Report on the Full Test Submission Assessment

TM2020-04

3D reconstructed human skin micronucleus assay (RSMN)

February 8th, 2023

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Test Method: **RSMN – TM2020-04**

**Summary of the Assessment**

The RSMN is an *in vitro* assay developed to address the toxicity to the genome. It is an adaptation of the well-established *in vivo* and *in vitro* micronucleus tests (OECD TG 474 (OECD, 2016c) and OECD TG487 (OECD, 2016a)).

The RSMN has been designed in a three-dimensional reconstructed human skin (RHS) model: the EpiDerm™ (MatTek, Ashland, USA). The EpiDerm™ model is cultured from normal human epidermal keratinocytes derived from neo-natal foreskin tissue on specially prepared tissue culture inserts. The EpiDerm™ resembles the structure, morphology, and xenobiotic metabolism of the human epidermis (Hu et al., 2010; Gotz et al., 2012; Hewitt et al., 2013; Yuki et al., 2013) and it is of high biological relevance when assessing the genotoxic potential of test items via dermal exposure route.

The RSMN is intended to be used within regulatory genotoxicity hazard identification testing strategies to follow up positive results from the classical *in vitro* test battery, which is used as a first step. It is proposed to be used instead of *in vivo* genotoxicity test methods (e.g. OECD TG 474 (OECD, 2016c) and TG 475 (OECD, 2016b)) in the context of test items that are causing clastogenic (chromosomal breaks and translocations) or aneugenic (abnormal number of chromosomes) effects *in vitro* and are primarily associated with dermal exposure.

The method has a potential impact on the 3Rs. It provides a viable follow-up solution of high human relevance for testing dermally exposed chemicals, both under regulations that allow animal testing and under regulations that prohibit *in vivo* testing, such as the EU cosmetics regulation.

The test method has undergone a full validation process thus, the Test Submitter requested EURL ECVAM to consider the RSMN assay for peer-review. The full test submission, received in 2021, was completed in all its parts. EURL ECVAM acknowledged that the submission included a detailed test protocol (SOP) together with data annexes and bibliography describing the validation study, the properties of skin model, and strategic use of the RSMN, including in regulatory context i.e., under Cosmetics Regulation.

EURL ECVAM was able to assess the test method’s readiness to enter peer review by evaluating the completeness and quality of the information provided for each of the following relevant modules: test definition, within-laboratory reproducibility (WLR), transferability, between-laboratory reproducibility (BLR), predictive capacity and applicability domain. Suggestions for performance standards (PS) were not provided at this stage. Nevertheless, the lack of information did not affect the EURL ECVAM assessment.

The Test Submitter highlighted that the RSMN can be used together with the RS Comet assay in order to cover all genotoxicity endpoints that usually need to be addressed for regulatory purposes (gene mutation, clastogenicity, and aneugenicity).

Based on the mechanistic and biological relevance of the test method and provided information, EURL ECVAM concludes that RSMN assay is ready to undergo to peer review.

**1. MODULE 1: TEST DEFINITION**

**1.1 Human health/environmental/biological effect addressed by the test method and biological/ mechanistic relevance**

The Test Submitter has adequately addressed the human health/environmental effect and the biological and mechanistic relevance of the RSMN assay, which are supported by the relevant references, including reference to OECD TG 474 and 487.

**1.2 Purpose and need of the test method**

The Test Submitter has addressed the intended purpose, the need for the test method, and its regulatory application.

The method has a potential impact on the 3Rs. It provides a viable follow-up solution of high human relevance for testing dermally exposed chemicals, both under regulations that allow animal testing, and under regulations that prohibit *in vivo* testing, such as the EU cosmetics regulation.

To be noted that animal reagents such as BSA (bovine serum albumin) is required for culturing the cells in the EpiDerm™ skin model and making the trypsin neutralising solution.

**1.3 Protocol(s) of the test method**

A detailed protocol, which describes all essential procedural elements, has been provided in Attachment 1 of the Test Submission Template (TST). Several critical protocol steps are also flagged in the protocol and in the TST (paragraph 2.1.6.i).

The protocol is based on the principles described in OECD TG 487 on the *in vitro* micronucleus assay. The protocol described in the submitted SOP is related to the latest validation phases (Phases 2c and 2d) (Pfuhler et al. 2021). The submitted protocol evolved from the one previously described by Dahl et al. (2011) and has been updated over different phases of the validation study, based on observations made in the early phases (Phases 1, 2a and 2b). The different phases of the study are schematised in paragraph 3.1 under Figure 4. Overall, the protocol amendments are transparently described under 2.1.6 of the TST.

The tissue used (EpidermTM) is a registered trademark and commercially available. No other elements of the protocol are covered by proprietary issues.

