EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

ESAC Request 2011-02

ECVAM Scientific Advisory Committee

(ESAC)

ECVAM REQUEST FOR ESAC ADVICE

on

the ECVAM-coordinated follow-up study to assess the predictive capacity of the already validated Neutral Red Uptake cytotoxicity assay for acute oral toxicity testing.

INSTRUCTIONS FOR IVM/ST STAFF:

Blue text: to be filled in by the ECVAM Scientific Officer completing the draft request in collaboration with ESAC Secretariat.

Green text: to be filled in by the ESAC Secretariat.

Title page information	
Abbreviated title of ESAC request	Follow-up study to assess the PC of the Neutral Red Uptake cytotoxicity assay for acute oral toxicity testing
ESAC REQUEST Nr.	2011-02
Template used for preparing request	EP 2.01
Date of finalising request	2011-03-07
Date of submitting request to ESAC	2011-03-09
Request discussed through	Plenary discussion at ESAC 34, 22-23 March 2011
ESAC WG report / opinion expected at (date)	ESAC 35, 4-5 October 2011
File name of this request	ER2011-02 3T3 NRU follow-up MANDATE.doc

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1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review of a Prevalidation Study or Validation Study	YES
If R1)applies please specify further:	
• Prevalidation Study	
• Prospective Validation Study	YES This study, finished in 2010, was planned and conducted as a follow-up to the previous full prospective validation study of the 3T3 NRU cytotoxicity assay conducted by NICEATM in collaboration with ECVAM and finalised in 2005. The study was designed to complement the information on predictive capacity of the 3T3 NRU assay for the specific purpose of identifying substances that do not need to be labelled for acute oral toxicity according to the EU CLP regulation (i.e. substances with LD_{50} doses above the limit dose of 2000 mg/kg body weight). Importantly, being a follow-up and complement exercise according to ECVAM's modular approach, the study deviates to some extent from the typical design of a full prospective validation study (e.g. no transferability/reproducibility assessment as these have been addressed in the previous study).
• Retrospective Validation Study	
• Validation Study based on Performance Standards	
R2 Scientific Advice on a test method submitted to ECVAM for validation (e.g. the test method's biological relevance etc.)	NO
R3 Other Scientific Advice (e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)	NO

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

Follow-up study on the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity assay to correctly identify substances not classified for acute oral toxicity under the EU CLP system (LD_{50} > 2000 mg/kg).

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

3.1 Summary of the follow-up study

This follow-up study was conducted as a complement to the previous full prospective validation study of the 3T3 Neutral Red Uptake (NRU) cytotoxicity assay (=the "3T3 NRU assay") conducted by NICEATM/ICCVAM in collaboration with ECVAM. The test exploits the correlation between the systemic toxicity (i.e. acute oral toxicity) of substances and their cytotoxicity exerted on 3T3 cells. Cytotoxicity is measured as reduction of uptake of the vital dye 'neutral red', which accumulates in lysosomes of healthy cells.

As a follow-up, the study deviates to some extent from the manner in which a full prospective validation exercise is typically conducted. The study was designed to specifically assess whether the 3T3 NRU assay is able to discriminate classified chemicals from non-classified ones (i.e. those beyond the limit dose of 2000mg/kg according to the EU CLP regulation implementing UN GHS). Thus, the study was intended to provide additional information on predictive capacity of the 3T3 NRU assay for this specific purpose, without addressing reproducibility/transferability of the protocol which had been previously demonstrated in the NICEATM/ECVAM validation study.

To assess the capacity of the 3T3 NRU to correctly identify chemicals not requiring classification, 56 test items (a sufficient number to analyse dichotomous classifications) with good in vivo reference data were selected and tested in one laboratory using the already validated protocol. In addition, the same chemicals were tested in two more laboratories using slight modifications of the protocol. These variations were 1) an abbreviated version of the validated protocol, and 2) a protocol modified for use on an automated platform. This additional testing was intended to provide information on the extent to which the original protocol is amenable to simplification (protocol variant 1), and automation (protocol variant 2).

