GENOTOXICITY ASSESSMENT – WHEN YOU NEED IT AND HOW TO DO THE EVALUATION

PART 2 – ASSESSMENT OF GENOTOXIC RISK

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TOPICS FOR TODAY

- Toxicological risk assessment of genotoxicity
  - Non targeted analysis (E&L)
  - Targeted analysis
  - QSAR
- Genotoxicity testing
  - Gene mutation
  - Chromosomal damage
  - *In vivo* testing
  - Sample prep

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GENOTOXICITY RISK ASSESSMENT

- ISO 10993-3, Section 5.1:
- Assessment of genotoxic risk should be made:
  - by conducting toxicological risk assessment based on chemical characterization according to ISO 10993-17 and ISO 10993-18;
  - **or**
  - by performing genotoxicity testing in the standard test battery (ISO 10993-3, Section 5.2.2).
- In certain cases (e.g. a material which has not been used in a legally marketed device for its intended use), **both toxicological risk assessment** of medical device constituents based on chemical characterization **and genotoxicity testing** of a medical device can be appropriate.
- *Genotoxicity testing can also be appropriate when chemical characterization data is insufficient for a toxicological risk assessment.*
- *If the device is made with and releases nanomaterials, additional evaluation shall be considered.*

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GENOTOXICITY RISK ASSESSMENT

The TSLs …. apply to systemic …., **genotoxicity**, carcinogenicity, …. for oral or parenteral routes, as well as adults, paediatrics …. and pregnant women



Material/manufacturing info



Chemical characterisation

Toxicological risk analysis (TRA)



Chemical analysis

- Aflatoxin-like compounds;
- N-Nitroso compounds;
- Azo compounds;
- Polyhalogenated –dibenzodioxins, -dibenzofurans, and -biphenyls;
- Strained heteronuclear rings;
- Heavy metals (e.g. elemental, ionic, or compounds) ;
- Alpha-nitro furyl compounds;
- Hydrazines/triazenes/azides/azoxy compounds;
- Polycyclic amines;
- Steroids;
- Organophosphorous compounds.

Identified constituent
Total quantity

- the nature of harm is irritation,
- the cohort of concern substances or excluded compounds, or
- substances that are not identified constituents (i.e. unknown or incomplete chemical identity

Toxicological screening limit (TSL)

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THRESHOLDS OF TOXICOLOGICAL CONCERN (TTC)

- Some evaluation schemes that employ the TTC concept use both non-cancer- and cancer-based threshold values, **only one set of TTC values is applied here to simplify the application of TTC values** for the safety assessment of constituents that may be released from medical devices.
- The TTC values used are based on the Acceptable Daily Intake values derived for individual mutagenic impurities in pharmaceuticals.
- A TTC value of 1.5 µg/person/day corresponds to a theoretical **excess lifetime cancer risk** of 1 in 100,000 (10^{-5}).
- The threshold value derived by ICH for mutagenic impurities in the longest exposure duration category (1,5 µg/d) is lower than the threshold value assigned to the most protective Cramer Class non-carcinogenic TTC value (90 µg/d), which is intended to be protective for chronic exposure to non-carcinogens.
- Carcinogens elicit cancer via genotoxic or non-genotoxic mechanisms. The TTC values used in ISO 10993 are intended to be protective for exposure to **carcinogens that exert their effect via either mechanism**.
- For limited and prolonged contacting medical devices, the recommended TTC value is 120 µg/d.
- The remaining TTC values (i.e. 20 µg/d, 10 µg/d, and 1,5 µg/d) apply to medical devices categorized as long-term contacting.
- When a medical device is categorized as long-term body contacting, but actual body contact is uncertain/plausible/actual, then the TTC value for > 10 years (**1,5 µg/d**) should be selected.

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TSL AND TTC

- Toxicological screening limits are based on mutagenic TTC values applicable to the duration of exposure to the constituent.
- The toxicological screening limit can be calculated according to ISO 10993-17

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INFORMATION FOR GENOTOXICITY RISK ASSESSMENT

- Genotoxicity can be evaluated in a toxicological risk assessment of chemical information.
- Any toxicological risk assessment shall be conducted in accordance with the requirements of ISO 10993-17 unless otherwise justified.



QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP – QSAR

Methods for prediction of biological effect of chemical compounds based on mathematical and statistical relationship

The methods are based on the assumption, that the activity of a certain chemical compound is related to its structure

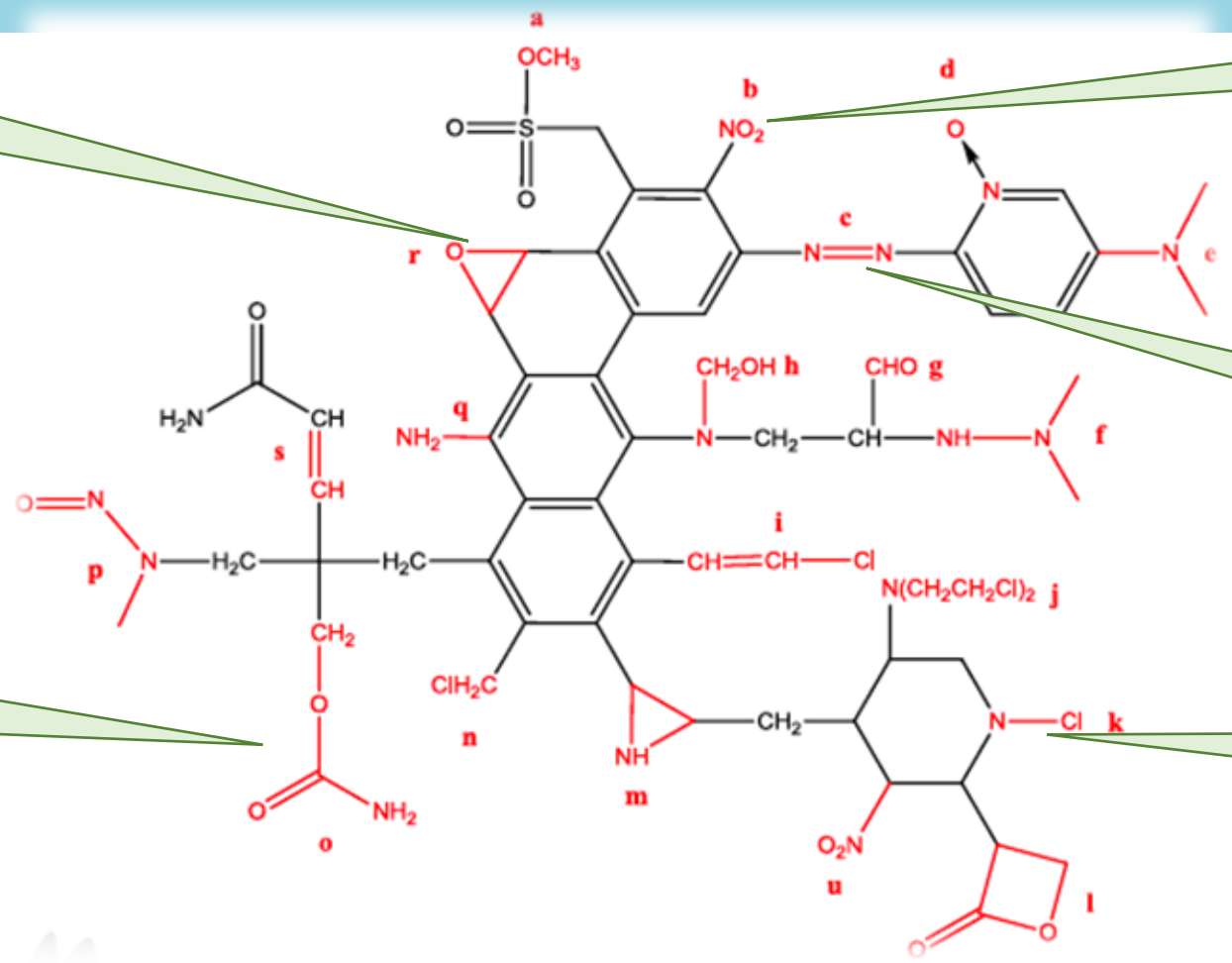
This approach says that the activity is related to the chemical structure through a certain mathematical algorithm

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THE ASHBY TENNANT POLY-CARCINOGEN

