Lecture 14.
Methods of genotoxicology. Test systems and test objects

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GENOTOXICITY

Genotoxicity can be defined as the ability of a test item to cause damage to the genetic material of cells.

A genotoxic agent may be:

- ✓ a mutagen (change the DNA code, induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms),
- ✓ a clastogen (damage or breaking the chromosomes)
- ✓ an aneugen (change the number of chromosomes).

The term genotoxicity is often confused with mutagenicity, but mutagens are only one class of genotoxin. In order to gain insight into the potential of a test, it is important to test for all three major mechanisms of genotoxicity.

Thus, Genotoxicity is a broader term and refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity.

GENOTOXICITY TESTING

The tests for genotoxicity include tests which provide an indication of induced damage to DNA (but not direct evidence of mutation) via effects such as unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE), DNA strand breaks, DNA adduct formation or mitotic recombination, as well as tests for mutagenicity.

The tests that do not detect mutagenicity but rather primary DNA damage are commonly termed "indicator" tests. DNA adduct formation, for example, occurs when a substance binds covalently to DNA, initiating DNA repair, which can either return the DNA to its original state or, in the case of mis-repair, result in a mutation.

Genotoxicity testing is performed with the following aims:

- to identify substances which could cause heritable damage in humans,
- to predict potential genotoxic carcinogens in cases where carcinogenicity data are not available, and
- to contribute to understanding of the mechanism of action of chemical carcinogens.

GENOTOXICITY TESTING

For an adequate evaluation of the genotoxic potential of a chemical substance, different end-points:

- ✓ induction of gene mutations,
- ✓ structural chromosome aberrations
- ✓ numerical chromosomal alterations

have to be assessed, as each of these events has been implicated in carcinogenesis and heritable diseases. An adequate coverage of all the above-mentioned end-points can only be obtained by the use of **multiple test systems (i.e. a test battery)**, as no individual test can simultaneously provide information on all end-points.

The screening strategy for mutagenicity testing is based on a battery of tests and includes both *in vitro* and *in vivo* assays, according to the results obtained. The above approach ensures that a wide variety of genetic damage such as gene mutation, chromosomal damage, and aneuploidy can be identified. Guidelines have been recommended internationally.

GENOTOXICITY TESTING

Genotoxicity assessment can be divided into three phases:

Phase 1 is based upon in vitro tests that are performed with cultured bacterial and mammalian cells;

Phase 2 involves the assessment of mutagenic activity in vivo in somatic cells;

Phase 3 assays screen for germ cell mutagens.

Recommended protocols for the suitable tests are given in the Organisation for Economic Co-operation and Development (OECD) guidelines and the International Workshops on Genotoxicity Testing (IWGT) guidance.

Table 3 Characteristics of the most popular bioassays used to assess the mutagenicity of compounds

Phase	Test name	Endpoint	Reference
1	*Salmonella typhimurium* reverse mutation test	Gene mutations in bacteria	OECD (1997a) Test Guideline 471
1	*Escherichia coli* WP2 reverse mutation test	Gene mutations in bacteria	OECD (1997a) Test Guideline 471
1	In vitro mouse lymphoma test	Gene mutations in mammalian cells	OECD (1997e) Test Guideline 476
1	Hypoxanthine guanine phosphorybosyl transferase (HPRT) gene mutation assay	Gene mutations in mammalian cells	OECD (1997e) Test Guideline 476
1	In vitro mammalian cell micronucleus test	Structural and numerical chromosome alterations	OECD (2010) Test Guideline 487
1	In vitro mammalian chromosome aberration test	Chromosome aberrations	OECD (1997b) Test Guideline 473
1	In vitro comet assay	DNA damage	Burlinson (2012)
1	*Saccharomyces cerevisiae* gene mutation assay	Gene mutations in yeast	OECD (1986a) Test Guideline 480
2	Mammalian erythrocyte micronucleus test	Structural and numerical chromosome alterations	OECD (1997c) Test Guideline 474
2	Mammalian bone marrow chromosome aberration test	Structural chromosome aberrations	OECD (1997d) Test Guideline 475
2	Transgenic animal assays for point mutations	Gene mutations	IWGT Test Guideline
2	In vivo comet assay	DNA damage	Burlinson et al. (2007) Burlinson (2012)
2	Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo	DNA damage	OECD (1997g) Test Guideline 486
3	Transgenic animal assays for point mutations	Gene mutations	IWGT Test Guideline
3	DNA mutation in expanded simple tandem repeat (ESTR) test		Singer et al. (2006)
3	Mammalian spermatogonial chromosome aberration test	Structural chromosome aberrations	OECD (1997f) Test Guideline 483
3	Mouse visible specific locus test	Gene mutations	Russell et al. (1981)
3	Mouse biochemical specific locus (MBSL) test	Gene mutations	Lewis et al. (1986)
3	Rodent dominant lethal test	Gene mutations and chromosome changes	OECD (1984) Test Guideline 478
3	Mouse heritable translocation assay	Structural and numerical chromosome changes	OECD (1986b)Test Guideline 485

PHASE 1

Phase 1 assays employ bacteria and mammalian cells and are used for the identification of gene mutations and chromosome alterations. In the early mutagenicity assessment, two or three different tests in bacteria and mammalian cells should be used.