The testing data of the validated protocol show that the 3T3 NRU identifies true positives with a sensitivity of 94%. Since the 3T3 NRU is able to correctly identify most positives, negative test results in the 3T3 NRU are very likely to represent either true negatives (non-classified chemicals) or false positives. In contrast the rate of false negatives is low. This is reflected by the high NPV (negative predictive value) of 92%. Therefore, the 3T3 NRU may be appropriate for identifying negatives as a first screening step in a tiered testing approach involving subsequent in vivo testing to 1) further categorise chemicals with positive results, 2) to identify false positive results of the 3T3 NRU (low specificity of 42%) and 3) to test, in specific cases where there is additional weight of evidence information, negative substances for confirmation. The two protocol variants gave similar predictive values suggesting that also these variants of the validated 3T3 NRU protocol may be used for the screening of non-classified chemicals according to EU CLP within a tiered testing strategy.

3.2 Detailed background

Several international projects have studied the possibility of using in vitro methods to predict acute oral toxicity.

The first of these studies was the Multicentre Evaluation of In Vitro Cytotoxicity (MEIC) programme. It showed that in vitro methods used in the study predicted human acute oral lethality better than did mouse and rat in vivo LD_{50} data.

In a second study based on information of the Registry of Cytotoxicity (RC, a database for rodent acute oral LD_{50} values and in vitro IC_{50} values), over 70% of the substances tested in vitro were able to predict the rodent acute oral lethality.

Third, the international NICEATM/ECVAM validation study (the In Vitro Basal Cytotoxicity Validation Study finished in 2005) used a human-derived cell model (primary normal human epidermal keratinocytes) and a mouse cell model (BALB/c 3T3 mouse fibroblasts) to evaluate the usefulness and limitations of the in vitro basal cytotoxicity test methods based on measuring cell viability trough neutral red uptake (NRU) for predicting starting doses for systemic (i.e. in vivo) acute oral toxicity test methods. In addition, this validation study assessed the accuracy of the two basal cytotoxicity test methods to estimate rodent oral LD_{50} values across the five categories of the Globally Harmonized System (GHS) for acute oral toxicity as well as unclassified toxicities. The study concluded that the two NRU test methods could be used in a weight-of-evidence approach to determine the starting dose for acute oral in vivo toxicity protocols. The validation study also showed that the overall accuracy of the 3T3 NRU test method for correctly predicting each of the GSH acute oral toxicity classification categories was low (around 30%), however, substances falling in the GHS 4 category (i.e. $300 < LD_{50} \bullet 2.000$ mg/kg) were predicted better, with 81% accuracy.

Taken together, the results of MEIC, the RC, and the NICEATM/ECVAM international validation study have all shown a correlation of around 60-70% between in vitro cytotoxicity data and oral rodent LD_{50} values. These studies indicated that the in vitro methods are able to predict low systemic toxicity with much greater precision than high systemic toxicity, suggesting the potential usefulness of these methods for identifying chemicals not requiring classification.

3.3 Purpose of the study

This follow-up study was initiated in 2008 by ECVAM and was finalised in October 2010. The aim of this study was to further explore, on the basis of the previous validation study, whether the predictive capacity (e.g. sensitivity, specificity, concordance) of the 3T3/NRU cytotoxicity assay is sufficient to correctly distinguish chemicals not requiring classification for acute oral toxicity according to provisions of the EU CLP regulation (i.e. $LD_{50} > 2000$ mg/kg b.w.) from those that require classification ($LD_{50} \bullet 2000$ mg/kg b.w.). The scientific and regulatory rationale embedded in study's objective was to assess whether the 3T3 NRU assay could be used as the first step of a tiered approach to identify unclassified chemicals so that subsequent testing in vivo would focus on confirmatory testing to classify positives according to the 4 classified classes of EU CLP and identify substances with positive test results in the 3T3 NRU that are actual negative (=3T3 NRU false positives).

