Three recently approved *in vivo* genotoxicity test guidelines

(Revised in February 2018)

a. Title of the test guideline (year of original adoption, year of update)

***In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene**, OECD: 490 (2015, 2016), EU: none at present;

Transgenic rodent (TGR) somatic and germ cell gene mutation assays, EU: B.58, OECD: 488 (2011, 2013);

***In vivo* alkaline single-cell gel electrophoresis assay for DNA strand breaks** (comet assay), OECD: 489 (2014, 2016), EU: none at present.

Note: the latest version of the OECD test guideline (TG) should always be used independent of whether it is published in the EU Test Method Regulation or not.

b. Link to the OECD site

http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

Link to the Health Effects test guidelines

http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

c. Which of the REACH information requirements may be met using the tests

The test methods covered in this document may be used to meet the REACH information requirements. These test methods usually need to be used within a testing strategy (see Table 1). The methods have often limitations and cannot be used for all kinds of substances. Therefore, registrants and test houses are advised to check the chapter "Specific scope and limitations of the test guideline" below before deciding on a new test/study.

According to **Annex VIII**, "*appropriate in vivo mutagenicity studies shall be **considered** in case of a positive result in any of the genotoxicity studies in Annex VII or VIII".*

When there is a positive result from an *in vitro* gene mutation study in bacteria (Ames test, OECD TG 471) or from an *in vitro* gene mutation study in mammalian cells (OECD TG 476 for tests using the *Hprt* and *xprt* genes; **OECD TG 490** for tests using the thymidine kinase gene), adequate somatic cell *in vivo* tests to investigate gene mutations are TGR (**OECD TG 488**), comet assay (**OECD TG 489**) or, if justified, Unscheduled DNA Synthesis (UDS) test with mammalian liver cells *in vivo* (OECD TG 486).

When there is a positive result from an *in vitro* cytogenicity study in mammalian cells (OECD TG 473) or from an *in vitro* micronucleus study (OECD TG 487), adequate somatic cell *in vivo* tests to investigate structural or numerical chromosome aberrations are the Mammalian Erythrocyte Micronucleus Test (OECD TG 474), Mammalian Bone Marrow Chromosomal Aberration Test (OECD TG 475) or comet assay (**OECD TG 489**).

According to **Annex IX**, "*[i]f there is a positive result in any of the* in vitro *genotoxicity studies in Annex VII or VIII and there are no results available from an* in vivo *study already, an appropriate* in vivo *somatic cell genotoxicity study shall be* ***proposed*** *by the registrant*". Consequently, a testing proposal must be submitted for *in vivo* tests intended to meet the information requirements of the REACH Regulation. Following examination of such a testing proposal, ECHA has to approve the test in its evaluation decision before it can be undertaken.

When there is a positive result from an *in vitro* gene mutation study in bacteria (Ames test, OECD TG 471) or from an *in vitro* gene mutation study in mammalian cells (OECD TG 476 or OECD TG 490), adequate somatic cell *in vivo* tests to investigate gene mutations are TGR (OECD TG 488), comet assay (OECD TG 489) or, if justified, UDS *in vivo* test (OECD TG 486). It is noteworthy that the TGR assay is the most appropriate and usually preferred test to follow up a positive result of an *in vitro* gene mutation test. The use of the UDS test should be justified on a case-by-case basis.

When there is a positive result from an *in vitro* cytogenicity study in mammalian cells (OECD TG 473) or from an *in vitro* micronucleus study (OECD 487), adequate somatic cell *in vivo* tests to investigate structural or numerical chromosome aberrations are the micronucleus test (OECD TG 474), the chromosome aberration test (OECD TG 475) or the comet assay (OECD TG 489).

Furthermore, according to Annex IX, "*if there is a positive result from an* in vivo *somatic cell study available, the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be* ***considered***." "Additional investigations" can potentially include *in vivo* studies.

At the **Annex X** tonnage level, the same information requirement applies as at Annex IX level.

In the case that results of a TGR or comet assay are already available, this is likely to affect how the information requirement that concerns the *in vitro* genotoxicity tests can be fulfilled. The *in vitro* mammalian cell gene mutation test will not usually be needed if adequate information is available from a reliable *in vivo* study capable of

detecting gene mutations. Such information may come from a **TGR** gene mutation assay.

