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The in vivo erythrocyte Pig-a gene mutation assay – Part 1 – Detailed Review Paper and Retrospective Performance Assessment

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NO. 315

THE IN VIVO ERYTHROCYTE PIG-A GENE MUTATION ASSAY – PART 1 – DETAILED REVIEW PAPER AND RETROSPECTIVE PERFORMANCE ASSESSMENT

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Foreword

This document is the Detailed Review Paper (DRP) of the *in vivo* erythrocyte *Pig-a* gene mutation assay (or *Pig-a* assay), developed by the US. It describes the state of the art knowledge on the *Pig-a* assay based on the outcome of several international or national inter laboratory trials that have been conducted between 2010 and 2019. The document also contains a Retrospective Performance Assessment of the test method.

This document is Part 1 of a series of two documents supporting the development of a Test Guideline (TG) for the *in vivo* erythrocyte *Pig-a* gene mutation assay. Part 2 of the series is the Retrospective validation study ENV/JM/MONO(2020)7.

Both documents served a as a basis for a peer review by an independent peer review panel in 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 Detailed Review Paper on the *in vivo* erythrocyte *Pig-a* gene mutation assay at its 32nd meeting in April 2020 and agreed to move forward with the development of a TG for this assay. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the Detailed Review Paper 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.

The *in vivo* erythrocyte *Pig-a* gene mutation assay

Part 1: Detailed review paper and performance assessment

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Executive Summary

1. Glycosylphosphatidylinositol (GPI) anchors tether >150 unique proteins to the outer surface of mammalian cells. The phosphatidylinositol glycan class A gene (*Pig-a*) is involved in an early step in the biosynthesis of these anchors and inactivating mutations in *Pig-a* result in loss of GPI anchors as well as loss of the proteins that GPI tethers to the cell surface. Along with *Pig-a*, approximately 30 genes are involved in the biosynthesis of GPI anchors; however, all the other genes are present in two copies, while the *Pig-a* gene is located on the X chromosome and present as one functional copy per cell. Thus, 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 (commonly referred to as 'the *Pig-a* assay'). Because the GPI biosynthesis pathway is highly conserved, the assay has been performed in several mammalian species, including humans. In addition, the assay has been conducted *in vitro*, using established mammalian cell lines. However, it is most commonly performed as an *in vivo* assay for gene mutation in rodents. In the rodent assay, flow cytometry is used to analyze erythrocytes from small samples of peripheral blood for the presence or absence of GPI-anchored proteins.

2. 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. This requirement is shared with commonly used *in vivo* tests for clastogenicity and aneugenicity in somatic cells, the erythrocyte micronucleus (MN) test (described in Organisation for Economic Cooperation and Development [OECD] Test Guideline [TG] 474) and the bone marrow chromosomal aberration test (TG 475). Other commonly used *in vivo* somatic cell genotoxicity tests, the transgenic rodent (TGR) gene mutation assay (TG 488) and the *in vivo* comet assay (TG 489), detect *in vivo* genotoxicity in virtually all animal tissues. The comet assay, however, measures only short-lived DNA damage, not heritable gene mutation. Also, the TGR assay measures gene mutation, but in bacterial transgenes rather than an endogenous mammalian

gene, and the assay is resource intensive and it only can be performed with specific genetically engineered rodents. Thus, the *Pig-a* assay fills an unoccupied niche of measuring *in vivo* gene mutation rapidly, in virtually any animal model, and in a manner that complements the blood-based cytogenetic assays. Additionally, as it is a peripheral blood-based assay, the animals need not be sacrificed to generate *Pig-a* gene mutation data. This property facilitates making mutation measurements in the same animals over time (longitudinal data collection) and integration of the assay into longer-term studies, including subchronic and chronic toxicology studies.

3. Mutagenicity responses in the *in vivo Pig-a* assay generally accumulate with repeat dosing, at least with strong mutagenic test substances. Thus, a dosing protocol similar to the one recommended for the TGR assay (TG 488) is also recommended for the *Pig-a* assay: repeat dosing for 28 consecutive days and mutant cell analysis following the final dose. As they provide the greatest degree of mutation assay sensitivity, longer-term dosing protocols, *e.g*. one employing 28 consecutive days of dosing, are recommended for virtually all regulatory applications. As induced mutant frequencies are persistent, the sampling time can be adjusted within a window of hours to several days after the final dose to provide opportunities for integration with other genotoxicity and general toxicity assays. Data indicate that short-term dosing protocols (especially those employing a single dose) do not detect a small number of weaker mutagens. Short-term dosing (≤ 14 consecutive days of dosing) is generally recommended only for nonregulatory applications (*e.g*., high-level screening for potent mutagens). Ideally assays are conducted with three appropriately spaced dose groups and a vehicle control, with the highest dose being the maximum tolerated dose (*e.g.,* as defined in TG 407) or the limit dose (1000 mg/kg/day for longer-term studies of ≥14 consecutive days of dosing and 2000 mg/kg/day for studies with shorter-term dosing schedules). Negative responses require confirmation with evidence of sufficient bone marrow exposure (see paragraph 93). Concurrent positive control animals typically are not used for the assay, although the mutant mimic control used to optimize flow cytometry conditions and frozen positive control samples from an animal treated with a genotoxicant and having an elevated mutant frequency may be used to lend confidence that the assay is capable of detecting a positive response. Laboratories are required to demonstrate proficiency in the assay before generating data intended for regulatory decision-making.

4. Mature erythrocytes are abundant in peripheral blood and thus very small blood volumes are sufficient to accurately measure mutant frequency. Newly formed reticulocytes (RETs) are only a few percent of total red blood cells (RBCs) in peripheral blood; however, it is recommended that both RETs and total RBCs

be analyzed for *Pig-a* mutant frequency. RET *Pig-a* mutant frequencies increase rapidly, over the first one or two weeks after dosing with a mutagen, and thus are a leading indicator of mutation. Because RBCs are more abundant than RETs, measurement of mutation in total RBCs produces highly robust data. In addition, the kinetics of mutant manifestation, RETs before RBCs, lends credence to the induction of small positive responses. The percent of RETs among erythrocytes (%RETs) in the blood samples should be monitored as an indication of toxicity to the erythropoietic system. As noted above, in the case of repeat dosing studies (*e.g.*, 28 or 90 consecutive days of dosing), the primary and essential blood harvest time is within a window of several hours to several days after the final dose. Also, because blood sampling can be conducted in a minimally invasive manner, it is useful (but not required) for *Pig-a* analyses to occur prior to the initiation of dosing in order to screen out rare rodents that exhibit unusually high mutant frequencies. Similarly, it can be useful (but not required) for *Pig-a* analyses to occur over the course of the dosing period (*e.g.*, at Day 15 of a 28-day dosing protocol), and/or, if feasible, at a later time point (*e.g.*, two to four weeks after cessation of a 28-day repeat dosing protocol). Although this protocol provides an opportunity to integrate the assay into standard 28-day or 90-day repeat dose general toxicology tests (TG 407), short-term dosing protocols (*e.g.*, single doses or doses given on three consecutive days) for many known test substances have yielded results qualitatively similar to those generated by repeat-dosing, and can be considered for nonregulatory studies. Whichever dosing and sampling protocol is employed, it is recommended that a minimum of 1 x 10^6 RETs and 1 x 10^6 total RBCs be assayed for *Pig-a* mutation. Assaying this many RETs normally requires enriching the blood samples for either RETs or mutants using immunomagnetic separation technologies.

5. The animal is the experimental unit for the statistical evaluation of *in vivo Pig-a* mutant frequency data. Transformations may be used to reduce any non-normality of data and the addition of a small value is often helpful to reduce complications associated with log-transforming ‘zero’ mutant frequencies (*e.g.*, 1.0, 0.1 or 0.01 added to the number of mutant cells per million). Positive responses exhibit a statistically significant, dose-related increase relative to the negative control, have at least one induced response that is significantly greater than the concurrent vehicle control, and have at least one induced response that exceeds the distribution of historical negative control frequencies (*e.g.*, historical vehicle controls evaluated concurrently with dosed animals and, when available, predosing frequencies). Negative responses do not fulfil these criteria. To conclude a substance is negative, however, there also must be evidence of sufficient bone marrow exposure. There is no requirement for verification of a clear positive or negative response, but further experimentation

may be necessary to resolve borderline or equivocal results. In cases where longitudinal mutant RET and mutant RBC data have been collected, the application of expert judgement that considers the kinetics of mutant RET and mutant RBC manifestation in the peripheral blood can be helpful for interpreting *Pig-a* data. Finally, statistical methods that take the kinetics of mutant cell manifestation into account can be helpful for interpreting *Pig-a* results.

6. Studies have been conducted to confirm that the *Pig-a* gene mutation assay measures gene mutation, and specifically mutation of the *Pig-a* gene. Analyses of *Pig-a* mutation have been conducted in erythroid and granulocyte precursor cells from the bone marrow of mice and rats. These analyses indicate that the increases in peripheral blood erythrocytes having the *Pig-a* mutant phenotype in rodents dosed with mutagens are due to mutation in the *Pig-a* gene of nucleated precursor cells. Compelling indirect evidence includes finding *Pig-a* mutations in mRNA from mutant RETs, and the consistent relationship between *Pig-a* mutation and the mutant phenotype in rat lymphocytes, in L5178Y/$Tk^{+/-}$ cells, and in hematopoietic cells from humans with paroxysmal nocturnal hemoglobinuria. Also, the positive relationships between *Pig-a* and TGR mutation and *Pig-a* mutation and genomic instability also are consistent with the assay measuring gene mutation. There is no evidence that alternative mechanisms, *e.g.*, gene silencing by methylation, are responsible for the phenotype measured in the *Pig-a* assay. Overall, the accumulated evidence indicates that the *Pig-a* phenotype, most commonly measured as loss of GPI-anchored proteins, is almost always due to mutations in the *Pig-a* gene. The rare instances when this was not the case have only been associated with human diseases or in particular cell lines; there is no indication that anything other than *Pig-a* mutation is responsible for mutants in the rodent *Pig-a* assay.

7. Ionizing radiation and over 80 chemicals, chemical mixtures, and nanomaterials have been tested for *Pig-a* mutation in rats, mice, or both. Several test substances have been tested in multiple laboratories and using different protocols. A committee was formed to establish criteria for evaluating *Pig-a* assay responses and categorize the responses as positive (P), negative (N), equivocal (E), or inconclusive (I). The committee used three sets of criteria that employed data from experiments conducted with short-term dosing protocols to different degrees. Although different definitions can be found in the literature, for the purpose of this document, short-term dosing protocols dosed animals on 13 or fewer consecutive days and longer-term dosing protocols dosed animals on 14 or more consecutive days. Using these criteria, data on 45 to 53 test substances were classified as producing P or N calls in the assay, and thus were useful for evaluating the performance of the assay.

8. Among the *Pig-a* positives, all were anticipated to be positive in the *Pig-a* assay based on their structure and/or responses in other assays. *Pig-a* negatives fell into several categories: 1) compounds that are generally regarded as negative for genotoxicity; 2) substances that are primarily or exclusively genotoxic through clastogenicity, aneugenicity, or mitotic recombination; and 3) substances that are genotoxic in other assays but appear to have insufficient bone marrow exposure by the dosing route employed in testing. The data indicate a mechanism-based complementarity between the types of genetic changes detected by the *in vivo Pig-a* and MN assays, where a few genotoxic compounds are positive for *Pig-a* and negative for MN and several are positive for MN and negative for *Pig-a*. In addition, studies have been conducted that discount the effects of potential confounders for the assay and that demonstrate the ability of the assay to distinguish between genotoxic and nongenotoxic pairs of structurally related compounds. Confounders that have been considered include toxicity to the erythropoietic system, and the effects of sex and of gene silencing.

9. In addition, many *Pig-a* studies include a mutant frequency analysis before exposure to any test substance or vehicle control substance. These data were used as a baseline control mutant frequency to evaluate the effect of dosing on 28 consecutive days with vehicle control substances using a longitudinal analysis of data. Data from ten of these substances were sufficient to make a call of P or N; all were evaluated as N.

10. An independent committee evaluated the test substances and vehicle control substances with either N or P responses in the *in vivo Pig-a* assay for genotoxicity in conventional genotoxicity assays and the rodent cancer bioassay. Responses in the *in vivo* TGR assay for bone marrow mutagenicity and mutagenicity in any tissue were used to define the *in vivo* mutagenicity of test substances; these defined responses were used to test the performance of the *Pig-a* assay as a test of *in vivo* gene mutation. Although the relatively small data sets employed for the analysis compromised differentiating between the variables that were evaluated, in general, the *Pig-a* assay showed a near perfect degree of accuracy when TGR mutagenicity in bone marrow or cancer in hematopoietic tissue was used to define true P and N responses. When TGR mutagenicity in any tissue or *in vivo* MN induction was used to define true P and N responses, the accuracy of the assay was slightly lower, with assay accuracy negatively impacted by the responses of test substances that were mutagenic in tissues other than bone marrow and/or mainly genotoxic by a mechanism that did not result in gene mutation. Lower accuracy when rodent cancer in any tissue was used to define P and N responses also was affected by several nongenotoxic carcinogens that were positive in the tumour bioassay

but negative in the *Pig-a* assay. Although positive responses for a small number of test substances are known to require multiple dosing protocols, the effect of dosing protocol (short-term *vs.* longer-term) was not a major factor in the overall accuracy of the assay.

11. When used to address regulatory requirements, the *Pig-a* assay is generally regarded as a follow-up assay for evaluating the *in vivo* mutagenicity of substances testing positive in bacterial or *in vitro* mammalian cell gene mutation assays. In this regard, the most recent International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use M7 (R1) guidance recommends the *Pig-a* gene mutation assay as a follow-up *in vivo* assay for drug impurities that are positive for bacterial mutagenicity as long as there is sufficient evidence for bone marrow exposure. 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. Other possible applications include using the human *PIG-A* assay as a direct confirmation of observations from animal studies and for human biomonitoring studies in potentially exposed populations. The TGR assay is not useful for these later applications as it requires the use of genetically modified animals, and it cannot be conducted with humans. *Pig-a* gene mutation assays also have been used in a variety of hypothesis-driven human and rodent studies that benefit from measurement of *in vivo* mutation.

12. GPI anchors are found on all mammalian cells that have been examined, along with cells from lower eukaryotes. Thus, *Pig-a* gene mutation assays, in theory, can be conducted in virtually all animal species of toxicological interest, making the *Pig-a* assay and *Pig-a* data highly translatable. The *Pig-a* assay began with work on measuring mutation in humans, and several recent studies have demonstrated the feasibility of a human erythrocyte *PIG-A* assay. Preliminary observations indicate that the assay can be conducted with erythrocytes from nonhuman primates; human granulocytes, lymphocytes, monocytes, and bone marrow progenitor cells; lymphocytes, granulocytes, and monocytes from rodents; and T lymphocytes from nonhuman primates. A *Pig-a* assay for male rat germ cells is in very early stages of development, with extensive work to be done. In addition to *in vivo* assays, assays measuring the *Pig-a* mutant phenotype (either loss of GPI anchors or GPI-anchored proteins) have been conducted *in vitro* with L5178Y/$Tk^{+/-}$ cells, TK6 human lymphoblastoid cells, and chicken DT-40 cells. There is a clear relationship between mutation in the *Pig-a* gene and loss of GPI-anchored proteins for L5178Y/$Tk^{+/-}$ cells. In DT-40 cells the mutant phenotype is due to mutation in the *Pig-o* gene (there are two copies

of *Pig-a* and one copy of *Pig-o* in chicken cells), and the mutant phenotype in TK6 cells is caused by mutation of either *PIG-A* or *PIG-L*, as both genes are present in one functional copy in these cells. None of these alternative assays has yet been developed to the extent of the rodent erythrocyte *Pig-a* gene mutation assay.

13. Sample collection in the rodent erythrocyte *Pig-a* assay is minimally invasive; samples can be collected over time from the same animal, and the assay lends itself to integration with other toxicology studies. Thus, even though it is an *in vivo* assay, these attributes make the assay consistent with the 3Rs principles, in that additional valuable data can be readily derived from animals already dedicated to toxicological testing. In addition, approaches have been described that use blood samples from one or a few animals to train personnel to conduct the assay and to establish laboratory proficiency in the assay as recommended by OECD test guidelines.

14. The Detailed Review Paper in Part 1 is accompanied by Part 2 (OECD, 2020a), which describes the state of *Pig-a* assay validation according to the principles set forth in OECD Guidance Document 34. Following review and acceptance of these documents by the OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT), a Test Guideline will be prepared for conducting the assay.

Introduction to Part 1

15. Lucio Luzzatto, David Araten and their colleagues were the first to suggest using the phosphatidylinositol glycan class A gene (*PIG-A*) as a reporter of *in vivo* mutation (Araten et al., 1999). As explained in Section 1, mutations in the *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. The Luzzatto and Araten labs subsequently conducted a number of studies using *PIG-A* as a reporter of mutation in human cells (*e.g*., Peruzzi et al., 2010; Rondelli et al., 2013); others built upon the basic principles used for their human *PIG-A* assay to develop assays for measuring gene mutation in rodent erythrocytes (Bryce et al., 2008; Miura et al., 2008a,b; Phonethepswath et al., 2008; reviewed in Dobrovolsky et al., 2010b). Unless otherwise specified, the term '*Pig-a* assay' (*Pig-a* is the rodent gene) is used in this report to refer to assays that measure gene mutation induced in the bone marrow erythroid cells of rats or mice. As explained below, these mutations are almost always detected by flow cytometric evaluation of the presence or absence of GPI-linked protein markers in peripheral blood erythrocytes.

16. The potential regulatory applications of the *Pig-a* assay were recognized early on by the Health and Environmental Sciences Institute (HESI) (until 2018, HESI was associated with the International Life Sciences Institute) (Schuler et al., 2011), and currently HESI sponsors a *Pig-a* workgroup as part of its Genetic Toxicology Technical Committee (GTTC). Additional support for development of the *Pig-a* assay came from a workgroup of the International Workshop on Genotoxicity Testing (IWGT) that met in 2013 in Foz do Iguaçu, Brazil. The review and recommendations of that workgroup (Gollapudi et al., 2015) form the basis for a major part of this report.

17. Encouraged by the IWGT recommendations, the GTTC *Pig-a* workgroup prepared an Organisation for Economic Cooperation and Development (OECD) Standard Project Submission Form (SPSF) proposing the development of a Test Guideline (TG) for the *in vivo Pig-a* assay (Test Guideline for the *Pig-a* assay: an *in vivo* gene mutation assay promoting the 3Rs principles). Following review and revision, that proposal was accepted by the OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in April

2015 (Project 4.93), with the understanding that a Detailed Review Paper (DRP) and a Validation Document should first be approved by the WNT before TG development. This DRP has been prepared in response to this WNT request. In particular, the WNT review of the SPSF emphasized supplying convincing evidence that the assay measures true gene mutation. Reviewers expressed enthusiasm about the integration potential and animal welfare benefits of the assay but were concerned that there were insufficient data for performing a useful Retrospective Performance Assessment (RPA) on the assay. They also expressed interest in the possibility of developing a *Pig-a* germ cell assay and an *in vitro* version of the assay. This report pays special attention to all these issues.

1 Principle of the *Pig-a* assay

18. Inactivating mutations and mutations that otherwise significantly affect the structure of an encoded protein often can alter the phenotype of the mutant cell. These phenotypic changes can result from shutdown, attenuation or repurposing of an endogenous metabolic process, accumulation or depletion of a specific metabolite, or disturbances in the networks involved in signal transduction, intracellular trafficking, gene expression and/or proliferation. Thus, many genes could potentially serve as a reporter of mutation. In practice, however, the number of useful reporter genes is quite limited because most mutations cause relatively subtle phenotypic changes in affected cells that are difficult to detect using common laboratory equipment. The *Pig-a* gene is particularly useful for mutant detection because rare mutant phenotype cells are easily detected and quantified among a large number of wild-type cells after minimal and relatively inexpensive sample processing. Also, the mutant phenotype, in rodents at least, does not affect the growth, viability, or persistence of mutant cells relative to wild-type cells.

a Genesis of the *Pig-a* assay

i. Glycosylphosphatidylinositol anchors and Pig-a

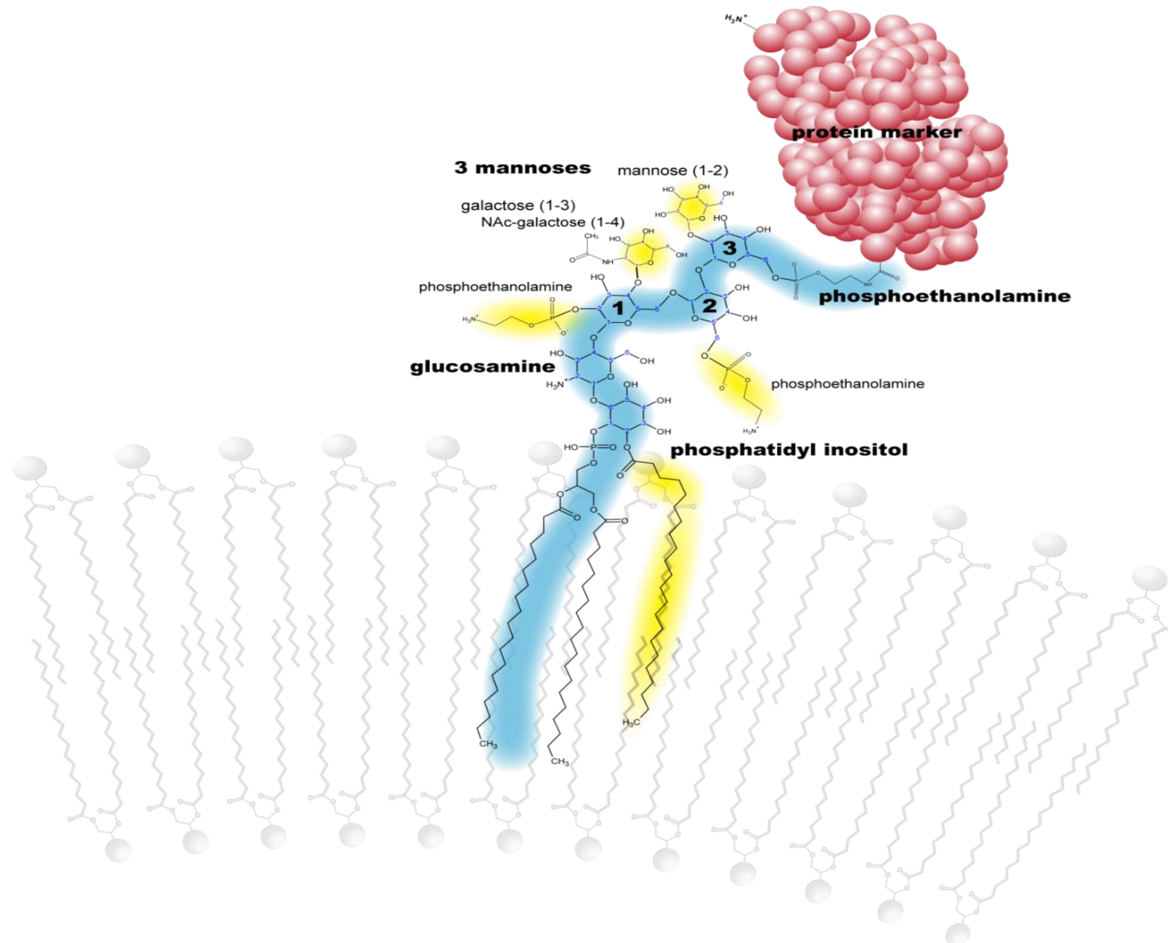
19. Most eukaryotic cells have evolutionally conserved mechanisms for displaying proteins on the exterior surface of their cytoplasmic membrane. A diverse subset of >150 of these proteins is maintained exoplasmically on the cytoplasmic membrane by the anchor molecule glycosylphosphatidylinositol (GPI) (Stevens, 1995; Brodsky and Hu, 2006; Brodsky, 2014; Kinoshita, 2014). The anchor is composed of a hydrophobic tail consisting of two or three fatty acid carbon chains and a charged three-mannose glycan core that covalently bridges to the protein *via* a phosphoethanolamine (Figure 1). The hydrophobic tail of the GPI molecule is embedded in the cytoplasmic membrane so that the attached protein does not require a lipophilic transmembrane domain to remain tethered to the cell membrane.

20. The syntheses of GPI and of GPI-anchored proteins are independent (Figure 2). The conjugation of the protein to the finished anchor occurs in the lumen of the endoplasmic reticulum at the ω-site of the peptide. The entire assembly is

exported via COP-II-mediated endocytosis to the Golgi apparatus, and then endosomically to the surface of the cell, presenting the GPI-anchored protein on the exoplasmic leaflet (Figure 2). At the surface, GPI-anchored proteins congregate within restricted domains, termed lipid rafts, which differ in lipid and protein content from the rest of the plasma membrane. Partial or complete disruption of GPI biosynthesis that results in GPI anchor deficiency causes a partial or complete deficiency of GPI-anchored proteins at the cellular surface.

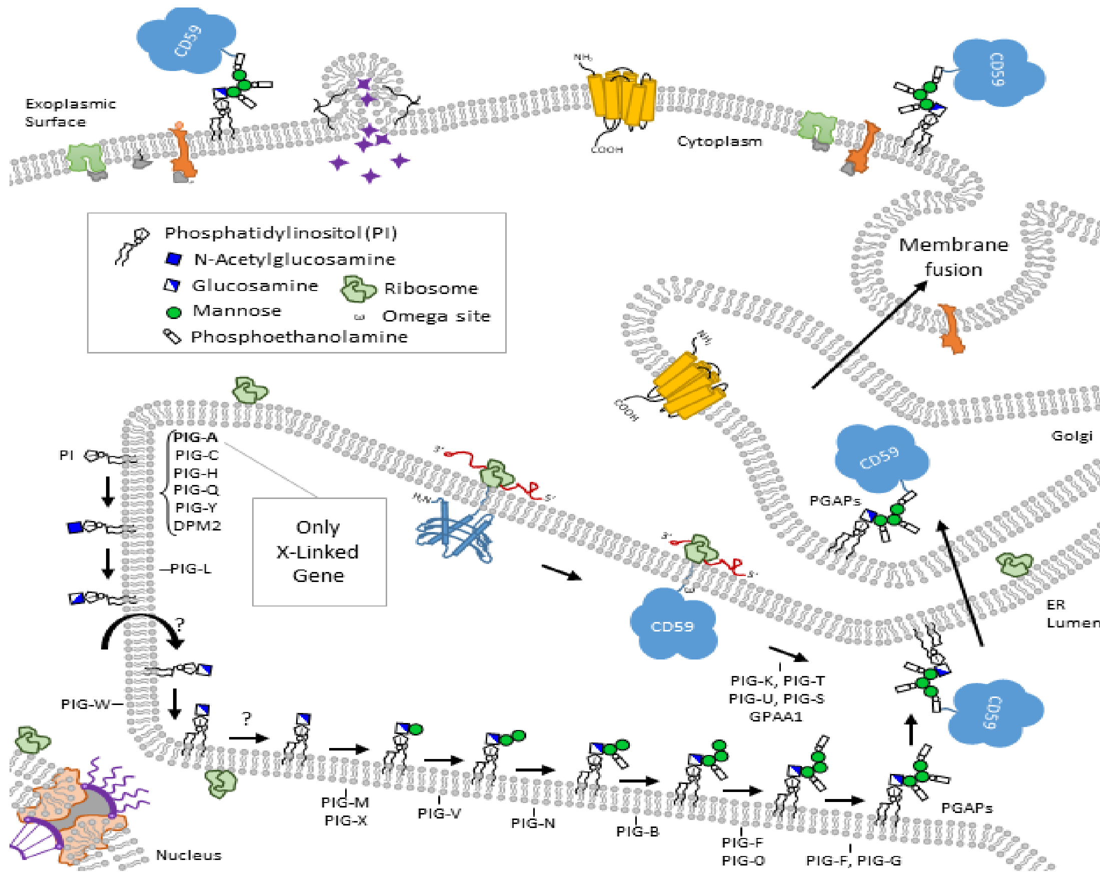
21. At least 31 genes are involved in the synthesis of the GPI anchor and the posttranslational modification of the anchor-protein complex (Figure 2) (Brodsky, 2014; Kinoshita, 2014). Inactivating mutations in several of these genes are known to result in a surface-protein-deficient phenotype, which can be detected by immunofluorescent labelling and flow cytometry (Figure 3). However, because *Pig-a* is the only one of these genes that is on the X chromosome in mammals (Takeda et al., 1993; Ware et al., 1994), it is the only gene involved in GPI biosynthesis that can reliably function as a reporter of mutation. The *PIG-A* gene in humans contains about 16 kilobases (Kb) of DNA sequence, coding for a protein of 484 amino acids. A single copy of *Pig-a* is present in male cells and a single functional copy in female cells (the second copy of the *Pig-a* gene is transcriptionally silenced in females). All other genes in the pathway are autosomal and are likely to be present in two functional copies. The probability of inducing a detectable mutation-associated phenotype by mutating the single functional copy of an X-linked gene is much higher than that of detecting a phenotype by independently mutating the two functional copies of an autosomal gene (Brodsky and Hu, 2006). Thus, the vast majority of 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.

Figure 1: Structure of the GPI anchor



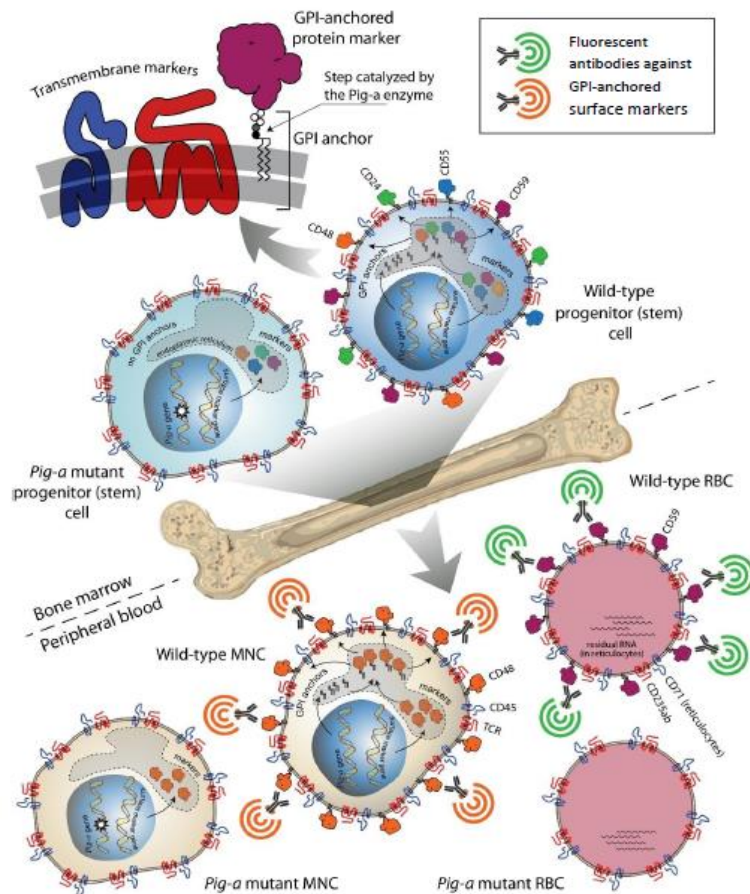
The anchor core (highlighted in blue) consists of a hydrophobic tail with two or three fatty acid carbon chains, and a charged three-mannose glycan core (with optional branching moieties) that covalently bridges to the protein marker via a phosphoethanolamine. Variable elements are highlighted in yellow. The hydrophobic tail of the GPI molecule is embedded in the cytoplasmic membrane so that the attached marker does not need its own lipophilic transmembrane amino acid motif in order to remain tethered to the cell surface. Figure prepared by V.N. Dobrovolsky.

Figure 2: Generic GPI-anchor biosynthesis pathway



Anchor synthesis begins on the outer surface of the endoplasmic reticulum (ER, in lower left of figure), and after the first few steps, 'flips' to the ER lumen (follow the arrows). The anchored protein (CD59) is synthesized independently and is added to the anchor in the ER lumen. The anchor-protein complex is then transported to the Golgi, for processing by Post GPI-Attachment to Proteins (PGAP) proteins, followed by translocation to its final location on the cytoplasmic membrane. *Pig-a*, the only X-linked gene, is part of the complex involved in the first anabolic step of GPI-anchor biosynthesis. Autosomal genes are responsible for the remainder of this complex and all downstream anabolic processes, the majority of which occur in the ER. Figure prepared by D.J. Roberts.

Figure 3: Principle of flow-cytometry-based *Pig-a* assays.



Upper left corner schematically shows the difference between transmembrane and GPI-anchored cell surface markers (see also Fig. 1). The basic GPI anchor consists of phosphatidylinositol, glucosamine and three mannoses. The Pig-a enzyme catalyzes the first step of GPI synthesis, transferring acetylglucosamine onto phosphatidylinositol (See more detailed scheme in Fig. 2). The anchor holds the attached marker by embedding long hydrophobic tails in the outer leaflet of the cell membrane. In wild-type bone marrow progenitor and precursor cells, GPI synthesis and the linking of multiple protein markers to the finished anchor happens at the surface of the endoplasmic reticulum (shown as grayed-out area with dashed border); from the endoplasmic reticulum, GPI-anchored markers get exported to the surface of the cell. In *Pig-a* mutant cells, GPI synthesis fails, and the protein markers cannot be exported to the surface; they remain trapped in the endoplasmic reticulum. Wild-type and *Pig-a* mutant progenitor cells in the bone marrow produce wild-type and mutant differentiated hematopoietic cells that are released into peripheral blood. In differentiated cells the expression pattern of different GPI-anchored markers depends on the cell type. In a typical cell type-specific *Pig-a* assay, cells of peripheral blood are labeled with fluorescent antibodies against one (or two) ubiquitously expressed GPI-anchored marker(s) (*e.g.*, antibody against CD59 in the human and rat red blood cell (RBC) *Pig-a* assays, or antibody against CD48 in the rat and mouse T-cell *Pig-a* assays). Under these conditions *Pig-a* wild-type cells will be fluorescent, but the *Pig-a* mutant cells will not fluoresce. Samples consisting of millions of labeled cells are processed with a high throughput flow cytometer and the fraction of non-fluorescent mutants can be determined accurately in a short period of time. Prior to performing the assays, the samples may be enriched for specific cell populations (*e.g.*, by density gradient centrifugation, for removal of mononuclear cells (MNCs) or for enrichment of the MNC fraction); also, additional antibodies can be used for identification of cells of interest, *e.g.*, antibodies against glycophorin (CD235ab for the human RBC assay) or glycophorin-associated proteins (HIS49 for the rat RBC assay and TER-119 for the mouse RBC assay), MNC-specific antibodies (anti

CD45), and anti T-cell receptor (TCR, or CD3) for positive identification of T-cells, etc. For performing a *Pig-a* assay on early erythrocytes, reticulocytes can be identified by staining for residual RNA with the SYTO family of nucleic acid stains (*e.g.*, SYTO13 or SYTO59) or by labelling with antibodies against the transferrin receptor, CD71. Figure prepared by V.N. Dobrovolsky.

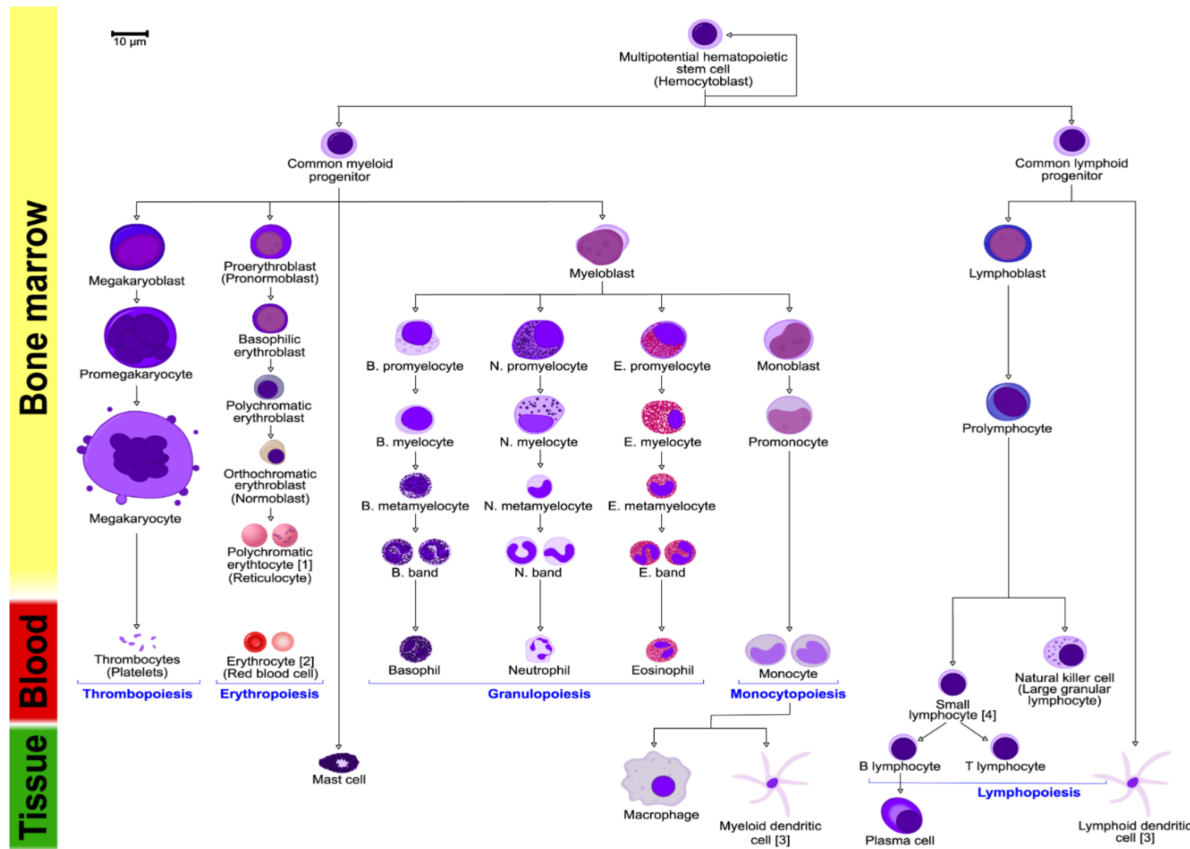
ii. PIG-A and paroxysmal nocturnal hemoglobinuria

22. Much of what is known about *Pig-a* mutation is derived from studies of a rare, acquired human disease, paroxysmal nocturnal hemoglobinuria (PNH), a bone marrow disorder that is characterized by hemolytic anemia, thrombosis, and peripheral blood cytopenia (Brodsky and Hu, 2006; Brodsky, 2014). The proximal cause of PNH is GPI deficiency, which is almost always caused by mutation of the *PIG-A* gene. The rare exceptions (sometimes referred as causing 'PNH-like' disease) involve mutations in both alleles of autosomal GPI biosynthesis genes (*e.g.*, *PIG-T* [Krawitz et al., 2013; Mason et al., 2019] or *PIG-S* [Nguyen et al., 2018]) or in genes for specific GPI-linked proteins (Yamashina et al., 1990; Nevo et al., 2013).

23. In PNH patients, the bone marrow is populated by one or a small number of expanded hematopoietic stem cell (HSC) clones that contain single *PIG-A* mutations. These clones give rise to the hematopoietic progenitor cell lineages (*e.g*., erythroid, lymphoid and myeloid; see Figure 4 for general scheme of the hemopoietic system), all having those same mutations. The *PIG-A* mutant progenitors, in turn, produce mature differentiated cells that transit into the peripheral circulation and that can result in specific cell populations that are nearly 100% deficient in GPI anchors and surface-tethered GPI-anchored proteins (Mortazavi et al., 2003; Schrezenmeier et al., 2014).

24. Deficiency of the GPI-anchored proteins CD55 and CD59 at the surface of peripheral RBCs results in a loss of complement regulation and triggers the lysis of mutant RBCs by the complement system (Wilcox et al., 1991). This accounts for the intravascular RBC lysis that is a primary clinical manifestation of PNH and the source of the pigmented urine (hemoglobinuria) that is often seen in PNH patients (Schrezenmeier et al., 2014).

Figure 4: Simplified scheme of hematopoiesis.



Erythropoiesis is part of the myeloid developmental pathway of hematopoiesis. The dividing, nucleated cells involved in erythropoiesis are found mainly in the bone marrow of adult animals. Multipotential hematopoietic stem cells and common myeloid precursor cells are very long lived and relatively few in number. Erythroblasts are derived from precursor cells and, with rounds of division, proceed through several developmental stages, culminating with enucleation to form short-lived reticulocytes or polychromatic erythrocytes and red blood cells. Mature red blood cells, which do not divide and lack DNA, have a half-life of several months in peripheral blood. Granulocytes are derived from common myeloid and stem cells but develop by a distinct pathway. See Sections 1b and 1c for further information. Figure reproduced from: https://en.wikipedia.org/wiki/Haematopoiesis#/media/File:Hematopoiesis_(human)_diagram_en.svg

iii.Analysis of Pig-a mutant cells as a reporter of gene mutation

25. A standard method for diagnosing PNH and for estimating the size of *PIG-A* mutant clones involves labelling the cells of peripheral blood with fluorescent monoclonal antibodies against one or more GPI-anchored proteins and subsequent quantitation of marker-deficient (nonfluorescent) *PIG-A* mutant cells by flow cytometry (Hall and Rosse, 1996; Madkaikar et al., 2009; Borowitz et al., 2010). Light scatter properties, sometimes in conjunction with additional antibodies against transmembrane proteins not anchored by GPI, can be used to identify the specific cell lineage. Granulocytes are often used for the diagnosis of PNH using anti-CD59 antibodies.

26. Besides the large clonal expansions of *PIG-A* mutant clones associated with PNH, healthy, non-PNH subjects have a low frequency (approximately 0.1-2 x 10^{-5}) of marker-deficient cells in the blood (Araten et al., 1999; Rawstron et al., 1999; Ware et al., 2001; Hu et al., 2005; Brodsky and Hu, 2006). This frequency is similar to the background mutant frequency of the *HPRT* gene in human peripheral blood lymphocytes (Robinson et al., 1994). Like *PIG-A*, *HPRT* is an X-linked gene, and the *HPRT* lymphocyte assay has been widely used to monitor mutant frequencies in humans. Based on their understanding of how *PIG-A* operates in GPI anchor synthesis, Araten et al. (1999) proposed that a flow cytometry-based PNH diagnostic protocol for detecting *PIG-A* mutant cells might form the basis of an assay for monitoring mutation in humans, specifically mutations induced in the bone marrow.

27. As the pathway for GPI synthesis is conserved in mammals (Kawagoe et al., 1994), similar flow cytometric methodology was designed for the quantitative detection of GPI-deficient and GPI-marker-deficient *Pig-a* mutant cells in rats, mice, and rhesus monkeys (Figure 3; Bryce et al., 2008; Miura et al., 2008a; Phonethepswath et al., 2008; Dobrovolsky et al., 2009). The unifying characteristic of all current flow cytometric assays detecting *Pig-a* (or *PIG-A*) mutant cells is the use of fluorescent antibody(s) against GPI-anchored protein marker(s), or (infrequently) a fluorescent, nontoxic version of aerolysin (FLAER) to distinguish between GPI-proficient and GPI-deficient cells (Miura et al., 2008a). FLAER is a fluorescent, nontoxic derivative of the bacterial toxin aerolysin that binds the GPI anchor rather than the anchored protein marker (Brodsky et al., 2000). With either labelling approach, wild-type cells will be fluorescent, and GPI-deficient cells or cells lacking surface-displayed GPI-anchored proteins, will be non-fluorescent and presumed to harbour *Pig-a* mutations.

28. Finally, native aerolysin is toxic to cells with GPI anchors. Aerolysin binding results in disruption of the cytoplasmic membrane and cell death; thus, aerolysin can be used as a selection agent for identifying GPI-deficient (presumably *Pig-a* mutant) cells. Aerolysin (more commonly as the protoxin, proaerolysin) has been used as a selection agent for limiting dilution cloning versions of the *Pig-a* assay, conducted both with rat and human T lymphocytes and with cell lines (*e.g*., Miura et al., 2008b; McDiarmid et al., 2011; Nakamura et al., 2012; Nicklas et al., 2015; Labash et al., 2015b; Y Wang et al., 2018). Although aerolysin selection is suitable for confirming the GPI-deficient phenotype and generating mutant clones for sequencing, it lacks the integration potential and convenience of the erythrocyte flow cytometry assay and thus is not considered useful as a routine regulatory assay for *in vivo* mutation.

b Considerations in assay design

29. Commercially available antibodies against GPI-anchored protein markers are usually species-specific (*e.g*., anti-rat CD59 antibody will not bind GPI-anchored CD59 on the surface of mouse or human cells) while FLAER is not species-specific (*i.e*., it will bind the anchors present at the surface of mouse, human and rat cells). But, in many cases, monoclonal antibodies are superior to the bacterial toxin-based FLAER for discriminating rare populations of GPI-deficient cells (*e.g*., Miura et al., 2008a); thus, FLAER is rarely used for routine *Pig-a* mutation assays.

30. At least 150 GPI-anchored proteins have been identified; often their expression is variable in different cell types and at different stages of maturation, which is something that should be considered for designing a reliable GPI-anchor detection method (Brodsky et al., 2000; Hernández-Campo et al., 2006, 2007). Just as not every GPI-anchored protein marker is suitable for diagnosis of PNH, not every marker is suitable for designing a flow cytometry-based *Pig-a* mutation detection assay. For specific cell types, it is necessary to choose a marker that is expressed ubiquitously and at a high level at the cell surface. Experience suggests that rat CD59 is suitable for rat erythrocyte assays while mouse CD24 is suitable for mouse erythrocyte assays.

31. Peripheral blood is the preferred source of samples for flow cytometry-based *Pig-a* assays: peripheral blood is easy to collect, and the samples are naturally single-cell suspensions amenable for labelling and processing on a flow cytometer. Importantly, enzymatic dissociation is typically used to generate individual cells from solid tissues. The need for this processing may limit the utility of the assay for mutation assessment in solid tissues due to the potential for damaging the surface-bound GPI-anchored markers employed in the

antibody labelling (but see Section 10d). Although *Pig-a* assays have been conducted with other cell types (Section 10c), erythrocyte-based *Pig-a* assays have a major advantage owing to the extraordinarily high concentration of erythrocytes in peripheral blood. Microliter volumes of blood contain sufficient erythrocytes for accurately measuring mutant cell fractions. The requirement for only small blood samples translates into an ability to collect samples repeatedly, even from small laboratory animals, thus allowing the acquisition of longitudinal data sets based on assays conducted with individual animals.

