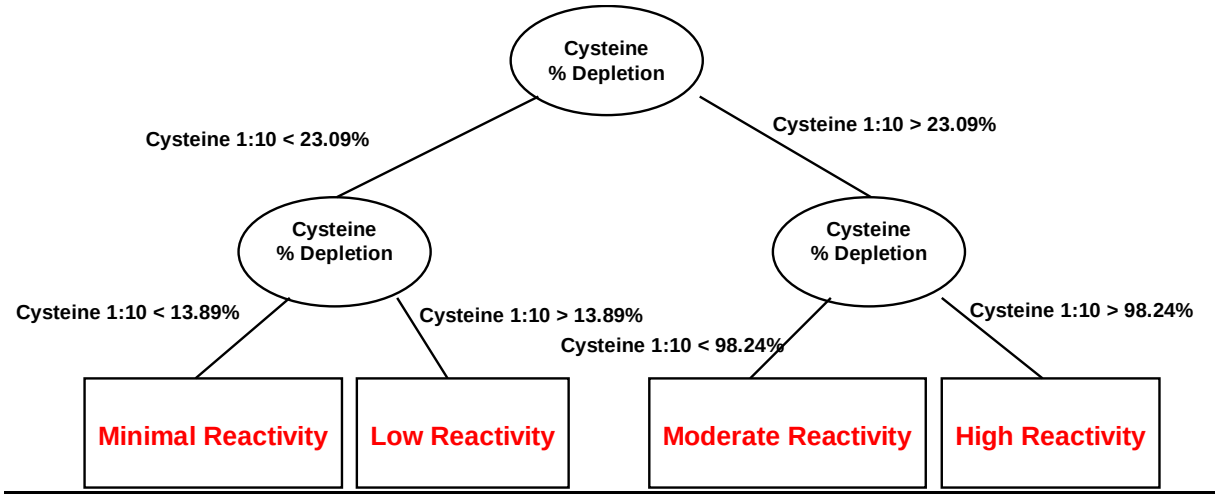

Figure 2 Cysteine 1:10 only prediction model

2.3.3 Between laboratory reproducibility

The between laboratory reproducibility was assessed on the basis of the data for the 24 chemicals tested (9 chemicals tested once and 15 chemicals tested 3 times in each laboratory). The main focus of the evaluation of the between-laboratory reproducibility was on the concordance of the predictions sensitisers (S) versus non-sensitisers (NS) and for the assignment to one of the four reactivity classes. As discussed in the WLR section, descriptive and inferential statistical analyses were also performed on the raw peptide depletion data (ANOVA) but the results of the inferential tests applied will only be considered as additional descriptive information.

2.3.4 Predictive capacity

The predictive capacity of the assay was also evaluated by comparing the prediction results with the existing proposed classification. Therefore 2x2 contingency tables (S versus NS) were constructed and sensitivity (probability of predicting S given the true state is S), specificity (probability of predicting NS given the true state is NS), false positive (FPR) and false negative rate (FNR) and accuracy were calculated (Table 1). The 95% confidence intervals (CI) reported for the sensitivity and specificity are the Wilson CI's based on the score test. The Wilson CI's based on the score test provide better coverage for small samples and estimates close to 1.0 (Agresti and Coull, 1998)

Although predictive values were requested they were not calculated because of the following reason. Positive and Negative Predictive Values (PPV and NPV) are not intrinsic to the test but depend on the prevalence (proportion of sensitizers among the population of chemicals which is an estimate of how common sensitizers are among chemicals). PV's are directly proportional to the prevalence (see equation for PPV [1]). Therefore, NPV and PPV can only be used in case the prevalence of the test condition is equivalent to the prevalence of the population.

PPV= sensitivity x prevalence / [(sensitivity x prevalence) + (1-specificity) x (1-prevalence)] [1]

Kappa coefficient was requested to compare prediction results obtained applying the 4-classes prediction model with the existing proposed classification. They were however not calculated because the use of the Kappa coefficient as a measure of agreement is associated with many problems and limitations. The Kappa coefficient was designed to measure correlation between nominal and not ordinal measures. Furthermore, the value of Kappa depends on the proportion of items in each category and on the number of categories used. The criteria for judging Kappa vary and are not completely objective.

Table 1 2x2 contingency table: evaluation of the predictive capacity

	DPRA prediction		
Reference	**S**	**NS**	**Total Reference**
S	$True_S$ (A)	FN (B)	S (A+B)
NS	FP (C)	$True_{NS}$ (D)	NS (C+D)
Total DPRA	S (A+C)	NS (B+D)	Total (A+B+C+D)

Accuracy (concordance) = (A+D)/(A+B+C+D)

Sensitivity = A/(A+B) → FNR = 1-sensitivity

Specificity = D/(C+D) → FPR = 1-specificity

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3 RESULTS AND DISCUSSION

3.1 Acceptance criteria controls

Before results can be accepted for further analysis, it was investigated if the acceptance criteria for the different reference controls and the positive control were met. The mean cysteine and lysine concentration for control A, control C in water and control C in acetonitrile should be between 0.45 mM and 0.55 mM. The mean cysteine depletion for the positive control should be between 60.8% and 100% and for the lysine depletion between 40.2% and 69.4%. The individual values for the cysteine and lysine controls in function of the independent experiments within each laboratory are shown in Figure 3 and Figure 4. The mean values are presented in Table 2 and Table 3.

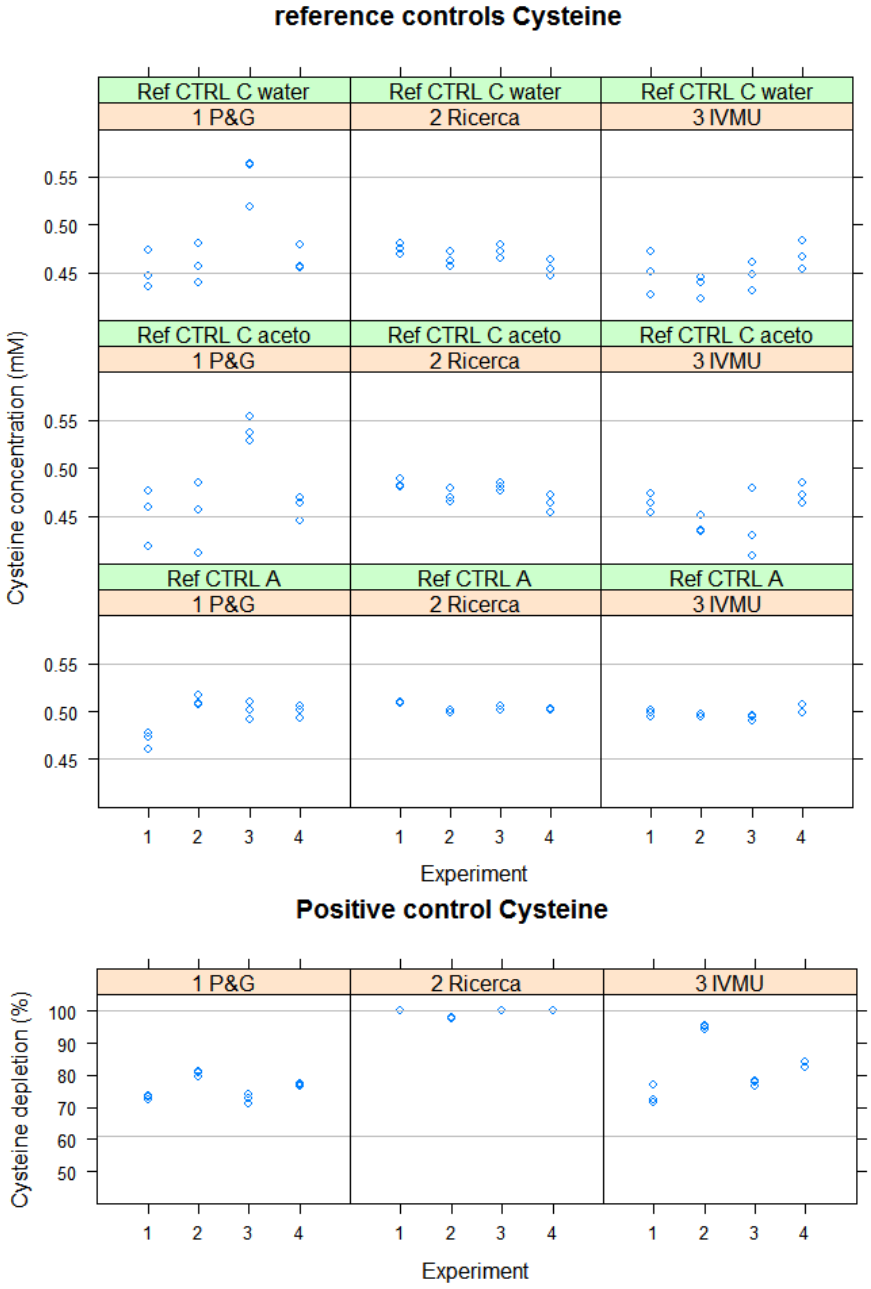

Figure 3 Cysteine concentration for the different reference controls and cysteine depletion for the positive control (individual data) for each of the individual runs performed at the 3

laboratories. Experiment 1 corresponds with phase B1 and experiment 2 up to 4 with phase B2. The grey lines correspond with the lower and upper threshold for the mean cysteine concentration (reference controls) or for the mean cysteine depletion (positive control).

