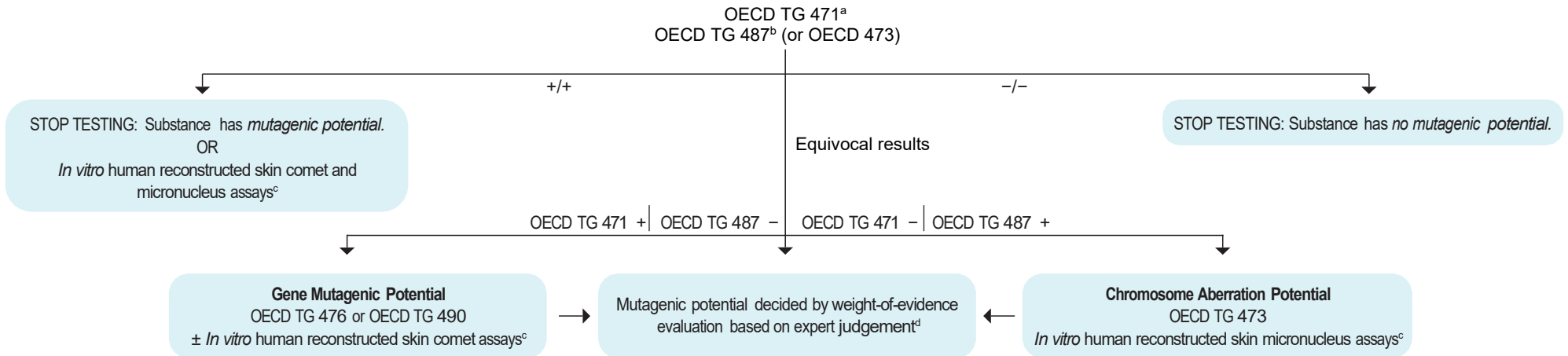


# METHODS FOR PREDICTING GENOTOXICITY



The proposed two-test battery consisting of OECD TG 471 and OECD TG 487 covers all three endpoints to be assessed in the standard testing battery (gene mutation, structural chromosomal aberration, and numerical chromosomal aberration). The set of tests used in the *in vitro* battery should be as small as scientifically justifiable to reduce the number of false positives.

a) Potential false positive results can be reduced by investigating the impact of bacteria-specific metabolism.

b) Potential false positive results can be reduced by using p53-competent human cells, choosing measures of cytotoxicity based on cell proliferation, and carefully checking the source and the characterisation of the cells.

c) Tier 2 assays include the *in vitro* human reconstructed skin micronucleus and comet assays, which are most applicable to testing dermally applied substances. For substances administered by other routes, methods based on 3-D liver or airway models or the HET-MN may be more relevant. (HET-MN uses animal embryos, thus, it should be used only to replace a regulatory requirement for a test on live animals at later life stages.)

d) This may include information on toxicodynamics, toxicogenomics, toxicokinetics, mechanism of toxicity, involvement of reactive oxygen species, saturation of detoxification mechanisms, gene expression profiles, and cell transformation assays. As an example, the European Chemicals Agency has waived tier 2 *in vivo* studies based on the ToxTracker assay and read-across data.

METHOD	TEST PRINCIPLE
<a href="#">OECD TG 471</a> : Bacterial Reverse Mutation Test (Ames Test)	The Ames test uses at least five strains of amino acid-requiring <i>Salmonella typhimurium</i> and <i>Escherichia coli</i> to detect point mutations by base substitutions or frameshifts. It detects mutations that revert other mutations present in the test strains, restoring the functional capability of the bacteria to synthesise an essential amino acid. A very large database of results for a wide variety of structures is available for bacterial reverse mutation tests, and well-established methodologies have been developed for testing chemicals with different physicochemical properties, including volatile compounds.
<a href="#">OECD TG 476</a> : <i>In Vitro</i> Mammalian Cell Gene Mutation Tests Using the HPRT and XPRT Genes	This test measures mutation at hypoxanthine-guanine phosphoribosyltransferase (HPRT) and at a transgene of xanthine-guanine phosphoribosyltransferase (XPRT). The HPRT and XPRT mutation tests detect different spectra of genetic events. Mutant frequency is determined by seeding known numbers of cells in a medium containing the selective agent in order to detect mutant cells and in a medium without the selective agent in order to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted.