32. In adult animals, most peripheral blood erythrocytes are derived from progenitors in the bone marrow (Sun et al., 2014; Figures 3 and 4). These progenitors generate a series of morphologically identifiable precursors that enucleate to form reticulocytes (RETs) and finally give rise to the mature red blood cells (RBCs) found in peripheral blood. As RETs mature into RBCs over a period of days, they progressively lose their non-GPI-anchored surface protein CD71 and their residual cytoplasmic RNA (Serke and Huhn, 1992), and so CD71 and residual RNA are convenient markers of the RET subpopulation of erythrocytes. The proportion of RETs among bone marrow cells is quite high (*e.g.*, 40-80 %; Fiedler et al., 2010); however, after RETs migrate to the periphery, they are rapidly converted into mature RBCs and their proportion among total erythrocytes is reduced to a few percent or less, depending on species, age, and RET labelling technique (*e.g.*, Serke and Huhn, 1992; Dertinger et al., 2015). RETs are important for the *Pig-a* assay because they are the earliest reporters of *Pig-a* mutations induced in erythroid precursors of the bone marrow that are accessible in peripheral blood. Since RETs are short-lived, the maximum frequency of mutant RETs after a single dose of mutagen is achieved rapidly. After a single dose of a potent mutagen to rats, for example, the maximum mutant frequency in peripheral RETs usually occurs within two weeks, while it may take six to eight 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).

33. Another important reason for studying mutation using RETs is that in human PNH patients, the complement system lyses CD59-deficient *PIG-A* mutant RBCs (Wilcox et al., 1991). Observations by Dertinger et al. (2015) suggest that this factor affects the erythrocyte *PIG-A* assay in humans. If this were to occur in mutagen-treated animals, then the longer a mutant erythrocyte remains in circulation, the more likely is the chance that it can be destroyed by complement-mediated lysis. In this respect, RET *Pig-a* assays not only convey information on mutagenicity in bone marrow earlier, data from RET assays also lend plausibility to small increases in mutant frequency measured in total RBCs. There is, however, accumulating evidence indicating that immune lysis does

not affect *Pig-a* mutant frequencies in mutagen-treated laboratory animals as it appears to do in humans (see below: Miura et al., 2009; Phonethepswath et al., 2010).

c Initial observations on the source of the mutants detected in the erythrocyte *Pig-a* assay

34. Relatively early observations about the rat erythrocyte *Pig-a* assay indicated that responses accumulate with repeated doses and once an elevated mutant frequency is achieved, it persists for a prolonged period of time. Miura et al. (2009) found that four smaller doses of *N*-ethyl-*N*-nitrosourea (ENU), given at weekly intervals, produced virtually the same RBC mutant frequency as when a single dose was given that was equivalent to the sum of the four small doses. Also, after the maximum mutant frequency was achieved in total RBCs (six-eight weeks after the single dose, or six-eight weeks after the final weekly dose), the response persisted until the experiment was terminated 16 to 20 weeks later. Phonethepswath et al. (2010) confirmed the persistence of elevated *Pig-a* mutant frequencies in an experiment that monitored ENU-induced mutant frequencies in rat total RBCs and RETs over a period of six months, and Dertinger et al. (2014a) observed a similar persistence of elevated *Pig-a* mutant frequencies in rats treated with cisplatin. Since the persistence of elevated mutant frequencies exceeded the lifetime of the differentiated peripheral blood erythrocytes, these observations suggested that a relatively stable, renewable cell population, *e.g.*, long-lived erythroid progenitors or perhaps even hematopoietic stem cells (HSCs), was the source of the induced mutants.

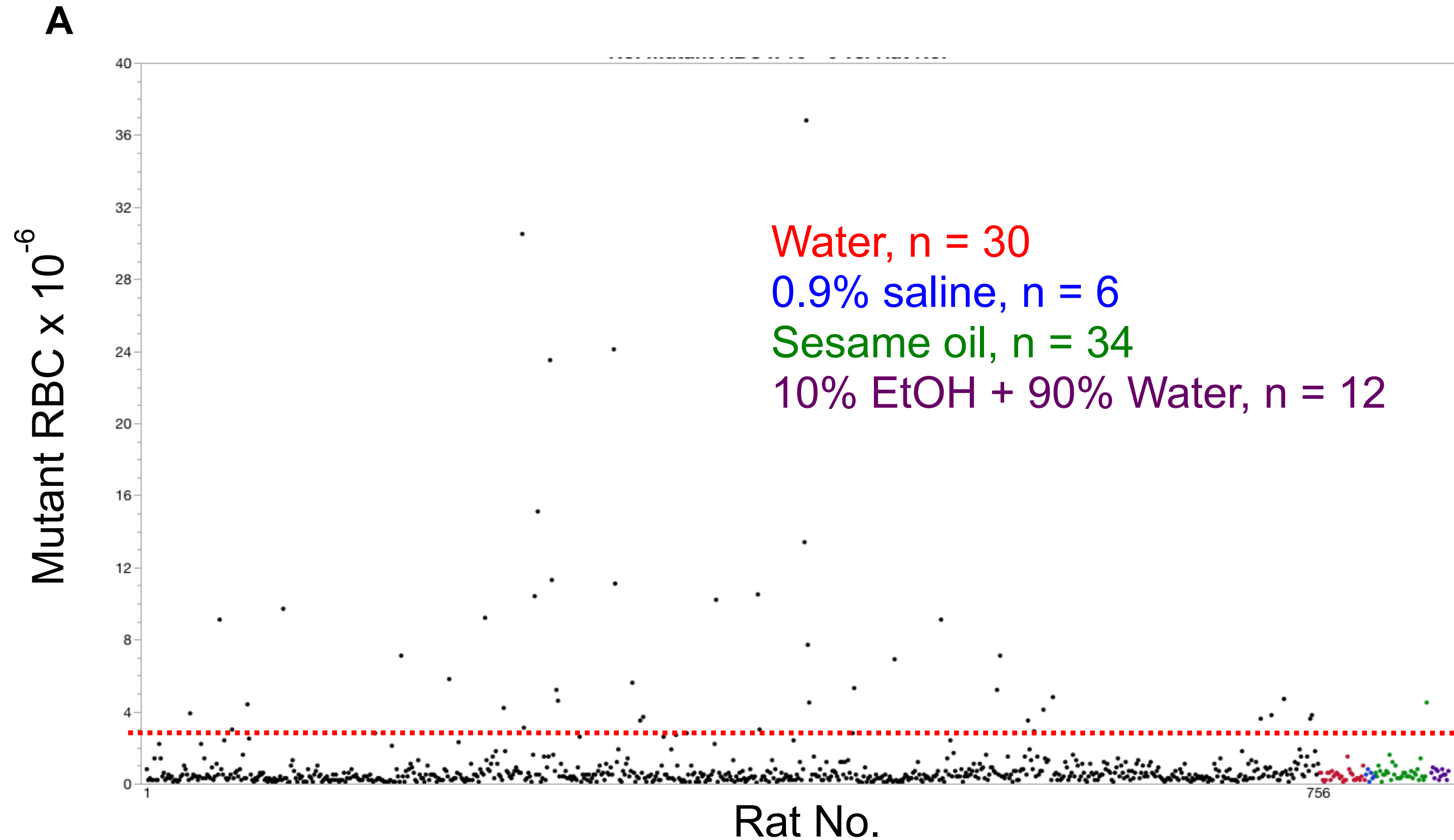
35. Bone marrow, the probable source of erythrocyte *Pig-a* mutants in the rodent *Pig-a* assay, contain a mixture of HSCs and multipotent and lineage-committed progenitor cells (Sun et al., 2014; Busch et al., 2015; Figure 4). Although all these cells are nucleated, and many are actively dividing, and thus could be the source of *Pig-a* mutations, it is unlikely that mutant HSCs make a major contribution to the mutant frequencies measured in the *Pig-a* assay. The number of ‘true’ HSCs in an animal is estimated to be 0.5-2 x 10^4 (Abkowitz et al., 2002; Busch et al., 2015); thus, assuming a neutral mutant phenotype, *Pig-a* mutant cell frequencies of less than 50 x 10^{-6} are unlikely if *Pig-a* mutation is restricted to HSCs. In humans with PNH, where the *PIG-A* mutations are most likely derived from HSCs, the mutant frequencies in all hematopoietic lineages are quite high, and can approach 100% (Mortazavi et al., 2003; Schrezenmeier et al., 2014). These extraordinarily high mutant frequencies are almost always due to the expansion of one, or a very few, mutant HSC clones (Brodsky, 2014;

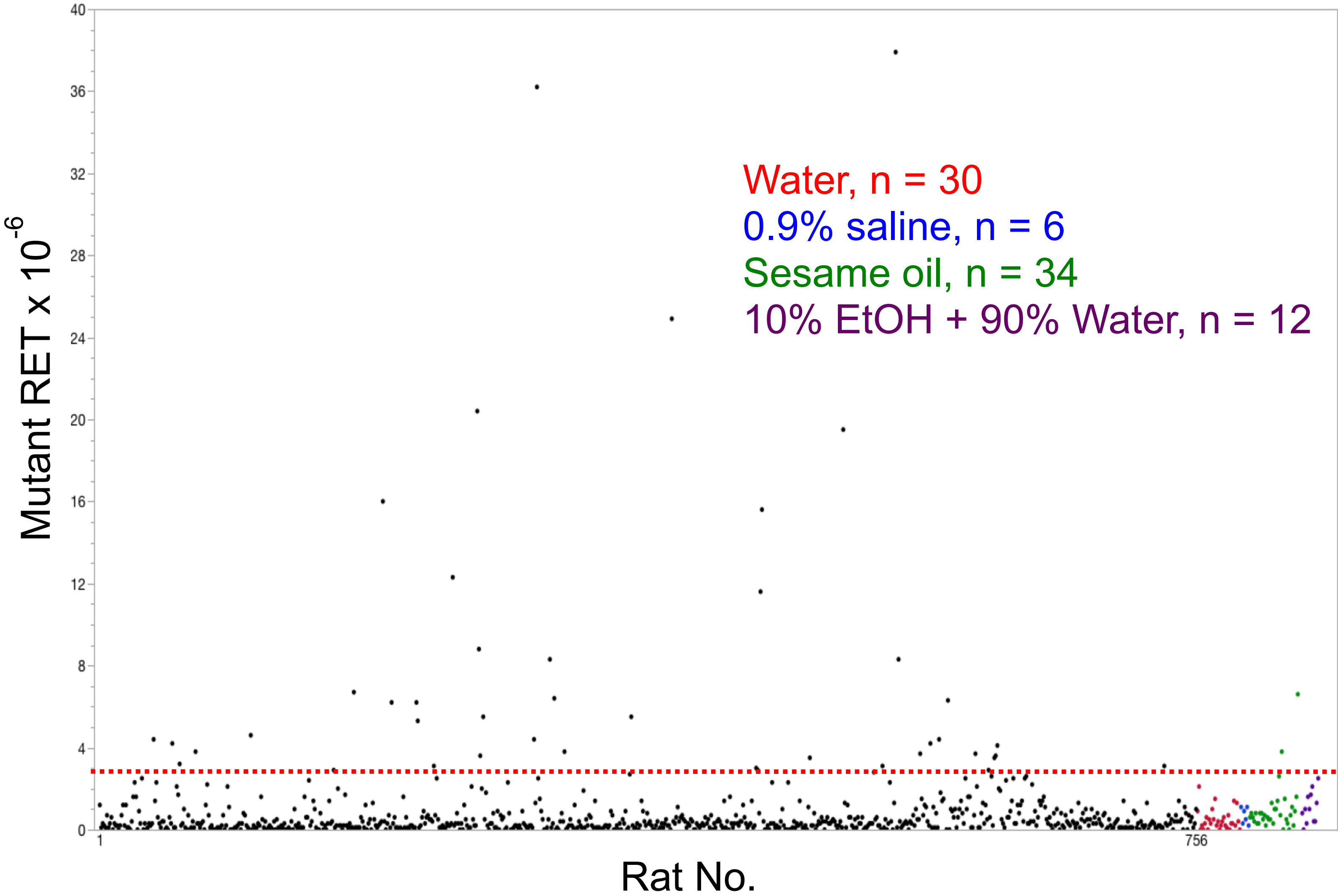
Kinoshita, 2014). Spontaneous *Pig-a* mutant frequencies in rodents, on the other hand, are almost always <5 x 10^{-6}, and often <1 x 10^{-6} (see predosing data in Figure 5); also, only potent mutagens produce *Pig-a* mutant frequencies in rodents of >50 x 10^{-6}. Finally, Busch et al. (2015) indicate that the time necessary for a *de novo* HSC mutation to be expressed fully in the periphery of an adult mouse may exceed the mouse's lifespan. Thus, it is unlikely that HSC mutation makes a major contribution to the *Pig-a* mutant phenotype measured in peripheral blood.

36. Bone marrow hematopoietic precursor cells are a more likely source of mutations in the assay. Recent studies indicate that much of steady-state erythropoiesis is driven not by true 'long-term' HSCs, but rather by relatively long-lived multipotent and lineage-specific progenitor cells in the bone marrow (Sun et al., 2014; Busch et al., 2015). The lifespans of these cells are consistent with the persistence of induced mutant frequencies seen in the assay. Although it is not clear how large these cellular compartments are, they are certainly larger than the HSC compartment, and thus minimum mutant frequencies resulting from mutations in these precursor cell populations could be of the order seen in the *Pig-a* gene mutation assay. The analysis of *Pig-a* mutation in rat bone marrow erythroid cells and granulocytes described in Section 6 is consistent with the *Pig-a* assay detecting mutations induced in these hematopoietic precursor cells.

Figure 5: Day 29 mutagenicity of solvent/vehicle controls in comparison with historical Day -1 mutant frequency data in individual adult male Sprague-Dawley rats.

A: Total RBC mutant frequencies; B: RET mutant frequencies. Black symbols: historical Day -1 RBC/RET mutant frequencies (n=756; RBCs, mean 1.1 x 10^{-6}; upper 95% TI, 2.9 x 10^{-6}; RETs: mean 1.0 x 10^{-6}; upper 95% TI, 2.9 x 10^{-6}). Colored symbols: Day 29 mutant frequencies from rats dosed for 28 days with vehicles, as indicated. Data courtesy of S.D. Dertinger; similar graphs in Avlasevich et al. (2018).




B
Mutant RET x 10^-6
40
36
32
28
24
20
16
12
8
4
0
1
756
Rat No.
Water, n = 30
0.9% saline, n = 6
Sesame oil, n = 34
10% EtOH + 90% Water, n = 12

2 Existing *in vivo* genetic toxicology assays

37. Establishing the utility of the *Pig-a* assay for evaluating *in vivo* genotoxicity requires understanding the principles, methodologies, and applications of the current tests used for that purpose. *In vivo* tests are used for hazard identification (hazard ID), both as parts of test batteries with *in vitro* tests and to follow-up *in vitro* findings (Cimino, 2006). *In vivo* genotoxicity tests, however, typically are focused on detecting a particular class of genotoxicity and/or detecting genotoxicity in a specific cell type. Thus, no one test is capable of reliably evaluating the genotoxicity of all test substances, which is the rationale for using batteries of tests (Eastmond et al., 2009). In addition to hazard ID, *in vivo* tests are used for risk characterization and dose-response analysis, as is the case when they are used for cancer mode-of-action studies or evaluating the potential health risks of low-dose exposures (Boverhof et al., 2011).

38. Of the many tests that have been developed over the years, eight tests for measuring *in vivo* genotoxicity currently have OECD TGs (OECD, 2013, 2016a,b,d,e,f). These tests have attained regulatory acceptance and have clear recommendations for their use and interpretation. Three of these *in vivo* assays are used only for somatic cell testing (TG 475 for detecting clastogenicity, TG 474 for detecting clastogenicity and aneugenicity, and TG 489, which detects DNA damage as single- and double-strand breaks), two are specifically for germ cell testing (TG 478 and TG 483, both of which mainly detect clastogenicity), and one can detect genotoxicity in both germ cells and somatic cells (TG 488, which detects gene mutation in transgenes). TGs for these assays are either new (TG 489, the *in vivo* alkaline comet assay) or recently revised (OECD, 2015). Two additional tests, a somatic cell test measuring DNA damage as unscheduled DNA synthesis (UDS) in rat liver (TG 486; OECD, 1997), and the heritable translocation assay for germ cell mutation in mice (TG 485; OECD, 1986b) are rarely used, and have not been updated recently. In addition, the OECD TG for the Mouse Spot Test (TG 484; OECD, 1986a), an *in vivo* test for somatic cell mutation, was deleted in 2014 because it has become obsolete and is no longer recommended. An extensive analysis of *in vitro* and *in vivo* genotoxicity tests is available in the review and DRP prepared

as part of TG 488 development (Lambert et al., 2005; OECD, 2009). The following, using language taken directly from the TGs, is intended to provide a general description of the tests that are most relevant to the *Pig-a* assay, the four most commonly used tests with OECD TGs that evaluate *in vivo* genotoxicity in somatic cells. Table I compares some of the salient features of these tests with those of the *Pig-a* assay.

Table I: Features of *in vivo* somatic cell genotoxicity assays

Test	**Response measured**	**Target tissue for response**
TG 474: Erythrocyte micronucleus test	Aneugenicity/clastogenicity	Bone marrow
TG 475: Bone marrow chromosome aberration test	Clastogenicity	Bone marrow
TG 488: Transgenic rodent assay	Gene mutation	Virtually any tissue (including male germ cells)
TG 489: *In vivo* comet assay	DNA damage	Virtually any tissue (but not germ cells)
Erythrocyte *Pig-a* assay	Gene mutation	Bone marrow

a TG 474: The mammalian erythrocyte micronucleus test

39. The following description is taken verbatim from the Introduction to OECD TG 474 (OECD, 2016a).

40. “The mammalian *in vivo* micronucleus test is especially relevant for assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA repair are active and contribute to the responses. An *in vivo* assay is also useful for further investigation of genotoxicity detected by an *in vitro* system.

41. “The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test chemical to the chromosomes or the mitotic apparatus of erythroblasts. The test evaluates micronucleus formation in erythrocytes

sampled either in the bone marrow or peripheral blood cells of animals, usually rodents.

42. “The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing either lagging chromosome fragments or whole chromosomes.

43. “When a bone marrow erythroblast develops into an immature erythrocyte (sometimes also referred to as a polychromatic erythrocyte or reticulocyte), the main nucleus is extruded; any micronucleus that has been formed may remain behind in the cytoplasm. Visualization or detection of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated immature erythrocytes in treated animals is an indication of induced structural or numerical chromosomal aberrations.

44. “Newly formed micronucleated erythrocytes are identified and quantitated by staining followed by either visual scoring using a microscope, or by automated analysis. Counting sufficient immature erythrocytes in the peripheral blood or bone marrow of adult animals is greatly facilitated by using an automated scoring platform.

45. “Although not normally done as part of the test, chromosome fragments can be distinguished from whole chromosomes by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA, both of which are characteristic of intact chromosomes. The absence of kinetochore or centromeric DNA indicates that the micronucleus contains only fragments of chromosomes, while the presence is indicative of chromosome loss.”

b TG 475: The mammalian bone marrow chromosomal aberration test

46. The following description is taken verbatim (with modifications as noted) from the Introduction to OECD TG 475 (OECD, 2016b).

47. “The mammalian *in vivo* bone marrow chromosomal aberration test is especially relevant for assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the responses. An *in vivo* assay is also useful for further investigation of genotoxicity detected by an *in vitro* system.”

48. “The mammalian *in vivo* chromosomal aberration test is used for the detection of structural chromosome aberrations induced by test chemicals in bone marrow cells of animals, usually rodents……Structural chromosomal aberrations may be of two types, chromosome or chromatid. While the majority of genotoxic chemical-induced aberrations are of the chromatid-type, chromosome-type aberrations also occur. Chromosomal damage and related events are the cause of many human genetic diseases and there is substantial evidence that, when these lesions and related events cause alterations in oncogenes and tumour suppressor genes, they are involved in cancer in humans and experimental systems. Polyploidy (including endoreduplication) could arise in chromosome aberration assays *in vivo*. However, an increase in polyploidy per se does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity. This test is not designed to measure aneuploidy. An *in vivo* mammalian erythrocyte micronucleus test (Test Guideline 474) or the *in vitro* mammalian cell micronucleus test (Test Guideline 487) would be the *in vivo* and *in vitro* tests, respectively, recommended for the detection of aneuploidy.”

c TG 489: The *in vivo* mammalian alkaline comet assay

49. The following description is taken verbatim (with modifications as noted) from OECD TG 489 (OECD, 2016f).

50. “The purpose of the comet assay is to identify substances that cause DNA damage. Under alkaline conditions (>pH 13), the comet assay can detect single and double stranded breaks, resulting, for example, from direct interactions with DNA, alkali labile sites or as a consequence of transient DNA stand breaks resulting from DNA excision repair. These strand breaks may be repaired, resulting in no persistent effect, may be lethal to the cell, or may be fixed into a mutation resulting in a permanent viable change. They may also lead to chromosomal damage which is also associated with many human diseases including cancer.”

51. “The *in vivo* alkaline comet assay is especially relevant to assess genotoxic hazard in that the assay’s responses are dependent upon *in vivo* ADME (absorption, distribution, metabolism and excretion), and also on DNA repair processes. These may vary among species, among tissues and among the types of DNA damage.”

52. “To fulfil animal welfare requirements, in particular the reduction in animal usage (3Rs -Reduction, Refinement, Replacement - principles), this assay can also be integrated with other toxicological studies, *e.g.*, repeated dose toxicity

studies……or the endpoint can be combined with other genotoxicity endpoints such as the *in vivo* mammalian erythrocyte micronucleus assay…….The comet assay is most often performed in rodents, although it has been applied to other mammalian and non-mammalian species."

53. "The selection of route of exposure and tissue(s) to be studied should be determined based on all available/existing knowledge of the test chemicals *e.g.* intended/expected route of human exposure, metabolism and distribution, potential for site-of-contact effects, structural alerts, other genotoxicity or toxicity data, and the purpose of the study. Thus, where appropriate, the genotoxic potential of the test chemicals can be assayed in the target tissue(s) of carcinogenic and/or other toxic effects. The assay is also considered useful for further investigation of genotoxicity detected by an *in vitro* system. It is appropriate to perform an *in vivo* comet assay in a tissue of interest when it can be reasonably expected that the tissue of interest will be adequately exposed."

54. "The technique is in principle applicable to any tissue from which analysable single cell/nuclei suspensions can be derived. Proprietary data from several laboratories demonstrate its successful application to many different tissues, and there are many publications showing the applicability of the technique to (many different) organs or tissues…"

55. "Cross-links cannot be reliably detected with the standard experimental conditions of the comet assay. Under certain modified experimental conditions, DNA-DNA and DNA-protein crosslinks, and other base modifications such as oxidized bases might be detected…..But further work would be needed to adequately characterize the necessary protocol modifications……The assay is not appropriate, even with modifications, for detecting aneugens."

d TG 488: The transgenic somatic and germ cell gene mutation assays

56. The following description is taken verbatim (with modifications as noted) from OECD TG 488 (OECD, 2013).

57. "(TGR gene mutation assays) use transgenic rats and mice that contain multiple copies of chromosomally integrated plasmid or phage shuttle vectors. The transgenes contain reporter genes for the detection of various types of mutations induced *in vivo* by test chemicals."

58. "Mutagenesis in the TGR models is normally assessed as mutant frequency; if required, however, molecular analysis of the mutations can provide additional information."

59. “These rodent *in vivo* gene mutation tests are especially relevant to assessing mutagenic hazard in that the assays’ responses are dependent upon *in vivo* metabolism, pharmacokinetics, DNA repair processes, and translesion DNA synthesis, although these may vary among species, among tissues and among the types of DNA damage. An *in vivo* assay for gene mutations is useful for further investigation of a mutagenic effect detected by an *in vitro* system, and for following up results of tests using other *in vivo* endpoints….."

60. “If there is evidence that the test chemical, or a relevant metabolite, will not reach any of the tissues of interest, it is not appropriate to perform a TGR gene mutation assay.”

61. “In the assays described above, the target gene is bacterial or bacteriophage in origin, and the means of recovery from the rodent genomic DNA is by incorporation of the transgene into a λ bacteriophage or plasmid shuttle vector……and subsequent detection of mutations in bacterial hosts under suitable conditions. The assays employ neutral transgenes that are readily recoverable from most tissues.”

62. “The basic TGR gene mutation experiment involves treatment of the rodent with a chemical over a period of time…..Administration is usually followed by a period of time, prior to sacrifice, during which the agent is not administered and during which unrepaired DNA lesions are fixed into stable mutations…….After the animal is sacrificed, genomic DNA is isolated from the tissue(s) of interest and purified.”

63. “The mutations scored in the *lacI*, *lacZ*, *cII* and *gpt* point mutation assays consist primarily of base pair substitution mutations, frameshift mutations and small insertions/deletions. The relative proportion of these mutation types among spontaneous mutations is similar to that seen in the endogenous *Hprt* gene. Large deletions are detected only with the Spi^{-} selection and the *lacZ* plasmid assays…..."

64. “It is anticipated that in the future it may be possible to combine a TGR gene mutation assay with a repeat dose toxicity study (TG 407). However, data are required to ensure that the sensitivity of TGR gene mutation assays is unaffected by the shorter one day period of time between the end of the administration period and the sampling time, as used in the repeat dose toxicology study, compared to the three days used in the TGR gene mutation assays. Data are also required to indicate that the performance of the repeat

dose assay is not adversely affected by using a transgenic rodent strain rather than traditional rodent strains."

3 Strengths and weaknesses of the *Pig-a* assay

65. The *Pig-a* assay has several compelling strengths for measuring gene mutation *in vivo*. Perhaps most importantly, it can be performed in most mammalian species, mutant analysis is rapid and relatively low in cost, and the assay integrates well into general toxicity testing, making maximum use of animal resources already committed to *in vivo* testing. The potential for integration with other assays may facilitate collection of data on multiple endpoints (*e.g.*, MN and comet as well as data from general toxicology endpoints), which can result in a more comprehensive analysis of *in vivo* genotoxicity hazard. In addition, the same endpoint that is monitored in rodents (*i.e.*, *Pig-a* mutation), can be measured in humans and mammalian cells in culture, making possible mechanistic studies *in vitro* and direct assessment of human effects *in vivo*. These advantages stem from the following characteristics of the assay:

- The assay measures a mutant phenotype dependent upon the functioning of a pathway conserved in mammalian species: it does not depend on the properties of any specific strain of animal.
- The assay requires only microliters of peripheral blood, which facilitates relatively non-invasive collection of serial data and integration into studies without the need for animal sacrifice.
- The assay measures mutation in unattached hematopoietic cells (*i.e.*, erythrocytes), which facilitates high throughput scoring *via* flow cytometric analysis without need for *ex vivo* culture or extensive manipulations.

66. These strengths are evident in comparison with the TGR assay, which is currently the only *in vivo* assay for gene mutation with an OECD Test Guideline (OECD, 2013). The costs associated with performing the TGR assay, in terms of time, labor, and materials, are often cited as a major barrier to its adoption for routine genotoxicity testing (Boverhof et al., 2011; Page et al., 2015). In addition, more than cost, the requirement for using specific strains of transgenic rodents and mutation sampling times that are incompatible with a 28-day repeat-dose general toxicology study reduce the potential for integration with other toxicology testing. Although efforts are on-going to address these

problems (Akagi et al., 2015; Nohmi et al., 2017; Hori et al., 2019), these barriers to integrating TGR mutation analysis with the measurement of other toxicology endpoints currently remain. In addition, using a transgene imposes limitations on the types of mutations that can be measured. Finally, the relatively complex structure of endogenous mammalian genes is such that they can recover a greater variety of mutation types than can a bacterial transgene, *e.g.*, megabase (Mb) deletions (Tao et al., 1993; Chen et al., 1998; Walker et al., 1999).

67. The use of erythrocytes for conducting the *Pig-a* assay facilitates rapid analysis, minimally invasive sample collection, and integration of the assay into general toxicity testing, which are all major advantages. Measuring mutation using erythrocytes, however, also results in the two greatest weaknesses of the assay (listed in order of significance).

- The assay only detects mutations induced in erythropoietic tissue (*i.e.*, mainly the bone marrow in adult animals; see Section 1c), reducing its sensitivity for substances that are strongly mutagenic only in other tissues, like liver. The effect of this limitation on the sensitivity of the assay may have resulted in some of the negative responses in the assay, as listed in Table IX, below. This limitation of measuring genotoxicity in hematopoietic tissue is also true for the somatic cell cytogenetic assays described in OECD TGs 474 and 475 (see Sections 2a and 2b). Related to this, a major advantage of the TGR assay is that it can be used to detect mutations in virtually any tissue including germ cells, while germ cell *Pig-a* assays are only in the beginning stages of development (see Section 10c).
- Measuring mutations induced only in bone marrow requires that the test substance and/or its metabolites expose the bone marrow to comprehensively evaluate its *in vivo* hazard. This requirement for bone marrow exposure is an important factor in the interpretation of negative responses (see discussion in paragraph 93).
- Because mutations are detected in cells that do not have DNA, it is challenging (but not impossible) to characterize the mutations measured in the *Pig-a* assay (see Section 6). By contrast, analysis of mutations in TGR assays can be conducted on any tissue with DNA, and mutation characterization is straightforward for most TGR systems, whose small bacterial transgene mutational targets are readily sequenced (Lambert et al., 2005), including by using newer high-throughput methods (Beal et al., 2015).

4 Assay protocol

68. Protocol recommendations for conducting the assay were given in the IWGT report (Gollapudi et al., 2015; summarized in Table II). The report indicated that the recommendations were based mainly on assays conducted using erythrocytes from male rats. More recently, however, considerable data have appeared on conducting the assay in male mice (Olsen et al., 2017; see Table VII and Annex II), and several studies have been published demonstrating similar responses to test substances in male and female rats (Chikura et al., 2014; Labash et al., 2015a,c) (see Section 7b). Thus, the available data indicate that protocol recommendations can be generalized for use with male and female rats and with male mice.

Table II: Summary of protocol recommendation from the IWGT report on the *Pig-a* assay (Gollapudi et al., 2015)

Recommendation	**Comment**
Use young adult animals, minimum of vehicle/negative control plus three dose levels with high dose being MTD or, for nontoxic test substances, 1000 mg/kg/day for longer-term studies (14 days of treatment or more) or 2000 mg/kg/day for short-term studies	Consistent with OECD TGs for repeat dose *in vivo* toxicology and genetic toxicology studies: 'Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering.' (TG 407; OECD 2008)
Six animals per group	Five analyzable per group acceptable if justified by power calculation
Positive control animals not needed if mutant-mimic flow cytometry control used	Running a positive control using an appropriate frozen sample is also possible
Prescreening animals for mutant frequency useful but not necessary	Data useful for establishing historical baseline mutant frequencies and

	eliminating animals with outlier mutant frequencies from assays
Longer-term dosing protocol preferred (*e.g.,* dosing for 28 consecutive days)	28-day dosing schedule is consistent with integration into standard toxicology studies
For short-term and 28-day dosing protocols, analyze samples at Day 28 to 31 after the first dose; additional analysis at earlier and later time points encouraged	Analyses conducted at ≥ Day 56 after the first dose may result in increased variability in responses (*e.g*., Miura et al. 2009; Mittelstaedt et al., 2019)
Analyze mutant frequencies in both RETs and total RBCs from peripheral blood samples	RET mutant frequencies respond sooner to dosing; however, many more total RBCs than RETs can be analyzed, potentially increasing the sensitivity of the assay
Analyze sufficient cells to detect at least one mutant cell per sample	In practice this means analyzing a minimum of 1-5 x 10^6 RETs or total RBCs per sample and usually requires the use of immunomagnetic separation techniques; this recommendation applies equally to assays conducted with rats and mice
To make maximum use of animal resources, *Pig-a* assays should be integrated into existing general toxicology or genetic toxicology tests whenever possible	

Nonstandard abbreviations and terms: OECD = Organization for Economic Cooperation and Development; TGs = Test Guidelines; MTD = maximum tolerated dose; RETs = reticulocytes; RBCs = total red blood cells

69. The recommendations in Table II for performing the in-life portion of the *in vivo Pig-a* assay in rodents follow the protocol recommendations in TG 488 for the TGR gene mutation assay (OECD, 2013), while the top dose selection follows recommendations from TG 407 (OECD, 2008). Dosing for approximately 28 consecutive days is strongly recommended both to take advantage of the accumulation of the *Pig-a* mutational response with the number of doses and to better integrate the assay with 28-day repeat-dose toxicity studies (OECD, 2008). However, many *Pig-a* assays have been performed with single dose protocols or short-term repeat-dose protocols, generally dosing on three

consecutive days (see Tables III and IV, Annexes I and II). Shorter dosing protocols can be economically attractive, or necessary when only limited amounts of the test substance are available, and acute or short-term dosing protocols may be justified for nonregulatory studies. For example, assuming all the recommendations in Table II and Section 4c are followed, short-term dosing may be justified if adequate dose levels can be achieved or if it is desirable to integrate the *Pig-a* assay with short-term genotoxicity (*e.g.*, micronucleus or comet), toxicity, or other *in vivo* tests (*e.g.*, single-dose pharmacokinetics study). It should be noted that the requirement for mutant manifestation for detecting mutants in the *Pig-a* assay generally will require holding animals for a longer period of time than required to perform short-term comet or micronucleus assays. If measuring as many genotoxicity endpoints as possible and making maximum use of dosed animals are goals of the study, it is possible to integrate MN and comet assay measurements into longer-term *Pig-a* assays, as has been done in many published studies (*e.g.*, Stankowski et al., 2011, 2015; Roberts et al., 2016).

70. *Pig-a* mutant frequencies have been analyzed using limiting-dilution cloning of spleen lymphocytes and proaerolysin as a selection agent (*e.g.*, Miura et al., 2008b; see Section 10c), but for the reasons stated in Section 1, *Pig-a* assays are almost always conducted by flow cytometric evaluation of erythrocytes from peripheral blood. Prior to flow cytometry, blood processing involves methods to reduce interference by other cells in the blood, either by enriching the sample for erythrocytes using density-gradient centrifugation (*e.g.*, Phonethepswath et al., 2010), or specifically labelling erythroid cells using fluorescent HIS49 antibody for rats (Dobrovolsky et al., 2010a; Kimoto et al., 2011a) or TER-119 for mice (Kimoto et al., 2011b). RETs are distinguished from mature RBCs, either by staining with a fluorescent nucleic acid dye that labels the residual RNA in RETs (*e.g.*, SYTO 13), or by using a fluorescently labeled antibody against the CD71 transferrin receptor, which is expressed in RETs but not in mature RBCs. Finally, cells that are wild-type for GPI anchor expression, and presumably descendant from cells having a wild-type *Pig-a* gene, are identified by labelling the blood sample with fluorescently labelled antibodies to a highly expressed GPI-anchored protein molecule, usually CD59 for rats or CD24 for mice. Samples are analyzed with a flow cytometer for light scatter as well as for the fluorescent signals produced by the various fluorochromes used for labelling. Mutants are typically distinguished from wild-type cells based on the fluorescence produced by the antibody specific for GPI-anchored protein (low fluorescence, mutant; high fluorescence, wild-type). Gates are usually set for mutant and wild-type cells based on the position of a mutant-mimic sample (erythrocytes not reacted with the GPI-anchored protein antibody; *e.g.*,

Phonethepswath et al., 2010), and the number of events in the mutant and wild-type gates counted to estimate a mutant frequency. Interlaboratory trials have demonstrated the transferability, reproducibility and relative sensitivity of the basic flow cytometric *Pig-a* assay (Dertinger and Heflich, 2011; Kimoto et al., 2013) (a description of interlaboratory trials is presented in Part 2, Section 7).

71. Once the labelling methodologies were sufficiently optimized, it became clear that spontaneous *in vivo* erythrocyte *Pig-a* mutant frequencies were relatively low, often 1 x 10^{-6} or less for both total RBCs and RETs. To avoid samples having mutant frequencies of 'zero', and thus compromising the power of the test to detect positives, the IWGT established a minimum number of cells that should be detected (1 x 10^{6} for both total RBCs and RETs; Table II). Directly detecting an adequate number of cells for mutation using a flow cytometer, especially in the case of RETs which are 20-100-fold less abundant in peripheral blood than mature RBCs, makes analysis times unacceptably long (*e.g.*, ≥15 min/sample). In order to overcome this problem, immunomagnetic enrichment procedures have been developed (Dertinger et al., 2011a; Kimoto et al., 2011a) as a practical solution to increasing the number of total RBCs and/or RETs interrogated for mutation in the assay.

72. The two major immunomagnetic enrichment protocols used for the *Pig-a* assay take different approaches to increase the number of mutants that are analyzed by the assay: one by reducing the number of wild-type erythrocytes in the sample, the other by increasing the number of RETs analyzed. The process devised by Dertinger and colleagues, commonly performed in North America and Europe and commercially known as the *In Vivo* MutaFlow® or HT (high throughput) method (hereafter referred to as the MutaFlow method), involves collecting approximately 100 µl samples of peripheral blood (typically from the tail vein), enrichment of the blood for erythrocytes, followed by labelling with phycoerythrin (PE) conjugated anti-CD59 (rats) or PE-conjugated anti-CD24 (mice) (Figure 6). A PE-conjugated antibody against platelets (CD61) also is incorporated into these assays to prevent platelets from being misread as mutant cells. The samples are then treated with immunomagnetic beads that bind to the PE fluorochrome and passed through a column in the presence of a strong magnet, so that the eluted cells are highly enriched for non-PE-labeled mutants (Figure 6). The cells are then stained with SYTO 13 to distinguish between RETs and mature RBCs and counting beads are added to estimate the total number of 'cell equivalents' analyzed. These enriched samples allow flow cytometric analysis of approximately 3 x 10^{6} RETs and >100 x 10^{6} total RBCs per sample in a flow cytometric run of approximately 5 min. The MutaFlow method has been extensively evaluated as described by Gollapudi et al. (2015) and Raschke et al. (2016).

73. The method developed by Kimoto and colleagues, and commonly used in Japan, is known as the PIGRET assay. In the PIGRET assay, 100-500 µl peripheral blood samples first are labeled with PE-conjugated anti-CD71, reacted with magnetic beads recognizing PE, and using a powerful magnet, a sample enriched for RETs is collected (Figure 7). This sample is then reacted with fluorescent antibodies for erythroid cells (HIS49 for rats, TER-119 for mice) and a highly expressed GPI-anchored protein (CD59 for rats, CD24 for mice), and processed by flow cytometry to evaluate the frequency of *Pig-a* mutants in RETs (CD71-positive, erythroid-marker-positive, GPI-anchored-protein-negative). In addition, fluorescent antibodies for erythroid and GPI-anchored protein markers are reacted with 10 µl blood samples without prior immunomagnetic separation and processed by flow cytometry to estimate a *Pig-a* mutant frequency in total RBCs (Chikura et al., 2019). As conducted in the most recent Japanese Environmental Mutagen Society/Mammalian Mutagenicity Study group (JEMS/MMS) interlaboratory trial (Kimoto et al., 2016; see Part 2, Section 7a), this approach analyzes at least 1 x 10^6 RETs and 1 x 10^6 total RBCs for estimating mutant frequency.

Figure 6. Scheme for the MutaFlow (also known as High Throughput, HT) version of the *in vivo Pig-a* assay.

See Section 4, paragraph 72 for description. Used with permission of Litron Laboratories.

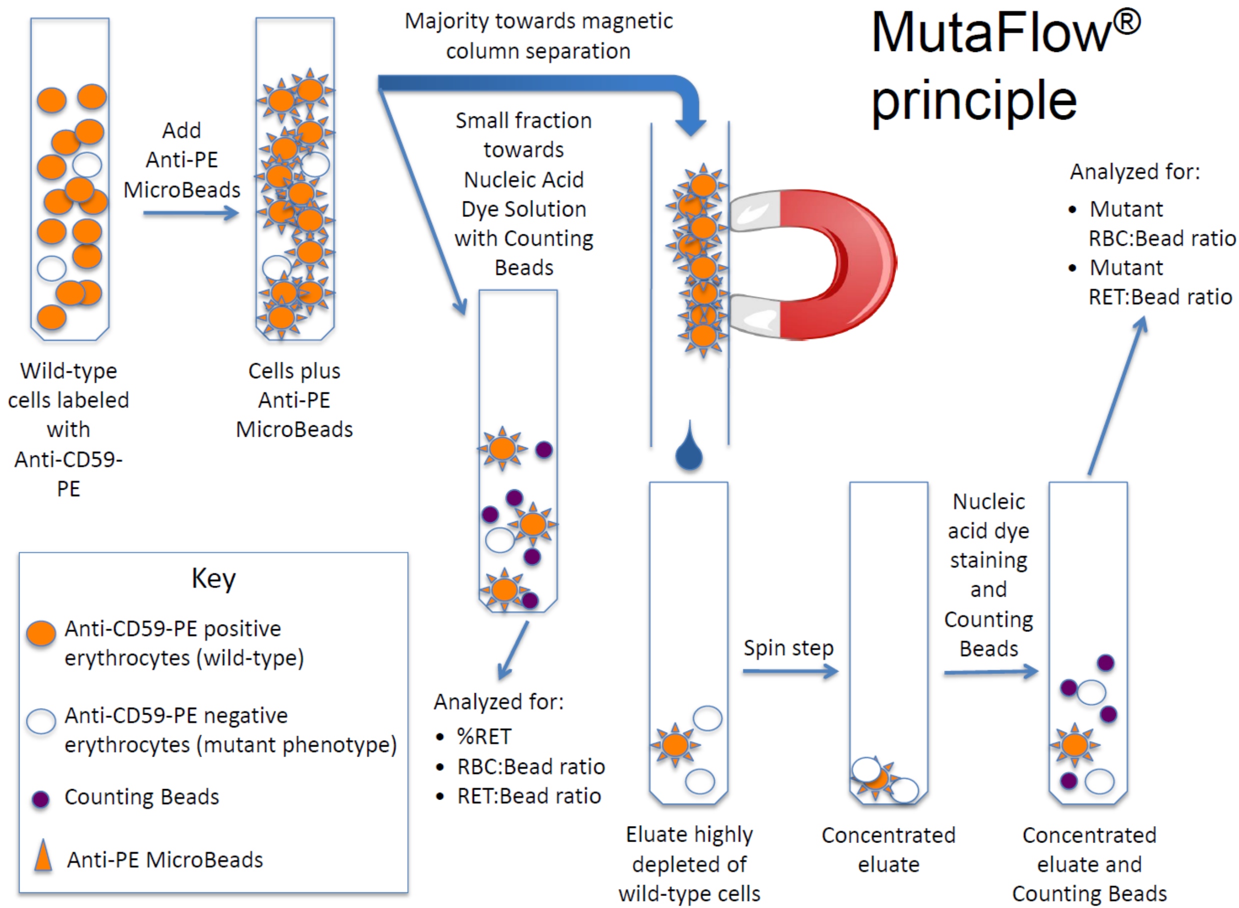
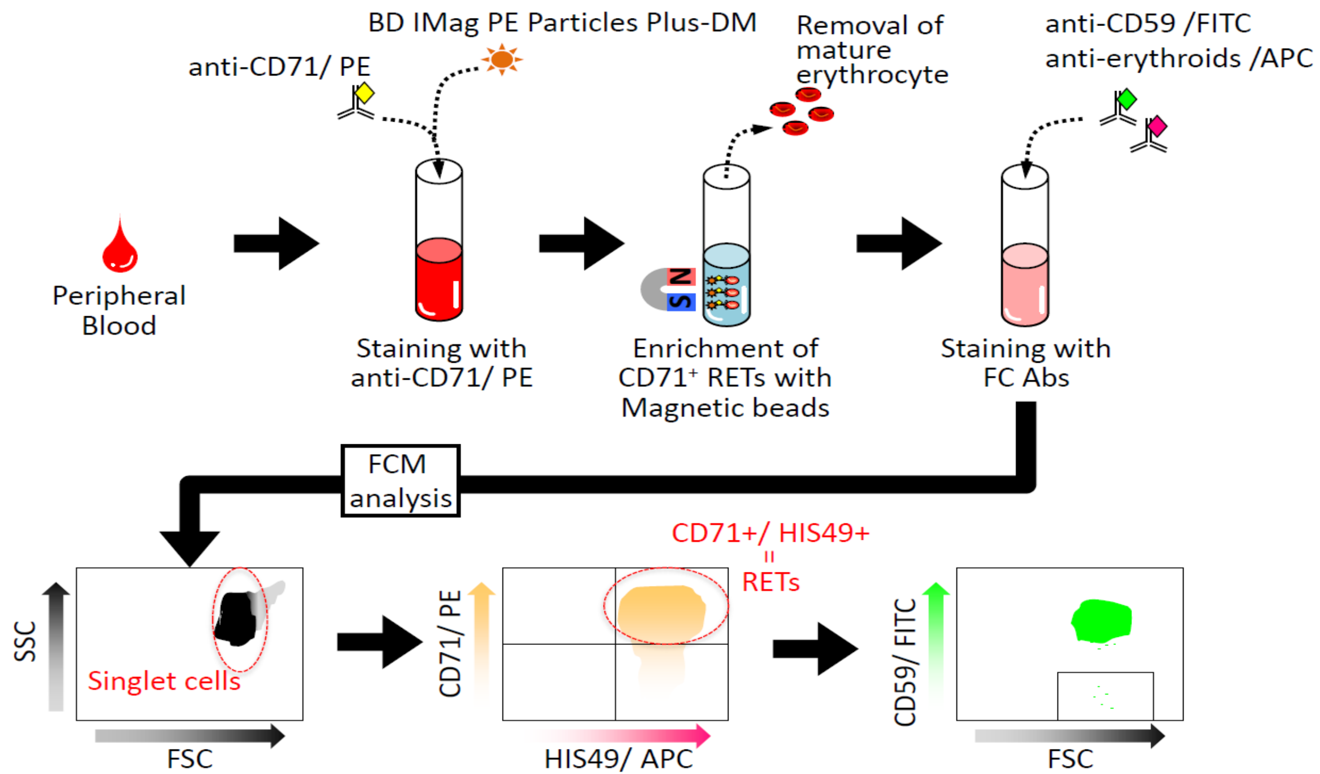


Figure 7: Scheme for conducting the PIGRET assay.

CD71-positive RETs are concentrated from whole blood using “Magnetic beads”, and the concentrated RETs are stained with antibodies for conducting the *Pig-a* assay. See Section 4 paragraph 73 for description. Figure prepared by K. Horibata.