The study used the test method protocol validated in the NICEATM/ECVAM validation study. In addition, two protocol modifications were assessed: one version of the 3T3/NRU protocol adapted to an automated platform and an abbreviated version of the validated protocol that was targeted at resolving acute oral toxicities around the 2000 mg/kg cut-off value. The aim of this additional testing

was to assess whether a simplified version and a version adapted for automated testing would generate similar data on the basis of the 56 test chemicals selected and to assess, therefore, to which extent these variants of the validated protocol may be used for purposes of identifying negatives (LD_{50} > 2000 mg/kg b.w.).

3.4 Organisation of the study

The study was coordinated and managed by a Validation Management Team composed of two ECVAM staff members. Although testing was performed in three laboratories, the core validation exercise (aiming at more detailed information on predictive capacity) concerned only laboratory Nr. 1 which worked with the validated protocol. Laboratories 2 and 3 produced additional data on the basis of two protocol variants supporting a comparative analysis of protocol performance. The laboratories were:

1) Health and Safety Laboratory (HSL), UK (under ECVAM sponsored contract) using the already validated manual test method protocol
2) JRC (IHCP), Italy using the automated version of the test method protocol
3) IIVS, US (sponsored by IIVS and PETA, People for the Ethical Treatment of Animals) using the abbreviated test method protocol

A set of 56 coded industrial chemicals (including cosmetic ingredients) were tested using each test method protocol. The chemicals were purchased from Sigma-Aldrich (Italy) and coded by ECVAM. The distribution of chemicals and respective material safety data sheets were done by Sigma-Aldrich Germany (for the two European laboratories) and Sigma-Italy for the laboratory in the US. The data from blind testing were de-coded and analysed independently by ECVAM.

3.5 Results and conclusions

The results of all three protocol variants show that the 3T3/NRU assay has high sensitivity (92-96%) and high negative predictive value (86-92%). This indicates that compounds identified as negatives by the method (40% - 44%) will most likely be correctly categorised as unclassified (LD50 > 2000 mg/kg b.w.). Therefore, if the above proposed tiered strategy is applied, negatives may not be required to be tested in subsequent confirmatory in vivo testing. Positives of the 3T3 NRU however would require confirmatory in vivo testing on the basis of a starting dose approach as validated in the NICEATM-ECVAM validation study.

A recent analysis of the New Chemicals Database showed that over 85% of new industrial chemicals do not require classification for acute oral toxicity according to EU CLP (LD50 > 2000 mg/kg b.w.). With the 3T3/NRU method, which was demonstrated of being able to correctly identify about 42% of all true negatives, a testing strategy could be developed, limiting animal testing to only those substances identified as "classified" by the 3T3/NRU assay.

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4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective Why does ECVAM require advice on the current issue?	The opinion of ESAC should support ECVAM with respect to the development of further recommendations regarding the ability of the 3T3/NRU test method to correctly identify substances not requiring classification for acute oral toxicity under the EU CLP system (LD50 > 2000 mg/kg b.w.) and the use of the test method in a tiered testing approach for acute oral toxicity testing.

4.2 QUESTION(S) TO BE ADDRESSED

Questions What are the questions and issues that should be addressed in view of achieving the objective of the advice?	1) DESIGN & CONDUCT OF STUDY: The ESAC is requested to review whether the validation study was conducted appropriately in view of the objective of the study, i.e. to assess the ability of the 3T3/NRU test method to correctly identify substances not requiring classification for acute oral toxicity under the EU CLP system (LD50 > 2000 mg/kg b.w.). In particular the following issues should be addressed: (a) Clarity of the definition of the study objective. (b) Appropriateness of the study design in view of study objective, inter alia: o Were the criteria for chemical selection appropriate? o Is the toxicity range of the selected chemicals appropriate for the purpose of the study (i.e. analysis of the ability to distinguish at the 2000mg/Kg b.w. threshold)? o In case of gaps (chemical class etc.) – are these justified? o Is the number of chemicals sufficient? o Is the number of laboratories sufficient? (c) Appropriateness of the study execution (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled, e.g. censoring of values, retesting etc?). (d) Appropriateness of the statistical analysis used for analysing predictive capacity. 2) CONCLUSIONS OF STUDY: The ESAC is requested to assess whether the conclusions, as presented in the Validation Study Report (VSR), are substantiated by the information generated during validation and are plausible with respect to existing information and current views (e.g. literature). In particular: (a) Do the data on the basis of these chemicals provide new information on applicability and possible limitations (in addition to the original information available upon completion of the original ICCAM/ECVAM study)? (b) Are the conclusions on predictive capacity justified and plausible with