METHOD	PRINCIPLE OF THE TEST
<a href="#">OECD TG 487</a> : <i>In Vitro</i> Mammalian Cell Micronucleus Test (MNvit)	The MNvit detects micronuclei in the cytoplasm of interphase cells. Micronuclei may originate in acentric chromosome fragments (i.e. those lacking a centromere) or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic test substances in cells that have undergone cell division during or after exposure to the test substance.
<a href="#">OECD TG 473</a> : <i>In Vitro</i> Mammalian Chromosomal Aberration Test	This method identifies agents that cause structural chromosomal aberrations in cultured mammalian somatic cells. At predetermined intervals after the exposure of cell cultures to the test substance, the cells are treated with a metaphase-arresting substance, harvested, and stained. Metaphase cells are then analysed microscopically for the presence of chromosomal aberrations.
<a href="#">OECD TG 490</a> : <i>In Vitro</i> Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	This TG includes two distinct <i>in vitro</i> mammalian gene mutation assays requiring two specific thymidine kinase (TK) heterozygous cell lines: L5178Y tk <sup>+</sup> /– 3.7.2C cells for the mouse lymphoma assay and TK6 tk <sup>+</sup> /– cells for the TK6 assay. Genetic events detected using the TK locus include both gene mutations and chromosomal events. Mutant frequency is determined by seeding known numbers of cells in a medium containing the selective agent to detect mutant cells and in a medium without the selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted.
<i>In Vitro</i> Human Reconstructed Skin Comet Assay	The comet assay detects the migration of DNA in an agarose gel following electrophoresis, which results in a “head” of intact DNA and a “tail” of fragmented DNA. Single cells are isolated from a highly differentiated three-dimensional tissue model consisting of normal, human-derived epidermal keratinocytes cultured on specially prepared tissue culture inserts and evaluated by fluorescence microscopy after staining with a DNA-binding fluorescent agent. The percentage of DNA in the tail of the comet is used as a measure of DNA damage.
<i>In Vitro</i> Human Reconstructed Skin Micronucleus (RSMN) Assay	Like the MNvit, the RSMN test detects micronuclei in the cytoplasm of interphase cells – in this case, a highly differentiated three-dimensional tissue model consisting of normal, human-derived epidermal keratinocytes cultured on specially prepared tissue culture inserts.

### Select Publications

- Groff K, Evans SJ, Doak SH, et al. *In vitro* and integrated *in vivo* strategies to reduce animal use in genotoxicity testing. *Mutagenesis*. 2021;36:389-400.
- Pfuhrer S, Downs T, Hewitt N, et al. Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays. *Mutagenesis*. 2021;36:1-17.
- Pfuhrer S, Pirow R, Downs TR, et al. Validation of the 3D reconstructed human skin comet assay, an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays. *Mutagenesis*. 2021;36(1):19-35.
- Pfuhrer S, van Benthem J, Curren R, et al. Use of *in vitro* 3D tissue models in genotoxicity testing: strategic fit, validation status and way forward. Report of the working group from the 7<sup>th</sup> International Workshop on Genotoxicity Testing (IWGT). *Mutat Res Genet Toxicol Environ Mutagen*. 2020;850-851:503135.
- Conway GE, Shah UK, Llewellyn S, et al. Adaptation of the *in vitro* micronucleus assay for genotoxicity testing using 3D liver models supporting longer-term exposure durations. *Mutagenesis*. 2020;35(4):319–330.
- Reisinger K, Dony E, Wolf T, Maul K. Hen’s egg test for micronucleus induction (HET-MN). *Methods Mol Biol*. 2019;2031:195-208.
- European Commission, Joint Research Centre. Zuang, V, Dura, A, eds. EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2019). EUR 30100 EN. Publications Office of the European Union; 2020.
- Scientific Committee on Consumer Safety. The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation 10<sup>th</sup> Revision. European Commission; 2018.
- Corvi R, Madia F. *In vitro* genotoxicity testing – can the performance be enhanced? *Food Chem Toxicol*. 2017;106:600-608.
- Organisation for Economic Co-operation and Development Environment, Health and Safety Division. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014–2015: series on testing and assessment no 238. OECD. Published 22 August 2017. Accessed 24 February 2022.
- Corvi R, Madia F, Worth A, Whelan M. EURL ECVAM Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing. EUR 26375. Publications Office of the European Union; 2013.
- European Chemicals Agency. N,N,4-trimethylpiperazine-1-ethylamine. European Chemicals Agency. Accessed 24 February 2022.