74. To summarize the differences between the two approaches: 1) the cell numbers analyzed for mutants by the PIGRET approach are less than with the Mutaflow method, but within the IWGT guidelines; 2) in contrast to the MutaFlow method, the PIGRET analysis is conducted directly on RETs and total RBCs, without the need for counting beads to estimate the total number of cells analyzed; and 3), the PIGRET protocol requires two labelling reactions and two flow cytometry analyses--one to score mutant RETs and one to score total RBCs, while the MutaFlow method utilizes one labelling reaction and two flow cytometric analyses. For the MutaFlow method, one of the flow analyses estimates the number of total RETs and total RBCs based on pre-immunomagnetic samples, and the second counts the number of mutant RETs and mutant RBCs in the post-immunomagnetic separation samples.

a Factors affecting *Pig-a* assay performance: dosing and sampling protocol and number of cells interrogated

75. To a certain extent, the IWGT protocol recommendations were based on theory, with a reliance on limited data from a small number of prototypical chemical substances. Table III shows results with ten test substances, where there is some evidence that employing a longer repeat-dose protocol and/or employing an enrichment technique that increases the number of cells analyzed for mutation affected the response in the assay. For 2-AAF and azathioprine, assays conducted using a single dose were negative, while assays conducted with a 3- or 28-day dosing protocol were positive (Table IV). With aflatoxin B1, assays conducted using 3-day and 15-day dosing protocols were negative, while aflatoxin B1 was positive using a 29-day repeat-dose protocol (Table IV). As for the number of erythrocytes interrogated for mutation, diethylnitrosamine and cisplatin were negative or inconsistent using low-throughput analysis techniques that did not use immunomagnetic enrichment, but positive when using an immunomagnetic enrichment protocol. While protocols not using immunomagnetic enrichment were occasionally negative for cyclophosphamide, MMS, and urethane mutagenicity, protocols using immunomagnetic enrichment were consistently positive.

76. Thus, the IWGT workgroup recommendations for using repeat-dose, approximately 28 consecutive-day dosing protocols, which generally result in the animal being dosed with a greater amount of test substance than can be delivered with a 1- or 3-day dosing protocol, and the analysis of the greatest number of erythrocytes practical, will increase assay performance. With current technologies, the latter means employing an immunomagnetic enrichment technique that facilitates examining a large number of erythrocytes. As

relatively robust, genotoxic carcinogens, like 2-AAF and aflatoxin B1, can be missed by using low throughput and/or short-term dosing protocols, establishing a negative response in the assay with confidence will require following these more stringent methods, as well as testing to the MTD or limit dose (Table II). One possible exception to these generalizations is described for a study on 4-nitroquinoline-1-oxide (4NQO) that was reported in two papers. 4NQO produced quantitatively higher responses when given in a short-term protocol than an equal total dose spread over 28 daily dosings (Stankowski et al., 2011; Roberts et al., 2016). Both short-term and longer-term dosing protocols were positive, but the magnitude of the response may have been greater with the short-term protocol.

77. While the IWGT recommended a single sampling time at Day 28-31 for both short-term and 28-day dosing protocols, it encouraged the use of additional, optional sampling at earlier and later times as an aid to data interpretation. Given the persistence of induced mutant frequencies in the assay (*e.g.*, Miura et al., 2009; Phonethepswath et al., 2010), the exact timing of Day 28-31 sampling can be adjusted to facilitate integration with other assays. Also, an additional dosing with the test substance can be added on, *e.g.*, Day 29, three hours before sampling, to facilitate integrating a comet endpoint into the study. The desirability of a later time point is based on the expectation that positive responses in total RBCs may be most fully manifested at a later time point, *e.g.*, Day 56, especially with longer repeat-dose protocols. This is because *Pig-a* mutant frequencies accumulate with multiple doses and are generally persistent (*e.g.*, Miura et al., 2009; Dertinger et al., 2010; Phonethepswath et al., 2010; Bhalli et al., 2011a). The desirability of an optional earlier time point is based on the expectation that positive total RBC responses will be preceded, or at least accompanied by, increases in RET mutant frequencies. In addition to conforming to erythropoiesis theory (Dobrovolsky et al., 2010b), this expectation has been confirmed in numerous assays, most recently in the JEMS/MMS interlaboratory trial comparing responses in the PIGRET and total RBC *Pig-a* assay (Kimoto et al., 2016). As an example of using these data for interpreting responses, Dobrovolsky et al. (2016) employed a weight-of-evidence approach that discounted positive RBC responses in occasional rats treated with acrylamide, in part because these responses were not preceded or accompanied by similar increases in RET *Pig-a* mutant frequencies. A positive response only in total RBCs, and not in RETs, would not be expected at any sampling time, and if it did occur, it should be scrutinized carefully for biological significance.

78. In addition to establishing the plausibility of a positive response, collecting total RBC data, in addition to RET data, can be useful in detecting small increases

in mutant frequency (*e.g.*, Khanal et al., 2018). This may be because the MutaFlow analysis method, in particular, interrogates 30-fold or more total RBCs for mutation than it does RETs, potentially increasing the precision with which RBC mutant frequencies are measured.

Table III: Tests where rat *Pig-a* assays conducted with different DOSING PROTOCOLS, total dose, and/or analysis method had different outcomes

First day of dosing designated Day 1. More information on these assays can be found in Annex I. Note that *Pig-a* response calls in this table generally are those of investigator and do not take into account the data evaluation criteria used for the consensus calls in Table VI.

Test Substance	Short-term (Single, 3-day, other up to 13-day) dosing protocol			Longer-term dosing (≥14 consecutive days)			Notes	References
	Days of dosing: daily doses	Response		Days of dosing: daily doses	Response			
		RETs: min dose P, max dose N ; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		RETs: min dose P, max dose N; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		
2-Acetylaminofluorene	1: 0, 125, 250, 500 mg/kg	N at 500 mg/kg on Day 29 (PIGRET)	N at 500 mg/kg on Day 29 (basic)				%RETs increased with dose	Shigano et al. (2016)
	3: 0, 125, 250, 500 mg/kg/day	P at 250 mg/kg/day on Day 15 (HT)	P at 125 mg/kg/day on Day 15 (HT)	28: 0, 37.5, 75, 150 mg/kg/day	P at 37.5 mg/kg/day on Day 15 (HT)	P at 75 mg/kg/day on Day 15 (HT)	%RETs decreased with dose	Dertinger et al. (2012)
Aflatoxin B1	3: 0, 0.25, 0.5, 1 mg/kg/day	N at 0.5 mg/kg/day on Days 16, 30, 45, 60 (HT)	N at 0.5 mg/kg/day on Days 16, 30, 45, 60 (HT)	15: 0, 0.06, 0.125, 0.25, 0.5 mg/kg/day; 29: 0, 0.125, 0.25, 0.5 mg/kg/day	15: N at 0.5 mg/kg/day on Days 16, 29 (HT); 29: P at 0.5 mg/kg/day on Day 16 (HT)	15: N at 0.5 mg/kg/day on Day 29 (HT); 29: P at 0.5 mg/kg/day on Day 57 (HT)	Some marginal (but judged negative) responses following 15-day treatment	Janssen, unpublished
Azathioprine	3: 0, 12.5, 0.25, 50 mg/kg/day	P at 12.5 mg/kg/day on Day 15 (HT)	P at 50 mg/kg/day on Day 29 (HT)	28: 0, 6.25, 12.5, 25 mg/kg/day	P at 12.5 mg/kg/day on Day 15 (HT)	P at 12.5 mg/kg/day at Day 29 (HT)	Dose-dependent reduction in %RETs	Dertinger et al. (2012)

Test Substance	Short-term (Single, 3-day, other up to 13-day) dosing protocol			Longer-term dosing (≥14 consecutive days)			Notes	References
	Days of dosing: daily doses	Response		Days of dosing: daily doses	Response			
		RETs: min dose P, max dose N ; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		RETs: min dose P, max dose N; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		
	1: 50, 100, 200 mg/kg	N at 200 mg/kg on Day 28 (PIGRET)	N at 200 mg/kg on Day 28 (basic)					Yoshida et al. (2016a)
Cisplatin	1: 0, 0.5, 1, 2 mg/kg	P at 2 mg/kg on Day 7 (PIGRET)	P at 2 mg/kg on Day 28 (basic)					Suzuki et al. (2016c)
				28: 0, 0.05, 0.1, 0.2, 0.4 mg/kg/day	P at 0.4 mg/kg/day on Day 15 (HT)	P at 0.1 mg/kg/day on Day 29 (HT)	MN P; mutants persist to 6 months	Dertinger et al. (2014a)
	3: 0, 0.5, 1, 2 mg/kg/day	P at 1 mg/kg/day on Day 14 (HT)	P at 0.5 mg/kg/day on Day 14 (HT)				PIGRET gave similar data to HT RETs; MN and *Hprt* lymphocyte P	Bhalli et al. (2013a)
	1: 0, 5 mg/kg i.p.	Not done	N at Day 28 (basic)					Pu et al. (2016)
	5: 0, 2 mg/kg/day	Not done	P at Day 28 (basic)					Pu et al. (2016)
Cyclophosphamide	1: 0, 20 mg/kg		N at 20 mg/kg on Day 70 (basic)				One P response at Day 38	Kimoto et al. (2012)

Test Substance	Short-term (Single, 3-day, other up to 13-day) dosing protocol			Longer-term dosing (≥14 consecutive days)			Notes	References
	Days of dosing: daily doses	**Response**		**Days of dosing: daily doses**	**Response**			
		RETs: min dose P, max dose N ; sampling time; (method used)	**RBCs: min dose P, max dose N; sampling time; (method used)**		**RETs: min dose P, max dose N; sampling time; (method used)**	**RBCs: min dose P, max dose N; sampling time; (method used)**		
	10: 0, 5 mg/kg/day		N at 5 mg/kg/day on Day 70 (basic)					
	3: 0, 7.5, 15, 30 mg/kg/day	P at 7.5 mg/kg/day on Day 15 (HT)	P at 15 mg/kg/day on Day 15 (HT)	28: 0, 2.5, 5 mg/kg/day	P at 5 mg/kg/day on Day 29 (HT)	P at 5 mg/kg/day on Day 29 (HT)	%RETs reduced, followed by rebound	Dertinger et al. (2012)
	1: 0, 10, 40, 80 mg/kg	'data unreliable' (basic)	P at 80 mg/kg on Day 42 (basic)				%RETs reduced, followed by rebound; MN and *Hprt* lymphocyte P	Bhalli et al. (2013a)
	1: 0, 20, 50 mg/kg	P at 20 mg/kg on Day 8 (PIGRET)	P at 50 mg/kg on Day 15 (basic)	28: 0, 2.5, 5, 10 mg/kg/day	P at 10 mg/kg/day on Day 29 (with removal of outlier control) (PIGRET)	N at 10 mg/kg/day on Day 29 (basic)	P for 28-day dosing requires removing an outlier control	Kimoto et al. (2014)
Diethylnitrosamine				28: 0, 5, 10 mg/kg/day	N at 10 mg/kg/day on Day 29 (basic)	N at 10 mg/kg/day on Day 29 (basic)	MN N; liver Comet P	Shi et al. (2011)
				28: 0, 3.1, 6.25, 12.5	P at 12.5 mg/kg/day on	P at 12.5 mg/kg/day on	MN N	Avlasevich et al. (2014)

Test Substance	**Short-term (Single, 3-day, other up to 13-day) dosing protocol**			**Longer-term dosing (≥14 consecutive days)**			**Notes**	**References**
	Days of dosing: daily doses	**Response**		**Days of dosing: daily doses**	**Response**			
		RETs: min dose P, max dose N ; sampling time; (method used)	**RBCs: min dose P, max dose N; sampling time; (method used)**		**RETs: min dose P, max dose N; sampling time; (method used)**	**RBCs: min dose P, max dose N; sampling time; (method used)**		
				mg/kg/day	Day 29 (HT)	Day 29 (HT)		
				28: 0, 5, 10, 15 mg/kg/day	P at 15 mg/kg/day on Day 29 (increases in majority of rats, but mean increase not significant) (HT)	P at 10 mg/kg/day on Day 29 (HT)	Blood MN N, liver MN P	Khanal et al. (2018)
	1: 0, 37.5, 75, 100 mg/kg	N at 100 mg/kg at Day 28 (PIGRET)	N at 100 mg/kg at Day 28 (basic)					Wada et al. (2016)
Melphalan	1: 0, 1.25, 2.5, 5 mg/kg	P at 1.25 mg/kg on Day 8 (PIGRET)	P at 5 mg/kg on Day 29 (basic)					Adachi et al. (2016)
	3: 0, 0.75, 1.5, 3 mg/kg/day	P at 1.5 mg/kg/day on Day 15 (HT)	P at 1.5 mg/kg/day on Day 15 (HT)	28: 0. 0.75 mg/kg/day	P at 0.75 mg/kg/day on Day 30 (HT)	P at 0.75 mg/kg/day on Day 15 (HT)		Novartis, unpublished
	3: 0, 1.25, 2.5 mg/kg/day	E at 1.25 mg/kg/day on Day 15 (HT)	P at 1.25 mg/kg/day on Day 15 (HT)	28: 0, 0.1875, 0.375, 0.75 mg/kg/day	P at 0.1875 mg/kg/day on Day 15 (HT)	P at 0.375 mg/kg/day on Day 15 (HT)	PB MN N	Dertinger et al. (2012)

Test Substance	Short-term (Single, 3-day, other up to 13-day) dosing protocol			Longer-term dosing (≥14 consecutive days)			Notes	References
	Days of dosing: daily doses	Response		Days of dosing: daily doses	Response			
		RETs: min dose P, max dose N ; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		RETs: min dose P, max dose N; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		
				28: 0, 0.032, 0.09, 0.28, 0.75 mg/kg/day	P at 0.0938 mg/kg/day on Day 15 (HT)	N at 0.75 mg/kg/day on Day 15 (HT)	PB MN P	Dertinger et al. (2014b)
4,4'-Methylenedianailine	1: 0, 40, 130, 240 mg/kg	N at 240 mg/kg on Day 28 (PIGRET)	N at 240 mg/kg on Day 28 (basic)	28: 0, 4.4, 13, 40, 120 mg/kg/day	P at 40 mg/kg/day on Day 14 (PIGRET)	P at 40 mg/kg/day on Day 14 (basic)	High dose N in 28-day study: possible hepatotoxicity	Sanada et al. (2014)
Methyl methanesulfonate	3: 0, 22.5, 45, 90 mg/kg/day	P at 45 mg/kg/day on Day 15 (HT)	P at 90 mg/kg/day on Day 15 (HT)	28: 0, 7.5, 15, 30 mg/kg/day	P at 30 mg/kg/day on Day 15 (HT)	P at 30 mg/kg/day on Day 29 (HT)	PB MN P	Dertinger et al. (2012)
	1: 0, 50, 100, 200 mg/kg	P at 200 mg/kg on Day 8 (PIGRET)	P at 200 mg/kg on Day 15 (basic)	28: 0, 7.5, 15, 30 mg/kg/day	P at 30 mg/kg/day on Day 8 (PIGRET)	N at 30 mg/kg/day on Day 29 (basic)	PB MN P	Muto et al. (2014)
				28: 0, 1.25, 2.5, 5, 10, 15, 30 mg/kg/day	P at 30 mg/kg/day on Day 28 (HT)	P at 30 mg/kg/day on Day 28 (HT)	P liver and blood Comet; P BM MN-PCE and PB MN RET; P liver gamma H2X	Zeller et al. (2016)
Urethane (ethyl carbamate)				28: 0, 250 mg/kg/day	P at 250 mg/kg/day on	P at 250 mg/kg/day on	PB MN P	Bemis et al. (2015)

Test Substance	Short-term (Single, 3-day, other up to 13-day) dosing protocol			Longer-term dosing (≥14 consecutive days)			Notes	References
	Days of dosing: daily doses	Response		Days of dosing: daily doses	Response			
		RETs: min dose P, max dose N ; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		RETs: min dose P, max dose N; sampling time; (method used)	RBCs: min dose P, max dose N; sampling time; (method used)		
					Day 15 (HT)	Day 29 (HT)		
				29: 0, 25, 100, 250 mg/kg/day	P at 100 mg/kg/day on Day 15 (HT)	P at 250 mg/kg/day on Day 15 (HT)	PB MN in RETs and BM MN in PCEs, Comet in PB and liver all P; N Comet in brain, kidney, spleen, lung and CA in PB	Stankowski et al. (2015)
	3: 0, 600 mg/kg/day	P at 600 mg/kg/day on Day 15 (HT)	P at 600 mg/kg/day on Day 15/ (HT)				Measured in males and females with similar results	Labash et al. (2015c)
	1: 0, 250, 500, 1000 mg/kg	P at 1000 mg/kg on Day 14 (PIGRET)	N at 1000 mg/kg on Day 28 (basic)					Narumi et al. (2016)

Nonstandard abbreviations and terms: N = negative; P = positive; E = equivocal; I = inconclusive; Min = minimum; max = maximum; RETs = reticulocytes; RBCs = total red blood cells; HT = high-throughput immunomagnetic enrichment protocol devised by Litron Laboratories, typically analyzing 1-3 x 10^6 RET and >100 x 10^6 total RBC equivalents; PIGRET = immunomagnetic enrichment protocol devised by Kimoto et al. (2011a), typically analyzing 1 x 10^6 RETs; HIS49 = antigen used to identify erythroid cells in PIGRET assay; basic = assay conducted without immunomagnetic enrichment, typically assaying 1 x 10^6 total RBCs and 3 x 10^5 RETs; MN = micronucleus; PB = peripheral blood; BM = bone marrow; CA = chromosome aberration; PCE = polychromatic erythrocyte.

Table IV: Test substances where length of DOSING may affect rat *Pig-a* responses.

Only responses shown conforming with Workgroup or extended data acceptance criteria (see Table VI; Section 7b).

Test agent	Single dose	2-13 daily doses (No. doses)	≥14 daily doses (No. doses)
2-Acetylaminofluorene	N	P (3)	P (28)
Aflatoxin B1		N (3)	N (15), P (29)
Azathioprine	N	P (3)	P (28)
Diethylnitrosamine	N		P (28), P (28)
4,4’-Methylenedianaline	N		E (28)

Nonstandard abbreviations and terms: N = negative; P = positive; E = equivocal

b Option of preserving peripheral blood samples for later analysis

79. A significant logistical challenge associated with erythrocyte-based *Pig-a* assays has been the inability to store samples for more than several days before processing and flow cytometric analysis. Recently, however, effective whole blood freezing and thawing reagents have been successfully tested at several facilities (Avlasevich et al., 2019). The method involves mixing peripheral blood samples with anticoagulant, maintaining this mixture at room temperature for approximately 10 min, followed by addition of a cryopreservative and then slowly reducing the temperature of the blood mixture to approximately -80° C. Blood processed in this manner can be maintained at -80° C for at least one-five years. Thawing is accomplished quickly by immersion in a 37° C water bath. This technical advance addresses several logistical considerations, such as 1) holding blood samples until a flow cytometer and trained technical staff are available; 2) accumulating samples so that longitudinal data can be collected on the same day using identical labelling reagents and instrument settings; and 3) using frozen samples from a genotoxicant-treated animal as a positive control rather than treating positive control animals for each study.

80. Aside from these considerations, perhaps the most consequential implication of effective freeze/thaw protocols is deferred decision-making. For example, if a decision is made to forgo *Pig-a* analysis in the conduct of a 28-day toxicology or other repeat-dose study, it may be advantageous to freeze and store whole blood samples at appropriate time point(s), for instance at the termination of the dosing phase. Whereas mutation analysis may not have been indicated based on data collected up to the time of study initiation, there may be occasions when data are subsequently generated that suggest the merit of assessing *in vivo* gene mutation potential. For example, observations such as hyperplasia, preneoplastic lesions, or even frank tumours could raise new concerns about possible carcinogenicity due to genotoxic mechanism(s). In these cases, rather than setting up a new study and dosing additional animals, it would be highly advantageous to thaw archived blood samples to investigate *in vivo* genotoxicity. In this 3Rs-friendly scheme, no additional animals or other additional costly resources would be necessary.

c Updated recommendations for conducting the *Pig-a* gene mutation assay

81. Based on recent experience, and the analysis in Section 4a, the following relatively minor additions, clarifications, and alterations can be made to the IWGT protocol recommendations of Gollapudi et al. (2015):

- Assays may be conducted in mice or rats: male and female rats and male mice may be used (limited data for female mice prevent their recommendation). For all dosing schedules, it may be advantageous (but not required) to perform *Pig-a* analyses prior to the first dose (*e.g.*, one to five days before the first dose) in order to identify animals having mutant frequencies outside of historical control limits so that they can be removed from the main study (see predosing mutant frequency data in Figure 5).

- When dosing occurs daily for several weeks, *e.g.*, using a 28-day repeat-dose protocol as recommended for regulatory hazard identification studies and as illustrated in Figure 8,

    - Collect blood for assay within day(s) of cessation of dosing (*e.g.*, Day 28-31 of 28-day repeat-dose protocol). Data collected from testing diverse genotoxicants suggest this is sufficient time for adequate manifestation of mutant RET and mutant RBC responses. This dosing and adjustable sampling protocol also has the advantage of facilitating integration of the *Pig-a* assay with general toxicology and other genetic toxicology assays. The addition of a dose on Day 29, followed by sample collection three hours later is permissible for facilitating tissue harvest for conducting the *in vivo* comet assay.

    - <u>Optionally</u>, and when logistically feasible, there may be merit to conducting *Pig-a* analyses on blood samples collected from a 28-day repeat dose protocol at an additional, later time point. For instance, some experiments include satellite “recovery groups” to evaluate whether toxic effects are diminished or increased upon discontinuation of dosing. Such blood samples, typically collected between two to four weeks after cessation of dosing, represent an opportunity to evaluate mutant RBC frequencies when a mutagen-induced effect is more fully manifested in the total RBC population.

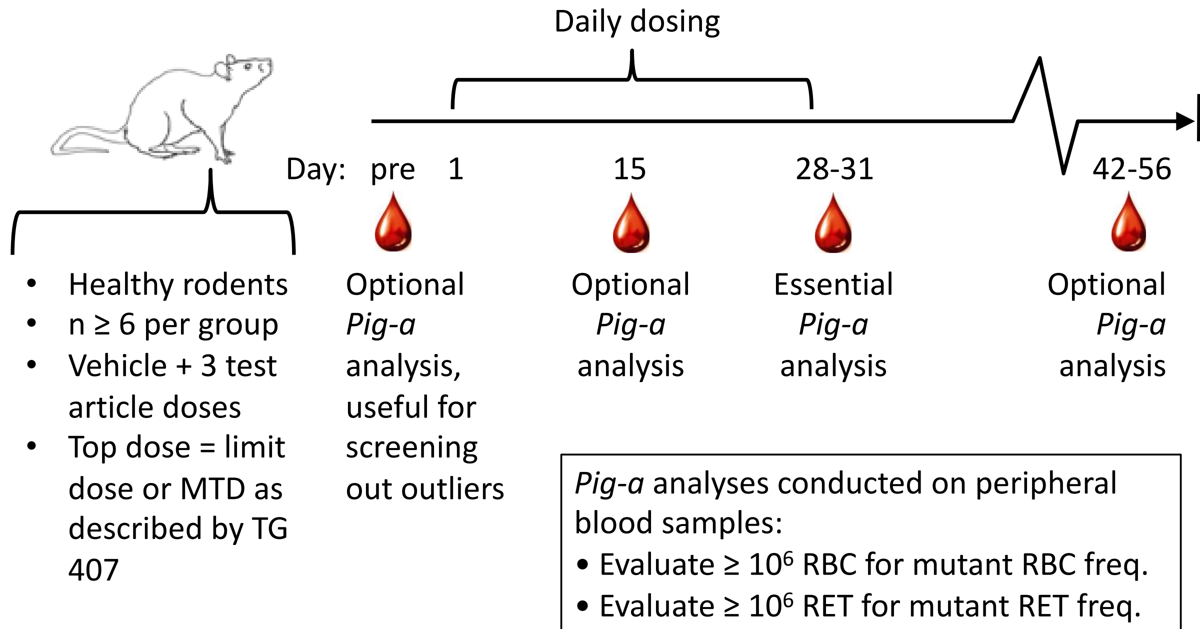
    - <u>Optionally</u>, and when logistically feasible, there may be merit to conducting an earlier blood sampling, for instance at Day 14 or 15 during a 28-day repeat-dose protocol. RET data from such samples, in particular, may be useful in supporting responses detected at the 28-31-day sampling point.

- Optionally, and when dosing occurs for more than 28 days (*e.g.*, 90 days), there may be merit in conducting assays at approximately 28 days (*e.g.*, at Day 28-31) in addition to at the cessation of dosing in order to mitigate the possible effect of clonal expansion on mutant frequency at extended sampling times (*e.g.*, Mittelstaedt et al., 2019).

- When test substance dosing occurs for one-three consecutive days, as with nonregulatory studies
  - Sampling at two times is recommended:
    - Collect blood and perform assays approximately one-two weeks after cessation of dosing. Data collected from testing on diverse genotoxicants suggest that this is sufficient time for adequate manifestation of mutant RET responses, and in the case of a very few potent mutagens, for mutant RBC responses.
    - It is important to include a second blood collection time, one at approximately Day 30. This provides time for the peripheral blood pool to more completely turn over, which enhances the sensitivity of the mutant RBC endpoint. Based on the data accumulated to date and differences between mutant RET and mutant RBC manifestation times, scientific justification would be required to support a negative result from a short-term-dosing study based on only one post-dosing blood collection time-point.

- Other dosing and sampling schedules may be scientifically justified for nonregulatory studies (*e.g.*, dosing on five consecutive days or once a week for four weeks); but as above, they should take into consideration the different manifestation times of mutant RET and mutant RBC responses.

Figure 8: Recommended scheme for performing the *in vivo Pig-a* gene mutation assay using a 28 consecutive day dosing protocol.



A preferred experimental design is described whereby laboratory animals (often rats) are exposed to 28 consecutive daily doses of the test substance. *Pig-a* mutant frequencies are determined by utilizing one of the immunomagnetic separation strategies followed by flow cytometric analysis. MTD = Maximum tolerated dose. Figure prepared by S.D. Dertinger.

5 Recommendations for reporting data, statistical analysis, and interpretation of results

82. For a valid test, data analysis is conducted only when the study fulfills all acceptability criteria in terms of animal number, dosing, sampling, mutant analysis, etc. described in Table II and Section 4. The recommendations for data analysis given in this section are based on those made in the IWGT *Pig-a* report (Gollapudi et al., 2015). In addition, they are supported by *Pig-a* data currently available and are aligned with the OECD Mammalian Erythrocyte Micronucleus Test Guideline (TG 474; OECD, 2016a). Although not all *Pig-a* data in the literature have been reported and analyzed in this way, the following recommendations are provided based on a current understanding of how *Pig-a* gene mutation data are generated and used.

83. Individual animal data should be reported or at least made available upon request. These data should include the number of cells evaluated, the number of mutant cells observed, and the frequency of mutant cells (typically reported as number per million). Furthermore, group means for like-dosed animals should be reported, and covariates such as blood sampling time, animal sex, age, etc. should be clearly indicated. A measure of statistical significance (*e.g.*, p-values) and within group variation (*e.g.*, standard deviation, standard error of the mean, and/or confidence intervals) should be reported. These can be helpful for interpreting the biological relevance of a statistical finding.

84. Data from *Pig-a* experiments are generally evaluated with the intention of determining whether a test substance affects the observed proportions of mutant RETs among all RETs and mutant RBCs among all RBCs. Such analyses should be accomplished by appropriate statistical methods, using the animal as the experimental unit. The statistical method(s) employed should be indicated. Providing that all the study acceptability criteria are fulfilled (Table II and Section 4; see also Section 7b), a test substance is considered clearly positive if it meets the following three criteria:

A) At least one of the experimental dosing groups exhibits a statistically significant increase in the frequency of *Pig-a* mutant phenotype cells compared with the concurrent negative control;

B) This increase is dose-related at least at one sampling time when evaluated with an appropriate trend test; and

C) At least one experimental dosing group mutant frequency exceeds the distribution of the historical negative control data.

A *Pig-a* experiment that does not meet any of the criteria (A, B or C) is a clearly negative test result. Negative results indicate that, under the test conditions, the test substance does not cause gene mutation in hematopoietic cells of the test species. In rare cases, even after further investigations (see paragraph 94, below), the data may preclude making a conclusion that the test substance produces either a clear positive or negative result, and the experimental result is concluded to be equivocal.

85. For Criterion A, appropriate statistical methods should be used to compare mutant frequencies in experimental dosing groups with the concurrent vehicle control group. When measured soon after cessations of dosing in a 28-day study, the expectation is that both RET and total RBC mutant frequencies will be elevated; only RET frequencies may be elevated if an earlier, mid-dosing, sampling is conducted. It is important to ensure that, whether a parametric or non-parametric statistical method is used, the underlying assumptions for the method are reviewed. In particular, it is important to consider the residual distribution and variance-homogeneity.

86. Transformation of the mutant frequencies may be used to reduce non-normality and/or heterogeneous variances. One example is a logarithmic transformation, using $\log_{10}$ or the natural log (ln). The complication of mutant frequencies of zero being observed occasionally can be overcome in the case of log transformation by adding a small value (such as 1.0, 0.1 or 0.01) to each mutant cell frequency (expressed as mutants x 10^{-6}).

87. When the objective of the analysis is to declare a comparison significant, the tests should be performed with a predefined alpha (α) value, and should generally be carried out as one-sided tests, with the expectation of detecting a dose-related increase in mutant cells. If pair-wise comparisons of dosing group means are carried out using methods which correct for multiple comparisons, then the p-values and confidence intervals should be adjusted appropriately. The method of multiple comparison must be clearly stated to allow this to be

considered in any assessment of the results.

88. In the case of Criterion B, at least three experimental dose groups should be analyzed to test for a dose-related increase. In general, a trend test can be applied to such data. These often test for a linear trend and care must be taken in interpreting the test when the highest dose level of a mutagen/genotoxicant does not result in the highest mutant cell frequency (*e.g.*, 2AAF; Dertinger et al., 2012). Care should be taken in the interpretation of results when the dose-response is non-linear, particularly when there is a downturn in mutant frequency at a high dose or a non-monotonic response. Use of trend tests incorporating downturn protection (*e.g.*, as proposed by Bretz and Hothorn, 2003) may be useful in such circumstances.

89. Criterion C is used for assurance that a statistically significant increase in mutant cell frequency has biological relevance. The experimental results are compared with an appropriate historical negative control distribution (*e.g.,* 95% Tolerance Interval), assuming that the laboratory performing the test has a good historical database (see data in Figure 5 for an example). The emphasis is on the distribution of the historical negative control data rather than the range (as determined by maximum and minimum values observed in the historical control data). The historical control distribution of mutant cell frequencies allows a comparison of data from the current experiment with metrics derived from the historical negative control database such as tolerance intervals, control limits and related values. This approach helps understand the likelihood of the observation of high mutant cell frequency value(s) instead of employing simple and less informative within- or out-of-range comparisons.

90. In some studies, background or baseline measures may be available, where blood samples were taken before the test substance was applied. It may be possible to include this covariate data into the analysis of the experiment.

91. Experimental designs that include multiple sampling times may be used for conducting longitudinal analysis. As indicated in Section 4 (paragraph 77), there are expectations regarding the manifestation of mutant RET and mutant total RBC frequencies with time, and these may contribute to an expert-judgement/weight-of-evidence analysis in establishing the biological relevance as well as statistical significance of a response. These experimental designs may involve dose groups consisting of sub-groups of animals being examined at different time points or, alternatively, the animals in the dose groups providing blood samples on multiple days. Only a few studies analyzing longitudinal data have appeared thus far in the literature (*e.g.*, Dertinger et al., 2010; Dobrovolsky et al., 2016; Igl et al., 2018) so that consensus recommendations cannot be

made at this time. However, designs with both dose and time as factors need to be analyzed by considering whether the blood samples are from independent animals or are measures from the same animal on different days. As is the case with all *Pig-a* studies, these studies need careful design to ensure that pre-specified effect sizes considered biologically relevant can be detected, if they exist, with high probability (*i.e.*, the design has sufficient power). Analysis of longitudinal data was used in the evaluation of vehicle control mutant frequencies as explained in Section 7b.

92. *Pig-a* assays collect data on both mutant RET and mutant total RBC frequencies and these endpoints usually have been analyzed separately in the literature. Statistical approaches capable of considering them simultaneously exist and these approaches may be useful in certain instances.

93. A negative result is considered relevant only when evidence is provided that the bone marrow has been exposed to the test substance. With toxic compounds, direct evidence of bone marrow exposure is provided by a depression of the immature to mature erythrocyte ratio. In the absence of toxicity to the erythropoietic system, other evidence (measurement of the plasma or blood levels of the test substance and its metabolites) can be useful for documenting exposure (a discussion of this topic is provided in EFSA [2017]). Alternatively, ADME (absorption, distribution, metabolism, and excretion) data, obtained in an independent study using the same dosing route and same species can be used to demonstrate bone marrow exposure. The issue of what plasma/blood levels are sufficient to support a negative result, especially when the negative *in vivo* test was performed as a follow-up to an *in vitro* positive, is currently unresolved and solutions are being actively pursued by organisations such as the HESI-GTTC. It is possible, for instance, that in some cases dose-response data from *in vitro* assays (see Section 10d) might be useful in setting target blood concentrations. In the case of intravenous administration, evidence of exposure is not needed.

94. There is no requirement for verification of a clear positive or clear negative response. In experiments where the results are neither clearly negative nor positive (*i.e.*, a possible equivocal response), and to assist in establishing the biological relevance of the result (*e.g.*, a small or borderline increase), the data should be evaluated by expert judgment and/or further investigations of the existing experiments. In some cases, analyzing more cells or performing a repeat experiment using modified experimental conditions could be useful.

6 Confirming that *Pig-a* mutation is responsible for the *Pig-a* mutant phenotype

95. There are several lines of evidence indicating that the phenotype measured in the rodent *Pig-a* assay, loss of GPI-anchored proteins, is due to *Pig-a* mutation. While there are rare instances associated with human diseases and assays conducted with *in vitro* cell cultures where mutations in another GPI-biosynthesis pathway gene caused GPI-anchor deficiency, there are no data indicating that this occurs in the rodent *Pig-a* gene mutation assay. As indicated in Section 1c, the mutations that are responsible for the phenotype measured in the erythrocyte *Pig-a* assay are induced primarily in nucleated erythroid precursor cells in the bone marrow. Thus, the most direct way of demonstrating that *Pig-a* mutations are being detected by the *Pig-a* assay is to evaluate GPI-marker-deficient bone marrow erythroid cells for *Pig-a* mutations. Other lines of evidence that relate to the mutational basis for the *Pig-a* phenotype are presented in approximate order of relevance.

a Evidence that GPI-marker-deficient bone marrow erythroid cells and granulocytes in rats and mice contain mutations in the *Pig-a* gene

i.Mouse studies

96. Kimoto et al. (2011b) treated mice with a single dose of 100 mg/kg ENU and measured *Pig-a* mutant frequencies in bone marrow erythroid cells and total RBCs from peripheral blood over a period of four weeks. Consistent with mutations being induced in the nucleated bone marrow erythroid cells and these cells differentiating and transiting, with time, into cells found in peripheral blood, mutant frequencies increased more rapidly in the bone marrow cells than in the peripheral blood RBCs. Pools of mutant bone marrow erythroids were prepared from seven treated mice by flow cytometric sorting. *Pig-a* cDNAs were synthesized from RNA recovered from each pool; the cDNAs were amplified by PCR and cloned into plasmid vectors. 18 of 25 cloned cDNA pools produced recombinant clones that were successfully analyzed by Sanger sequencing. All

recombinants contained *Pig-a* mutations, with the same mutation detected multiple times in clones from four of the mice. The mutations were either basepair substitutions (70% of which were at A:T basepairs, with the mutated T on the nontranscribed DNA strand), or exon deletions that could have been produced by basepair substitutions. The findings demonstrated that all the *Pig-a* sequences that could be recovered from phenotypically mutant erythroid cells contained *Pig-a* mutations. In addition, the types of mutations that were detected were consistent with those expected to be induced by ENU in a transcribed mammalian gene.

ii.Rat studies

97. A series of studies have been conducted to evaluate mutation induction in the *Pig-a* gene of bone marrow erythroid cells and granulocytes of rats. Rats were treated with either vehicle or three doses of 40 mg/kg ENU, given every other day, and assayed for *Pig-a* mutation in nucleated bone marrow erythroid cells 10 or 12 days following the last dose (on Day 17 or 19; Revollo et al., 2018). Bone marrow was stained with Hoechst33342, to identify DNA-containing cells, allophycocyanin (APC) conjugated anti-CD71, to identify early erythroid cells, and PE anti-CD59, to identify cells with GPI-anchored protein markers. The average erythroid cell CD59-deficient mutant frequency in six vehicle control rats was 17 x 10^{-6}, while the mutant frequency increased to an average of 406 x 10^{-6} in six ENU-treated rats. Phenotypically mutant cells were sorted from each of the ENU-treated rats and a next generation sequencing (NGS) method called MAML (Mutation Analysis with Multiplexed Libraries; Revollo et al., 2017a) was used to identify *Pig-a* mutations in pools of either five cells or ten genome equivalents. A total of 116 different *Pig-a* mutations were detected in the mutant erythroid cells, with one mutation found in three different rats and 16 mutations found in two different rats; the remaining 99 mutations were found in only one rat. The spectrum of mutations was typical of that produced by ENU in a mammalian gene (*e.g.*, Mittelstaedt et al., 1995): 73% of mutations occurred at A:T basepairs, with the mutated T located on the nontranscribed DNA strand of the gene; T→A transversion was the most frequent mutation followed by T→C transition.

98. *Pig-a* mutant frequencies also were measured in bone marrow granulocytes. These studies were performed 1) to provide additional evidence that *Pig-a* mutation was responsible for the *Pig-a* mutant phenotype using a cell type closely related to erythroid cells; and 2) to gain insight into the timing of mutation induction in hematopoietic cells, *i.e.,* in multipotent or myeloid precursor cells before commitment to the erythroid and granulocyte lineages and/or in lineage-committed cells (see Figure 4). Rats were treated with ENU

and bone marrow harvested as described for the erythroid studies outlined above, with some rats being evaluated both for bone marrow erythroid and granulocyte mutation (Dad et al., 2018). Mutant granulocytes were quantified by flow cytometry using a combination of APC anti-CD11b, for identifying granulocytes, and PE anti-CD48, for identifying cells with GPI-anchored protein markers. CD48 is a GPI-linked marker that is highly expressed in granulocytes and is much more useful than CD59 for identifying GPI-deficient granulocytes. CD48-deficient mutant frequencies increased from an average of 8.4 x 10^{-6} in eight vehicle control rats to 567.1 x 10^{-6} in eight ENU-treated rats. Mutants were sequenced by MAML in pools of 25 cells; a total of 133 mutations were identified in cells from four of the ENU-treated rats (granulocytes from the other four treated rats were not sequenced). As was the case with the erythroid ENU-induced mutation spectrum, most of the mutations were at A:T basepairs, with the mutated T located on the nontranscribed DNA strand. The most common mutations were T→A transversion, followed by T→C transition.

99. Both granulocyte and erythroid mutants were analyzed for *Pig-a* mutations in bone marrow harvested from three of the eight ENU-treated rats, yielding a total of 61 granulocyte mutations and 69 erythroid cell mutations. Although the overall mutation spectra for granulocytes and erythroid cells were very similar, only three mutations were common to the two cell populations from a single rat.

100. *Pig-a* mutation induction in erythroids also was evaluated in rats treated with 7,12-dimethylbenzo[*a*]anthracene (DMBA), a compound that requires metabolic activation to produce DNA adducts and that forms bulky DNA adducts with A and, to a lesser extent, with G residues (Revollo et al., 2019). The design of the experiment was similar to that of the ENU study described in paragraphs 97-99, above. In this case, male F344 rats were treated by gavage with a total of 75 mg/kg DMBA and bone marrow was collected for mutation analysis 11 to 13 days later. The frequency of *Pig-a* mutant BMEs increased from 72 x 10^{-6} in control rats to 364 x 10^{-6} in dosed rats, and MAML analysis identified 8-11 independent *Pig-a* mutations in mutant erythroids from nine DMBA-dosed rats. The mutations were predominantly base pair substitutions at A:T, with the A on the non-transcribed stand of DNA; 13 % of all mutations were at G:C and 6 % of mutations were indels having either a one base pair addition or deletion.

iii.Summary of bone marrow Pig-a studies

101. The observations made in these studies indicate that *Pig-a* mutations can be found in GPI-anchored-marker-deficient erythroid cells isolated from rat and

mouse bone marrow, thus confirming that the erythrocyte *Pig-a* assay is measuring *Pig-a* mutation. The rat observations also shed light on the source of the mutations. Early after dosing, a variety of mutations can be detected in the erythroid cells and granulocytes of mutagen-treated rats, indicating that the mutant phenotype cells were caused by many independent mutations. In addition, 10-12 days following ENU dosing there was little overlap between the mutations detected in bone marrow erythroid cells and granulocytes from the same rat. This suggests that a small number of *Pig-a* mutations may have been induced in the bone marrow precursors that produce both erythroid and granulocyte cells, but that the majority of the mutations detected by the erythrocyte *Pig-a* assay occur after the common myeloid progenitor cells commit to the erythroid lineage (see Figure 4). The spectrum of *Pig-a* mutations in rats treated with either ENU or DMBA also reflects the effects of strand-specific DNA repair on the adducts produced by these two substances (ethyl adducts at T for ENU and bulky adducts at A and G for DMBA). Thus, the mutations were consistent with the types of DNA damage inducing them and the biological processing that DNA damage undergoes in expressed genes. The mutational data from these rat erythroid studies also indicate that, at a minimum, the erythrocyte *Pig-a* assay is capable of detecting base pair substitutions at both A:T and G:C base pairs and frameshifts involving both types of base pairs. Finally, all the mutable sites capable of producing GPI-deficient mutants in rodent erythroid cells have not been identified; however, the available information from studies conducted on rat and mouse erythroid cells described here, as well as on PNH patients (section 6c), rat lymphocytes (section 6e), and on *in vitro Pig-a* assays (section 6f) indicate that *Pig-a* mutations causing GPI anchor deficiency occur throughout the coding sequence of the gene, with no apparent hot spots (Rossi, 1997; Miura et al., 2008b, 2011; Dobrovolsky et al., 2015; Nicklas et al., 2015; Revollo et al., 2015, 2016, 2017a, 2017b, 2018, 2019; Bemis et al., 2018; Wang et al., 2018). Annex VII shows the distribution of *Pig-a* mutations induced in rat erythroid cells in the studies of Revollo et al. (2018, 2019).

102. Section 4b recommends that peripheral blood be assayed for *Pig-a* mutation at least twice for 1-3-day dosing protocols, at Days 7-14 and at approximately Day 30. For 28-day repeat-dose studies, it is recommended that mutants be evaluated at least once, at approximately Day 30. The findings from these bone marrow mutation studies imply that, at these sampling times, the mutant phenotype measured in peripheral blood erythrocytes will be due to a variety of mutations that were induced independently, primarily in bone marrow progenitor cells committed to the erythroid developmental pathway.

b Rat bone marrow cells with GPI-anchored protein markers contain wild-type *Pig-a* genes

103. In the experiments performed by Revollo et al. (2018, 2019) phenotypically wild-type bone marrow erythroid cells (*i.e*., cells positive for the CD59 GPI-anchored protein) also were sorted for *Pig-a* sequence analysis by MAML. The wild-type cells that were examined contained no *Pig-a* mutations at all. The same result was found for pools of wild-type granulocytes in the studies conducted by Dad and colleagues (2018): no *Pig-a* mutations were found in phenotypically wild-type bone marrow granulocytes. These results serve to confirm that analysis of GPI-anchored protein status (*i.e*., performing the flow cytometric erythrocyte *Pig-a* assay) distinguishes between cells having mutant and wild-type *Pig-a* genes.

c GPI-deficient cells in PNH patients are almost always associated with *PIG-A* mutations

104. As described in Section 1, classical hemolytic PNH almost always is associated with the expansion of a mutant HSC clone or occasionally a limited number of mutant HSC clones, each clone containing a *PIG-A* mutation (Nafa et al., 1995, 1998; Rosse, 1997; Brodsky and Hu, 2006; Brodsky, 2014). The mutations typically result in GPI deficiency in a substantial fraction of all hematopoietic cell lineages descendent from HSCs, including erythroid cells (Brodsky and Hu, 2006). *PIG-A* mutant clones also can be found in patients that develop PNH following aplastic anemia, although the development of GPI-deficient erythrocytes sometimes is not as apparent in these patients (Mortazavi et al., 2003).

105. The *PIG-A* mutations in PNH may have originated as spontaneous mutations, and although several possibilities exist accounting for their expansion (*e.g*., Luzzatto and Besseler, 1996; O'Keefe et al., 2011; Sugimori et al., 2012; Luzzatto, 2016), why particular mutations develop into expanded PNH clones is not totally clear (Brodsky and Hu, 2006). However, the important observation is that PNH, with rare exception, is due to GPI-deficient hematopoietic cells that harbor mutations in the *PIG-A* gene. The rare exceptions, described in Section 1, include patients with other mutations that either result in anchor deficiency or in a deficiency in an anchored protein (Krawitz et al., 2013; Brodsky, 2014). Thus, the PNH literature indicates that GPI-deficiency and GPI-anchored-protein deficiency is due to mutation, almost always to mutation in the *PIG-A* gene (Brodsky and Hu, 2006). It should be noted that even though hemolysis is a hallmark of the classical disease, the association of PNH with *PIG-A* mutation is almost entirely based on mutations

detected in granulocytes and lymphocytes, and rarely with bone marrow erythroid cells (*e.g.*, Nafa et al., 1995, 1998; Mortazavi et al., 2003; Hu et al., 2005).

d Analysis of *Pig-a* mRNA in mutant RETs from mice

106. Since RETs contain RNA, they may contain *Pig-a* mRNA descendant from mutated nucleated erythroid precursors that can be analyzed for mutations. Even though mRNA analysis doesn't evaluate the *Pig-a* gene directly, mRNA sequencing potentially can be conducted with a cell type, RETs, that is used for detecting mutants in the assay. Unfortunately, efforts to amplify *Pig-a* mRNA by RT-PCR of RNA from the mutant RETs of rats and mice largely have been unsuccessful (D.J. Roberts, V.N. Dobrovolsky, J.R. Revollo, R.A. Mittelstaedt personal communications).