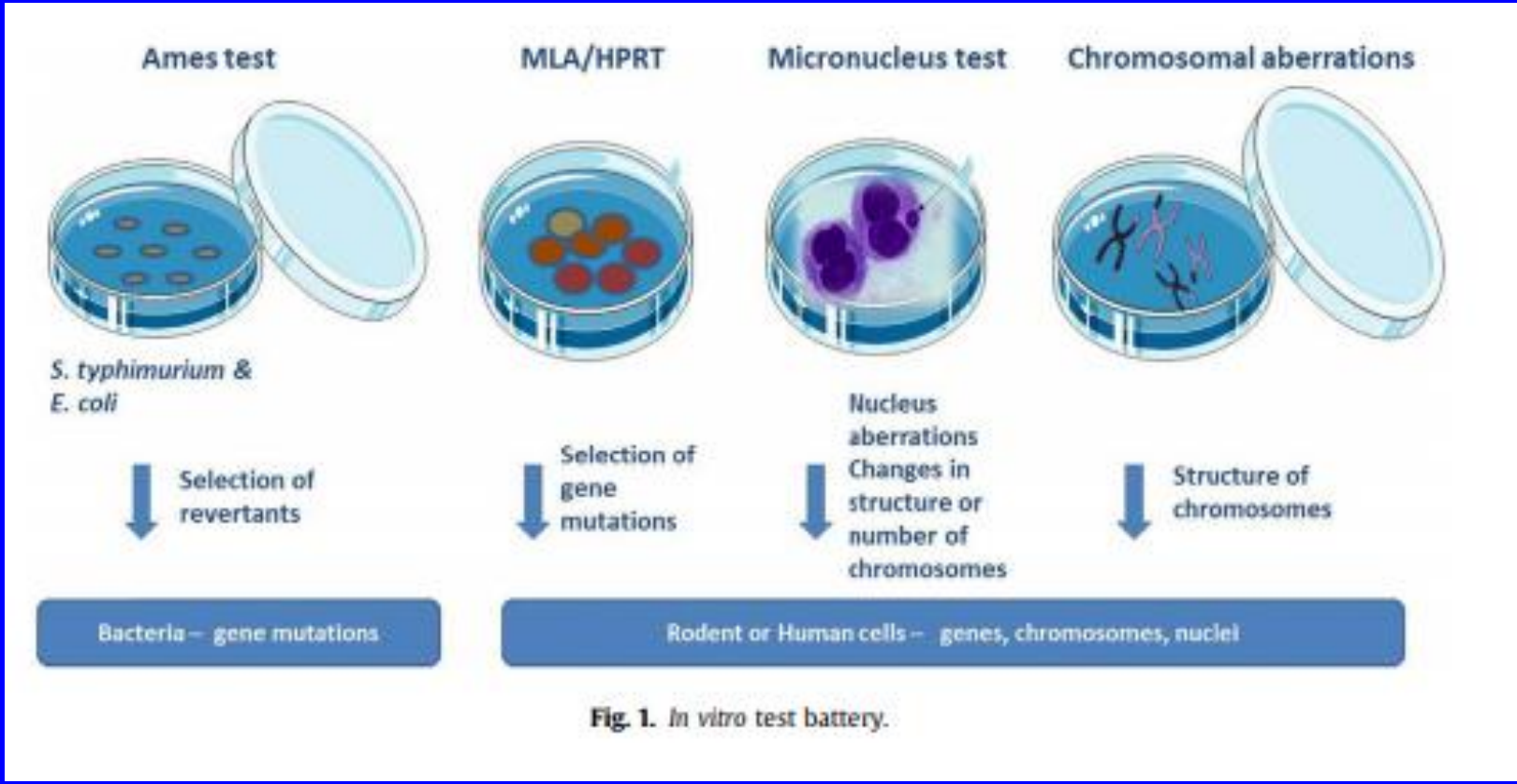
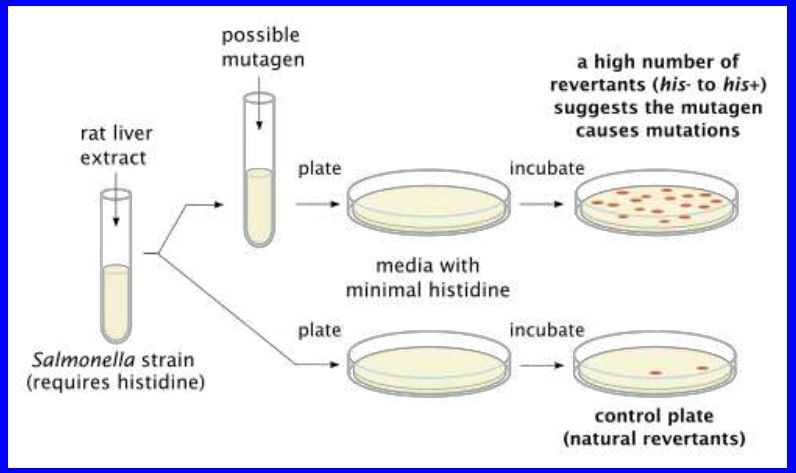


Fig. 1. *In vitro* test battery.

PHASE 1

The bacterial reverse mutation test (Ames test) is the most widely used assay to detect gene mutations. The test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. It has the ability to differentiate between frame-shift and base-pair substitutions with the use of different bacterial strains.

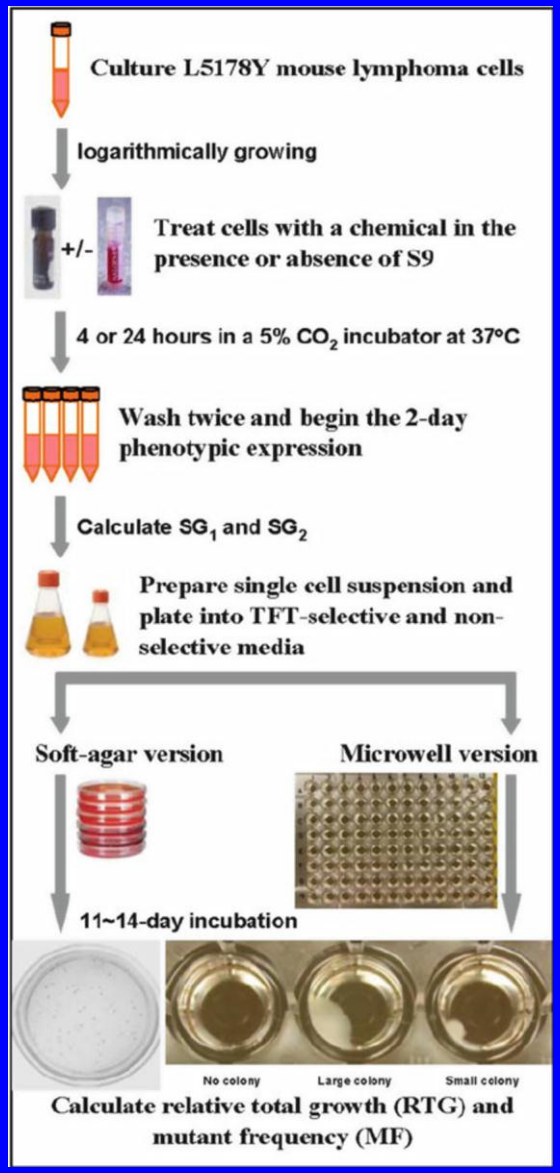
The principle of this test is that it detects mutations which revert mutations originally present in the test strains and which restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.