	respect to existing information (c) Is the information on the two protocol variants (abbreviated and automated version) sufficient in view of supporting their standardized use alongside the already validated protocol? (d) Are there possible gaps between study design and study conclusions which remain to be addressed in view of the suggested conclusions / use (see also point 3)? 3) SUGGESTED USE OF THE TEST METHOD: The ESAC is requested to review the suggested use of the validated method within a strategy to identify only unclassified chemicals (LD50 > 2000 mg/kg b.w.) as proposed by the Validation Management Team. (a) Is the suggested use of the test method, based on the information generated in the Validation Study, plausible and scientifically justified? (b) Is there additional information required (i.e. are there gaps) to be able to conclude on the plausibility of the suggested use?

4.3 TIMELINES

Timelines concerning this request When does ECVAM require the advice?	Timeline	Indication
	Draft/finalised ESAC Opinion required by:	ESAC 35, 4-5 October 2011
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	NO
	Request to be presented to ESAC at ESAC plenary meeting	ESAC 34, 22-23 March 2011

5. ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request Does the advice require an ESAC working group, an ESAC rapporteur etc.?	Structure(s) required	Required according to ECVAM? (YES/NO)
	S1 ESAC Rapporteur	NO
	S2 ESAC Working Group	YES Proposals from (a) ECVAM, (b) ESAC members and (c) ICATM partner organisations are listed in a separate document
	S3 Invited Experts	NO
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	

5.2 DELIVERABLES AS PROPOSED BY ECVAM

Deliverables What deliverables (other than the ESAC opinion) are required for addressing the request?	Title of deliverable other than ESAC opinion	Required? (YES/NO)
	D1 ESAC Rapporteur Report and draft opinion	NO
	D2 ESAC Peer Review Report and draft opinion	YES
	If other than above (D1-D2):	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Available (YES/NO)	File name
1	Final Study Report	yes	Final 3T3 NRU study report_March 2011
2	Study protocol HSL	yes	Annex 1_Study protocol of HSL
3	Study protocol JRC	yes	Annex 2_Study protocol of JRC
4	Study protocol IIVS	yes	Annex 3_Study protocol of IIVS
5	Solubility protocol	yes	Annex 4_ Solubility protocol
6	Seidle et al._2010_Cross sector review drivers and available 3Rs approaches acute toxicity testing	yes	2010_Seidel et al._ Toxicological Sciences
7	Creton et al._2010_Acute toxicity testing of chemicals—Opportunities to avoid redundant testing and use alternative approaches	yes	2010_Creton et al., Critical Reviews in Toxicology
8	Bulgheroni et al._2009_Estimation of acute oral toxicity using NOAEL	yes	2009_Bulgheroni et al._Toxicology In Vitro

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 34th meeting on 22./23. March the ESAC plenary unanimously decided to establish an ESAC Working Group charged with the detailed scientific review of the ECVAM follow-up study on the predictive capacity of the validated 3T3 NRU assay for acute toxicity testing.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:
ESAC Working Group for the detailed scientific peer review of the ECVAM follow-up study of the 3T3 NRU assay for acute toxicity testing

Abbreviated title:
ESAC WG 3T3 NRU

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the ECVAM-conducted follow-up study concerning the predictive capacity of the 3T3 NRU assay. The review needs to address the questions put forward to ESAC by ECVAM and the more detailed questions developed by the ESAC members of the ESAC WG in collaboration with the ESAC Chair, Vice Chair and Secretariat.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions drawn in the Validation Study Report are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Secretariat a detailed ESAC Working Group Report outlining its analyses and conclusions. A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.