107. A single report (Byrne et al., 2014), however, describes the detection of *Pig-a* mutations in GPI-deficient RETs from mice. This study used the PIGRET assay to evaluate genomic instability in a transgenic myelodysplastic syndrome (MDS) mouse model. The results indicated higher *Pig-a* mutant frequencies in peripheral blood RETs from MDS mice than from wild-type mice. Mutations were analyzed by sorting 2000 mutant RETs from each of three MDS mice (analyses were not done with mutants from wild-type mice), extracting RNA from the three mutant pools, and amplifying *Pig-a* cDNAs by RT-PCR. Amplified cDNAs were cloned into a plasmid vector and four recombinant clones from each mouse sequenced. All 12 recombinant clones contained *Pig-a* mutations, with all but one of the clones containing multiple mutations--as many as seven mutations in a single clone. For one mouse, the one to four mutations in each of the four sequenced clones were all different from one another; another mouse had the same basepair substitution in three of the four clones (along with several unique mutations); while in the third mouse, all clones contained four identical mutations. Although mutant frequencies in the transgenic mice were quite high, approaching 1×10^{-2}, it is difficult to rationalize the existence of multiple mutations in 11 of the 12 clones that were sequenced. Although mRNA sequencing has been useful for characterizing mutational spectra both *in vitro* and *in vivo*, direct analysis of DNA generally has been more successful in producing useful *Pig-a* mutational spectra.

e Evidence that GPI-deficient rat T lymphocytes contain *Pig-a* mutations

108. Each mammal contains several thousand HSCs from which the various cell types in peripheral blood are derived (Abkowitz et al., 2002; see Figure 4).

Thus, PNH, a disease that is caused by *PIG-A* mutation in HSCs, is characterized by large mutant clones in the differentiated cell types formed by HSCs, including lymphocytes, granulocytes, and erythrocytes (*e.g.,* Mortazavi et al., 2003). As indicated in Section 6c, even though hemolytic lysis is a major pathogenic manifestation of classical PNH, the association between *PIG-A* mutation and the disease largely rests on mutation analyses conducted with nucleated hematopoietic cells, most usually granulocytes. Mutation induction in the *Pig-a* assay may not be perfectly analogous to *PIG-A* mutation in PNH; however, the persistence of the mutant phenotype and the bone marrow sequencing data described in Section 6a indicate that mutations in early precursor cells likely are involved in the responses. Thus, evaluating mutation induction in nucleated myeloid and lymphoid cells has relevance to mutation induction in the erythrocyte *Pig-a* assay.

109. In this regard, several studies have been conducted to evaluate *Pig-a* mutation in GPI-deficient and GPI-anchored-protein-deficient lymphocytes from rats. Miura et al. (2008b, 2011) treated rats with ENU, and four weeks later, assayed for GPI-deficient splenic lymphocytes using proaerolysin selection. *Pig-a* cDNA prepared from the mutant clones was used for sequence analysis. Including putative splicing mutations, approximately 70% of the mutant clones contained clear alterations of *Pig-a* cDNA sequence, and about 80% of these changes were clearly independent. A total of 76 *Pig-a* mutations were identified in 66 clones analyzed. Most mutations were basepair substitutions at A:T, with the mutated T on the nontranscribed DNA strand (36 of 38 mutations at A:T). The spectrum was consistent with the types of mutations produced by an ethylating agent in a transcribed mammalian cell gene, and indeed was very similar to a published spectrum for ENU-induced mutation in the rat lymphocyte *Hprt* gene (Mittelstaedt et al., 1995).

110. The *Pig-a* assay, however, is normally conducted by identifying the mutant phenotype using flow cytometric evaluation of GPI-anchored proteins, not by proaerolysin selection of mutants. Flow cytometric analysis also has the potential to speed up molecular analysis as it rapidly identifies mutants for analysis. Revollo and colleagues (2015) flow-sorted rat spleen lymphocytes deficient in the GPI-anchored marker CD48 from ENU-dosed rats, expanded the sorted cells into clones, and then performed *Pig-a* cDNA sequencing on RNA extracted from the mutant clones. In this study, the rats were dosed with ENU, and analyzed for mutant lymphocytes in small groups (to accommodate flow sorting) four to eight weeks later. Approximately 88% of the sorted mutant clones contained *Pig-a* mutations, and at least 87% of the mutations were induced independently. The mutation spectrum was characteristic of that described for ENU above: a predominance of basepair substitutions at A:T, with

the mutated T on the nontranscribed strand of DNA. This spectrum was very similar to the ENU-induced *Pig-a* mutant spectrum induced in proaerolysin-selected lymphocyte mutants (Miura et al., 2011) and in rat lymphocyte *Hprt* mutants (Mittelstaedt et al., 1995). To increase the efficiency of the analysis, Revollo et al. also used massively parallel DNA sequencing to analyze a pool of cDNAs from 64 of the clones derived from the flow-sorted mutants. The distribution (spectrum) of mutations using this approach was virtually identical to that derived by clone-by-clone Sanger sequencing analysis.

111. Similar mutation analysis, clone-by-clone analysis using Sanger sequencing and NGS sequencing on a pool of mutant cDNAs, produced a distinct *Pig-a* mutation spectrum for flow-sorted lymphocyte mutants from DMBA-treated rats. Rats were treated with DMBA and assayed for lymphocyte mutants six weeks later. In this case, approximately 78% of mutants had *Pig-a* mutations, at least 60% of which contained independent basepair substitutions (other mutants contained large deletions and indels). The spectrum was dominated by basepair substitutions at A:T with the mutated A on the nontranscribed DNA strand (Dobrovolsky et al., 2015; Revollo et al., 2016). The *Pig-a* mutation spectrum from DMBA-treated rats also was very similar to *Hprt* lymphocyte mutation spectra for DMBA-treated rats that were reported previously (Heflich et al., 1996; Mittelstaedt et al., 1998). Analysis of flow-sorted pools of *Pig-a* mutant T cells from procarbazine-treated rats indicated that at least 75% had *Pig-a* mutations, with the mutations caused by adducts with dA (Revollo et al., 2017a).

112. Like the erythroid cell and granulocyte mutation spectra that were detected in bone marrow soon after ENU dosing (Section 6a), the lymphocyte spectra consisted of many independent (different) mutations. For instance, of the 249 mutant lymphocyte clones from DMBA-treated rats that were analyzed by Dobrovolsky et al. (2015), 197 contained mutations in the *Pig-a* gene, and 117 of the basepair substitution mutations were unique. Unlike the bone marrow mutants, the lymphocyte mutations were analyzed in the peripheral circulation (spleen), and in a long-lived cell (T lymphocytes), whose mutations may have reflected both the few mutations that potentially were induced in early precursor cells plus mutations induced in the intermediate/progenitor cells that had transited from the bone marrow (see Section 1c).

f Evidence that *Pig-a* mutations cause GPI deficiency in mammalian cells *in vitro*

113. As indicated in Section 10d, several *in vitro* gene mutation assays have been devised that measure deficiency in GPI or GPI-anchored protein markers

as an endpoint. Sequence analysis of the mutants detected in these assays has shown that mutation in a GPI-biosynthesis gene is the cause of the phenotype. However, in some biological systems mutations in genes other than *Pig-a* can contribute to the GPI-deficient phenotype. The reasons why this is the case generally support the model for how the *Pig-a* assay detects mutation *in vivo*.

114. An assay for gene mutation in chicken DT-40 cells identifies mutants by clonal selection in the presence of proaerolysin (Nakamura et al., 2012). Analysis of 18 MMS-induced DT-40 mutants found that all had mutations in the *Pig-o* gene. In chickens, *Pig-a* is an autosomal gene, while chickens have a single copy of *Pig-o* on the Z chromosome. Thus, the same logic that is used to indicate that mutations in the *Pig-a* gene are the most likely cause of GPI-deficiency in mammals can be used to argue that mutations in *Pig-o* are the likely cause of GPI deficiency in DT-40 cells.

115. Assays for detecting GPI-deficient mutants in human lymphoblastoid TK6 cells have been described by several researchers. Nicklas et al. (2015) were the first to report that *PIG-L* was heterozygous in these cells, so that a '*PIG-A* assay' based on detecting GPI-anchor deficiency actually detects mutants that are mutated in either *PIG-A* or *PIG-L*. Sequence analysis indicated that almost all spontaneous proaerolysin-resistant mutants (59 out of 60 analyzed) contained mutations in the *PIG-L* gene, and none in the *PIG-A* gene, while mutants induced by EMS were almost equally divided between those with *PIG-A* and *PIG-L* mutations. These general observations were subsequently confirmed in sequencing studies conducted by Krüger et al. (2016).

116. Although the genome of mouse L5178Y/*Tk*$^{+/-}$ cells has literally millions of sequence alterations, both large and small, relative to the mouse genome (Sawyer et al., 2006; McKinzie and Revollo, 2017), *Pig-a* mutants, determined by both proaerolysin resistance and flow cytometric identification of cells deficient in GPI-anchored proteins, almost always contain mutations in the *Pig-a* gene. Wang et al. (2018) found that 38 of 41 spontaneous mutants, 18 of 18 ENU-induced mutants, and 27 of 27 benzo[*a*]pyrene (BaP) induced mutants contained mutations in the *Pig-a* gene. In subsequent studies, Revollo et al. (2017b) found that all 81 of the BaP-induced mutants that they evaluated contained mutations in the *Pig-a* gene, while Bemis et al. (2018) found all 38 of their EMS-induced *Pig-a* mutants contained *Pig-a* mutations. The induced mutants contained mainly *Pig-a* basepair substitution mutations reflecting the mutational specificity of the test substance, while mutants from negative control cultures contained a high proportion of frameshifts and large deletions.

117. Taken together, these studies indicate that the *Pig-a* assay phenotype, loss of GPI-anchored proteins, is caused by mutation. In mammalian cells where *Pig-a* is the only single-copy GPI-biosynthesis gene (*i.e.*, in L5178Y/$Tk^{+/-}$ cells), the phenotype is caused by mutation of the *Pig-a* gene.

g Evidence that *Pig-a* gene mutation responses are consistent with TGR and endogenous reporter gene mutation responses in the same animals

118. *Pig-a* mutant frequencies have been assayed in conjunction with transgene mutant frequencies in studies conducted with TGRs (Table V). The rationale for this combination of assays is that the *Pig-a* assay serves as a rapid and sensitive approach for determining systemic (or bone marrow) mutation responses while the transgenes can be used to evaluate tissue-specific gene mutation. In most cases, the *Pig-a* and tissue-specific transgene responses were similar: EMS, BaP, ENU, and procarbazine were positive in the *Pig-a* and transgenic assays, while carbon nanotubes, 3-monochloropropane-1,2-diol and various ester conjugates of 3-monochloropropane-1,2-diol, dichloromethane, dichloropropane, and TiO_2 nanoparticles were negative in both types of assays. The discordant responses are for a mixture of dichloromethane and dichloropropane and for 4NQO where these compounds tested negative for *Pig-a* mutation and positive in the liver of *gpt*-delta mice (Horibata et al., 2013; Suzuki et al., 2014). These 'discordant' responses, however, are only superficially discordant, and consistent with the known tissue specificity of the *Pig-a* assay. In the Horibata et al. study, 4NQO was also tested in the bone marrow *gpt* assay, where it was negative. Thus, these data are consistent with both assays detecting a similar response, *i.e*., gene mutation, with the *Pig-a* assay being limited to mutations induced in the bone marrow. Note that many of these negative *Pig-a* responses were generated using protocols that were judged by the team reviewing *Pig-a* data for the RPA as insufficient to support a negative call (see Section 7b), and were classified as Inconclusive (I) (see Table VII, Annexes I and II).

119. *Hprt* lymphocyte mutation assays also have been paired with the *Pig-a* assay in *in vivo* gene mutation studies. Both *Pig-a* and *Hprt* are endogenous genes residing on the X chromosome, and assays for mutation in both genes are conducted using hematopoietic cells (*i.e*., erythrocytes and T lymphocytes), so it is anticipated that mutational responses in the two assays will be similar. That has proven to be the case in a limited number of studies conducted with ENU and BaP (Bhalli et al., 2011b; Cammerer et al., 2011). As was the case for the TGR-*Pig-a* data, these results are consistent with the *Pig-a* and *Hprt* assays both measuring gene mutation.

Table V: Studies measuring both *Pig-a* and transgenic gene mutation *in vivo*

Test substance	TGR model	TGR response/tissue	*Pig-a* response	Notes	References
Ethyl methanesulfonate	*gpt*-delta mouse	P *gpt* in liver, lung, bone marrow, intestine, kidney	P for RBCs and RETs	28-day oral dosing; Basic assay	Cao et al. (2014)
Benzo[*a*]pyrene	Mutamouse	P *lacZ* in liver, glandular stomach, bone marrow, small intestine	P for RBCs and RETs	28-day daily oral dosing up to 75 mg/kg/day; adducts and MN also P	Lemieux et al. (2011)
	gpt-delta mouse	P in liver and lung	P for RBCs	4 daily i.p. doses of 50 mg/kg/day	L Wang et al. (2017)
	gpt-delta mouse	P *gpt* in bone marrow and liver	P for RBCs	Single oral doses of 100 and 200 mg/kg; basic assay	Horibata et al. (2013)
Multi-walled carbon nanotubes	*gpt*-delta rat	N in lung	N for RBCs	Single doses of 0.25 to 1 mg/kg by intratracheal instillation; basic assay	Horibata et al. (2017)
N-Ethyl-*N*-nitrosourea	*gpt*-delta mouse	P *gpt* in liver and bone marrow	P for RBCs	Single oral dose of 40 mg/kg; basic assay	Horibata et al. (2013)
	gpt-delta rat	P *gpt* in liver and bone marrow	P for RBCs and RETs	Single oral dose of 40 mg/kg; PIGRET assay	Horibata et al. (2014)
Furan	Big Blue rat	N *cII* in liver	N for RBCs	Oral doses 5-times/week for 8 weeks; basic method	McDaniel et al. (2012)
4-Nitroquinoline-1-oxide	*gpt*-delta rat	P *gpt* in liver, N in bone marrow	Nonsignificant increase for RBCs	Single oral dose of 50 mg/kg;	Horibata et al. (2013)

Test substance	**TGR model**	**TGR response/tissue**	***Pig-a* response**	**Notes**	**References**
				basic assay	
3-Monochloropropane-1,2-diol	*gpt*-delta rat	N *gpt* and spi in kidney and testis	N for RBCs	5-times per week for 4 weeks intragastric instillation; basic assay; N for PB MN	Onami et al. (2014)
3-Monochloropropane-1,2-diol palmitate diester					
3-Monochloropropane-1,2-diol palmitate monoester					
3-Monochloropropane-1,2-diol oleate diester					
Dichloromethane (DCM)	*gpt*-delta mice	N for *gpt* in liver	N	Delivered by inhalation; PB MN also N; liver comet increase by DCP, not DCM	Suzuki et al. (2014)
Dichloropropane (DCP)	*gpt*-delta mice	N for *gpt* in liver	N		
Mixture of DCP and DCM	*gpt*-delta mice	Significant 2.6-fold increase in *gpt* for liver	N		
Procarbazine	Mutamouse	P for *lacZ* mutation in bone marrow	P	28-day daily oral dosing; up to 25 mg/kg; P for blood MN 2 days after last dose	Maurice et al. (2019)
TiO_2 nanoparticles	*gpt*-delta mice	N in liver for *gpt* and spi^-	N for RBCs	Up to 50 mg/kg by i.v. injection once/week for 4 weeks; basic method; MN, liver comet also N	Suzuki et al. (2016a)

Nonstandard abbreviations and terms: N = negative; P = positive; E = equivocal; I = inconclusive; TGR = transgenic rodent; MN = micronucleus; PB = peripheral blood; RETs = reticulocytes; RBCs = total red blood cells; basic = assay conducted without immunomagnetic enrichment, typically assaying 1 x 10^6

total RBCs and 3 x 10^5 RETs; PIGRET = immunomagnetic enrichment protocol devised by Kimoto et al. (2011a), typically analyzing 1 x 10^6 RETs

h Evidence that animals with genomic instability have elevated *Pig-a* mutant frequencies

120. If the *Pig-a* assay is responding to mutation, then the expectation is that mutant frequencies will increase in mice with a mutator phenotype or genomic instability. Byrne et al. (2014) detected increased erythrocyte (as well as T cell and monocyte) *Pig-a* mutant frequencies in a mouse model for MDS, a HSC/progenitor cell disease associated with increased levels of genomic instability. *Pig-a* mutant frequencies in RETs increased with the age of the mice and were approximately 50-fold greater in MDS than wild-type mice. RT-PCR analysis of the mutant RETs revealed multiple *Pig-a* mutations (see Section 6d). MDS mouse *Pig-a* mutant frequencies were also greater in T cells and monocytes, but the differences with wild-type mice were not nearly as great as with erythrocytes.

121. Graupner et al. (2014, 2015, 2016) reported on *Pig-a* assays conducted in *Ogg* knockout, heterozygous, and wild-type mice. Ogg is involved in the repair of oxidative lesions and the expectation is that decreased lesion repair will result in increased levels of DNA damage and mutation. Higher levels of DNA damage were detected in nucleated blood cells from *Ogg* mice; however, higher *Pig-a* mutant frequencies were not detected. These studies, however, were compromised by the use of an early mouse *Pig-a* mutant analysis protocol that is now known to result in the lysis (and thus loss) of GPI-deficient RETs. These results, therefore, should be treated with caution.

i Lack of evidence that loss of GPI anchors and GPI-anchored proteins is due to something other than mutation, *e.g.,* epigenetic gene silencing.

122. An early observation in patients treated with CAMPATH-1H for lymphoid malignancies was the emergence of large clones of GPI-anchor-deficient cells (Hertenstein et al., 1995; Taylor et al., 1997). CAMPATH-1H is a humanized monoclonal antibody against CD52, a GPI-anchored protein that is expressed in differentiated lymphoid cells, but not in stem cells; importantly, CAMPATH-1H is unlikely to be genotoxic. The expanded cells sometimes contained *PIG-A* mutations, but sometimes no mutation could be found, an observation that planted the idea that *Pig-a* mutant cells could, under the right conditions, be caused by non-genotoxic mechanisms. Further investigation with more

sensitive mutation analysis tools, however, indicated that the CAMPATH-1H most likely results in *in vivo* selection of the progeny of pre-existing *PIG-A* mutant HSCs (Rawstron et al., 1999), producing large increases in *PIG-A* mutant frequency in peripheral blood.

123. As *Pig-a* is an endogenous gene on the X chromosome, and proper gene expression is necessary for the assay to correctly discriminate between mutant and wild-type cells, aberrant gene silencing has been cited as a possible mechanism for generating GPI-deficient cells. This possibility was discussed in the IWGT *Pig-a* report (Gollapudi et al., 2015), but without any evidence to support such a mechanism. Since then, studies have been conducted comparing *Pig-a* mutant frequencies in male and female rats (Chikura et al., 2014; Labash et al., 2015a,c). Because one of the X chromosomes in females is inactivated during development (*i.e.*, by lyonization), it might be hypothesized that inappropriate inactivation (methylation) might occasionally inactivate both *Pig-a* alleles, which might elevate mutant frequencies. Even though differences in metabolism, weight and %RETs can occur between males and females, there is no evidence that sex affects induced *Pig-a* mutant frequencies (Chikura et al., 2014; Labash et al, 2015a,c). Inappropriate X-chromosome inactivation might affect spontaneous mutant frequencies with higher mutant frequencies occurring in females than in males. However, there also is no evidence that male and female spontaneous mutant frequencies differ (Labash et al., 2015a).

124. Aberrant gene silencing may also affect *Pig-a* responses when testing substances known to modulate endogenous methylation. This may be seen most clearly with tests conducted with substances not expected to induce gene mutation but that are known to hypermethylate DNA. Hydroxyurea is one of several compounds known to cause hypermethylation of DNA (Townsend et al., 2017), a process that might be hypothesized to silence the *Pig-a* gene and increase *Pig-a* mutant frequencies. However, independent studies conducted in two laboratories, which used the most sensitive immunomagnetic enrichment technologies, up to 28-day dosing protocols, and dosing up to the MTD, found that rats exposed to hydroxyurea had no increase in *Pig-a* mutant frequency, although hydroxyurea did increase the frequency of micronuclei (Dertinger et al., 2012; Adachi et al., 2016). Also, furan, phenobarbital, and caffeic acid alter endogenous methylation (both hypomethylation and hypermethylation) (Watson and Goodman, 2002; Lee and Zhu, 2006; Phillips and Goodman, 2008; de Conti et al., 2014, 2016), but all these compounds have tested negative (or negative but inconclusive) in the *Pig-a* assay (see Table VII, and Annexes I and II). Caffeic acid, however, was positive for peripheral blood micronucleus induction (Bhalli et al., 2016).

125. Dietary folate deficiency might lead to uracil incorporation and hypomethylation of DNA. Hypomethylation could decrease *Pig-a* mutant frequencies by causing expression of silenced genes; the opposite is also conceivable, where hypomethylation could lead to an increase in *Pig-a* mutant frequency. RET and total RBC *Pig-a* mutant frequencies did not differ in mice fed a folate-deficient diet and mice fed a control diet; however, total RBC mutant frequencies for folate-deficient mice were greater than for folate-supplemented mice (MacFarlane et al., 2015). Thus, there was an effect but the differences in mutant frequency were quite small, and the data in some groups quite variable. In addition, similar differences were not seen for RET mutant frequencies. A follow-up study found no effect on baseline or ENU-induced RET or total RBC *Pig-a* mutant frequency in MutaMice fed a folate-deficient diet or a diet supplemented with additional folate (LeBlanc et al., 2018). In contrast, baseline erythrocyte micronucleus frequencies and bone marrow *lacZ* mutant frequencies were increased in folate-deficient mice. The increased micronucleus frequency was expected; the authors suggested that the differential effect of folate deficiency on baseline *lacZ* and *Pig-a* mutation was due to transcription-coupled DNA repair mitigating the effects of folate deficiency on the transcribed *Pig-a* gene (*lacZ* is not transcribed in MutaMouse). The relevant conclusion from these studies is that *Pig-a* mutant frequency was not affected by the amount of folate in the diet.

126. In addition to DNA methylation, methylation and acetylation of histones bound at gene regulatory regions are important for controlling transcriptional status. Thus, aberrant gene silencing might also be mediated by chemicals that modulate histone modifications. The only chemical known to exert effects on histone methylation or acetylation evaluated in the *Pig-a* assay is caffeic acid, which as mentioned above, tested negative for *Pig-a* mutation.

127. In summary, to date, there is no convincing experimental evidence for epigenetic silencing as a mechanism for producing GPI-anchored-protein-deficient cells.

7 Evaluation of *Pig-a* assay results

a Identification and cataloging *Pig-a* testing data

128. *Pig-a* assay data were identified in two ways: 1) in studies reported in the published, peer-reviewed literature, and 2) data from a variety of less formal routes*, e.g.,* posters, platform presentations, and personal communications. In all cases efforts were made to secure the original data by directly contacting the scientists that were involved with the studies. The responsible scientists were asked to populate a spread sheet (Excel file) that was developed by Stephen D. Dertinger (Litron Laboratories), Takafumi Kimoto (Teijin Pharma), and Vasily N. Dobrovolsky (USFDA/NCTR), with important help from Paul White (Health Canada), George Johnson (Swansea University, UK), and Wout Slob (RIVM, The Netherlands). Completed spread sheets contain animal-by-animal and sampling-point-by-sampling-point data derived from the study, and include details about the test article, vehicle, dosing protocol and analysis methods, the results, observations of associated toxicity, other assays conducted as part of the study, conclusions about responses, and any reports generated that describe the findings. These spread sheets were collected and curated by a group at the USFDA/NCTR that included L. Patrice McDaniel, Roberta A. Mittelstaedt, and Jennifer M. Shemansky. As part of this process, statistical evaluations were performed on the data, as necessary, for confirming or establishing the significance of test substance responses and the mutagenicity of vehicle controls in the assay (see Section 7b). The curated data spread sheets then were transferred to a group at the University of Maryland, School of Pharmacy (Baltimore USA) that included Christopher Klimas and James Polli. This group developed the design and search functions of a public website on which the data files were posted (Shemansky et al., 2019). This website can be accessed through the following link: http://www.pharmacy.umaryland.edu/centers/cersi-files/

129. A second website was established for data that are not available for public distribution (mainly data that are intended for inclusion in future publications), but that could be useful for evaluating the performance of the assay. The sources of the data used for evaluating each of the test substance

responses in the assay (publication citation and/or website postings) are indicated in Annexes I and II.

b Analysis of *Pig-a* test responses

i.Criteria for evaluating Pig-a test responses

Test substances

130. A subcommittee of the HESI GTTC *Pig-a* Assay Workgroup was formed to provide advice on criteria for *Pig-a* data analysis and to form consensus where necessary on assay responses. The subcommittee consisted of Robert H. Heflich (USFDA/NCTR), Javed A. Bhalli (Covance and BioReliance), B. Bhaskar Gollapudi (Exponent), and Daniel J. Roberts (IUVO Biosciences and Charles River Laboratories).

131. The subcommittee defined the following response categories:

- Positive (P): overall positive call
- Negative (N): overall negative call
- Inconclusive (I): weakness in study design or other considerations makes it impossible to interpret results with a reasonable degree of certainty
- Equivocal (E): results from well-designed studies are insufficient for supporting a P or N call---often involves multiple studies having differing conclusions or single studies having data points supporting differing conclusions

132. The Workgroup subcommittee also established a set for rules for analyzing *Pig-a* assay data and arriving at a response call referred to as the ‘Workgroup (or WG) Criteria’ in Table VI. The subcommittee designed the criteria in a manner to produce the greatest possible certainty in the positive and negative *Pig-a* response calls, at the expense of evaluating many studies as inconclusive. Key elements in these criteria included the use of a repeat-dose, longer-term (typically 28-day) dosing protocol; dosing up to the MTD or limit dose; assaying both RETs and total RBCs for *Pig-a* mutation; and assaying at least 1 x 10^6 of both RETs and RBCs (a requirement that typically involved using an immunomagnetic enrichment protocol). For the purpose of the data analysis, ‘short-term’ was defined as a single dose protocol plus repeated dose protocols of up to 13 days, with dosing most often conducted on Days 1-3. All longer dosing protocols were considered ‘longer-term’, which were, most commonly, dosing on 28 consecutive days.

133. A great deal of latitude was applied to responses that were clearly positive. For example, clearly positive responses that were generated from short-term dosing protocols, or by assaying only total RBCs, typically were judged to be positive. The WG Criteria described in paragraph 132 and Table VI were applied in a stricter manner for declaring a negative and resulted in many nominally negative responses being evaluated as inconclusive because of various protocol deficiencies.

134. When preliminary results using the WG Criteria were reviewed by the *Pig-a* Expert Group (EG) formed by the OECD, it was strongly suggested that the criteria be modified to include all data from studies using short-term dosing protocols that otherwise conformed with the WG Criteria. It was recognized that a few test substances were positive using longer-term dosing protocols and not using short-term dosing protocols; however, the number of test substances falling into this category were relatively few, and mainly involve single doses (see Tables III and IV). Even though short-term dosing may not be suitable for regulatory hazard identification assessments, it was felt that the practical advantages of conducting a short-term dosing study made it advisable to at least document the effect of accepting data from short-term and longer-term dosing protocols as equivalent. Thus, the evaluations summarized in Table VII and Annexes I and II were conducted both using the WG Criteria and the 'Extended Criteria' described in Table VI.

Table VI: Workgroup and extended criteria for evaluating Responses in the *Pig-a* gene mutation assay.

Workgroup (WG) Criteria guidelines for making *Pig-a* calls

1. Consider data from all ‘reliable’ sources communicated through peer-reviewed publications, or unpublished submissions to the *Pig-a* data base
2. Consider only compounds having at least some primary data (submitted or derived) in the database
3. Consider recommendations in Section 4c as the ideal for study design and interpretation, but deviations can be made based on scientific judgement
4. Trust the statistical evaluations in papers when available---except when scientific judgement indicates some inconsistency
5. Conduct statistical analysis on data from database when statistical analysis is not available elsewhere
6. Generally, ignore route of administration and form of the test substance in making overall call with the understanding that negatives can be dependent on the method of dosing (*e.g.*, by inhalation).
7. Assume that short-term (≤13 days, generally one-three day) dosing to the MTD or limit dose (2000 mg/kg) is not sufficient for making a negative call
8. Negative calls require testing at least 1 x 10^6 RETs and 1 x 10^6 RBCs for mutant frequency soon after the 28-day dose for longer-term dosing protocols
9. Any uncertain calls are reviewed by a group of experts to generate a consensus call: positive (P), negative (N), equivocal (E), inconclusive (I).

Extended Criteria Guidelines for making *Pig-a* calls

1. Assume that short-term dosing protocols are as valid as longer-term protocols. Assays for short-term dosing protocols must be conducted at least on approximately Day 15 and Day 30
2. Follow all other WG Criteria guidelines

Vehicle controls

135. A practical consideration in analyzing the performance of the *in vivo Pig-a* assay was the availability of sufficient negative data in comparison to positive responses in the assay. This was a problem not only for the *Pig-a* assay, where much more test data are available on presumed genotoxicants than data on presumed nongenotoxicants, but also for the reference assays used to perform an RPA. For the validation of the TGR assay, which led to establishing TG 488, this problem was partially overcome by using vehicle controls as negative substances (OECD, 2009).

136. The RPA for the TGR assay assumed that commonly used vehicles are negative in both the TGR assay and reference assays without testing that assumption (OECD, 2009). In the case of the *Pig-a* assay, this assumption was verified by analyzing vehicle controls for their mutagenicity using a modification of the analysis method commonly used for test substance data. Many *Pig-a* studies use a predosing sampling point (*e.g.*, on Day -1) to establish the baseline mutant frequencies of the animals (and perhaps eliminate outliers; see Gollapudi et al., 2015). As this data point was generated before the animals were exposed to the vehicle, it can serve as a negative control for assays conducted subsequently on vehicle control animals (see Figure 5 for examples of predosing mutant frequency data in comparison with mutant frequencies from animals dosed for 28 days with different vehicles).

137. Several deviations from the approaches used for analyzing data on test substances were necessary for evaluating the mutagenicity of vehicle controls. The dose was generally limited by the volume administered (*e.g.*, 10-20 ml/kg/rat for aqueous vehicles), rather than by the mass or toxicity of the test substance, and only one test volume was generally used. These volumes are governed by established guidelines on the volumes that can be tolerated by animals during experimentation (*e.g.*, Gad et al., 2016; IQ 3Rs Leadership Group, 2016). It is also the case that the same limit is generally applicable both to repeat dose studies and single dose studies, so that the animal experiences amounts of the vehicle control in direct proportion to the number of doses conducted.

138. A review of appropriate test data in the *Pig-a* database was conducted by the data identification and cataloging subcommittee. All data for this analysis were required to be available in the on-line *Pig-a* database (Shemansky et al., 2019). A preliminary screen identified studies that evaluated predosing samples as well as performed *Pig-a* assays after various short-term and longer-term dosing protocols with vehicle controls (Annex III). For the analysis conducted

here, only studies were considered that were conducted with immunomagnetic enrichment, where 28 or more daily doses with the vehicle control were conducted, and where *Pig-a* assays were conducted within one week of the final dose. This design provides the maximum exposure to the vehicle substance, uses the most sensitive methods for detecting *Pig-a* mutant frequencies, and, from a practical standpoint, eliminated from consideration only three of the vehicles shown in Annex III (corn oil, the designated etoposide solvent, and a mixture of 1.25% HPMC, 0.18% methylparaben, 0.02% polyparaben, and 0.1% docusate sodium in water).

139. Vehicle control data were analyzed by comparing predosing mutant frequencies with postdosing mutant frequencies from individual studies. This involved employing a modified longitudinal analysis of *Pig-a* mutant frequency data as discussed in Section 5. As also described in Section 5, a small value was added to each mutant frequency, the data were $\log_{10}$ transformed, and the frequencies were tested for increases using either a one-tailed Student's t-test or Dunn's test, as appropriate. Rare dosing-associated decreases were considered irrelevant, and where significant increases were detected, they were compared with historical controls established for the individual laboratories. With the exception of Litron Laboratories, which have published historical controls (*e.g.*, Avlasevich et al., 2018), laboratory historical controls were generated *ad hoc* when sufficient predosing data were available.

140. Vehicle control mutant frequencies with significant postdosing increases relative to the predosing frequency and that exceeded the historical control for the laboratory were considered positive. Vehicle controls that did not have significantly increased mutant frequencies or did not exceed the range of historical negative control data for the laboratory were considered negative. This approach to evaluating vehicle control responses was reached by consensus agreement of the *Pig-a* data analysis subcommittee.

ii.Pig-a test responses in rats and mice

141. Ionizing radiation and over 80 chemicals, chemical mixtures, and nanomaterials have been tested for *Pig-a* mutation in either rats or mice. Seventy-two have been assayed in rats, 23 in mice, and 13 test substances have been assayed in both rodent species. Responses for the test substances evaluated in rats are listed in Annex I, substances evaluated in mice are listed in Annex II, and a summary of the *Pig-a* responses in mice and rats and overall consensus calls are given in Table VII. Annexes I and II provide details about most of the tests that have been conducted (not all tests in rats involving ENU

are listed), the sources of the information used for making response calls, and the reasons for the rat and mouse response E and I calls.

142. The 13 test substances that were tested in both mice and rats produced qualitatively similar *Pig-a* assay results. Thus, for most of the following discussion and analysis, data from mice and rats were combined, and the overall responses shown in the three right columns of Table VII were used for analyzing assay performance.

143. Several test substances have been tested in multiple laboratories and using different protocols (details given in Annexes I and II; see also Table III). In addition to its use as a positive control, ENU is the first compound most laboratories use when establishing the assay. Including assays conducted as part of interlaboratory trials, ENU has been tested in more than 20 laboratories, and only a fraction of these tests are listed in the tables and annexes (but many additional data sets can be found on the web-based database). Other compounds tested for *in vivo Pig-a* mutation in three or more laboratories include 1,3-propane sultone: seven labs; DMBA and 4-nitroquinoline-1-oxide (4NQO): five labs; acrylamide, benzo[*a*]pyrene (BaP), and ethyl methanesulfonate (EMS): four labs; 2-acetylaminofluorene (2-AAF), aristolochic acids, chlorambucil, cisplatin, cyclophosphamide, *N*-propyl-nitrosourea, ionizing radiation, melphalan, methyl methanesulfonate (MMS), mitomycin C, *N*-nitroso-*N*-methyl urea (MNU), and ethyl carbamate/urethane: three labs.

144. As described above, all response calls were made using two sets of criteria. In general, strict application of the Extended Criteria had the effect of changing some (*i.e.*, four) of the P responses using the WG Criteria into E calls (Table VII, Summary of calls), as there were several test substances that tested P only with repeat-dose protocols (see Tables III and IV and analysis in Section 4a). In addition, the Extended Criteria evaluated five compounds that tested negative, but only used short-term dosing protocols, as N, while with the WG Criteria, they were evaluated as I (Table VII). Thus, the Extended Criteria resulted in fewer P and I calls, and a greater number of N and E calls. As only P and N calls were used for the performance assessment, the WG Criteria produced 52 useful data calls (34 P and 18 N), while the Extended Criteria produced 53 (30 P and 23 N).

145. Table VII also lists overall calls for data from the studies conducted using short-term dosing protocols (*i.e.*, 13 or fewer daily doses). Altogether, 56 test substances were assayed using short-term protocols. The summary data in Table VII indicate that results from studies with these shorter dosing protocols

had a lower fraction of I calls (11% as opposed to approximately 30% when all studies were considered), with the result that the fraction of P and N calls was increased from approximately 60% when all *Pig-a* data were considered to 80% when only studies with short-term dosing protocols were considered. The significance of this observation is not clear and may reflect the relatively small number of test substances in the *Pig-a* database.

146. The 14 vehicle controls that were evaluated for their mutagenicity are listed in Annex III. Eleven of these vehicle controls were used in studies that had predosing data, 28 or more daily doses, immunomagnetic separation protocols, and assays conducted at Day 28 to Day 35 of the study (Table VIII). Expert data curation indicated that there were sufficient primary data to evaluate *Pig-a* mutant frequency responses for ten of these vehicle controls, including phosphate buffered saline, water, and vegetable oil. All these substances were evaluated as negative in the *Pig-a* assay. There were insufficient data available to establish a *Pig-a* mutant frequency response for 0.5% methylcellulose (Table VIII). For the purpose of the performance assessment, vehicle controls that were negative after 28-days of dosing were assumed to be negative with short-term protocols.

Table VII: Summary of substances tested in the rat and/or mouse *Pig-a* assay, with responses evaluated using both the Workgroup (WG) and Extended Criteria

Short-term studies employed treatments on 13 or fewer consecutive days

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
3R4F cigarette smoke	Complex mixture	I	I	N	N	N	N	N
Acetaminophen	Hydroxyaniline/ 103-90-2			N	N	N	N	N
2-Acetylaminoflourene	Aromatic amide/ 53-96-3			P	E	P	E	E
Acrylamide	α,β-unsaturated amide /79-06-1	N	N	E	E	E	E	E
Aflatoxin B1	Mycotoxin			P	E	P	E	N
4-Aminobiphenyl	Aromatic amine/92-67-1			P	P	P	P	P
o-Anthranilic acid	Aromatic amine/ 118-92-3			N	N	N	N	N
Aristolochic acids	Aromatic nitro compound/313-67-7			P	P	P	P	P
Azathioprine	Aromatic nitro compound/446-86-			P	E	P	E	E

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
	6							
Azidothymidine	Nucleoside analog/ 30516-87-1	I	I	I	N	I	N	N
Azoxymethane	Alkylating agent/ 24843-45-2			I	E	I	E	E
$BaSO_4$ NM-220	Metal sulfate nanomaterial			I	I	I	I	
Benzo[a]pyrene	Polycyclic aromatic hydrocarbon/50-32-8	P	P	P	P	P	P	P
2-Butoxyethanol	Ethylene glycol derivative/111-76-2			N	N	N	N	N
Caffeic acid	Hydroxycinnamic acid/331-39-5			I	N	I	N	N
Carbon nanotubes (MWCNTs)	Multi-walled carbon nanotubes/308068-56-6			I	I	I	I	I
Carboplatin	Antineoplastic crosslinker/41575-94-4			P	P	P	P	P

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
CeO_2 NM-212	Rare earth nanomaterial			N	N	N	N	
Chlorambucil	Nitrogen mustard alkylator/305-03-3			P	P	P	P	P
4-Chloro-1,2-diaminobenzene	Aromatic amine/ 95-83-0			N	N	N	N	
5-(2-Chloroethyl)-2'-deoxyuridine (CEDU)	Pyrimidine analogue/95-83-0			P	P	P	P	P
4-Chloro-*ortho*-toluidine-HCl	Aromatic amine/ 3165-93-3			I	I	I	I	
Cisplatin	Antineoplastic crosslinker/15663-27-1			P	P	P	P	P
Clofibrate	Fibric acid derivative/ 637-07-0			N	N	N	N	
Compound X**	?			N	N	N**	N**	
Compound X-A**	Nucleoside analog			P	P	P**	P**	
Cyclophosphamide	Nitrogen mustard alkylator/6055-19-2			P	P	P	P	P

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
Deoxynivalenol	Mycotoxin/51481-10-8	I	I			I	I	N
Dibenzo[a,l]pyrene	Polycyclic aromatic hydrocarbon/191-30-0			P	P	P	P	P
Dichloromethane	Chlorinated organic solvent/75-09-2	I	I			I	I	
Dichloromethane plus dichloropropane	Chlorinated organic solvent mixture/ 75-09-2 plus 78-87-5	I	I			I	I	
Dichloropropane	Chlorinated organic solvent/78-87-5	I	I			I	I	
Diethylnitrosamine	Nitrosamine/ 55-18-5			P	E	P	E	N
3,5-Difluoroboronic acid	Aryl boronic acid/ 156545-07-2			N	N	N	N	
[1-(3-Dimethylamino-propyl)-3-ethylcarbodiimide Hydrochloride (EDAC)	Carbodiimide/25952-53-8			N	N	N	N	
7,12-Dimethylbenz[a]-anthracene	Polycyclic aromatic hydrocarbon/57-97-6	P	P	P	P	P	P	P
1,2-Dimethylhydrazine	Hydrazine/ 306-37-6			I	N	I	N	N

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
Ethyl carbamate (urethane)	Carbamate/ 51-79-6	P	P	P	P	P	P	P
Ethyl methanesulfonate	Alkylating agent/62-50-0	P	P	P	P	P	P	P
N-Ethyl-*N*-nitrosourea	Alkylating agent/759-73-9	P	P	P	P	P	P	P
Etoposide	Topoisomerase inhibitor; 33419-42-0			E	E	E	E	E
5-Fluorouracil	Pyrimidine analogue/ 51-21-8			I	N	I	N	N
Fullerene	Nanomaterial	I	I			I	I	I
Furan	Heterocycle/110-00-9			I	I	I	I	
Glycidamide	Oxirane amide/5694-00-8			P	P	P	P	P
Gycidyl methacrylate	Methacrylate ester/106-91-2			P	P	P	P	
2-Hydroxypyridine-N-oxide (HOPO)	Pyridine derivative/ 13161- 30-3			N	N	N	N	
Hydroxyurea	DNA replication			N	N	N	N	N

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
	inhibitor/127-07-1							
Ionizing radiation		P	P	P	P	P	P	P
Isopropyl methanesulfonate	Alkylating agent/ 926-06-7			P	P	P	P	
Isopropyl toluenesulfonate	Alkylating agent/ 2307-69-9			P	P	P	P	P
Melamine	Triaminotriazine/ 108-781			I	N	I	N	N
Melphalan	Nitrogen mustard alkylating agent/148-82-3			P	P	P	P	P
Methyl carbamate	Carbamate / 598-55-0	I	N	N	N	N	N	N
Mitomycin C	Alkylating crosslinking agent/50-07-7			P	P	P	P	P
4,4’-methylenedianaline	Aromatic amine/ 101-77-9			E	E	E	E	N
Methyl methanesulfonate	Alkylating agent/66-27-3			P	P	P	P	P
4-(Methylnitrosamino)-1-(3-pyridyl)-1-	Nitrosamine/64091-91-4			N	N	N	N	

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
butanone (NNK)								
N-Methyl-*N*-nitrosourea	Alkylating agent/684-93-5			P	P	P	P	P
3-Monochloropropane-1,2-diol	Chloropropane/96-24-2			I	I	I	I	
3-Monochloropropane-1,2-diol palmitate diester	Chloropropane derivative			I	I	I	I	
3-Monochloropropane-1,2-diol palmitate monoester	Chloropropane derivative			I	I	I	I	
3-Monochloropropane-1,2-diol oleate diester	Chloropropane derivative			I	I	I	I	
Methylphenidate	Phenylethylamine derivative/ 113-45-1			I	I	I	I	
4-Nitroquinoline-N-oxide	Aromatic N-oxide/56-57-5	I	I	P	P	P	P	P
Nivalenol	Mycotoxin/23282-20-4	I	I			I	I	I

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	**Extended Criteria**	**WG Criteria**	**Extended Criteria**	**WG Criteria**	**Extended Criteria**	**Extended Criteria: short-term studies only**
Phenobarbital	Barbiturate/50-06-6			I	I	I	I	
Procarbazine	Alkylating agent/671-16-9	P	P	P	P	P	P	P
1,3-Propane sultone	Alkylating agent/1120-71-4			P	P	P	P	P
N-Propyl-nitrosourea	Alkylating agent/816-57-9			P	P	P	P	P
Pyrene	Polycyclic aromatic hydrocarbon/129-00-0	I	N	N	N	N	N	N
Silver nanoparticles	Nanomaterial	I	I			I	I	I
Sodium chloride	Salt/7647-14-5			N	N	N	N	N
Sulfisoxazole	Aryl sulfonamide/127-69-5			N	N	N	N	N
Temozolomide	Imidazotetrazine alkylating agent/85662-93-1			P	P	P	P	P
2,2',4,4'-tetrabromodiphenyl ether	Polybrominated biphenyl ether/ 5436-43-1	I	I			I	I	
Thiotepa	Aziridine/52-24-4			P	P	P	P	P
TiO_2 nanoparticles	Metallic nanomaterial	I	I			I	I	I
Triclosan	Polychloro phenoxy phenol/ 3380-34-5	I	I			I	I	I

Test substance	Chemical species/ CAS	Mouse response*		Rat response*		Overall response*		
		WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	WG Criteria	Extended Criteria	Extended Criteria: short-term studies only
Trimethoprim-sulfamethoxazole	Dihydrofolate reductase inhibitor plus sulfonamide antibiotic/738-70-5 plus 723-46-6			P	P	P	P	P
Triptolide	Diterpenoid epoxide/ 38748-32-2			I	I	I	I	
Vinblastine	Alkaloid/865-21-4			N	N	N	N	
Summary of calls		P = 7 N = 1 E = 0 I = 15	P = 7 N = 3 E = 0 I = 13	P = 34 N = 18 E = 3 I = 17	P = 30 N = 23 E = 8 I = 11	P = 34 N = 18 E = 3 I = 27	P = 30 N = 23 E = 8 I = 21	P = 27 N = 18 E = 5 I = 6

Nonstandard abbreviations and terms: WG = Workgroup; N = negative; P = positive; E = equivocal; I = inconclusive

**P,N>I, E>N **Not useful for performance analysis*

TABLE VIII: Summary of solvent/vehicle control data in rats

All studies analyzed using immunomagnetic separation with at least five rats per group, approximately 28 days of dosing. All usable data (with predosing and approx. 28-day data points) are from rats. Most results compare predosing with approx. 28-day responses for vehicle controls; propylene glycol used concurrent sham-dosed rats for comparison. Historical predosing 95% one-sided tolerance intervals were available or were calculated for four labs: Litron, Janssen, Novartis, and NCTR.

Solvent/vehicle	Number of studies	Number of labs	Number of daily treatments	Route of administration	Rat strains tested: all males, except males and females marked with *	Studies with significantly increased RBCs/ number of studies	Studies with significantly increased RETs/ number of studies
0.9% saline/physiological saline	2	1	28	*i.p.*	SD	0/2	0/2
10% Ethyl alcohol	1	1	28	*p.o.*	SD	0/1	0/1
Phosphate buffered saline (pH 6.0)	1	1	28	*p.o.*	CD	0/1	0/1
Phosphate buffered saline (neutral or not specified)	3	2	28	*p.o.*	F344, SD	1/3[a]	1/3[a]
Water	19	7	28	*p.o.*	SD, HW, CD, Wistar	1/19[b]	3/19[b]
Propylene glycol[c]	1	1	35	Inhalation	SD*	0/1	0/1
Sesame oil	6	1	28	*p.o.*	SD	0/6	1/6[a]
Olive oil	2	2	28	*p.o.*	HW, Wistar	0/2	0/2

0.5% Hydroxypropyl methylcellulose/0.1% Tween 80	1	1	28	*p.o.*	HW	0/1	0/1
0.5% Methylcellulose	1	1	28	*p.o.*	SD	0/1	1/1[d]
0.5% Hydroxypropyl methylcellulose	2	1	29	*p.o.*	SD	0/2	0/2

Abbreviations: RBCs, red blood cells; RETs, reticulocytes; i.p., intraperitoneal; p.o., per os (gavage), SD, Sprague-Dawley, F344, Fisher 344; CD, Cesarean-derived; HW, Han Wistar

[a]All significant RET and total RBC results had no more than one rat exceeding the historical pretreatment 95% one-sided tolerance interval for the laboratory that generated the response.

