Transgenic Rodent Gene Mutation Assays:

Current State of Validation

Prepared for Organization for Economic Cooperation and Development (OECD) by

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Introduction

The Detailed Review Paper (DRP) on Transgenic Rodent (TGR) Gene Mutation Assays, which has been approved and published by OECD (OECD, 2009), provides significant information and analysis pertinent to this review of the state of existing validation. This report also follows closely the validation criteria established by the OECD (OECD, 2005), which are used as the headings in the following text.

1) **The rationale for the test method.**
This should include a clear statement of the scientific basis, regulatory purpose and need for the test.

Scientific basis. The scientific basis for TGR mutation assays has been described exhaustively in the DRP on TGR Assays. Briefly, TGR mutation assays are well-established assays that employ transgenic rats and mice which contain multiple copies of chromosomally integrated plasmid or phage DNA that harbour reporter genes for the detection of mutation. Mutations arising in a rodent are scored by recovering the vector and analysing the phenotype of the reporter gene in a bacterial host. TGR gene mutation assays allow mutations induced in a genetically neutral transgene (Cosentino and Heddle, 1996; 1999; Swiger et al., 2001) to be scored in any tissue of the rodent, and therefore circumvent many of the existing limitations associated with the study of *in vivo* gene mutation. TGR models for which sufficient data are available to permit evaluation include Muta^{tm}mouse, Big Blue® mouse and rat, *lacZ* plasmid mouse, and *gpt* delta mouse and rat. Mutagenesis in the TGR models is normally assessed as a mutant frequency; if required, however, molecular analysis (i.e. DNA sequencing) can provide additional information on the exact nature of the mutations detected. The assays provide a unique opportunity to determine the ability of test agents to induce gene mutations *in vivo* in any tissue. Thus, TGR assays provide quick and reliable methods for detecting and quantifying gene mutations *in vivo*. They are useful for determining whether or not *in vitro* assays have produced false positive results, and for providing a sensitive and statistically reliable confirmation of marginal results in cancer bioassays of potentially genotoxic carcinogens.

Regulatory purpose and need for the test. The TGR test detects gene mutations in virtually any rodent tissue from which DNA can be obtained. While OECD Test Guidelines exist for a range of *in vitro* mutation assays that are capable of detecting chromosomal alterations or gene mutations, *in vivo* assays are required components of many genetic toxicity testing strategies.

For somatic cells *in vivo*, OECD Test Guidelines are currently available for assays capable of assessing induced chromosomal damage (TG 474 and 475). Yet, for gene mutations *in vivo*, there are no OECD Test Guidelines, aside from the little-used (Wahnschaffe et al., 2005) rodent Spot Test (TG 484). While there are some non-transgenic assays that can be used for analysis of gene mutations *in vivo*, none of these assays have an OECD test guideline, and they are limited by factors such as: the number of tissues in which genotoxicity can be measured; the state of understanding of the endpoint; the nature of the chemicals that can be detected; and cost. The TGR assay is the furthest advanced and most well characterized of the *in vivo* tests for gene mutation.

The OECD Working Group of National Coordinators of the Test Guidelines Program (WNT) has

accepted the DRP (OECD, 2009) that describes and analyzes TGR mutation assays, and it has accepted the proposal to proceed to the development of a Test Guideline, subject to demonstration of sufficient validation.

TGR assays can fulfill three different regulatory needs or strategies. 1) TGR assays could be used as the primary *in vivo* test system for confirming positive *in vitro* gene mutation results. This strategy would have immediate application in some regulatory jurisdictions (e.g. Canadian Environmental Protection Act, New Substances Notification regulations) where it can be integrated immediately into existing regulatory frameworks. However, other regulatory testing jurisdictions may need to revise their testing requirements to accommodate this approach. 2) A more easily implemented approach would be to use TGR assays as the second *in vivo* test when the primary test [normally the *in vivo* micronucleus test (TG 474) or chromosomal aberration assay (TG 475)] is negative and the substance is positive *in vitro*. 3) TGR assays also can be used in case of tumour findings to determine if carcinogenicity in the target tissue is due to genotoxic or nongenotoxic mechanisms, knowing that further understanding of the mode of action impacts risk assessment approaches, and to confirm marginal responses with an assay of much greater statistical power and reproducibility. Furthermore, TGR studies can be performed simultaneously with TG 474 on the same animals, thus minimizing the use of animals and reducing the cost of the studies

2) **The relationship between endpoint(s) and the *in vivo* biological effect, and the toxicity of interest should be described.**
This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.

