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REPORT OF THE PEER REVIEW OF THE VALIDATION STATUS OF THE IN VIVO ERYTHROCYTE PIG-A GENE MUTATION ASSAY

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NO. 319

REPORT OF THE PEER REVIEW OF THE VALIDATION STATUS OF THE IN VIVO ERYTHROCYTE PIG-A GENE MUTATION ASSY

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FOREWORD

This document contains the report of the Peer review panel on the validation status of the *in vivo* erythrocyte *Pig-a* gene mutation assay. Between 2010 and 2019, several international or national inter laboratory trials have been conducted with the *in vivo* erythrocyte *Pig-a* gene mutation assay. The available information on this assay has been collected by the United States in a Detailed Review Paper (DRP) and a Retrospective validation study which served a as a basis for the peer review.

The report was completed by the Peer review panel on November 15th, 2019. The Peer review report, the DRP and the Retrospective validation report of the *in vivo* erythrocyte *Pig-a* gene mutation assay were circulated to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) for comments in December 2019.

The WNT endorsed the Peer review report at its 32nd meeting in April 2020. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the Peer review report on 9 July 2020. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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1. Summary

1. This document presents the Peer Review Report (PRR) of the retrospective performance assessment of the in vivo erythrocyte *Pig-a* gene mutation assay. In the last ten years, several international or national inter laboratory trials have been conducted with the *in vivo* erythrocyte *Pig-a* gene mutation assay. The available information on the *Pig-a* gene mutation assay has been collected by the United States in a Detailed Review Paper (DRP) in order to serve as a basis for the development of a Test Guideline on the *Pig-a* gene mutation assay.

2. The draft DRP, complemented by a Retrospective Performance Assessment (RPA) and a Validation report on the *in vivo* erythrocyte *Pig-a* gene mutation assay was submitted for review to an independent Peer Review Panel (PRP) who conducted a retrospective assessment of the validation status of the *Pig-a* gene mutation assay. The work of the Panel was coordinated by the OECD Secretariat.

3. The PRP was asked to evaluate how the retrospective validation approach addresses the principles outlined in the OECD Guidance Document 34 on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (GD 34).

4. The PRP concluded that the above documents meet the validation principles of OECD GD 34. The Panel agreed that the data available is sufficient to conclude that the development of the *Pig-a* assay towards a Test Guideline should move forward. However, some elements require additional discussion and/or analysis of the data.

5. The PRP made a number of general and specific recommendations to strengthen the robustness of the validation study and clarify technical aspects of the protocol, for careful consideration during the development of a Test Guideline for regulatory use.

2. Background

6. Mutations in the phosphatidylinositol glycan class A (*Pig-a*) gene are known to disrupt the synthesis of glycosylphosphatidylinositol (GPI) anchors, resulting in the loss of cell-surface proteins. These losses can be identified by immunofluorescent staining and flow cytometric analysis of peripheral blood cells.

7. This is the basis for the rodent *Pig-a* gene mutation assay, first described in 2008, and which has emerged as a potential regulatory test for evaluating in vivo gene mutation.

8. The development of the *Pig-a* assay has benefited from several grants from the U.S. National Institutes of Health and Japan Health Sciences, and research conducted by industry. In addition, important expertise and organisational support has come from workgroups formed by the International Workshop on Genotoxicity Testing (IWGT) and the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute. Consensus documents published by these organisations has formed the basis for the development of the current Detailed Review Paper (DRP) and validation report.

9. The project for developing a Test Guideline for the in vivo erythrocyte *Pig-a* gene mutation assay was proposed by the United States and included in the work plan of the Test Guidelines Programme in 2015 (Project 4.93: Test Guideline for the *Pig-a* assay: an in vivo gene mutation assay promoting the 3Rs principles), with the understanding that a DRP and a validation document should first be approved by the Working Group of the National Coordinators for the Test Guidelines Programme (WNT) before TG development. Considering the available data, the conduction of new experimental work was not deemed necessary and in the interest of reducing animal use, a retrospective assessment was preferred over a prospective validation study. An OECD expert group for the development of the *Pig-a* gene mutation assay was established in January 2018, made of experts nominated by the WNT.

10. In February 2018 a draft DRP was submitted to this expert group for comments. The DRP was subsequently revised by the lead country and a full peer review package, made of the DRP, a Retrospective Performance Assessment (RPA) and a validation report, was submitted for peer review to a panel established in June 2019.

3. The peer review process

11. The Peer Review Panel (PRP) was established in order to provide an independent review of the validation status of the *in vivo* erythrocyte *Pig-a* gene mutation assay for the detection of mutagens. The review was conducted between June and October 2019, coordinated by the OECD Secretariat.

12. To establish the panel, the Secretariat contacted the members of the OECD expert group for the development of the *Pig-a* gene mutation assay, nominated in January 2018 by the WNT.

13. Only experts who had not participated in the development of the Detailed Review Paper or Retrospective Performance Assessment were invited to apply to the Panel. The selected members of the Panel are listed in Annex 1.

14. Furthermore, the Secretariat invited two members of the expert group who had participated in the development of the validation report to serve as observers and support the panel to clarify potential issues, if necessary.

15. The Peer Review Panel (PRP) was asked to review the validation status of the *in vivo* erythrocyte *Pig-a* gene mutation assay, and respond to charge questions based on the OECD Guidance Document 34.

16. Panel members were asked to base their review on two documents:

- The in vivo erythrocyte *Pig-a* gene mutation assay Part 1: Detailed review paper and performance assessment, May 20, 2019, and
- The in vivo erythrocyte *Pig-a* gene mutation assay Part 2: Validation report, March 1, 2019

17. These documents were publicly available on the OECD public website: http://www.oecd.org/env/ehs/testing/chemicalstestingdraftoecdguidelinesforthetestingofchemicals-sections1-5.htm

18. In addition, the following articles (referred to in the validation study report) were provided to the PRP as background documents for the review:

Dertinger et al., Environmental and Molecular Mutagenesis. 2011[1]

Kimoto et al., Mutation Research. 2013[2]

Godin-Ethier et al., Mutagenesis. 2015[3]

[1] Dertinger SD, S Phonethepswath, P Weller J Nicolette, J Murray, P Sonders, H-W Vohr, J Shi, J Krsmanovic, C Gleason, L Custer, A Henwood, K Sweder, LF Stankowski Jr, DJ Roberts, A Giddings, J Kenny, AM Lynch, C Defrain, F Nesslany, B-jM van der Leede, T Van Doninck, A Schuermans, K Tanaka, Y Hiwata, O Tajima, E Wilde, A Elhajouji, WC Gunther, CJ Thiffeault, TJ Shutsky, RD Fiedler, T Kimoto, JA Bhalli, RH Heflich, JT MacGregor (2011b) International Pig-a gene mutation assay trial: Evaluation of transferability across 14 laboratories, Environ Mol Mutagenesis 52:690-698.

[2] Kimoto T, K Horibata, S Chikura, K Hashimoto, S Itoh, H Sanada, S Muto, Y Uno, M Yamada, M Honma (2013) Interlaboratory trial of the rat Pig-a mutation assay using an erythroid marker HIS49 antibody, Mutat Res 755:126-134.

[3] Godin-Ethier J, F Leroux, N Wang, S Thébaud, F Merah, A Nelson (2015) Characterizsation of an in vivo Pig-a gene mutation assay for use in regulatory toxicology studies. Mutagenesis 30:359-363.

Gollapudi et al., Mutation Research. 2015[4]

Kimoto et al., Mutation Research. 2016[5]

Raschke et al., Environmental and MolecularMutagenesis.2016[6]

Chung et al., Mutant Res Gen Tox En. 2018[7]

19. The charge to the Panel was to assess to what extent the eight OECD validation criteria set out in the OECD Guidance Document 34 had been met. The charge questions, adapted to the retrospective evaluation of the *Pig-a* gene mutation assay, are listed in Annex 2.

20. Each Panel member provided written responses to the charge questions to the Secretariat by 22 July 2019. For transparency, the individual comments from the Panel members are provided anonymously in Annex 3.

21. Based on these responses, the Secretariat prepared a draft summary of the PRP's responses to the individual questions and the initial report was circulated to the panel on 25 September 2019. The issues identified were discussed at a teleconference on 1st October 2019.

22. The initial peer review report was updated based on the discussions at the first teleconference and provided to the Panel for review and comments on 8 October 2019. The panel commented on the draft report until 23 October 2019

23. The final report was updated by the Secretariat and sent to the PRP for comments on 4 November, 2019 and final approval was reached on 15 November 2019.

24. This report presents the summary of the assessment of the draft Detailed Review Paper (DRP), Retrospective Performance Assessment (RPA) and the Validation report on the *in vivo* erythrocyte *Pig-a* gene mutation assay and the resulting agreed responses of the PRP to each of the charge questions.