## IN SILICO TOOLS FOR PREDICTING GENOTOXICITY

*In silico* strategies for genotoxicity often rely on two complementary modelling approaches, used in tandem to maximise predictive confidence. **Expert rule-based systems** use "structural alerts" (e.g., specific molecular substructures, such as aromatic amines or nitro groups) known to react with DNA to inform predictions. These systems also provide a mechanistic basis for why a chemical is flagged as potentially mutagenic. Complementing this approach, **statistical models** employ machine learning algorithms trained on vast datasets of experimental results (such as the Ames test) to statistically correlate physicochemical properties with genotoxic activity.

As an example, the International Council for Harmonisation (ICH) [M7](#) guidance on the assessment and control of DNA-reactive (mutagenic) impurities in pharmaceuticals calls for the use of *in silico* models, including an expert rule-based model (e.g., Derek Nexus or Toxtree) and a complementary statistical model (e.g., Sarah Nexus) (See Figure 1).

An important consideration in predicting genotoxicity is bioactivation, the process by which a non-toxic parent chemical (pro-mutagen) is converted into a DNA-reactive metabolite by the body's enzymes. Advanced *in silico* models integrate metabolic simulators with QSAR models to predict the genotoxicity of both the parent compound and its likely metabolites.

A non-exhaustive list of *in silico* tools for predicting genotoxicity is provided below. Please contact Kyle Martin at [kmartin@thepsci.eu](mailto:kmartin@thepsci.eu) to include additional resources on this list or with any questions.

TOOL	DEVELOPER	METHOD/APPROACH	AVAILABILITY
<a href="#">ADMET Predictor</a>	Simulations Plus	Statistical	Commercial
<a href="#">CASE Ultra</a>	MultiCASE Inc.	Statistical	Commercial
<a href="#">Derek Nexus</a>	Lhasa Limited	Expert rule-based	Commercial
<a href="#">Leadscope Model Applier</a>	Instem	Statistical	Commercial
<a href="#">OECD QSAR Toolbox</a>	OECD/Laboratory of Mathematical Chemistry	Statistical (read-across/QSAR)	Open-source
<a href="#">Sarah Nexus</a>	Lhasa Limited	Statistical	Commercial
<a href="#">Toxtree</a>	IDEAconsult Ltd./European Commission	Expert rule-based	Open-source
<a href="#">VEGA Hub</a>	Istituto di Ricerche Farmacologiche Mario Negri	Consensus/hybrid	Open-source

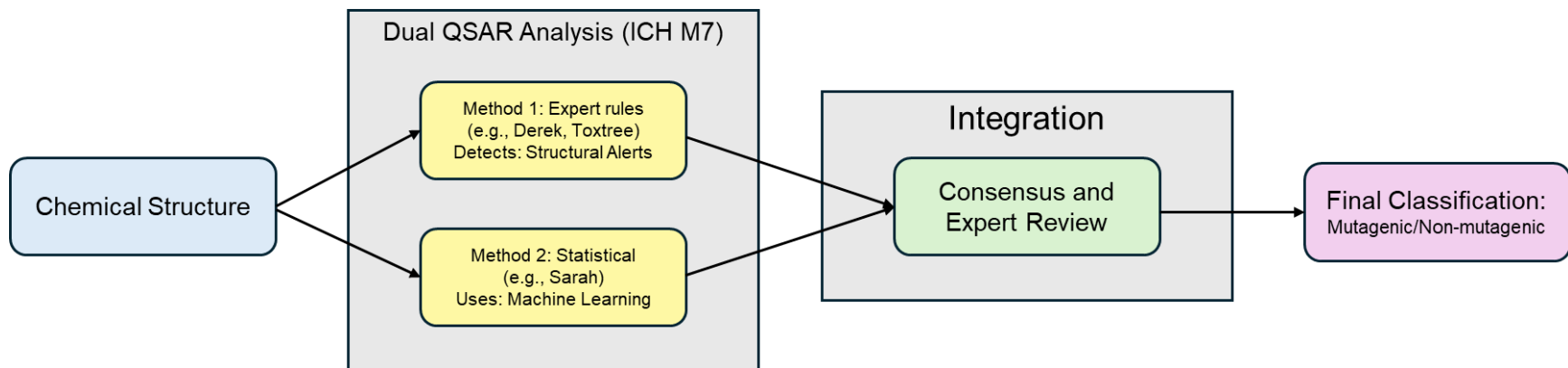


Figure 1. Shows the complementary use of an expert rule-based and a statistical model as described in ICH M7 guidance for predicting the mutagenic potential of pharmaceutical impurities.