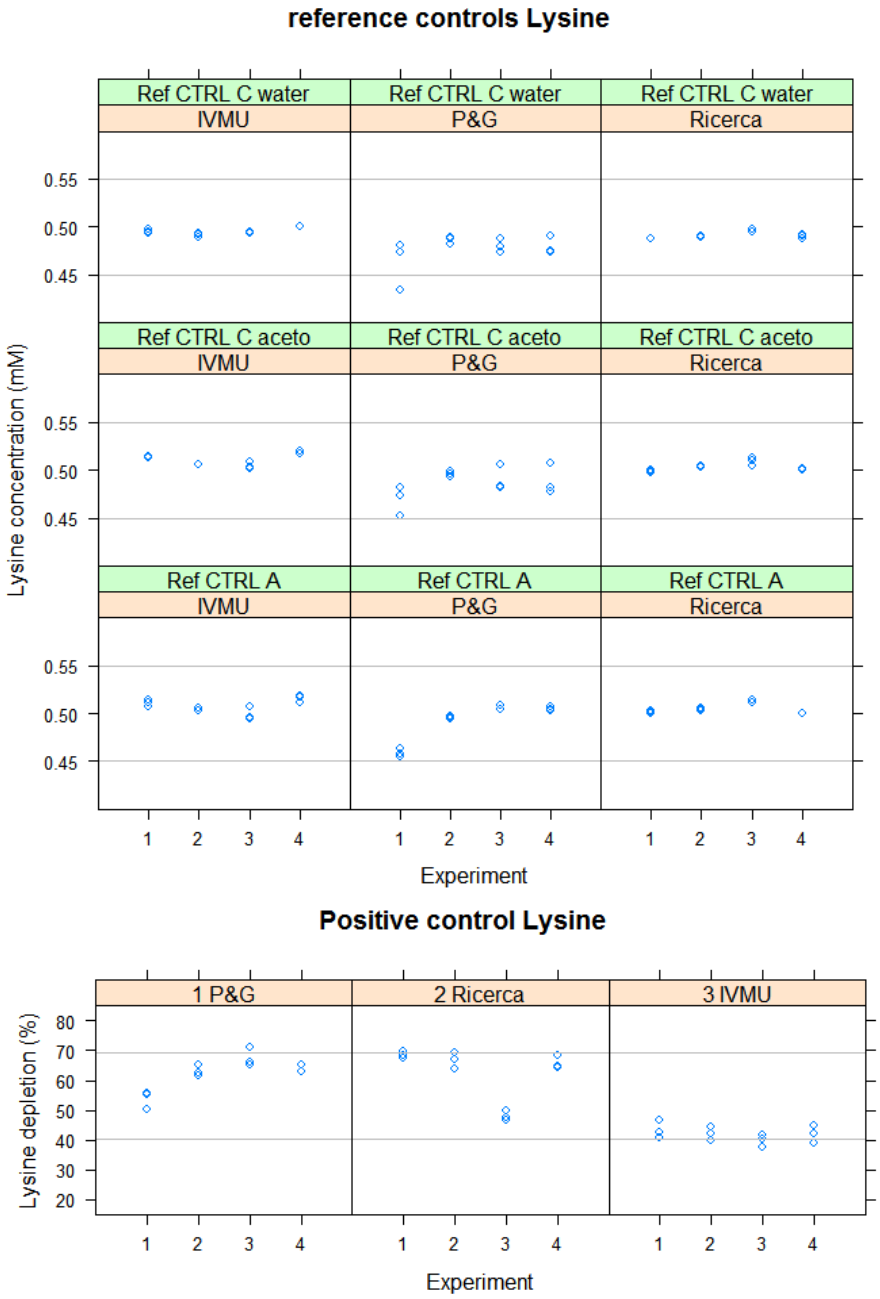

Figure 4 Lysine concentration for the different reference controls and lysine depletion for the positive control (individual data) for each of the individual runs performed at the 3 laboratories. Experiment 1 corresponds with phase B1 and experiment 2 up to 4 with phase B2. The grey lines correspond with the lower and upper threshold for the mean lysine concentration (reference controls) or for the mean lysine depletion (positive control).

The acceptance criteria for all the reference and positive controls for both peptides were always met for P&G and Ricerca. At IVMU, the mean cysteine concentration for reference control C in water and in acetonitrile were respectively 1 time and 2 times just below the threshold value of 45 mM. The mean lysine depletion was also for 1 experiment just below the threshold value of 40.2%.

In fact, all the data for the cysteine reference control C (in water and acetonitrile) and lysine depletion for the positive control generated at IVMU were systematically very close to the lower limit of the acceptance range which indicates that the validity of the results generated can occur just by chance. During the VMT meeting held at ISPRA on the 6th of October 2011, it was felt that minimal value would be gained by generating numerous additional (possibly) invalid runs at IVMU when it was already clear that the acceptance criteria would need to be revised at the end of the study (since the range had been defined solely on P&G's historical data which may not always be appropriate for other laboratories). Therefore, it was decided to accept all the results of the experiments performed at IVMU.

Table 2 Cysteine concentration of the reference controls and cysteine depletion of the positive control for the independent experiments in the three laboratories

Lab	Experiment	Cysteine concentration (mM)			Cysteine depletion
		Ctrl A	Ctrl C water	Ctrl C acetonitrile	Postive control
P&G	Phase B1-1	0.47 ± 0.01	0.45 ± 0.02	0.45 ± 0.03	72.99 ± 0.6
P&G	Phase B2-2	0.51 ± 0.01	0.46 ± 0.02	0.45 ± 0.04	80.33 ± 1.0
P&G	Phase B2-3	0.50 ± 0.01	0.55 ± 0.03	0.54 ± 0.01	72.67 ± 1.6
P&G	Phase B2-4	0.50 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	76.82 ± 0.4
Ricerca	Phase B1-1	0.51 ± 0.00	0.47 ± 0.01	0.48 ± 0.00	100.00 ± 0.0
Ricerca	Phase B2-2	0.50 ± 0.00	0.46 ± 0.01	0.47 ± 0.01	97.80 ± 0.4
Ricerca	Phase B2-3	0.50 ± 0.00	0.47 ± 0.01	0.48 ± 0.00	100.00 ± 0.0
Ricerca	Phase B2-4	0.50 ± 0.00	0.45 ± 0.01	0.46 ± 0.01	100.00 ± 0.0
IVMU	Phase B1-1	0.50 ± 0.00	0.45 ± 0.02	0.46 ± 0.01	73.50 ± 2.9
IVMU	Phase B2-2	0.50 ± 0.00	0.44 ± 0.01	0.44 ± 0.01	95.00 ± 0.6
IVMU	Phase B2-3	0.49 ± 0.00	0.45 ± 0.02	0.44 ± 0.04	77.50 ± 0.8
IVMU	Phase B2-4	0.50 ± 0.00	0.47 ± 0.02	0.47 ± 0.01	83.50 ± 1.1

Values are presented as mean ± SD, n=3
Values in red: acceptance criteria not met

Table 3 Lysine concentration of the reference controls and lysine depletion of the positive control for the independent experiments in the three laboratories

Lab	Experiment	Lysine concentration (mM)			Lysine depletion
		Ctrl A	Ctrl C water	Ctrl C acetonitrile	Postive control
P&G	Phase B1-1	0.46 ± 0.00	0.46 ± 0.02	0.47 ± 0.02	53.7 ± 3.1
P&G	Phase B2-2	0.50 ± 0.00	0.49 ± 0.00	0.50 ± 0.00	63.1 ± 1.8
P&G	Phase B2-3	0.51 ± 0.00	0.48 ± 0.01	0.49 ± 0.01	67.6 ± 3.3
P&G	Phase B2-4	0.50 ± 0.00	0.48 ± 0.01	0.49 ± 0.02	64.4 ± 1.1
Ricerca	Phase B1-1	0.50 ± 0.00	0.49 ± 0.00	0.50 ± 0.00	68.5 ± 1.2
Ricerca	Phase B2-2	0.50 ± 0.00	0.49 ± 0.00	0.50 ± 0.00	66.9 ± 2.8
Ricerca	Phase B2-3	0.51 ± 0.00	0.50 ± 0.00	0.51 ± 0.00	47.9 ± 1.8
Ricerca	Phase B2-4	0.50 ± 0.00	0.49 ± 0.00	0.50 ± 0.00	65.8 ± 2.1
IVMU	Phase B1-1	0.51 ± 0.00	0.50 ± 0.00	0.51 ± 0.00	43.4 ± 3.0
IVMU	Phase B2-2	0.50 ± 0.00	0.49 ± 0.00	0.51 ± 0.00	42.0 ± 2.2
IVMU	Phase B2-3	0.50 ± 0.01	0.49 ± 0.00	0.50 ± 0.00	39.8 ± 2.1
IVMU	Phase B2-4	0.52 ± 0.00	0.50 ± 0.00	0.52 ± 0.00	41.9 ± 3.0

Values are presented as mean ± SD, n=3
Values in red: acceptance criteria not met

3.2 Within laboratory reproducibility

A subset of 15 chemicals was tested in three independent experiments in each laboratory. The main focus of the test method's reliability was on the concordance of the predictions between the independent experiments within a laboratory. Additionally, descriptive and inferential statistical analyses were performed on the raw peptide depletion data (ANOVA).

3.2.1 Lead laboratory: P&G

3.2.1.1 Concordance in predictions

In relation to the primary aim, the reproducibility in terms of the classification S versus NS, for 11 of the 15 chemicals the same prediction was obtained in the 3 independent experiments resulting in a WLR of 73.3% (Table 4). For the assignment to a reactivity class, 10 of 15 chemicals (66.7%) were assigned the same reactivity class in all 3 experiments. Furthermore, in all cases of disagreement, the difference in the reactivity class assignment was only of one class, e.g. chemical 12 (Table 4, right side), was classified as either 'LOW' (twice) or 'MODERATE' (once)).

