DB illustrated the experimental design of the current study, discussed during the morning session: 24 (16+, 8-) chemicals for the BLR and 15 (10+, 5-) chemicals for the WLR, and that there was a solid statistical rationale behind those numbers.

The discussion then moved to debate about the exact scope of the study, and to where exactly the work of the current study stands in the vision of a full-fledged validation study. WS in particular commented that considerations should be taken to identify how much work will be missing after this prevalidation phase in order to fully validate the methods and the chemicals that will be required to do so. According to PA, The COLIPA perspective in developing these approaches was not to develop stand alone replacements but to understand how these methods would be combined in a battery of tests. He agreed to prepare a short presentation on Colipa's strategy for the next VMT meeting.

Session 8: Chemical Selection

The representatives from the lead laboratories were not present for the discussion on chemical selection for the validation study. A record of the presentation and discussion on chemical selection will be provided to the VMG members only in a separate appendix to the meeting minutes that will not be posted on the shared section of the CIRCA website.

Summary of Actions

	Responsible	Action	Deadline
1	Kao Shiseido	Clarify that the aim of the method is to test chemicals in the SOP and revise the SOP to incorporate troubleshooting	5/01/2010
2	Kao Shiseido	Include in the SOP a section on EC150 and EC200 (how to calculate them, how to report them...)	5/01/2010
3	ECVAM	Revise the h-CLAT SOP with tracked changes and send to TA and HS	1/3/2010
4	L'Oreal	Modify the table on page 2 of the SOP to make the time schedule more general + general SOP corrections	5/01/2010
5	ECVAM	provide as a starter the list for materials and methods done for the call for tender to JMO	4/12/2009
6	L'Oreal	Provide and present analysis of data according to GHS classification 1A and 1B	12/01/2010
7	ECVAM	assess concordance in the in vivo classification between the three tests for GHS	12/01/2010
8	ECVAM	revise the MUSST SOP with tracked changes and send to JMO	1/3/2010
9	ECVAM	check the results of Action 18 of first meeting and comment before next meeting.	5/01/2010
10	ECVAM	harmonize "pre-validation" or "prevalidation" in the study plan	10/12/2009
11	ECVAM	have internal discussions about a transparent explanation about the choice of MUSST instead of h-CLAT as the method validated in-house.	12/01/2010

12	All labs	communicate the names of study directors, quality assurance and safety officers	5/01/2010
13	L'Oreal	find a study director different from the quality assurance officer	5/01/2010
14	ECVAM	replace the words "should" with the words "shall" in the spirit of GLP minimum requirements	5/01/2010
15	Lead laboratories	Prepare a transfer protocol/plan	5/01/2010
16	ECVAM	Find an example of transfer protocol	11/12/2009

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

3rd Validation Management Team Meeting minutes

January 12th and 13th 2010, ECVAM, Ispra, Italy

Meeting participants:

David Basketter (DB; chair), Silvia Casati (SC; co-chair), Alexandre Angers (AA; ECVAM representative), Pierre Aeby (PA; external expert), , Thomas Cole (TC; chair of the Chemicals Selection Group (CSG)), Jon Richmond (JR; external expert), Andre Kleensang (AK; ECVAM biostatistician), Anna Compagnoni (AC, ECVAM biostatistician), Frank Gerberick (FG; DPRA lead laboratory representative), Takao Ashikaga (TA; h-CLAT lead laboratory representative), Jean-Marc Ovigne (JMO; MUSST lead laboratory representative), Abigail Jacobs (AJ; ICCVAM)

Sebastian Hoffmann (SH, external expert), Hitoshi Sakaguchi (HS; h-CLAT lead laboratory representative), William Stokes (WS, NICEATM), Dave Allen (DA; NICEATM) and Eleni Salicru (ES; NICEATM) participated at the meeting via teleconference during various sessions.

Day 1

Introduction

DB started the meeting by summarizing the current position and explained the aims of the meeting: the finalization of the protocols for the test methods, the preparation the forthcoming training and transfer phases, the introduction of the third laboratories involved, the drafting of the study timelines and the finalization of the chemical selection.

Session 1: Review of Minutes and Actions

No comments were made on the minutes of the 2nd VMT meeting and they were adopted. Going through the list of Actions showed that all of them have been addressed by the responsible parties.

Session 2: DPRA – Review of progress done

ECVAM has worked with FG and Leslie Foertsch (LF) to make a version of the DPRA SOP on which they agree. The main changes compared to the submitted version were

sections concerning the solubilisation procedures, the co-elution and the run sequences when using multiple solvents.

The only comment raised was on page 3 where acetonitrile is sometimes labeled "no trifluoroacetic acid" and sometimes "without trifluoroacetic acid"

Action (P&G): Harmonize this.

There was no other comment, and the VMG endorsed the DPRA SOP.

ECVAM suggested labeling the approved protocols as "ECVAM Skin Sensitisation Prevalidation Study Version 1" to dissociate the protocols from those used internally by the companies and, in the case of the DPRA, in the skin irritation validation study.

FG suggested that since ECVAM developed the reporting section, it would be appropriate for them to propose the first draft of the reporting template.

Action (ECVAM): Prepare a reporting template for the DPRA and send it to FG for comments.

Finally, FG clarified the names of the personnel involved in the study, to be included in the study plan. ECVAM requested further contact details information for each of these individuals, a comment that applies to all the laboratories involved.

Action (All laboratories): Send contact details for the personnel involved (Study Director, Quality Assurance Officer, Safety Officer and Experimental Team).

ECVAM will be in charge of obtaining these details from the contract laboratories, and to compile all the information in the Study Plan document.

Session 3: h-CLAT – Review of progress done

For this session, HS joined by teleconference.

ECVAM clarified that although they started to comment on the older version of the SOP, the elements of the newer version were incorporated when they received it, so the file that was sent to them should be up-to-date. The lead laboratories should now verify the comments and decide if they are appropriate. The aim would be to have a final SOP at the end of January, so that it can be approved by the VMG (through e-mail exchanges) by the middle of February

Action (ECVAM, Kao and Shiseido): Finalize the SOP for the h-CLAT for VMG approval

TA asked if the SOP could be released to other companies that approach them to learn the method. The VMT clarified that there were no reason why Kao and Shiseido should be

prevented to release the SOP, as long as the version is not officially labeled "ECVAM Skin Sensitisation Prevalidation Study" AJ suggest the addition of a disclaimer to this label that the SOP might change by the end of the validation study. JR suggested the disclaimer "not yet scientifically validated".