The endpoint of the TGR assays is *in vivo* gene mutation. Transgenes serve as surrogates for the genes that are targets for diseases resulting from mutation. Mutation cause both somatic diseases (Erikson, 2005) and heritable genetic diseases, and is the primary biological phenomenon of interest in the induction of cancer (Cleaver, 1969). The DRP contains an exhaustive analysis of the predictive value of the assay for the detection of mutations. This analysis showed that over 96% of the almost 33,000 mutant phenotypes detected in TGR assays are true genotypic mutations by DNA sequence analysis, thus, confirming and validating the exceptional positive predictivity of the assay for the mutation endpoint.

3) **A formal detailed protocol for the test method should be readily available in the public domain.**
The protocol should be sufficiently detailed and should include, e.g., a description of the materials needed, such as specific cell types or construct or animal species that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analysed, decision criteria for evaluation of data and what are the criteria for acceptable test performance.

Details on the assay principles and the description of the multi-step experimental procedures are described in the literature and have been summarized in the DRP. A detailed internationally-harmonized protocol has been developed and published by the International Workshops on Genotoxicity Testing (IWGT:; Heddle et al., 2000; Thybaud et al., 2003). Recommendations were made regarding the proper conduct of the TGR assay, which relate to treatment protocols, accepted characteristics, and post-treatment sampling procedures. Of particular importance in

optimizing TGR protocols are two experimental variables, the administration time and the sampling time. Based on observations that mutations accumulate with each consecutive treatment, a repeated-dose regimen for a period of 28 days is recommended, with sampling at 3 days following the final treatment. If slowly proliferating tissues are of particular importance, then a longer sampling time of 28 days may be more appropriate. This protocol forms the basis for the TGR OECD Test Guideline.

4) **The intra-test variability, repeatability, and reproducibility of the test method within and amongst laboratories should be demonstrated.**
Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed.

As of December 2007, according to the data that were available, 228 chemicals had been tested in TGR assays. While the identity of these chemicals included virtually all the common mutagens and a number of nonmutagens, the majority of these exposures did not use the newer IWGT protocol, with the majority of exposures being for 1-5 days with varying sampling times (OECD, 2009; Table 1; Table 4.6). Nevertheless, from the data-based mechanistic considerations underlying the IWGT recommendations it is highly unlikely that any positive outcomes derived from a non-IWGT protocol would be negative using the IWGT protocol. In contrast, since mutations accumulate with repeated treatments (because the reporter genes are neutral resulting in no selection for or against a mutant), it is possible that non-IWGT protocols with shorter treatment times could yield false negative outcomes for weak mutagens. The 28 day treatment time proposed in the IWGT protocol overcomes this hypothetical possibility. For example, as described in the DRP, two weak mutagens, acrylamide (Thybaud *et al*., 2003) and ethyl carbamate (urethane; Singer, unpublished), have been identified correctly using the IWGT protocol, confirming the advantage of this protocol.

The fact that the assay can be performed effectively over a wide variety of treatment and sampling times attests to its adaptability and plasticity. Accordingly, studies on inter- and intra-laboratory variation, and the sources of variability that were conducted prior to the establishment of the IWGT protocol (described below) can be considered as representative of the assay's performance, since these studies were conducted on chemicals that were known to be positive under the protocol conditions studied.