Table 4 Phase B2 P&G: concordance in predictions between the three independent experiments for the subset of 15 chemicals

Chemical	P&G (mean pept depl, %)[A]			Agreement	P&G (4 reactivity classes)			Agreement
	Exp 2	Exp 3	Exp 4	2 classes	Exp 2	Exp 3	Exp 4	4 classes
CHEM 10	100.0	100.0	100.0	Yes	$HIGH_{Lys}$	$HIGH_{Lys}$	$HIGH_{Lys}$	Yes
CHEM 11	10.5	12.3	15.9	Yes	LOW	LOW	LOW	Yes
CHEM 12	24.5	19.9	22.2	Yes	MODERATE	LOW	LOW	No
CHEM 13	100.0	100.0	100.0	Yes	$HIGH_{Lys}$	$HIGH_{Lys}$	$HIGH_{Lys}$	Yes
CHEM 14	3.3	3.3	2.8	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 15	100.0	100.0	100.0	Yes	$HIGH_{Lys}$	$HIGH_{Lys}$	$HIGH_{Lys}$	Yes
CHEM 16	3.9	2.2	7.9	No	MINIMAL	MINIMAL	LOW	No
CHEM 17	5.1	1.6	3.1	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 18	7.9	1.9	6.6	No	LOW	MINIMAL	LOW	No
CHEM 19	5.1	3.1	7.1	No	MINIMAL	MINIMAL	LOW	No
CHEM 20	5.2	3.6	5.8	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 21	6.3	1.1	5.1	Yes	$MINIMAL_{Lys}$	$MINIMAL_{Lys}$	$MINIMAL_{Lys}$	Yes
CHEM 22	5.6	2.4	5.6	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 23	0.6	1.0	0.8	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 24	5.2	2.3	6.9	No	MINIMAL	MINIMAL	LOW	No

[A] Values represent the mean cysteine and lysine peptide depletion. In case of co-elution with the lysine peptide (indicated by the LYS subscript in the columns with the reactivity classes) the value represents the mean cysteine peptide depletion. Mean depletion values with an orange background correspond to a sensitiser prediction, those with a green background correspond to a non-sensitiser prediction.

3.2.1.2 Reproducibility of the depletion values for cysteine and lysine

The mean cysteine and lysine peptide depletions for each chemical were compared between the 3 independent experiments by one-way ANOVA. The results for the cysteine depletion are presented in Table 5 and the results for lysine depletion are presented in Table 6. ANOVA revealed no differences in mean cysteine or lysine depletion between the three independent experiments, except for chemical 12. For this chemical, the mean cysteine depletion differed significantly between the independent experiments. However this had no impact on the final prediction as the chemical was predicted as sensitiser in all three independent experiments. The variability within an experiment was very small, therefore minor differences between the experiments resulted in a significant difference in mean cysteine depletion for this chemical.

Table 5 Phase B2 P&G: within laboratory variability of the cysteine depletion

Chemical	Cysteine depletion (%)			Cysteine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 11	20.8 ± 10.4	24.6 ± 5.5	31.0 ± 7.6	25.5 ± 5.1	0.362
CHEM 12	44.7 ± 1.0 ^{C}	36.6 ± 1.2 ^{A}	40.6 ± 1.2 ^{B}	40.6 ± 4.0	<0.001*
CHEM 13	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 14	6.6 ± 8.2	6.4 ± 11.0	5.5 ± 5.2	6.2 ± 0.5	0.988
CHEM 15	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 16	7.8 ± 7.2	2.8 ± 4.2	14.4 ± 6.1	8.3 ± 5.8	0.134
CHEM 17	10.0 ± 9.8	2.0 ± 3.4	5.8 ± 5.9	5.9 ± 4.0	0.414
CHEM 18	15.5 ± 4.1	2.5 ± 2.4	12.2 ± 6.2	10.1 ± 6.8	0.029
CHEM 19	9.1 ± 8.0	3.4 ± 5.1	11.3 ± 4.9	7.9 ± 4.1	0.330
CHEM 20	9.9 ± 9.2	5.0 ± 5.8	10.8 ± 4.0	8.5 ± 3.1	0.551
CHEM 21	6.3 ± 8.3	1.1 ± 2.0	5.1 ± 4.6	4.2 ± 2.7	0.534
CHEM 22	10.7 ± 8.4	3.9 ± 4.2	10.6 ± 8.1	8.4 ± 3.9	0.457
CHEM 23	1.2 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.7	0.422
CHEM 24	9.6 ± 8.4	2.5 ± 4.1	12.1 ± 7.6	8.1 ± 5.0	0.289

Values presented as mean ± SD, n=3

[1] One-way ANOVA critical α-level = 0.027 (corrected for the number of hypothesis tested), *mean values with a different subscript are significantly different from each other (Tukey post-hoc all comparisons)

NA: ANOVA testing not performed since all individual values were equal

Table 6 Phase B2 P&G: within laboratory variability of the lysine depletion

Chemical	Lysine depletion (%)			Lysine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	4.8 ± 5.0	11.1 ± 5.4	3.5 ± 3.6	6.5 ± 4.1	0.190
CHEM 11	0.1 ± 0.1	0.0 ± 0.0	0.8 ± 1.3	0.3 ± 0.4	0.450
CHEM 12	4.3 ± 4.7	3.2 ± 1.2	3.8 ± 1.5	3.8 ± 0.5	0.906
CHEM 13					
CHEM 14	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.1	0.183
CHEM 15	3.5 ± 2.8	8.7 ± 5.2	9.0 ± 6.7	7.1 ± 3.1	0.392
CHEM 16	0.0 ± 0.0	1.6 ± 1.3	1.5 ± 0.7	1.0 ± 0.9	0.115
CHEM 17	0.2 ± 0.4	1.1 ± 1.2	0.3 ± 0.3	0.6 ± 0.5	0.309
CHEM 18	0.3 ± 0.5	1.3 ± 0.9	1.0 ± 0.4	0.9 ± 0.5	0.207
CHEM 19	1.0 ± 0.8	2.9 ± 1.5	3.0 ± 0.2	2.3 ± 1.1	0.086
CHEM 20	0.5 ± 0.8	2.3 ± 3.2	0.7 ± 0.6	1.2 ± 1.0	0.502
CHEM 21					
CHEM 22	0.4 ± 0.7	0.9 ± 0.6	0.6 ± 0.2	0.6 ± 0.3	0.488
CHEM 23	0.1 ± 0.2	2.0 ± 2.3	1.5 ± 1.8	1.2 ± 1.0	0.410

CHEM 24	0.8 ± 1.4	2.1 ± 1.9	1.6 ± 1.5	1.5 ± 0.6	0.641

Values presented as mean ± SD, n=3

[1] One-way ANOVA, critical α-level = 0.027 (corrected for the number of hypothesis tested)

Chemicals with a grey background correspond with lysine co-elution

3.2.2 Ricerca

3.2.2.1 Concordance in predictions

In relation to the primary aim, the reproducibility in terms of the classification S versus NS, for all of the 15 chemicals the same prediction was obtained in the 3 independent experiments resulting in a WLR of 100% (Table 7, left side). For the assignment to a reactivity class, the 15 chemicals were assigned the same reactivity class in all 3 experiments (Table 7, right side).

Table 7 Phase B2 Ricerca: concordance in predictions between the three independent experiments for the subset of 15 chemicals

Chemical	Ricerca (mean pept depl, %)[A]			Agreement	Ricerca (4 reactivity classes)			Agreement
	Exp 2	Exp 3	Exp 4	2 classes	Exp 2	Exp 3	Exp 4	4 classes
CHEM 10	53.3	52.6	54.1	Yes	HIGH	HIGH	HIGH	Yes
CHEM 11	1.1	0.4	1.4	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 12	26.0	26.9	25.3	Yes	MODERATE	MODERATE	MODERATE	Yes
CHEM 13	100.0	100.0	100.0	Yes	$HIGH_{Lys}$	$HIGH_{Lys}$	$HIGH_{Lys}$	Yes
CHEM 14	2.0	3.7	2.7	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 15	51.2	51.3	50.8	Yes	HIGH	HIGH	HIGH	Yes
CHEM 16	0.2	0.0	0.3	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 17	1.0	1.4	0.3	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 18	7.1	10.2	9.2	Yes	LOW	LOW	LOW	Yes
CHEM 19	0.8	0.9	0.7	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 20	0.4	0.8	3.1	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 21	2.5	0.8	2.5	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 22	2.4	1.1	0.4	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 23	7.0	10.2	11.5	Yes	LOW	LOW	LOW	Yes
CHEM 24	0.4	0.3	0	Yes	MINIMAL	MINIMAL	MINIMAL	Yes

[A] Values represent the mean cysteine and lysine peptide depletion. In case of co-elution with the lysine peptide (indicated by the LYS subscript in the columns with the reactivity classes) the value represents the mean cysteine peptide depletion. Mean depletion values with an orange background correspond to a sensitiser prediction, those with a green background correspond to a non-sensitiser prediction.

3.2.2.2 Reproducibility of the depletion values for cysteine and lysine

The cysteine and lysine peptide depletions for each chemical were compared between the three independent experiments by one-way ANOVA. The results for the cysteine depletion are presented in Table 8 and the results for lysine depletion are presented in Table 9. One-way ANOVA yielded no statistically significant differences in mean cysteine depletion between the three independent experiments, with the exception of chemical 20 (Table 8). For the lysine depletion, ANOVA revealed statistically significant differences in mean depletion for three chemicals (Table 9). Again, the variability within an experiment was very small, therefore minor differences between the experiments resulted in a significant difference in mean peptide depletion for these four chemicals. However this had no impact on the final classifications of the chemicals or on the assigned reactivity class.