Aliphatic epoxides and aromatic oxides

Aromatic nitro groups



Aromatic azo groups

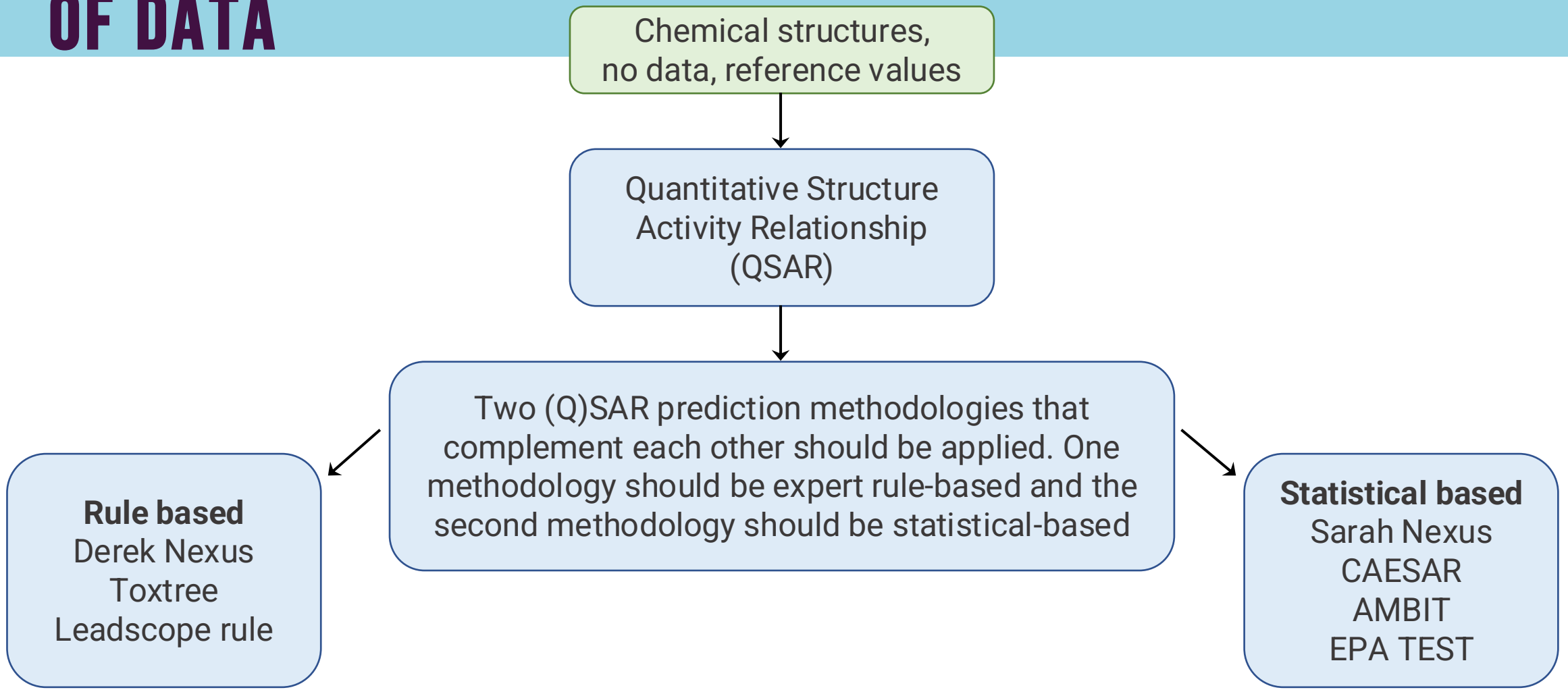
Derivatives of urethane (carbamates)

N-chloramines

Ashby's poly-carcinogen model, modified after Ashby and Tennant (1988) and Tennant and Ashby (1991)

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TOXICOLOGICAL RISK ASSESSMENT IN THE ABSENCE OF DATA



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QSAR TOOLS

Rule based

System Name	Vendor	Commercial or non-Commercial	Type
DEREK Nexus	Lhasa ltd	Commercial	Rule-Based
DEREK Sarah	Lhasa ltd	Commercial	Statistical-based
Leadscope	Leadscope	Commercial	Rule- and Statistical-Based
CASE Ultra	MultiCASE	Commercial	Rule- and Statistical-Based
TIMES-SS	OASIS	Commercial	Rule-Based
REACH*Across*	UL	Commercial	Statistical-based
ToxTree	JRC	Non-Commercial	Rule-Based
OECD QSAR Toolbox	ECHA	Non-Commercial	Rule-Based
VEGA	VEGA HUB	Non-Commercial	Statistical-Based