Test name	Main advantages	Main disadvantages
Salmonella typhimurium assay	- Very extensive database available - Easy to perform - No special equipment is necessary	- Tester organism is a potentially pathogenic bacterium - Several tester strains should be used - A relatively long time necessary to perform the analysis - Will not detect mutagens that interact with eukaryote-specific targets
Escherichia coli WP2 assay	- Easy to perform - No special equipment is necessary - Only one tester strain is needed	- A relatively long time necessary to perform the analysis - Will not detect mutagens that interact with eukaryote-specific targets

PHASE 1

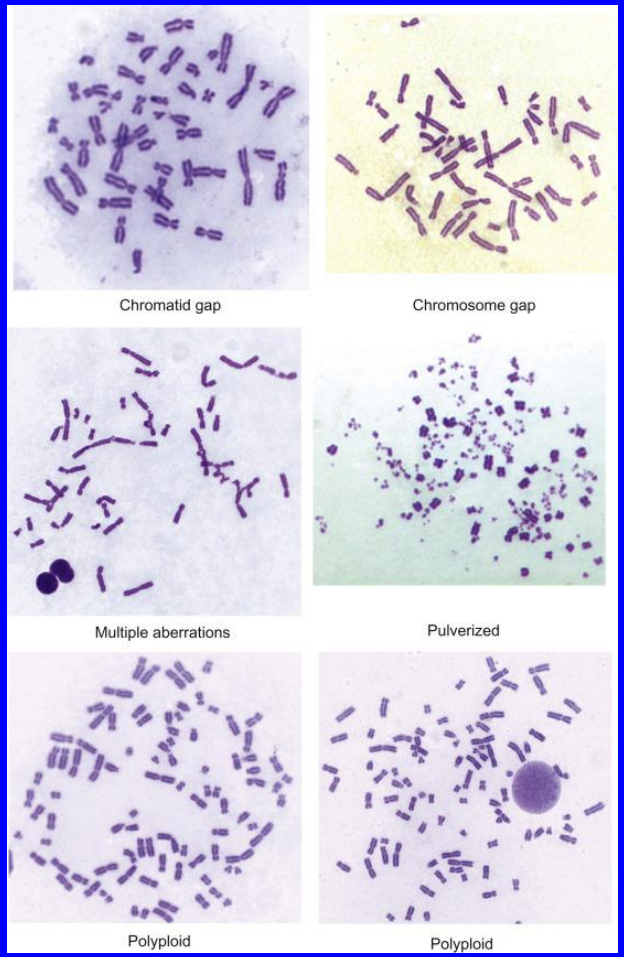
Mammalian mutation ***assays*** are useful especially in case of bactericidal compounds and agents acting preferentially on the replication system in mammals. The ***in vitro mammalian cell gene mutation test*** can detect gene mutations, including base pair substitutions and frame-shift mutations. Common Phase 1 in vitro mammalian tests include: the ***mouse lymphoma thymidine kinase (TK) gene mutation assay***, which detects compounds that induce forward gene mutations in the tk gene of the L5178Y mouse lymphoma cell line, and the ***hypoxanthine guanine phosphorybosyl transferase (HPRT) gene mutation assay***, which identifies agents that cause gene mutations in the hprt gene of a suitable cell line, such as Chinese hamster cells.



PHASE 1

With reference to chromosomal abnormalities detection, both structural and numerical changes can be identified in vitro in metaphase-spread preparations from exposed mammalian cells. Common in vitro chromosomal damage tests include the mammalian chromosome aberration test and the micronucleus test.

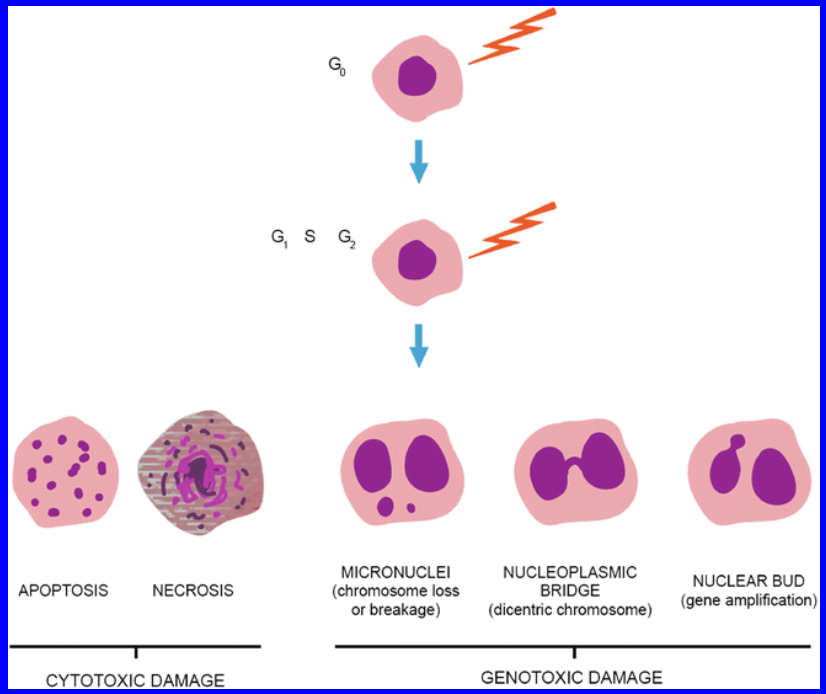
The ***in vitro chromosomal aberration*** (CAvit) ***test*** detects structural aberrations and may give an indication for numerical chromosome aberrations (polyploidy) in cultured mammalian cells caused by the test substance. The in vitro chromosomal aberration test may employ cultures of established cell lines or primary cell cultures. The test has been widely used for many decades but it is resource intensive, time consuming and it requires good expertise for scoring.