[4] Gollapudi BB, AM Lynch, RH Heflich, SD Dertinger, VN Dobrovolsky, R Froetschl, K Horibata, MO Kenyon, T Kimoto, DP Lovell, LF Stankowski Jr, PA White, KL Witt, JY Tanir (2015) The in vivo Pig-a assay: a report of the International Workshop on Genotoxicity Testing (IWGT) Workgroup, Mutat Res 783:23-35.

[5] Kimoto T, K Horibata, D Miura, S Chikura, Y Okada, A Ukai, S Itoh, S Nakayama, H Sanada, N Koyama, S Muto, Y Uno, M Yamamoto, Y Suzuki, T Fukuda, K Goto, K Wada, T Kyoya, M Shigano, H Takasawa, S Hamada, H Adachi, Y Uematsu, E Tsutsumi, H Hori, R Kikuzuki, Y Ogiwara, I Yoshida, A Maeda, K Narumi, Y Fujiishi, T Morita, M Yamada, M Honma (2016) The PIGRET assay, a method for measuring Pig-a gene mutation in reticulocytes, is reliable as a short-term in vivo genotoxicity test: Summary of the MMS/JEMS-collaborative study across 16 laboratories using 24 chemicals, Mutat Res 811:3-15.

[6] Raschke M, B-W Igl, J Kenny, J Collins, SD Dertinger, C Labash, JA Bhalli, CCM Tebbe, KM McNeil, A Sutter (2016) In vivo Pig-a gene mutation assay: Guidance for 3Rs-friendly implementation, Environ Mol Mutagenesis 57:678-686.

[7] Chung Y-S, B Pak, S Han, J Lee, J Kim, S-M Back, C-R Park, S-H Kim, J-K Lee (2018) Multi-laboratory evaluatin of 1,3-propane sultone, N-propyl-N-nitrosourea, and mitomycin C in the Pig-a mutation assay in vivo, Mutat Res 831:62-68.

4. Validation Principle 1 – The rationale for the test method should be available

Charge Question 1: *Do you consider that the rationale for the Pig-a gene mutation assay test method is clearly elaborated in the detailed review paper / validation report in terms of its scientific basis, regulatory purpose and need?*

25. Overall, the PRP considered that the rationale for the *Pig-a* gene mutation assay test protocol is clearly detailed in the DRP in terms of its scientific basis, regulatory purpose and need.

26. The scientific basis is well described in the reviewed material which provides the mechanistic basis of the proposed test method in terms of:

- How the biological functions of the protein expressed from *Pig-a* gene are related to GPI-anchors tethering cell surface proteins,
- How the measuring the loss of cell surface proteins predicts mutation in the endogenous X-linked *Pig-a* gene,
- How the mutation in *Pig-a* gene causes PNH (paroxysmal nocturnal hemoglobinuria) in human,
- How the phenotypic mutant cells as a reporter of gene mutation are quantitatively evaluated using flow cytometric assay,
- How mutant erythrocytes, mutant reticulocytes (RET) and mutant Red blood cells (RBC), respond with treatment and how erythroid progenitors or HSCs are related to accumulated mutant frequencies.

27. In terms of the regulatory purpose, several peer reviewers noted that the test is particularly interesting to follow up positive in vitro finding. The PRP also noted that the assay can have additional applications in conducting weight-of-evidence genotoxicity assessments, quantitative measurements of in vivo mutation, extended-time monitoring of mutant frequencies, and can be used as a routine complement to micronucleus testing that affords more information on in vivo genotoxicity. A reviewer noted that data generated from *Pig-a* assay can be useful for hazard/risk assessment purposes for human health due to the similarity in GPI-anchored systems.

28. It was noted that although many in vivo genotoxicity tests exist, currently, only the transgenic rodent (TGR) assay (TG 488) evaluates in vivo gene mutation. The PRP considered that the *Pig-a* gene mutation assay can be seen as an alternative test to TG 488 and has several advantages e.g. (i) it doesn't require the use of transgenic animals, (ii) only small amounts of blood can be sampled, (iii) integration to repeated dose toxicity test may be easy. Regarding this latter point, caution was expressed since the assay has not been validated in combination with a repeated dose toxicity study.

29. The PRP however raised some limitations to the assay, which derive from the design of the test system:

- The erythrocyte *Pig-a* can only be used to detect gene mutations induced in the bone marrow since the mutants measured in the erythrocyte *Pig-a* assay are formed in nucleated erythroid progenitor cells that, in adult rodents, are mainly located in the bone marrow. The test substance or its metabolites must be able

to reach the bone marrow for an adequate evaluation of in vivo hazard. Thus, caution must be exercised in interpreting negative results.

- For the time being, the assay only detects mutations induced in erythropoietic tissue (i.e. mainly the bone marrow in adult animals), reducing its sensitivity for substances that are mutagenic only in other tissues, like liver.

30. The main concern that was expressed was about the choice of the treatment schedule. It was noted that a short term dosing period (i.e. 1-3 days), used in some protocols may be less sensitive than the longer term dosing period (i.e. 28 days), used in other protocols, since some positive mutagens compounds failed to be detected in the short treatment protocol.

Overall, the PRP agreed that the Validation Principle 1 has been met. However, the PRP recommends that the treatment schedule be thoroughly discussed and data analysed to support the choice of an optimal treatment schedule in the development of a standardised test method.

5. Validation Principle 2 – The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described

Charge Question 2: *Is the relationship between the test method's endpoint(s) and the (biological) phenomenon of interest described adequately, using relevant references for the scientific relevance of the effect(s) measured (in terms of their mechanistic (biological) or empirical (correlative) relationship) to the specific type of effect/toxicity of interest?*

31. Overall, the PRP considered that there are sufficient evidence and data that suggest or demonstrate the link between the test method's endpoint (i.e. mutagenicity) and the biological phenomenon of interest (i.e. loss of GPI anchor).

32. The relationship between *Pig-a* mutation and the loss of GPI anchors and their associated proteins is the basis for the *Pig-a* gene mutation assay. It was noted that only a few studies clearly demonstrate this relationship and only limited analyses have been performed to determine the types of gene mutations. But those that have been conducted confirm that the assay detects similar mutations that the test agents induce in other assays.

33. The following elements are described and referenced in the DRP and provide some strength to the relationship:

- The *Pig-a* mutations were confirmed by DNA sequencing of DNA extracted from GPI-marker-deficient bone marrow erythroid progenitor cells and granulocytes in rats using NGS.
- *Pig-a* cDNAs were synthesised from mouse mutant bone marrow erythroids and analysed by Sanger sequencing. Base pair substitutions were observed.
- Wild type bone marrow erythroid cells as well as wild-type granulocytes contain no *Pig-a* gene mutation.
- GPI-deficient cells in PNH patients are almost always associated with *Pig-a* mutations.
- One successful report containing analysis of *Pig-a* mRNA in mouse mutant RETs by amplification of cDNA using RT-PCR demonstrated *Pig-a* gene mutation.
- GPI-deficient rat T lymphocytes containing *Pig-a* gene mutations were observed in many studies.
- *Pig-a* gene mutations cause GPI deficiency in mammalian cells in vitro.
- *Pig-a* gene mutation responses are consistent with TGR and endogenous reporter gene mutation responses in the same animals.
- Lack of evidence that loss of GPI anchors and GPI-anchored protein is due to something other than mutation, e.g., epigenetic gene silencing.

Overall, the peer review panel agreed that the Validation Principle 2 has been met.

6. Validation Principle 3 – A detailed protocol for the test method should be available

Charge Question 3: *Do you consider that the protocol description in the general recommendations for conducting the assay that were made in the IWGT report (Gollapudi et al., 2015) is sufficiently detailed, including a description of all the materials needed (e.g. specific cell types or construct or animal species), a description of what is measured and how it is measured, a description of appropriate data analysis, decision criteria for evaluation of data and what are the criteria for acceptable test performance? Determine whether the use of the IWGT general recommendations for conducting this assay is best. If not, what updated protocol in Part1, Section 4 would you recommend?*

34. Overall, the PRP found the protocol description to be quite detailed and clear. It was noted however, that some parts of the protocol need to be further discussed.

35. The reviewers reported that there are multiple study designs/treatment schedules described in the literature, as acknowledged in tables VI and VII of the DRP. Regarding the treatment duration, 2 protocols have mainly been used, the short term (1 day to 3 day treatment) and the long term (28 day treatment). Regarding the sampling time, this has been much more variable across the various studies, going from a few days before the end of a long term treatment to several weeks after the end of the treatment (either short or long).

36. It was noted that the way the DRP refers to the sampling time may be confusing, since it doesn't correspond to the duration between the end of the treatment and the day of sampling but to the number of days since the experience has started, i.e. a 28 day treatment followed by 28 days without treatment before the sampling day would read "sampling day: 56", similarly, 28 day treatment followed by 3 days without treatment before the sampling day would read "sampling day: 31". Since 'Sampling time' may also be understood as a duration after treatment, clarification may be needed in the DRP.

37. Considering that some weak mutagens would not be detected after a short term treatment, the reviewers recommended that the choice of the treatment schedule be based on the toxicokinetic properties of the substance and that the assay be conducted using a 28-day repeat-dose protocol per default, in case of absence of data of bioavailability on the compound and in case weak mutagenic properties are observed in vitro.