Table 1: Test methods to be used within the testing strategy

Latest update	Test method	EU test method / OECD Test Guideline	REACH requirement according to Annex	Test on somatic / germ cells	
In vitro					
1997	Bacterial-reverse mutation test	B.13/14 / TG471	VII	-	
2016	*In vitro* mammalian chromosome aberration test	B.10* / TG 473	VIII	-	
2016	*In vitro* mammalian cell micronucleus test	B.49* / TG 487	VIII	-	
2016	*In vitro* mammalian cell gene mutation test using the *Hprt* and *xprt* genes	B.17* / TG 476	VIII[a]	-	
2016	*In vitro* mammalian cell gene mutation test using the thymidine kinase gene	None / TG 490	VIII[a]	-	
In vivo					
2016	Mammalian erythrocyte micronucleus test	B.12* / TG 474	IX[b], X[b]	somatic cells	
2016	Mammalian bone marrow chromosome aberration test	B.11* / TG 475	IX[b], X[b]	somatic cells	
2016	*In Vivo* Mammalian Alkaline Comet Assay	None / TG 489	IX[b,c], X[b,c]	somatic cells [f]	
2013	Transgenic Rodent (TGR) Somatic and Germ Cell Gene Mutation Assays	B.58 / TG 488	IX[c,d], X[c,d]	somatic and germ cells	
1997	Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*	B.39* / TG 486	IX[e], X[e]	somatic cells	
2016	Rodent dominant lethal test	B.22* / TG 478	IX[d], X[d]	germ cells	

2016	Mammalian spermatogonial chromosome aberration test	B.23* / TG 483	IX[d], X[d]	germ cells	

* The EU method is outdated and does not reflect the latest update of the corresponding OECD method. Any new test should be performed following the updated OECD TG.
[a] To be performed only if Ames (TG 471) and cytogenicity test (TG 473 or TG 487) are negative.
[b] Test required only to follow up a positive result in chromosome aberration *in vitro*, if no results are already available from an *in vivo* study.
[c] Test required only to follow up a positive result in gene mutation *in vitro*, if no results are already available from an *in vivo* study
[d] Test on germ cells to be considered only if positive result were obtained in *in vivo* somatic cell study (if toxicokinetic data are not conclusive).
[e] Less suitable but may be required, under certain conditions, to follow up a positive result in gene mutation *in vitro*, if no results are already available from an *in vivo* study.
[f] Comet assay may be performed on gonadal cells (which contain a mixture of somatic and germ cells).

d. How to use these methods

As indicated above, the TGR assay can be used as follow-up to positive results from an *in vitro* gene mutation study to examine, whether the effect seen is relevant also *in vivo*, i.e. in animals and, ultimately, in humans. TGR is considered to be the most appropriate test for that type of follow-up testing because this assay does measure mutations, i.e. permanent transmissible changes in the DNA.

The comet assay can be used to investigate whether the gene mutations or chromosomal aberrations observed *in vitro* are relevant also *in vivo.* It is noteworthy that the comet assay is considered to be an indicative test detecting DNA lesions and alone may not allow a definitive conclusion to be drawn about germ cell mutagenicity. Moreover, the *in vivo* chromosome aberration test and the *in vivo* micronucleus test are considered to be the most appropriate tests to follow up the concern of chromosomal aberrations.

Before any decisions are made about the need for *in vivo* testing, a review of the *in vitro* test results and all available information on the toxicokinetic and toxicodynamic profile of the test substance is needed. A particular *in vivo* test should be conducted only when it can be reasonably expected from all the properties of the test substance and the proposed test protocol (including the route of exposure) that the **specific target tissue will be adequately exposed to the test substance and/or its metabolites**. If necessary, a targeted investigation of **toxicokinetics** should be conducted before progressing to *in vivo* testing (e.g. a preliminary studies, *in vitro*, if possible) to confirm that absorption occurs and that an appropriate administration route is used. In the case that the aim of the genotoxicity study is to address effects at the site of contact, toxicokinetic information would usually not be needed.

For substances inducing gene mutation or chromosomal aberration *in vitro*, and for which no indication of sufficient systemic availability has been presented, or that are short-lived or reactive, an alternative strategy involving studies to focus on tissues at initial sites of contact with the body should be considered. Expert judgement should be used on a case-by-case basis to decide which tests are the most appropriate. The available options are the *in vivo* **comet assay**, the **TGR** gene mutation assays, and

DNA adduct studies. For any given substance, expert judgement, based on all the available toxicological information, will indicate which of these tests are the most appropriate. The route of exposure should be selected that best allows assessment of the hazard posed to humans. For insoluble substances, the possibility of release of active molecules in the gastrointestinal tract may indicate that a test involving the oral route of administration is particularly appropriate.

Another use of TGR assays is to examine whether a substance may induce genotoxic effects in germ cells. It is to be noted that the first step of the evaluation of the effects on germ cells is to consider the available toxicokinetic information, in order to understand whether the substance may reach the germ cells (*in vivo)*.