The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The Secretariat has proposed timelines which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	Mid April	Teleconference to discuss/decide 1. the list of proposed external (non-ESAC) experts for the ESAC WG 3T3 NRU 2. the more detailed questions to put forward to the ESAC WG	1. List with 3 preferred options (3 external experts + 3 ESAC members = 6 experts in total) 2. Consolidated list of questions
2	Mid April	Both deliverables of item 1 to go to the ESAC for approval / amendment	Amended deliverables as listed under item 1 (if appropriate)
3	Kick-off teleconference in May or June	Discuss the organisation of review and drafting of report, distribution of work. Discuss the studies. Agree on the meeting date and further timelines.	Minutes and agreed meeting date/timelines, work organisation.
	WG meeting in September 2011	Finalisation of draft WG report. Preparation of presentation to ESAC.	1. Preliminary draft report. 2. Presentation of key elements (ESAC)

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the three questions posed to the ESAC which have been broken down further in more specific questions by the ESAC chair, the chair of the ESAC WG and the Secretariat (see section 4.2).

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Secretariat will provide guidance if necessary.

APPENDIX 1 REPORTING STRUCTURE FOR THE ESAC WG REPORT

The following suggested structure follows the ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions. A template has been created on the basis of the structure below and this template will be made available to the ESAC.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

1. Data collection
    - 1.1 Information / data sources used
    - 1.2 Search strategy
    - 1.3 Selection criteria applied to the available information

2. Study objective and design
    - 2.1 Clarity of the definition of the study objective
    - 2.2 Analysis of the scientific rationale provided
    - 2.3 Analysis of the regulatory rationale provided
    - 2.4 Appropriateness of the study design
    - 2.5 Appropriateness of the statistical evaluation

3. Test definition (Module 1)
    - 3.1 Quality and completeness of the overall test definition
    - 3.2 Quality of the background provided concerning the purpose of the test method
    - 3.3 Quality of the documentation and completeness of (a) standardised protocols (SOPs) and (b) prediction models

4. Data quality
    - 4.1 Overall quality of the evaluated data
    - 4.2 Sufficiency of the evaluated data in view of the study objective
    - 4.3 Quality of the reference data for evaluating reliability and relevance[1]

5. Test materials
    - 5.1 Sufficiency of the number of evaluated test items in view of the study objective
    - 5.2 Representativeness of the test items with respect to applicability

6. Within-laboratory reproducibility (Module 2)
    - 6.1 Assessment of repeatability and reproducibility in the same laboratory
    - 6.2 Conclusion on within-laboratory reproducibility as assessed by the study

[1] OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It sis the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

7. Transferability (Module 3)
    7.1 Quality of design and analysis of the transfer phase
    7.2 Conclusion on transferability to a second laboratory/other laboratories as assessed by the study

8. Between-laboratory reproducibility (Module 4)
    8.1 Assessment of reproducibility in different laboratories
    8.2 Conclusion on reproducibility as assessed by the study

9. Predictive capacity (Module 5)
    9.1 Adequacy of the assessment of the predictive capacity in view of the purpose
    9.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

10. Applicability domain (Module 6)
    10.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions
    10.2 Quality of the description of applicability domain, limitations, exclusions

11. Performance standards (Module 7)
    11.1 Adequacy of the proposed Essential Test Method Components
    11.2 Adequacy of the Reference Chemicals
    11.3. Adequacy of the defined Accuracy Values

12. Readiness for standardised use
    12.1 Assessment of the readiness for regulatory purposes
    12.2. Assessment of the readiness for other uses (in house screening etc.)
    12.3 Critical aspects impacting on standardized use
    12.4 Gap analysis

13. Other considerations

14. Conclusions on the study
    14.1 Summary of the results and conclusions of the study
    14.2 Extent to which conclusions are justified by the study results alone
    14.3 Extent to which conclusions are plausible in the context of existing information

15. Recommendations

    15.1 General recommendations concerning the study
    15.2 Recommendations concerning the test method (test system, protocol, prediction model)

16. References

17. Annexes