38. Regarding the duration before sampling, the PRP could not make a particular recommendation but suggested that further analysis be conducted in relation with the stability of the induced RCB and RET *Pig-a* mutant frequency (MF) and time-response relationship of *Pig-a* MF.

39. Additional elements in the protocol would also need to be clarified/discussed:

- Acceptable or recommended route of exposure
- Minimum number of dosing groups
- Acceptable animal age range

It was questioned if the background RBC and RET *Pig-a* MF increase with age like TGR assay, and if too old (with low %RET) or too young (with high %RET) animals can be used for *Pig-a* assay.

- Method of blood sampling, the volumes to be collected in particular, depending on specific cases

Several questions need to be addressed such as (i) the volume of peripheral blood that should be sampled in rat and mouse to conduct both RBC- and RET-*Pig-a* assay under the recommendation (ii) if the same amount of blood is enough for both 1 day and 28 day sampling time (because %RET is higher in young animal and decreases in older animal).

- Option for long time storage of blood
- Minimum number of cells to be analysed

It was noted that although the IWGT report focuses primarily on the *Pig-a* assay in rats, other mammalian species used in other toxicological/investigational studies can be used and this is supported by the literature. The DRP thus describes both rats and mice. However, background *Pig-a* MF could be lower in mice than that in rats. This may have an impact on the minimum number of cells to be analysed. If a greater number of cells is needed, it may affect blood sampling.

Overall, the PRP agreed that the Validation Principle 3 has been met. However, a recommendation is made for specific clarifications in the protocol description of various elements, such as the treatment and sampling schedule, route of exposure, number of dosing group, animal age, volume of blood sampling, option for long time storage of blood, as well as the number of cells to be analysed.

7. Validation Principle 4 – The intra-, and inter-laboratory reproducibility of the test method should be demonstrated

Charge Question 4: *Determine whether there are sufficient data from the various international validation studies to conclude acceptable intra- and inter-laboratory reproducibility. Do you consider that the intra-, and inter-laboratory reproducibility of the Pig-a gene mutation assay test method is adequately demonstrated, considering availability of the data over time, as well as the degree to which biological variability affects the test method reproducibility?*

40. Overall, based on the review of the various international interlaboratory trials as well as additional intra- and inter-laboratory studies referenced in the validation report, the reviewers considered that the technical transferability as well as the intra- and inter-laboratory reproducibility of the *Pig-a* gene mutation assay were good and adequately demonstrated. It was noted however that the assessment of intra-laboratory reproducibility relies on a limited number of studies some of which were performed using an earlier version of the assay, not involving immunomagnetic separation.

41. The PRP considered that the factors that contribute to variability were well explained. As discussed in Charge Questions (CQ) 1 and 3, it was noted that the most important factor that affects the test method reproducibility is the treatment schedule, i.e. short term or long term treatment. In addition, the following elements were also identified as critical variables of the test system which need to be well controlled:

- the blood collecting process, which should be carefully performed to preserve cell surface protein;
- immunomagnetic separation which is needed to obtain sufficient mutant erythrocytes.

42. The variations in background mutation frequencies among the laboratories was considered normal and did not appear to affect the analysis.

43. The PRP agreed that the classes of compounds that have been tested do not cover the full spectrum of chemical space but considered that the available data constitute a sufficient assessment of the ability of the assay to detect mutagens among various chemical classes.

The PRP agreed that the Validation Principle 4 has been met, despite the limited number of studies related to the assessment of intra-laboratory reproducibility and the fact that the classes of compounds that have been tested do not cover the full spectrum of chemical space. More experiences with time could address these issues.

8. Validation Principle 5 – Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.

Charge Question 5: *Check that the applicability domain has been appropriately defined. Are the reference chemicals used to demonstrate the performance of this test method representative of the types of substances for which the test method will be used and have they been tested under code to exclude bias?*

44. The PRP considered that the applicability domain has been appropriately defined. The types of chemicals used for the validity of the *Pig-a* assay were various in terms of structure and functions and represent a variety of mutagenic (carcinogenic mutagens, noncarcinogenic mutagens, epigenic carcinogens), clastogens, aneugens, promutagenic and non-mutagenic chemicals that are typically employed to evaluate genetic toxicity endpoints.

45. As mentioned in CQ 1, it was noted that, like for the in vivo micronucleus test, the *Pig-a* assay only detects mutations induced in erythropoietic tissues (mainly the bone marrow), reducing its sensitivity for substances that are mutagenic only in other tissues.

46. Although it appears that none or almost none of the *Pig-a* data that were used for the performance analysis were produced using coded samples, the PRP was confident that this didn't alter the evaluation of the results, due in particular to the fact that most of the data generation in the *Pig-a* assay is automated by the flow cytometric analysis.

The PRP agreed that the Validation Principle 5 has been met, although the majority of the data was obtained from non coded compounds.

9. Validation Principle 6 – The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.

Charge Question 6: *In the context of this retrospective performance assessment, do you consider the approach employed to assess the performance of the assay is relevant? Do you consider that the performance of the test method has been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data?*

47. Overall the PRP considered that the retrospective performance assessment evaluating studies in the published peer-reviewed literature and other routes, where original data could be obtained, was highly relevant and provided essential support for the performance of the *Pig-a* assay.

48. For describing accuracy, sensitivity, specificity, positive predictivity, negative predictivity, and concordance were evaluated, and accuracy of *pig-a* gene mutation assay considered high enough to be used for regulatory test. It is appreciated that these descriptive statistics are based upon comparisons between test results obtained from experiments carried out by different laboratories, using protocols that may present some differences, and that the DRP cautions that the relatively small data sets result in wide 95% confidence intervals.

49. The PRP concluded that (i) the assay is highly accurate when TGR mutation in bone marrow is used to define true positive and negative responses, and (ii) the assay is also reasonably accurate when TGR mutation in any tissue, in vivo MN induction, and rodent cancer in any tissue is used to define true positive and negative responses. Overall, the PRP approved the statement of the RPA that indicates that the assay is highly accurate for detecting rodent mutagens and carcinogens that affect the bone marrow and the hematopoietic system, respectively.

50. The PRP considered that the review has demonstrated the assay can be applicable to rat and mice. It was noted that any performance using other species was not included. One reviewer however was of the opinion that the assay can be applicable across species, including human monitoring.

51. It was noted that the testing dataset has a bias in that it includes many positive chemicals but a small number of negative chemicals. The same problem was also met with the dataset used for the TGR assay. To mitigate this bias in the *Pig-a* assay, solvent/vehicle control data were added for negative data. The same approach was used in DRP of TGR assay (OECD 2009).

The PRP agreed that the Validation Principle 6 has been met, despite the fact that the data analysed were generated using protocols that presented some differences and that a small number of negative chemicals was used in the dataset.

10. Validation Principle 7 – Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP

***Charge Question** 7: Have all of the data supporting the validity of a test method been obtained in accordance with the principles of GLP? If not, has an adequate consideration been given to the potential impact on the validation status of the test method?*

52. The PRP agreed that it is difficult to know to which extent the data supporting the validity of the test method have been obtained in accordance with the principles of Good Laboratory Practices (GLP). However, the PRP agreed that it is reasonable to assume that data were generated following good scientific practices, including extensive training of laboratory personnel and extensive planning of the studies. The PRP was confident that although the majority of the work may not have been conducted in compliance with GLP, this does not preclude the robustness of the assay.

The PRP agreed that the Validation Principle 7 has been partly met.

11. Validation Principle 8 – All data supporting the assessment of the validity of the test method should be available for expert review

Charge Question 8: *Do you consider that all the data supporting the assessment of the validity of the test method are easily available for expert review? These include: a detailed and readily available test method protocol to the public and independent laboratories; organised and easily accessible data to permit independent review(s); benchmarks by which an independent laboratory can itself assess its proper adherence to the protocol.*

53. The PRP considered that most of the data supporting the assessment of the test method validation are available for expert review. The assay protocol and recommendations are described in the DRP (section 4 and 5). In addition, all of the data and methods supporting this document are in the open, peer-reviewed literature and all *Pig-a* data that were identified and served in the analysis were included in a data collection spreadsheet publicly available.

54. Minor concerns were noted such as the fact that the tables of studies available in the DRP are a bit complex to follow, that there is no section or paragraph about proficiency criteria by which an independent laboratory can assess its proper adherence to the protocol in DRP or that some data were not available for public distribution. Regarding this latter point though, it was noted that it apparently consisted mainly of data for future publication.

The PRP agreed that the Validation Principle 8 has been met.

12. Conclusions and recommendations

55. The PRP concluded that the retrospective assessment of the in vivo erythrocyte *Pig-a* gene mutation assay validation has been performed according to the validation principles described in OECD GD 34.