Session 4: MUSST – Review of progress done

JMO updated the situation for the MUSST SOP which, after a round of comments and teleconference discussions, is close to a final first version. He mentioned his desire to make a few final changes before submitting it for approval to the VMG.

Action (ECVAM): Send a modified version of the SOP to JMO so that he can make his final modifications

Concerning the assignment of GHS classes using the MUSST, there was a suggestion that a certain EC150 threshold could be established (17 μg/ml) to distinguish the chemicals which would belong to class 1A and 1B. In any case, more data are needed before any conclusion can be drawn.

JMO mentioned that the reporting template included in the submission is not the latest version, and that he has been working on it since.

Action (L'Oreal): send the updated version of the template to ECVAM for comments

TA clarified that the reporting template included in the h-CLAT submission is the latest version

Action (ECVAM): Look at the reporting templates and make comments/modifications

The need for a standard nomenclature for filing the results generated by the different laboratories was raised. ECVAM will develop this for Phase B Stages I and II.

Session 5: Project Plan Update

Experimental Design:

AA and AC discussed the changes made in the current version of the Experiment Design document (version 3), which include mainly the update in the calculations of sample size as it was discussed and agreed at the last meeting, and some biostatistics clarifications related to comments sent by ICCVAM/NICEATM in December.

FG demanded a clarification of the definitions of "success" we are expecting for this study, and was referred to the minutes of the previous meeting where this subject was discussed. AC offered to include a sentence in the Experimental Design which would

make explicit the expectations for reproducibility which are currently implicit in the calculations of the sample size.

Study plan:

At this point, SC introduced the third laboratories that will be involved in the study, the contracts being signed at the end of the last year. MDS Pharma will be performing the DPRA, and the CRO Bioassay will perform the h-CLAT and the MUSST. ECVAM promised to distribute the contact information to the lead laboratories by the end of the week so that they initiate contacts with them.

For the second laboratory involved in the MUSST, the current suggestion is to involve FICAM, the Finnish Center for Alternative Methods, who have expressed interest in participating in the study. Further enquiries will be made to ensure that the laboratory will satisfy all the conditions required for the proper performance of the assay.

Action (ECVAM) Compile and send contact details of the participating personnel to the lead laboratories

The responsibilities of the Quality Officer as described in the Study Plan will be amended since it currently includes a close monitoring of the study.

It was decided that it will be better if both naïve laboratories are trained by the lead laboratories at the same time. For the h-CLAT, TA clarified that Shiseido and Kao will perform together the training. He proposed that the location of the training would be the JaCVAM facilities located in Tokyo.

The 9 chemicals tested once in each laboratory were proposed for the Phase B Stage 1 phase, Stage 2 thus involving the other chemicals tested three times in each laboratory.

FG mentioned that if the coded chemicals include respiratory sensitisers, it would be important to know since the laboratories handle these differently. JMO repeated his suggestion to have the individual MSDS sealed in different envelopes.

The laboratories will not be told which solvent to use.

JR agreed to re-write the section on "Intellectual property Agreement", which will be re-discussed early on day 2.

Session 6: Project Planning, Training and Transfer.

For this session, SH, WS, DA and ES joined by teleconference.

Training report:

P&G has an example of training report made by LF for the training of the DPRA in the Eye irritation study, which she e-mailed to ECVAM. It was printed and shared with the other test submitters as guidance for their own training reports.

The VMG will approve the training reports, but the responsibility for determining the success of the training belongs to the lead laboratories.

Transfer report:

Transferability is one of the modules of the ECVAM modular approach, and the information used to document fulfillment of this module includes those contained in the Transfer report. One report should be produced by each trained laboratory. Results for the chemicals tested during the transfer phase should be reported according to the SOP instructions and using the agreed reporting template. The lead laboratories will be responsible for closely monitoring the transfer phase. JMO suggested that the lead laboratories give their "green light" for the submission of the reports to the VMG since they have final word on the outcome of this phase.

The chemicals for the transfer phase (and, for the DPRA, the peptides) should be acquired by each of the trained laboratory. The data should be sent to one contact person in ECVAM (AA), who will then distribute them to the biostatisticians.

The reports should include a section on where and how the SOPs were modified based on the input of the trainees during the training. A reporting template for the study phases including the transfer phase will be annexed to the Study Plan.

SH suggested to change the words "data will be analysed after decoding", since he would prefer the data to be analysed before decoding. The code will be broken only for the analysis of the test method accuracy.

About the DPRA Transfer plan, AK commented that the "all chemicals should be assigned the proper category or one off" might be too liberal, since if all the chemicals are categorized one category too high, the criteria would be fulfilled but the VMG would not consider this transfer successful. He suggested setting a number of chemicals that should be identified in the right category and a fraction that can be one-off.

When asked about the value of incorporating analyses of the quantitative data to evaluate the transfer, FG answered that it was not necessary for him to get a feel whether the lab is ready to perform the assay.

The lead laboratories clarified that they expected the persons that perform the assays to be present at the training, a request that will be communicated to the contract laboratories by ECVAM. If more than one person from the same site is trained, only one report is requested by the VMG.

DA asked whether there were plans to develop historical data for the positive controls at each site to develop the acceptance criteria. It was answered that the SOPs already incorporated control acceptance criteria based on the historical data of the test submitters.

For the h-CLAT, TA clarified that each sensitiser assessed in the transfer phase, should be correctly classified as positive (2 out of 3 runs), although unlike the Prediction Model it should be positive for both CD54 and CD86

Action (Kao, Shiseido, P&G) Re-arrange the transfer plan into a word document

Day 2

Session 7: Consolidation of day 1

The discussions started on the "Intellectual Property" section of the Study Plan (re-titled "Documents and Data Property") written by JR. Precisions were added to the nature of the parties concerned by each clause.