Table 8 Phase B2 Ricerca: within laboratory variability of the cysteine depletion

Chemical	Cysteine depletion (%)			Cysteine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 11	2.0 ± 2.2	0.7 ± 1.3	2.6 ± 1.9	1.8 ± 1.0	0.418
CHEM 12	49.6 ± 1.6	51.4 ± 2.0	48.6 ± 2.8	49.9 ± 1.4	0.347
CHEM 13	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 14	3.9 ± 2.4	7.4 ± 1.9	5.4 ± 4.4	5.5 ± 1.7	0.435
CHEM 15	100.0 ± 0.0	98.7 ± 2.2	100.0 ± 0.0	99.6 ± 0.7	0.422
CHEM 16	0.2 ± 0.3	0.0 ± 0.1	0.7 ± 1.2	0.3 ± 0.3	0.553
CHEM 17	1.4 ± 1.3	2.5 ± 1.6	0.7 ± 1.2	1.5 ± 0.9	0.314
CHEM 18	13.6 ± 2.0	18.8 ± 2.5	18.4 ± 1.1	16.9 ± 2.9	0.030
CHEM 19	0.3 ± 0.6	0.2 ± 0.4	0.4 ± 0.6	0.3 ± 0.1	0.953
CHEM 20	0.3 ± 0.5[A]	1.0 ± 1.0[A]	6.1 ± 1.2[B]	2.5 ± 3.2	<0.001*
CHEM 21	0.7 ± 0.8	0.4 ± 0.7	0.2 ± 0.4	0.4 ± 0.2	0.697
CHEM 22	4.6 ± 2.1	1.4 ± 1.2	0.9 ± 1.4	2.3 ± 2.0	0.590
CHEM 23	13.9 ± 4.7	20.4 ± 5.7	22.9 ± 6.2	19.1 ± 4.6	0.204
CHEM 24	0.5 ± 0.9	0.3 ± 0.6	0.0 ± 0.0	0.3 ± 0.3	0.603

Values presented as mean ± SD, n=3

[1] One-way ANOVA critical α-level = 0.027 (corrected for the number of hypothesis tested), *mean values with a different subscript are significantly different from each other (Tukey post-hoc all comparisons)

NA: ANOVA testing not performed since all individual values were equal

Table 9 Phase B2 Ricerca: within laboratory variability of the lysine depletion

Chemical	Lysine depletion (%)			Lysine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	6.6 ± 0.7 [B]	5.2 ± 0.8 [A B]	8.2 ± 0.4 [B]	6.7 ± 1.5	0.005*
CHEM 11	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.340
CHEM 12	2.3 ± 0.3	2.5 ± 0.4	2.0 ± 0.3	2.3 ± 0.2	0.213
CHEM 13					
CHEM 14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
CHEM 15	2.3 ± 0.5 [A]	3.9 ± 0.2 [B]	1.6 ± 0.2 [A]	2.6 ± 1.2	<0.001*
CHEM 16	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.079
CHEM 17	0.6 ± 0.1	0.2 ± 0.3	0.0 ± 0.0	0.3 ± 0.3	0.038
CHEM 18	0.5 ± 0.3	1.5 ± 1.0	0.0 ± 0.1	0.7 ± 0.7	0.073
CHEM 19	1.3 ± 0.3	1.6 ± 0.3	1.0 ± 0.0	1.3 ± 0.3	0.059
CHEM 20	0.5 ± 0.1	0.6 ± 0.5	0.0 ± 0.0	0.4 ± 0.3	0.096
CHEM 21	4.2 ± 0.7 [B]	1.2 ± 0.5 [A]	4.7 ± 0.5 [B]	3.4 ± 1.9	<0.001*
CHEM 22	0.1 ± 0.1	0.7 ± 0.4	0.0 ± 0.0	0.3 ± 0.4	0.030
CHEM 23	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.422
CHEM 24	0.2 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.171

Values presented as mean ± SD, n=3

[1] One-way ANOVA, *mean values with a different subscript are significantly different from each other (Tukey post-hoc all comparisons)

NA: ANOVA testing not performed since all individual values were equal

Chemicals with a grey background correspond with lysine co-elution

3.2.3 IVMU

3.2.3.1 Concordance in predictions

In relation to the primary aim, the reproducibility in terms of the classification S versus NS, for 13 of the 15 chemicals the same prediction was obtained in the three independent experiments resulting in a WLR of 86.7% (Table 10, left side). For the assignment to a reactivity class, 11 of the 15 chemicals (66.7%) were assigned the same reactivity class in all three experiments (Table 10, right side). Note that in case of disagreement, all reported differences in the reactivity class assignment were only of one class.

Table 10 Phase B2 IVMU: concordance in predictions between the three independent experiments for the subset of 15 chemicals

Chemical	IVMU (mean pept depl, %)[A]			Agreement	IVMU (4 reactivity classes)			Agreement
	Exp 2	Exp 3	Exp 4	2 classes	Exp 2	Exp 3	Exp 4	4 classes
CHEM 10	46.8	46.5	93.5	Yes	HIGH	HIGH	$MODERATE_{Lys}$	No
CHEM 11	1.7	1.5	1.2	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 12	19.5	15.4	19.0	Yes	LOW	LOW	LOW	Yes
CHEM 13	79.5	79.0	77.1	Yes	HIGH	HIGH	HIGH	Yes
CHEM 14	4.3	2.0	5.7	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 15	50.0	50.0	50.0	Yes	HIGH	HIGH	HIGH	Yes
CHEM 16	8.5	5.7	6.9	No	LOW	MINIMAL	LOW	No
CHEM 17	9.4	2.5	4.9	No	LOW	MINIMAL	MINIMAL	No
CHEM 18	26.0	16.8	17.4	Yes	MODERATE	LOW	LOW	No
CHEM 19	12.8	12.2	11.6	Yes	LOW	LOW	LOW	Yes
CHEM 20	0.9	0.4	4.8	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 21	2.4	0.0	2.1	Yes	$MINIMAL_{Lys}$	$MINIMAL_{Lys}$	$MINIMAL_{Lys}$	Yes
CHEM 22	2.3	0.4	0.5	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 23	4.0	3.2	4.3	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 24	1.3	0.0	0.6	Yes	MINIMAL	MINIMAL	MINIMAL	Yes

[A] Values represent the mean cysteine and lysine peptide depletion. In case of co-elution with the lysine peptide (indicated by the LYS subscript in the columns with the reactivity classes) the value represents the mean cysteine peptide depletion. Mean depletion values with an orange background correspond to a sensitiser prediction, those with a green background correspond to a non-sensitiser prediction.

3.2.3.2 Reproducibility of the depletion values for cysteine and lysine

The mean cysteine and lysine peptide depletions for each chemical were compared between the three independent experiments by one-way ANOVA. The results for the cysteine depletion are presented in Table 11 and the results for lysine depletion are presented in Table 12. One-way ANOVA yielded significant differences in mean cysteine depletion between the three independent experiments for four chemicals (Table 11). For the lysine depletion, ANOVA revealed significant differences in mean peptide depletion for four chemicals (Table 12), two of them showed also significant differences in mean cysteine depletion (chemical 12 and 18). For chemical 17, the significant difference in mean cysteine depletion resulted in different S/NS prediction. For chemical 18 the significant difference in mean cysteine and lysine depletion resulted in the assignment of a different reactivity class.

Table 11 Phase B2 IVMU: within laboratory variability of the cysteine depletion

Chemical	Cysteine depletion (%)			Cysteine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	93.5 ± 0.2	93.0 ± 0.5	93.5 ± 1.3	93.4 ± 0.3	0.702
CHEM 11	2.6 ± 2.2	3.0 ± 3.9	2.4 ± 2.6	2.7 ± 0.3	0.962
CHEM 12	37.3 ± 1.2 ^{B}	30.5 ± 0.8 ^{A}	36.7 ± 0.5 ^{B}	34.8 ± 3.8	<0.001*
CHEM 13	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 14	8.5 ± 3.2	3.9 ± 3.8	11.4 ± 2.6	7.9 ± 3.8	0.077
CHEM 15	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
CHEM 16	4.5 ± 3.8	0.0 ± 0.0	2.4 ± 2.4	2.3 ± 2.2	0.185
CHEM 17	13.2 ± 4.1 ^{B}	0.0 ± 0.0 ^{A}	3.9 ± 3.6 ^{A}	5.7 ± 6.8	0.005*
CHEM 18	51.8 ± 3.4 ^{B}	33.5 ± 3.0 ^{A}	33.0 ± 2.3 ^{A}	39.5 ± 10.7	<0.001*
CHEM 19	1.0 ± 1.3	0.0 ± 0.0	0.4 ± 0.8	0.5 ± 0.5	0.390
CHEM 20	1.5 ± 1.8 ^{A}	0.0 ± 0.0 ^{A}	9.3 ± 2.4 ^{B}	3.6 ± 5.0	0.001*
CHEM 21	2.4 ± 2.1	0.0 ± 0.0	2.1 ± 2.2	1.5 ± 1.3	0.281
CHEM 22	4.6 ± 3.5	0.7 ± 1.3	1.0 ± 1.0	2.1 ± 2.2	0.135
CHEM 23	8.1 ± 4.9	6.3 ± 5.9	8.5 ± 5.8	7.6 ± 1.1	0.883
CHEM 24	2.7 ± 2.3	0.0 ± 0.0	1.1 ± 1.5	1.3 ± 1.3	0.198

Values presented as mean ± SD, n=3

[1] One-way ANOVA critical α-level = 0.027 (corrected for the number of hypothesis tested), *mean values with a different subscript are significantly different from each other (Tukey post-hoc all comparisons)