[b]The significant RBC result and two of three significant RET results had no more than one rat exceeding the historical pretreatment 95% one-sided tolerance interval for the lab that generated the response; the remaining significant results could not be confirmed due to the lack of an historical pretreatment database.

[c]The non-exposed controls for this study were concurrent sham-exposed animals.

[d]Significant results could not be confirmed because an historical pretreatment 95% one-sided tolerance interval could not be generated for the lab

iii.Discussion of test responses

Testing relevant to establishing the chemical space and applicability domain of the assay

147. Testing has been conducted on substances from a wide range of chemical classes, although in many cases the number of substances from each group is not large. Of the most commonly evaluated chemical classes, data have been collected on ten alkylating agents, six aromatic nitro/amino compounds, six nanomaterials, four polycyclic aromatic hydrocarbons (PAHs), and three mycotoxins (Table VII and Annexes I and II).

148. Other than vehicles, 18 (WG Criteria) or 23 (Extended Criteria) test substances tested negative in the assay (Table VII). These test substances can be divided into several categories in order to account for the negative responses (Table IX). Note that the data underlying this categorization was not the result of a formal rigorous literature review; such a review, however, can be found for select conventional assays used for the performance assessment described in Section 8, below.

TABLE IX: Analysis of test substances negative in the *Pig-a* assay.

Vehicle/solvent controls from Table VIII not included.

Nongenotoxic compound	Primarily clastogen/ aneugen	Possible insufficient bone marrow exposure
Acetaminophen	Azidothymidine*	3R4F cigarette smoke**
o-Anthranilic acid	Caffeic acid*	4-Chloro-*ortho*-toluidine**,***
2-Butoxyethanol	1,2-Dimethylhydrazine	3,5-Difluorophenyl-boronic acid **
Clofibrate	5-Fluorouracil*	1-(3-Dimethylamino-propyl)-3-ethylcarbo-diimide hydrochloride (EDAC)**
Melamine	Hydroxyurea*	2-Hydroxypyridine-N-oxide (HOPO)
Methyl carbamate	Vinblastine sulfate*	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butone (NNK)**

Nongenotoxic compound	Primarily clastogen/ aneugen	Possible insufficient bone marrow exposure
Pyrene		4-Chloro-1,2-diaminobenzene*,****
Sodium chloride		CeO_2
Sulfisoxazole		

**Bone marrow/blood MN and/or comet P in same animals*

***Bone marrow/blood MN and/or comet N in same animals*

****Blood MN P, but presumed to be artifact*

*****Possible sex effect*

149. First, there are nine substances that are generally regarded as nongenotoxic based on their structure and previous testing and are expected to be negative in any genotoxicity assay (see analysis of test substances in conventional genetic toxicity assays in Annex IV). Second, there are six genotoxic compounds that were negative in the *Pig-a* assay, but the primary (or only) known mechanism of genotoxicity for these compounds is clastogenicity, aneugenicity, or mitotic recombination (or gene conversion). These compounds are expected to be difficult to detect using a mutation reporter gene located on the X chromosome (DeMarini et al., 1989).

150. Third, there are eight compounds that are positive in other *in vitro* and *in vivo* gene mutation and genotoxicity assays but tested negative in the *in vivo Pig-a* assay. For these compounds, it is possible that adequate amounts of the reactive metabolites that caused them to be mutagenic in other assays did not reach the bone marrow of the test animals. It is possible, for instance, that a reactive metabolite produced in the liver may not be sufficiently stable to reach the bone marrow.

151. The third group includes 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone (NNK), which induced gene mutations *in vivo* using other assays (von Pressentin et al., 1999, 2001) and was positive for *Pig-a* mutation in an *in vitro* assay (Table XIV; Mittelstaedt et al., 2019). In the *in vivo Pig-a* assay, NNK was delivered by inhalation, and the limit dose by inhalation (determined by nasal tissue toxicity) was considerably lower than doses producing positive responses in the mouse *in vivo* MN assay, where the compound was administered by intraperitoneal injection (Padma et al., 1989). Thus, it is likely

that insufficient amounts of the test substance reached the bone marrow to produce a positive response *in vivo* (Mittelstaedt et al., 2019).

152. Insufficient exposure of the bone marrow also could be the reason for the negative *Pig-a* response for CeO_2. Although the best way of testing the genotoxicity of nanomaterials is currently uncertain (Elespuru et al., 2018), the negative *Pig-a* response, like the NNK response, was generated in an inhalation experiment, where relatively low doses of the test substance were tolerated, and where exposure of bone marrow is often questionable (Cordelli et al., 2017). Relatively higher doses used for gavage dosing resulted in positive bone marrow MN responses (Kumari et al., 2014a,b). In contrast, the negative response produced by 1-(3-dimethylamino-propyl)-3-ethylcarbo-diimide hydrochloride (EDAC) has been attributed to a specific detoxification pathway or process present *in vivo* (Custer, 2012), and the negative produced by 4-chloro-1,2-diaminobenzene may be due to a sex-specific effect (see discussion in Section 8).

Evidence for complementarity between the Pig-a assay and other in vivo genetic toxicity tests

153. Many of the studies in Annexes I and II and summarized in Table VII were evaluated in other *in vivo* genotoxicity endpoints in the same animals used to conduct the *Pig-a* gene mutation assay (see notes in Annexes I and II). Comparison of the responses in the different assays is informative for determining if combining assays could give a more complete evaluation of the genotoxicity of a test substance. For example, the second group of *Pig-a* negatives listed in Table IX includes seven compounds that have tested positive for blood or bone marrow MN induction in the same animals used for the *Pig-a* assay. It can be concluded that the genotoxicity produced by these test substances is more readily detected with assays measuring aneugenicity and/or clastogenicity (like the MN assay) than gene mutation (like the *Pig-a* assay).

154. Among other compounds with differential responses in different genetic toxicology assays, acrylamide was negative for *Pig-a* in mice but positive for MN induction (Hobbs et al., 2016). In addition, acrylamide was equivocal for *Pig-a* and negative for MN induction in rats, but positive for liver comet (Dobrovolsky et al., 2016; Hobbs et al., 2016). In separate studies, NNK was positive for TGR gene mutation in tissues other than blood or bone marrow (*e.g.*, oral tissue; von Pressentin et al., 1999, 2001); presumably similar positive

responses might occur if these assays were conducted in the animals used for the *Pig-a* assay.

155. Among *Pig-a* positives, a few have been negative in the *in vivo* MN or comet assays conducted in the same animals. For example, aristolochic acids have tested positive for *Pig-a* gene mutation in rat studies conducted independently in three labs (Bhalli et al., 2013b; Elhajouji et al., 2014; Koyama et al., 2016). In the Bhalli *et al.* study, which used both 3-day and 28-day dosing protocols, aristolochic acids were consistently positive for the *Pig-a* endpoint, but at best produced an equivocal response for peripheral blood MN and were weakly positive with no dose response for liver comet. Other compounds that appear to fall into this category of a positive *Pig-a* response and small or negative responses in blood or bone marrow-based assays for clastogenicity and DNA strand breakage include diethylnitrosamine (DEN, using immunomagnetic enrichment; Avlasevich et al., 2014; Kanal et al., 2018), a proprietary compound tested by BioReliance (Compound X-A, Annex I; Dutta, 2017), and 5-(2-chloroethyl)-2'-deoxyuridine (CEDU) (Annex I; Elhajouji et al., 2018), with the latter two compounds being nucleoside analogues. (Note that DEN is sometimes classified as positive for MN induction, but this positive response is for the liver MN assay.) A more formal analysis of the apparent complementarity between the blood-based *Pig-a* and MN assays can be found in the performance assessment described in Section 8.

156. Some of the sensitivity of the *Pig-a* mutation assay relative to other genotoxicity assays may be due to its ability to accumulate mutations with repeat dosing. While 4NQO is positive for *Pig-a* gene mutation using both short-term and longer-term dosing protocols (Annex I and Table VII), it is reliably positive for the MN and comet endpoints only using relatively high doses in short-term dosing protocols, and not using doses that can be tolerated for a 28-day repeat-dose study (Stankowski et al., 2011; Roberts et al., 2016). With regards to this latter observation, because of the large dynamic range for *Pig-a* mutation and its extremely low background frequency, it is often the case that greater fold-increases are noted for *Pig-a* mutation than for other genotoxicity endpoints, even when both endpoints are considered positive (*e.g.*, Dertinger et al., 2010, 2012). This suggests that the *Pig-a* endpoint may have an intrinsic advantage in sensitivity over other *in vivo* genotoxicity endpoints, at least for gene mutagens.

Studies of genotoxicant/nongenotoxicant pairs

157. In studies intended to test the specificity of the assay, *Pig-a* assays have been conducted with structurally related genotoxicant/nongenotoxicant pairs. The genotoxicant BaP and the nongenotoxicant pyrene have been tested in both rats and mice, and only BaP has induced *Pig-a* mutation (Table VII, Annexes I and II; Torous et al., 2012; Labash et al., 2016). A similar distinction can be made between the genotoxic carcinogen ethyl carbamate (urethane) and the nongenotoxic carcinogen methyl carbamate These two test substances have tested positive and negative, respectively, in both mice and rats (Table VII, Annexes I and II; Bemis et al., 2015; Labash et al., 2016; Stankowski et al., 2015). Although regarded as a genotoxic carcinogen, the genotoxicity of ethyl carbamate is difficult to detect *in vitro*, since it requires CYP2E1 activation, which is not present at sufficient levels in many *in vitro* genotoxicity assays (Tweets et al., 2007).

Assays conducted to evaluate confounders and limitations of the assay

158. Additional testing has been conducted with protocols, and test substances, designed to discover any limits on how the assay can be performed and interpreted. Cytotoxicity is regarded as a major confounder for genotoxicity assays and can produce nonrelevant responses. This is especially clear for *in vitro* assays that measure chromosome breakage (Greenwood et al., 2004; Fellows et al., 2008; Galloway et al., 2011) and it is a suspected confounder of the *in vivo* comet assay (*e.g.*, Rothfuss et al., 2011). However, most genotoxicants are cytotoxic and cytotoxicity is also a valuable indicator of exposure, so there is always a question of how much cytotoxicity is sufficient to demonstrate exposure without producing an artefactual response. Recommendations on dose selection and cytotoxicity in the *Pig-a* assay (Table II; Gollapudi et al., 2015) are consistent with existing recommendations for conducting other *in vivo* genotoxicity assays (*i.e.*, TG 474; TG 475; TG 488; TG 489). Two studies have been conducted in rats to stress the hematopoietic system and induce a wave of compensatory erythropoiesis. However, no effect was seen on the *Pig-a* mutant frequencies (Kenyon et al., 2015; Nicolette et al., 2018).

159. Another possible complication for the assay is its reliance on an endogenous gene located on the X-chromosome as a reporter of mutation. One of the two copies of *Pig-a* in female animals is silenced during development, resulting in one functional copy of the gene in both males and females. Inappropriate silencing of the ‘expressed allele’ in females (or the single allele

in males) could increase *Pig-a* mutant frequencies in untreated animals. Also, if a test substance had the effect of altering methylation patterns or inducing gene silencing in the animals used for a *Pig-a* assay, increases in *Pig-a* mutant frequency might result that do not represent increases in *Pig-a* mutation. Testing conducted to explore the possibility that epigenetic effects confound the *Pig-a* assay is discussed in Section 6i.

Additional studies

160. The *Pig-a* assay also has been used as a mutational reporter in studies evaluating the effect of nutritional factors and genotype on genomic stability. The primary objective of these studies was to answer basic science questions and not to test substances for *in vivo* mutagenicity; thus, many are not included in Annexes I or II. Elevated *Pig-a* mutant frequencies have been detected in a mouse model of leukemia (Byrne et al., 2014); as a result of dietary protein deficiency (Pacheco-Martinez et al., 2016), dietary selenium deficiency (Graupner et al. 2014, 2015, 2016), diet-induced obesity (Wickliffe et al., 2016); and in BaP-treated P450 reductase knockout mice (L Wang et al., 2017). A study that varied the concentration of dietary folate given to mice found the well-known effect of folate deficiency increasing MN frequency, but its effects on *Pig-a* mutant frequency were questionable (MacFarlane et al., 2015). Finally, a study using knock-out mice for DNA glycosylases NEIL1, NEIL2, and NEIL3 found no effect of the genotype on either cancer predisposition or spontaneous *Pig-a* mutant frequency (Rolseth et al., 2017).

8 Performance assessment of the *Pig-a* assay

a Approach employed

161. OECD Guidance Document 34 recommends evaluating the performance of a new assay primarily by describing the '….. accuracy (*e.g.*, sensitivity, specificity, positive and negative predictivity, false positive and false negative rates) of the proposed test method', indicating that '….it should be compared to that obtained for the reference test method currently accepted by regulatory agencies…….' (OECD, 2005).

162. As described in Sections 1 and 6, the *Pig-a* assay measures *in vivo* gene mutation, specifically mutations induced in the *Pig-a* gene of rat and mouse bone marrow cells. Thus, the primary approach chosen to evaluate the performance of the *Pig-a* assay was to compare its accuracy in evaluating *in vivo* gene mutation relative to the mutagenicity of test substances whose mutagenicity responses are defined by the TGR assay. The TGR assay is the only *in vivo* gene mutation assay widely accepted by regulatory agencies and the only *in vivo* gene mutation assay currently with an OECD TG (TG 488). As described in Section 2d, the TGR assay is capable of measuring mutation in the bone marrow cells of rats and mice, as well as in virtually any other tissue of transgenic rodents. As indicated in Section 7b above, *Pig-a* assay results meeting test criteria have been established for over 50 test substances. The accuracy of the *Pig-a* assay was evaluated by comparing these *Pig-a* responses with responses in the TGR assay for these same test substances. Separate comparisons were made between *Pig-a* responses and TGR responses in rodent bone marrow and in any tissue of rats and mice. In order to make these comparisons, responses in rats and mice were merged into single data sets for each endpoint. As is evident from Table VII and Annexes I and II, most *Pig-a* data are from experiments conducted with rats, while most TGR data are from studies with mice (Lambert et al., 2005).

163. In addition, the most widely used *in vivo* genotoxicity assay is the *in vivo* erythrocyte MN assay, described in OECD TG 474 (see Section 2a). Section 7b describes data indicating that, even though they measure genotoxicity in the same basic target tissue, the *in vivo Pig-a* assay may complement the *in vivo* MN assay because the assays measure mechanistically different genotoxic responses. In order to determine the extent to which this mechanistic specificity affects the predictive value of these assays for evaluating genotoxicity, the accuracy of the *in vivo Pig-a* assay was evaluated using responses in the *in vivo* MN assay as the reference.

164. Finally, cancer is a primary health concern addressed by *in vivo* gene mutation data. Gene mutation provides regulatory agencies with a practical, relatively short-term method for identifying potential genotoxic carcinogens. Therefore, in addition to evaluating the accuracy of the *Pig-a* assay as a test for *in vivo* gene mutation, we evaluated its accuracy for identifying rodent carcinogens.

b Establishing responses in reference assays

165. A separate subcommittee, that included members of the HESI-GTTC *Pig-a* Assay Workgroup as well as other genetic toxicology experts, was formed to provide a curated set of responses in conventional genotoxicity tests and rodent cancer bioassays for the test substances evaluated in the *Pig-a* gene mutation assay. Besides establishing mutation responses in the TGR assay for bone marrow and all tissues, and responses in the rodent cancer bioassay (separating cancer in hematopoietic tissue from cancer in all tissues), responses also were determined for the *in vitro* MN and chromosome aberration assays, the *in vivo* MN assay, and the Ames' bacterial gene mutation assay. This subcommittee, which operated independently of the *Pig-a* assay subcommittee described above, consisted of Michelle O. Kenyon (Pfizer), Stephen D. Dertinger (Litron Laboratories), Leon F. Stankowski (Charles River Laboratories), Martha M. Moore (Rambol), Katsuyoshi Horibata (NIHS, Japan), and Takafumi Kimoto (Teijin Pharma). The same P, N, E, and I categories used for describing *Pig-a* responses were used to describe responses in the conventional assays.

166. First, genotoxicity and carcinogenicity data for the test agents were obtained from the Vitic Database (Lhasa, Ltd). Where all results for a test substance in an assay were in agreement, the result was considered to be accurate. When there were different test results in an assay for a given test substance, the results and citations were provided to an independent reviewer for assessment. Additional databases were consulted as needed. The reviewer

provided a suggested call for discussion by the subcommittee. If a consensus call could not be made, that test substance was documented as Equivocal (E) in the assay.

167. Annex IV provides the consensus calls developed by the subcommittee for data from conventional *in vivo* genetic toxicology assays and cancer bioassays.

c Performance assessment results

168. Table X compares *Pig-a* assay responses generated by applying both the WG and Extended Criteria with responses defined by TGR responses in bone marrow and in all tissues, by responses in the *in vivo* MN assay, and by rodent cancer bioassay responses in the hematopoietic system and in all tissues. Only test substances producing either P or N overall responses in the *Pig-a* assay were used for this analysis.

TABLE X: Comparison of *Pig-a* assay responses with conventional *in vivo* genotoxicity assay responses and with rodent cancer bioassay responses in hematopoietic tissue and in all tissues.

Only test substances either P or N in the *Pig-a* assay are evaluated. Response calls are those of the committees described in Section 8.

Test substance	Overall *Pig-a* assay response			TGR assay responses		Rodent cancer bioassay responses		*In vivo* MN response
	WG Criteria	Extended Criteria	Extended criteria: short-term studies only	Bone marrow	All tissues	Hematopoietic system	All tissues	
3R4F cigarette smoke	N	N	N					
Acetaminophen	N	N	N	N	N	P	P	P
2-Acetylaminoflourene	P	E	E		P		P	P
Aflatoxin B1	P	E	N		P		P	P
4-Aminobiphenyl	P	P	P	P	P		P	P
o-Anthranilic acid	N	N	N			N	N	
Aristolochic acids	P	P	P	P	P		P	N
Azathioprine	P	E	E	P	P	P	P	P
Azidothymidine	I	N	N				P	P
Benzo[a]pyrene	P	P	P	P	P		P	P

Test substance	Overall *Pig-a* assay response			TGR assay responses		Rodent cancer bioassay responses		*In vivo* MN response
	WG Criteria	**Extended Criteria**	**Extended criteria: short-term studies only**	**Bone marrow**	**All tissues**	**Hematopoietic system**	**All tissues**	
2-Butoxyethanol	N	N	N				P	
Caffeic acid	I	N	N				P	P
Carboplatin	P	P	P					P
CeO_2 NM-212	N	N						
Chlorambucil	P	P	P	P	P	P	P	P
4-Chloro-1,2-diaminobenzene	N	N			P		P	P
5-(2-Chloroethyl)-2'-deoxyuridine (CEDU)	P	P	P	P	P			
Cisplatin	P	P	P		P	P	P	P
Clofibrate	N	N		N	N		P	
Cyclophosphamide	P	P	P				P	P
Deoxynivalenol	I	N	N			N	N	
Dibenzo[a,l]pyrene	P	P	P					P
Diethylnitrosamine	P	E	N	N	P		P	P
3,5-Difluoroboronic acid	N	N						
[1-(3-Dimethylamino-propyl)-3-	N	N						

Test substance	Overall *Pig-a* assay response			TGR assay responses		Rodent cancer bioassay responses		*In vivo* MN response
	WG Criteria	Extended Criteria	Extended criteria: short-term studies only	Bone marrow	All tissues	Hematopoietic system	All tissues	
ethylcarbodiimide Hydrochloride (EDAC)								
7,12-Dimethylbenz[a]-anthracene	P	P	P	P	P	P	P	P
1,2-Dimethylhydrazine	I	N	N				P	P
Ethyl carbamate (urethane)	P	P	P	P	P	P	P	P
Ethyl methanesulfonate	P	P	P	P	P		P	P
N-Ethyl-*N*-nitrosourea	P	P	P	P	P	P	P	P
5-Fluorouracil	I	N	N				P	P
Glycidamide	P	P	P		P		P	P
Gycidyl methacrylate	P	P			I	N	N	P
2-Hydroxypyridine-N-oxide (HOPO)	N	N						
Hydroxyurea	N	N	N		P		P	P
Ionizing radiation	P	P	P		P		P	P
Isopropylmethane	P	P			P		P	P

Test substance	**Overall *Pig-a* assay response**			**TGR assay responses**		**Rodent cancer bioassay responses**		***In vivo* MN response**
	WG Criteria	**Extended Criteria**	**Extended criteria: short-term studies only**	**Bone marrow**	**All tissues**	**Hematopoietic system**	**All tissues**	
sulfonate								
Isopropyltoluene sulfonate	P	P	P					
Melamine	I	N	N				P	N
Melphalan	P	P	P			P	P	P
Methyl carbamate	N	N	N				P	N
Mitomycin C	P	P	P	P	P		P	P
Methyl methanesulfonate	P	P	P	P	P	P	P	P
4-(Methylnitrosamino) - 1-(3-pyridyl)-1-butanone (NNK)	N	N			P		P	I
N-Methyl-*N*-nitrosourea	P	P			P		P	P
4-Nitroquinoline-N-oxide	P	P	P	P	P		P	P
Procarbazine	P	P	P				P	P
1,3-Propane sultone	P	P	P				P	P
N-Propyl-	P	P	P	P	P	P	P	P

Test substance	Overall *Pig-a* assay response			TGR assay responses		Rodent cancer bioassay responses		*In vivo* MN response
	WG Criteria	Extended Criteria	Extended criteria: short-term studies only	Bone marrow	All tissues	Hematopoietic system	All tissues	
nitrosourea								
Pyrene	N	N	N			N	N	N
Sodium chloride	N	N	N			N	N	N
Sulfisoxazole	N	N	N			N	N	N
Temozolomide	P	P	P				P	P
Thiotepa	P	P	P		P		P	P
Trimethoprim-sulfamethoxazole	P	P						
Vinblastine	N	N				N	N	P
0.9% saline/physiological saline	N	N	(N)	N	N	N	N	N
10% Ethyl alcohol	N	N	(N)	N	N	N	N	N
Phosphate buffered saline (pH 6.0)	N	N	(N)	N	N	N	N	N
Phosphate buffered saline (neutral or not specified)	N	N	(N)	N	N	N	N	N
Water	N	N	(N)	N	N	N	N	N
Propylene glycol	N	N	(N)	N	N	N	N	N

Test substance	**Overall *Pig-a* assay response**			**TGR assay responses**		**Rodent cancer bioassay responses**		***In vivo* MN response**
	WG Criteria	**Extended Criteria**	**Extended criteria: short-term studies only**	**Bone marrow**	**All tissues**	**Hematopoietic system**	**All tissues**	
Sesame oil	N	N	(N)	N	N	N	N	N
Olive oil	N	N	(N)	N	N	N	N	N
0.5% Hydroxypropyl methylcellulose/0.1 % Tween 80	N	N	(N)					
0.5% Hydroxypropyl methylcellulose	N	N	(N)	N	N	N	N	N

Nonstandard abbreviations and terms: WG = Workgroup (Criteria; see Table VI); MN = micronucleus; N = negative; P = positive; E = equivocal; I = inconclusive

169. The performance evaluation of the *Pig-a* assay was conducted by Robert H. Heflich (USFDA/NCTR) in consultation with David P. Lovell (St. Georges's University, London). *Pig-a* assay performance was evaluated by defining true positive and negative responses as the responses in the reference assay and calculating the following metrics:

- Sensitivity: the percent of all the positives in the reference assay detected as positives in the *Pig-a* assay
- Positive predictivity: the percent of positives in the *Pig-a* assay that are positive in the reference assay
- Specificity: the percent of all negatives in the reference assay detected as negatives in the *Pig-a* assay
- Negative predictivity: the percent of negatives in the *Pig-a* assay that are negatives in the reference assay
- Concordance: the number of true negatives and positives (defined by their responses in the reference assay) correctly identified in the *Pig-a* assay divided by the total number of test substances x 100.

170. The calculation of these metrics from data arrayed in two-by-two tables is shown in Annex V.

171. In addition, performance was evaluated by the application of two tests of agreement between two different measures:

- Cohen's kappa test: an estimate of the inter-assay agreement (*i.e.*, the agreement between the *Pig-a* assay and a reference assay) for qualitative (categorical) items. The kappa statistic ranges from -1 (perfect disagreement) to 0 (no agreement) to +1 (perfect agreement); among positive agreements, 0.01-0.20 is considered slight agreement, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial, and 0.81 to 1.00 almost perfect.
- PABAK (prevalence and bias adjusted kappa): since Cohen's kappa is affected by prevalence (*e.g.*, unbalanced data sets having more positives than negatives), tests like the PABAK have been devised that adjust the statistic for prevalence and distribution of the marginal totals. However, even with adjustment, none of the association tests is perfect, and must be view in context with other quantitative measures.

172. Calculations were made with the DAG Stat statistical suite for Microsoft Excel, available at: https://www.biostats.com.au/DAG_Stat/. The two-by-two tables and performance statistics, including 95% confidence intervals for some of the statistics and a list of nonconcordant test results, are shown in Annex V.

Note that the performance metrics that were calculated for the assay have large 95% confidence intervals, a function of the relatively small data sets (n = 25-53) that were used for the calculations. Thus, any perceived differences in the performance of the assay must be tempered by the level of confidence in the estimates.

i.Performance assessment using Pig-a responses evaluated using the WG Criteria

173. Table XI summarizes the results of the performance analysis conducted on *Pig-a* assay responses determined using the WG Criteria. The responses with the *Pig-a* assay displayed a high degree of accuracy when the bone marrow TGR assay defined P and N test substances, with only DEN producing discordant results. DEN is a genotoxic carcinogen whose genotoxicity is difficult to detect in blood-based *in vivo* assays (Kanal et al., 2018). DEN was positive in the *Pig-a* assay using immunomagnetic separation and negative in the bone marrow TGR assay, suggesting that, at least for bone marrow mutation, the *Pig-a* assay was more sensitive to the mutagenicity of this compound than the TGR assay.

TABLE XI: Summary of performance measurements for *Pig-a* assay data analysed with the Workgroup (WG) Criteria and using conventional *in vivo* genetic toxicity assay responses and rodent cancer bioassay responses to define positive and negative responses

Metric	*Pig-a* vs. bone marrow TGR response (n = 28)[a]	*Pig-a* vs. any tissue TGR response (n = 40)[b]	*Pig-a* vs *in vivo* MN response (n = 52)[c]	*Pig-a* vs rodent hematopoietic cancer response (n = 25)[d]	*Pig-a* vs. rodent cancer in any tissue response (n = 49)[e]
Sensitivity	100%	90%	85%	90%	79%
Positive predictivity	94%	100%	94%	90%	96%
Specificity	92%	100%	89%	93%	93%
Negative predictivity	100%	79%	76%	93%	67%
Concordance	96%	93%	87%	92%	84%
Cohen's kappa	0.93	0.83	0.71	0.83	0.65
PABAK	0.93	0.85	0.73	0.84	0.67

Abbreviations: TGR, transgenic rodent; PABAK, prevalence and bias adjusted kappa
Discordant test substances:
[a]*DEN*
[b]*Hydroxyurea, 4-chloro-1,2-diaminobenzene, NNK*
[c]*Acetaminophen, aristolochic acids, 4-chloro-1,2-diaminobenzene, hydroxyurea, vinblastine, CeO_2, CEDU*
[d]*Acetaminophen, glycidyl methacrylate*
[e]*Acetaminophen, 2-butoxyethanol, 4-chloro-1,2-diamonobenzene, glycidyl methacrylate, hydroxyurea, clofibrate, methyl carbamate, NNK*

174. When TGR responses in any tissue were used as the reference assay, the accuracy of the *Pig-a* mutation assay was only slightly lower. Discordant results were found for three test substances, hydroxyurea, NNK, and 4-chloro-1,2-diaminobenzene, which were negative in the *Pig-a* assay but were mutagenic in the TGR assay in tissues other than bone marrow. In addition, as discussed in Section 7b, hydroxyurea is known mainly as a clastogen (Ames-test positive only at concentrations that exceed the current recommendation of 5000 µg/plate), and the *Pig-a* study in which NNK was evaluated used inhalation dosing, which produced limited exposure of bone marrow (Mittelstaedt et al., 2019). Finally, when 4-chloro-1,2-diaminobenzene, an Ames' positive mouse liver and rat bladder carcinogen, was tested in the mouse TGR assay, it was only positive for liver mutation induction in female mice, and it was negative in male mice (Suter et al., 1996). Since the *Pig-a* data were generated in male rats, it is possible that the discordance between the positive TGR response and negative *Pig-a* response for 4-chloro-1,2-diaminobenzene not only could be due to its tissue specificity, but also its species and sex specificity. Note that DEN, which produced discordant responses when *Pig-a* and TGR responses were evaluated in bone marrow, was positive for other tissues in the TGR assay. Thus, DEN was positive in both the *Pig-a* and TGR assays when all tissues were considered for the TGR assay.

175. When the *in vivo* MN assay was used as the reference assay, the accuracy of the *Pig-a* assay was still good ('substantial' using the Kappa test interpretation guidelines described above), but lower that when the TGR assay was used to define P and N responses. Seven discordant compounds were detected: acetaminophen, aristolochic acids, CEDU, 4-chloro-1,2-diaminobenzene, CeO_2, hydroxyurea, and vinblastine. As noted in Section 7b acetaminophen, hydroxyurea, and vinblastine are mainly genotoxic through mechanisms resulting in clastogenicity or aneugenicity, and thus were positive in the MN assay and negative for *Pig-a* mutation. On the other hand, aristolochic acids and CEDU, which are known to be genotoxic mainly through the induction of gene mutation, were positive in the *Pig-a* assay and negative for MN induction. Thus, the mechanistic complementarity of the MN and *Pig-a* assays noted in Section 7b can be detected in this quantitative assessment.

176. When cancer in hematopoietic tissues was used to define positive and negative responses, responses in the *Pig-a* assay had a very high degree of accuracy ('near perfect' in the terminology described above). Only two test substances produced discordant results: acetaminophen, whose genotoxicity is most readily observed as clastogenicity and not gene mutation, and glycidyl methacrylate, which is positive in many conventional gene mutation and clastogenicity assays (see Annex IV) and may be an example of a genotoxic

noncarcinogen. Note that this high degree of precision was based on observations with very few compounds (25), and that the precision benefited greatly from using vehicle control data in the analysis.

177. Defining positive and negative responses by the ability of a test substance to induce cancer in any rodent tissue markedly reduced the accuracy of the *Pig-a* assay. This was mainly reflected in a large reduction in the negative predictivity of the assay, *i.e.*, there were several compounds negative in the *Pig-a* assay that were rodent carcinogens. In all, there were eight discordant responses, including responses from test substances that are often classified as nongenotoxic carcinogens (methyl carbamate, 2-butoxyethanol), compounds that are mainly clastogens (acetaminophen, hydroxyurea) and compounds that may have poor bone marrow exposure (NNK) (see Table IX and associated discussion). As indicated above, 4-chloro-1,2-diaminobenzene may exhibit several possible specificities in its mutagenic response, and it is possible that this influences its carcinogenicity. In addition, glycidyl methacrylate was positive in the *Pig-a* assay, while it was negative for rodent cancer. As mentioned above, this compound may be an example of a genotoxic noncarcinogen.

ii.Performance assessment using Pig-a responses evaluated using the Extended Criteria

178. Table XII summarizes the results of the performance analysis conducted with *Pig-a* assay responses determined using the Extended Criteria. Using these criteria, there was perfect agreement between the responses in the *Pig-a* assay and the bone marrow TGR assay. Note that in strictly applying the Extended Criteria, DEN (which produced the only discordant results using the WG Criteria) was not used for the analysis since it was scored as equivocal (two well conducted assays producing different results, discussed further in Section 4a). Also, two compounds, azathioprine and 2-acetylaminofluorene, were scored as equivocal because studies with well-conducted single-dose protocols were negative while these test substances were positive when animals were treated with 3-day or 28-day dosing protocols (see discussion in Section 4a). Thus, results with these two substances were not used for the performance evaluation. All other responses were the same using both the WG and Extended Criteria.

179. The accuracy of the *Pig-a* mutation assay was slightly lower (but still substantial, applying the Kappa test interpretation guidelines) when TGR responses in any tissue were used as the reference assay to define positives and negatives. Discordant results were found for the same three test

substances that were discordant using the WG Criteria: hydroxyurea, NNK, and 4-chloro-1,2-diaminobenzene. All were negative in the *Pig-a* assay, regardless of including or excluding short-term dosing studies, but were mutagenic in the TGR assay in tissues other than bone marrow. Note that DEN, which produced discordant responses when *Pig-a* and TGR responses were evaluated in bone marrow and *Pig-a* responses were evaluated using the WG Criteria, was scored as equivocal in the *Pig-a* assay using the Extended Criteria since a well-conducted *Pig-a* study with a single dose was negative and *Pig-a* studies having 28-day dosing protocols were positive.

180. When the *in vivo* MN assay was used as the reference assay, the performance of the *Pig-a* assay fell to ‘moderate’, lower than when the TGR assay was used to define P and N responses. When analyzed using the Extended Criteria, 11 discordant compounds were detected: acetaminophen, aristolochic acids, CEDU, 4-chloro-1,2-diaminobenzene, CeO_2, hydroxyurea and vinblastine, as with the WG Criteria, plus caffeic acid, 1,2-dimethylhydrazine, 5-fluorouracil, and AZT. As noted in Section 7b these later four compounds are considered to operate primarily through mechanisms that produce clastogenic/aneugenic responses. As was the case when *Pig-a* responses were evaluated using the WG Criteria, the mechanistic complementarity of the MN and *Pig-a* assays noted in Section 7b is evident in this assessment.

181. When cancer in hematopoietic tissues was used to define positive and negative responses, responses in the *Pig-a* assay, analyzed with the Extended Criteria, displayed a very high degree of accuracy. As was the case when the WG Criteria were used to evaluate *Pig-a* responses, only two test substances produced discordant results, acetaminophen and glycidyl methacrylate. Again, this high degree of accuracy was based on observations with very few compounds (26 in this case) and benefited greatly from using vehicle control data for the analysis.

182. Defining positive and negative responses by the ability of a test substance to induce cancer in any rodent tissue reduced the accuracy of the *Pig-a* assay to ‘moderate’. This was mainly reflected in a large reduction in the negative predictivity of the assay, from 94% when only hematopoietic cancer data were used to 56% when cancer in any tissue was used. There were 13 discordant responses among 50 test substances with relevant data. Along with the eight discordant test substances identified with the WG Criteria, discordant test substances included four powerful clastogens that are weak or negative in gene mutation assays (caffeic acid, AZT, 1,2-dimetylhydrazine, and 5-

fluorouracil) and melamine, which is believed to be a nongenotoxic carcinogen (see Table IX and associated discussion).

TABLE XII: Summary of performance measurements for *Pig-a* assay data analysed with the Extended Criteria and using conventional *in vivo* genetic toxicity assay responses and rodent cancer bioassay responses to define positive and negative responses

Metric	*Pig-a* vs. bone marrow TGR response (n = 26)[a]	*Pig-a* vs. any tissue TGR response (n = 37)[b]	*Pig-a* vs *in vivo* MN response (n = 53)[c]	*Pig-a* vs rodent hematopoietic cancer response (n = 26)[d]	*Pig-a* vs. rodent cancer in any tissue response (n = 50)[e]
Sensitivity	100%	84%	74%	90%	65%
Positive predictivity	100%	100%	93%	90%	96%
Specificity	100%	100%	89%	94%	94%
Negative predictivity	100%	79%	65%	94%	56%
Concordance	100%	89%	79%	92%	74%
Cohen's kappa	1.00	0.77	0.58	0.84	0.50
PABAK	1.00	0.78	0.58	0.85	0.48

Abbreviations: TGR, transgenic rodent; PABAK, prevalence and bias adjusted kappa
Discordant test substances:
[a] *None*
[b] *Hydroxyurea, 4-chloro-1,2-dimanobenzene, NNK, 1,2-dimethylhydrazine*
[c] *Acetaminophen, aristolochic acids, 4-chloro-1,2-diaminobenzene, AZT, caffeic acid, 1,2-dimethylhydrazine, 5-flourouracil, hydroxyurea, vinblastine,* CeO_2*, CEDU*
[d] *Acetaminophen, glycidyl methacrylate*
[e] *Acetaminophen, 2-butoxyethanol, caffeic acid, AZT, 4-chloro-1,2-diaminobenzene, clofibrate, 1,2-dimethylhydrazine, 5-fluorouracil, glycidyl methacrylate, hydroxyurea, melamine, methyl carbamate, NNK*

iii.Performance assessment using only Pig-a responses from assays conducted with short-term dosing protocols

183. Table XIII summarizes the results of the performance analysis conducted with *Pig-a* assay responses determined using data only from studies conducted using short-term dosing protocols and analyzed using the Extended Criteria. Under these conditions, there was perfect agreement between the responses in the *Pig-a* assay and the bone marrow TGR assay. Note that a study employing a single dose of DEN produced a negative response, the same as the response in the bone marrow TGR assay. Also, two compounds, azathioprine and 2-acetylaminofluorene, were scored as equivocal using short-term protocols because well-conducted studies using a single dose were negative while these test substances were positive when treated with three daily doses (see discussion in Section 4a). Thus, data from these two substances were not used for the performance evaluation.

TABLE XIII: Summary of performance measurements for *Pig-a* assay data from short-term dosing studies analysed with the Extended Criteria using conventional *in vivo* genetic toxicity assay responses and rodent cancer bioassay responses to define positive and negative responses

Metric	*Pig-a* vs. bone marrow TGR response (n = 27)[a]	*Pig-a* vs. any tissue TGR response (n = 34)[b]	*Pig-a* vs *in vivo* MN response (n = 49)[c]	*Pig-a* vs rodent hematopoietic cancer response (n = 24)[d]	*Pig-a* vs. rodent cancer in any tissue response (n = 46)[e]
Sensitivity	100%	83%	73%	90%	66%
Positive predictivity	100%	100%	92%	100%	100%
Specificity	100%	100%	89%	100%	100%
Negative predictivity	100%	71%	68%	93%	56%
Concordance	100%	88%	80%	96%	76%
Cohen's kappa	1.00	0.75	0.59	0.91	0.54
PABAK	1.00	0.77	0.59	0.92	0.52

Abbreviations: TGR, transgenic rodent; PABAK, prevalence and bias adjusted kappa
Discordant test substances:
[a]*None*
[b]*Hydroxyurea, aflatoxin B1, DEN, 1,2-dimethylhydrazine*
[c]*Acetaminophen, aristolochic acids, hydroxyurea, aflatoxin B1, AZT caffeic acid, DEN, 1,2-dimethylhydrazine, 5-fluorouracil, CEDU*
[d]*Acetaminophen*
[e]*Acetaminophen, aflatoxin B1, AZT, 2-butoxyethanol, caffeic acid, 5-fluorouracil, hydroxyurea, melamine, methyl carbamate, DEN, 1,2-dimethylhydrazine*

184. The accuracy of the *Pig-a* mutation assay using only short-term dosing protocols was slightly lower (but still substantial) when TGR responses in any tissue were used as the reference assay to define test substances with positive and negative responses. Discordant results were found for four test substances, hydroxyurea, NNK, 1,2-dimethylhydrazine, and DEN, all of which were negative in the *Pig-a* assay and positive in the TGR assay in a tissue other than bone marrow. Reasons why hydroxyurea and NNK might be negative for the *Pig-a* assay are discussed above for *Pig-a* data evaluated by both the WG and Extended Criteria. Although it wasn't evaluated in the bone marrow, the mutagenicity of 1,2-dimethylhyrazine in the TGR assay was strongly associated with the colon (Newell and Heddle, 2004). DEN was discordant because a *Pig-a* mutation study employing a single dose was negative (it hasn't been tested with other short-term testing protocols), whereas DEN is positive for TGR mutation in a tissue other than bone marrow.

185. When the *in vivo* MN assay was used as the reference assay, the performance of the *Pig-a* assay was still good, but lower than when the TGR assay was used to define P and N responses (falling into the moderate range). The lower accuracy is reflected in the lower negative predictivity value, meaning that a number of negatives in the *Pig-a* assay were positive in the MN assay. When data from short-term *Pig-a* studies were analyzed using the Extended Criteria, ten discordant compounds were detected: acetaminophen, aristolochic acids, CEDU, hydroxyurea, aflatoxin B1, caffeic acid, 1,2-dimethylhydrazine, 5-fluorouracil, and AZT. As noted above, several of these compounds are considered primarily to be aneugens/clastogens (acetaminophen, hydroxyurea, caffeic acid, 1,2-dimethylhydrazine, 5-flourouracil, AZT), while aristolochic acids and CEDU operate mainly though a pathway resulting in gene mutation. As was the case when *Pig-a* responses were evaluated using the WG or Extended Criteria, the mechanistic complementarity of the MN and *Pig-a* assays noted in Section 7b was apparent in this assessment. The negative response using aflatoxin B1 with short-term dosing protocols is notable because it is one of the few compounds whose mutagenicity is detected only with longer-term dosing protocols (see Table IV); this limitation in the gene mutation response is not true for the MN assay. Finally, DEN was classified as positive for *in vivo* MN by the conventional assays subcommittee, but this call was based on strong responses in the liver MN assay. DEN was found to be negative in several erythrocyte MN studies that were conducted in conjunction with *Pig-a* gene mutation assays (Shi et al., 2011; Avlasevich et al., 2014; Kanal et al., 2018).

186. When cancer in hematopoietic tissues was used to define positive and negative responses, responses in the *Pig-a* assay, analyzed with the Extended

Criteria (including responses in assays conducted with short-term dosing protocols) displayed a very high degree of accuracy. In this case, only acetaminophen produced discordant results, and, as explained above, this may be because the genotoxicity of acetaminophen is characterized by clastogenicity and not gene mutation. Again, this high degree of accuracy was based on observations with very few compounds (24, the smallest data set used in this analysis), and the precision benefited greatly from using vehicle control data in the analysis.

187. As was the case when the results from longer-term dosing studies were included in the analysis, defining positive and negative responses by the ability of a test substance to induce cancer in any rodent tissue markedly reduced the precision of the *Pig-a* assay (into the moderate range). This was mainly reflected in a large reduction in the negative predictivity of the assay, from 93% when only hematopoietic cancer data were used to 56% when cancer in any tissue was used. This analysis resulted in 11 discordant responses among 46 test substances with relevant data, and in each case the discordant finding involved a negative *Pig-a* response for a rodent carcinogen. Ten of the discordant compounds were also discordant using the WG or Extended data analysis criteria on the full set of *Pig-a* data responses. The situation with aflatoxin B1 is discussed above; aflatoxin B1 was only positive in the *Pig-a* assay when a 29-day daily dosing protocol was used.

9 Positioning of the assay relative to existing *in vivo* genetic toxicology assays

188. The IWGT report described placement of the assay relative to existing genotoxicity testing strategies (Gollapudi et al., 2015), and those recommendations are essentially unchanged in this document. The major use currently envisioned for the *Pig-a* assay is as a follow-up for evaluating test substances that are positive for gene mutation *in vitro* using either bacterial or mammalian cell tests. The IWGT report pointed out that the assay is a gene mutation assay and therefore may not detect substances that are primarily clastogens or aneugens. It also noted that negative tests should only be accepted if there was an indication of bone marrow exposure. Bone marrow exposure could be demonstrated by a reduction in the frequency of RETs or by demonstrating adequate plasma levels of the test substance or its metabolites. Table IX contains examples confirming the wisdom of these recommendations: genotoxic compounds like hydroxyurea that are primarily clastogens, and gene mutagens like NNK that have poor bone marrow exposure, have produced negative results in the *in vivo Pig-a* assay.

189. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) M7(R1) guidance (ICH, 2017) recommends the *Pig-a* assay as a follow-up *in vivo* assay for drug impurities positive for bacterial mutagenicity. The ICH and IWGT recommendations are quite similar for this follow-up application, although the ICH recommendations do not consider clastogens/aneugens at all and suggest demonstration of adequate exposure to metabolite(s) only for indirect (S9-mediated) bacterial mutagens. To our knowledge, the assay has been used in follow-up studies for at least two types of Ames-positive drug impurities. In addition, the *Pig-a* assay has been used as a follow-up test for a nucleoside analog active pharmaceutical ingredient producing large colony mutants in the MLA. These examples, however, come from meeting presentations or personal communications and are not in the published literature (Custer, 2012; Dutta, 2017; Joel Bercu, personal communication).

190. The IWGT report also indicated that the *Pig-a* assay has value in developing weight-of-evidence arguments for the genotoxicity of a substance. The quantitative assessment of genotoxicity data has shown potential for estimating human risk (Johnson et al., 2014, 2015), and the properties of the *Pig-a* assay may be useful for this application. Its large dynamic range, its ability to respond to repeated dosing in a cumulative manner, its ability to repeatedly sample test animals over time, and its potential to analyze the same endpoint in multiple species, including humans, may be beneficial to generating quantitative gene mutation data for human risk assessment.

191. In addition, the IWGT report suggested, on mainly theoretical grounds, that the *Pig-a* gene mutation assay can serve as a complement to the *in vivo* peripheral blood MN assay for many routine *in vivo* genotoxicity testing applications. While both measure genotoxicity in bone marrow cells, each measures a different spectrum of genotoxicity responses (gene mutation vs. clastogenicity/aneugenicity). Especially when used in conjunction with repeat dose studies, both could be integrated readily into standard toxicity protocols without impacting other testing. The analyses in Sections 7b and 8 provide evidence of the complementarity of the two tests for detecting the genotoxicity of several compounds. This includes clastogens like hydroxyurea, aneugens like vinblastine, and gene mutagens like CEDU and aristolochic acids, where one test was positive and the other negative for these known genotoxic substances.

192. Another potential regulatory application, as the human *PIG-A* assay becomes more widely used, is direct confirmation of findings in animal studies with data from humans. This ability is unique to assays like the *Pig-a*/*PIG-A* assay that measure genotoxicity in endogenous genes and thus provide the potential for translation between laboratory animals and humans. This degree of translation is not possible with TGR assays that employ genetically engineered rodents and exogenous transgenes.