Regarding the Experimental Design the text will be changed so that:

- It is made clear that comparing the prediction obtained is more important than comparing the raw data
- It is made explicit that the negative and positive predictivity values will be affected by the unbalance between the number of sensitisers and non-sensitisers.
- For the MUSST and the h-CLAT, when an EC value can be calculated, the analyses of reliability might be performed on these values rather than the raw induction
- It is clear we are not limiting the analyses to the ones included in the document (eg. "The analyses will include, but not be limited to:")
- The DPRA section is corrected so that the results are referred to as "reactivity classes", not "sensitisation classes".
- It is clarified that the last sentence on significance relates to the whole analyses.

Session 8: Timelines

For this session and all the following, SH joined by teleconference

In order to achieve the expected timeline for the Final Draft of the Study Plan, the document (including the Experimental Design) will be corrected and circulated, and approved by the VMG through e-mails.

Action (ECVAM): Correct and circulate the Study Plan (including Experimental Design) final draft version for approval.

The deadline for submitting the final SOPs to the VMG has been set to end of January, with an approval deadline of middle of February.

The outcome of training and transfer phases needs to be approved by the VMT. Since the methods will end this phase at different times, and to avoid unnecessary delays, the process of this approval will be determined ad hoc (e-mail exchanges, teleconference, VMT meeting), depending on the problems identified (or lack thereof) during these phases.

The Study Plan will be amended to satisfy JMO's request to have real-time access to the data generated during the transfer phase by the naive laboratories.

The lead laboratories should not start Phase B before the other two naïve laboratories are ready to do so. There should be no supervision of the naïve laboratories by the lead laboratory during this phase since the testing phases will have to be conducted in an independent fashion.

The coding of the chemicals will be different for each laboratory and for the same chemicals when they are tested three times. Each laboratory will then receive 54 coded samples.

If the DPRA ends Phase B before the other methods, the results can still be analysed without breaking the code. The final report could be issued without revealing the names of the chemicals tested.

Future VMT meetings:

The following dates were reserved for potential future VMT meetings:

- Week of 21-25 of June. The exact dates will be set later, based on the dates of the COLIPA meeting. The major points of discussion would be the concerns arising during the training and transfer phases.
- 22nd-23rd-24th of September
- 4th-5th of November

The meeting in March is cancelled.

Reasonable delivery points of the data for the Phase B stages if the transfer is completed at the end of June:

DPRA: July 15th for Stage I, September 15th for the rest.
MUSST: end of September for Stage I, end of June 2011 for the rest
h-CLAT: end of September for Stage I, end of July 2011 for the rest

Session 9: Comments from Liaison Representatives

For this session and the following, WS, DA and ES joined by teleconference.

WS reiterated his concerns about the future of these methods and the work to be done to sufficiently evaluate their predictive capacity. He also showed interest in the future use of these methods for screening purposes by combining the results, as this could be considered for OECD guidelines. DB agreed that these discussions should (and will) occur once information becomes available in order to build a scientific consensus on these issues. However, these activities fall outside the scope of the current study.

The following meetings were discussed as potential forums to raise awareness on the work performed in the study:

- The ESCD meeting in Strasbourg in September,
- The IIVS meeting in October,
- The EPAA annual congress in November,
- Eurotox in Paris in August 28-31st 2011,
- The SOT meeting next year.

DB promised to draft an abstract for the ESCD meeting to distribute for comments and approval, and WS offered to circulate the proposal for SOT from last year.

Session 10: Chemical Selection

The representatives from the lead laboratories were not present for the discussion on chemical selection for the validation study. A record of the presentation and discussion on chemical selection will be provided to the VMG members only in a separate appendix to the meeting minutes that will not be posted on the shared section of the CIRCA website.

Summary of Actions:

			Deadline
1	P&G	**Minor editorial corrections to SOP.**	31/01/2010
2	ECVAM	**Prepare a reporting template for the DPRA and send it to FG for comments.**	12/02/2010
3	All laboratories	**Send contact details for the personnel involved (SD, QA, SO).**	31/01/2010
4	ECVAM	**Compile all the details and distribute.**	31/01/2010
5	ECVAM, L'Oréal, Kao and Shiseido	**Finalize the SOP for the h-CLAT and the MUSST.**	31/01/2010
6	L'Oréal	**Send the updated version of the reporting template to ECVAM for comments.**	31/01/2010
7	ECVAM	**Look at the reporting templates from all methods and make comments and modifications.**	26/02/2010
8	P&G, Kao and Shiseido	**Reformat the training and transfer plan into a word document and send ECVAM.**	31/01/2010
9	L'Oréal	**Update the training and transfer plan and send ECVAM.**	12/02/2010
10	ECVAM	**Correct and circulate the Study Plan and Experimental Design final draft.**	31/01/2010

Time schedule (2010.3.1 ~ 3.5)

Time	3/1	3/2	3/3	3/4	3/5
7:00					
8:00					
9:00	Start at 10:00	Introduction	Introduction	Introduction	
10:00	Introduction		Overview of the options - Thawing of the cells - Buffer preparing - calculation of the doubling time	Exp. I (Training) treatment	On this day, we have a tight schedule. So we have to have lunch in our spare time. General discussion Qs and As
11:00					Exp. I (Training) analysis
12:00	Lunch	Lunch		General discussion Qs and As Exp. II (Training) treatment	
13:00	PI assay (Demo) treatment	Overview of flowcytometry	Lunch and "Hakone" tour (plan)		Exp. II (Training) analysis
14:00		PI assay (Demo) analysis		Lunch	
15:00	Sub-culture (Demo)			General discussion Qs and As	
16:00	Sub-culture (Training)	Data analysis			Data analysis
17:00					Final discussion
18:00	Welcome dinner				Deperture
19:00					
20:00					
21:00					
22:00					
23:00					