56. The PRP considered that the rationale for the *Pig-a* gene mutation assay test protocol is clearly detailed in the DRP in terms of its scientific basis, regulatory purpose and need. It was recognised that the *Pig-a* assay has several benefits in terms of regulatory use. The main disadvantage though, compared to the TGR, is that the *Pig-a* assay only detects mutations induced in the erythropoietic tissue (i.e. mainly the bone marrow in adult animals), which exclude from it applicability domain substances that are mutagenic only in other tissues. In addition, kinetics of test chemicals, especially bone marrow exposure affects the mutant cell responses. It was thus recommended that for an adequate evaluation of the in vivo hazard, it should be demonstrated that test substance or its metabolites are able to reach the bone marrow since this is where the mutants are formed.

57. The PRP considered that there are sufficient evidence and data that suggest or demonstrate the link between the test method's endpoint (i.e. mutagenicity) and the biological phenomenon of interest (i.e. loss of GPI anchor).

58. Overall, the PRP found the protocol description to be quite detailed and clear, however some specific additions and clarifications to the protocol description were recommended. The treatment schedule and sampling time in particular are not standardised among studies and need to be further discussed and optimised. Other parameters need to be further described such as the route of exposure, number of dosing groups, animal age, volume of blood sampling, option for long time storage of blood and the number of cells to be analysed.

59. The PRP considered that the technical transferability, the intra- and inter-laboratory reproducibility of the *Pig-a* gene mutation assay were good and adequately demonstrated. In addition, the factors that contribute to variability were well explained.

60. The PRP considered that the applicability domain has been appropriately defined and appears to represent the type of chemicals typically employed to evaluate genetic toxicity endpoints.

61. Despite the small number of negative chemicals used in the dataset, the PRP agreed that the assay is highly accurate for detecting rodent mutagens and carcinogens that affect the bone marrow and the hematopoietic system, respectively, and is thus relevant to be used for regulatory purposes.

62. The PRP agreed that it is difficult to know if the data supporting the validity of the test method have been obtained in accordance with the principles of GLP. The PRP was however confident that although it is likely that the majority of the work has not been conducted in compliance with GLP, there is evidence that this does not preclude the robustness of the assay.

63. The PRP confirmed that most of the data supporting the assessment of the test method validation were available for expert review.

64. Overall, the PRP recommends the development of an internationally agreed test method for a regulatory use of the *Pig-a* assay. The development of such OECD Test

Guideline though should carefully take into consideration the caveats expressed by the PRP.

12.1. Acknowledgements:

The OECD Secretariat thanks Peer Review Panel for their review and valuable comments.

Annex 1- Peer Review Panel Composition

Ludovic Le Hegarat	Toxicology of contaminant Unit, Agence Nationale Sécurité Sanitaire Alimentaire Nationale (ANSES), France
Kenichi Masumura	Division of Genetics and Mutagenesis, National Institute of Health Sciences, Japan
John J. Nicolette	AbbVie Inc, USA
Young-Shin Chung	Department of Biotechnology, Hoseo University, Korea
David Michael DeMarini	Integrated Systems Toxicology Division, US Environmental Protection Agency, USA
Timothy W. Robison	Division of Pulmonary and Allergy Products, Center of Drug Evaluation and Research, Food and Drug Administration, USA

Peer Review co- Managers	Nathalie Delrue (OECD Secretariat) and Kanako Ito (OECD Secretariat)
Observer to the peer review:	Dr. Vasily Dobrovolsky (Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, US FDA) and Dr. David Lovell (St. George's University of London, UK)

Annex 2 - Charge Questions for the Peer Review of the Validation Status of the in vivo erythrocyte Pig-a gene mutation assay

Questions based on the validation principles and criteria in OECD GD34 (link)

Q1: Do you consider that the rationale for the Pig-a gene mutation assay test method is clearly elaborated in the detailed review paper / validation report in terms of its scientific basis, regulatory purpose and need?
Q2: Is the relationship between the test method's endpoint(s) and the (biological) phenomenon of interest described adequately, using relevant references for the scientific relevance of the effect(s) measured (in terms of their mechanistic (biological) or empirical (correlative) relationship) to the specific type of effect/toxicity of interest?
Q3: Do you consider that the protocol description in the general recommendations for conducting the assay that were made in the IWGT report (Gollapudi et al., 2015) is sufficiently detailed, including a description of all the materials needed (e.g. specific cell types or construct or animal species), a description of what is measured and how it is measured, a description of appropriate data analysis, decision criteria for evaluation of data and what are the criteria for acceptable test performance? Determine whether the use of the IWGT general recommendations for conducting this assay is best. If not, what updated protocol in Part1, Section 4 would you recommend?
Q4: Determine whether there are sufficient data from the various international validation studies to conclude acceptable intra- and inter-laboratory reproducibility. Do you consider that the intra-, and inter-laboratory reproducibility of the Pig-a gene mutation assay test method is adequately demonstrated, considering availability of the data over time, as well as the degree to which biological variability affects the test method reproducibility?
Q5: Check that the applicability domain has been appropriately defined. Are the reference chemicals used to demonstrate the performance of this test method representative of the types of substances for which the test method will be used and have they been tested under code to exclude bias?
Q6: In the context of this retrospective performance assessment, do you consider the approach employed to assess the performance of the assay is relevant? Do you consider that the performance of the test method has been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data?
Q7: Have all of the data supporting the validity of a test method been obtained in accordance with the principles of GLP? If not, has an adequate consideration been given to the potential impact on the validation status of the test method?
Q8: Do you consider that all the data supporting the assessment of the validity of the test method are easily available for expert review? These include: a detailed and readily available test method protocol to the public and independent laboratories; organised and easily accessible data to permit independent review(s); benchmarks by which an independent laboratory can itself assess its proper adherence to the protocol.

Annex 3 - Response Compilation

Peer Review of the Validation Status of the in vivo erythrocyte *Pig-a* gene mutation assay

Responses to the charges questions were collected in June-July 2019.

<table>
<tr>
<td rowspan="3">Q1: Do you consider that the rationale for the Pig-a gene mutation assay test method is clearly elaborated in the detailed review paper / validation report in terms of its scientific basis, regulatory purpose and need?

Validation principle a: The rationale for the test method should be available.
This should include a clear statement of the scientific basis, regulatory purpose and need for the test.</td>
<td>PR1
Yes, I consider that the rationale for the Pig-a gene mutation assay test protocol is clearly detailed in this review paper in terms of its scientific basis, regulatory purpose and need. This test is particularly interesting to perform for in vitro positive mutagenic compounds. In fact, the bone marrow micronucleus test is not enough sensitive to dectect this kind of compounds, the TGR test is too expensive, and the comet assay detect only primary DNA damage.
The major concern is the choice of treatment schedules. Have we enough data to consider that a negative result obtained with a positive in vitro mutagenic compound in the 3d+28 pig-a assay is really negative or a 28d treatment must to be recommended for all pig-a assay in order to be sure that all mutagens (weaker in particular, will be detected). In fact, some positive mutagens compounds failed to be detected in the short treatment protocol, even if the large majority of high potent mutagens are easily detected in the 3d+38 protocol, we have to be sure that this short treatment duration is useful for all mutagens.</td>
</tr>
<tr>
<td>PR2
Principle and rationale of the Pig-a gene mutation assay are clearly written in the DRP/VR. The assay detects Pig-a mutant erythrocytes and reticulocytes in peripheral blood by flow cytometry. Pig-a mutant cells are lack of GPI-anchored cell surface proteins and immunofluorescent labelling method are used to distinguish wild-type and mutant cells. Pig-a assay has several benefits on regulatory purpose: 1) no need to use specific transgenic rodents, 2) small amount of blood sampling, 3) integration to repeated dose toxicity test may be easy.</td>
</tr>
<tr>
<td>PR3
Yes to all considerations</td>
</tr>
</table>

	PR4 In the detailed review paper/validation report (DRP/VR), the rationale for the *Pig-a* gene mutation assay test method is clearly explained. In terms of scientific basis, the following knowledge is well elaborated; - how the biological functions of the protein expressed from *pig-a* gene are related to GPI-anchors tethering cell surface proteins, - how the measuring the loss of cell surface proteins predicts mutation in the endogenous X-linked *pig-a* gene, - how the mutation in *PIG-A* gene causes PNH (paroxysmal nocturnal hemoglobinuria) in human, - how the phenotypic mutant cells as a reporter of gene mutation are quantitatively evaluated using flow cytometric assay, - how mutant erythrocytes, mutant RET and mutant RBC, respond with treatment and how erythroid progenitors or HSCs are related to accumulate mutant frequencies. In terms of regulatory purpose and need, the followings are explained; - In vivo tests are used for hazard identification as a part of battery tests or to follow-up in vitro findings. Many in vivo genotoxicity tests exist, but only TGR is registered as an OECD TG for evaluation of in vivo gene mutation. - Other three in vivo genotoxicity tests (OECD TG 474, 475, 489) are focused on a particular class of genotoxicity. - Strength and weakness of the *Pig-a* assay are compared with those of TGR assay. - *Pig-a* gene mutation assay is recommended as an alternative test for TGR. - A follow-up *in vivo* assay for drug impurities is recommended by ICH with M7 guidance. - The assay has additional applications in conducting weight-of-evidence genotoxicity assessments, quantitative measurements of *in vivo* mutation, extended-time monitoring of mutant frequencies, and as a routine complement to MN testing that affords more information on *in vivo* genotoxicity without using additional animals. - Data generated from *Pig-a* assay are useful for hazard/risk assessment purposes for human health due to the similarity in GPI-anchored systems.
	PR5 The rationale for the assay is described clearly, with a review of the history of the discovery of the gene. The details of the assay are described in words and pictures, and of course, the relevant references are included. Details of the pros and cons of the assay are described, as well as details of the methods. Validation includes evaluation of 70 chemicals and ionizing radiation, across numerous laboratories, largely in North America and Europe, with a comparable assay evaluated in Japan. The scientific basis for the assay is clearly described, as well as the regulatory purpose and regulatory need for the assay. (I discuss these issues further below.)