193. The *Pig-a* assay also has applications in studies that go beyond genotoxicity testing. As outlined in Section 7b, the assay has been used to evaluate the effect of diet and genotype on genomic stability in rodents and the effect of these factors on responses to mutagens (Byrne et al., 2014; Graupner et al., 2014, 2015; Pacheco-Martinez et al., 2016; Wickliffe et al., 2016; Rolseth et al., 2017; L Wang et al., 2017). Because the assay can be performed with human cells, it has been used to evaluate mutant frequencies and mutation rates in humans and in cells cultured from humans with diseases associated with genomic instability (Olsen et al., 2017; see Sections 10a and 10c). For

example, Araten et al. (2010) have used the *PIG-A* assay to demonstrate genomic instability in cells derived from patients with T cell acute lymphoblastic leukemia, mantle-cell lymphoma, follicular lymphoma, and in some plasma cell neoplasms.

10 Potential for expanding the applicability of the assay

194. The potential for translation is one of the strongest features of the *Pig-a* assay. Given the conservation of the *Pig-a* gene, GPI anchors, and the function of GPI anchors across species and cell types, the assay can potentially be adopted to other species of toxicological relevance as well as to different cell types *in vivo* and to *in vitro* mammalian cell systems.

a Erythrocyte *PIG-A* assays conducted in humans

195. The most important species for evaluating human risk is humans, and the genesis of the rodent *Pig-a* assay began with studies using the *PIG-A* gene as a reporter of somatic cell mutation in humans (Araten et al., 1999; see Section 1a). Several investigators have followed up on the initial observations that reported a 'background' frequency of 8 x 10^{-6} CD59 plus CD55 doubly-deficient total RBCs in one normal donor (Araten et al., 1999). Dobrovolsky et al. (2011) greatly expanded the number of healthy volunteers assayed for CD59-deficient erythrocyte *PIG-A* mutant frequency and found a frequency of 5.1 ± 4.9 x 10^{-6} for total RBCs from 95 of 97 volunteers; there were no significant relationships between mutant frequency and age or smoking habits. Two individuals had markedly increased mutant frequencies of approximately 100 and 300 x 10^{-6}, with the frequencies remaining elevated upon resampling. In addition, ten cancer patients receiving cytotoxic/mutagenic chemotherapy were monitored for total RBC mutant frequency before and for approximately six months after beginning therapy. Only one patient, treated with a combination therapy that included cisplatin, displayed an increase in *PIG-A* mutant frequency with time.

196. More recently Cao et al. (2016) used the total RBC *PIG-A* assay to measure mutant frequencies in 217 Chinese volunteers. The mean mutant frequency, 5.25 ± 3.6 x 10^{-6}, was remarkably similar to that detected by Dobrovolsky et al. (2011) in a U.S. population, and again, no relationship between age and mutant frequency was found. Both study populations, however, were skewed towards individuals over 30 years of age, and age-

related increases in *HPRT* lymphocyte mutant frequency are most clearly seen in individuals under 30 (Robinson et al., 1994). In the larger Cao et al. population, a positive relationship was found between mutant frequency and smoking expressed as cigarette-pack-years. In a subsequent pilot study, Cao et al. (2017) reported increased erythrocyte *PIG-A* mutant frequencies in some (but not all) of 20 inflammatory bowel disease patients treated with azathioprine and in 17 barbeque restaurant workers potentially exposed to elevated levels of PAHs.

197. In addition, Horibata et al. (2016) monitored total RBC *PIG-A* mutant frequencies in ten heathy volunteers and 27 patients undergoing cancer chemotherapy. Although the study did not include pre-chemotherapy mutant frequency determinations, and the times of sampling were often too early to expect dose-related increases in mutant frequency, two of the cancer patients had *PIG-A* mutant frequencies significantly greater than the healthy controls.

198. These foregoing studies provide evidence that an erythrocyte *PIG-A* assay can be deployed in humans. There has been a question, however, about the extent to which complement-mediated lysis of GPI-deficient cells may affect the sensitivity of the assay (Peruzzi et al., 2010). Complement-mediated lysis of *PIG-A* mutants is a well-established pathology for PNH, and the sizes of PNH clones can be considerably greater for RETs than for mature RBCs (Iwamoto et al., 1995; Ware et al., 1995). In addition, none of the earlier studies measuring erythrocyte *PIG-A* mutation in humans took advantage of immunomagnetic enrichment to increase the number of cells evaluated. With this in mind, Dertinger et al. (2015) have adopted the immunomagnetic enrichment techniques used for the rodent *Pig-a* assay for measuring mutant frequencies in human RETs and total RBCs from 52 healthy volunteers. As anticipated, the frequency of CD59 plus CD55 mutants was approximately two-fold greater in RETs than in total RBCs (mean frequencies, 6.0×10^{-6} vs. 2.9×10^{-6}, respectively), indicating a modest selection pressure in humans against mutant erythrocytes. The relatively large inter-individual variability in RET mutant frequencies (30-fold) and the consistency in RET mutant frequency with repeat sampling was similar to that noted with previous studies measuring *PIG-A* mutant frequencies in total RBCs. These studies using immunomagnetic enrichment, however, detected age-related increases in both RET and total RBC mutant frequencies.

199. Methods for measuring RET *PIG-A* mutant frequencies have not been applied widely to populations at risk for elevated genotoxicity. However, a preliminary report presented as a meeting poster provided evidence that combination therapies that included cisplatin increased peripheral blood *PIG-A*

and/or micronucleated RET frequencies (n=3; Dertinger et al., 2016). Importantly, these chemotherapy patients provided predosing blood samples to address the fact that humans exhibit wide baseline variations in *PIG-A* mutant frequency.

200. In addition, human *PIG-A* assays have been conducted with cells other than erythrocytes, where complement-mediated selection against *PIG-A* mutants is expected to be less of an issue. These studies are described in Section 10c, below.

b Erythrocyte *PIG-A* assays conducted in nonhuman primates

201. A single study by Dobrovolsky et al. (2009) evaluated *PIG-A* (CD59-deficient) mutant frequencies in total RBCs in ten controls and in one male rhesus monkey (*Macaca mulatta*) treated twice with 50 mg/kg ENU. The ENU was given by *i.p.* injection at approximately two years and 3.5 years of age. Total RBC mutant frequencies measured six months after the last dose of ENU indicated a mutant frequency of 46.5 x 10^{-6} vs a mean frequency of 7.8 x 10^{-6} in the controls.

c *In vivo Pig-a*/*PIG-A* assays conducted in germ cells and other tissues

i.Germ cells

202. Mutations transmitted though germ cells are of concern since they will occur in every cell of the resulting offspring and can have a profound impact on the phenotype. Thus, the analysis of germ cell genotoxicity and gene mutation is sometimes recommended under particular circumstances, *e.g*., when there is evidence of germ cell exposure whether or not there is evidence of genotoxicity in somatic cells (Yauk et al., 2015). In addition to cells of the bone marrow, male germ cells are actively dividing in adults, and can be made into single cell suspensions, making them potentially amenable for the detection of gene mutation using the *Pig-a* assay. TG 488 describes the use of the TGR assay for measuring gene mutation in male germ cells (OECD, 2013; note that this TG is currently being updated for measuring germ cell mutation). While there may be advantages to analysis of germ cell mutation using a *Pig-a* assay (*e.g*., it could be conducted in non-transgenic animals), it is not practical to repeatedly sample animals for germ cell mutation as can be done for the erythrocyte *Pig-a* assay.

203. Initial work has been conducted on a male germ cell *Pig-a* assay in two laboratories. In a study conducted by Bhalli and coworkers and presented only

in a poster (Bhalli et al., 2017), male rats were treated with procarbazine. Assays conducted with epidydimal sperm approximately 45 days after the dosing detected a dose-related increase in the frequency of CD59-negative sperm, with a relatively high background frequency that the authors attributed to the preliminary stage of methods development. Another study, using a similar approach, found positive responses with a germ cell mutagen (ENU), negative responses for a nonmutagen (clofibrate), and a high CD59-negative sperm frequency in samples from vehicle controls (84 and 88 x 10^{-6}) (Ji and LeBaron, 2017). These data are quite preliminary, and much more characterization will be necessary to confirm that these assays are actually measuring mutations in true male germ cells. When the current problems and uncertainties are resolved, a modified version of the *Pig-a* assay could be useful for evaluating male germ cell mutagens.

ii.Other cell types

204. Several human *PIG-A* studies have used blood cells, other than erythrocytes, to measure *PIG-A* mutant frequencies. These studies have used granulocytes, lymphocytes, monocytes, and bone marrow progenitor cells, but many of these studies have been in the context of evaluating PNH or making other disease diagnoses rather than measuring small increases in mutant frequency (*e.g*., Brodsky et al., 2000; Mukhina et al., 2001; Mortazavi et al., 2003). As reviewed in Olsen et al. (2017), however, low-level mutant frequencies have been measured in humans in several contexts: understanding the significance of low-level spontaneous *PIG-A* mutant frequencies and their connection to PNH and other diseases (Araten et al., 1999; Ware et al., 2001; Hu et al., 2005; Pu et al., 2012); using *PIG-A* mutant frequency as a reporter of genomic instability (Araten and Luzzatto, 2006; Araten et al., 2005, 2010, 2012, 2013; Grasso et al., 2014; Rondelli et al., 2013); using *PIG-A* as a biomarker of human exposure to potential genotoxins (McDiarmid et al., 2011); and using *PIG-A* for establishing mutational rates in humans. The mutation rate studies can be viewed as a hybrid *in vivo*/*in vitro* analysis, as B lymphocytes were taken from humans, transformed into lymphoblastoid cultures, cleansed of existing *PIG-A* mutants by flow sorting, and then measuring mutant frequencies over a period of time to establish a mutation rate (Araten et al., 2005; Peruzzi et al., 2010).

205. McDiarmid et al. (2011) described using limiting-dilution cloning of proaerolysin-resistant peripheral blood T lymphocyte mutants for evaluating mutations in Gulf War veterans exposed to munitions containing depleted uranium. Since human blood samples don’t have the volume limitations of rodent samples, the use of T lymphocytes for evaluating *PIG-A* mutant

frequencies may have some advantages for biomonitoring over the use of erythrocytes. T Lymphocytes from humans can be analyzed for *PIG-A* mutation either by flow cytometry or by limiting dilution cloning (*e.g*., Rawstron et al., 1999; Ware et al., 2001), lymphocytes expand readily and contain DNA so the mutations can be analyzed directly (*e.g*., Revollo et al., 2015; Dobrovolsky et al., 2017), and the use of lymphocytes avoids the problem of immune lysis that may complicate human erythrocyte assays (Section 10a). Cao et al. (2016) reported on the feasibility of using FLAER to monitor the frequency of GPI-negative white blood cells from a small number (5) of human volunteers.

206. Several studies also evaluated *Pig-a* mutant frequencies in rodents using blood cells other than erythrocytes. *Pig-a* mutant frequencies have been measured in peripheral blood T lymphocytes and monocytes from mice (Byrne et al., 2014) and in peripheral blood T cells from rats (Miura et al., 2008a,b, 2011; Bhalli et al., 2011b; Cammerer et al., 2011; Dobrovolsky et al., 2015; Revollo et al., 2015, 2016). The mouse study measured the effects of a presumed mutator phenotype on mutant frequency, while the rat studies detected the *in vivo* mutagenicity of known mutagens (*i.e*., ENU, DMBA, and BaP). In addition, several studies have evaluated *Pig-a* mutation in nucleated rat and mouse bone marrow erythrocyte and granulocyte precursor cells (Kimoto et al., 2011a,b; Revollo et al., 2018; Dobrovolsky et al., 2017; Dad et al., 2018; Kimoto and Miura, 2017), with the primary objective of directly demonstrating that *Pig-a* mutation is responsible for the mutant phenotype measured in the assay (*i.e.*, loss of GPI-anchored protein markers; see Section 6).

207. A single study evaluated *PIG-A* mutation in T lymphocytes from *Macaca mulatta* (rhesus monkeys) (Dobrovolsky et al., 2009). Increases in proaerolysin-resistant T lymphocytes were detected in a single monkey given two doses of ENU more than a year apart and monitored over a period of 21 months.

d *In vitro Pig-a* assays

208. Flow cytometric analysis of *Pig-a* mutation may benefit *in vitro* analysis of gene mutation from a throughput standpoint and in developing high-density dose response data (Johnson et al., 2015). An *in vitro* version of an *in vivo* assay also may be envisioned as having value in testing hypotheses about results in the *in vivo* assay (*e.g*., confirming negative *in vivo* responses, see paragraph 93 and [Mittelstaedt et al., 2019]) and in prescreening compounds for *in vivo* testing (Bemis and Heflich, 2019). Most of the work on developing *in vitro* versions of the *Pig-a* assay has been conducted using TK6 human lymphoblastoid cells and L5178Y/$Tk^{+/-}$ mouse lymphoma cells. These cell lines

are grown in suspension, lending themselves to flow cytometry analysis, and they already are used for regulatory *in vitro* gene mutation assays (OECD, 2016c; OECD 2016g). Therefore, the characteristics of these cells are relatively well known, and standardized cultures suitable for genetic toxicology evaluations are available through cell repositories (Lorge et al., 2016).

209. TK6 cells were used in one of the first papers describing *PIG-A* as a reporter of mutation (Chen et al., 2001). Although there have been problems with extraordinarily high spontaneous mutant frequencies in these cells (>1,000 x 10^{-6}), methods have been devised to obtain lower, more stable background mutant frequencies (*e.g.*, Rees et al., 2017). Using cleansed cultures, Krüger and colleagues (Krüger et al., 2015, 2016; Piberger et al., 2017) demonstrated that EMS, 4-NQO, BaP diol epoxide, and UVC induced strong mutational responses in TK6 cells (Table XIV). It should be noted that the TK6 cell '*PIG-A'* mutation assay detects mutants that have mutations in either *PIG-A* or *PIG-L,* as *PIG-L* is heterozygous in these cells (Nicklas et al., 2015; Krüger et al., 2016; see Section 6f for more information). Thus, although the TK6 cell assay may not be totally analogous to the *in vivo Pig-a* assay, it has been suggested that the sensitivity of the TK6 cell assay may benefit from having both an X-linked and autosomal reporter of mutation, thereby increasing the types of mutations the assay can detect (Nicklas et al., 2015).

210. Recent reports indicate that the *Pig-a* assay in L5178Y/$Tk^{+/-}$ cells is sensitive to standard mutagens and appropriately discriminates between mutagens and nonmutagens (Table XIV; Bemis et al., 2018; David et al., 2018; Y Wang et al., 2018). Also, Bemis et al. (2018), Y Wang et al. (2018), and Revollo et al. (2017b) found that mutants detected in the assay almost always contain *Pig-a* mutations (see Section 6f, above), making it unlikely that any gene, other than *Pig-a*, is involved in the GPI-deficient mutational response detected in this cell line.

211. A recent study described a flow cytometric *PIG-A* assay conducted with HepG2 cells, an attached human liver cell line that retains some capability for promutagen activation (Kopp et al., 2018). If these mutants are confirmed as containing *PIG-A* mutations, this observation suggests that the long-held presumption that the *Pig-a* assay was only suitable for unattached cells (see Section 1b) may not be totally correct.

212. An additional *Pig-a*-type *in vitro* assay has been described by Nakamura et al. (2012) (Table XIV). Like *Pig-a* assays, the assay measures GPI-anchor deficiency as the mutant phenotype. Because the assay is conducted in chicken DT-40 cells, however, the GPI biosynthesis gene that is present as a single

copy on a sex chromosome (Z in chickens) is *Pig-o*, whereas *Pig-a* is located on an autosome. This means that *Pig-o* rather than *Pig-a* is the primary reporter for mutation in this assay. The assay also is conducted by limiting dilution cloning and proaerolysin selection rather than flow cytometry. MMS produced an increase in proaerolysin-resistant mutants in DT-40 cells, and these mutants were shown to contain mutations in the *Pig-o* gene.

TABLE XIV: ***In vitro Pig-a*** **gene mutation studies**

Cell system	Substances tested/response	Notes	Reference
DT-40	MMS positive	Chicken cells: *Pig-o* reporter; limiting dilution cloning/proaerolysin selection; mutation spectrum established in *Pig-o*	Nakamura et al. (2012)
TK6		Compared responses with other cell lines; assay details unclear	Chen et al. (2001)
		Cells were almost 100% *PIG-A* mutant	Morris et al. (2006)
	EMS positive	Limiting dilution cloning/proaerolysin selection; cells found to have large deletion in one allele of *PIG-L* (confirmed in isolates from other labs); EMS-induced GPI-deficient mutants had either a *PIG-A* or *PIG-L* mutation in the functional allele; spontaneous mutants contained only *Pig-L* mutations; spontaneous GPI	Nicklas et al. (2015)

Cell system	Substances tested/response	Notes	Reference
		mutant frequency 4-26 x 10^{-6}	
	EMS positive	Flow cytometric assay; used various strategies to reduce background and increase viability	Rees et al. (2017)
	EMS, 4NQO, UVC positive; pyridine, cycloheximide negative	Flow cytometric and limiting dilution cloning/proaerolysin selection assays; cleansed of spontaneous mutants (>3000 x 10^{-6}) to 18-45 x 10^{-6}; confirmed heterozygosity in *PIG-L* and mutations in both *PIG-A* and *PIG-L* causing GPI-anchor deficient mutants	Krüger et al. (2015; 2016)
	BaP diol epoxide positive	Flow cytometric assay; positive for BaP adducts and induction of DNA damage genes	Piberger et al. (2017)
WI-L2-NS	EMS, UVC positive	Flow cytometric assay; lower spontaneous mutant frequency and less sensitive to mutagens than TK6 cells; speculate *PIG-L*	Krüger et al. (2016)

Cell system	Substances tested/response	Notes	Reference
		homozygous wild-type	
MCL-5	EMS positive; MNU, ENU, 2.4-DNP negative	Flow cytometric assay; included membrane integrity marker, modified gating strategies	Rees et al. (2017)
L5178Y*Tk*$^{+/-}$	EMS, ENU, 4NQO, 1,3-Propane sultone, MMS, cisplatin positive; phenformin, D-mannitol, dexamethasone, diethanolamine, cycloheximide negative	Flow cytometric assay	Bemis et al. (2018)
	ENU, EMS, BaP (+S9), DMBA (+S9) all positive	Flow cytometric and limiting dilution cloning/proaerolysin selection assays; 18 of 18 ENU-induced mutants have *Pig-a* mutations	Y Wang et al. (2018)
	EMS positive	Flow cytometric assay	David et al. (2018)
	NNK (+S9), BaP (+S9), EMS positive	Flow cytometric assay; most NNK mutants have *Pig-a* mutations	Mittelstaedt et al. (2019)

Nonstandard abbreviations: MMS = methyl methanesulfonate; EMS = ethyl methanesulfonate; 4NQO = 4-ntroquinoline-1-oxide; BaP = benzo[a]pyrene; MNU = N-methyl-N-nitrosourea; ENU = N-ethyl-N-nitrosourea; 2,4-DNP = 2,4-dinitrophenol; DMBA = 7,12-dinitrobenz[a]anthracene; S9 = 9000 x g supernatant of Aroclor-1254-pretreated rat liver homogenate; NNK = (methylnitrosamino)- 1-(3-pyridyl)-1- butanone.

11 Animal welfare issues associated with the *Pig-a* assay

213. A compelling argument for developing an OECD TG for the erythrocyte *Pig-a* assay is its ability to make better use of existing animal resources devoted to toxicology testing, thus conforming with the principles commonly referred to as the 3Rs (replacement, reduction, refinement). The 3Rs principles recommend adopting non-animal methods (replacement), methods to obtain comparable information using fewer animals or gain more information from the same number of animals (reduction), and using methods to alleviate or minimize potential pain, suffering or distress (refinement) (Russell and Burch, 1959). Invasive tissue sampling or animal sacrifice is not necessary to conduct the erythrocyte *Pig-a* assay, consistent with refinement, and repeated sampling of the same animals greatly reduces the number of animals necessary for establishing the temporality of a response, as might occur when evaluating the cancer mode of action of a test substance (Moore et al., 2008), consistent with reduction. Section 3 describes how the characteristics of the assay make it easier to integrate the *Pig-a* assay into existing general toxicology studies than most other genotoxicity assays, which would have the effect of reducing animal usage. As an example, the U.S. National Toxicology Program has recently decided to incorporate the *Pig-a* assay along with the peripheral blood MN assay into their routine rodent toxicity tests to make better use of the animals (K Witt, personal communication).

214. Data generated with the *Pig-a* assay indicate that, with proper training, the reproducibility and transferability of the *Pig-a* assay are impressively high (Gollapudi et al., 2015; a discussion of interlaboratory trials is in Part 2). Training and a subsequent demonstration of laboratory proficiency are important elements to implementing any assay, and the OECD has established guidelines for how that should be done for various genetic toxicology assays, including *in vivo* assays (OECD, 2015). This can be especially challenging since adopting a new assay into a laboratory typically requires several experimental trials to learn the methodology, educate laboratory staff, and demonstrate that the performance of the assay is adequate for generating acceptable data. With *in vivo* tests other than *Pig-a*, this learning and ‘laboratory

validation' period is likely to be at direct odds with the 3Rs (Russell and Burch, 1959).

215. An in-house validation procedure that involved only ten rats was described by Godin-Ethier et al. (2015). Raschke and colleagues (2016) described reconstruction experiments (also known as spiking experiments) that enable laboratories to measure mutant phenotype cells of varying frequencies, and to compare the observed results to expected frequencies. These experiments minimize the focus on steps that are not of high importance for laboratory proficiency (*e.g.*, ability to successfully dose animals with test substances), and focus on those elements that are considered critical to conducting the assay — sample handling/labelling and flow cytometric analysis.

216. The Raschke study described two approaches that were employed by four laboratories – (a) CD59 masking of blood from an untreated animal, and (b) using blood from a mutagen-treated animal. The CD59 masking technique was developed and utilized by investigators at Bayer Pharma and did not require prior dosing of animals; thus, there was no need to wait for mutant manifestation. The whole experimental procedure could be performed in one day, and performed repeatedly, providing flexibility for training purposes. Mutant-mimic samples were generated by blocking the CD59 epitope using non-fluorescently labelled anti-CD59 antibody (CD59 masking), thereby preventing any CD59-associated fluorescence during flow cytometry. Varying amounts of the fully CD59-masked sample were spiked into aliquots of unmasked blood (peripheral blood from an untreated animal) to achieve very low to moderately elevated erythrocyte mutant frequencies. A second approach, which used two animals, was utilized by GSK, Covance, and Litron Laboratories. In this approach, single rats were administered 0 or 20 mg ENU/kg/day via oral gavage for three consecutive days. Approximately 1.0 mL blood was collected from each animal approximately four weeks after the dosing and the blood samples were mixed in different ratios to create samples with minimum to moderately elevated erythrocyte mutant frequencies.

217. These reconstruction experiments were conducted independently in four laboratories and showed good overall precision (correlation coefficients >0.99) and accuracy (estimated slope: 0.71–1.09) of mutant cell scoring measured using the Bland and Altman method (Bland and Altman, 1999). These data strongly support the use of reconstruction experiments for training purposes and demonstrating laboratory proficiency with very few animals.

218. Finally, although the current recommended protocol described in Section 4 does not include running a separate positive control, positive controls are

often included in genetic toxicology assays. The method for preserving samples described in Section 4b allows including a positive control in addition to a mutant-mimic standard in the flow cytometric analysis of *Pig-a* assay samples without including additional positive control animals in a study. Preserving samples also has the potential advantage of generating *Pig-a* data from previous studies where a measure of mutation was not considered important at the time the study was conducted.

12 Summary and conclusions

219. An *in vivo* gene mutation assay has been developed that is based on measuring the loss of cell surface proteins caused by mutation in the endogenous, X-linked *Pig-a* gene.

220. In theory, the assay can be conducted in any animal of toxicological interest. To date, it has been conducted in mice, rats, rhesus monkeys and humans; it is most fully developed in rodents. The ability to conduct the assay in humans, the species of greatest toxicological interest, is a major advantage. The assay evaluates hazard, but together with exposure data, it may be applicable for risk evaluation and assessment.

221. Most *Pig-a* assay data are for peripheral blood erythrocytes. The use of erythrocytes for conducting the assay imparts advantages in terms of speed, economy, and the efficient use of animals.

222. The gene mutations measured in the assay are induced in nucleated bone marrow erythroid cells. Because many of these cells are long-lived and have extensive replication potential, mutations induced in the assay result in elevated frequencies of mutant peripheral blood erythrocytes that persist over many months.

223. The mutations detected in the rodent assay appear to have a neutral phenotype; mutant frequencies accumulate with repeated dosing.

224. Consensus protocols have been developed and evaluated in several interlaboratory trials.

225. More than 90 test substances and vehicle controls have been evaluated in rats and mice using the assay. Negative responses were generated either by known nongenotoxicants or by substances not likely to produce gene mutations in an X-linked gene. All positive responses were induced by test substances known to be gene mutagens. The assay detected the mutagenicity of a few genotoxic substances that are difficult to detect in blood-based MN assays (DEN, CEDU, aristolochic acids), and several genotoxicants that are primarily known as clastogens (cisplatin, ionizing radiation, cyclophosphamide).

226. Test data support consensus protocol recommendations, including using longer-term repeat dose protocol, assaying both total RBCs and RETs, and assaying as many RETs and RBCs as practical for regulatory applications. Alternative short-tem dosing protocols have been used, and in most cases performed well; therefore, short-term (including single dose) dosing protocols can generally be used with justification for non-regulatory applications, like screening for strong mutagenic substances.

227. The test data also indicate that the assay is complementary to the erythrocyte *in vivo* MN assay, a combination that results in a more comprehensive analysis of *in vivo* genotoxicity than can be done using either test by itself.

228. A retrospective performance assessment conducted with curated *Pig-a* and conventional test response data indicated that the *Pig-a* assay is highly accurate when responses in the bone marrow TGR assay and rodent cancer in hematopoietic tissues are used to establish true positive and negative responses. The accuracy of the assay was lower, most clearly seen as lower for negative predictivity, when *in vivo* MN responses, TGR responses in all tissues, and cancer responses in all tissues were used to define true positive and negative responses. In general, within the limits of the available data, the assay performed well as an assay for detecting *in vivo* gene mutation.

229. The results of a recent evaluation of curated TGR and comet assay data contrasted with the findings of the current performance analysis for *Pig-a* gene mutation. While *Pig-a* mutation was highly accurate for identifying compounds that were positive and negative in the bone marrow TGR assay, the comet assay was relatively poor (Kirkland et al., 2019). Although the amount of data available for evaluating comet assay performance was even lower than for evaluating *Pig-a* assay performance (15 compounds for comet *vs* 26-28 for *Pig-a*), the comparison highlights the value of using a gene mutation assay for identifying mutagenic substances.

230. The current regulatory applications of the *Pig-a* assay are: 1) a test for evaluating the *in vivo* risks associated with positive responses in *in vitro* gene mutation screening assays, and 2) an *in vivo* gene mutation test that can be integrated with the *in vivo* MN test into repeat-dose toxicology studies to provide information about gene mutation in combination with chromosomal damage and loss. Current guidances and other documents anticipate use of *Pig-a* data for regulatory decision-making.

231. *In vitro Pig-a* assays have been described that should be useful for evaluating substances for *in vivo* testing and addressing hypotheses that are difficult or unethical to test *in vivo* (*e.g*., Bemis et al, 2018; David et al., 2018; Y Wang et al., 2018). For instance, *in vitro Pig-a* assays could be used to rapidly establish *Pig-a* mutation spectra for substances of interest or quantitative analysis of *in vitro Pig-a* data could be used to set plasma concentration targets for evaluating negative *in vivo* responses (see paragraph 93). Much more work will be necessary to develop these assays: Bemis and Heflich (2020) have recommended additional research that should be conducted before these assays can be used confidently for regulatory purposes.

232. A *Pig-a* assay for male rat germ cells is in very early stages, with extensive developmental work necessary before it can be considered a reliable test.

233. It is envisioned that, as the human *PIG-A* assay becomes more widely used, laboratory studies that interface with human studies using this same gene as a reporter of mutation will become increasingly important for regulatory decision-making.

234. The erythrocyte *Pig-a* assay can be readily integrated into *in vivo* general toxicology tests, making the assay consistent with the 3Rs principles of reduction and refinement of animal use. Responses in the *Pig-a* assay are dependent on the cumulative dose of the test substance, rather than the daily dose, making integration into repeat-dose studies (*e.g*., 28- or 90-day general toxicology studies) a particularly attractive approach to maximizing the sensitivity of measuring gene mutation *in vivo*.

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ANNEX I: Substances tested in the rat *Pig-a* assay with consensus calls.

Consensus calls for *Pig-a* responses using different evaluation criteria shown in Table VI. Green highlight = consensus call made by expert committee. 13 consecutive days of dosing or less, short-term; ≥14 consecutive days of dosing, longer-term.

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
3R4F cigarette smoke	Complex mixture	1-30	1 or 2 hr/day (inhalation); histpath P, lung comet P	30 (Both M and F tested)	RBCs, RETs	HT	Dalrymple et al. (2016)	Yes	Yes	N	N	Only two doses, but well conducted study/MN N
Acetaminophen	Hydroxyaniline/ 103-90-2	1-3	0, 500, 1000, 2000 mg/kg/day; limit dose	-4, 15, 30, 45	RBCs, RETs	HT	Janssen, unpublished	No	Yes	I (short-term dosing with RBCs only sig at Day 45 due to outlier)	N	N in both short-term and 29-day dosing protocols/potent liver toxicant
		1-15	0, 250, 500, 1000 mg/kg/day; MTD by weight, WBC reduction	16	RBCs, RETs	HT	Janssen, unpublished	No	Yes	I (N with short-term dosing)	N	
		1-29	0, 250, 500, 1000 mg/kg/day;	-1, 15, 29 (recovery animals,	RBCs, RETs	HT	Janssen, unpublished	No	Yes	N	N	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of columns: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I of E)**	**Notes**
			recovery animals 0, 1000 mg/kg; limit dose	up to 57)								
		1	0, 500, 1000, 2000 mg/kg	-3, 7, 14, 28	RBCs, RETs	PIGRET	Y Suzuki et al. (2016b)	Yes	Yes	I (N with short-term dosing)	N	
2-Acetylaminoflourene	Aromatic amide/ 53-96-3	1	0, 125, 250, 500 mg/kg; literature	8,15,29	RBCs, RETs	PIGRET	Shigano et al. (2016)	Yes	Yes	I (N call with 1-day dosing)	N	Longer dosing protocols all P/requires metabolic activation, MN + in concurrent assays
		1-3	0, 125, 250,500 mg/kg; reduced weight gain, altered %RETs	-1,15,29	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
		1-28	0, 37,5, 75, 150 mg/kg; reduced weight gain, altered %RETs	-1,15,30	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
Acrylamide	α,β-unsaturated amide /79-06-1	1-28	0, 0.33, 0.66, 1.32, 2.7, 5, 10, 20 mg/kg; hind limb paralysis	-1,15,29, 56	RBCs, RETs	HT	Dobrovolsky et al. (2016)	Yes	Yes	E (high dose gives sig RBCs only at Day 56 vs. low dose)	E	Sporadic sig increases for all studies, both short-term and longer-term dosing protocols;

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		1-30	0, 0.5, 1.5, 3, 6, 12 mg/kg; literature	31	RBCs, RETs	HT	Hobbs et al. (2016)	Yes	Yes	E (high dose gives sig RETs only)	E	4 labs; TGR, *Hprt* lymphocyte P (2-month treatment)
		1-3	0, 10, 50 mg/kg; none given	-1,15,29	RBCs, RETs	HT	Novartis, unpublished	Yes	Yes	E (sig RBCs only at high dose on Day 29)	E	
		1-28	0, 5, 10, 20 mg/kg; none given	-1,15,33, 60	RBCs, RETs	HT	Novartis, unpublished	Yes	Yes	E (sig RETs and RBCs at Days 15, 60)	E	
		1	0, 25, 50, 100, 137.5, 175 mg/kg; body weight reduction, altered %RETs	7,14,28	RBCs, RETs	PIGRET	Horibata et al. (2015)	Yes	Yes	E (sig ANOVA for RETs only Day 28)	E	
Aflatoxin B1	Mycotoxin/1162-65-8	1-3	0, 0.25, 0.5, 1 mg/kg/day; MTD estimated from previous 3-day study, deaths in high dose, %RETs	-4, 16, 30, 45, 60	RBCs, RETs	HT	Janssen, unpublished	No	Yes	I (N with short-term dosing protocol)	N	P with longer-term dosing, not with short-term dosing; short-term: Day 4 MN P, Day 3 blood comet N, liver comet I; 29-day dosing *Pig-a*

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			reduced									response weak but sig, Day 4 and 29 MN P, Day 29 liver comet I
		1-15	0, 0.0625, 0.125, 0.25. 0.5 mg/kg/day; MTD estimated from previous 3-day study; body weight, %RETs decrease	-4, 16, 29	RBCs, RETs	HT	Janssen, unpublished	No	Yes	N	N	
		1-29	0, 0.125, 0.25, 0.5 mg/kg/day; MTD estimated from 15-day study; decreases in body weight and %RETs	-4, 16, 29 (recovery animals 57)	RBCs, RETs	HT	Janssen, unpublished	No	Yes	P	P	
4-Aminobiphenyl	Aromatic amine/92-67-1	1-14	0, 5, 10, 15, 25, 50, 75	-1, 15/16, 36/37	RBCs, RETs	HT	F. Hoffmann-LaRoche, unpublished	No	Yes	P	P	Weak P response; severe toxicity at high dose; methemoglobinemia, mild at lower doses, may be confounder

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
o-Anthranilic acid	Aromatic amine/ 118-92-3	1-28	0, 250, 500, 1000 mg/kg; limit dose	-1,15.29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	N	N	N using both longer-term and short-term dosing protocols; non-alerting structure, concurrent MN N
		1-3	0, 500, 1000, 2000 mg/kg/day; limit dose	-1,15,30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	yes		I (N call with 3-day dosing protocol)	N	
Aristolochic acids	Aromatic nitro compound/313-67-7	1	0, 15, 30, 6 mg/kg; dose range finder, decreased body weight	'before dosing', 7,14,28	RBCs, RETs	PIGRET	Koyama et al. (2016)	No	No	P	P	
		1-3	0, 11, 22, 30 mg/kg/day; dose range finder, decreased weight gain	-1, 15, 29, 42	RBCs, RETs	HT	Bhalli et al. (2013b)	Yes	Yes	P	P	Weak concurrent MN
		1-3	0, 10, 20, 30 mg/kg/day; decreased weight gain	-1, 15, 30, 45	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 2.75, 5.5, 11 mg/kg/day; none given	-1, 15, 29, 56	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 2.75, 5.5, 11 mg/kg/day;	-1, 15, 29, 56	RBCs, RETs	HT	Bhalli et al. (2013b)	Yes	Yes	P	P	N concurrent MN

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of columns: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I of E)**	**Notes**
			14-day dose range finder									
Azathioprine	Aromatic nitro/ 446-86-6	1	0, 50, 100, 200 mg/kg; dose range finder, reduced weights and deaths in 200 mg/kg group	-1,7,14, 21,28	RBCs, RETs	PIGRET	Yoshida et al. (2016a)	Yes	Yes	I (N call with 1-day dosing)	N	P with dosing >1 day; Immuno-toxicant; TGR P, lymphocyte *Hprt* N, MN P
		1-3	0.12.5, 25, 50 mg/kg; weight gain reduction, alterations in %RETs	-1,15,29, 42	RBCs, RETs	HT	Dertinger et al. (2016)	Yes	Yes	P	P	
		1-28	0, 6.25, 12.5, 25 mg/kg; reduction in weight gain, alterations in %RETs	-1,15,29, 42	RBCs, RETs	HT	Dertinger et al. (2016)	Yes	Yes	P	P	
Azidothymidine	Nucleoside analog/ 30516-87-1	1	0, 500, 1000, 2000 mg/kg; limit dose	-1, 7, 14, 28	RBCs, RETs	PIGRET	Sanada et al. (2016)	Yes	Yes	I (N call with short-term dosing)	N	N with short-term dosing only; in vivo MN P
		1-7	0-1000 mg/kg/ twice per day;	22, 50	RBCs only	Basic	Guérard et al. (2013)	Yes	Yes	I (N call with insufficient	I (N call with insufficien	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of columns: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I of E)**	**Notes**
			limit dose							protocol)	t protocol)	
Azoxymethane	Alkylating agent/ 24843-45-2	1-3	0, 2.5, 5, 10 mg/kg/day; depression in %RETs, based on dose range finder	- 1,15,29,42	RBCs, RETs	HT	Covance, unpublished	No	Yes	I (3-day dosing protocol; P for RBCs only at Days 15,29,42 driven by single animals)	E (sporadic P responses)	No longer-term dosing data; occasional P for RBCs; P MN on Day 4
$BaSO_4$ NM-220	Metal sulfate nanomaterial	5 day/week for 6 months	50 mg/m^3, 6 hr/day (inhalation); no effect in range finder, no effect on %RETs	At 6 months only	RBCs, RETs	HT	Cordelli et al. (2017)	Yes	Yes	I (N response but only one dose, no toxicity at high dose)	I (N response but only one dose, no toxicity at high dose)	Not clear if single dose is practical limit for inhalation exposure
Benzo[*a*]pyrene	Polycyclic aromatic hydrocarbon/50-32-8	1	0, 75, 150, 300 mg/kg; doses based on literature	0, 7, 14, 28	RBCs, RETs	PIGRET	Kikuzuki et al. (2016)	Yes	Yes	P	P	Consistently P response regardless of dosing protocol; ENU P control
		1-3	0, 125, 250 mg/kg/day; %RET reduction at Day 4	-1, 4, 15, 30, 45, 90	RBCs, RETs	Basic	Phonethpswath et al. (2010)	Yes	Yes	P	P	Concurrent MN-RET P

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		1-3	0, 125 mg/kg twice/day; dose range-finding study	-1, 15, 30, 45	RBCs, RETs	HT	Torous et al. (2012)	Yes	Yes	P	P	MN-RETs P on Day 4
		1-28	0, 125 mg/kg/day; dose range-finding study	-4, 15, 29, 42	RBCs, RETs	HT	Torous et al. (2012)	Yes	Yes	P	P	MN-RETs P on Days 4 and 29
		1-28	0, 37.5, 75, 150 mg/kg/day; MTD estimated from dose range-finder	-1, 15, 29, 56	RBCs, RETs	Basic	Dertinger et al. (2010)	Yes	Yes	P	P	MN-RET P on Days 4 and 29
		1-28	0, 37.5, 75, 150 mg/kg/day; literature	-1, 15, 29, 56	RBCs, RETs	Basic	Bhalli et al. (2011b)	Yes	Yes	P	P	Done in two labs, with different rat strains and comparable results; MN-RETs P Day 4 and 29
2-Butoxyethanol	Ethylene glycol derivative/ 111-76-2	1	0, 10, 35, 100, 250, 450 mg/kg; RBC depression	15, 33	RBCs, RETs	HT	Kenyon et al. (2015)	Yes	Yes	I (N result with short-term dosing protocol)	N	N regardless of dosing protocol; hemolytic substance, Ames N
		1-28	0, 10, 100, 250, 450 mg/kg/day;	15, 29, 43, 57	RBCs, RETs	HT	Kenyon et al. (2015)	Yes	Yes	N	N	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			reduced body weight, RBC depression									
Caffeic acid	Hydroxycinnamic acid/ 331-39-5	1-3	0, 500, 1000, 2000 mg/kg/day; limit dose	-1, 14, 29	RBCs, RETs	HT	Covance, unpublished	Yes	Yes	I (N result with short-term dosing)	N	Concurrent MN P
Carbon nanotubes (MWCNTs)	Multi-walled carbon nanotubes/308068-56-6	1	0, 0.25, 0.5, 1 mg/kg; not clear—no toxicity noted for high dose	28 day only	RBCs	Basic	Horbibata et al. (2017)	Yes	Yes	I (N with one day dosing, basic method, RBCs only, possible insufficient high dose)	I (basic method, RBCs only, possible insufficien t high dose)	Intratracheal administration; concurrent *gpt* TGR assay N in lung
Carboplatin	Antineoplastic crosslinker/ 41575-94-4	1-3	0.75, 15, 30 mg/kg/day; decrease in %RETs	-1, 15, 29	RBCs, RETs	HT	Covance, unpublished	No	Yes	P	P	P MN-RETs at Day 4; better clinical effectiveness than cisplatin
CeO_2 NM-212	Metallic nanomaterial	5 days/wee k for 6 months	0, 0.1, 0.3, 1, 3 mg/m^3 6 hr/day (inhalation); MTD estimated from range-finder	At 6 months	RBCs, RETs	HT	Cordelli et al. (2017)	Yes	Yes	N	N	N result in assay conforming with most criteria; outlier responses removed/ concurrent MN and blood comet N, ENU P control

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		5 days/week for 3 months	0, 0.1, 0.3, 1 mg/m^3 6 hr/day (inhalation); MTD uncertain due to sample loss	At 3 months	RBCs, RETs	HT	Cordelli et al. (2017)	Yes	Yes	I (N with many samples lost due to clotting, MTD uncertain)	I (N with many samples lost due to clotting, MTD uncertain)	
Chlorambucil	Nitrogen mustard alkylator/305-03-3	1	0, 10, 20, 40 mg/kg/day; based on toxicity in 3-day study	-4, 8, 15, 29	RBCs, RETs	PIGRET	Maeda et al. (2016)	Yes	Yes	P	P	Consistently P response, regardless of dosing protocol
		1-3	0, 7.5, 15 mg/kg/day; 30 mg/kg/day was extremely toxic	-1, 15, 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	Concurrent MN-RET P at Day 4
		1-3	0, 3, 6, 12 mg/kg/day; none given	-1, 15, 30, 52	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 1.25, 2.5, 5 mg/kg/day; MTD set by dose range-finding study, reduced %RETs	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	Concurrent MN-RET P at Days 4 and 29
		1-28	0, 0.299, 0.896, 2.69, 5	-5, 16, 29,	RBCs,	HT	Dertinger et	Yes	Yes	P	P	Concurrent MN-RET P at Days 4

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			mg/kg/day; previous studies and tumorigenic dose	57	RETs		al. (2014b)					and 29
		1-28	0, 1.5, 3, 6 mg/kg/day; reduced weight gain	-1, 15, 29, 62	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
4-Chloro-1,2-diaminobenzene	Aromatic amine/ 95-83-0	1-28	0, 30, 100, 200, 300 mg/kg/day; MTD established in dose range finder	-5, 15,29	RBCs, RETs	HT	GSK, unpublished	Yes	Yes	N	N	N result in assay conforming with most criteria/in vivo MN P on Day 4; males only tested
5-(2-Chloroethyl)-2'-deoxyuridine (CEDU)	Pyrimidine analogue/ 90301-59-0	1-5	0, 500, 1000, 2000 mg/kg/day; limit dose	-1, 14, 28, 56	RBCs, RETs	HT	Elhaljouji et al. (2018)	No (but data availab le, awaiti ng postin g)	No (but data availab le, awaitin g posting)	P	P	Consistent P response/ MN-RETs N at Day 6
		1-28	0, 250, 500, 1000; limit dose	-1, 15, 29, 40	RBCs, RETs	HT	Elhaljouji et al. (2018)	No (but data availab le, awaiti	No (but data availab le, awaitin g	P	P	MN-RETs N at Day 29

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
								ng postin g)	posting)			
4-Chloro-*ortho*-toluidine-HCl	Aromatic amine/ 3165-93-3	1-28	0, 25, 50, 100, 150, 200, 250 mg/kg/day; 14-day range-finder	29, 52	RBCs, RETs	HT	Guérard et al. (2018)	No	Yes	I (RBCs P only on Day 29 for all doses—dose response, Day 52 RBCs & all RETs N; possible methema-globin-emia con-founder)	I (RBCs P only on Day 29 for all doses—dose response, Day 52 RBCs & all RETs N; possible methema-globin-emia con-founder)	Inconsistent results/new flow-cytometer used for analysis: possible confounder?
Cisplatin	Antineoplastic crosslinker/15663-27-1	1	0, 0.5, 1, 2 mg/kg; high dose nephrotoxic in previous study	-2, 7, 17, 28	RBCs, RETs	PIGRET	Suzuki et al. (2016c)	Yes	Yes	P	P	Consistent P response in better conducted studies ENU P control;
		1	0, 5 mg/kg; based on clinical range, severe toxicity noted	-1, 7, 14, 28	RBCs	Basic	Pu et al. (2016)	No	No	I (N with single dose, basic method, RBCs only)	I (N with basic method, RBCs only)	
		1-3	0, 0.5, 1, 2 mg/kg/day;	-1, 14, 35,	RBCs,	HT	Bhalli et al.	Yes	Yes	P	P	Day 4 MN-RETs

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			non-lethal toxicity expected	56, 113	RETs		(2013a)					P
		1-3	0, 2 mg/kg/day; non-lethal toxicity expected	-1, 14, 35, 56	RBCs, RETs	PIGRET	Bhalli et al. (2013a)	Yes	Yes	P	P	
		1-3	0, 0.5, 1, 2 mg/kg/day; non-lethal toxicity expected	-1, 14, 35, 56	RBCs, RETs	HT	Bhalli et al. (2013a)	Yes	Yes	P	P	
		1-5	0, 2 mg/kg/day; doses based on clinical range, severe toxicity noted	-1, 7, 14, 28	RBCs	Basic	Pu et al. (2016)	No	No	I (N with short-term dose, basic method, RBCs only)	I (N with basic method, RBCs only)	
		1-28	0, 0.05, 0.1, 0.2, 0.4 mg/kg/day; cancer treatment doses, weight gain, %RETs reduction	-4, 15, 29, 56, 84, 112, 139, 168, 196	RBCs, RETs	HT	Dertinger et al. (2014a)	Yes	Yes	P	P	MN-RETs P at Days 4 and 29
Clofibrate	Fibric acid derivative/ 637-07-	1-28	0, 300 mg/kg/day; MTD based	-5, 29 (only treated	RBCs, RETs	HT	Ji et al. (2019)	No	Yes	N	N	Single dose N, but evaluated as sufficient for WG

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
	0		on carcinogenesis dose in rats; liver weight increased relative to control	animals tested on Day -5)								criteria; MTD indicated by increase in liver weight; same dose negative in sperm *Pig-a*
Compound X	?	28 days – Total of 26 exposure days – 7d/week (except 2 days) – 6hr/day	0, 5000, 15000, 50000 ppm (inhalation); dose range finder indicates tox at higher dose	0, 28	RBCs, RETs	HT	Nesslany (Pasteur-Lille), Unpublished	No	Yes	N	N	Study conforms with WG criteria but not useful for RPA
Compound X-A	Nucleoside analog	?	0, 30, 50, 100 mg/kg/day; reached MTD	-1, 29	RBCs, RETs	HT	BioReliance, unpublished	No	No (but data available, awaiting posting)	P	P	Study conforms with WG criteria but not useful for RPA (also are unpublished genetox data---MLA large colony P)
Cyclophosphamide	Nitrogen mustard alkylator/6055-19-2	1	0, 10, 40, 80 mg/kg; expected to produce non-lethal toxicity	-1, 28, 42, 56, 63, 84, 112	RBCs	Basic	Bhalli et al. (2013a)	Yes	Yes	P	P	Weak P in studies using robust analysis methods regardless of treatment protocol; MN-RET P on Day 3,