Time	3/1	3/2	3/3	3/4	3/5
8:00		9時集合			
9:00	10時集合	導入 スケジュールの確認	導入 スケジュールの確認	導入 スケジュールの確認 試験1 処理 培養室にて 二つに分かれて実施 準備＆片付け含め2時間ほど	導入 スケジュールの確認
10:00	導入 自己紹介 トレーニングの流れ確認		その他の説明 細胞の解凍 Bufferの作成、 倍加時間の計算など 培養室？ → 会議室		確認 質疑応答
11:00	SOPの説明 実験内容				試験1 解析 培養室 → 測定室 二つに分かれて実施 測定中にもうひとつのラボが解析 出来るようにPCを測定室へ移動 休憩は玄関
12:00	昼食	昼食(中華弁当？)		質疑応答 または 昼食 会議室	
13:00	PI assayデモ 処理 培養室にて 準備＆片付け含め2時間	フローサイトメーターの説明 測定室にて 一般情報とセッティングの仕方	昼食 → 箱根ツアー	試験2 処理 培養室にて 二つに分かれて実施 準備＆片付け含め2時間ほど	
14:00	休憩時には実験室別部屋	PI assayデモ 解析 培養室→14時半過ぎに測定室へ 培養室の作業は40分ほどか 測定までに1時間、測定に30分 説明しながら解析に30分 計2時間強		昼食 または 質疑応答 会議室	試験2 解析 培養室 → 測定室 試験1とかぶってくる可能性がある 一人が培養室に試験2のために移動 測定中にもうひとつのラボが解析 出来るようにPCを測定室へ移動 休憩は玄関
15:00	継代デモ 培養室にて 30 分もかからず終了 続いて練習			流れの確認 質疑応答 細胞が余っているので、場合によっては継代や処理のトレーニングをやっていただく可能性 培養室にて	
16:00	継代 練習 培養室にて 各施設に2つの中フラを用意	データの解析 会議室にて PCを用いて 生データを移動させ 解析 WS作成まで			データ解析 二つのラボが終了したら会議室で 最終議論 会議室にて
17:00	各人が継代を行えるように 休憩時には実験室別部屋				
18:00	Welcome dinner				出発 18:00-18:30頃
19:00					

h-CLAT トレーニング報告書

背景

2010 年 3 月より ECVAM において 3 つの in vitro 皮膚感作性試験法のプレバリデーションが開始される事が決まった。この 1 つである human Cell Line Activation Test (h-CLAT) は、株式会社資生堂と花王株式会社により開発された試験で、今回 JaCVAM のサポートも受けて ECVAM でプレバリデーションが実施される。このプレバリデーション実施に向けて、3 月 1 日から 5 日の 5 日間、新たに参加する施設に対して h-CLAT のトレーニングを実施することとなった。

１、トレーニング

今回の ECVAM のプレバリデーションにおいては、試験法を開発したリード施設に加え、その試験法を行った事の無い新たな施設 2 つを加えて実施される。h-CLAT においては、上記 2 施設がリード施設となるため、新たにヨーロッパの 2 施設を加えた 4 施設で評価が行なわれる事になった。プレバリデーションを適切に行なうためには、すべての参加施設が適切に試験を実施する必要があるため、試験法の概要説明および実際の評価フローを体験するトレーニングが今回の ECVAM のプレバリデーションの 1 つのステップとして組み込まれている。

２．参加施設及び参加者

リード施設１：株式会社資生堂
　足利太可雄、薗さき子
リード施設２：花王株式会社
　額田佑子、安保孝幸、坂口　斉
施設３：In-Vitro Methods Unit (ECVAM)
　Dr. Ingrid Langezaal、
　Dr. Ann-Charlotte (Lotta) Bostroem
施設４：Bioassay（ドイツの受託機関）
　Dr. Axel Hohenstein、Ms. Kirstin Young

３．トレーニング期間及び場所

3 月 1 日から 3 月 5 日の 5 日間、神奈川県秦野市の食品薬品安全センター、秦野研にて実施した。

４．トレーニング内容

今回の 5 日間では h-CLAT の実際の評価手順に基づき、以下の項目に関して実施した。

１） h-CLAT の概要と詳細な SOP の説明
２） THP-1 細胞の前培養
３） DNCB と SLS を用いた試験濃度設定のための PI assay
４） DNCB と SLS での反応性試験
５） 試験結果の解析

これらトレーニングを行なうために、細胞培養施設での細胞の取り扱いからフローサイトメトリーを用いた細胞の解析まで h-CLAT の一通りの試験を実施した。また、これら評価は、初めにリード施設のメンバーが実施するのを見て手順を理解してもらい、その後施設３と４の方々に実施してもらった。なお、今回のトレーニングのスケジュールを資料１として添付する。

５．トレーニング結果

１）PI assay による DNCB 及び SLS の CV75

リード施設のデモにより得られた値とほぼ同じ値が施設３及び施設４の実験でも得られた。このことより、基本的な操作はマスターできたと考える。

２）DNCB の反応性

施設３及び４共に、CD86 と CD54 の両方が陽性判断基準値を超え、陽性と判断できた。そのため、陽性物質の検出において、基本的な操作はマスターできたと考える。

３）SLS の反応性

施設３及び４共に、CD86 と CD54 の両方が陽性判断基準値を超えず、陰性と判断できた。ただし、試験最高濃度においても細胞生存率が 90％以上であったため、試験成立条件から逸脱したため、今回の施設３及び４の結果は、採用できないものと判断された。なお、デモとして行なったリード施設の SLS 反応試験においても同様に、試験最高濃度で細胞生存率が 90％を超えており、その意味では、リード施設の結果を良く再現していた。このことより、基本的な操作はマスターできたと考える。なお、今回 SLS の試験最高濃度で細胞生存率が 90％以上となった理由に関しては、通常よりも高い CV75 の値が得られたためと考えられた。なぜこのような CV75 の値が得られたかに関しては、明確にはわからなかったが、SLS のように生存率が濃度により急激に減少する化合物を評価する際は適用溶液の希釈系列作成時に公比を小さくして行うとうまくいく場合が多いことを伝えた。