NA: ANOVA testing not performed since all individual values were equal

Table 12 Phase B2 IVMU: within laboratory variability of the lysine depletion

Chemical	Lysine depletion (%)			Lysine depl (%)	p-value[1]
	Experiment 2	Experiment 3	Experiment 4	Between experiment	
CHEM 10	0.0 ± 0.0	0.0 ± 0.0		0.0 ± 0.0	NA
CHEM 11	0.9 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5	0.081
CHEM 12	1.8 ± 0.2 ^{C}	0.3 ± 0.2 ^{A}	1.3 ± 0.1 ^{B}	1.1 ± 0.7	<0.001*
CHEM 13	59.0 ± 0.4 ^{B}	58.1 ± 0.9 ^{B}	54.2 ± 1.1 ^{A}	57.1 ± 2.5	<0.001*
CHEM 14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
CHEM 15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
CHEM 16	12.5 ± 1.8	11.4 ± 2.7	11.3 ± 0.9	11.7 ± 0.7	0.728
CHEM 17	5.6 ± 1.3	5.0 ± 1.8	6.0 ± 0.8	5.5 ± 0.5	0.682
CHEM 18	0.1 ± 0.2 ^{A}	0.0 ± 0.0 ^{A}	1.8 ± 0.2 ^{B}	0.6 ± 1.0	<0.001*
CHEM 19	24.6 ± 0.5 ^{B}	24.4 ± 0.6 ^{B}	22.9 ± 0.4 ^{A}	24.0 ± 1.0	0.009*
CHEM 20	0.2 ± 0.3	0.9 ± 0.3	0.2 ± 0.1	0.4 ± 0.4	0.038
CHEM 21					NA
CHEM 22	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
CHEM 23	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA
CHEM 24	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.1	0.079

Values presented as mean ± SD, n=3

[1] One-way ANOVA critical α-level = 0.027 (corrected for the number of hypothesis tested), *mean values with a different subscript are significantly different from each other (Tukey post-hoc all comparisons)

NA: ANOVA testing not performed since all individual values were equal

Chemicals with a grey background correspond with lysine co-elution

3.2.4 Conclusions

The main focus of the within laboratory reproducibility (WLR) for the subset of 15 chemicals in each laboratory was on the concordance of the predictions sensitiser (S) versus non-sensitiser (NS) between the three independent experiments. The WLR for P&G, Ricerca, and IVMU for the S/NS predictions was 73.3%, 100%, and 86.7% respectively. The average WLR for sensitiser (S) versus non-sensitiser (NS) from the three laboratories was 87%. When 4 reactivity classes were considered, the WLR was 66.7% for P&G, 100% for Ricerca, and 73.3 for IVMU% with an average WLR of 80% for the three laboratories. Notably, in case of inconsistency in the assignment to a reactivity class, the difference was never by more than one class.

Furthermore, the results of the ANOVA showed that statistically significant differences in mean peptide depletion between the three independent runs within each laboratory did not always have an impact on the final classifications of the chemicals or on the assigned reactivity class.

3.3 Between laboratory reproducibility

The between laboratory reproducibility (BLR) was assessed on the basis of the data of the 24 chemicals: a subset of 9 chemicals was tested once in each laboratory (Phase B1 – Experiment 1) and a subset of 15 chemicals was tested 3 times in each laboratory (Phase B2 – Experiment 2, 3, and 4). The main focus of the evaluation of the between-laboratory reproducibility was on the concordance of the predictions sensitisers (S) versus non-sensitisers (NS) and for the assignment to one of the four reactivity classes. As discussed in the WLR section, descriptive and inferential statistical analyses were also performed on the raw peptide depletion data (ANOVA) but because of the small samples size (three replicates) they were only used in an exploratory way.

3.3.1 Concordance in predictions

For the evaluation of the BLR, the final prediction for the chemicals that were tested three times (chemicals 10 to 24) in each laboratory was based on the classification obtained using the median depletion values (Table 13). For example for chemical 18 (P&G) the depletion values were 1.9, 6.6, and 7.9 (Table 14). The median equals 6.6 which corresponds to a sensitiser (S) classification (Table 13) and the assignment to a low reactivity class.

Eighteen of the 24 chemicals were consistently classified (S/NS) by the three laboratories resulting in a BLR of 75%. The BLR for the pair-wise comparisons was 87% for P&G/Ricerca (13/15 chemicals), and 67% for P&G/IVMU and Ricerca/IVMU (10/15 chemicals). For 15 out of the 24 chemicals the laboratories assigned the same reactivity class resulting in a BLR of 62.5%. As for the within-laboratory reproducibility in case of disagreement, the difference in the reactivity class assignment was only of one class (Table 13, right side).

An overview of the mean peptide depletions for the individual experiments and the assigned reactivity classes (4 classes) is shown in Table 14. Note that for the 15 chemicals tested three times the assigned reactivity classes were always the same or one-off with the only exception chemical 18 which was assigned to three different classes (minimal, low and moderate reactivity).

Table 13 Phase B1: concordance in S versus NS predictions and in assignment of reactivity class between the laboratories

Chemical	Peptide depletion (%)[A]			Agreement 2 classes	4 reactivity classes			Agreement 4 classes
	P&G	Ricerca	IVMU		P&G	Ricerca	IVMU	
	Mean of 1 experiment				Mean of 1 experiment			
CHEM 1	92.3	99.4_{LYS}	92.5	Yes	HIGH	$HIGH_{LYS}$	HIGH	Yes
CHEM 2	53.6	52.3_{CL}	65.0	Yes	HIGH	$HIGH_{CL}$	HIGH	Yes
CHEM 3	4.4	4.0_{LYS}	9.2	No	MINIMAL	MINIMAL	LOW	No
CHEM 4	23.7_{LYS}	17.0_{CL}	16.7_{CL}	Yes	$MODERATE_{LYS}$	$\geq LOW_{CL}$	$\geq LOW_{CL}$	No
CHEM 5	42.1	38.5	31.9	Yes	MODERATE	MODERATE	MODERATE	Yes
CHEM 6	12.3	29.8_{CL}	23.7	Yes	LOW	$\geq MODERATE_{CL}$	MODERATE	No
CHEM 7	1.7	0.6	0.9	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 8	3.9	3.3	1.7	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 9	1.4	1.1	14.0	No	MINIMAL	MINIMAL	LOW	No
	Median of 3 experiments				Median of 3 experiments			
CHEM 10	100.0_{LYS}	53.3	46.8	Yes	$HIGH_{LYS}$	HIGH	HIGH	Yes
CHEM 11	12.3	1.1	1.5	No	LOW	MINIMAL	MINIMAL	No
CHEM 12	22.2	26.0	19.0	Yes	LOW	MODERATE	LOW	No
CHEM 13	100.0_{LYS}	100.0	79.0_{LYS}	Yes	$HIGH_{LYS}$	$HIGH_{LYS}$	HIGH	Yes
CHEM 14	3.3	2.7	4.3	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 15	100.0_{LYS}	51.3	50.0	Yes	$HIGH_{LYS}$	HIGH	HIGH	Yes
CHEM 16	3.9	0.2	6.9	No	MINIMAL	MINIMAL	LOW	No
CHEM 17	3.1	1.0	4.9	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 18	6.6	9.2	17.4	Yes	LOW	LOW	LOW	Yes
CHEM 19	5.1	0.8	12.2	No	MINIMAL	MINIMAL	LOW	No
CHEM 20	5.2	0.8	0.9	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 21	5.1_{LYS}	2.5_{LYS}	2.1	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 22	5.6	1.1	0.5	Yes	MINIMAL	MINIMAL	MINIMAL	Yes
CHEM 23	0.9	10.2	4.0	No	MINIMAL	LOW	MINIMAL	No
CHEM 24	5.2	0.4	0.6	Yes	MINIMAL	MINIMAL	MINIMAL	Yes

[A] Values represent the mean peptide depletion (Chemical 1 to 9) or median peptide depletion of 3 experiments (chemical 10 to 24). In case of co-elution with the lysine peptide (indicated by the LYS subscript in the columns with the reactivity classes) the value represents the cysteine peptide depletion. CL indicates co-elution with both peptides. Mean depletion values with an orange background correspond with an S prediction, those with a green background correspond with an NS prediction.

Table 14 Concordance in reactivity class (4 classes) within and between laboratories

Chemical	Mean peptide depletion (%)[A]									Agreement
	P&G			Ricerca			IVMU			
	Exp 1/2	Exp 3	Exp 4	Exp 1/2	Exp 3	Exp 4	Exp 1/2	Exp 3	Exp 4	4 classes
CHEM 1	92.3			99.4_{LYS}			92.5			Yes
CHEM 2	53.6			52.3_{CL}			65.0			Yes
CHEM 3	4.4			4.0_{LYS}			9.2			No
CHEM 4	23.7_{LYS}			17.0_{CL}			16.7_{CL}			No
CHEM 5	42.1			38.5			31.9			Yes
CHEM 6	12.3			29.8_{CL}			23.7			No
CHEM 7	1.7			0.6			0.9			Yes
CHEM 8	3.9			3.3			1.7			Yes
CHEM 9	1.4			1.1			14.0			No
CHEM 10	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	53.3	52.6	54.1	46.8	46.5	93.5_{LYS}	Yes
CHEM 11	10.5	12.3	15.9	1.1	0.4	1.4	1.7	1.5	1.2	No
CHEM 12	24.5	19.9	22.2	26.0	26.9	25.3	19.5	15.4	19.0	No
CHEM 13	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	79.5	79.0	77.1	Yes
CHEM 14	3.3	3.3	2.8	2.0	3.7	2.7	4.3	2.0	5.7	Yes
CHEM 15	100.0_{LYS}	100.0_{LYS}	100.0_{LYS}	51.2	51.3	50.8	50.0	50.0	50.0	Yes
CHEM 16	3.9	2.2	7.9	0.2	0.0	0.3	8.5	5.7	6.9	No
CHEM 17	5.1	1.6	3.1	1.0	1.4	0.3	9.4	2.5	4.9	Yes
CHEM 18	7.9	1.9	6.6	7.1	10.2	9.2	26.0	16.8	17.4	Yes
CHEM 19	5.1	3.1	7.1	0.8	0.9	0.7	12.8	12.2	11.6	No
CHEM 20	5.2	3.6	5.8	0.4	0.8	3.1	0.9	0.4	4.8	Yes
CHEM 21	6.3_{LYS}	1.1_{LYS}	5.1_{LYS}	2.5	0.8	2.5	2.4_{LYS}	0.0_{LYS}	2.1_{LYS}	Yes
CHEM 22	5.6	2.4	5.6	2.4	1.1	0.4	2.3	0.4	0.5	Yes
CHEM 23	0.6	1.0	0.8	7.0	10.2	11.5	4.0	3.2	4.3	No
CHEM 24	5.2	2.3	6.9	0.4	0.3	0	1.3	0.0	0.6	Yes

[A] Values represent the mean peptide depletion. In case of co-elution with the lysine peptide (indicated by the LYS subscript in the columns with the reactivity classes) the value represents the cysteine peptide depletion. CL indicates co-elution with both peptides. The background of the mean depletion values corresponds with the following reactivity classes: dark orange = severe, orange = moderate, light orange = low and green = minimal reactivity.