Furthermore, since the DRP data cut-off date of December 2007, there have been at least 23 additional studies (data from additional studies exist but were not available for this analysis) performed on standard mutagens and molecules with unknown genotoxic potential that attest to the performance of the IWGT protocols, i.e. 28 + 3 and 28 + 28 (Table 2). These data are important because many of these studies (i.e. those performed by Contract Research Organizations (CROs) were conducted at the request of regulatory authorities, and/or were submitted to regulatory authorities, thereby demonstrating their existing routine regulatory use. Of particular significance in this regard are data on ethylmethanesulphonate (Gocke et al., 2009) that were used as the basis for a regulatory decision in the EU (Müller and Singer, (2009).

Intralaboratory variability as a measure of repeatability.

Sources of variability in the experimental protocol that can affect the performance of the assay have been examined (Piegorsch *et al*., 1994, 1997). Such sources of variability include plate-to-plate (within package), package-to-package (within animal) and animal-to-animal variability.

Data from five laboratories were evaluated in detail. The results suggested that only scattered patterns of excess variability below the animal-to-animal level occur, but that, generally, excess variability is observed at the animal-to-animal level (Piegorsch *et al*., 1997). Statistical tests that may be used to reduce variability have been suggested (Carr and Gorelick, 1994, 1995; Piegorsch *et al*., 1995) and are components of the IWGT-recommended test protocol described by Thybaud *et al.* (2003).

Fung *et al*. (1998) studied the sources of variation from a tightly controlled experimental design. Sources of variation, including plates (within packaging reactions), packaging reactions (within animal) and between animals, were evaluated for extrabinomial variation. Although hardly any evidence of overdispersion was detected at the plate level, limited evidence of extra-binomial variation was observed at the packaging reaction level. There was, nevertheless, much stronger evidence of overdispersion at the animal level. Statistical tests for increasing trend in mutant frequency with increasing dose were also performed at the animal level. A significant dose-relationship following exposure to *N*-nitrosodibenzylamine was detected by trend analysis in liver but not in bone marrow. A logistical model was used to further describe the dose-response relationship observed in N-nitrosodibenzylamine-treated liver tissue.

The DRP compared results for several chemicals in Muta™mouse liver and bone marrow and in Big Blue® mouse liver. These results are obtained from querying the DRP database (TRAID) and are not the result of any collaborative study. Muta™mouse liver and bone marrow and Big Blue® mouse liver are the only combinations where significant experimental data are currently available to allow comparisons among studies. The majority of the chemicals examined are strong mutagens and produce, as expected, positive results in the TGR assays. Among these are only a small number of instances in which ENU returned inconsistent results when inadequate sampling times were used. Nevertheless, overall, the data in the DRP strongly indicate that similar qualitative results are obtained in different studies and that the results are reproducible.

A nonstatistical comparison (Table 2) of intralaboratory reproducibility of data from studies using the IWGT protocols shows a qualitatively reproducible response from multiple experiments showing the mutagenicity of the weak mutagen ethyl carbamate (urethane).

A statistical analysis of ‘old’ and IWGT protocols was performed on data from a Muta^{tm}mouse study carried out using, ethyl carbamate, a weak mutagen (Douglas and Williams, 2011, Table 1s). A weak mutagen was chosen for analysis in order to provide a rigorous test of the robustness of the protocols in different tissues. The analysis showed that while the IWGT protocols yielded similar (positive) results in both rapidly (bone marrow) and slowly dividing (liver) tissue, the ‘old’ protocols did not produce positive results in a slowly dividing tissue (liver). Similar results were found previously for acrylamide, another weak mutagen (Thybaud *et al*., 2003). The lack of a positive response in slowly dividing tissue is likely the result of insufficient accumulation of (weak) mutagenic DNA lesions, and their fixation as mutations through cell division when using a short administration time. In contrast, the longer 28 day administration time in the IWGT protocols provide sufficient accumulation and fixation of mutations in both slowly and rapidly dividing tissues.

In addition to demonstrating the robustness of the IWGT protocols, these results show that data produced with older protocols that used shorter administration times (OECD, 2009) are still relevant to the consideration of overall TGR assay performance if performed on strong mutagens, or when positive results are reported.