	PR6 Yes. The Detailed Review Paper (DRP) provides a clear description of the GPI-deficient cells or cells lacking surface-displayed GPI-anchored proteins, which can be measured, and Pig-a mutation. The in vivo Pig-a mutation assay can be used as a follow-up to a positive in vitro bacterial reverse mutation assay or in vitro mammalian cell mutation assay (e.g., HGPRT forward mutation). Antibodies against GPI-anchored protein markers are commercially available and usually species-specific. The mutants measured in the erythrocyte Pig-a assay are formed in nucleated erythroid progenitor cells that, in adult rodents, are mainly located in the bone marrow. Thus, when used as a test for mutagenicity, the test substance or its metabolites must be able to reach the bone marrow for an adequate evaluation of in vivo hazard. The assay only detects mutations induced in erythropoietic tissue (i.e., mainly the bone marrow in adult animals), reducing its sensitivity for substances that are strongly mutagenic only in other tissues, like liver.”

Q2: Is the relationship between the test method's endpoint(s) and the (biological) phenomenon of interest described adequately, using relevant references for the scientific relevance of the effect(s) measured (in terms of their mechanistic (biological) or empirical (correlative) relationship) to the specific type of effect/toxicity of interest? ***Validation principle b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.*** This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.	PR1 Yes, there are sufficient evidence and data that suggested or demonstrated the link between the absence of GPI and the mutation on the Pig-a gene. Even if few studies clearly demonstrated the absence of GPI and the mutation of Pig-a gene on bone marrow cells, but considering that only mutagenic compounds induced the loss of cell surface proteins and all negative controls and non-genotoxic compounds failed to increase the proportion of RET or RBC without GPI markers, we can considered that the link between loss of cell surface proteins and pig-a gene mutation sufficiently high.

<table>
<tr><td rowspan="3"></td><td>PR2
The assay detects Pig-a mutant erythrocytes (and reticulocytes) in peripheral blood as a reporter of gene mutation. Pig-a mutants are lack of GPI-anchored cell surface proteins. Loss of GPI anchors and GPI-anchored proteins are almost always due to mutation of the Pig-a gene. This relationship between Pig-a mutation and loss of anchors and their associated proteins is the basis for the Pig-a gene mutation assay. In the erythrocyte Pig-a assay, the mutants are formed in nucleated erythroid progenitor cells that are mainly located in the bone marrow. So, the erythrocyte Pig-a assay is used to detect gene mutations induced in bone marrow. DRP confirmed that Pig-a gene mutation is responsible for the Pig-a mutant phenotype with evidences of DNA sequencing studies using bone marrow cells, T lymphocytes, in vitro studies, etc.</td></tr>
<tr><td>PR3
Yes to all considerations</td></tr>
<tr><td>PR4
Clear relationship between the test endpoint(s) and gene mutation is most important to establish the test method. These relationships are explained in different ways with references in Part I, Section 6.
- The pig-a mutations were confirmed by DNA sequencing of DNA extracted from GPI-marker-deficient bone marrow erythroid progenitor cells and granulocytes in rats using NGS.
- Pig-a cDNAs were synthesized from mouse mutant bone marrow erythroids and analyzed by Sanger sequencing. Base pair substitutions were observed.
- Wild type bone marrow erythroid cells as well as wild-type granulocytes contain no pig-a gene mutation.
- GPI-deficient cells in PNH patients are almost always associated with PIG-A mutations.
- One successful report containing analysis of pig-a mRNA in mouse mutant RETs by amplification of cDNA using RT-PCR demonstrated pig-a gene mutation.
- GPI-deficient rat T lymphocytes contain pig-a gene mutations were observed in many studies.
- Pig-a gene mutations cause GPI deficiency in mammalian cells in vitro.
- Pig-a gene mutation responses are consistent with TGR and endogenous reporter gene mutation responses in the same animals.
- Lack of evidence that loss of GPI anchors and GPI-anchored protein is due to something other than mutation, e.g., epigenetic gene silencing.</td></tr>
</table>

	PR5 Yes, the endpoint is described in great detail, and adequate supporting evidence is provided to conclude that the endpoint is largely mutation within the Pig-a gene and not altered gene expression or mutation in other genes. The data also support the view that the assay detects exclusively gene mutation and not chromosomal mutation. Only limited analyses have been performed to determine the types of gene mutations, but those that have been conducted confirm that the assay detects the same classes of base substitutions that the test agents induce in other assays.
	PR6 Yes. Most mutant cells that lack the GPI anchor and acquire a surface-protein-deficient phenotype are expected to result from mutation in the Pig-a gene. This association of GPI-anchored protein loss with Pig-a mutation is the basis for the Pig-a assay. The linkage between surface-protein-deficient phenotype and a causative gene mutation (Pig-a) is clearly described in the DRP. Detection of in vivo gene mutation is the effect/toxicity of interest. Unless bone marrow exposure to the parent compound or to its metabolite(s) can be demonstrated directly or indirectly via plasma or by a reduction in the percentage of RETs, caution must be exercised in interpreting negative results.

Q3: Do you consider that the protocol description in the general recommendations for conducting the assay that were made in the IWGT report (Gollapudi et al., 2015) is sufficiently detailed, including a description of all the materials needed (e.g. specific cell types or construct or animal species), a description of what is measured and how it is measured, a description of appropriate data analysis, decision criteria for evaluation of data and what are the criteria for acceptable test performance? Determine whether the use of the IWGT general recommendations for conducting this assay is best. If not, what updated protocol in Part1, Section 4 would you recommend? ***Validation principle c) A detailed protocol for the test method should be available.*** The protocol should be sufficiently detailed and should include, *e.g.,* a description of the materials needed, such as specific cell types or construct or animal species that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analysed, decision criteria for evaluation of data and what are the criteria for acceptable test performance.	PR1 Yes, all these points are clearly defined. Treatment schedules should more precise considering that some weak mutagens would not be detected in 3d+28 schedule. It will be good to add that the choose of treatment schedule will be based on the toxicokinetic properties of the substance and assay must be conducted using a 28-day repeat-dose protocol per defaut, in case of absence of data of bioavailability on the compound and in case of weak mutagenic propertie observed in vitro.

	PR2 DRP presented updated recommendations with minor additions, clarifications and alterations of that in the IWGT report. Most of general recommendations are based on consistency with available OECD TGs (in vivo general- or genetic-toxicology studies) and acceptable for Pig-a assay. Specific comments about Pig-a assay protocol/method are shown below. 1) Acceptable or recommended route of exposure should be described. 2) Method of blood sampling could be written more detail. To conduct both RBC- and RET-Pig-a assay under the recommendation, how much volume of peripheral blood should be sampled in rat and mouse? Is the same amount of blood enough for both -1 day and 28 day sampling time? Because %RET is higher in young animal and decreases in older animal. 3) "Analyzing a minimum of 1-5 x 10^6 RETs or total RBCs per sample" (from IWGT report) is not clear. Background Pig-a mutant frequency (MF) could be lower in mice than that in rats. A minimum number of cells required could be calculated from background MF data (like Fig. 8 in DRP). Otherwise, practical reason could be described. If a greater number of cells is needed, it may affect blood sampling.
	PR3 Suggest in the table of summarization of the method to suggest a minimum of 3 dosing groups. Specifically indicating 3 dose group then submissions containing 4 dosing groups can be questioned by regulators even if the science demanded an additional group. I would also like to see a suggested statistical methodology at least as an example in the table.

<table>
<tr><td rowspan="2"></td><td>PR4
This DRP in Section 4 is explaining the detailed protocol for Pig-a assay;
- animal species and sex: male or female mice and rats,
- animal number: 6 animals per group or 5 analyzable per group acceptable if justified by power calculation,
- test endpoint: mutant frequencies of RETs and RBCs as number per million
- method for measurement of test endpoint: specific antibodies against GPI-anchored proteins and flow cytometer with immunomagnetic separation,
- duration of test chemical dosing and blood collecting times: 28-31 days after the first treatment with short-term (≤ 13 days) or longer-term (≥14) treatment

Section 5 explains the reporting data, statistical analysis, interpretation of results and acceptance criteria.