3.3.2 Within and between laboratory reproducibility of the depletion values for cysteine and lysine

Summary statistics for the mean cysteine and lysine depletion are shown in Table 15 and Table 16. The mean peptide depletions for chemical 1 to chemical 9 were compared between the three laboratories by one-way ANOVA. Two-way ANOVA was used for the evaluation of the within and between laboratory reproducibility of the peptide depletions values for the chemicals that were tested three times independently in each laboratory (chemical 10 to 24).

Two-way ANOVA revealed a significant interaction between the experiments and laboratories, for two chemicals in the mean cysteine peptide depletion values and for two chemicals in the mean lysine peptide depletion values. This means that the mean peptide depletion values of the three independent experiments may differ from each other in one lab, whereas in another lab the mean values are more or less of the same magnitude. Therefore the overall mean values cannot be compared as such between the laboratories (main effect of the laboratory). Chemical 18 for example at P&G resulted in a mean cysteine depletion of 15.5% and 12.2% in 2 experiments whereas the mean of experiment 3 was 2.5% (Table 15). The mean cysteine depletions for Ricerca were closer to each other (13.6%, 18.8%, and 18.4%) and for IVMU a higher variability was observed within the lab (51.8%, 33.5%, and 33%). There was also substantial variability in mean cysteine depletion between the labs. This variability within and between the laboratories was also reflected in the assignment of the reactivity classes of chemical 18 to three different classes (minimal, low and moderate reactivity). When the interaction is not significant, the mean depletion values between the laboratories can compared (main effect of the laboratory).

For 9 chemicals no significant differences were observed between the mean cysteine depletions obtained by the different laboratories. Chemical 13 and chemical 15 resulted in 100% depletion in all experiments with one exception; therefore ANOVA testing was not appropriate.

The lysine depletion values were generally very low for the different chemicals (Table 16). The variability within the experiments was also low in the majority of the cases which resulted in significant differences between and within the laboratories for 17 chemicals. For 4 chemicals no significant differences were observed between the mean lysine depletions obtained by the different laboratories. Chemical 14 resulted in 0% depletion in all experiments with one exception; therefore ANOVA testing was not appropriate.

3.3.3 Conclusions

The main focus of the between laboratory reproducibility (BLR) for the 24 chemicals was on the concordance of the predictions sensitisers (S) versus non-sensitisers (NS) between the three laboratories. The BLR for the S/NS prediction was 75%. When 4 reactivity classes where considered the BLR was 62.5%

Statistical significant differences in mean peptide depletion between or within and between laboratories were observed for 13 chemicals (cysteine depletion) and 17 chemicals (lysine depletion) of the 24 chemicals. These differences in mean peptide depletion did not always correspond with differences in reactivity class.

Table 15 Within and between laboratory variability of the cysteine depletion

Chemical	P&G									Ricerca										IVMU										Between lab		p-value	
	Exp 1			Exp 2		Exp 3		Exp 4		Exp 1			Exp 2		Exp 3		Exp 4			Exp 1			Exp 2		Exp 3		Exp 4						
	Mean	SD		Mean	SD	Mean	SD	Mean	SD	Mean±	SD		Mean	SD	Mean	SD	Mean	SD		Mean±	SD		Mean	SD	Mean	SD	Mean	SD			Mean	SD		BL[1]
CHEM 1	94.8	6.7								99.4	0.1									99.9	0.2										98.0	2.8		0.28
CHEM 2	92.3	10.1								85.1	10									100	0.0										92.5	7.5		0.173
CHEM 3	6.9	6.1								4.0	1.2									11.2	5.2										7.4	3.6		0.248
CHEM 4	23.7	5.3								12.3	3.8										(n=1)										18.0	8.1		0.037 (t)
CHEM 5	58.5	5.1	B							55.9	1.1	B								47.8	2.8	A									54.1	5.6		0.020*
CHEM 6	21.0	9.6	A							45.1	4.4	B								42.1	4.5	B									36.1	13.1		0.008*
CHEM 7	2.5	4.4								1.2	0.5									1.8	1.6										1.8	0.7		0.831
CHEM 8	6.9	4								6.5	1.5									3.5	3.9										5.6	1.9		0.432
CHEM 9	2.8	3.8								2.0	0.9									2.3	2.5										2.4	0.4		0.924
	Between exp									Between exp										Between exp												Interact[2]	BL[2]
CHEM 10	100	0.0	B	100	0.0	100	0.0	100	0.0	100	0.0	B	100	0.0	100	0.0	100	0.0		93.3	0.3	A	93.5	0.2	93.0	0.5	93.5	1.3		97.8	3.8	0.823	<0.001*
CHEM 11	25.5	5.2	B	20.8	10.4	24.6	5.5	31.0	7.6	1.8	1.0	A	2.0	2.2	0.7	1.3	2.6	1.9		2.7	0.3	A	2.6	2.2	3.0	3.9	2.4	2.6		10.0	13.4	0.428	<0.001*
CHEM 12	40.6	4.1	Int	44.7	1.0	36.6	1.2	40.6	1.2	49.9	1.4	Int	49.6	1.6	51.4	2.0	48.6	2.8		34.8	3.8	Int	37.3	1.2	30.5	0.8	36.7	0.5		41.8	7.6	<0.001*	
CHEM 13	100	0.0		100	0.0	100	0.0	100	0.0	100	0.0		100	0.0	100	0.0	100	0.0		100	0.0		100	0.0	100	0.0	100	0.0		100	0.0	NA	
CHEM 14	6.2	0.6		6.6	8.2	6.4	11	5.5	5.2	5.6	1.8		3.9	2.4	7.4	1.9	5.4	4.4		7.9	3.8		8.5	3.2	3.9	3.8	11.4	2.6		6.6	1.2	0.560	0.640
CHEM 15	100	0.0		100	0.0	100	0.0	100	0.0	99.6	0.8		100	0.0	98.7	2.2	100	0.0		100	0.0		100	0.0	100	0.0	100	0.0		99.9	0.3	NA	
CHEM 16	8.3	5.8	B	7.8	7.2	2.8	4.2	14.4	6.1	0.3	0.4	A	0.2	0.3	0.0	0.1	0.7	1.2		2.3	2.3	A	4.5	3.8	0.0	0.0	2.4	2.4		3.6	4.2	0.115	<0.001*
CHEM 17	5.9	4.0	B	10	9.8	2.0	3.4	5.8	5.9	1.5	0.9	A	1.4	1.3	2.5	1.6	0.7	1.2		5.7	6.8	B	13.2	4.1	0.0	0.0	3.9	3.6		4.4	2.5	0.129	<0.001*
CHEM 18	10.1	6.8	Int	15.5	4.1	2.5	2.4	12.2	6.2	16.9	2.9	Int	13.6	2.0	18.8	2.5	18.4	1.1		39.4	10.7	Int	51.8	3.4	33.5	3.0	33.0	2.3		22.1	15.4	<0.001*	
CHEM 19	7.9	4.1	B	9.1	8.0	3.4	5.1	11.3	4.9	0.3	0.1	A	0.3	0.6	0.2	0.4	0.4	0.6		0.5	0.5	A	1.0	1.3	0.0	0.0	0.4	0.8		2.9	4.4	0.344	<0.001*
CHEM 20	8.6	3.1	B	9.9	9.2	5.0	5.8	10.8	4.0	2.5	3.2	A	0.3	0.5	1.0	1.0	6.1	1.2		3.6	5.0	AB	1.5	1.8	0.0	0.0	9.3	2.4		4.9	3.2	0.539	0.011*
CHEM 21	4.2	2.7		6.3	8.3	1.1	2.0	5.1	4.6	0.4	0.3		0.7	0.8	0.4	0.7	0.2	0.4		1.5	1.3		2.4	2.1	0.0	0.0	2.1	2.2		2.0	1.9	0.772	0.083
CHEM 22	8.4	3.9	B	10.7	8.4	3.9	4.2	10.6	8.1	2.3	2.0	A	4.6	2.1	1.4	1.2	0.9	1.4		2.1	2.2	A	4.6	3.5	0.7	1.3	1.0	1.0		4.3	3.6	0.660	0.011*
CHEM 23	0.4	0.7	A	1.2	2.0	0.0	0.0	0.0	0.0	19.1	4.6	C	13.9	4.7	20.4	5.7	22.9	6.2		7.6	1.2	B	8.1	4.9	6.3	5.9	8.5	5.8		9.0	9.4	0.325	<0.001*
CHEM 24	8.1	5.0	B	9.6	8.4	2.5	4.1	12.1	7.6	0.3	0.3	A	0.5	0.9	0.3	0.6	0.0	0.0		1.3	1.4	A	2.7	2.3	0.0	0.0	1.1	1.5		3.2	4.2	0.299	0.001*

[1]One-way or [2]two-way ANOVA critical α-level = 0.024 (corrected for number of hypothesis tested), BL: between laboratories, mean values with a different capital letter (A, B, C) are significantly different BL (Tukey post-hoc all comparisons); Int: interaction, the main effect of the laboratory cannot be interpreted separately because there was a significant interaction between laboratory and experiment; NA: ANOVA testing not performed since all individual values were equal; Chemicals with a grey background correspond with cysteine co-elution