The transgenic rodent (TGR) somatic and germ cell gene mutation assays (**OECD TG 488**) and the comet assay (**OECD TG 489**) employ methods by which **any tissue** (containing nucleated cells) of an animal can in theory be examined for effects on the genetic material. This gives the possibility to examine target tissues (including germ cells) and site-of-contact tissues (i.e. skin, epithelium of the respiratory or gastro-intestinal tract). However, differences can exist regarding the number and type of tissues for which the use of a specific test has been scientifically validated. For instance, the TGR assays as described in OECD TG 488 **can be used to examine germ cells**, whereas the comet assay as described in the OECD TG 489 is, at present, **not appropriate** to measure DNA strand breaks in mature germ cells. On the other hand, the OECD TG 489 does state that the comet assay has been performed in gonadal/testicular cells at different stages of differentiation and that "gonads contain a mixture of somatic and germ cells. For this reason, positive results in whole gonad (testis) are not necessarily reflective of germ cell damage, nevertheless, they suggest that tested chemicals have reached the gonad."

The ability to include sampling of somatic and germ/gonadal cells in a single study (be it TGR or comet assay) significantly reduces the need to perform additional studies to obtain such information, thereby conforming to the 3Rs principle[1]. The TGR assays can also be used to investigate the possibility of transmission of mutations to the offspring since treatment of transgenic male mice can result in offspring carrying mutations.

If the TGR assay is to be conducted on somatic tissues, germ cell samples should also be collected if possible, frozen and analysed for mutagenicity only in case of a positive result in somatic cells.

e. Status of the validation by EURL ECVAM, when necessary

These tests have been validated before the OECD (and EU) approval.

f. Specific scope and limitations of the test guidelines

For example, limitations on chemical categories covered, if any, and limitation on classification and labelling are addressed below.

TGR (OECD TG 488): Gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi- assay models). / Since the transgenes are

[1] The 3Rs refer to replacement, reduction and refinement of animal experiments.

transmitted by the germ cells, they are present in every cell. Therefore, gene mutations and/or chromosomal rearrangements can be detected in virtually all tissues of an animal, including target tissues and specific site-of-contact tissues, provided that the substance reaches that tissue.

TGR can be used directly for providing evidence of *in vivo* gene mutagenicity. Therefore, for substances that appear preferentially to induce gene mutations, the TGR assays are the most appropriate and usually preferred tests to follow up an *in vitro* gene mutation positive result and to detect substances that induce gene mutation *in vivo*.

The comet assay (OECD TG 489): DNA strand breaks. / The DNA strand breaks may result from direct interactions of the substance with DNA, alkali labile sites or as a consequence of incomplete excision repair. Therefore, the comet assay recognises **primary DNA damage that would lead to gene mutations and/or chromosome aberrations**, but will also detect DNA damage that may be effectively repaired or lead to cell death. The comet assay can be applied to almost every tissue of an animal from which single cell or nuclei suspensions can be made, including specific site-of-contact tissues.

The comet assay requires specific supporting information, for example results from *in vitro* mutagenicity studies, to be used for making definitive conclusions about *in vivo* mutagenicity and lack thereof.

Certain substances in addition to those already noted may need special consideration, such as **highly electrophilic substances** that give positive results *in vitro*, particularly in the absence of metabolic activation. Although these substances may react with proteins and water *in vivo,* and thus may be rendered inactive towards many tissues, they may be able to express their mutagenic potential at the initial site of contact with the body. Consequently, the use of test methods such as the **comet assay (OECD TG 489)** or **TGR (OECD TG 488)** that can be applied to the respiratory tract, upper gastrointestinal tract and skin may be appropriate. It is possible that specialised test methods will need to be applied in these circumstances, and that these may not have recognised, internationally valid, test guidelines. The validity and utility of such tests and the selection of protocols should be assessed by appropriate experts or authorities on a case-by-case basis.

***In vitro* mammalian cell gene mutation tests using the thymidine kinase gene (OECD TG 490):** The purpose of these tests is to detect gene mutations induced by chemicals. It should be noted that these tests can provide some insight into the type(s) of damage (mutagens vs. clastogens) induced by the test chemical. It is not appropriate to use these tests to detect aneugens.

OECD TG 490 is intended for use with two cell lines: the L5178Y mouse lymphoma cell line and the TK6 human lymphoblastoid cell line. Although the two cell lines vary because of their origin, cell growth, p53-status, etc., the thymidine kinase gene mutation tests can be conducted in a similar way in both cell types as described in OECD TG 490.

g. Reference to the relevant guidances

1) Information toolkit

http://echa.europa.eu/en/support/information-toolkit

This web page provides practical information and tools in relation to help using existing information and non-test methods as a first step to meeting the REACH information requirements.

2) Guidance on information requirements and chemical safety assessment, Chapter R.7.7.1 Mutagenicity
http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf.

Further information: Please note that in some parts of this website section, reference to other genotoxicity test methods/guidelines is made, depending on the context. Please note that this section does not aim to provide guidance on the use of these other methods. This website section primarily covers guidance on the recently approved tests using the thymidine kinase gene (OECD TG 490), TGR (OECD TG 488) and comet assay (OECD TG 489), as according to the titles given in part a.