PHASE 1

The purpose of the ***in vitro micronucleus test*** (MNvit) is to identify substances that cause structural and numerical chromosomal damage in cells that have undergone cell division during or after the exposure to the test substance. In the MNvit, micronuclei in the cytoplasm of cultured mammalian cells during inter- phase is detected. The micronucleus test is a procedure for the detection of both aneuploidy and clastogenicity in cultured mammalian cells. The MNvit is rapid and easy to conduct and it is the only in vitro test that can efficiently detect both clastogens and aneugens.

In the both tests, cytotoxicity needs to be controlled to avoid false positive results, as with other in vitro genotoxicity tests conducted in mammalian cells.



PHASE 2

Studies to investigate gene mutations:
→ Gene mutation assays in transgenic models

Studies to investigate chromosome damage:
→ Mammalian erythrocyte micronucleus test;
→ Mammalian bone marrow chromosomal aberration test

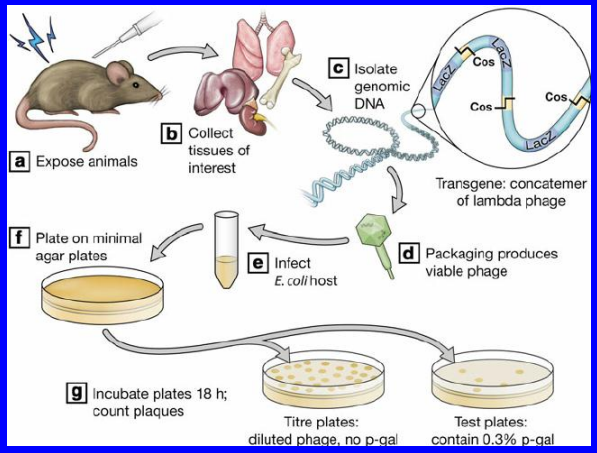
Studies to investigate primary DNA damage:
→ Comet assay (the single-cell gel electrophoresis assay)
→ Mammalian unscheduled DNA synthesis (UDS) assay *in vivo*

PHASE 2

In vivo transgenic rodent (TGR) gene mutation assay

The transgenic rodent mutation assay (TGR) is based on transgenic rats and mice that contain multiple copies of chromosomally integrated phage or plasmid shuttle vectors that harbour reporter genes for detection of mutation and/or chromosomal rearrangements (plasmid model and Spi-assay) induced *in vivo* by test substances. TGR mutation assays measure mutations induced in genetically neutral marker genes (i.e. genes that have no immediate consequence to the animal) recovered from virtually any tissue of the rodent. These neutral transgenes are transmitted by the germ cells. Mutations arising in a rodent are scored by recovering the transgene and analysing the phenotype of the reporter gene in a bacterial host deficient for the reporter gene.

The transgenic mice models respond to mutagens in a similar manner to endogenous genes and are suitable for the detection of point mutations, insertions and small deletions. The Spi-assay and the plasmid model can detect large deletions. The transgenic rodent models could also be used in repeated-dose toxicity studies as the transgenes are neutral genes.



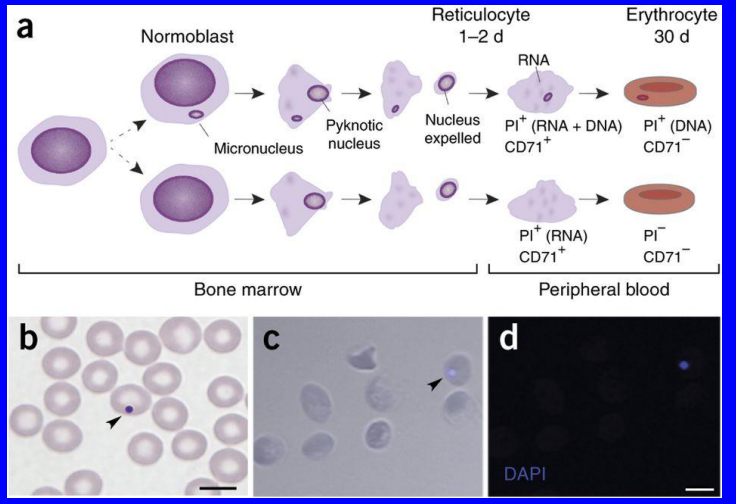
PHASE 2

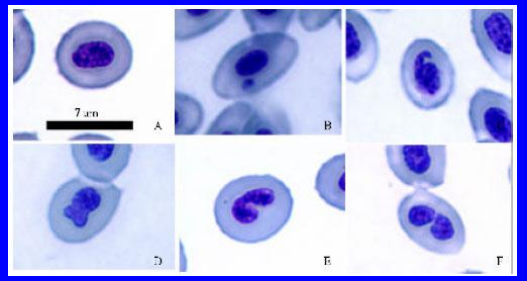
In vivo mammalian erythrocyte micronucleus test

The purpose of the in vivo mammalian erythrocyte micronucleus test (MNviv) is to identify substances that cause structural and numerical chromosomal damage in somatic cells in vivo. The damage results in the formation of micronuclei, containing chromosome fragments or whole chromosomes in young (polychromatic) erythrocytes sampled in bone marrow and/or reticulocytes of peripheral blood cells of animals, usually rodents.