Table 16 Within and between laboratory variability of the lysine depletion

Chemical	P&G									Ricerca										IVMU										Between lab		p-value	
	Exp 1			Exp 2		Exp 3		Exp 4		Exp 1			Exp 2		Exp 3		Exp 4			Exp 1			Exp 2		Exp 3		Exp 4						
	Mean	SD		Mean	SD	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD		Mean	SD		Mean	SD	Mean	SD	Mean	SD			Mean	SD		BL[1]
CHEM 1	89.7	0.5	B							13.2	2.5	A								85.0	4.8	B									62.6	42.9		<0.001*
CHEM 2	14.9	3.4	A							19.6	0.9	A								29.9	3.4	B									21.5	7.7		0.002*
CHEM 3	1.8	1.9	A							3.3	0.3	A								7.1	1.6	B									4.1	2.7		0.011*
CHEM 4	28.4	2.3	B							21.8	1.4	A								33.4	1.1	C									27.9	5.8		<0.001*
CHEM 5	25.7	0.5	C							21.1	0.4	B								15.9	0.9	A									20.9	4.9		<0.001*
CHEM 6	3.7	2.5	A							14.5	0.4	B								5.3	0.7	A									7.8	5.9		<0.001*
CHEM 7	0.8	0.7								0.0	0.1									0.1	0.2										0.3	0.4		0.129
CHEM 8	0.8	1.4								0.0	0.0									0.0	0.0										0.3	0.5		0.422
CHEM 9	0.0	0.0	A							0.1	0.2	A								25.6	0.6B	B									8.6	14.8		<0.001*
	Between exp									Between exp										Between exp												Interact[2]	BL[2]
CHEM 10	6.5	4.1	B	4.8	5.0	11.1	5.4	3.5	3.6	6.7	1.5	B	6.6	0.7	5.2	0.8	8.2	0.4		0.0	0.0	A	0.0	0.0	0.0	0.0					4.4	3.8	0.056	0.006*
CHEM 11	0.3	0.4		0.1	0.1	0.0	0.0	0.8	1.3	0.1	0.1		0.1	0.1	0.1	0.1	0.2	0.1		0.3	0.5		0.9	0.8	0.0	0.0	0.0	0.0			0.2	0.1	0.150	0.747
CHEM 12	3.8	0.6	B	4.3	4.7	3.2	1.2	3.8	1.5	2.3	0.3	A	2.3	0.3	2.5	0.4	2.0	0.3		1.1	0.8	A	1.8	0.2	0.3	0.2	1.3	0.1			2.4	1.3	0.926	0.015*
CHEM 13																				57.1	2.6		59.0	0.4	58.1	0.9	54.2	1.1			57.1		NA	
CHEM 14	0.0	0.1		0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0			0.0	0.0	NA	
CHEM 15	7.1	3.1	B	3.5	2.8	8.7	5.2	9.0	6.7	2.6	1.2	A	2.3	0.5	3.9	0.2	1.6	0.2		0.0	0.0	A	0.0	0.0	0.0	0.0	0.0	0.0			3.2	3.6	0.348	<0.001*
CHEM 16	1.0	0.9	A	0.0	0.0	1.6	1.3	1.5	0.7	0.0	0.1	A	0.1	0.1	0.0	0.0	0.0	0.0		11.7	0.7	B	12.5	1.8	11.4	2.7	11.3	0.9			4.3	6.5	0.355	<0.001*
CHEM 17	0.5	0.5	A	0.2	0.4	1.1	1.2	0.3	0.3	0.3	0.3	A	0.6	0.1	0.2	0.3	0.0	0.0		5.5	0.5	B	5.6	1.3	5.0	1.8	6.0	0.8			2.1	3.0	0.393	<0.001*
CHEM 18	0.9	0.5		0.3	0.5	1.3	0.9	1.0	0.4	0.7	0.8		0.5	0.3	1.5	1.0	0.0	0.1		0.6	1.0		0.1	0.2	0.0	0.0	1.8	0.2			0.7	0.1	<0.001*	
CHEM 19	2.3	1.1		1.0	0.8	2.9	1.5	3.0	0.2	1.3	0.3		1.3	0.3	1.6	0.3	1.0	0.0		24.0	0.9		24.6	0.5	24.4	0.6	22.9	0.4			9.2	12.8	<0.001*	
CHEM 20	1.2	1.0		0.5	0.8	2.3	3.2	0.7	0.6	0.4	0.3		0.5	0.1	0.6	0.5	0.0	0.0		0.4	0.4		0.2	0.3	0.9	0.3	0.2	0.1			0.7	0.4	0.747	0.253
CHEM 21										3.4	1.9		4.2	0.7	1.2	0.5	4.7	0.5													3.4		NA	
CHEM 22	0.6	0.3	B	0.4	0.7	0.9	0.6	0.6	0.2	0.3	0.4	A	0.1	0.1	0.7	0.4	0.0	0.0		0.0	0.0	A	0.0	0.0	0.0	0.0	0.0	0.0			0.3	0.3	0.384	0.003*
CHEM 23	1.2	1.0	B	0.1	0.2	2.0	2.3	1.5	1.8	0.0	0.0	A	0.0	0.0	0.0	0.1	0.0	0.0		0.0	0.0	A	0.0	0.0	0.0	0.0	0.0	0.0			0.4	0.7	0.422	0.025*
CHEM 24	1.5	0.7	B	0.8	1.4	2.1	1.9	1.6	1.5	0.1	0.1	A	0.2	0.1	0.2	0.2	0.0	0.0		0.0	0.1	A	0.0	0.0	0.0	0.0	0.1	0.1			0.6	0.8	0.717	0.006*

[1]One-way or [2]two-way ANOVA critical α-level = 0.024 (corrected for number of hypothesis tested), BL: between laboratories, mean values with a different capital letter (A, B, C) are significantly different BL (Tukey post-hoc all comparisons); NA: ANOVA testing not performed since all individual values were equal; Chemicals with a grey background correspond with lysine co-elution

3.4 Predictive capacity

An overview of the predicted classification and the reference classification is presented in Table 17. The predictive capacity was assessed separately for each laboratory (Table 18). For chemicals that were tested 3 times in a laboratory, the median was selected as the final conclusion for each laboratory, i.e. for example in the case of chemical 18 (P&G) that was two times predicted S and once NS by P&G, the final prediction was S. The sensitivity and specificity for P&G and Ricerca were 68.7% and 100%, respectively. Twenty of the 24 chemicals were predicted the same by P&G and Ricerca. The sensitivity and specificity for IVMU was 75%.

Three of the 24 chemicals (chemical 3, 11, and 23) selected for the current study were known when the test chemicals were selected to fall outside the applicability domain of the DPRA. Therefore, the analysis of the predictive capacity in relation to the putative applicability domain was done after discarding test data on these chemicals (Table 19). This resulted in an overall sensitivity (cumulative over the 3 labs) of 79.5%, a specificity of 91.5% and an accuracy of 84.1%. The sensitivity and specificity for P&G and Ricerca were 76.9% and 100%, respectively. The sensitivity and specificity for IVMU were 84.6% and 75% respectively.

Note that these numbers are for a total of 63 results, representing the results from the 21 chemicals in each of the three laboratories. Alternatively, the predictive parameters were calculated by assigning the median classification of the three laboratories to each chemical. The respective results are shown in table 20. In this case the overall accuracy was 85.7% with a sensitivity of 76.9% and a specificity of 100%. While the accuracies of both approaches are very similar, sensitivity and specificity are balanced differently. While the median approach has the advantages that it maintains the sample size of 21 and that it allows calculating confidence intervals, it reduces the available information to some extent. The cumulative approach increases the sample size by considering the individual, but dependent laboratory classifications per chemical. Therefore, confidence intervals are not reported.

Furthermore, for the previously tested substances (i.e. chemicals reported as being previously tested by the DPRA in the original submission to ECVAM) 6 sensitizers (which correspond with 36 studies) were always identified correctly (100%) and 3 non-sensitizers (which correspond with 21 studies) were predicted correctly in 81% of the studies. The accuracy of the predictions based on majority voting was 100% (9/9). Focusing on the chemicals that were not tested previously, the accuracy for majority voting was 75% (9/12). The five non-sensitisers (which correspond with 33 studies) were correctly identified in 97% of the studies. Three of the seven sensitisers were classified as non-sensitiser, in fact a sensitizers which was not tested previously, was identified correctly in 47% of the studies (51 studies).