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
												4
		1	0, 20 mg/kg; decrease in %RETs	'pre', 7, 14, 21, 28, 42, 70	RBCs	Basic	Kimoto et al. (2012)	No	No	I (N with short-term dose, basic method, RBCs only)	I (N with basic method, RBCs only)	MN-RETs on Days 2 and 15
		1	0, 20, 50 mg/kg; test to MTD: blood in urine on Day 3	-4, 8, 15, 29	RBCs, RETs	PIGRET	Kimoto et al. (2014)	Yes	Yes	P	P	
		1-3	0, 7.5, 15, 30 mg/kg/day; MTD estimated from dose-range finder, decrease weight gain	-1, 15, 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	Day 4 MN-RETs P
		1-5 plus 8-12	0, 5 mg/kg/day; decrease in %RETs	'pre', 7, 14, 21, 28, 42, 70	RBCs	Basic	Kimoto et al. (2012)	No	No	I (N with short-term dose, basic method, RBCs only)	I (N with basic method, RBCs only)	MN-RETs P on Day 2 and 15
		1-28	O, 2.5, 5, 10 mg/kg/day; reduction in	-4, 8, 15, 29	RBCs, RETs	PIGRET	Kimoto et al. (2014)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			body weight									
		1-28	0, 2.5, 5 mg/kg/day; MTD estimated from dose-range finder, decrease weight gain	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	Days 4 and 29 MN-RETs P; 1.25 mg/kg/day group excluded because of extraordinarily high responses
Dibenzo[*a,l*]pyrene	PAH/ 191-30-0	1-2	0, 7, 14, 28 mg/kg/day; dose range-finder	15, 28	RBC, RETs	HT	F. Hoffmann LaRoche, unpublished	No	Yes	P	P	Strong positive, regardless of dosing protocol; a few outlier responses
		1-28	0, 0.03125, 0.0625, 0.125, 0.25, .0.5, 1 mg/kg/day; dose range finder: toxicity in 1 mg/kg/day animals	Pre, 29, 42	RBCs, RETs	HT	F. Hoffmann LaRoche, unpublished	No	Yes	P	P	
Diethylnitrosamine	Nitrosamine/ 55-18-5	1-28	O, 5, 10 mg/kg; animals at 20 mg/kg died	-1,15,29	RBCs, RETs	basic	Shi et al. (2011)	No	No	I (N call with basic method)	I (N call with basic method)	Generally P with 28-day dosing, HT method and N with short-term dosing protocol; 4 labs, requires metabolic
		1-28	0, 3.125, 6.25, 12.5 mg/kg;	15,29,42	RBCs, RETs	HT	Avlasevich et al. (2014)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			reduced body weight gain; altered %RETs									activation, blood/BM MN weak-N, BM TGR N, liver MN P
		1-28	0, 5, 10, 15 mg/kg; literature plus reduced body weight and %RETs	29	RBCs, RETs	HT	Khanal et al. (2018)	No	No	P (sig for RBCs only)	P (sig for RBCs only)	
		1	0, 37.5, 75, 150 mg/kg; high dose decreased motor activity and weight	-3,7,14, 28	RBCs, RETs	PIGRET	Wada et al, (2016)	No	No	I (N call with 1-day dosing)	N	
3,5-Difluoroboronic acid	Aryl boronic acid/ 156545-07-2	1-28	0, 30, 100, 300 mg/kg/day; MTD estimated from 7-day study- %RETs reduced	-6, 15, 29	RBCs, RETs	HT	GSK, unpublished	Yes	Yes	N	N	N in study conforming with WG criteria; concurrent ENU control P; concurrent MN and liver comet N; Weak Ames and MLA P
[1-(3-Dimethylamino-propyl)-3-ethylcarbodiimide Hydrochloride (EDAC)	Carbodiimide/ 25952-53-8	1-28	0, 150, 300, 600, 800 mg/kg/day; dose range	-4, 15, 30	RBCs, RETs	HT	BMS, unpublished	No	Yes	N	N	N in study conforming with WG criteria; Ames and *in vitro* MN P; tested up

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			finder									to MTD, rapid degradation into non-mutagenic metabolite in acid conditions and in aqueous solutions (e.g., in SD rats)
7,12-Dimethylbenz[a]-anthracene	PAH/ 57-97-6	1	0, 20, 60 mg/kg	Pre, 7, 14, 28	RBCs, RETs	PIGRET	Kimoto et al. (2013)	Yes (Teijin only)	Yes (Teijin only)	P (PIGRET in 3 labs, RBCs in 4 labs, RBC in transported samples in 1 lab)	P (PIGRET in 3 labs, RBCs in 4 labs, RBC in transported samples in 1 lab)	Consistently P, regardless of protocol; P in 11 labs, twice at one lab (SD and Wistar rats); Requires metabolic activation; P *Pig-a* lymphocyte, MN, comet data
		1-3	0, 40 mg/kg/day	28	RBCs, RETs	Early method	Bryce et al. (2008)	No	No	P	P	
		1-3	0, 40 mg/kg/day	42	RBCs	Early method	Bryce et al. (2008)	No	No	P	P	
		1-3	0, 25 mg/kg/day	35	RBCs	Basic	Dobrovolsky et al. (2015)	No	No	P	P	
		1-3	O, 25, 50 mg/kg/day; reduced %RETs	-1, 15, 30, 45, 90	RBCs, RETs	Basic	Phonethepswath et al. (2010)	Yes	Yes	P (two rat strains)	P	
		1-3	0, 40 mg/kg/day;	-1, 7, 14, 28, 35, 56	RBCs	Basic	Pu et al. (2016)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			reduced body weight									
		1-3	0, 12/5, 25 mg/kg/day	-1, 15, 30, 45	RBCs, RETs	HT	Janssen, unpublished	Yes	Yes	P	P	
		1-3	0, 25 mg/kg/day	-5, 15, 29	RBCs, RETs	HT	GSK, unpublished	No	Yes	P	P	
		1-28	0, 2.5, 5, 10 mg/kg/day; toxicity at 10 mg/kg/day	-1, 15, 29, 56	RBCs, RETs	Basic	Dertinger et al. (2010)	Yes	Yes	P	P	
		1-28	0, 2.5, 5, 10 mg/kg/day; literature	-1, 15, 29	RBCs, RETs	Basic	Shi et al. (2011)	No	No	P	P	
1,2-Dimethylhydrazine	Hydrazine/ 306-37-6	1	0, 25, 50, 100 mg/kg/day; MTD estimated from dose range finder	-4, 7, 14, 28	RBCs, RETs	PIGRET	Chikura et al. (2016a)	Yes	Yes	I (N call with short-term dosing)	N	
Ethyl carbamate (urethane)	Carbamate/ 51-79-6	1	0, 250, 500, 1000 mg/kg/day; %RETs reduced	-1, 7, 14,28	RBCs, RETs	PIGRET	Narumi et al. (2016)	Yes	Yes	P	P	Consistent P conforming with regardless of dosing protocol; concurrent in vivo MN P; *Pig-a* consistently P only for RETs
		1-3	0, 600 mg/kg/day;	-4, 15, 30,	RBCs,	HT	Labash et al.	Yes	Yes	P	P	Male and female rats tested

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			based on previous P results	46	RETs		(2015c)					similarly
		1-3	0, 400, 800 mg/kg/day; claimed to be MTD, decreases in %RETs at Day 4	-1, 16, 30, 45	RBCs, RETs	HT	G Chen et al. (2019)	No (but data available, awaiting posting)	No (but data available, awaiting posting)	P	P	Concurrent MN and blood comet P on Day 4
		1-28	0, 250 mg/kg/day; previous P results	-4, 15, 29, 43	RBCs, RETs	HT	Bemis et al. (2015)	Yes	Yes	P	P	Used as P control for methyl carbamate study
		1-28	0, 25, 100, 250 mg/kg/day; decreased body weight gain, toxicity to bone marrow	-3/-5, 15, 29	RBCs, RETs	HT	Stankowski et al. (2015)	No	No	P	P	Concurrent MN, and blood and liver comet P; blood chrom abs N
		1-28	0, 150, 300 mg/kg/day; body weight reduced, %RETs reduced at	-1 15, 28, 43, 57	RBCs, RETs	HT	G Chen et al. (2019)	No (but data available, awaiting	No (but data available, awaiting posting	P	P	Concurrent MN and blood, liver, kidney, lung comet P

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			Day 4					postin g)	)			
Ethyl methanesulfonate	Alkylating agent/ 62-50-0	1	180, 360 mg/kg	-1, 14, 28, 42, 56	RBCs	Basic	Kimoto et al. (2012)	Yes	Yes	P	P	Consistent P regardless of protocol; P in multiple labs using different methods
		1	0, 100, 175, 350, 700 mg/kg/ MTD from literature	-1, 15, 29, 57	RBCs, RETs	Basic	Dobo et al. (2011)	No	No	P	P	
		1	0, 360, 720 mg/kg; literature MTD	0, 7, 14	RETs	PIGRET	Itoh et al. (2014)	No	No	P	P	
		1	0, 180, 360, 720 mg/kg; literature MTD	-1, 7, 14, 28	RBCs, RETs	PIGRET	Itoh et al. (2016b)	Yes	Yes	P	P	
		1-7	0, 120 mg/kg/day	23, 50	RBCs, RETs	Basic	Guérard et al. (2013)	Yes	Yes	P	P	
		1-28	0, 6.25, 12.5, 25, 50, 100 mg/kg/day; MTD from literature	-1, 15, 29, 55	RBCs, RETs	Basic	Dobo et al. (2011)	No	No	P	P	
		1-28	0, 12,20 mg/kg/day	-1, 14, 28, 42, 56	RBCs	Basic	Kimoto et al. (2012)	Yes	Yes	P	P	
		1-28	0, 6.26, 12.5, 25, 50, 100	-1, 15, 28,	RBCs,	Basic	Gunther et	No	No	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			mg/kg/day; literature, %RETs reduction	55, 105	RETs		al. (2014)					
		1-28	25, 50, 100 mg/kg/day; literature MTD	0, 7, 14, 28	RBCs	Basic	Itoh et al. (2014)	No	No	P	P	
N-Ethyl-*N*-nitrosourea*	Alkylating agent/ 759-73-9	1	0, 10, 40 mg/kg; literature	Before, 14, 28	RBCs	basic	Kimoto et al. (2013)	No	No	P	P	Interlab trial with 4 labs with 5th lab using samples from another lab
		1	0, 10, 40	Pretreatment, 7, 14, 28	RBCs, RETs	PIGRET	Kimoto et al. (2016)	Yes		P	P	Step 1 of JEMS/ MMS study; 13 labs participated
		1-3	0, 20, 40 mg/kg/day; reduced %RETs	-1, 15, 30, 45, 90, 180	RBCs, RETs	Basic	Phonethepswath et al. (2010)	Yes		P	P	MN-RETs P at Day 4
		1-3	0, 20, 40 mg/kg/day (plus others); literature	-1, 15, 30 (plus others)	RBCs, RETs	Basic	Dertinger et al. (2011b)	No	No	P	P	14 lab validation study demonstrating transferability, reproducibility
		1-3	0, 1, 5, 25 mg/kg/day; literature	-4, 15, 29, 46	RBCs, RETs	HT	Labash et al. (2015a)	Yes		P	P	Males and female rats produce comparable results; MN-RETs P

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		1-28	0, 2.5, 5, 10 mg/kg/day; preliminary range-finding study; reduced weight gain	-1, 15, 29, 56	RBCs, RETs	Basic	Dertinger et al. (2010)	Yes		P	P	MN-RETs P at Days 4 and 29
		1-28	0, 2.5, 5, 10 mg/kg/day; literature	-1, 15, 29 or 31, 56 or 57	RBCs, RETs	Basic	Cammerer et al. (2011)	Yes		P	P	Performed in two labs; MN-RETs, blood, liver, colon comet P, *Hprt* lymphocyte mutation P; colon, liver MN N
Etoposide	Topoisomerase inhibitor; 33419-42-0	1	0, 5, 10, 20 mg/kg (i.v.); severe myelode-pression	-6, 2, 7, 14, 28	RBCs, RETs	PIGRET (but no enrichment on Day 2)	Yamamoto and Wakada (2016)	Yes	Yes	I (N in short-term study)	N	Sporadic P in some studies; MN P
		1-3	0, 14, 25, 28.5, 57 mg/kg/day; none given	-1, 15	RBCs, RETs	HT	Novartis, unpublished	No	No (but data available, awaiting posting)	E (called N but Day 15 RBCs all sig)	E	
		1-28	0, 14.25, 28.5, 57	-1, 15, 29	RBCs,	HT	Novartis,	No	No (but data	E (called N but Day 15	E	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of columns: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I of E)**	**Notes**
			mg/kg/day; none given		RETs		unpublished		available, awaiting posting)	high-dose RETs P, Day 29 RBCs P for 28.5 and 57 mg/kg)		
5-Fluorouracil	Pyrimidine analogue/ 51-21-8	1-3	0, 11.5, 23, 46 mg/kg/day; MTD estimated from 3-day dose range finder	-1, 15, 29, 45	RBCs, RETs	HT	Zhou et al., (2014)	Yes	Yes	I (N with short-term dosing protocol)	N	N with short-term dosing only conforming with extended criteria; concurrent MN P
Furan	Heterocycle/110-00-9	5/week for 8 weeks	0, 2, 8, 16, 30 mg/kg/day; reduced body weight	7, 56	RBCs	Basic	McDaniel et al. (2012)	Yes	Yes	I (N call with basic method, RBCs only)	I (N call with basic method, RBCs only)	N call with basic method, RBCs only; requires metabolic activation by CYP2E1; concurrent spleen lymphocyte *Hprt* and *Pig-a* and *cII* liver mutation assays N
Glycidamide	Oxirane amide/	1-3	0, 10, 50, 100	-1, 15, 28	RBCs,	HT	Novartis,	No	Yes	P	P	P response with

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
	5694-00-8		mg/kg/day; none given		RETs		unpublished					short-term dosing; high dose clearly elevated for RETs and RBCs
Gycidyl methacrylate	Methacrylate ester/ 96-24-2	1-28	0, 50, 100, 150 mg/kg; doses based on dose-range-finder, reduction in weight gain	-1, 15, 29. 56	RBCs, RETs	HT	Dobrovolsky et al. (2016)	Yes	Yes	P	P	P in study conforming with WG criteria; some mix ups in dosing; concurrent MN-RET P on Day 4
2-Hydroxypyridine-N-oxide (HOPO)	Pyridine derivative/ 13161- 30-3	1-28	0, 50, 150, 500 mg/kg/day; 2-week dose range finder	-4, 28	RBCs, RETs	HT	BMS, unpublished	No	Yes	N	N	N in study confirming with WG criteria; concurrent ENU plus cyclophosphamide P control: concurrent MN and liver/intestine comet (no result given)
Hydroxyurea	Inhibits DNA replication/ 127-07-1	1	0, 250, 500, 1000 mg/kg; dose range finder	Pre, 8, 15, 29	RBCs, RETs	PIGRET	Adachi et al. (2016)	No	No	I (N with short-term dosing (outlier ignored))	N	N in study conforming with WG criteria; 2 labs; *in vivo* MN P
		1-3	0, 62.5, 125, 250	-4, 15, 30,	RBCs,	HT	Dertinger et	Yes	Yes	I (N with short-term	N	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			mg/kg/day; dose range finder	45	RETs		al. (2012)			dosing)		
		1-28	0, 31.25, 62,5, 125 mg/kg/day; dose range finder	-4, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	N	N	
Ionizing radiation	X-ray	1-3	0, 0.33, 0.66, 1 Gy/day; reduced %RETs	-1, 7, 14, 21, 35, 42, 56, 63, 84, 112	RBCs	Basic	Bhalli et al. (2013a)	Yes	Yes	P	P	Consistently P regardless of protocol; concurrent MN, lymphocyte *Hprt* assays P
	Cs137	1	0, 0.5, 2 Gy; literature value	15, 29, 43, 60, 81	RBCs, RETs	HT	Litron, unpublished	Yes	Yes	P	P	Females tested
		1/week for 4 weeks	0, 0.5 Gy /treatment	15, 29, 43, 60, 81	RBCs, RETs	HT	Litron, unpublished	Yes	Yes	P	P	Females tested
Isopropyl methanesulfonate	Alkylating agent/ 926-06-7	1	0, 3.5, 7, 14, 28, 56 mg/kg; Literature and dose range-finding	-2, 15, 22, 28, 56	RBCs, RETs	HT	Coffing et al. (2015)	Yes	Yes	P	P	P in most robust studies; 2 labs
		1	0, 50, 100, 200 mg/kg; dose range finder	-1, 7, 14, 28	RBCs, RETs	PIGRET	Itoh et al. (2016a)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		1-28	0, 0.125, 0.25, 0.5, 1, 2 mg/kg/day; Literature and dose range-finding	-2, 15, 21, 29, 49, 56, 77	RBCs, RETs	HT	Coffing et al. (2015)	Yes	Yes	P	P	
		1-28	0, 0.1, 0.22, 0.3, 0.4, 1 mg/kg/day	29, 56	RBCs, RETs	HT	Coffing et al. (2015)	No	No	E (no trend for RETs, RBCs P)	E (no trend for RETs, RBCs P	
Isopropyl toluenesulfonate	Alkylating agent/ 2307-69-9	1	0, 125, 250, 500 mg/kg; dose range-finder, weight loss	7, 14, 28	RBCs, RETs	PIGRET	Chikura et al. (2016b)	Yes	Yes	P	P	Strong, dose related P
Melamine	Triaminotriazine/ 108-781	1-3	0, 500, 1000, 2000 mg/kg/day; body weight reduced, RET toxicity, limit dose	-1, 15, 29, 60	RBCs, RETs	HT	Tu et al. (2015)	Yes	Yes	I (N with short-term dosing protocol only)	N	N with short-term dosing only; in vivo MN N, Ames and in vitro MN N, probable non-genotoxic carcinogen
Melphalan	Alkylating agent; nitrogen mustard/ 148-82-3	1	0, 1.25, 2.5, 5 mg/kg; dose range finder: reduced %RETs	Pre, 8, 15, 29	RBCs, RETs	PIGRET	Adachi et al. (2016)	No	No	P (RETs only)	P (RETs only)	P in studies regardless of protocol; 3 labs; concurrent MN P
		1-3	0, 1.25, 2.5 mg/kg; dose range finder: altered	-1, 15. 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			%RETs; 5 mg/kg extremely toxic									
		1-3	0, 0.75, 1/5, 3 mg/kg	-1, 15, 30, 45	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	O, 0.1875, 0.375, 0.75; dose range finder: altered %RETs	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
		1-28	O, 0.1875, 0.375, 0.75; dose range finder: altered %RETs	-1, 15, 30, 45	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 0.0321, 0.0938, 0.28, 0.75 mg/kg/day; literature, cancer doses, weight loss	-4, 15, 29, 56	RBCs, RETs	HT	Dertinger et al. (2014b)	Yes	Yes	P	P	
Methyl carbamate	Carbamate / 598-55-0	1-28	0, 125, 250, 500 mg/kg/day; MTD established by dose-range finder	-4, 15, 29, 43	RBCs, RETs	HT	Bemis et al., (2015)	Yes	Yes	N	N	N conforming with WG criteria; concurrent MN N; generally N genetox, but P in Drosophila; carcinogenic in

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
												rats but not mice
Mitomycin C	Alkylating/ crosslinking agent/ 50-07-7	1-3	0, 0.5, 1, 2 mg/kg/day; toxicity to high dose group	-1, 15, 29	RBCs, RETs	HT	Chung et al. (2018)	No (but data available, awaiting posting)	No (but data available, awaiting posting)	P	P	P in all 3 labs of interlab trial; concurrent blood MN P, blood comet N
4,4'-Methylenedianaline	Aromatic amine/ 101-77-9	1-28	0, 4.4, 13. 40, 120 mg/kg/day; not clear if MTD reached	7, 14, 28	RBCs, RETs	PIGRET	Sanada et al. (2014)	No	No (but data available, awaiting posting)	E (RBCs sig for 40 on Day 14, and for 40 and 120 on Day 28)	E (RBCs sig for 40 on Day 14, and for 40 and 120 on Day 28)	Occasional P responses in longer-term assay, but rat to rat frequencies very variable
		1	0, 40, 130, 240; not clear if MTD reached	7, 14, 28	RBCs, RETs	PIGRET	Sanada et al. (2014)	No	No (but data available, awaiting posting)	I (N with short-term dosing protocol)	N	
Methyl methanesulfonate	Alkylating agent/ 66-27-3	1	0, 50, 100, 200 mg/kg; reduction in %RETs;	Pre, 8, 15, 29	RBCs, RETs	PIGRET	Muto et al. (2014)	No	No	P	P	Consistently P responses in 3 labs; concurrent comet, MN in

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			literature									bone marrow and peripheral blood, p-H2AX in liver tissue P
		1-3	0, 22.5, 45, 90 mg/kg/day; dose range-finder	-4, 15, 30,45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
		1-28	0, 7.5, 15, 30 mg/kg/day; dose range-finder	-5, 15, 30, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
		1-28	0, 7.5, 15, 30 mg/kg/day; reduced %RETs; literature	8, 15, 29	RBCs, RETs	PIGRE T	Muto et al. (2014)	No	No	P	P	
		1-28 + 63-65	0, 1.25, 2.5, 5, 10, 15, 30 mg/kg/day; reduced body weight gain, alterations in %RETs	-1, 15, 28, 44, 65	RBCs, RETs	HT	Zeller et al. (2016)	Yes	Yes	P	p	
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Tobacco specific nitrosamine/ 64091-91-4	1-90	O (sham), 0 (vehicle), 0.2, 0.8, 3.2, 7.8; preliminary dose range-finder	7, 35, 63, 98, 140	RBCs, RETs	HT	Mittelstaedt et al. (2019)	Yes	Yes	N	N	NNK P for *Pig-a* mutation *in vitro* at $\geq$100 µg/ml (+S9)
N-Methyl-*N*-	Alkylating agent/	1	0, 25, 50, 100	8, 15, 29	RBCs,	PIGRE	Muto et al.	No	No	P	P	Consistent P at

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
nitrosourea	66-27-3		mg/kg		RETs	T	(2016)					higher doses in 4 labs; concurrent MN, blood and liver comet P
		1-3	0, 15, 30 mg/kg/day; reduced %RETs	-1, 15, 30, 45, 90	RBCs, RETs	Basic	Phonethepswath et al. (2010)	No	No	P	P	
		1-28	0, 2.5, 5, 10 mg/kg/day; range-finder, reduced weight gain in high dose	-1, 15, 29, 56	RBCs, RETs	Basic	Dertinger et al. (2010)	No	No	P	P	
		1-28 (conducted at 2 labs)	0, 2.5, 5, 10 mg/kg/day; literature, reduced %RETs	-1, 15, 29 (GSK) and -1, 15, 29, 43, 57 (BMS)	RBCs, RETs	Basic	Lynch et al. (2011)	Yes (GSK and BMS data)	Yes (GSK and BMS data)	P	P	
		1-28	0, 0.1, 0.3, 0.6, 0.9, 1.25, 2.5 mg/kg/day; low dose study	15. 29	RBCs, RETs	Basic	Lynch et al. (2011)	No	No	I (N response but basic method, not tested to MTD)	I (N response but basic method, not tested to MTD)	
		1-28	0, 0.9, 1.3, 2.5, 5 mg/kg/day;	-4, 15, 29	RBCs, RETs	HT	BMS, unpublished	Yes	Yes	P	P	
3-Monochloropropane-1,2-diol	Chloropropane/96-24-2	5/week for 4 weeks	No treatment, 5mL/kg/BW oil, 40 mg/kg/BW;	0, 15, 29	RBCs	Basic	Onami et al. (2014)	Yes	Yes	I (N with single dose, basic method,	I (N with single dose, basic method,	In vitro genotoxic food contaminant; IARC Group 2B

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			literature cancer assay: no body weight or major tox changes, no alteration in %RETs							RBCs only not clear MTD reached)	RBCs only, not clear MTD reached)	carcinogen; in vivo MN, *gpt*, Spi, and comet N
3-Monochloropropane-1,2-diol palmitate diester	Chloropropane derivative	5/week for 4 weeks	No treatment, 5mL/kg/BW oil, 220 mg/kg/BW; equal molar to parent: no body weight or major tox changes, no alteration in %RETs	0, 15, 29	RBCs	Basic	Onami et al. (2014)	Yes	Yes	I (N with single dose, basic method, RBCs only, not clear MTD reached)	I (N with single dose, basic method, RBCs only, not clear MTD reached)	MN, *gpt*, and Spi N
3-Monochloropropane-1,2-diol palmitate monoester	Chloropropane derivative	5/week for 4 weeks	No treatment, 5mL/kg/BW oil, 130 mg/kg/BW; equal molar to parent: no body weight or major tox changes, no alteration in %RETs	0, 15, 29	RBCs	Basic	Onami et al. (2014)	Yes	Yes	I (N with single dose, basic method, RBCs only, not clear MTD reached)	I (N with single dose, basic method, RBCs only, not clear MTD reached)	MN, *gpt*, and Spi N
3-Monochloropropane-	Chloropropane	5/week for 4	No treatment, 5mL/kg/BW	0, 15, 29	RBCs	Basic	Onami et al.	Yes	Yes	I (N with single	I (N with single	MN, *gpt*, and

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
1,2-diol oleate diester	derivative	weeks	oil, 240 mg/kg/BW; equal molar to parent: no body weight or major tox changes, no alteration in %RETs				(2014)			dose, basic method, RBCs only, not clear MTD reached)	dose, basic method, RBCs only, not clear MTD reached)	Spi N
Methylphenidate	Phenylethylamine derivative/ 113-45-1	3/day for 21 days	0, 9 mg/kg/day; reduction in %RETs	Only 62	RBCs both M and F	Basic	Dobrovolsky et al. (2010a)	Yes	Yes	I (N with single dose, basic methods, RBCs only)	I (N with single dose, basic methods, RBCs only)	
4-Nitroquinoline-*N*-oxide	Aromatic N-oxide/ 56-57-5	1	0, 50, 100 mg/kg; severe toxicity at 200 mg/kg	Pre, 8, 14, 21, 28, 42, 56, 70	RBCs	Basic	Kimoto et al. (2012)	No	No	P	P	P in 9 labs, E in one with basic method; concurrent MN and comet P and N, and chromosome aberrations N
		1	0, 25, 50 100 mg/kg; severe toxicity at 200 mg/kg	-1, 8, 15, 29	RBCs, RETs	PIGRET	Kimoto et al. (2013)	Yes	Yes	P (P in 4 labs)	P (P in 4 labs)	
		1	0, 17.5, 35, 70, 105, 140 mg/kg; literature	-4, 15, 29	RBCs, RETs	Basic	Roberts et al. 2016)	No	No	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		1-3	0, 12.5, 25 mg/kg/day	-1, 15, 30, 45, 90	RBCs, RETs	Basic	Phonetheps wath et al. (2010)	Yes	Yes	P	P	
		1-3	0, 5.8, 11.7, 23.3, 35, 46.7 mg/kg/day	-1, 17, 31	RBCs, RETs	Basic	Roberts et al. (2016)	No	No	P	P	
		1-28	0, 2.5, 3.75, 5 mg/kg/day; previous data, reduced body weight gain	0, 15, 29	RBCs, RETs	Basic	AbbVie, unpublished	No	Yes	E (considerable variation, and high backgrounds)	E (considerable variation, and high backgrounds)	
		1-28	0, 1.25, 2.5, 5 mg/kg/day; reduced weight gain	-1, 15, 29, 56	RBCs, RETs	Basic	Dertinger et al. (2010)	Yes	Yes	P	P	
		1-28	0, 1.25, 2.5, 3.75, 5 mg/kg/day; severe toxicity at 7.5 mg/kg/day	-1, 15, 29	RBCs, RETs	Basic	Stankowski et al. (2011)	Yes	Yes	P	P	
		1-28	0, 1.5, 3, 6 mg/kg/day; reduction in weight gain, %RETs	-1, 15, 29	RBCs, RETs	HT	Zhou et al. (2014)	Yes	Yes	P	P	
Phenobarbital	Barbiturate/50-06-6	1-28	0, 100	-5, 29	RBCs,	HT	Ji et al.	No	Yes	I (N with	I (N with	Only tested half

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			mg/kg/day; no effect on %RETs		RETs		(2019)			single dose and no indication of MTD)	single dose and no indication of MTD)	animals at day -5 (treatment group only); CYP inducer
Procarbazine	Alkylating agent/ 671-16-9	1-3	0, 50, 100, 200 mg/kg/day	-1, 15, 29	RBCs, RETs	HT	Covance, unpublished	Yes	Yes	P	P	Consistently P in 3 labs, regardless of protocol; requires metabolic activation; RET-MN and Comet P, *Pig-a* lymphocytes P, lymphocyte mutant sequencing data
		1-3	0, 100 mg/kg/day	29	RBCs, RETs	HT	Revollo et al. (2017a)	No	No	P	P	
		1-3	0, 75, 150 mg/kg/day; dose range finding study	-1, 16, 30, 45	RBCs, RETs	HT	G Chen et al. (2019)	No (but data available, awaiting posting)	No (but data available, awaiting posting)	P	P	
		1,3,5	0, 100 mg/kg/day; body weight decease	-1, 7, 14, 28, 35, 56	RBCs	Basic	Pu et al. (2016)	Yes	Yes	P	P	
		1-28	0, 30, 60 mg/kg/day	-1, 15, 28, 43, 57	RBCs, RETs	HT	G Chen et al. (2019)	No (but data available, awaiting postin	No (but data available, awaiting posting	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
								g)	)			
1,3-Propane sultone	Alkylating agent/ 1120-71-4	1	0, 150, 300, 600 mg/kg; preliminary range-finder, reduced body weight gain	Pre, 8, 15, 29	RBCs, RETs	PIGRET	Shigano et al. (2016)	Yes	Yes	P	P	Consistently positive, regardless of protocol; 7 labs; concurrent blood MN and blood comet P
		1-3	0, 20, 40, 80 mg/kg/day; dose range finders, reduced body weight gain	-1, 15, 30,45	RBCs, RETs	HT	Dertinger et al. (2011c)	Yes	Yes	P	P	
		1-3	0, 80 mg/kg/day; Literature	-4, 15, 30, 46	RBCs, RETs	HT	Labash et al. (2015c)	Yes	Yes	P (in both males and females)	P (in both males and females)	
		1-3	0, 20, 40, 80 mg/kg/day	-1, 15, 29	RBCs, RETs	HT	Chung et al. (2018)	No (but data available, awaiting posting)	No (but data available, awaiting posting)	P (in all 3 labs of interlab trial)	P (in all 3 labs of interlab trial)	
		1-28 (high dose, 1-21)	0, 5, 15, 25 mg/kg/day; Literature values, reduced body	-5, 15, 29	RBCs, RETs	HT	GSK, unpublished	No	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			weight gain									
		1-28	0, 12.5, 25, 50/37.5 mg/kg/day; dose-range-finding studies, severe toxicity by 50 mg/kg dose	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2011c)	Yes	Yes	P	P	
		1-28	0, 12.5, 25, 50 mg/kg/day	-1, 15, 29, 45	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 1.28, 3.84, 11.52, 37.5 mg/kg/day; based on TD50, decrease in %RETs, body weight gain	-4, 16, 29, 56	RBCs, RETs	HT	Dertinger et al. (2014b)	Yes	Yes	P	P	
N-Propyl-nitrosourea	Alkylating agent/ 816-57-9	1-3	0, 30. 60, 120 mg/kg/day; literature and dose range-finding	-1, 15, 29	RBCs, RETs	HT	Chung et al. (2018)	No (but data available, awaiting posting)	No (but data available, awaiting posting)	P	P	P in all 3 labs of interlab trial; concurrent blood MN and blood comet P

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of columns: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I of E)**	**Notes**
Pyrene	PAH/ 129-00-0	1	0, 500, 1000, 2000 mg/kg; limit dose: severe toxicity at 2000	7, 14, 21, 28	RBCs, RETs	PIGRET	Yoshida et al. (2016b)	Yes	Yes	I (N with 1-day dosing protocol)	N	N regardless of protocol; concurrent MN assay N
		1-3	0, 500, 1000 mg/kg/day; extreme toxicity at 2000	-1, 15, 30, 45	RBCs, RETs	HT	Torous et al. (2012)	Yes	Yes	I (N with 3 day dosing protocol)	N	
		1-28	0, 125, 250, 500 mg/kg/day;	-4, 15, 29, 42	RBCs, RETs	HT	Torous et al. (2012)	Yes	Yes	N	N	
Sodium chloride	Salt/ 7647-14-5	1-3	0, 500, 1000, 2000 mg/kg/day; limit dose	-5, 15, 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	I (N in 3-day study)	N	N regardless of protocol; concurrent MN N
		1-28	0, 250, 500, 1000 mg/kg/day; limit dose	-5, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	No: (but data available, awaiting posting)	No (but data available, awaiting posting)	N	N	
Sulfisoxazole	Aryl sulfonamide/ 127-69-5	1-3	0, 500, 1000, 2000 mg/kg/day;	-1, 15, 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	I (N with 3-day dosing	N	N regardless of protocol; concurrent MN N

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			limit dose							protocol)		or without biological significance
		1-28	0, 250, 500, 1000 mg/kg/day; limit dose	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	N	N	
Temozolomide	Imidazotetrazine; (Alkylating agent)/ 85662-93-1	1	0, 25, 50, 100 mg/kg; preliminary dose range-finder	8, 15, 29	RBCs, RETs	PIGRET	Muto et al. (2016)	No	No	P	P	Concurrent MN and liver, blood, and jejunum comet P
		1-5	0, 0.375, 0.75, 1.5, 3.75, 7.5, 15 mg/kg/day; dose range-finder	-5, 29, 44	RBCs, RETs	HT	Guérard et al. (2017)	No	Yes	P	P	
Thiotepa	Aziridine/ 52-24-4	1-3	0, 3.75, 7.5, 15 mg/kg/day; preliminary dose range-finder: reduced weight gain, altered %RETs	-1, 15, 30, 45	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	Consistent positive in two labs, some samples assayed in 3rd lab; concurrent MN P
		1-3 (males and	0, 7.5 mg/kg/day; literature	-4, 15, 30, 46	RBCs, RETs	HT	Labash et al. (2015c)	Yes	Yes	P	P	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
		females)										
		1-3	0, 3.75, 7.5 mg/kg/day	-1, 15, 29, 45	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
		1-28	0, 2.5, 5, 10 mg/kg/day; preliminary dose range-finder: reduced weight gain, altered %RETs	-1, 15, 29, 42	RBCs, RETs	HT	Dertinger et al. (2012)	Yes	Yes	P	P	
		1-28	0, 0.0547, 0.164, 0.492, 10 mg/kg/day; literature, high dose reduced to 5 mg/kg/day due to toxicity	-4, 15, 29, 56	RBCs, RETs	HT	Dertinger et al. (2014b); Gollapudi et al. (2015)	Yes	Yes	P	P	
		1-28	0, 2, 4, 8 mg/kg/day	-1, 15, 30, 56	RBCs, RETs	HT	Novartis, unpublished	No	Yes	P	P	
Trimethoprim-sulfamethoxazole	Dihydrofolate reductase inhibitor plus sulfonamide antibiotic/ 738-70-5 + 723-46-6	1-5, 1-10	0, 10 + 50 mg/kg/day; therapeutic doses, reductions to	-1, 15, 30, 45	RBCs, RETs	Basic	Pacheco-Martinez et al. (2018)	Yes	Yes	P	P	Clear P with short-term dosing/ combination therapeutic; Studies

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of columns: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I of E)	Notes
			body weight									conducted in well-nourished and malnourished rats, males and females tested
Triptolide	Diterpenoid epoxide/ 38748-32-2	1-7	5 mL/kg/day saline, 450 µg/kg/day; no evidence of toxicity	-1,7,14, 28,35, 56	RBCs	Basic	Pu et al. (2016)	Yes	Yes	I (N with RBCs only, basic protocol, one dose, problems with control)	I (N with RBCs only, basic protocol, one dose, problems with control)	Control dosed differently than treatment i.g. (ctrl: i.p. for 1-3 days); botanical
Vinblastine	Alkaloid/ 865-21-4	1-28 (dosing ending early for some high-dose animals)	0, 0.016, 0.031, 0.063, 0.125 mg/kg/day; range finder; effects on %RETs, toxicity to high dose	-4, 15, 29, 46	RBCs, RETs	HT	Avlasevich et al. (2018)	Yes	Yes	N	N	Occasional high responses limited to one rat/group/time point: overall N; concurrent MN P

**Select assays shown: assays used solely as positive controls not included.*

Abbreviations: PAH, polycyclic aromatic hydrocarbon; P, positive; N, negative; E, equivocal; I, inconclusive; MTD, maximum tolerated dose; RBCs, total red blood cells; RETs, reticulocytes; MN, micronucleus; Pre, pre-dosing (sample); basic = assay conducted without immunomagnetic enrichment, typically assaying 1 x 10^6 total RBCs and 3 x 10^5 RETs; HT = high-throughput immunomagnetic enrichment protocol devised by Litron Laboratories, typically analyzing 1-3 x 10^6 RET and >100 x 10^6 total RBC equivalents

ANNEX II: Substances tested in the mouse *Pig-a* assay with consensus calls

Consensus calls for *Pig-a* responses using different evaluation criteria shown in Table VI. Green highlight = consensus call made by expert committee. 13 consecutive days of dosing or less, short-term; ≥14 consecutive days of dosing, longer-term.

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of collums: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I or E)**	**Notes**
3R4F cigarette smoke	Complex mixture	1-28 (5-times/ week for 4 weeks)	40 and 80 min/day, 5-times/week for 4 weeks; top dose justification unclear	30	RBCs	Basic	Shanghai Jiao Tong University, Public Health Core Laboratories, unpublished	Yes	Yes	I (stats N but RBCs only, small groups (as few as 3), basic method)	I (stats N but RBCs only, small groups (as few as 3), basic method)	Inhalation study; MN on Day 30
Acrylamide	α,β-unsaturated amide/79-06-1	1-30	0, 1.5, 3, 6, 12, 24 mg/kg/day; cancer bioassay doses—body	31	RETs, RBCs	HT	Hobbs et al. (2016)	Yes	Yes	N (author says N-ANOVA N; 3 mg/kg RBCs P, but frequency	N	Concurrent MN assay P

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of collums: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I or E)	Notes
			weight unchanged							<1x10^{-6})		
Azidothymidine (AZT)	Nucleoside analog/30516-87-1	1-7 (2-times/day)	0, 250, 500, 1000 mg/kg; highest dose was toxic	-12/-6, 13, 20, 34/35, 48, 62,	RBCs	Basic	NCTR unpublished	Yes	Yes	I (N response with basic assay, RBCs only; short-term treatment, Day 62 ANOVA sig, but no sig treatments)	I (N response with basic assay, RBCs only; Day 62 ANOVA sig, but no sig treatment)	Wild-type and Cd24 heterozygous (HZ) mice tested—HZ mice higher and more variable mutant frequencies; Concurrent MN assay P
Benzo[a]pyrene	PAH/ 50-32-8	1	0, 100, 200 mg/kg; literature	14, 28, 49	RBCs	Basic	Horibata et al. (2013)	Yes	Yes	P	P	Consistent P in 5 labs; concurrent TGR , DNA adducts and MN assays P, comet N; conducted in *Ogg1* and *HRN* KO mice
		1-3	0, 50 mg/kg/day; dose P for germ cell mutation	-5, 16, 34	RBCs, RETs	Early HT	Graupner et al. (2014)	Yes	Yes	P	P	
		1-3	0, 62.5, 125, 250 mg/kg/day; literature	15, 30	RBCs, RETs	HT	Labash et al. (2016)	Yes	Yes	P (high dose repeated in 2nd study)	P (high dose repeated in 2nd study)	

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of collums: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I or E)	Notes
		1-4	0, 50 mg/kg	-1, 15	RBCs	Basic	L Wang et al. (2017)	Yes	Yes	P (males and females)	P (males and females)	
		1-28	0, 25, 50, 75 mg/kg/day; literature, slight alteration in %RETs	31	RBCs, RETs	Basic	Lemieux et al. (2011)	Yes	Yes	P	P	
Deoxynivalenol	Mycotoxin/51481-10-8	1-3	0, 2, 4, 8 mg/kg/day; toxicity	28, 45	RBCs, RETs	HT (early method)	Le Hegarat et al. (2014)	No	Yes	I (generally N, but early HT method) short-term dosing; low-dose RBCs at 28 days sig but 3 of 5 animals <1 $x10^{-6}$, some outliers)	I (early HT method may have lost mutants)	Concurrent *in vivo* MN and comet N; N Ames, weak P *in vitro* CA; *in vitro* comet N
Dichloromethane	Chlorinated organic solvent/75-09-2	6 weeks	5 days/week, 6 hr/day at 400, 800, 1,600 ppm; reprotox doses	21, 42	RBCs	Basic	Suzuki et al. (2014)	Yes	Yes	I (N but RBCs only, basic method, outliers)	I (N but RBCs only, basic method)	Concurrent/ parallel MN, liver TGR, liver comet N; ENU (i.p.) positive control P; administered via inhalation; rodent carcinogen; weak *in vitro* genotoxin

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of collums: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I or E)	Notes
Dichloromethane + dichloropropane	Mixture of chlorinated organic solvents/75-09-2 + 78-87-5	6 weeks	5 days/week, 6 hr/day at 150 + 400 ppm, 300 + 800 ppm DCP + DMP; reprotox doses	21, 42	RBCs	Basic	Suzuki et al. (2014)	No	No	I (N but RBCs only, basic method)	I (N but RBCs only, basic method)	Concurrent MN N but concurrent/ parallel liver comet and TGR P ; ENU (i.p.) positive control P; administered via inhalation; rodent carcinogen; genotoxic *in vitro*
Dichloropropane	Chlorinated organic solvent/78-87-5	6 weeks	5 days/week, 6 hr/day at air, 150, 300, 600 ppm; reprotox doses	21, 42	RBCs	Basic	Suzuki et al. (2014)	No	No	I (N, but RBCs only, basic method)	I (N, but RBCs only, basic method)	Concurrent/ parallel MN and liver TGR N, but liver comet P; administered via inhalation; ENU (i.p.) positive control P; rodent carcinogen; genotoxic *in vitro*
7,12-Dimethylbenz[*a*]-anthracene	PAH/ 57-97-6	1,3,5	0, 75 mg/kg/day	14	RBCs, RETs	Basic	Phonethepswath et al. (2008)	No	No	P	P	Early study, but clearly P
Ethyl carbamate (urethane)	Carbamate/ 51-79-6	1-3	0, 100, 200, 400 mg/kg/day; dose range-finder	15, 30	RBCs, RETs	HT	Labash et al. (2016)	Yes	Yes	P (top dose repeated in 2nd study)	P (top dose repeated in 2nd study)	P in well conducted studies; Concurrent MN P

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of collums: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I or E)**	**Notes**
Ethyl methanesulfonate	Alkylating agent/ 62-50-0	1-28	0, 5, 13, 20, 55, 100 mg/kg/day; literature	29	RBCs, RETs	Basic	Cao et al. (2014)	No (data available, but not posted)	No (data available, but not posted)	P	P	Dose-response; concurrent MN and TGR P
N-Ethyl-*N*-nitrosourea*	Alkylating agent/ 759-73-9	1	0, 100 mg/kg	7, 14, 28	RBCs	Basic	Kimoto et al. (2011b)	No	No	P	P	Consistently P in multiple labs; TGR, MN, *Pig-a* in CD24 heterozygous KO mice all P
		1	0, 10, 25, 45, 70, 100, 140 mg/kg; literature	-1, 14, 28, 42, 56, 84, 140, 182	RBCs, RETs	Basic	Bhalli et al. (2011a)	No	No	P	P	
		1	0, 32, 160 mg/kg; literature	-1, 14, 28, 42, 56, 84, 140, 182	RBCs, RETs	Basic	Bhalli et al. (2011a)	No	No	P	P	
		1	0, 10, 40, 160 mg/kg	-1, 14, 28, 42, 56	RBCs	Basic	NCTR, unpublished	Yes	Yes	P	P	
		1	0, 40 mg/kg; literature	14, 28, 49	RBCs	Basic	Horibata et al. (2013)	Yes	Yes	P	P	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of collums: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I or E)**	**Notes**
		1	0, 40	15	RBCs	Basic	Faculty of Public Health Shanghai Jiao Tong University School of Medicine, unpublished	Yes	Yes	P	P	
		1, 3, 5	0, 20 mg/kg/day	7, 14, 21, 28, 35	RBCs, RETs	Early basic method	Phonethepswath et al. (2008)	No	No	P	P	
		1-3	0, 12.5, 25, 50 mg/kg/day; literature	15, 30	RBCs, RETs	HT	Labash et al. (2016)	Yes	Yes	P	P	
		1, 7, 14, 21	0, 8, 40 mg/kg/day	-1, 14, 28, 42, 56, 84, 140, 182	RBCs, RETs	Basic	Bhalli et al. (2011a)	No	No	P	P	
Fullerene nanomaterials	Nanomaterial/ 99685-96-8	1	3 mg/mouse; cancer dose	14, 56	RBCs	Basic	Horibata et al. (2011)	Yes	Yes	I (N but single dose, RBCs only, basic method)	I (N but single dose, RBCs only, basic method)	Concurrent ENU positive control P; lung TGR and comet P for intratracheal instillation; *in vivo* MN N by gavage
Ionizing radiation		1	0, 0.5, 1, 2 Gy; depletion in RETs	0 (treatment day), 2, 7, 14, 21, 28, 35, 42, 49,	RBCs, RETs	Basic	Ohtani et al. (2012)	Yes	Yes	P	P	Consistent P in two labs; studies conducted with *p53* KO mice and with Se deficiency in

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of collums: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I or E)	Notes
				56, 70, 84, 105, 126, 148, 203, 232, 267, 297								*Ogg1* KO mice; P (RBC only) using old prototype mouse Microflow kit with 37C incubation step
		0 (first exposure), 7, 14, 21	0, 0.5 Gy; depletion in RETs	0 (first exposure), 2, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 105, 126, 148, 203, 232, 267, 297	RBCs, RETs	Basic	Ohtani et al. (2012)	Yes	Yes	P	P	
		1	0, 1 Gy; bone marrow suppression	0 (treatment day), 2, 7, 14, 21, 28, 35, 42, 49, 56, 63, 77, 91, 112, 133, 225, 323, 413, 713	RBCs	Basic	Ohtani et al. (2014)	No: 2012 study listed twice	No: 2012 study listed twice	P	P	
		45-day continuous	0, 1.4 mGy/h; human low-dose exposure	59	RBCs, RETs	Early HT	Graupner et al. (2016)	No	No	P (RBCs only)	P (RBCs only)	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of collums: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I or E)**	**Notes**
Methyl carbamate	Carbamate/ 598-55-0	1-3	0, 500, 1000, 2000; limit dose, dose range-finder	15, 30	RBCs, RETs	HT	Labash et al. (2016)	Yes	Yes	I (N with short-term dosing protocol)	N	
4-Nitroquinoline-1-oxide	Aromatic N-oxide/ 56-57-5	1	0, 50 mg/kg; literature	14, 28, 49	RBCs	Basic	Horibata et al. (2013)	Yes	Yes	I (N with basic method, short-term, single dose only)	I (N with basic method, single dose only)	Concurrent TGR N (bone marrow) and P
Nivalenol	Mycotoxin/ 23282-20-4	1-3	0, 5, 10, 20 mg/kg/day; preliminary range-finder: 40 mg/kg/day toxic	28, 45	RBCs, RETs	Early HT	Le Hegarat et al. (2014)	No	Yes	I (N with short-term , early HT protocol used)	I (N with early HT protocol used)	Early HT protocol may have lost mutant cells; comet, oxidative comet, MN in various tissues N
Procarbazine	Alkylating agent/ 671-16-9	1-3	0, 37.5, 75, 150 mg/kg/day; literature	15, 30	RBCs, RETs	Early HT	Phonethepswath et al. (2013)	No	No	P	P	P but magnitude questionable because of methods used; concurrent MN P
		1-28	0, 6.25, 12.5, 5, 25 mg/kg/day; dose range finder	31, 98	RBCs, RETs	HT	Maurice et al. (2019)	Yes	Yes	P	P	

Test substance	**Chemical species/ CAS**	**Days of dosing**	**Daily doses/Top dose justification**	**Sampling days**	**Cells analyzed**	**Method**	**Source of data, in order of collums: Publication, Public database, Restricted database**			***Pig-a* call using WG Criteria (reason for I or E)**	***Pig-a* call using Extended Criteria (reason for I or E)**	**Notes**
Pyrene	PAH/ 129-00-0	1-3	0, 125, 250, 500; dose range-finder, slight reduction in body weight gain	15, 30	RBCs, RETs	HT	Labash et al. (2016)	Yes	Yes	I (N response with 3-day dosing protocol)	N	Concurrent MN N
Silver nanoparticles, PVP-coated	Metallic nanomaterial	1	0, 0.5, 1, 2.5, 5, 10, 20 mg/kg	-1, 15, 29, 42	RBCs, RETs	Basic	Li et al. (2014)	No (but available, not yet posted)	No (but available, not yet posted)	I (N with short-term dosing, basic method)	I (N with basic method)	
2,2',4,4'-tetrabromodiphenyl ether	Halogenated hydrocarbon; 5436-43-1	1-42 (6 times/ week)	0, 0.0015, 1.5, 10, 30 mg/kg/day; multiple of environ-mental exposure, %RETs increased, plasma levels established	-2, 18, 37	RBCs	Basic	You et al. (2018)	Yes	Yes	I (N but RBCs only, basic method, not clear if MTD reached)	I (N but RBCs only, basic method, not clear if MTD reached)	Concurrent MN N, liver and testes *gpt* N
TiO_2 nanoparticles	Metallic nanomaterial	Once weekly for 4 weeks	0, 2, 10, 50 mg/kg/day iv	30?	RBCs	Basic	Suzuki et al. (2016a)	No	No	I (N but RBCs only, basic	I (N but RBCs only, basic	Concurrent MN N, liver *gpt*, Spi- and comet N

Test substance	Chemical species/ CAS	Days of dosing	Daily doses/Top dose justification	Sampling days	Cells analyzed	Method	Source of data, in order of collums: Publication, Public database, Restricted database			*Pig-a* call using WG Criteria (reason for I or E)	*Pig-a* call using Extended Criteria (reason for I or E)	Notes
										method)	method)	
		1-3	0, 0.5, 5, 50 mg/kg/day.; reduction in %RETs	-1, 7, 14, 28, 42	RBCs, RETs	Basic	Sadiq et al. (2012)	Yes	Yes	I (N but short-term dosing protocol, basic method)	I (N but basic method)	
Triclosan	Polychloro phenoxy phenol/ 3380-34-5	1-5	0, 31.25, 62.5, 125, 250, 500, 1000 mg/kg/day	-1, 15	RBCs	Basic	Unpublished Shanghai Jiaotong University	Yes	Yes	I (N but short-term protocol, only 15-day sample, only RBCs tested, basic method)	I (N but only 15-day sample, only RBCs tested, basic method)	MTD uncertain
		1-90	0 (water), 0 (vehicle), 1, 10, 50 mg/kg/day in diet	80	RBCs	Basic	Unpublished, Shanghai Jiaotong University	No (but available, not yet posted)	No (but available, not yet posted)	I (N but only 80-day sample, RBCs tested, basic method)	I (N but only 80-day sample, RBCs tested, basic method)	

**Assays used solely as positive controls not included.*

Abbreviations: P, positive; N, negative; E, equivocal; I, inconclusive; MTD, maximum tolerated dose; RETs = reticulocytes; RBCs = total red blood cells;

basic = assay conducted without immunomagnetic enrichment, typically assaying 1 x 10^6 total RBCs and 3 x 10^5 RETs; HT = high-throughput immunomagnetic enrichment protocol devised by Litron Laboratories, typically analyzing 1-3 x 10^6 RET and >100 x 10^6 total RBC equivalents; Ames = Salmonella reversion assay; MN = micronucleus; CA = chromosome aberration; BM, bone marrow; TGR = transgenic rodent; PAH = polycyclic aromatic hydrocarbon; KO = knockout (mouse)

ANNEX III: Summary of solvent/vehicle control data

All studies analyzed used immunomagnetic separation for mutant analysis and at least 5 rats per group. All usable studies had primary data in the *Pig-a* data base and had at least predosing mutant frequencies and an approx. 28-day data point. All useful studies were conducted with rats. Most results compare predosing mutant frequencies with approx. 28-day responses for vehicle controls; propylene glycol used concurrent sham-dosed rats for comparison. Historical predosing 95% one-sided Tolerance Intervals were available for four labs: Litron, Janssen, Novartis, and NCTR.