This assay has a long history of use and it is also potentially applicable in tissues other than the bone marrow or the peripheral blood. The MNviv is still the most widely used in vivo genotoxicity test that detects both clastogens and aneugens.

Possible confounding effects like hypo- and hyperthermia may affect the formation of micronuclei and therefore the scoring. The MNviv can be combined with FISH to provide additional mechanistic information.



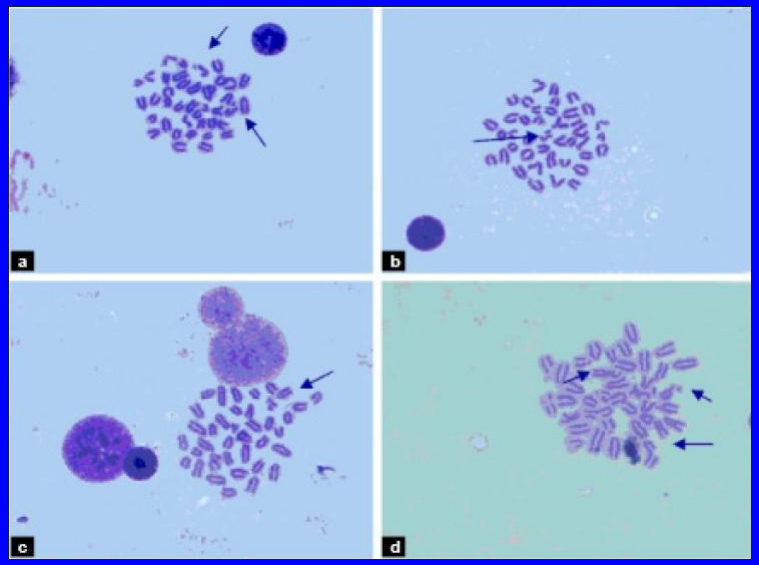


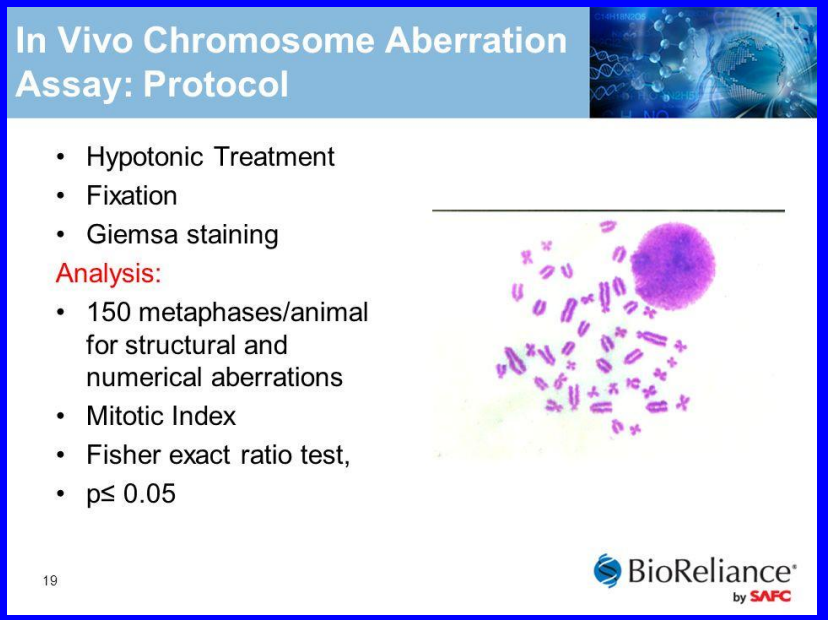
PHASE 2

In vivo mammalian bone marrow chromosomal aberration test

The mammalian *in vivo* chromosomal aberration (CA) test is used for the detection of structural chromosomal aberrations induced by test substances in bone marrow cells of animals, usually rodents. Bone marrow is the target tissue of this test, therefore if there is evidence that the test substance or the reactive metabolite does not reach the bone marrow, it would not be appropriate to use this test.

It requires experienced scientists for the scoring of metaphases. It might not detect organ-specific compounds and unstable compounds or metabolites. This assay is potentially applicable also to tissues other than the bone marrow.