In updated protocol, the different manifestation times of mutant RETs and mutant RBCs responses are emphasized.
- Rats and mice with both sexes may be used.
- In dosing daily for several weeks, blood samples are better to be collected on the day of cessation of the treatment. Optionally blood samples may be collected during 2 to 4 weeks after cessation of exposure to evaluate mutant RBC frequencies. Another optional blood sampling at earlier time before cessation of treatment may be useful for mutant RET analysis.
- In short-term dosing, sampling at two collection times is recommended; at 1-2 weeks after cessation of treatment and at approximately Day 30 after the first exposure.</td></tr>
<tr><td>PR5
The Gollapudi et al. (2015) IWGT paper is now essentially 5 years old, and although it describes the basic details of how to perform the assay, which have largely not changed, there are more recent refinements, such as freezing the blood sample prior to analysis, as well as other minor technical developments, that have occurred since then that are useful to know for people starting to use the assay for the first time. Nonetheless, the 2015 paper is largely adequate as the basis for doing the assay. Perhaps the time has come for a methods paper to be published by Dertinger et al. in a methods journal that would present a slightly revised protocol from the 2015 paper. Another possibility is for the current document by Heflich et al. (dated December 2017) be slightly updated and published because it provides the most comprehensive overview of the assay as it is known at this point and could provide a useful document for those starting to use the assay. Nonetheless, the general recommendations in the 2017 IWGT paper are still valid and, in the absence of some of the newer (largely minor) additions to the protocol, would serve adequately as a protocol at this time.</td></tr>
</table>

	PR6 Yes. In general, the IWGT report (Mutation Research 783: 23-35, 2015) provides sufficient detail to conduct the assay, evaluation of the data, and judging test performance. The IWGT report focuses primarily on the Pig-A assay in rats, although other mammalian species used in other toxicological/investigational studies can be used. The references in the report are useful for finding additional details.

<table>
<tr>
<td rowspan="4">Q4: Determine whether there are sufficient data from the various international validation studies to conclude acceptable intra- and inter-laboratory reproducibility. Do you consider that the intra-, and inter-laboratory reproducibility of the Pig-a gene mutation assay test method is adequately demonstrated, considering availability of the data over time, as well as the degree to which biological variability affects the test method reproducibility?

Validation principle d)The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.

Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed.</td>
<td>PR1
Yes</td>
</tr>
<tr>
<td>PR2
International interlaboratory trial (US and Europe) was conducted with HESI consortium’s Pig-a WG and the results were published in several papers. Another collaborative study organized by MMS/JEMS in Japan was conducted and the results were published in papers. Additional interlaboratory studies are described in VR. Some intralaboratory studies are also described in VR with references. Those studies performed total RBC and RET assays, MutaFlow scoring approach, PIGRET assay, different dosing or sampling protocol, off-site analyses, etc. Technical transferability and reproducibility were demonstrated well.</td>
</tr>
<tr>
<td>PR3
Yes the method appears adequately assessed for reproducibility between labs as well as within laboratories and factors that can contribute to variability well explained.</td>
</tr>
<tr>
<td>PR4
Inter-laboratory reproducibility had been demonstrated from the independent in vivo Pig-a assay with various international validation studies among the European countries and North America, among Japanese institutes and among Korean laboratories. Two to 16 laboratories were involved in each inter-laboratory studies. In addition, the various aliquots of same blood samples collected from animals treated with chemicals had been transferred and analyzed in different laboratories on a same day.

In addition, high accuracy in comparison with other in vivo tests like TGR or rodent cancer was proven using limited Pig-a test results. Most important factor to affect the test method reproducibility is study design containing single or multiple dose, and dosage of treatment. And kinetics of test chemicals, especially bone marrow exposure affects the mutant cell responses. The blood collecting process is carefully performed to preserve cell surface protein. In addition, immunomagnetic separation is needed to obtain sufficient mutant erythrocytes.</td>
</tr>
</table>

	PR5 Ionizing radiation and >70 chemicals, mixtures, and nanomaterials have been tested in the assay, many of them by multiple labs, with consistent results among the labs. For example, ENU has been tested in >20 laboratories; 1,3-propane sultone in 7 labs; DMBA and 4NQA in 5 labs; acrylamide, B[a]P, and EMS in 4 labs; and 2-AAF, aristolochic acids, chlorambucil, cisplatin, cyclophosphamide, IR, melphalan, MMS, mitomycin C, MNU, and urethane in 3 labs. Although these classes of compounds do not cover the chemical space likely to be tested in the assay by pharmaceutical companies, it is a sufficient assessment of the ability of the assay to detect mutagens among various chemical classes. The reproducibility of the assay based on the compounds noted above that have been tested across laboratories appears to be good, indicating that the technical aspects of the assay are able to be performed well in different laboratories. Background mutation frequencies varied among the labs, but this is normal and did not appear to affect the analysis.

	PR6 Studies of inter-laboratory reproducibility of the Pig-a assay appear to be adequate. - The multi-laboratory study of the PIGRET assay organized by the Mammalian Mutagenicity Study Group of the Japanese Environmental Mutagen Society (MMS/JEMS) was an extensive multi-laboratory study of the PIGRET assay that consisted of 16 collaborators and 24 test chemicals and reported good concordance/reproducibility and transferability (Kimoto et al., Mutation Research 811: 3-15, 2016). - Dertinger et al., (Environmental and Molecular Mutagenesis 52:690698, 2011) reported results from 14 laboratories with ENU, which indicated good transferability. Subsequently, 5 agents were studied using a 28-day treatment period with the results published in a special issue of Environmental and Molecular Mutagenesis: ENU (Cammerer et al., EMM 52: 721-730, 2011), dimethylbenz[a]anthracene (DMBA; Shi et al., EMM 52: 711-720, 2011), N-methyl-N-nitrosourea (Lynch et al., EMM 52: 699-710, 2011), benzo[a]pyrene (Bhalli et al., EMM 52: 731-737, 2011), and 4-nitroquinoline-1-oxide (4NQO; Stankowski et al., EMM 52: 738-747, 2011). Each chemical was studied in at least 2 laboratories and results demonstrated good reproducibility across sites. The rarity of mutants (1 per 10^6 RBCs and RETs) indicated that the assay could be enhanced by increasing the number evaluated per sample. Dertinger et al., EMM 52: 748-755, 2011 reported results with the in vivo MutaFlow scoring approach. - An inter-laboratory trial was conducted by the Korean Ministry of Food and Drug Administration (MFDA) using three laboratories (Chung et al., Mutation Research 831: 62-68, 2017) and demonstrated good reproducibility and transferability. - Additional inter-laboratory validations have been reported by Raschke et al. using four laboratories (Environmental and Molecular Mutagenesis 57: 678-686, 2016) and Gollapudi et al. (Mutation Research 783: 23-35, 2015) using two laboratories. - These studies in Japan, the United States, Europe, and South Korea clearly demonstrated good inter-laboratory reproducibility and transferability. There are limited of studies of intra-laboratory reproducibility. Gollapudi *et al.* (Mutation Research 783: 23-35, 2015) describe an example indicating a high degree of reproducibility when the same laboratory (Litron) analyzed technical replicates from both mutagen-treated and negative control animals on the same day and on different days. These assays were performed using an earlier version of the assay, not involving immunomagnetic separation

Q5: Check that the applicability domain has been appropriately defined. Are the reference chemicals used to demonstrate the performance of this test method representative of the types of substances for which the test method will be used and have they been tested under code to exclude bias? ***Validation principle e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.*** A sufficient number of the reference chemicals should have been tested under code to exclude bias (see paragraphs on "Coding and Distribution of Test Samples").	PR1 Yes, partially, false positive-results are not expected in this test so even if the number of non-genotoxic compounds tested are low, we can considered that the number of reference chemicals tested are enough.
	PR2 DRP clearly shows that the Pig-a assay is designed to measure in vivo gene mutation originating in erythroid precursor cells of bone marrow. The assay only detects mutations induced in erythropoietic tissue (mainly the bone marrow), reducing its sensitivity for substances that are mutagenic only in other tissues. This limitation is also true for the somatic cell cytogenetic assays described in OECD TG 474 (in vivo MN test). Over 80 chemicals, chemical mixtures, and nanomaterials have been tested for Pig-a mutation in either rats or mice (listed in DRP, Table VI and VII). Seventy-two have been assayed in rats, 23 in mice, and 13 test substances have been assayed in both rodent species. Although none of the Pig-a data used for the performance analysis were produced using coded samples, most of the data generation in the Pig-a assay is automated by the flow cytometric analysis under criteria of data acceptance and data interpretation standards. It is reasonable that VR says "Thus, significant efforts were made to minimize any bias in the assay performance evaluation."
	PR3 Given the retrospective nature of the evaluation a broad applicability domain seems inferred which is reasonable given the nature of the endpoint being evaluated under the conditions. The types of chemicals assessed represent a variety of mutagenic, promutagenic and non-mutagenic chemicals that are typically employed to evaluate genetic toxicity endpoints. It was not clear if some of the experimentation was done with coded compounds but the majority of the works were not done in this manner. However, for the considerations of in vivo animal testing as well as safety of those working with these chemicals, it is reasonable that the chemicals were known in most cases, as the evaluation criteria for positive/negative/equivocal by the committee(s) were well established to make objective decisions.