Based on the above, EURL ECVAM considers that the protocol is clearly described and seems adequate to be performed by a naïve laboratory, where a standard technical expertise in 3D cultures and *in vitro* genotoxicity testing is ensured, including the interpretation of data and results. In addition, expertise in integrating statistical results with an assessment of the biological relevance of effects is required to come to an overall conclusion for the test items.

**1.4 Data analysis and prediction model**

The Test Submitter has described the data analysis procedure within the attached protocol (Attachment 1), in the TST, and in the following reference: Pfuhler et al 2021.

EURL ECVAM acknowledges that data interpretation, as described within the TST text, was applied consistently to the data as reported in Attachment 9*.*

Based on the attached data files, literature references, and *ad hoc* supplementary information, the procedures described for experimental data processing, data analysis, and the prediction model/s seem clear and sufficiently well detailed to be reproduced.

**1.5 Test items used for developing and optimising the test method (protocol and prediction model)**

The test items used for developing and optimising the RSMN are reported in Attachment 2. For each test item, CAS number, EC numbers (which also contains information on the commercial supplier), purity, physical form, chemical class/organic functional groups, molecular weight, octanol-water partition coefficient, water solubility, vapour pressure, as well as the links to websites from which the physical-chemical properties were retrieved.

Attachment 14 illustrates in which phases of the development, optimisation or validation the different items were used. However, the related results are spread across the different following publications: Curren et al. (2006), Mun et al. (2009) and Hu et al. 2009).

In total 9 genotoxic and 5 non-genotoxic test items were used in the development and optimisation phase.

It should be noted that the protocol was further optimised during the course of the validation study.

The information provided in paragraph 2.1.7 of the TST is consistent with the history of the test method development Attachment 14.

Overall, EURL ECVAM considers that the test items used for developing and optimising the test method (protocol and prediction model) were clearly reported.

**1.6 Occurrence of non-qualified test**

Three experiments out of 110 were considered invalid, based on the validity criteria reported in the SOP. The reasons for invalidity are reported in Attachment 9.

**1.7 Issues and drawbacks**

No issues and drawbacks have been reported.

**1.8 Essential information for a Validation Study based on Performance Standards [for similar or updated test method(s)]**

Not applicable.

**1.9 Overall conclusions on Module 1**

The information provided in Module 1 is complete and clear.

The RSMN assay is well defined. Background information on the design and development of the test method has been provided. The test protocol is clearly described. The Test Submitter has provided a detailed SOP and conspicuous number of references.

The quality of the information reported is adequate for the purpose of assessing Module 1.

**2. MODULE 2: WitHin-LaBoratory reproducibility**

**2.1 Test items used for assessing within-laboratory reproducibility**

No specific criteria to select test items for WLR were applied.

A total of 38 test items were used by the Test Submitter to demonstrate within-laboratory reproducibility.

The test items used for assessing the WLR are listed in Attachment 4 together with their commercial supplier, purity, physical form, chemical class/organic functional groups, molecular weight, octanol-water partition coefficient, water solubility, vapour pressure and relative experiment results. Chemical structures, EC-numbers, and links to websites from which the physical-chemical properties were retrieved, were included in the first worksheet of Attachment 9. The test items used cover almost the entire applicability domain that has been explored so far.

**2.2 Assessment of within-laboratory reproducibility**

The assessment of WLR was derived from the available data, since the study was not specifically designed to address WLR. This was possible because for most of the test items at least two experiments were available.

WLR was considered for a total of 69 repeated experiments within a single laboratory (the Test Submitter used the term “instances”) and covered 38 test items. For this analysis, the experiments conducted at 48h were considered independently from those at 72h, even if conducted by the same laboratory with the same test item. The WLR has been assessed in terms of concordance between predictions.

The overall WLR was 84%, and varied across the individual laboratories from 74% to 93%. A probability calculus was also applied and reported in Fig. 1 of the TST.

Some potential sources of variability have been identified by the Test Submitter but measures to control them were not proposed.

**2.3 Identification and discussion of outlying values**

Eleven experiments considered for WLR gave discordant results. A possible reason for outlying values was identified only for four of these experiments. Experiments with equivocal results were not considered in the WLR.

**2.4 Overall conclusions on Module 2**

Overall, the information provided by the Test Submitter in Module 2 seems sufficient to assess within-laboratory reproducibility of the RSMN.

**3. MODULE 3: TRANSFERABILITY**

**3.1 Test items used for assessing the transferability**

Seven test items used to assess transferability were listed in Attachment 5. They include three true positives, three true negatives, and one misleading positive. Information about commercial supplier, purity, physical form, chemical class/organic functional groups, molecular weight, octanol-water partition coefficient, water solubility, vapour pressure as well as links to websites, from which the physical-chemical properties were retrieved, was reported in Attachment 5.

Information provided in this section of the TST is consistent with what is reported in Attachment 14.