４)質疑応答

プロトコールの正しい理解のため、トレーニング中に質疑応答の時間を設け、可能な限り疑問点を解消してもらうようにした。

６．今後の予定

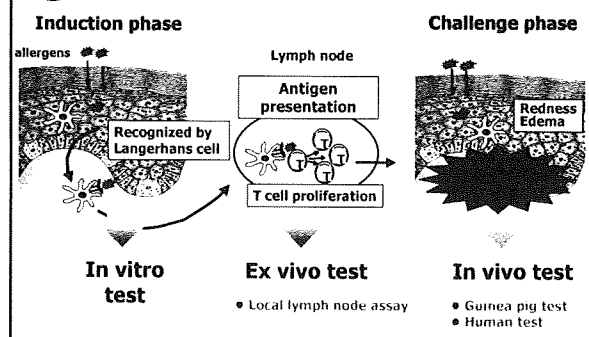
ECVAM に対して、今回のトレーニングの報告書を作成し、提出する。また、施設３及び４は、リード施設が定めた 5 被験物質を用いて、h-CLAT で適切に評価できるか検討を行なう。それが問題ないと判断された場合、正式なプレバリデーションを開始する予定である。

human Cell Line Activation Test : h-CLAT (In Vitro Skin Sensitization Test).

H. Sakaguchi[1], T. Ashikaga[2],

1. Kao Corporations,
2. Shiseido Co., Ltd.,

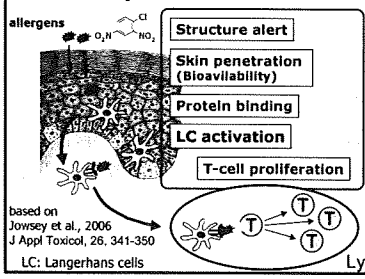
Mechanisms of Skin Sensitization and Current Tests



Approach for Developing of in vitro Methods

It is imperative to understand the mechanisms the sensitization (induction) phase of contact hypersensitivity (Vandebriel et al., 2005)

Induction phase



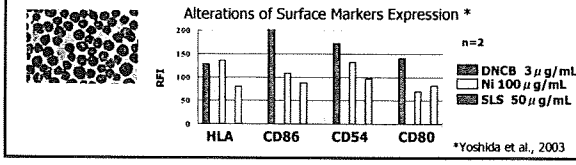
- Capture key biological and phys-chem aspects of skin sensitization
- Provide integrated view of skin sensitization activity

Selection of appropriate cell for in vitro test

Langerhans Cells (LC) / Dendritic Cells

- Limited number of cells (Ryan et al., 2007)
- Donor-to-donor variability in response (Aiba et al., 1997; Pichowski et al., 2000)
- Time & cost for culture procedures (Aiba et al., 1997; Pichowski et al., 2000)

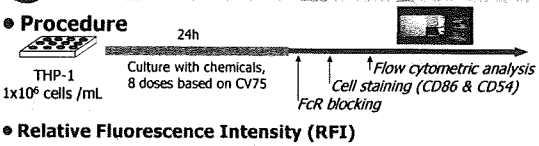
THP-1 (human monocytic leukemia cell line)



*Yoshida et al., 2003

human Cell Line Activation Test (h-CLAT)*

- **Procedure**



- **Relative Fluorescence Intensity (RFI)**

$$RFI = \frac{\text{MFI of chemical treated cells - MFI of chemical treated Isotype control cells}}{\text{MFI of vehicle control cells - MFI of vehicle Isotype control cells}} \times 100$$

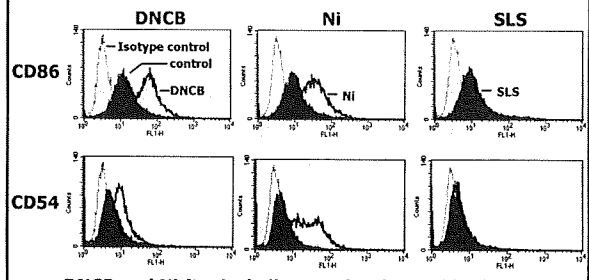
MFI = geometric mean fluorescence intensity

- **Prediction Model**

Viability ≥ 50% by Propidium Iodide
Positive criteria: CD86 RFI ≥ 150% and/or CD54 RFI ≥ 200%
Positive: 2 of 3 independent data at any dose should exceed the positive criteria

*: Ashikaga et al., 2006 Toxicol In Vitro 767-73., Sakaguchi et al., 2006 Toxicol In Vitro 774-84.

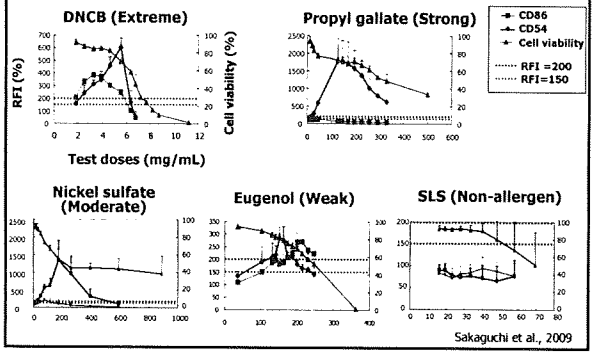
Allergen-specific augmentation of CD86 / CD54 expression



DNCB and Ni (typical allergens) enhanced both CD86 and CD54 expressions but SLS (non-allergen) did not.

Miyazawa et al., 2007

The alteration of CD86 and CD54 expression

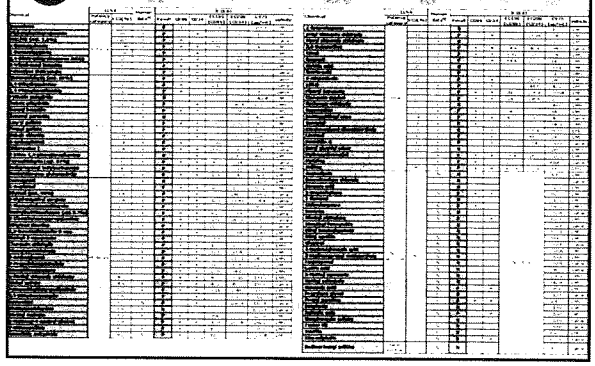


Sakaguchi et al., 2009

The purpose of this study

- **To confirm the prediction performance**
  - Evaluation of 100 chemicals by the h-CLAT to compare with LLNA
- **To clarify the applicability domain**
  - Applicability domain based on the data base
- **To classify of skin sensitization potency**
  - Using EC150 and EC200 values as the indicator
- **To evaluate the inter-laboratory reproducibility**
  - Ring Trials in the COLIPA (5 labs) and Japan (8 labs)