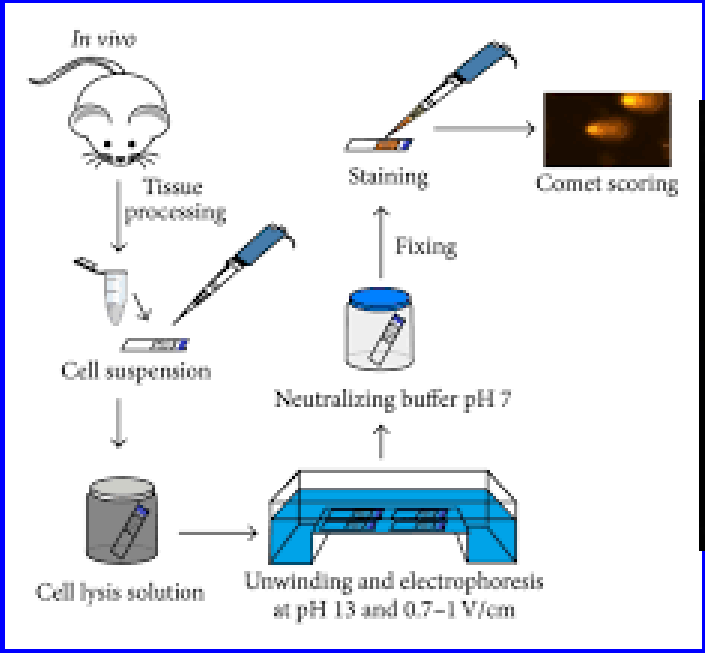


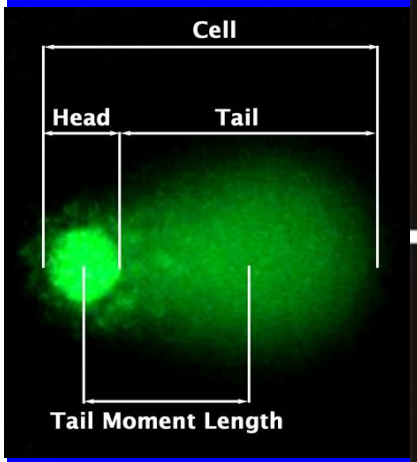
PHASE 2

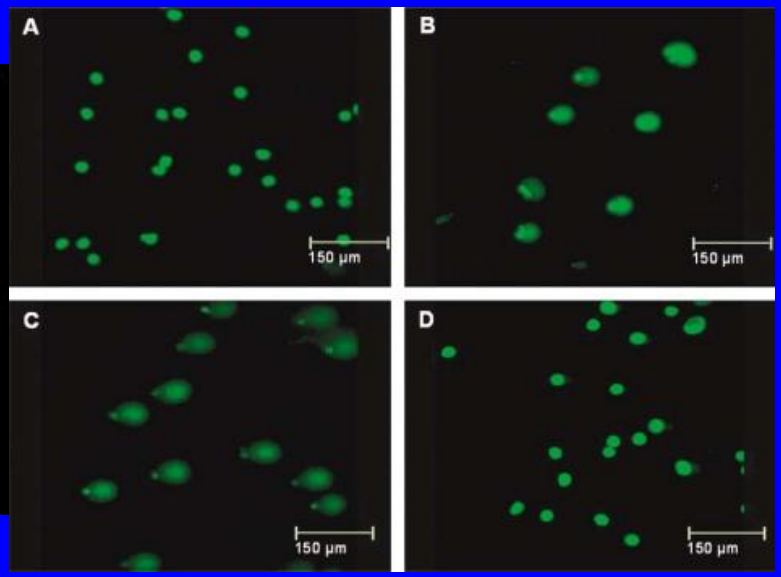
In vivo Comet assay (the single-cell gel electrophoresis assay)

The *in vivo* Comet assay detects DNA single and double strand breaks, alkali-labile lesions, as well as DNA strand breaks arising during the repair of DNA lesions.

The *in vivo* Comet assay has the advantage of being rapid and easy to conduct and may be applied to any tissues that can be subcultured. Cell division is not required and a low number of cells is sufficient for the analysis. It is considered an indicator test detecting pre-mutagenic lesions and can be used for mechanistic studies.





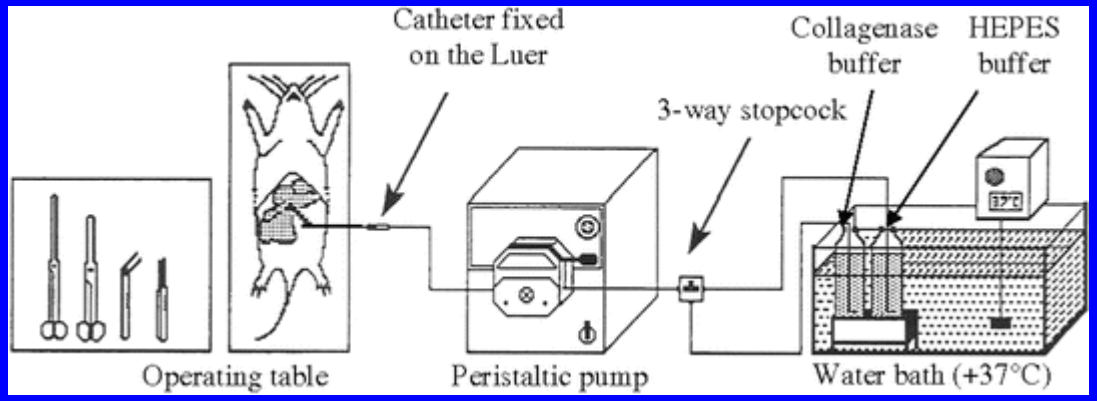


PHASE 2

In vivo mammalian unscheduled DNA synthesis (UDS) test

The *in vivo* UDS test allows the investigation of genotoxic effects of substances in the liver. The endpoint measured is indicative of DNA adducts removal by nucleotide excision repair in liver cells and it is measured by determining the uptake of labelled nucleosides in cells that are not undergoing scheduled (S-phase) DNA synthesis.

It has to be considered as an indicator test for DNA damage and not a surrogate test for gene mutations per se. The UDS assay has a long history of use but it is useful only for some classes of substances. Tissues other than the liver may in theory be used. However, UDS has a limited use for cells other than liver and its sensitivity has been questioned. It is resource intensive and the scoring time consuming. Moreover, radio-labelled substances are required when performing this test.



PHASE 3

Compounds that give positive results for mutagenic potential in somatic cells in vivo should be further tested with germ cells. Germ cell assays available in Phase 3 fall into two classes.

Class 1 includes assays in germ cells *per se*:

- ✓ gene mutation tests in transgenic animals;
- ✓ DNA mutation in expanded simple tandem repeat (ESTR) test;
- ✓ mammalian spermatogonial chromosomal aberration tests.

Class 2 contains assays used for the identification of alterations in the offspring of exposed animals:

- ✓ mouse visible specific locus test;
- ✓ mouse biochemical specific locus (MBSL) test;
- ✓ rodent dominant lethal test;
- ✓ mouse heritable translocation assay

PHASE 3

Rodent dominant lethal test

Dominant lethal (DL) effects cause embryonic or foetal death. Induction of a dominant lethal event after exposure to a test substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal aberrations (structural and numerical anomalies), but gene mutations and toxic effects cannot be excluded.

Generally, male animals are exposed to the test substance and mated to untreated virgin females. The most widely used is the single administration of the test substance by oral or by intraperitoneal injection. Normally, three dose levels should be used. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time, and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos.