	PR4 First applicability domain is the follow-up study after positive results in vitro Ames test. In second, Pig-a assay can serve as a complement to the in vivo peripheral blood MN assay for many routine in vivo genotoxicity testing applications. Third potential regulatory application is direct confirmation of findings in animal studies with data from humans. The reference chemicals employed for the validity of *Pig-a* assay were various in aspect of structure and of functions, and representative to various specific class of genetic toxicants (carcinogenic mutagens, noncarcinogenic mutagens, epigenic carcinogens, clastogens, and aneugens). It is difficult to confirm if the most of those chemicals have been coded for testing.
	PR5 The most commonly evaluated chemical classes tested were 10 alkylating agents, 6 aromatic nitro/amino compounds, 6 nanomaterials, 4 PAHs, and 3 mycotoxins. Of the 71 agents tested, 31 were positive, and 40 were negative. Of the 40 negative agents, 13 were known chromosomal mutagens (clastogens) and would be predicted to be negative in the Pig-a assay. Although it appears that none of the agents were tested under code, this does not detract from the validity of the results, nor does there appear to be any bias in the interpretation of the data. As noted above, consistent results in terms of molecular changes (base substitutions) were found for various agents in the assay compared to the mutations induced by those agents in other assays.
	PR6 Ionizing radiation and over 80 chemicals, chemical mixtures, and nanomaterials have been tested for Pig-a mutation in rats, mice, or both. Pig-a assay results meeting test criteria have been established for over 50 test substances. Several test substances have been tested in multiple laboratories and using different protocols. The applicability domain appears to be representative of general chemicals. The number of drugs tested, outside of oncology indications, appeared to be small (<10). It was unclear if compounds were tested under code to exclude bias. Conceivably with adoption of a DRP, there might be greater interest in testing drugs in the Pig-a assay, although the Pig-a assay is not currently recommended in the ICH S2 (R1) Guidance, although we have recently recommended the assay for a few drugs found to be in the in vitro bacterial mutagenicity assay. Under the ICH M7 (R1) Guidance, the Pig-a assay can be used as a follow-up assay to a positive in vitro bacterial mutagenicity assay with a genotoxic impurity.

Q6: In the context of this retrospective performance assessment, do you consider the approach employed to assess the performance of the assay is relevant? Do you consider that the performance of the test method has been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data? ***Validation principle f)The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.*** In the case of a substitute test method adequate data should be available to permit a reliable analysis of the performance and comparability of the proposed substitute test method with that of the test it is designed to replace.	PR1 Yes
	PR2 In the RPA, Pig-a assay were compared with mutation responses in the TGR assay for bone marrow and all tissues, responses in the in vivo MN assay, and in the rodent cancer bioassay (cancer in hematopoietic tissue and cancer in all tissues). Selected reference tests are relevant and reasonable. Testing dataset has a bias that there are many positive agents but small number of negative agents. This is a problem of the dataset in both Pig-a assay and TGR assay. To improve this bias, solvent/vehicle control data were added for negative data. The same approach was used in DRP of TGR assay (OECD 2009). In the performance measurements for Pig-a assay using each reference test, total number of agents (n) was n=26~28 (vs. bone marrow TGR), n=34~40 (vs. any tissue TGR), n=49~53 (vs. in vivo MN), n=24~26 (vs. rodent hematopoietic cancer) and n=46~50 (vs. rodent cancer in any tissue). RPA indicates that the assay is highly accurate for detecting rodent mutagens and carcinogens that affect the bone marrow and the hematopoietic system, respectively. The assay is highly accurate when TGR mutation in bone marrow is used to define true positive and negative responses. The assay is also reasonably accurate when TGR mutation in any tissue, in vivo MN induction, and rodent cancer in any tissue is used to define true positive and negative responses.
	PR3 Yes on all matters. The review has demonstrated the assay can be applicable across species including human monitoring.
	PR4 In the performance assessment of the *Pig-a* assay, reference test methods (TGR, in vivo MN and rodent cancer) were used for comparison to describe accuracy. Only the performances using rodents (mice and rats) in *Pig-a* assay was employed and all of reference test methods were using rodents. Any performance using other species were not included. For describing accuracy, sensitivity, specificity, positive predictivity, negative predictivity, and concordance were evaluated, and accuracy of *pig-a* gene mutation assay are high enough to be used for regulatory test.

	PR5 Despite the limited chemical classes of compounds assessed in the assay, the chemical spaced covered, and most importantly, the mutagenic mechanisms by which these agents function, is sufficient to characterize the validity of the assay. The finding that that those agents that induce exclusively chromosomal mutations (clastogens) are negative, whereas the known gene mutagens are positive, is compelling evidence that the assay would fill the niche of an in vivo assay that can be performed in any rodent species to detect gene mutations. Obviously, the assay also has relevance to humans and could eventually be considered as a biomonitoring assay, similar to the human micronucleus assays in blood or buccal cells. Thus, the assay is clearly relevant to humans and consistent with mechanistic aspects of the agents tested. Thus, I think that the performance of the assay has been evaluated sufficiently.

	PR6 Yes. The retrospective performance assessment of evaluating studies in the published peer-reviewed literature and other routes, where original data could be obtained, was highly relevant and provided essential support for the performance of the Pig-a assay. Both short-term studies (<13-day [Days 1-3]) and longer treatment protocols (i.e., 28 days) were evaluated. Caution was applied to short-term studies as it was recognized that a few test substances were more readily detected as positive using longer-term rather than short-term dosing protocols given that mutations were known to accumulate with longer treatment; however, the number of test substances falling into this category was small. Several chemicals were tested in multiple laboratories. Both data from rats and mice was considered. It was noted that using a longer repeat-dose treatment protocol and/or employing an enrichment technique that increased the number of cells analyzed for mutation affected the response in the assay (i.e., for 2-AAF and azathioprine, assays conducted using a single treatment were negative, while assays conducted with a 3- or 28-day treatment protocol were positive, and with aflatoxin B1, assays conducted using 3-day and 15-day treatment protocols were negative, while aflatoxin B1 was positive using a 29-day repeat-dose treatment protocol). The DRP and IWGT Report appropriately direct testing laboratory toward a longer repeat-dose treatment protocol and use of an enrichment technique. In general, most positives observed in the Pig-a assay were generally consistent with the compound's known mechanism that would be expected to result in in vivo gene mutation. There was good concordance for positive and negative calls for chemicals tested in both rats and mice. From the retrospective performance analysis, the high degree of accuracy between the results of Pig-a assay and bone marrow TGR assay for defined P and N test substances, although limited in size, adds support to the performance of the Pig-a assay. Further, the high degree of accuracy between responses in the Pig-a assay and cancer in hematopoietic tissues lends further support to performance of the Pig-a assay.

<table>
<tr><td rowspan="6">Q7: Have all of the data supporting the validity of a test method been obtained in accordance with the principles of GLP? If not, has an adequate consideration been given to the potential impact on the validation status of the test method?

Validation principle g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.

Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated.</td><td>PR1
Yes</td></tr>
<tr><td>PR2
VR noted that “A minority of the Pig-a data evaluated in this report were generated as part of studies formally complying with the principles of Good Laboratory Practice (GLP).” “However, many Pig-a studies, because of the extensive planning and resources involved in executing them, are conducted ‘in the spirit of GLP’ to maximize the value of the results.”</td></tr>
<tr><td>PR3
It appears the majority of the work may not have been conducted in compliance with GLP and likely was done without the intention to adhere to the spirit of GLP. It seems reasonable to assume that the data were generated following Good Scientific Practices in many cases, and the committees identified the testing conditions and outcomes in a consistent clear manner throughout the paper.</td></tr>
<tr><td>PR4
It is difficult to consider if all of the data supporting the validity of a test method have been obtained in accordance with the principles of GLP. However, some of participants were working in GLP institutes and performed a Pig-a assay in compliance with GLP principles. The most inter-lab validation trials had demonstrated their proficient in the performance of Pig-a assay through their own practice or through transferability test.
In WG evaluation, invalid studies were exempt for evaluating the validity of the test method.</td></tr>
<tr><td>PR5
No, not all of the studies were performed under GLP, but this, in fact, provides further evidence as to how robust the assay is—in that it has been performed in a variety of laboratories not necessarily under GLP, and given similar results. So, to me, the absence of GLP for some of the studies strengthens the assessment and shows that the assay can be done under less-than-stringent laboratory conditions and still provide valid results.</td></tr>
<tr><td>PR6
In general, most laboratories participating in the method validation were not GLP-compliant; however, most of the studies were conducted “in the spirit of GLP”. From the description, it appeared that there was extensive training of laboratory personnel as well as extensive planning of studies. The studies conducted “in the spirit of GLP” can be anchored (supported) by GLP-compliant studies in that there was close agreement of study results.</td></tr>
<tr><td></td><td></td></tr>
</table>