Results of 100 test chemicals



Comparative evaluation with LLNA and human

h-CLAT vs LLNA

		h-CLAT	
		+(70)	-(30)
LLNA	+(72)	63	9
	-(28)	7	21

Sensitivity: 63/72 (88%)
Specificity: 21/28 (75%)
Positive predictivity: 63/70 (90%)
Negative predictivity: 21/30 (70%)
Accuracy: 84/100 (84%)

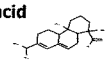
h-CLAT vs human

		h-CLAT	
		+(49)	-(20)
Human	+(53)	44	9
	-(16)	5	11

Sensitivity: 44/53 (83%)
Specificity: 11/16 (69%)
Positive predictivity: 44/49 (90%)
Negative predictivity: 11/20 (55%)
Accuracy: 55/69 (80%)

Good accuracy, but some false negative / positive

False negative (1) : Limited solubility

	Applying dose based on cytotoxicity	Estimated maximum water solubility*
Hexyl cinnamic aldehyde 	44-12 μg/mL	> 2.75 μg/mL
	↳ Precipitation or cloudy solution	
Abietic acid 	154-43 μg/mL	> 0.09 μg/mL
	↳ Precipitation or cloudy solution	
Phthalic anhydride 	Max applying dose (400 μg/mL) with no-cytotoxicity	
	↳ To be dissolved in appropriate sol.	

Solubility is important issue to predict correctly

*: Calculated with "Water freq" software.

False negative (2): Pro(Pre)-hapten

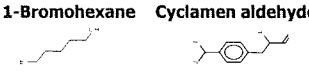
Benzoyl peroxide 	Metabolic activity can change the structure (Nacht et al., J. Am. Acad. Dermatol., 4, 31-37, 1981).
Geraniol 	• Metabolic activity or air oxidation can change the structure (Basketter et al., Contact Dermatitis, 47(3), 161-164, 2002). • Oxidation products of geraniol (Geranial and Neral) augmented CD54 expression (Kosaka et al., SOT 2008).
Isoeugenol 	Oxidation involes senstitising potencial (Bertrand et al., Chem Res Toxicol., 10(3), 335-343, 1997).
Abietic acid 	Air oxidation involves expression of sensitizing potencial (Basketter et al., Food Chem Toxicol 33, 1051-1056, 1995).

THP-1 cells may not have enough enzyme for metabolism

False negative (3) : Sensitivity

Weak sensitizers by LLNA classification

1-Bromohexane **Cyclamen aldehyde** **Butyl glycidyl ether**

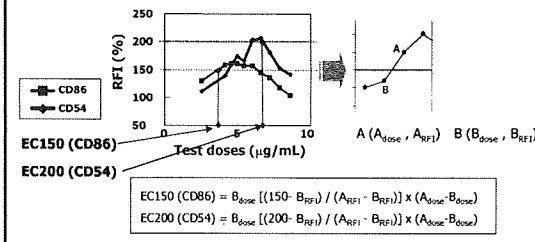


LLNA class	Number of tested chemicals	Number of false negatives	Sensitivity (%)
Extreme	8	0	100
Strong	16	2	88
Moderate	24	2	92
Weak	23	6	74

Several weak sensitizers did not affect CD86/CD54 expression at protein level.

Correlation between h-CLAT and in vivo data

EC150 / 200 (Estimated concentration of RFI150 / 200)



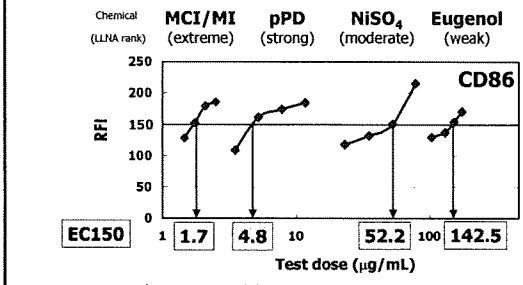
$EC150\ (CD86) = B_{dose}\ [(150 - B_{RFI}) / (A_{RFI} - B_{RFI})] \times (A_{dose} - B_{dose})$

$EC200\ (CD54) = B_{dose}\ [(200 - B_{RFI}) / (A_{RFI} - B_{RFI})] \times (A_{dose} - B_{dose})$

The intermediate value of three experiments was defined as EC150 or EC200.

Calculated based on the calculational procedure of LLNA EC3

Difference of EC150 and EC200

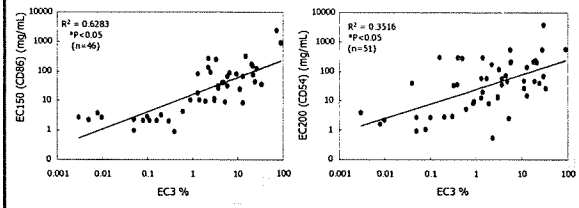


EC values were different between chemicals

MCI/MI; Methylchloroisothiazolinone / methylisothiazolinone
pPD; para-phenylenediamine $NiSO_4$; Nickel sulfate

Relationship between EC3(LLNA) and EC150/200

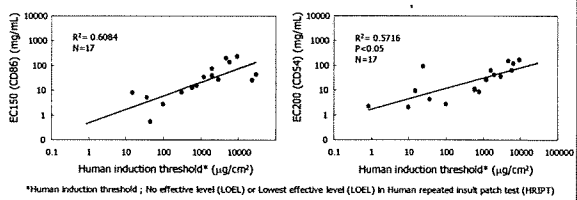
EC150 (CD86) *EC200 (CD54)*



Both EC150 (CD86) and EC200 (CD54) have significant correlations with EC3 value of LLNA.