The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group.

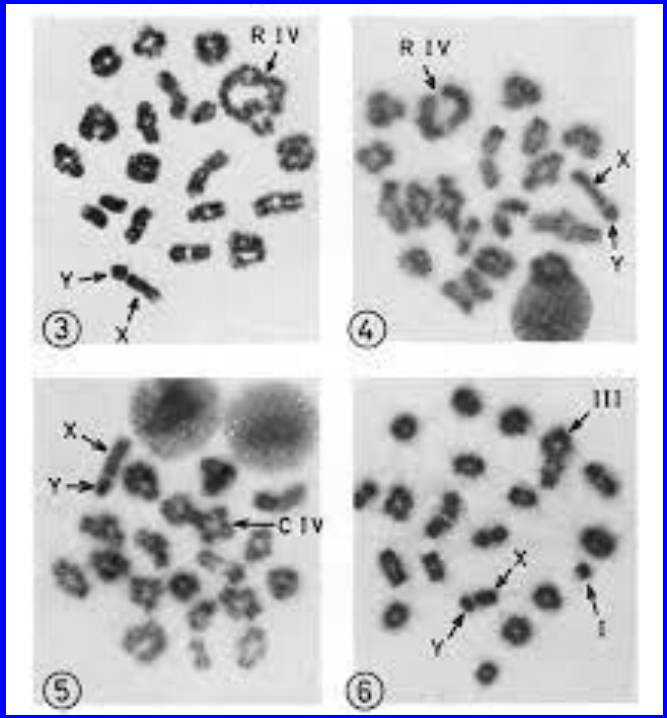
PHASE 3

Mouse heritable translocation test

The mouse heritable translocation test detects structural and numerical chromosome changes in mammalian germ cells as recovered in first generation progeny. The types of chromosome changes detected in this test system are reciprocal translocations. Carriers of translocations and XO-females show reduced fertility which is used to select first generation progeny for cytogenetic analysis.

Translocations are cytogenetically observed in meiotic cells at diakinesis metaphase I of male individuals.

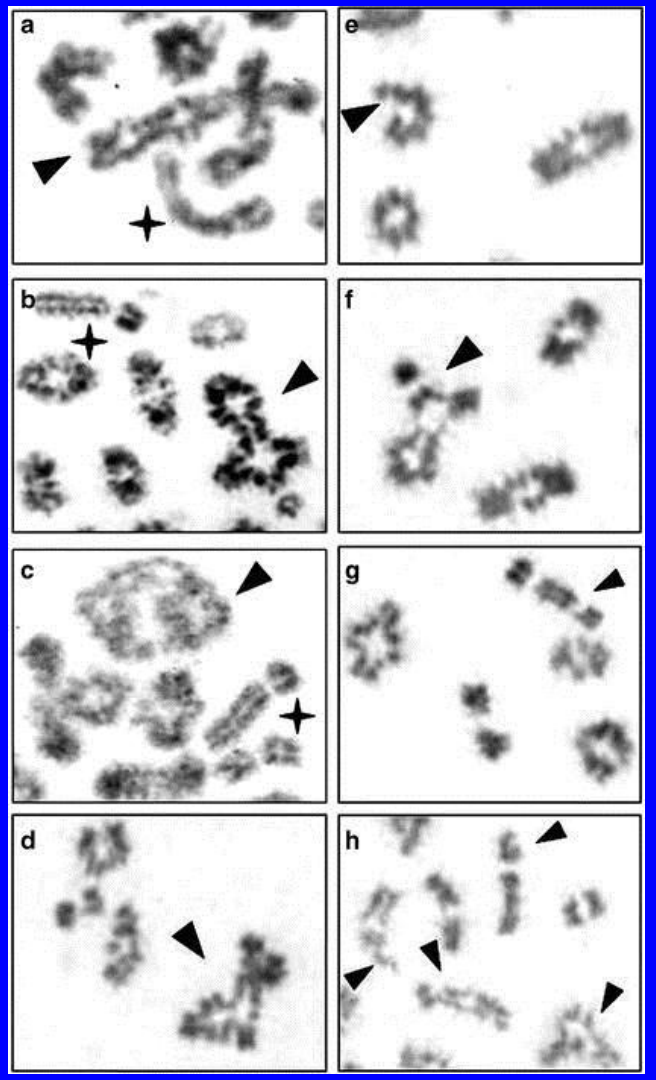
For translocation heterozygosity one of two possible methods is used: fertility testing of first generation progeny; or cytogenetic analysis of all male first generation progeny are possible.