Interlaboratory variability as a measure of reproducibility

A collaborative study involving 26 laboratories examined ENU mutagenesis in eight organs of Muta™mouse liver, spleen, bone marrow, brain, lung, kidney, urinary bladder and heart following a single intraperitoneal injection of 150 mg ENU/kg bw (Collaborative Study Group for the Transgenic Mouse Mutation Assay, 1996). Many of the laboratories involved in this study had no prior experience using TGR assays, and the study was preceded by a 1-day training session for all participants. A standard DNA sample was analysed by all laboratories; the results from only two of the laboratories varied over two-fold from the mean mutant frequency, and there was an overall high level of concordance in the mutant frequencies obtained using this standard sample. Among the organs tested, similar conclusions were reached by most laboratories regarding whether a positive or negative result was obtained. As expected, the potent mutagen ENU increased the mutant frequency in all organs except brain. However, the study design did not allow a rigorous statistical evaluation of the data or the extent of interlaboratory variation (Collaborative Study Group for the Transgenic Mouse Mutation Assay, 1996).

The mutagenicity of DMN was evaluated in three laboratories using common liver samples from Muta™mouse and Big Blue® mice. The liver samples compared were obtained from mice treated once with either saline or DMN (10 mg/mL, 14-day sampling time). Each assay gave an increased mutant frequency for the DMN-treated livers when compared with the saline control mutant frequencies (Tinwell *et al*., 1995).

A collaborative study examined mouse germ cell mutagenesis of ENU, isopropylmethanesulphonate (iPMS) and methylmethanesulphonate (MMS) in both Muta™mouse and Big Blue® mice. Both testicular DNA and epididymal sperm DNA were evaluated, and a range of sampling times, from 3 to 100 days, was examined. ENU and iPMS were found to be mutagenic to both testicular DNA and epididymal sperm DNA. MMS was not mutagenic under any test condition. The authors concluded that a good level of qualitative agreement was obtained for the two assays and for the same assays conducted in different laboratories (Ashby, 1995; Ashby, Gorelick and Shelby, 1997). In addition, the comparability of the *gpt* delta assay with these two assays has also been established (Swiger *et al*., 2001)

With respect to recent studies employing the IWGT protocol (28 + 3) a Japanese interlaboratory study on the performance of the *gpt* delta rat assay for detecting the mutagenicity of 2,4-diaminotoluene, 2,6-diaminotoluene, and aristolochic acid has recently been completed. The study confirms the interlaboratory comparability of this protocol (Table 2; Douglas and Williams, 2010, Table 2s, Table 3s).

In summary, the results of a number studies carried out using a variety of experimental protocols with a number of mutagens suggest that the TGR assays show good qualitative reproducibility in both somatic and germ cells and quantitative reproducibility over a range of conditions covering protocols employing shorter exposure conditions, as well as studies using the IWGT protocol across different laboratories.

5) T**he test method's performance must be demonstrated using a series of reference chemicals preferably coded to exclude bias.**
A sufficient number of the reference chemicals should have been tested under code to exclude bias.

The DRP (OECD, 2009) shows that a full range of representative and reference agents have been tested under a variety of experimental conditions in TGR assays. Analysis of predictive statistics

in the DRP showed that TGR assays perform similarly to, or better than, other assays for gene mutation; for example, the positive and negative cancer predictivities for the TGR and *Salmonella* assays were almost identical. Furthermore, Table 2 demonstrates a range of reference and representative agents tested recently using the IWGT protocol, including many tested by contract research organizations for regulatory purposes.

Guidance Document 34 (OECD, 2005) states that it is "preferable", but it is not a requirement, that agents be coded so that their identity is not known during the testing and data collection, in order to avoid bias in the results due to prior knowledge of the expected responses. While most of the data available are not from coded samples, there is little danger of bias because the methodology is mostly analytical (i.e. not subjective), and there is adequate duplication across laboratories among the chemicals tested, which provides a check against the introduction of bias.

6) **The performance of the test method should have been evaluated in relation to relevant toxicity data as well as information from the relevant target of concern.**
In the case of a substitute test method adequate data should be available to permit a reliable analysis of the performance and comparability of the proposed substitute test method with that of the test it is designed to replace.