Gives
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Statistical based

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you accurate mutagenicity

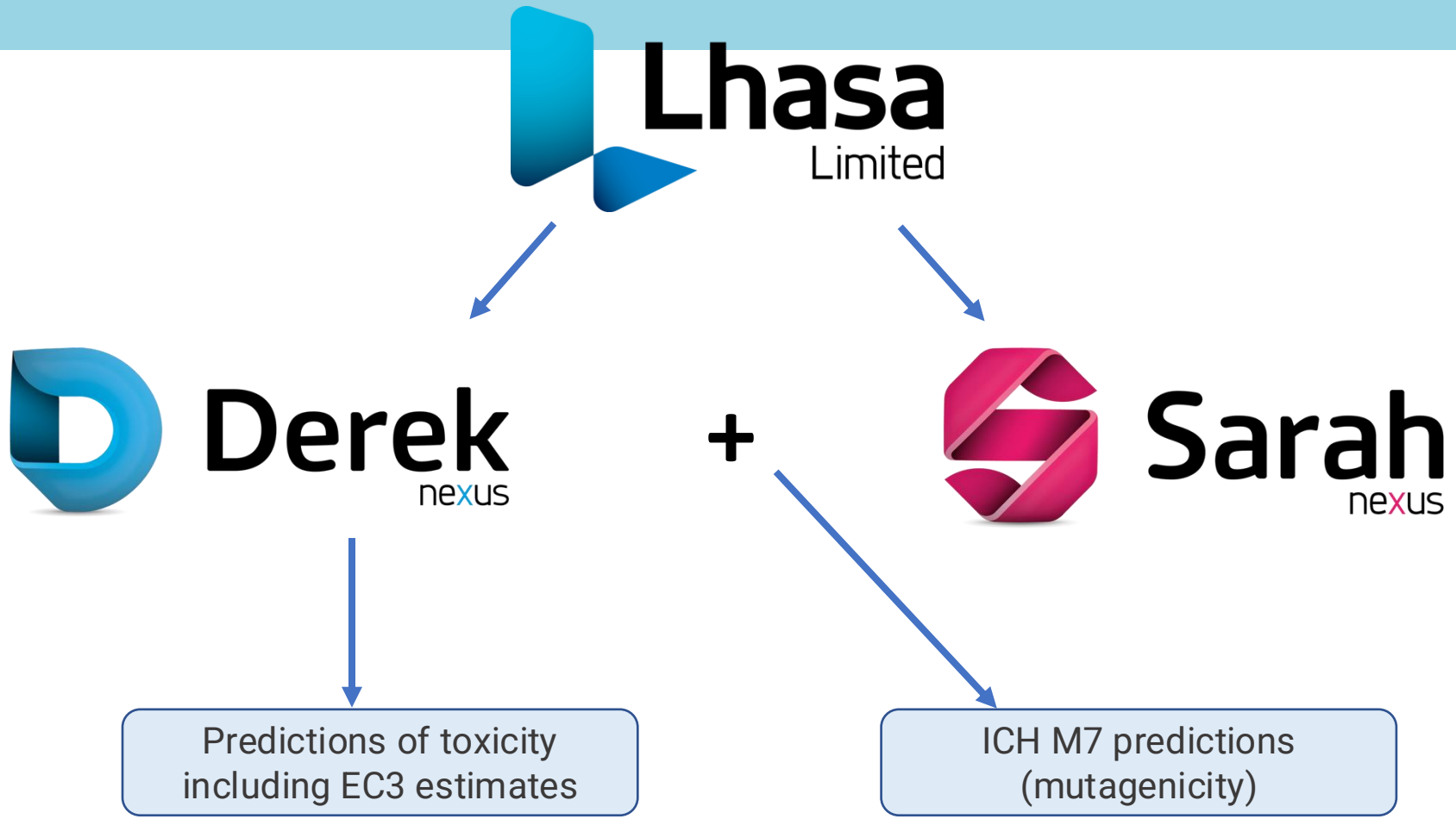
MultiCASE

redicting toxicity of chemicals
sed

TEST
50)

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LHASA

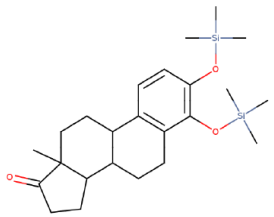


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EXAMPLE LHASA OUTPUTS

No toxicity data identified from literature sources

Derek analysis for genotoxicity



Smiles: CC12CCC3C(C1CCC2=O)CCC4=C3C=CC(=C4O[Si](C)(C)C)O[Si](C)(C)C

Exact Mol Mass	430.2359
Average Mol Mass	430.73 (Source: Lhasa Limited, version 1.0)
Log Kp	-0.71 (Source: Potts & Guy, version 1.0)
Log P	6.53 (Source: BioByte Corp., version 5.9)