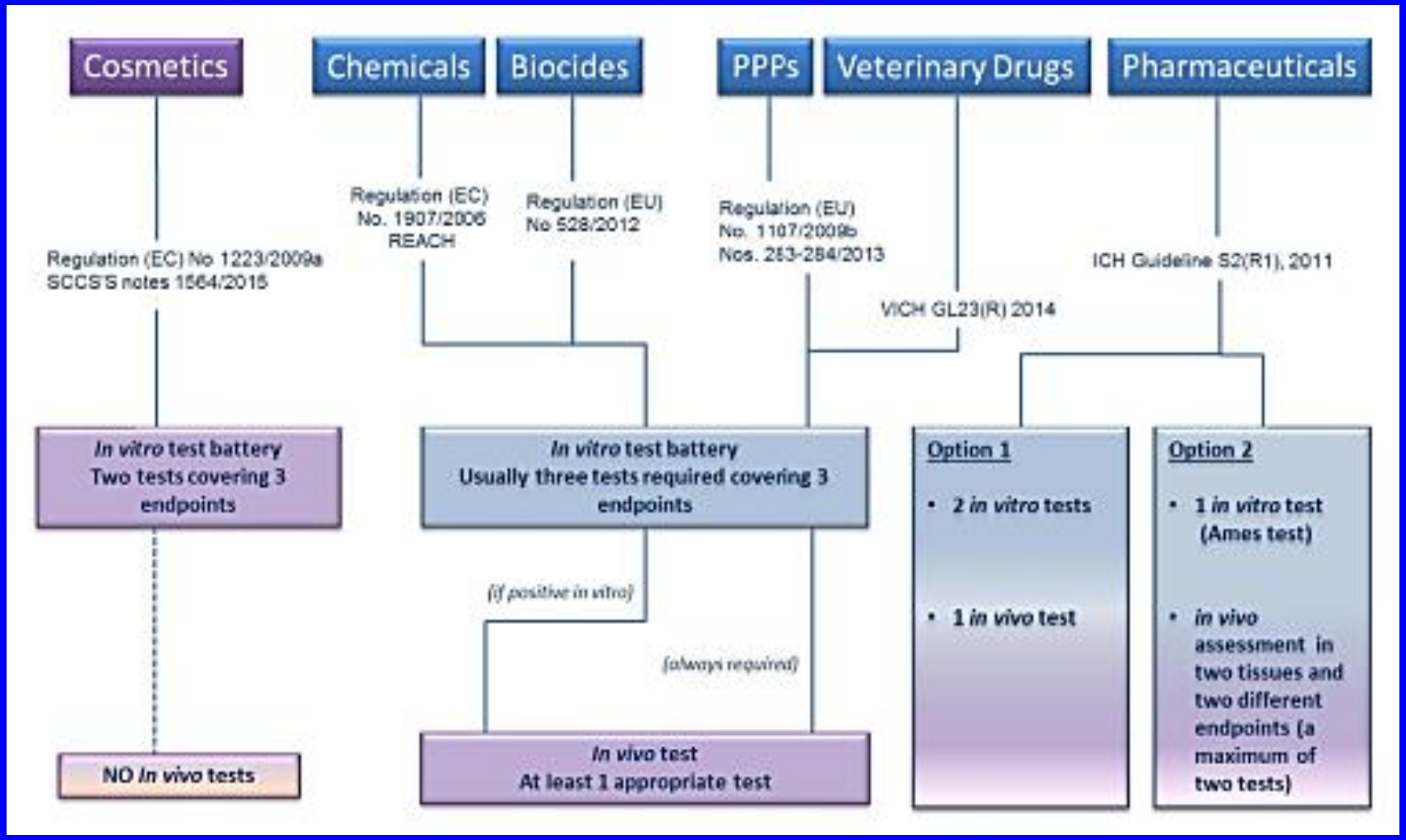
PHASE 3

Mammalian spermatogonial chromosomal aberration test

This test measures structural chromosomal aberrations (both chromosome- and chromatid-type) in dividing spermatogonial germ cells and is, therefore, expected to be predictive of induction of heritable mutations in these germ cells. The purpose of the in vivo mammalian spermatogonial chromosomal aberration test is to identify those chemicals that cause structural chromosomal aberrations in mammalian spermatogonial cells. In addition, this test is relevant to assessing genetoxicity because, although they may vary among species, factors of in vivo metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response.



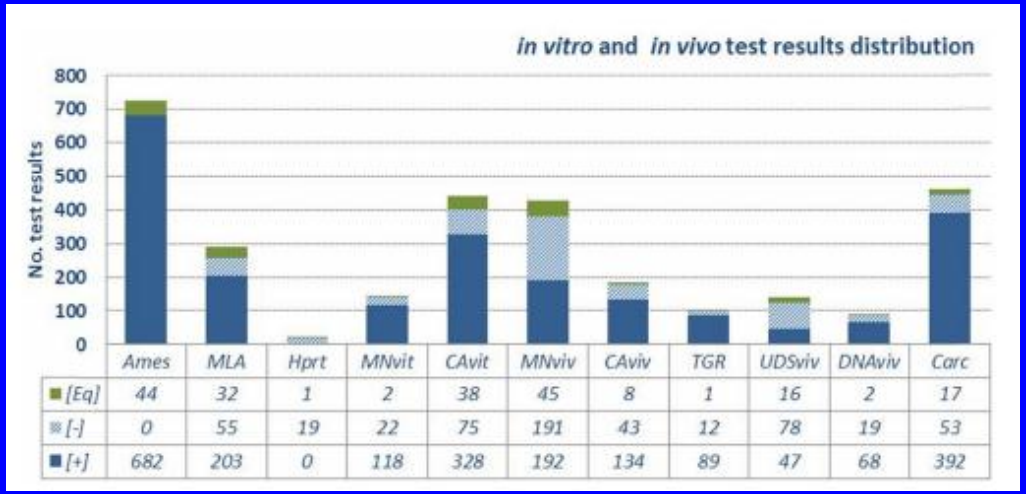
Summary of testing requirements and guidance documents for genotoxicity assessment in the EU



PPP - plant protection products

THE GENOTOXICITY & CARCINOGENICITY CONSOLIDATED DATABASE (DB) constructed following the recommendation of the EURL ECVAM workshop (EU Reference Laboratory for Alternatives to Animal Testing) was launched at the end of 2014 (https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicitycarcinogenicity-db).

It represents a structured master database that compiles available genotoxicity and carcinogenicity data for Ames positive chemicals originating from different sources and complemented by literature search. Data were collected for the following tests: in vitro tests (Ames, mouse lymphoma Tk+/- [MLA] or Hprt, micronucleus [MN], chromosome aberration [CA]); in vivo tests (MN, CA, UDS, transgenic models [TGR], DNA breakage [Comet and alkaline elution assay]); rodent carcinogenicity.



	Ames	MLA	Hprt	MNvit	CAvit	MNviv	CAviv	TGR	UDSviv	DNAviv	Carc
[Eq]	44	32	1	2	38	45	8	1	16	2	17
[-]	0	55	19	22	75	191	43	12	78	19	53
[+]	682	203	0	118	328	192	134	89	47	68	392

[+] positive, [-] negative and [Eq] equivocal

Thank you for attention!