Table 17 Agremeent between the predicted class and the reference class

Chemical	Test prev[A]	Reference	Major[B]	P&G				Ricerca				IVMU			
				Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
CHEM 1	Y	+ (1A)	S	S				S_{LYS}				S			
CHEM 2	Y	+ (1A)	S	S				S_{CL}				S			
CHEM 3[C]	N	+ (1B)	NS	NS				NS_{LYS}				S			
CHEM 4	N	+ (1B)	S	S_{LYS}				S_{CL}				S_{CL}			
CHEM 5	Y	+ (1B)	S	S				S				S			
CHEM 6	N	+ (1B)	S	S_{CL}				S_{CL}				S			
CHEM 7	Y	- (NC)	NS	NS				NS				NS			
CHEM 8	N	- (NC)	NS	NS				NS				NS			
CHEM 9	N	- (NC)	NS	NS_{LYS}				NS				S			
				Major[B]				Major[B]				Major[B]			
CHEM 10	Y	+ (1A)	S	S	S_{Lys}	S_{Lys}	S_{Lys}	S	S	S	S	S	S	S	S
CHEM 11[C]	N	+ (1A)	NS	S	S	S	S	NS	NS	NS	NS	NS	NS	NS	NS
CHEM 12	Y	+ (1A)	S	S	S	S	S	S	S	S	S	S	S	S	S
CHEM 13	N	+ (1A)	S	S	S_{Lys}	S_{Lys}	S_{Lys}	S	S_{Lys}	S_{Lys}	S_{Lys}	S	S	S	S
CHEM 14	N	+ (1A)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHEM 15	Y	+ (1A)	S	S	S_{Lys}	S_{Lys}	S_{Lys}	S	S	S	S	S	S	S	S
CHEM 16	N	+ (1B)	NS	NS	NS	NS	S	NS	NS	NS	NS	S	S	NS	S
CHEM 17	N	+ (1B)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	S	NS	NS
CHEM 18	N	+ (1B)	S	S	S	NS	S	S	S	S	S	S	S	S	S
CHEM 19	Y	- (NC)	NS	NS	NS	NS	S	NS	NS	NS	NS	S	S	S	S
CHEM 20	Y	- (NC)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHEM 21	N	- (NC)	NS	NS	NS_{Lys}	NS_{Lys}	NS_{Lys}	NS	NS	NS	NS	NS	NS_{Lys}	NS_{Lys}	NS_{Lys}
CHEM 22	N	- (NC)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CHEM 23[C]	N	+ (NA)	NS	NS	NS	NS	NS	S	S	S	S	NS	NS	NS	NS
CHEM 24	N	- (NA)	NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS	NS	NS

[A] Indication if the chemical was tested previously (Y: yes) or not (N: no)

[B] final predicted class based on majority voting

[C] outside the applicability domain

LYS subscript or CL subscript corresponds with co-elution for Lysine and co-elution with CYS/LYS

Table 18 Overall predictive capacity of the DPRA (cumulative over the three laboratories) and predictive capacity for each laboratory

Reference result	Cumulative		P&G		Ricerca		IVMU	
	+	-	+	-	+	-	+	-
+ (n=16)	34	14	11	5	11	5	12	4
- (n=8)	2	22	0	8	0	8	2	6
Total	36	36	11	13	11	13	14	10
Sensitivity (95% CI[A])	70.8		68.7 (44.4-85.8)		68.7 (44.4-85.8)		75 (50.5-89.8)	
Specificity (95% CI[A])	91.7		100 (67.6-100)		100 (67.6-100)		75 (40.9-92.9)	
Accuracy	77.8		79.2		79.2		75	

[A] Wilson confidence interval based on the score test

Appendix 15

Table 19 Overall predictive capacity of the DPRA (cumulative over the three laboratories) and predictive capacity for each laboratory for the chemicals falling into the applicability domain

Reference result	Cumulative		P&G		Ricerca		IVMU	
	+	-	+	-	+	-	+	-
+ (n=13)	31	8	10	3	10	3	11	2
- (n=8)	2	22	0	8	0	8	2	6
Total	33	30	10	11	10	11	13	8
Sensitivity (95% CI [A])	79.5		76.9 (49.7-91.8)		76.9 (49.7-91.8)		84.6 (57.8-95.7)	
Specificity (95% CI [A])	91.7		100 (67.6-100)		100 (67.6-100)		75 (40.9-92.9)	
Accuracy	84.1		85.7		85.7		81.0	

[A] Wilson confidence interval based on the score test

Table 20 Overall predictive capacity of the DPRA (considering the median of the results in each laboratory) for the chemicals falling into the applicability domain

Reference result	Median	
	+	-
+ (n=13)	10	3
- (n=8)	0	8
Total	10	11
Sensitivity (95% CI)	76.9 (49.7-91.8)	
Specificity (95% CI)	100 (67.6-100)	
Accuracy	85.7	

3.4.1 Conclusion

The accuracy of the DPRA (cumulative over the 3 labs), having removed the chemicals to fall outside the claimed applicability domain, was 84.1% with a sensitivity of 79.5% and specificity of 91.5%.

4 CONCLUSIONS

The main focus of the within and between laboratory reproducibility (WLR and BLR) was on the concordance of the predictions, sensitiser (S) versus non-sensitiser (NS). For the subset of 15 chemicals that were tested in three independent experiments in each laboratory, the average WLR for sensitiser (S) versus non-sensitiser (NS) from the three laboratories was 87%. When 4 reactivity classes were considered, the average WLR was 80% for the three laboratories. Notably, in case of inconsistency in the assignment to a reactivity class, the difference was never by more than one class. The BLR for the 24 chemicals for the S/NS prediction was 75%. When 4 reactivity classes where considered the BLR was 62.5%

The accuracy of the DPRA (cumulative over the 3 labs), after having removed the three chemicals that fall outside the claimed applicability domain, was 84.1% with a sensitivity of 79.5% and specificity of 91.5%.

5 REFERENCES

Agresti A. and Coull B.A. (1998). Approximate is better than 'exact' for interval estimation of binomial proportions. *The American Statistician* **52**, 119–126.

EU (2008b) Regulation (EC) No 1272/2008 (16 December 2008) of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. *Official Journal of the European Union **L 353**, (31/12/2008) p. 1-1355*.

Appendix 16

Additional available documents

List of additional documents filed for the study and available on request

1 VMT meetings minutes

Minutes from each VMT meetings, including the list of actions identified, were drafted by ECVAM and distributed to the whole VMT, including the liaisons and the lead laboratories representatives. When the meeting included a session on the chemical selection, which were closed to the lead laboratories representatives, the minutes for this session were prepared in a separate document, which was distributed only to the VMG and the liaisons.

Final minutes were drafted based on all the comments received, and approved in the early sessions of at the subsequent VMT meeting.

The final approved minutes are found in the folder \Minutes

Documents	Meeting held
SSPS_VMG_MIN_Meeting_1.pdf SSPS_VMG_MIN_Meeting_1_Chemical.pdf	30/9/2009-01/10/2009
SSPS_VMG_MIN_Meeting_2.pdf SSPS_VMG_MIN_Meeting_2_Chemical.pdf	02/12/2009-03/12/2009
SSPS_VMG_MIN_Meeting_3.pdf SSPS_VMG_MIN_Meeting_3_Chemical.pdf	12/01/2010-13/01/2010
SSPS_VMG_MIN_Meeting_4.pdf SSPS_VMG_MIN_Meeting_4_Chemical.pdf	24/06/2010-25/06/2010
SSPS_VMG_MIN_Meeting_5.pdf	18/11/2010-19/11/2010
SSPS_VMG_MIN_Meeting_6.pdf	24/03/2011-25/03/2011
SSPS_VMG_MIN_Meeting_7.pdf	30/06/2011-01/07/2011

2 Transfer reports

Transfer Reports were drafted by the trained laboratories once the lead laboratories had given them their approval that the method has been successfully transferred and the transfer results met the criteria included in the Transfer Plans. The reports described the transfer plan and the relevant transfer acceptance criteria, as well as the transfer results obtained. Any difficulties encountered during the transfer, and the measures implemented to overcome them, were also described.

These reports were sent for VMG for approval, as this approval was needed to allow the laboratory to proceed to the testing phase (Phase B).

Laboratory	Report received	Response sent	Final report received
Ricerca	29/06/2010	**Comments on report:** 15/07/2010 Ares(2010)430290 **Permission to proceed:** 14/10/2010 Ares(2010)696306	18/11/2010 Reports\Transfer Reports and Evaluations\AA89404 DPRA report Phase A_Stage II_v02.pdf
IVMU	02/03/2011	17/03/2011 Ares(2011)297773	21/03/2011 \Reports\Transfer Reports and Evaluations\IVMU_DPRA_Transfer_Study_report_version_01.pdf

3 Phase B Stage I and Stage II reports

Phase B Stage I reports were drafted by all testing laboratories following the completion of the testing of the first 9 chemicals. Phase B Stage II reports were drafted following the completion of the testing of the rest of the chemicals (15 chemicals tested three times each).

The reports contain a summary of the results obtained, and the final conclusions for each of the coded chemicals Any difficulties encountered during the testing, and the measures implemented to overcome them, should also be described.

The report itself were not requited to include all the filled reporting templates containing the results of the testing phase. ECVAM requested that these templates were sent regularly during the testing period in order to follow the progress of the testing and to perform the quality checks of the templates as they were generated. The correspondence of the reported summary results with the results sent in the reporting templates were checked by ECVAM prior to distribution of the reports to the VMG.

These reports were sent for VMG for approval, as approval of the Phase B Stage I report was needed to allow the laboratory to proceed to Phase B Stage II, and the approval of the Phase B Stage II report was needed to consider the testing completed at this testing laboratory.

Phase B Stage I reports:

Laboratory	Report received	Response sent	Final report received
P&G	21/12/2010	05/01/2011 Ares(2011)10620	N/A (no changes requested) \Reports\Phase B Stage I reports\DPRA\SSPS_DPRA_B1_PG_REPORT.pdf
Ricerca	23/12/2010	05/01/2011 Ares(2011)10701	19/01/2011 \Reports\Phase B Stage I reports\DPRA\AA89404 DPRA report Phase B_Stage I_v03.pdf
IVMU	31/05/2011	17/06/2011 Ares(2011)656108	21/06/2011 \Reports\Phase B Stage I reports\DPRA\ST-16_Final study report_signed.pdf

Phase B Stage II reports:

Laboratory	Report received	Response sent	Final report received

P&G	20/06/2011	30/06/2011 (VMT meeting)	07/12/2011 \Reports\Phase B Stage II reports\DPRA\P&G\SSPS_DPRA_B2_PG_REPORT_REVISED.pdf
Ricerca	10/06/2011	05/07/2011 Ares(2011)728666	26/09/2011 \Reports\Phase B Stage II reports\DPRA\Ricerca\AA89404 DPRA report Phase B_Stage II_v03.pdf
IVMU	16/11/2011	01/12/2011 Ares(2011)1293705	09/01/2012 \Reports\Phase B Stage II reports\DPRA\IVMU\Final report