The TGR assay is not a substitute assay; it is a new definitive test or adjunct test method (depending on the use context) that is designed to fill a gap in the *in vivo* mutation assays that are available for regulatory testing. As described above in (section 2), it faithfully detects the endpoint that it is designed to detect.

7) **Normally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.**
Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated.

Much of the data in Table 2 were derived from full GLP studies, or studies conducted in the spirit of GLP. However, it should be recognized that because this assay is not a rapid screening test, and is experimentally complex, the experiments require a significant planning effort which results in a high level of procedural and formal quality even when not conducted under full GLP. In the foreseeable use for regulatory purposes, GLP compliance will be an obvious prerequisite, and many studies have been conducted in that way already.

8) **All data supporting the assessment of the validity of the test method should be available for expert review.**
The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organised and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol.

The detailed IWGT test method protocol is readily available and in the public domain (Heddle *et al*., 2000; Thybaud et al., 2003). The test method description is sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data (see Table 2 and Supplementary Analysis). The recommended protocol contains sufficient benchmark indicators by which a laboratory can assess its proper adherence to the protocol and the quality of the results obtained. The DRP (OECD, 2009) contains well-organized, extensive data supporting the overall

validity of the test as performed prior to the IWGT protocol modifications. Table 2 contain additional summary information demonstrating the routine use of the new IWGT protocol, some of which cannot be released in more detail because the data are unpublished or proprietary. The Supplementary Analysis contains more detailed data for some of the studies in Table 2, but which are available on a restricted basis until published.

Conclusion

The level of information supporting the validation of the TGR assay is at least as extensive as was available prior to the establishment of other OECD Test Guidelines. Based on the above summary, it is concluded that there is more than sufficient evidence of validation of the TGR mutation assay to support the establishment of a Test Guideline.

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Table 1. Summary of administration time versus sampling time extracted from records assembled in the DRP (OECD, 2009).

		Sampling time (days following the last dose)						
Administration time (days)	Total no. of records*	0	≤3	3.5–7	8–14	15–28	29–60	61–420
1	1 422	13	192	203	350	336	164	164
2–5	645	7	71	66	276	126	65	34
6–14	265	43	26	2	102	38	53	1
15–28	336	84	16	86	53	56	25	16
29–60	166	77	15	27	23	3	15	6
≥61	352	228	51	18	36	0	0	19
Total	3 186	452	371	402	840	559	322	141

• Record = database record for a single animal group

Table 2. Recent TGR mutation studies conducted using IWGT-recommended protocols (28 + 3, 28 + 28)

ITEM #	SUBSTANCE or CODE	TRANSGENIC MODEL	MUTATION MARKER	INVESTIGATOR OR LABORATORY	VEHICLE	SEX	TISSUE	ADMIN TIME (DAYS)	SAMP TIME (DAYS)	ROUTE OF ADMIN.	NUMBER OF TREATMENT GROUPS (EXCLUDING VEHICLE CONTROL)	DOSE RANGE (mg/kg/day)	TEST RESPONSE (+/-), in progress)	CONCURRENT POSITIVE CONTROL (mg/kg/day)	POSITIVE CONTROL RESPONSE (+/-)	PUBLISHED, UNPUBLISHED, or PROPRIETARY
1	Acrylamide	Muta^{tm}mouse	*lacZ*	Douglas, Canada	DMSO+PBS	M	Bone Marrow	28	3	i.p.	1	25	+			Thybaud et al., 2003
								28	28		1	25	+			
							Liver	28	3		1	25	+			Unpublished
								28	28		1	25	+			
2	ENU	Muta^{tm}mouse	*lacZ*	Singer, Canada	PBS	M	Bone Marrow	28	3	i.p.	2	3-5	+			Unpublished
							Liver	28	3		2	3-5	+			
3	ENU	Muta^{tm}mouse	*lacZ*	Gocke, Switzerland	PBS	M	Bone Marrow	28	3	Oral	3	1.35-22.25	+			Gocke et al., 2009
							Liver	28	3		3	1.35-22.25	+			
							Small Intestine	28	3		3	1.35-22.25	+			
4	EMS	Muta^{tm}mouse	*lacZ*	Gocke, Switzerland	Water	M	Bone Marrow	28	3	Oral	7	1.56-100	+			Gocke et al., 2009
							Liver	28	3		7	1.56-100	+			
							Small Intestine	28	3		7	1.56-100	+			
5	Urethane	Muta^{tm}mouse	*lacZ*	Singer, Canada	PBS	M	Bone Marrow	28	3	Oral	2	25-100	-			Unpublished
								28	28		2	25-100	+			
							Liver	28	3		2	25-100	-			
								28	28		2	25-100	+			
							Small Intestine	28	3		2	25-100	-			
								28	28		2	25-100	+			
6	Benzo(a)pyrene	Muta^{tm}mouse	*lacZ*	Douglas, Canada	PBS	M	Bone Marrow	28	3	Oral	3	25-75	+			Unpublished
							Liver	28	3		3	25-75	+			
							Small Intestine	28	3		3	25-75	+			
							Stomach	28	3		3	25-75	+			