**3.2 Assessment of the test method transferability to other laboratories**

Transferability was initially demonstrated with seven test items by Hu et al. (2009) and subsequently assessed in Phase 1 of the validation study with two test items (Aardema et al., 2010). The transferability assessment is therefore based on the original protocol. As explained in paragraph 2.1.6 of the TST, this protocol did not yet include the measurement of viable cell count as second readout for cytotoxicity, the specification that only the lowest precipitating concentration hasto be included in the statistical analysis, and the addition of the 72h time point.

The conduct of a RSMN requires standard technical laboratory skills as well as expertise in interpreting data and results of genotoxicity test methods. In addition, experience in handling 3D tissue and in defining the dose range for *in vitro* genotoxicity tests is important.

Moreover, based on the experience acquired during the validation study, the Test Submitter has suggested that a naïve laboratory should conduct at least two experiments with three test items (cyclohexanone, mitomycin C and n-ethyl-n-nitrosourea) and additional experiments to establish a reliable historical control.

The Test Submitter did not recommend any specific training protocol. Therefore no training protocol, neither a transfer report have been provided. Critical aspects of the SOP have been properly emphasised in paragraph 2.1.6.i and some recommendations were provided by the Test Submitter.

**3.3 Overall conclusions on Module 3**

Overall, the information provided by the Test Submitter in Module 3 seems sufficient to assess transferability of the RSMN.

**4. MODULE 4: BETWEEN-LaBoratory reproducibility**

**4.1 Test items used for assessing between-laboratory reproducibility**

The test items used to assess between-laboratory reproducibility (BLR) are listed in Attachment 8, including information on the commercial supplier, purity, physical form, chemical class/organic functional groups, molecular weight, octanol-water partition coefficient, water solubility and vapour pressure, and relative experiment results. Chemical structures, EC-numbers and links to websites from which the physical-chemical properties were retrieved are included in the first worksheet of Attachment 9.

Twenty-two test items were selected to cover various MoA. They include 11 true positives, 7 true negatives, and 4 misleading positives for which positive *in vitro* findings were reported, but not confirmed in *in vivo* studies.

The information provided in this section is consistent with Attachment 8 and Attachment 14.

**4.2 Assessment of between-laboratory reproducibility of experimental data**

The BLR on the concordance of predictions was assessed between 2, 3 and 4 laboratories respectively for 7, 12 or 3, and 3 test items.

The overall BLR was reported to be 77.3% (17/22) (Attachment 8).

It should however be noted that only 16 test items (out of 22) had concordant calls, while 4 were discordant and 2 were negative in one or two laboratories and equivocal in the other. If the BLR is considered equivocal for these two test items, then the overall BLR should be 72.7%. Instead, the Test Submitter has considered a positive concordance for 17 items in the overall BLR assessment with the following explanation “*Considering resorcinol and tolbutamide due to the remaining uncertainty that led to equivocal results to be between-laboratory reproducible with a likelihood of 50%, the overall BLR, when weighing all test items equally, was 17/22 = 77.3%."*

Potential sources of variability have been identified in paragraph 2.4.3. No measure to control the sources of variability was proposed by the Test Submitter.

**4.3 Identification and discussion of outlying values**

Potential experimental sources of variability that can result in reduced BLR were identified as follows: experiments with borderline results, differences in dose selection, and cytotoxicity between laboratories.

**4.4 Overall conclusions on Module 4**

Overall, the information provided by the Test Submitter in Module 4 seems sufficient to assess between-laboratory reproducibility of the RSMN.

**5. MODULE 5: predictive capacity**

**5.1 Test items used for assessing predictive capacity**

The full list of 43 test items used to assess the predictive capacity of the RSMN assay, including their names, CAS numbers, commercial source, purity, physical form, and any other relevant physical/chemical properties (e.g. chemical classes/organic functional groups, MW, LogP), is presented in the first worksheet of Attachment 9.

Information on MoA, reference *in vitro* and *in vivo* genotoxicity data, as well as carcinogenicity data are presented in Attachment 10.

Test items have been selected to cover a variety of MoA and to include three categories: true negative (10) and true positive (21) chemicals, with concordant *in vitro* and *in vivo* data, and misleading positives (12) for which positive *in vitro* findings were reported but not confirmed in *in vivo* studies.

The information provided in paragraph 2.5.1 is consistent with Attachment 9 and Attachment 14.

Two of the test items used for the optimisation of the test method were also used in the assessment of predictivity.

**5.2 Assessment of the predictive capacity of the test method**

The predictive capacity of the RSMN was analysed using two different approaches and the related results were reported in the TST as well as in Attachment 9 (predictive capacity worksheet). The prediction model appears to be applied consistently. In a few cases expert judgement was applied to come to a final lab call, e.g. tolbutamide, laboratory 2.