Relationship between Human threshold and EC150/200

EC150 (CD86) *EC200 (CD54)*



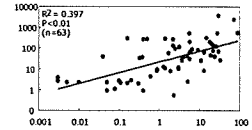
*Human induction threshold ; No effective level (LOEL) or Lowest effective level (LOEL) in Human repeated insult patch test (HRIPT)

Both EC150 (CD86) and EC200 (CD54) have significant correlations with human induction threshold.

Example of classification of skin sensitization using h-CLAT

Minimum Induction Threshold of h-CLAT – MIT (h-CLAT) -

determined as a minimum value, smallest of either EC150 or EC200.



Significant correlation with LLNA EC3

Might be useful to predict...

LLNA EC3 ...?
Proposed GSH subcategories ...?

Ref. Proposed GHS subcategories for skin sensitization based on LLNA EC3 and the example of prediction

Subcategory	Animal test results (using LLNA data)	Cut off (h-CLAT)	Accuracy(%)
1A (Strong sensitizer)	EC3 ≤ 2%	MIT 10 μg/mL	78.8
1B (Weak sensitizer)	EC3 > 2%		

Summary of evaluation for 100 test chemicals

- Total 100 chemicals were evaluated with a single h-CLAT protocol.
- Good prediction performance (accuracy: 82%/80%) between the h-CLAT and LLNA/human data was observed.
- Possible applicability domain of the h-CLAT (solubility, metabolic activity, sensitivity, etc.) was suggested.
- Correlation between h-CLAT and in vivo data
  - EC150 and EC200 of h-CLAT correlate with LLNA EC3
  - both values correlate with human induction threshold of HRIPT
  - h-CLAT values might be useful to predict the allergic potency of chemicals classified in GSH

Purpose of COLIPA and Japanese Ring Trials

- To evaluate
  - Protocol transferability
  - Inter-laboratory reproducibility
  - Predictive capacity
- Goals
  - Identify unexpected problems with either test design or procedures
    - Protocol optimization/standardization
  - Identify problems with data analysis/ interpretation
    - Prediction model refinement
- Members
  - COLIPA: P&G, L'Orel, Henkel-Phnion, Shiseido and Kao
  - Japan: Kanebo Cosmetics, Kose, Lion, Nippon Menard Cosmetic, Pola Chemical Industries, NIHS, Shiseido and Kao

Summary of the COLIPA 3rd Ring Trial data

8 test chemicals (5 allergens, 3 non-allergens), 5 labs

(# of positive experiments / # of total experiments)

Chemical	Potency	Lab A	Lab B	Lab C	Lab D	Lab E
MCI/MI	Extreme	*	- (2/3)	- (2/3)	+ (3/3)	+ (2/3)
DNCB	Extreme	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Hydroquinone	Strong	+ (3/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)
HCA	Weak	- (1/3)	- (1/3)	- (0/3)	- (1/3)	- (1/3)
Eugenol	Weak	+ (2/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (3/3)
SLS	NS	- (1/3)	- (0/3)	- (0/3)	- (0/3)	- (0/3)
BKC	NS	+ (2/3)	- (0/3)	- (0/3)	- (0/3)	- (1/3)
Vanillin	NS	- (0/3)	- (0/3)	- (1/3)	- (1/3)	- (0/3)

[*] : not good qualified data (cell viability of the highest dose > 90%)

- MCI/MI, DNCB, HQ, EU, SLS, VN were identified correctly
- HCA: false negative in all labs, BKC: one false positive data
- Basically good inter-lab reproducibility

COLIPA 4th Ring Trial summary data

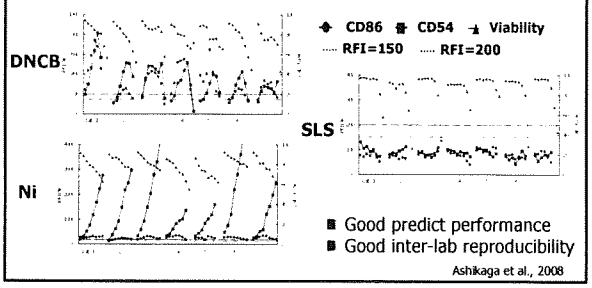
7 test chemicals (5 allergens, 2 non-allergens), 4 labs

Chemical	Potency	Lab B	Lab C	Lab D	Lab E
PPD	Strong	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Methyldibromo glutaronitrile	Strong	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)
2-Mercaptobenzothiazole	Strong	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Cinnamic Aldehyde	Moderate	- (1/3)	+ (3/3)	+ (2/3)	+ (3/3)
Tetramethylthiuram Disulfide	Moderate	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Glycerol	NS	- (0/3)	- (0/3)	- (0/3)	- (0/3)
Salicylic Acid	NS	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)

- Cinnamic Aldehyde : one false negative data
- Salicylic acid : false positive in all labs
- Almost good predict performance
- Good inter-laboratory reproducibility

Japanese 1st Ring Trial

- 3 test chemicals (2 allergens, 1 non-allergens), 7 labs
- Test doses were same in all labs



- Good predict performance
- Good inter-lab reproducibility

Ashikaga et al., 2008

Japanese 3rd Ring Trial summary data

5 test chemicals (4 allergens, 1 non-allergen), 7 labs

Chemical	Potency	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
p-Benzoquinone	Extreme	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Glutaraldehyde	Strong	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)
Ethylene diamine	Moderate	+ (3/3)	+ (3/3)	+ (2/3)	- (1/3)	+ (3/3)	+ (3/3)	+ (2/3)
Eugenol	Weak	+ (3/3)	- (0/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (2/3)
Lactic acid	NS	- (0/3)	- (0/3)	- (0/3)	- (0/3)	- (0/3)	- (0/3)	- (1/3)

- Ethylene diamine had one false negative
  → ?? Oxidation ??
- Eugenol had one false negative
  → solubility is problem?
- Basically good predict performance and inter-lab reproducibility

Ashikaga et al., 2008

Summary of h-CLAT Ring Trials and Next step

- COLIPA : 15 Test Materials
  - ..approx 85% predicted correctly
- Japan : 8 Test Materials
  - ..approx 96% predicted correctly

- Good predictive performance and inter-lab reproducibility
- Not good reproducibility for some chemicals
  - → by chemical property ? (ex. low solubility; HCA, Eugenol)