Table 2 (cont.). Recent TGR mutation studies conducted using IWGT-recommended protocols (28 + 3, 28 + 28)

ITEM #	SUBSTANCE or CODE	TRANSGENIC MODEL	MUTATION MARKER	INVESTIGATOR OR LABORATORY	VEHICLE	SEX	TISSUE	ADMIN TIME (DAYS)	SAMP TIME (DAYS)	ROUTE OF ADMIN.	NUMBER OF TREATMENT GROUPS (EXCLUDING VEHICLE CONTROL)	DOSE RANGE (mg/kg/day)	TEST RESPONSE (+/-), in progress)	CONCURRENT POSITIVE CONTROL (mg/kg/day)	POSITIVE CONTROL RESPONSE (+/-)	PUBLISHED, UNPUBLISHED, or PROPRIETARY
7	Agent 244	Bigblue® mouse	*cII*	SRI, USA	Methylcellulose	F	Liver	28	28	Oral	3	50-500	-	Urethane (50)	+	Proprietary
						F	Colon	28	28		3	50-500	-	Urethane (50)	+	
						F	Ovary	28	28		3	50-500	-	Urethane (50)	+	
						M	Liver	28	28	Oral	3	50-500	-	Urethane (50)	+	Proprietary
						M	Colon	28	28		3	50-500	-	Urethane (50)	+	
8	Agent 249	Bigblue® mouse	*cII*	SRI, USA	Methylcellulose	M	liver	28	28	Oral	3	125-500	-	Urethane (50)	+	Proprietary
							colon	28	28		3	125-500	-	Urethane (50)	+	
							kidney	28	28		3	125-500	-	Urethane (50)	+	
						F	liver	28	28	Oral	3	125-500	-	Urethane (50)	+	Proprietary
							colon	28	28		3	125-500	-	Urethane (50)	+	
							kidney	28	28		3	125-500				
9	Agent 255	Bigblue® mouse	*cII*	SRI, USA	Methylcellulose	M	liver	28	28	Oral	3	62.5-125	-	Urethane (75)	+	Proprietary
							colon	28	28		3	62.5-125	-	Urethane (75)	+	
							kidney	28	28		3	62.5-125	-	Urethane (75)	+	
							Spleen	28	28							
						F	liver	28	28	Oral	3	62.5-125	-	Urethane (75)	+	Proprietary
							colon	28	28		3	62.5-125	-	Urethane (75)	+	
							kidney	28	28		3	62.5-125	-	Urethane (75)	+	
							Spleen									
10	DMN	Bigblue® mouse	*cII*	SRI, USA	Water	M	Liver	28	28	Oral	2	2-6	+			Unpublished
11	Agent 281A	Bigblue® mouse	*cII*	SRI, USA	Water	M	Liver	28	28	Oral	3	5-60	-	DMN (2)	+	Proprietary
12	Agent 281B	Bigblue® mouse	*cII*	SRI, USA	Water	M	Liver	28	28	Oral	2	100-300	-			Proprietary