**5.3 Identification and discussion of false predictions**

False predictions have been identified and extensively discussed in paragraph 2.5.3. Thirteen test items were not correctly predicted in at least one laboratory. In about half of the cases, a possible explanation for misclassification has been suggested by the Test Submitter.

**5.4 Quality/variability/uncertainty of the reference data used to calculate predictive capacity**

All relevant information on the reference data has been summarised in Attachment 10. Reference data for true positive (TP), misleading positive (MP), and true negative (TN) chemicals were submitted by the Test Submitter.An analysis of the possible uncertainty of these data has been presented identifying a few test items that have a slightly higher uncertainty when compared to the others.

**5.5 Overall conclusions on Module 5**

Overall, the information provided in Module 5 by the Test Submitter should be sufficient to allow an assessment of the predictive capacity of the RSMN.

**6. MODULE 6: APPLICABILITY DOMAIN**

**6.1 Limitations of the test method**

Based on the data generated during the study, no apparent limitation of the applicability domain has been identified.

The Test Submitter has recognised that the applicability domain of the RSMN may need further re-evaluation once more data are published.

The only technical limitation of the test method is related to the solubility of the substances and the suitability of the solvent used (acetone or 70% ethanol).

**6.2 Overall conclusions on Module 6**

The information provided in Module 6 by the Test Submitter appears to be sufficient for assessing the applicability domain of the RSMN.

**7. MODULE 7: PERFORMANCE STANDARDS**

**7.1 Suggestions for essential test method components, possible reference items, accuracy and reliability values**

Not applicable. The Test Submitter will provide information on performance standards in a later step.

**7.2 Overall conclusions on Module 7**

Not applicable.

**8. ADDITIONAL INFORMATION**

**8.1 Study organisation and management**

The study organisation and the different phases of the studies have been reported in paragraph 3.1 by the Test Submitter. The original composition of the steering committee roles is reported in Table 4. Although the Test Submitter mentioned that involved institutions and roles changed during the study, an updated list was not provided. It should be noted that representatives from each laboratory were present in the steering committee.

**8.2 Collection of existing (historical) information**

Published work on the development of the RSMN assay undertaken prior to the validation are well reported in paragraph 3.2.

**8.3 Study conclusions**

The Test Submitter has showed that the RSMN assay works with the MatTek Epi-200TM Skin model. According to the Test Submitter, the assay can be transferred to other laboratories and is reproducible within and between laboratories. Moreover, the Test Submitter has claimed that the method has high predictive capacity and it could be used in combination with the RS Comet assay.

**8.4 Study recommendations**

The Test Submitter has not provided any real recommendations, rather an encouragement to use the method for hazard assessment in order to extend the applicability domain, as already recommended in the SCCS note of guidance.

**9. OTHER**

The RSMN assay has already been included into the OECD’s test guideline work program.

An EURL ECVAM representative was part of the validation study steering committee only in the initial phase of the study, but not during phase 2.However, an expert from EURL ECVAM participated in the selection of chemicals to be used in Phase 2.

**10. OVERALL CONCLUSIONS ON THE OUTCOME OF THE ASSESSMENT**

Following the evaluation of the RSMN assay Submission, EURL ECVAM concludes that Module 1, 2, 3, 4, 5 and 6 of the EURL ECVAM modular approach appear complete.

The supplementary application of expert judgment for some test results highlights the need of highly curated analysis for the correct interpretation of results. As such, EURL ECVAM identifies the reliance on specific expertise as a critical factor for the qualification of tests and correct interpretation of test results.

EURL ECVAM considers the test method submission suitable to progress to peer review.

**11. CLARIFICATIONS/ADDITIONAL INFORMATION TO BE REQUESTED TO THE TEST SUBMITTER**

The Test Submitter has provided additional information and clarifications by mail (see the following word file in Email\_Exchange\_withSubmitter: RSMN RS Comet TST clarifications).

**ANNEX: FOR INTERNAL USE ONLY (TO BE DELETED IN THE FINAL ASSESSMENT REPORT)**

Please complete the following table by ticking for each module (M) the appropriate box (where feasible) with regard to the completeness and quality of the information provided in the Test Submission.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Completeness and quality of the information provided for each module** | **Insufficient** | **Requires substantial additional work** | **Requires minor additional work** | **Complete** |
| M1 - Test Definition |  |  |  |  |
| M2 - Within-Laboratory Reproducibility |  |  |  |  |
| M3 - Transferability |  |  |  |  |
| M4 - Between-Laboratory Reproducibility |  |  |  |  |
| M5 - Predictive Capacity |  |  |  |  |
| M6 - Applicability Domain |  |  |  |  |
| M7 - Performance Standards |  |  |  |  |

***Any additional comments from the assessment team:***

Raw data were missing in the initial submission but were then provided upon request.