Vehicle/solvent	**Number of studies**	**Number labs**	**Number of daily doses**	**Treatment route**	**Rat strains tested: all males, except males and females marked with ***	**Significantly increased RETs/no. of studies**	**Significantly increased RBCs/no. of studies**
0.9% saline/physiological saline	9	4	1, 3, 28	Iv, ip	SD, F344	0/9	0/9
10% Ethyl alcohol	4	3	1, 3, 28	po	SD, HW	0/4	0/4
Phosphate Buffered Saline (pH 6.0)	5	4	5+3, 1, 3, 28	po	F344, SD*, HW/*gpt*-delta,	0/5	0/5

Vehicle/solvent	Number of studies	Number labs	Number of daily doses	Treatment route	Rat strains tested: all males, except males and females marked with *	Significantly increased RETs/no. of studies	Significantly increased RBCs/no. of studies
					CD		
Phosphate Buffered Saline (neutral or not specified)	6	3	1, 3, 28	po, ip	F344, CD, SD	2/6[a]	2/6[a]
Water	34	14	1, 3, 28	po, ip	SD*, HW, CD, F344, Wistar	5/34[b]	4/34[b]
Propylene glycol	1	1	7, 35, 63, 90	Inhalation	SD*	0/5	0/5
Sesame oil	15	4	1, 3, 28	po	SD, CD	2/15[a]	1/15[c]
Corn oil	3	2	1	po	SD, CD	0/3	0/3
Olive oil	3	3	1, 28	po	HW, SD, Wistar	0/3	0/3
0.5% HPMC/0.1% Tween 80	1	1	28	po	HW	0/1	0/1

Vehicle/solvent	Number of studies	Number labs	Number of daily doses	Treatment route	Rat strains tested: all males, except males and females marked with *	Significantly increased RETs/no. of studies	Significantly increased RBCs/no. of studies
0.5% Methyl Cellulose	2	2	1, 28	po	SD	1/2[c]	0/2
0.5% HPMC	6	2	3, 15, 29	po	SD	1/6[a]	1/6[a]
1.25% HPMC, 0.18% methylparaben, 0.02% polyparaben, 0.1% docusate sodium in water	1	1	5	po	Wistar	1/1[c]	0/1
10% designated solvent for etoposide	1	1	1	iv	SD	0/1	0/1

Abbreviations: HPMC, hydroxypropyl methyl cellulose; iv, intravenous; ip, intraperitoneal; po, per os (gavage); SD, Sprague Dawley; F344, Fisher 344; HW, Han Wistar; CD, Cesarean derived; RET, reticulocytes; RBC, red blood cells.

[a]All significant RET and total RBC results had no more than one rat exceeding the historical predosing 95% one-sided Tolerance Interval for the lab that generated the response.

[b]4 of 5 significant RET results and 3 of 4 significant RBC results had no more than one rat exceeding the historical predosing 95% one-sided Tolerance Interval for the lab that generated the response; the remaining significant results could not be checked due to a lack of historical predosing databases.

ANNEX IV: Conventional genotoxicty assay results for compounds tested in the *in vivo* rat or mouse *Pig-a* gene mutation assay.

Basis for the calls given in Section 8b. Substances used as solvents/vehicles are listed as such. Additional results from *in vitro* genotoxicity assays also gathered and available upon request.

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
3R4F cigarette smoke		Complex mixture					
2-Acetylaminofluorine	53-96-3	Aromatic amine		P	P		P
Acetaminophen	103-90-2	Hydroxyaniline		N	P	P	P
Acrylamide	79-06-1	α,β-unsaturated amide	P	P	N		P
Aflatoxin B1	1162-65-8	Mycotoxin		P	P		P
4-Aminobiphenyl	92-67-1	Aromatic amine	P	P	P		P
o-Anthranilic acid	118-92-3	Aromatic amine					N
Aristolochic acids	313-67-7	Aromatic nitro		P	E/N		P
Azathioprine	446-86-6	Aromatic nitro	P	P	P	P	P

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
Azidothymidine	30516-87-1	Nucleoside analog			P		P
Azoxymethane	24843-45-2	Alkylating agent					
$BaS0_4$ NM-220		Metal sulfate nanomaterial					
Benzo[*a*]pyrene	50-32-8	PAH	P	P	P		P
2-Butoxyethanol	111-76-2	Ethylene glycol derivative					P
Caffeic acid	331-39-5	Hydroxycinnamic acid			P		P
Carboplatin	41575-94-4	Coordination complex of Pt (antineoplastic; crosslinker)			P		
CeO_2 NM-212		Rare earth nanomatieral					
Chlorambucil	305-03-3	Alkylator; nitrogen mustard	P	P	P	P	P
4-Chloro-1,2-diaminobenzene	95-83-0	Aromatic amine		P	P		P
5-(2-Chloroethyl)-2’-deoxyuridine (CEDU)	90301-59-0	Pyrimidine analog	P	P			

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
4-Chloro-*ortho*-toluidine HCl	95-69-2	Aromatic amine					P
Cisplatin	15663-27-1	Square planar coordination complex of Pt (antineoplastic; crosslinker)		P	P	P (leukemia)	P
Clofibrate	637-07-0	Fibric acid derivative		N			P
Compound X		(Inhaled)					
Compound X-A		Nucleoside analog					
Corn oil	8001-30-7	(Solvent/vehicle)	N	N	N	N	N
Cyclophosphamide	6055-19-2	Alkylator; nitrogen mustard			P		P
Deoxynivalenol (vomitoxin)	51481-10-8	Mycotoxin					N
Dibenzo[*a*,*l*]pyrene	191-30-0	PAH			P		
Dichloromethane	75-09-2	Halogenated hydrocarbon		P	P		P
1,2-Dichloropropane	78-87-5	Halogenated hydrocarbon		P	I		P
Diethylnitrosamine	55-18-5	Nitrosamine	N	P	P		P

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
3,5-Difluorophenylboronic acid	156545-07-2	Aryl boronic acid					
[1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC)	25952-53-8	Carbodiimide					
7,12-Dimethylbenz[*a*]-anthracene	57-97-6	PAH	P	P	P	P	P
1,2-Dimethylhydrazine hydrochloride	306-37-6	Hydrazine			P		P
10% Ethanol	64-17-5	Solvent/vehicle	N	N	N	N	N
Ethylmethane sulfonate	62-50-0	Alkylating agent	P	P	P		P
N-Ethyl-*N*-nitrosourea	759-73-9	Alkylating agent	P	P	P	P	P
Etoposide	33419-42-0	Podophyllotoxin derivative (topoisomerase inhibitor)	N	N	P		P
5-Fluorouracil	51-21-8	Pyrimidine analog			P		P
Fullerene nanomaterials							
Furan	110-00-9	Heterocycle			E	P	P
Glycidamide	5694-00-8	Oxirane amide		P	P		P
Glycidyl methacrylate	106-91-2	Methacrylate ester		I	P		N

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
0.5% Hydroxypropyl methylcellulose	9004-65-3	(Solvent/vehicle)	N	N	N	N	N
0.5% Hydroxypropyl methylcellulose, 0.18% methylparaben, 0.02% polyparaben, 0.1% docusate sodium in water		(Solvent/vehicle)					
2-Hydroxypyridine-N-oxide (HOPO)	13161-30-3	Aromatic N-oxide					
Hydroxyurea	127-07-1	Nucleoside analog (antimetabolite)		P	P		P
Isopropylmethane sulfonate	926-06-7	Alkylating agent		P	P		P
Isopropyltoluene sulfonate	2307-69-9	Alkylating agent					
Melamine	108-78-1	Triaminotriazine			N		P
Melphalan	148-82-3	Alkylating agent; nitrogen mustard			P	P	P
Methyl carbamate	598-55-0	Carbamate			N		P
0.5% Methyl cellulose	9004-67-5	(Solvent/vehicle)	N	N	N	N	N
4,4’-Methylenedianiline	101-77-9	Aromatic amine			P		P

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
Methylmethane sulfonate	66-27-3	Alkylating agent	P	P	P	P	P
Methylphenidate	113-45-1	Phenylethylamine derivative					E
Mitomycin C	50-07-7	Methylazirino-pyrroloindoledione antibiotic (crosslinking agent)	P	P	P		P
3-Monochloropropane-1,2-diol	96-24-2	Chloropropanol			N		P
3-Monochloropropane-1,2-diol palmitate diester		Chloropropanol					
3-Monochloropropane-1,2-diol palmitate monoester		Chloropropanol					
3-Monochloropropane-1,2-diol oleate diester		Chloropropanol					
4-Nitroquinoline-1-oxide	56-57-5	Aromatic N-oxide	P	P	P		P
N-Nitroso-*N*-methylurea	684-93-5	Alkylating agent		P	P		P

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
Nivalenol	23282-20-4	Mycotoxin					
4-[Methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone (NNK)	64091-91-4	Nitrosamine		P	I		P
Olive oil	8001-25-0	(Solvent/vehicle)	N	N	N	N	N
Phenobarbital	50-06-6	Barbiturate		I	E		P
Phosphate-buffered saline (pH 6.0)		(Solvent/vehicle)	N	N	N	N	N
Phosphate-buffered saline (neutral)		(Solvent/vehicle)	N	N	N	N	N
Procarbazine	671-16-9	Alkylating agent			P		P
1,3-Propane sultone	1120-71-4	Alkylating agent			P		P
Propylene glycol	57-55-6	(Solvent/vehicle)	N	N	N	N	N
N-Propyl-nitrosourea	816-57-9	Alkylating agent	P	P	P	P (leukemia)	P
Pyrene	129-00-0	PAH			N		N
0.9% Saline	7647-14-5	(Solvent/vehicle)	N	N	N	N	N
Sesame oil	8008-74-0	(Solvent/vehicle)	N	N	N	N	N
Silver nanomaterials (PVP-coated)		Coated metallic nanomaterial					
Sodium chloride	7647-14-5	Salt			N		N

Test Substance	**CAS Number**	**Chemical class (other information)**	***In vivo* TGR/bone marrow**	***In vivo* TGR/any tissue**	***In vivo* micronucleus**	**Rodent cancer/ hematopoietic system**	**Rodent cancer/any tissue**
Sulfisoxazole	127-69-5	Aryl sulfonamide			N		N
Temozolomide	85662-93-1	Imidazotetrazine; alkylating agent			P		P
2,2’,4,4’-Tetrabromodiphenyl ether	5436-43-1	Polybrominated biphenyl ether (endocrine disrupter)					
Thiotepa	52-24-4	Aziridine		P	P		P
Titanium dioxide nanomaterials		Metallic nanomaterial					
Triclosan	3380-34-5	Polychloro phenoxy phenol (antimicrobial)			N		
Trimethoprim – sulfamethoxazole	738-70-5 plus 723-46-6	Dihydrofolate reductase inhibitor plus sulfonamide antibiotic					
Triptolide	38748-32-2	Diterpenoid epoxide					
Urethane (ethyl carbonate)	51-79-6	Carbamate	P	P	P	P	P
Vinblastine	865-21-4	Alkaloid			P		N
Water	7732-18-5	(Solvent/vehicle)	N	N	N	N	N

Test Substance	CAS Number	Chemical class (other information)	*In vivo* TGR/bone marrow	*In vivo* TGR/any tissue	*In vivo* micronucleus	Rodent cancer/ hematopoietic system	Rodent cancer/any tissue
X-rays/gamma rays		Ionizing radiation			P		P

Abbreviations: P, positive; N, negative; E, equivocal; I, inconclusive; TGR, transgenic rodent (gene mutation assay); PAH, polycyclic aromatic hydrocarbon

ANNEX V: *Pig-a* assay performance analysis calculations

Definitions for select metrics:

	Pig-a P	Pig-a N
Conventional assay P	a	b
Conventional assay N	c	d

% Sensitivity: a/ (a + b) x 100
% Positive predictivity: a/ (a + c) x 100
% Specificity: d/ (c + d) x 100
% Negative predictivity: d/ (b + d) x 100
% Concordance: (a + d)/(a +b + c + d) x 100

Performance of *Pig-a* assay responses in relation to different *in vivo* genetic toxicity assay and rodent cancer endpoints: *Pig-a* responses determined using the Working Group (WG) criteria, I and E calls not used.

Pig-a vs BM/blood TGR

	Pig-a P	Pig-a N
BM TGR P	16	0
BM TGR N	1	11

Sensitivity	100% (79%-100%)
Positive predictivity	94% (71%-100%)
Specificity	92% (62%-100%
Negative predictivity	100% (72%-100%)
Concordance	96% (82%-100%)
Cohen's kappa (95% CI)	0.93 (0.78-1.07)
PABAK	0.93
Discordant test articles	DEN

Pig-a vs TGR

	Pig-a P	Pig-a N
TGR P	26	3
TGR N	0	11

Sensitivity	90% (73%-98%)
Positive predictivity	100% (87%-100%)
Specificity	100% (72%-100%)
Negative predictivity	79% (49%-95%)
Concordance	93% (80%-98%)
Cohen's kappa (95% CI)	0.83 (0.64-1.01)
PABAK	0.85
Discordant test articles	Hydroxyurea, 4-chloro-1,2-diaminobenzine, NNK

Pig-a vs *in vivo* MN

	Pig-a P	Pig-a N
In vivo MN P	29	5
In vivo MN N	2	16

Sensitivity	85% (69%-95%)
Positive predictivity	94% (79%-99%)
Specificity	89% (66%-99%)
Negative predictivity	76% (53%-92%)
Concordance	87% (74%-94%)
Cohen's kappa (95% CI)	0.71 (0.52-0.91)
PABAK	0.73
Discordant test articles	Acetaminophen, aristolochic acids, 4-Chloro-1,2-diaminobenzene, hydroxyurea, vinblastine, CeO_2, CEDU

Pig-a vs rodent hematopoietic system cancer

	Pig-a P	Pig-a N
Hematopoietic cancer P	9	1
Hematopoietic cancer N	1	14

Sensitivity	90% (56%-100%)
Positive predictivity	90% (56%-100%)
Specificity	93% (68%-100%)
Negative predictivity	93% (68%-100%)
Concordance	92% (74%-99%)
Cohen's kappa (95% CI)	0.83 (0.61-1.05)
PABAK	0.84
Discordant test articles	Acetaminophen, glycidyl methacrylate

Pig-a vs all rodent cancer

	Pig-a P	Pig-a N
Cancer P	27	7
Cancer N	1	14

Sensitivity	79% (62%-91%)
Positive predictivity	96% (82%-100%)
Specificity	93% (68%-100%)
Negative predictivity	67% (43%-85%)
Concordance	84% (70%-93%)
Cohen's kappa (95% CI)	0.65 (0.44-0.87)
PABAK	0.67
Discordant test articles	Acetaminophen, 2-butoxyethanol, 4-chloro-1,2-diaminobenzene, glycidyl methacrylate, hydroxyurea, clofibrate, methyl carbamate, NNK

Performance of *Pig-a* assay responses in relation to different *in vivo* genetic toxicity assay and rodent cancer endpoints: *Pig-a* responses determined using Extended Criteria, I and E calls not used.

Pig-a vs BM/blood TGR

	Pig-a P	Pig-a N
BM TGR P	15	0
BM TGR N	0	11

Sensitivity	100% (78%-100%)
Positive predictivity	100% (78%-100%)
Specificity	100% (72%-100%)
Negative predictivity	100% (72%-100%)
Concordance	100% (87%-100%)
Cohen's kappa (95% CI)	1.00 (1.00-1.00)
PABAK	1.00
Discordant test articles	

Pig-a vs TGR

	Pig-a P	Pig-a N
TGR P	22	4
TGR N	0	11

Sensitivity	84% (65%-96%)
Positive predictivity	100% (85%-100%)
Specificity	100% (72%-100%)
Negative predictivity	79% (45%-92%)
Concordance	89% (75%-97%)
Cohen's kappa (95% CI)	0.77 (0.56-0.98)
PABAK	0.78
Discordant test articles	Hydroxyurea, 4-chloro-1,2-diaminobenzine, NNK, 1,2-dimethylhydrazine

Pig-a vs *in vivo* MN

	Pig-a P	Pig-a N
In vivo MN P	25	9
In vivo MN N	2	17

Sensitivity	74% (56%-87%)
Positive predictivity	93% (76%-99%)
Specificity	89% (67%-99%)
Negative predictivity	65% (44%-83%)
Concordance	79% (66%-89%)
Cohen's kappa (95% CI)	0.583 (0.37-0.79)
PABAK	0.58
Discordant test articles	Acetaminophen, aristolochic acids, 4-Chloro-1,2-diaminobenzene, AZT, caffeic acid, 1,2-dimethylhydrazine, 5-fluorouracil, hydroxyurea, vinblastine, CeO_2, CEDU

Pig-a vs rodent hematopoietic system cancer

	Pig-a P	Pig-a N

Hematopoietic cancer P	9	1
Hematopoietic cancer N	1	15

Sensitivity	90% (56%-100%)
Positive predictivity	90% (56%-100%)
Specificity	94% (70%-100%)
Negative predictivity	94% (70%-100%)
Concordance	92% (75%-99%)
Cohen's kappa (95% CI)	0.84 (0.621-1.05)
PABAK	0.85
Discordant test articles	Acetaminophen, glycidyl methacrylate

Pig-a vs all rodent cancer

	Pig-a P	Pig-a N
Cancer P	22	12
Cancer N	1	15

Sensitivity	65% (46%-80%)
Positive predictivity	96% (78%-100%)
Specificity	94% (70%-100%)
Negative predictivity	56% (35%-75%)
Concordance	74% (60%-85%)
Cohen's kappa (95% CI)	0.50 (0.28-0.71)
PABAK	0.48
Discordant test articles	Acetaminophen, 2-butoxyethanol, caffeic acid, AZT, 4-chloro-1,2-diaminobenzene, clofibrate, 1,2-dimethylhydrazine, 5-fluorouracil, glycidyl methacrylate, hydroxyurea, melamine, methyl carbamate, NNK

Performance of *Pig-a* assay responses in relation to different *in vivo* genetic toxicity assay and rodent cancer endpoints: *Pig-a* responses using only short-term treatment protocols and determined using Extended Criteria, I and E calls not used.

Pig-a vs BM/blood TGR

	Pig-a P	Pig-a N
BM TGR P	13	0
BM TGR N	0	14

Sensitivity	100% (75%-100%)
Positive predictivity	100% (75%-100%
Specificity	100% (77%-100%)
Negative predictivity	100% (77%-100%)
Concordance	100% (87%-100%)
Cohen's kappa (95% CI)	1.00 (1.00-1.00)
PABAK	1.00
Discordant test articles	

Pig-a vs TGR

	Pig-a P	Pig-a N
TGR P	20	4
TGR N	0	10

Sensitivity	83% (63%-95%)
Positive predictivity	100% (83%-100%)
Specificity	100% (69%-100%)
Negative predictivity	71% (42%-92%)
Concordance	88% (73%-97%)
Cohen's kappa (95% CI)	0.75 (0.52-0.97)
PABAK	0.77
Discordant test articles	Hydroxyurea, DEN, aflatoxin B1, 1,2-dimethylhydrazine

Pig-a vs *in vivo* MN

	Pig-a P	Pig-a N
In vivo MN P	22	8
In vivo MN N	2	17

Sensitivity	73% (54%-88%)
Positive predictivity	92% (73%-99%)
Specificity	89% (67%-99%)
Negative predictivity	68% (47%-85%)
Concordance	80% (66%-90%)
Cohen's kappa (95% CI)	0.59 (0.38-0.81)
PABAK	0.59
Discordant test articles	Acetaminophen, aristolochic acids, hydroxyurea, aflatoxin B1, AZT, caffeic acid, DEN, dimethylhydrazine, 5-fluorouracil, CEDU

Pig-a vs rodent hematopoietic system cancer

	Pig-a P	Pig-a N
Hematopoietic cancer P	9	1
Hematopoietic cancer N	0	14

Sensitivity	90% (56%-100%)
Positive predictivity	100% (66%-100%)
Specificity	100% (77%-100%)
Negative predictivity	93% (68%-100%)
Concordance	96% (79%-100%)
Cohen's kappa (95% CI)	0.91 (0.75-1.08)
PABAK	0.92
Discordant test articles	Acetaminophen

Pig-a vs all rodent cancer

	Pig-a P	Pig-a N
Cancer P	21	11
Cancer N	0	14

Sensitivity	66% (47%-81%)
Positive predictivity	100% (84%-100%)
Specificity	100% (77%-100%)
Negative predictivity	56% (35%-76%)
Concordance	76% (61%-87%)
Cohen's kappa (95% CI)	0.54 (0.33-0.75)
PABAK	0.52
Discordant test articles	Acetaminophen, aflatoxin B1, AZT, 2-butoxyethanol, caffeic acid, 5-fluorouracil, hydroxyurea, melamine, methyl carbamate, DEN, 1,2-dimethylhydrazine

ANNEX VI: Follow-up to the peer review of the validation status of the *in vivo* erytrocyte *Pig-a* assay: responses to issues raised by the peer review report and explanation of subsequent changes made to the detailed review paper (Part 1)

A Peer Review of the validation status of the in vivo erythrocyte Pig-a gene mutation assay was conducted in 2019 (OECD, 2020b), The Peer Review Panel (PRP) found that the Detailed Review Paper (DRP) and Validation Document '…..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' (para 4 of the Report). However, 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… (para. 5 of the Report). The PRP went on to indicate that these issues should be given '……careful consideration during the development of a Test Guideline for regulatory use' (para. 5).

In anticipation of developing a Test Guideline for the in vivo *Pig-a* gene mutation assay, the following comments and alterations to DRP and validation document are offered in response to the findings of the PRP Report. Note that references that are cited below are listed in the Reference section of the DRP. The major request for clarification and additional information was stated in paragraph 58 of the Report:

'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.'

Response: We agree that the recommendations made in the DRP for dosing and sampling schedule are subject to refinement during OECD Test Guideline (TG) development, and once a TG is established, subject to periodic updating based on advances in scientific knowledge. However, the assertion that the dosing schedule and sampling times are not standardized for the *Pig-a* assay is somewhat misleading.

Strong recommendations have been made for both dosing duration and sampling time in the DRP (Section 4) that are based on those made by the International Workshop on Genotoxicity Testing (IWGT) report on the assay (Gollapudi et al., 2015). The IWGT is an international consensus-forming group whose evaluations and recommendations often serve as a precursor to OECD TG development. Note that the International Council for Harmonization (ICH) believes that the *Pig-a* assay is sufficiently well-developed and protocols standardized to recommend it for evaluating the mutagenicity of drug impurities, a recommendation that was made most recently in ICH M7 (R1) (ICH, 2017). With regard to this recommendation, an influential genetic toxicologist, Sheila Galloway, indicates that ‘Although an OECD guideline does not yet exist for the *Pig-a* assay, there is extensive experience, and protocol recommendations have been published by an IWGT Workgroup [Gollapudi et al., 2015]’ (Galloway, 2017). We contend the dosing schedule and sampling are reasonably well standardized for the assay and that recommendations are clearly stated in the DRP.

As described in the DRP, protocol optimization, including standardization of the dosing and sampling schedules, is based on several important elements:

1. Taking advantage of the accumulation of mutations to increase the sensitivity of the *Pig-a* assay by strongly recommending the use of repeat-dosing designs (e.g., 28 days of daily dosing) for hazard identification
2. Optimizing animal use for toxicology testing consistent with the 3Rs principles by using experimental designs that are consistent with integration of the *Pig-a* assay into other in vivo genotoxicity and general toxicity testing
3. Providing some flexibility in sample collection to enhance integration potential without compromising assay accuracy.
4. Collecting sufficient data to provide a robust evaluation of in vivo mutagenicity while maintaining a practical protocol to promote use of the assay: recommendations are made for the minimum number sampling times (e.g., a single sample taken soon after the completion of a 28-day daily dosing schedule) and the minimum number of cells assayed (one million each of reticulocytes and total red blood cells) consistent with maintaining assay accuracy.
5. Providing additional options for sampling (e.g., collecting samples before, during, and/or several weeks after the dosing period) that, when practical to perform, may enhance response evaluation.
6. Providing additional options for dosing protocols (e.g., shorter-term dosing schedules), but only when sufficiently justified. The option for additional dosing protocols, like all recommendations, is subject to modification during TG development.
7. Prospective and retrospective analyses of protocol options (e.g., number of days of dosing, number of erythrocytes analyzed, sex of animals) to document their effect on test performance.
8. A Retrospective Performance analysis indicating that the recommended protocol provides an accurate test of in vivo mutation.

While there is extensive experience conducting the *Pig-a* assay that conforms with the recommended guidelines (e.g., see number of studies in Table VI and VII (now Annexes I and II) with P, N, or E calls using the Work Group or Extended criteria), Tables VI and VII (now Annexe I and II) in the DRP list **ALL** *Pig-a* assays that have been conducted, some of which were conducted before immunomagnetic separation techniques were developed and/or before the IWGT recommendations were made. The comprehensive listing of all studies may give the false impression that strong recommendations of conducting the assay have not been established. Note that experience with performing assays with alternative, less-than-ideal protocols has been useful in establishing the criteria for conducting a useful assay and the protocol recommendation made in the DRP.

Recommendations on animal age, number of experimental groups, and the minimum number of RETs and total RBCs for conducting the assay are given in Table I (now Table II) of the DRP. The recommendations on animal age and number of experimental groups are derived from other OECD in vivo TGs with the overall intention of maximizing the integration of the *Pig-a* assay with other in vivo general toxicology and genotoxicity assays. The rationale for the recommended minimum number of RETs and total RBCs assayed is given in Section 4a and mainly is based on a combination of practicality, the background mutant frequencies detected in the assay, and power analyses. The option of freezing blood samples for delayed analysis is covered in Section 4b of the DRP. The volume of blood necessary for conducting the various *Pig-a* assays has been added to the DRP (p 33).

There are no recommendations made in the DRP on the route of exposure as the endpoint itself is agnostic with respect to exposure route. Note also that route of exposure was ignored in conducting the RPA. In practice, however, most studies have been conducted using gavage dosing since this route of exposure is relevant for human exposure and typically provides a combination of adequate bone marrow exposure and test substance metabolism; presumably, i.v. dosing would provide even better exposure (as implied in Section 5). Other routes of exposure may not be nearly as effective (e.g., all studies conducted using inhalation exposure, even using known genotoxicants, have been negative); thus, the most important factors in choosing a route of exposure for a hazard identification *Pig-a* assay may be bone marrow exposure, adequate metabolism and possibly the route of human exposure. No changes have been made to the DRP; we will deal with this subject more thoroughly in drafting the TG.

Annex 4 of the peer review report contains a number of additional comments. Responses are given in bold below; the references used in the responses are listed in the DRP (Part 1 of the submission package).

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.
	Response: The assay was not 'validated' specifically for integration into general toxicology assays. But there are several publications that describe its use in this manner, most recently in Mittelstaedt et al. (2019), where the assay was incorporated into a 90-day subchronic GLP tox assay (using samples released from the main study). As mentioned above, protocol recommendations were made with facilitating integration into other in vivo toxicology studies in mind.
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?
	Response: This is probably not a major issue for regulatory hazard ID assays as young adult animals are recommended for these assays (Table 1 (now Table II)). An important factor in making this recommendation on the age of animals used in the assay is compatibility with the age range recommended for other in vivo genetic toxicology and general toxicology assays into which the *Pig-a* assay might be integrated. That being stated, *Pig-a* assays have been performed successfully on at least one-year-old rats and mice; however, it is possible that there is an age limit due to aging of the erythropoietic system. This has not been studied in any detail. Also, very little data have been generated using very young rodents; the source of the mutants in the assay may be a complicating factor in very young rodents, as it seemed to be in an in utero exposure study conducted by Dobrovolsky et al. (2012). In addition, Horibata and colleagues, in a study reported only in a poster (K Horibata, A Ukai, M Honma (2017) Mice mutagenicity on the next generation and effect on the differences of both age and sex detected by the Pig-a assay, 2017 ICEM meeting, poster P002-110), found that erythrocytes from fetal and 4-day-old mice were partially deficient in GPI-anchored protein expression. Dertinger et al. (2019), however, successfully measured mutation in a study that used 4-week-old rats, which are slightly younger than the young adult animals recommended by the DRP for performing hazard ID studies. TG development may consider a recommendation for testing younger and older animals when the experimental question would be addressed by such data. **Excluding exceptionally young animals, an age effect for the rodent *Pig-a* assay has not been described, although it is quite possible that one exists. For example, an age effect has been described for the human assay (Dertinger et al., 2015; described in Section 10a of the DRP), where a wide age range was evaluated, with RET and total RBC frequencies increasing slowly with age. As indicated above, most rodent assays have been conducted with young adult animals, and there are extensive data on assay variably for rats of this age (see, for example, Avlasevich et al. (2018) and Figure 8 (now Figure 5) in the DRP).**

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.
	Response: The message of this comment is correct. When it has been measured in long-term mutant manifestation studies, the persistence of the mutational response has been remarkable. However, the factors mentioned could impact persistence, which is likely to vary to a certain degree with the mutagen. **In fact, it has been established that sampling 28-31 days following the first dose is not ideal for either 1-3 day or 28-day dosing protocols, especially for total RBC frequencies. However, the mutant manifestation studies that have been conducted and the performance analysis described in the DRP suggest that it is a adequate compromise for measuring *Pig-a* erythrocyte mutant frequencies. Note that a 1-2 week sampling point is also recommended when 1-3-day dosing studies are conducted to improve the analysis of RET mutants (Section 4c). Note also these are the minimum recommended sampling times. More samples and more data mean more confidence in results and potentially a greater chance of detecting small effects.**
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?
	Response: Yes: the Avlasevich et al. (2019) paper cited in Section 4b describes a method for preserving blood samples (up to 7 months of storage were evaluated) whose robustness was tested in three different laboratories. Although this is a relatively recent development, the available data look very encouraging.

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.
	Response: Reference added.
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?
	Response: More detail on blood preservation has been added to the DRP. As indicated in the DRP, the blood is stable for at least 7 months. The method described in the reference given in Section 4b has been evaluated in three labs for its ability to accurately evaluate mutant frequency relative to unfrozen samples. The method described is only available commercially using proprietary reagents (so we are hesitant to recommend it as a default---only a possible alternative), but other similar methods can be deduced from the published literature.

P39, line16:	“animal sex, etc.” Please add “age” of animal.
	Response: added.
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.
	Response: Yes, *Pig-a* sequence data on mutants from control animals and cells are available, but only for T lymphocytes from rats (Dobrovolsky et al., 2015) and for in vitro mutants from L5198Y/*Tk*$^{+/-}$ cells (Revollo et al., 2017b; Mittelstaedt et al., 2019). Because of technical limitations, mutation analysis has not been done using *Pig-a* mutant erythroid cells from control rats. **The size of the human *PIG-A* gene has been added to Section 1a of the DRP. It might be possible to compare mutation spectra in transgenes and the *Pig-a* gene for a few test substances, but there are many differences in sequence, expression (which affects repair), the presence of splice sites, etc. which make only general comparisons reasonable. That being said, the spectra are reasonably coincident for ENU- and DMBA-induced mutants: characteristic mutational signatures are evident for both the *Pig-a* and TGR reporters.**
P77, line 16:	“ii)” should be “iii)”.
	Response: The numbering has been corrected
PR3	

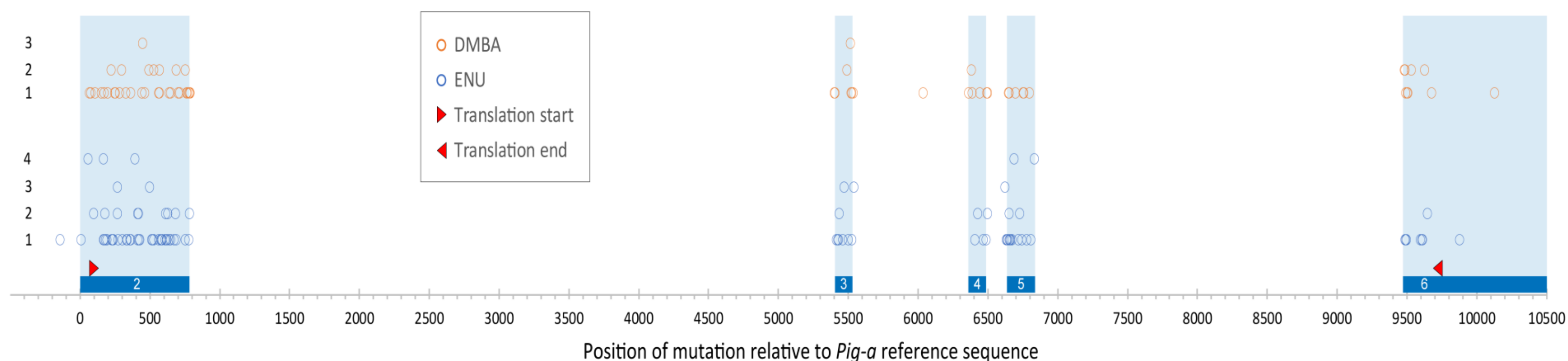
P7, line 19-22	Significant dose-related increases compared to the control implies that at least one dose must be significant. Use "dose" not 'treatment". You're not 'treating' disease, you're 'dosing' animals.
	Response: We don't think this is necessarily the case, but positive responses have both significant increases relative to the control (using pair-wise tests) and significant dose-related increases. **'dose' didn't quite fit here, but the 'treatments' were replaced. The words 'treatment' and 'exposure' were replaced throughout the DRP.**
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?
	Response: No, we are not. The eventual development methods to measure *Pig-a* mutations in tissues other than blood, including solid tissues, would benefit the assay, increasing its applicability.
P19, line 24	Define acute here if meaning more than one dose
	Response: Here it is meant to refer to a single dose. 'Acute' was replaced with 'single'.
P19, line 25	After acute dosing with a potent mutagen, the maximum mutant frequency..
	Response: Wording changed as suggested, except 'single' was used instead of 'acute'.
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?
	Response: This is correct; thus (as indicated in the text), the time it takes to turn over the existing RBC pool and approximately balance the mutant frequency in the periphery with the incoming mutant frequency from mutagenized progenitor cells (which takes about 6-8 weeks) typically corresponds to the time it takes to achieve the maximum total RBC mutant frequency. Before this maximum is reached, the existing pool of peripheral erythrocytes would 'dilute out' mutagenized cells in the periphery, a phenomenon that is especially evident with total RBC frequencies.
P22, line 8	In vivo genotoxicity tests are typically used for hazard identification....
	Response: The sentence was altered but the use for hazard ID is given in the preceding sentence, so we have not repeated it here.
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.
	Response: the individual paragraphs are from the same source, but from different places in the source. We have taken out the close quote marks for all but the last paragraph of each section. Perhaps the OECD copy-editors could help here!
P34, line 26-P35 line 2	I don't think a true Haber's law extrapolation should ever be expected in limited animal experimentation.

	Response: We generally agree, but in some instances the responses for split doses have been remarkably additive (see Miura et al., 2009); sometimes not so much (Bhalli et al., 2011a). Overall, we are on safe ground to conclude that there is a benefit to the sensitivity of the assay from repeat-dose protocols.
P35, line 4-7	Are these also similar concerns with doing single or three dose TGR assays?
	Response: 2-AAF and AFB1 are generally difficult to detect in genetic toxicology assays, and the recommendation for employing a 28-day dosing schedule in TGR assays applies to these compounds. Note that these are both liver carcinogens so the TGR assay has the advantage of being able to measure mutation in a target tissue. Thus, without evaluating TGR responses in detail for these compounds, it is possible that the necessity for a longer dosing protocol may not be as obvious for the TGR assay as for the *Pig-a* assay.
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
	Response: This point is well taken. The relatively high predictive value of the assay conducted with short-term dosing schedules in Table XIV (now Table XIII) is undoubtedly influenced by the nature of the substances tested. This is a major reason for the recommendation that careful justification is necessary to support any testing performed with short-term dosing schedules, especially when negative responses are detected. However, it is conceivable that the assay may be used, for instance, at an early stage of drug development for preliminary data on potential genotoxicity where a short-term dosing protocol may be employed (e.g., integration with a PK study). This would occur prior to a definitive test for regulatory decision making. Therefore, for the time being at least, we prefer to leave this option of doing shorter-term dosing studies with the knowledge that the TG development team will undoubtedly revisit this issue.
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.
	Response: Point taken; however, 'weak' is meant to refer to a small effect that is difficult to detect in a particular assay, which may or may not mean that the overall genotoxic potential of a substance is weak. The offending word has been replaced with 'small increases in mutant frequency'.
P38, line 13	Are there scenarios where one dosing regimen may be recommended over the other?
	Responses: No: not recommended, especially for regulatory assays. The analysis in Section 4a clearly indicates the problems associated with a single dose schedule. But in the interests of maintaining flexibility, the DRP concedes that there may be situations where a single dose protocol may be justified. The recommendation for a repeat dose schedule as the default is indicated in several places in the DRP, including in Table I (now Table II) and on p 6 and pp 34-35. The TG development team will undoubtedly revisit this issue and may decide to remove the current flexibility in assay design.

P42, line 22-25	Are the same concerns expressed related to TG474 and TG475?
	Response: Yes, these concerns are similar to those for the bone-marrow-based cytogenetic assays. This is mentioned at the end of Section 3 of the DRP.
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.
	Response: this is a major question for all in vivo genetic toxicology assays, not just the *Pig-a* assay, and we handle it similarly to the recommendations for other assays. We do not directly suggest using any additional animals to resolve questionable responses. With the *Pig-a* assay, animals are not necessarily sacrificed for sampling, and data from intermediate sampling points may be available, so employing additional sampling times or assaying additional erythrocytes to resolve a questionable result is possible. With the *Pig-a* assay, it should be kept in mind that responses at some sampling points could be positive whereas others are negative, and that the timing is consistent with what is known about the effects of erythropoiesis on mutant manifestation for RETs and total RBCs. This variability in mutant manifestation could be evidence for a positive response. True equivocal responses are also possible.
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.
	Response: Point taken. If the same tissues were being assayed, the discordant compounds may well have been concordant. This is a limitation in the *Pig-a* assay in detecting in vivo mutagens that target tissues other than bone marrow. We think the DRP is clear on this point (see ll 16-18). Note that the performance analysis used both TGR data from bone marrow and TGR data from any tissue as the reference, and there was a small degradation in *Pig-a* assay performance with the latter (see Tables XII-XIV; now Tables XI-XIII).
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.
	Response: The key point is that there are a few compounds that required a longer-term dosing schedule to produce a positive response, referring the Tables II and III (now Tables III and IV). This section has been altered to make it clearer.
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.
	Response: Agreed. This was part of the rationale for including this section in the DRP that compares potentially complimentary endpoints.

P74, line 23	"are genotoxic through mechanisms resulting in"
	Response: change made.
P77, line 24	was this conventional assay comparator a single or short-term dose test as well?
	Response: The reference genotoxicity assays were conducted as recommended in OECD TGs or other consensus documents. This means that at least the negative TGR responses were generated using 28-day dosing protocols. Various dosing protocols are possible for the MN assay.
P80, line 9	"Bone marrow exposure could bb"
	Response: change made.
P80, line 15	Consider ICH M7(R1) as the most current version
	Response: Version updated.
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.
	Response: these points are a major advantage of the assay. These issues are reviewed in Section 11 of the DRP.
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.
	Response: This is a rather novel use of the assay for regulatory evaluations that may prove to be quicker, more definitive, and/or more cost effective than running the *Pig-a* and/or TGR assays separately. These sorts of recommendations, however, may be more properly made in regulatory agency-specific guidances.

ANNEX VII: Distribution of mutations induced in the *Pig-a* gene of rat erythroid cells.



Data have been compiled from two studies: the blue circles show the position of mutations induced in rats dosed with *N*-ethyl-*N*-nitrosourea (ENU) in the study of Revollo et al. (2018); the orange circles show the location of mutations induced in rats dosed with 7,12-dimethylbenz[*a*]anthracene (DMBA) in Revollo et al. (2019). The X-axis shows the base numbering of the *Pig-a* reference sequence; the dark blue numbered rectangles immediately above the reference sequence numbers show the positions of *Pig-a* protein coding exons 2-6 (*Pig-a* exon 1 is not translated and not shown). The Y-axis shows the number of different rats a particular ENU- or DMBA-induced mutation was identified in. Figure prepared by V.N. Dobrovolsky.