Table 2 (cont.). Recent TGR mutation studies conducted using IWGT-recommended protocols (28 + 3, 28 + 28)

ITEM #	SUBSTANCE or CODE	TRANSGENIC MODEL	MUTATION MARKER	INVESTIGATOR OR LABORATORY	VEHICLE	SEX	TISSUE	ADMIN TIME (DAYS)	SAMP TIME (DAYS)	ROUTE OF ADMIN.	NUMBER OF TREATMENT GROUPS (EXCLUDING VEHICLE CONTROL)	DOSE RANGE (mg/kg/day)	TEST RESPONSE (+/-), in progress)	CONCURRENT POSITIVE CONTROL (mg/kg/day)	POSITIVE CONTROL RESPONSE (+/-)	PUBLISHED, UNPUBLISHED, or PROPRIETARY
13a	2,4-diaminotoluene	gpt delta rat	*gpt*	Hatano Research Institute, Food and Drug Safety Center, Japan	Water	M	Liver	28	3	Oral	2	10-30	+	ENU (50 x5day)	+	Unpublished
13b	2,4-diaminotoluene	gpt delta rat	*gpt*	Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Japan	Water	M	Liver	28	3	Oral	2	10-30	+	ENU (50 x5day)	+	Unpublished
13c	2,4-diaminotoluene	gpt delta rat	*gpt*	Biosafety research Center, Foods, Drugs and Pesticides, Japan	Water	M	Liver	28	3	Oral	2	10-30	+	ENU (50 x5day)	+	Unpublished
14a	2,6-diaminotoluene	gpt delta rat	*gpt*	Hatano Research Institute, Food and Drug Safety Center, Japan	Water	M	Liver	28	3	Oral	1	60	-	ENU (50 x5day)	+	Unpublished
14b	2,6-diaminotoluene	gpt delta rat	*gpt*	Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Japan	Water	M	Liver	28	3	Oral	1	60	-	ENU (50 x5day)	+	Unpublished
14c	2,6-diaminotoluene	gpt delta rat	*gpt*	Biosafety Research Center, Foods Drugs and Pesticides	Water	M	Liver	28	3	Oral	1	60	-	ENU (50 x5day)	+	Unpublished
15a	Aristolochic acid	gpt delta rat	*gpt*	Kirin Holdongs Co.,Ltd., Japan	Water	M	Liver	28	3	Oral	2	0.3-1.0	+	ENU (50 x5day)	+	Unpublished
							Kidney	28	3	Oral	2	0.3-1.0	+	ENU (50 x5day)	+	Unpublished
15b	Aristolochic acid	gpt delta rat	*gpt*	Suntory Business Expert Ltd, Japan	Water	M	Liver	28	3	Oral	2	0.3-1.0	+	ENU (50 x5day)	+	Unpublished
						M	Kidney	28	3	Oral	2	0.3-1.0	+	ENU (50 x5day)	+	Unpublished

Table 2 (cont.). Recent TGR mutation studies conducted using IWGT-recommended protocols (28 + 3, 28 + 28)

ITEM #	SUBSTANCE or CODE	TRANSGENIC MODEL	MUTATION MARKER	INVESTIGATOR OR LABORATORY	VEHICLE	SEX	TISSUE	ADMIN TIME (DAYS)	SAMP TIME (DAYS)	ROUTE OF ADMIN.	NUMBER OF TREATMENT GROUPS (EXCLUDING VEHICLE CONTROL)	DOSE RANGE (mg/kg/day)	TEST RESPONSE (+/-), in progress)	CONCURRENT POSITIVE CONTROL (mg/kg/day)	POSITIVE CONTROL RESPONSE (+/-)	PUBLISHED, UNPUBLISHED, or PROPRIETARY
16	SR 46349B	Muta^{tm}mouse	*lacZ*	Covance Laboratories Ltd., UK	0.5% MC	M	Liver	28	28	Oral	3	10-125	-			Proprietary
17	Nifursol	Muta^{tm}mouse	*lacZ*	Covance Laboratories Ltd., UK	0.5% MC	M	ileum/jejunum	28	3	Oral	2	550-850	-			Proprietary