Charge Q8: Do you consider that all the data supporting the assessment of the validity of the test method are easily available for expert review? These include: a detailed and readily available test method protocol to the public and independent laboratories; organised and easily accessible data to permit independent review(s); benchmarks by which an independent laboratory can itself assess its proper adherence to the protocol. ***Validation principle h) All data supporting the assessment of the validity of the test method should be available for expert review.*** The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organised and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol.	PR1 Yes,
	PR2 Assay protocol and recommendations are described in DRP (section 4 and 5). Detailed protocols for different methods are published in papers (for example, Kimoto et al., 2016) or supplied by commercial company (In Vivo MutaFlow method by LITRON). Data used for performance analyses and validation are presented in DRP (section 8, Table VII~XIV, Annex III). There is no section or paragraph about criteria by which an independent laboratory can assess its proper adherence to the protocol in DRP. Some criteria could be described to confirm that the assay was successfully performed.
	PR3 Yes. The tables of studies are a bit complex to follow. The authors may wish to consider expanding abbreviations that may be more commonly used (like DRF for dose rangefinding) and spell out others such Positive or Negative to allow the reader's eye easily spot results.
	PR4 For independent reviews, data were organized and provided using spread sheets containing test article, vehicle, treatment protocol, analysis methods, results, associated toxicity, other assays conducted as part of the study, conclusions about responses, and any reports with findings. Those data sets are available at two different websites; one at public distribution and the other only at persons to evaluating the performance of the assay. Thus, an independent laboratory can itself assess the validity of the test method.
	PR5 With almost no exceptions, all of the data and methods supporting this document are in the open, peer-reviewed literature. As such, they are accessible and available for independent review and comparison to results one might obtain in one's own laboratory. Again, there might be merit in Heflich et al. publishing an updated version of this document to provide a state-of-the art assessment of the assay and recommended protocols.
	PR6 Yes. In general, most of the data supporting the assessment of the test method validation are available for expert review. I was able to easily access the data on the website at the School of Pharmacy, University of Maryland, Baltimore and found it to be extensive and understandable. It is noted that some data was not available for public distribution, which apparently consisted mainly of data for future publication.

Annex 4 - Additional Comments

PR1	
	The paper indicated that this test could be performed in parallel with a repeat dose toxicity study (28days), but this statement is only speculative since the assay was not validated by using the kind of test.
PR2	
General:	Does background RBC and RET Pig-a MF increase with age like TGR assay? Can too old (with low %RET) or too young (with high %RET) animals be used for Pig-a assay? What is acceptable range of week-old?
P19, line25:	"After an acute treatment of rats, for example, the maximum mutant frequency in peripheral RETs usually occurs within two weeks, while it may take 6 to 8 weeks before the maximum frequency is reached in mature RBCs (which corresponds to the approximate lifetime of RBCs in the periphery) (Miura et al., 2009)." It may suggest that the recommended 28-31 day-sampling point may not give the maximum response in RBC Pig-a MF in both short (1-3 days) and 28-day treatment protocol. DRP noted persistency of elevated RBC and RET Pig-a MF induced by ENU and cisplatin over several months (P20). On the other hand, some mutagens showed the maximum MF in peripheral RETs at 14 day-sampling time and the MF decreased at 28-31 day-sampling time (For example, Dertinger et al., Tox Sci 2012). More discussion about time-response relationship of Pig-a MF could be added. How long stable is the induced RBC and RET Pig-a MF? It may be affected by lifespan of Pig-a mutant cells, erythroid progenitor cells and hematopoietic stem cells. Toxicity in bone marrow during dosing period may be another factor to be concerned. The 28-31 day sampling time point may be reasonable, even if it is not the timing of maximum response. Because integration to repeat dose toxicity test is one of the benefits of the Pig-a assay. However, performance analyses presented in DRP were based on an overall call (P, N, E, I) from database including different tests and different sampling points. Could the performance analyses suggest reliability of the 28-31 day sampling point? Multiple sampling points (for example, day -1, 14, 28 and later (if possible)) may be appropriate to catch positive responses in both RBC and RET Pig-a assay.
P30, line 6:	One weakness is that fresh blood must be used for analyses within 1-2 days after sampling. Was an option for long-time storage of blood (P36) validated?
P30, section 4 Assay Protocol:	An additional reference should be cited. Chikura S, Kimoto T, Itoh S, Sanada H, Muto S, Horibata K. Standard protocol for the total red blood cell Pig-a assay used in the interlaboratory trial organized by the Mammalian Mutagenicity Study Group of the Japanese Environmental Mutagen Society. Genes Environ. 2019;41:5. doi: 10.1186/s41021-019-0121-z.
P36, b:	If possible, the option of preserving peripheral blood samples should be described in detail. How long can blood samples be stored? Is there any difference in

	analyses between fresh and stored samples? Can this method be widely available and recommended as a default for the assays?
P39, line16:	"animal sex, etc." Please add "age" of animal.
P43, section 6:	Is there any sequencing data of Pig-a gene from vehicle control animals/cells? Mutational characteristics of Pig-a gene (type of mutations, hotspots) could be compared with those of endogenous reporter gene or TGR assay, if possible. Size of Pig-a gene could be noted in introduction or somewhere.
P77, line 16:	"ii)" should be "iii)".
PR3	
P7, line 19-22	Significant dose-related increases compared to the control implies that at least one dose must be significant. Use "dose" not 'treatment". You're not 'treating' disease, you're 'dosing' animals.
P18, line 25-P19, line 6	While these are advantages of the peripheral blood evaluation, are the authors indicating that solid tissue assessment should not be pursued in the future?
P19, line 24	Define acute here if meaning more than one dose
P19, line 25	After acute dosing with a potent mutagen, the maximum mutant frequency..
P19, line 25-28	Is there an issue here too related to dilution of mutant events in the RBC by matured RBC already present that masks meaningful mutant frequency increases in the standard assay?
P22, line 8	In vivo genotoxicity tests are typically used for hazard identification....
P23, line16-P24, line14 section a paragraph 1	If the quotation continues from the same source I don't think you need to end quote after each paragraph.
P34, line 26-P35 line 2	I don't think a true Haber's law extrapolation should ever be expected in limited animal experimentation.
P35, line 4-7	Are these also similar concerns with doing single or three dose TGR assays?
P35, line 7-12	It should also be pointed out that most experimental mutation tests with new chemicals will likely NOT be potent mutagens as described here. Therefore, the expectation that a single or three day dosing regimen would be likely to uncover true mutation concerns from less potent Ames positives is probably unrealistic
P36, line 2	It may be good to explain what is meant by 'weak positive' here, since this is a bad term passed down through generations. In traditional genetox testing, whether weak or not, a positive is a positive (can't be 'weakly pregnant'). If what is meant here is that based on interrogation of many more events than traditional genetox work leading to statistical phenomenon, rather than biologically relevant findings, it should be made clear.
P38, line 13	Are there scenarios where one dosing regimen may be recommended over the other?

P42, line 22-25	Are the same concerns expressed related to TG474 and TG475?
P42, line 26-P43, line 3	In the cases of multiple time points that give conflicting outcomes with some not clearly positive/negative and others clearly negative, how is it suggested to handle. While a simple assay, thought should be considered for reducing the need to do repeat animal experiments.
P53, line 11-18	I think this is an important point: if the TGR assay does not detect a positive response in the same tissue as the Pig-a, they are actually concordant; the 'discordant' result points to the limitation of Pig-a analysis as previously described, not an insensitivity in this tissue vs TGR in this case.
P59, line 15-18	This is a bit confusing; indicating chemicals as positive with longer term protocols, but then saying that the number of substances were few and mainly involved single treatments.
P66, line 7-9	It is however becoming increasingly important for our audiences to understand that 'genotoxicity' is the range of responses, where this test specifically is detecting mutagenicity, thus it is not a fault of the test nor is there explanation needed if a clastogenic substance (for example) that is not mutagenic by other existing tests, is negative in this assay.
P74, line 23	"are genotoxic through mechanisms resulting in"
P77, line 24	was this conventional assay comparator a single or short-term dose test as well?
P80, line 9	"Bone marrow exposure could bb"
P80, line 15	Consider ICH M7(R1) as the most current version
PR4	
	The most benefit of a Pig-a assay is the animal welfare in consistent with refinement and reduction of 3Rs principles. In other words, a Pig-a assay can be integrated with general toxicology and genetic toxicology tests. In conclusion, a Pig-a assay is the appropriate and robust test method to evaluate in vivo gene mutation for regulatory purposes.
PR6	
	The Pig-a assay offers an alternative to the transgenic rodent mutation (TGR) assay, which is expensive and rarely performed to support regulatory submissions. The Pig-a assay can be conducted as an additional endpoint within the TGR assay if desired. If the Pig-a endpoint were found to be positive, there would be potentially little need to conduct the tissue evaluation to identify mutations in bacterial transgene. If the Pig-a endpoint was negative, the tissue evaluation to identify mutations in the bacterial transgene should proceed.