Next step

Test Development → **Pre Validation** → **Validation** → Peer Review → OECD Test Guideline

LIST OF PUBLICATIONS (1)

1. Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol.
   Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama , K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., and Toyoda, H.
   Toxicology in Vitro, 20 (5), 767-773, 2006.
2. Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT.
   Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama , K., Hirota, M.,Itagaki, H., Toyoda, H., and Suzuki, H.
   Toxicology in Vitro, 20 (5), 774-784, 2006.
3. Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1.
   Sakaguchi, H., Miyazawa, M., Yoshida, Y., Ito, Y., Suzuki, H.
   Archives of Dermatological Research, 298 (9), 427-437, 2007.
4. Assessment of the human Cell Line Activation Test (h-CLAT) for Skin Sensitization; Results of the First Japanese Inter-laboratory Study.
   Ashikaga, T., Sakaguchi, H., Okamoto, K., Mizuno, M., Yamada, T., Yoshida, M., Sato, J., Kodama, T., Ohta, N., Hasegawa, S., Okamoto, Y., Kuwahara, H., Kosaka, N., Sono, S., Ohno, Y.
   Alternatives to Animal Testing and EXperimentation, 13, (1), 27-35, 2008.

LIST OF PUBLICATIONS (2)

5. The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test – human cell line activation test (h-CLAT).
   Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N, Ito, Y., Yoneyama , K., Sono, S., Itagaki, H., Toyoda, H., and Suzuki, H.
   Cell Biology and Toxicology, 25, 109-126, 2009
6. A Study for the Criteria for Selection of THP-1 Cells in the human Cell Line Activation Tset (h-CLAT): Results of 2nd Japanese Inter-Laboratory Study
   Kosaka, N., Okamoto, K., Mizuno, M., Yamada, T., Yoshida, M., Kodama,T., Sono, S., Ashikaga, T., Sato, J., Ohta, N., Hasegawa, S., Okamoto, Y., Kuwahara, H., Sakaguchi, H., andOhno, Y.
   Alternatives to Animal Testing and EXperimentation, 13, (2), 55-62, 2008.
7. A Study on serum difference on test results in the human Cell Line Activation Tset (h-CLAT): Results of 3rd Japanese inter-laboratory Study
   Sono, S., Yamada, T., Kosaka, N., Okamoto, K., Mizuno, M., Sato, J., Yoshida, M., Ohta, N., Kodama,T., Okamoto, Y., Kuwahara, H., Sakaguchi, H., Hasegawa, S., Ashikaga, T.,and Ohno, Y.
   Alternatives to Animal Testing and EXperimentation, 13, (2), 63-69, 2008.
8. Effect of pre-culture conditions on the human Cell Line Activation Tset (h-CLAT) results: Results of the 4th Japanese inter-laboratory Study
   Mizuno, M., Yoshida, M., Kodama,T., Kosaka, N., Okamoto, K., Sono, S., Yamada, T., Hasegawa, S., Ashikaga, T., Kuwahara, H., Sakaguchi, H., Sato, J., Ohta, N., Okamoto, Y., andOhno, Y.
   Alternatives to Animal Testing and EXperimentation, 13, (2), 70-82, 2008.

How to do "h-CLAT"

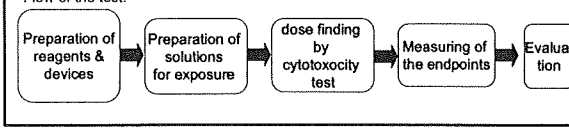
—based on our protocol & experiences—

Shiseido & Kao

1. Topics for today

1. Reagents & Devices
2. Cell culture
3. Maintenance & setting of Flow cyotmetry
4. Vehicle selection & Preparation of solutions
5. Dose finding assay (cytotoxicity test)
6. Measuring of CD86 and CD54 expression
8. Data analysis & interpretation

Flow of the test:



1. Necessary Reagents & Devices

Cells THP-1 (purchased from cell bank like ATCC (TIB-202))

Culture medium: RPMI1640 supplemented 10% FBS, 0.05mM 2-mercaptoethanol and anitibiotics

Flowcytometry:
FACS Calibur (Becton Dickinson), EPICS XL-MSL (Beckman Coulter), etc.

Antibodies (specified!):
1. For CD86 : BD-Pharmingen, #555657 (Clone: Fun-1)
2. For CD54 : DAKO, #F7143 (Clone: 6.5B5)
3. For Isotype control: FITC labeled-mouse IgG1, DAKO, #X0927

FcR blocking buffer:
Globulins Cohn fraction II, III, Human: SIGMA, #G2388-10G

Note:
- The quality of materials and disposals should be confirmed before use because something impurities (e.g.,LPS) may affect THP-1.
- You need to use antibodies specified in the SOP, because reactivity is different according to products.

Only cells and serum which passed a reactivity check should be used.

2. Cell culture

Lot check:
The quality of each lot of THP-1 cells should be checked two weeks after thawing a new batch.

- Viability of the cells are 90% or more (usually around 97%).
- Pass the reactivity check: Both DNCB and Ni should produce a positive response of both CD86 and CD54. SLS should produce a negative response of both CD86 and CD54.

Doubling time:
In order to obtain reliable results, it is important that properly growing cell cultures are used. You should accumulate your own historical data of a doubling time and set the acceptance range according to these.

In our experiences, an average doubling time is approximately 43 hr and it varies widely (approximately 30-55 h).

2. Cell culture

Maintenance of THP-1 cells:
- THP-1 cells should be maintained at densities from 0.1×10^6 to 0.8x106 cells/mL. Cells are routinely passaged every 2-3 days at the density of 0.1 to 0.2×10^6 cells/mL. Do not allow the cell density to exceed 1×10^5 cells/mL.

- Cells can be used up to two month after thawing (Passage number should not exceed 30).

Pre culture:
To keep the condition of cells constant, pre culture should be done before assay.

For example, Before any assay THP-1 cells are seeded between 0.1×10^6 and 0.2×10^6 cells/mL, and pre-cultured for 48 h or 72 h in culture flasks. You should keep the setting condition in every test.

3. Flow cytometry analysis (control)