Predictions

Knowledge Base: Derek KB 2020 1.0

Version	Last Modified Date	Certified by
1.0	26/03/2020 08:28:54	Lhasa Limited, Leeds, Yorkshire, UK

Reasoning Summary

- ◆ **Hepatotoxicity in mammal is PLAUSIBLE**
  - Alert matched: 650 Organosilicon compound
- ◆ **Mutagenicity in vitro in bacterium is INACTIVE**
  - No misclassified or unclassified features
- ◆ **Nephrotoxicity in mammal is EQUIVOCAL**
  - Alert matched: RapidPrototype056 Organosilicon compound
- ◆ **Skin sensitisation in mammal is NON-SENSITISER**
  - No misclassified or unclassified features

Sarah analysis for genotoxicity

Prediction

For the Mutagenicity in vitro endpoint with the Sarah Model - 2020.1 model, the compound is predicted to be

Negative

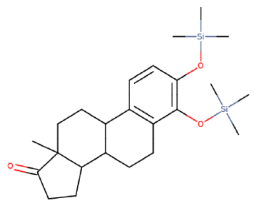
with 55% confidence in the prediction

Hypotheses Summary

The compound is predicted to be negative with 55% confidence for the 'Mutagenicity in vitro' endpoint in the model: 'Sarah Model - 2020.1'. Supporting hypotheses containing similar examples from the training set have been found.

Results	Number of Hypotheses
Positive	0
Negative	2
Positive (Overruled by Training Set Example)	0
Negative (Overruled by Training Set Example)	0
Total Count	**2**

Prediction Summary - Structure



Derek: Inactive
Sarah: Negative

Smiles:
CC12CCC3C(C1CCC2=O)CCC4=C3C=CC(=C4O[Si](C)(C)C)O[Si](C)(C)C

Average Mol Mass:	430.73
Exact Mol Mass:	430.2359
Log Kp:	-0.71
Log P:	6.53

Non genotoxic

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IN VITRO GENOTOXICITY TESTING

- All in vitro assays produce misleading positive results, but some produce more than others.
- The performance of in vitro genotoxicity assays is commonly described in terms of sensitivity and specificity.
  - Sensitivity is the proportion of genotoxic carcinogens that produces positive results in a given test
  - Specificity is the proportion of non-carcinogens (and ideally non-genotoxic carcinogens) that produces negative results.
- A low specificity figure therefore identifies a test that produces a high proportion of misleading positive results.

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IN VITRO GENOTOXICITY ASSAY PERFORMANCE

Test* name	Sensitivity (%)	Specificity (%)
Bacterial reversion (Ames)	60	77
Chromosome aberrations	70	55
Mammalian mutation	81	48

* Regulatory standard assays only

Walmsley RM, Billinton N. How accurate is in vitro prediction of carcinogenicity?
Br J Pharmacol. 2011 Mar;162(6):1250-8. doi: 10.1111/j.1476-5381.2010.01131.x. PMID: 21091657; PMCID: PMC3058158.

Kirkland D, Aardema M, Henderson L, Müller L. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity.
Mutat Res. 2005 Jul 4;584(1-2):1-256. doi: 10.1016/j.mrgentox.2005.02.004. Erratum in: Mutat Res. 2005 Dec 7;588(1):70. PMID: 15979392.

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GENOTOXICITY TESTING – ISO 10993-3

- No single test is capable of detecting all types of genotoxic agents. Therefore, the usual approach is to conduct a battery of *in vitro* and under certain circumstances also *in vivo* tests.
- Genotoxicity tests are designed to detect the two major classes of genetic damage:
  - gene mutations
    - DNA single base pair changes
    - small deletions or additions)
  - chromosomal damage
    - structural aberrations such as translocations, small or large deletions and insertions
    - gain or loss of whole chromosomes (aneuploidy)
    - gain or loss of parts of chromosomes

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GENOTOXICITY TESTING – ISO 10993-3

- When genotoxicity testing is performed, the test battery shall include:
  - a test for gene mutations in bacteria (see OECD 471*), modified for medical devices, and
  - one of the following three validated in vitro mammalian cell tests:
    - an in vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells (see
    - OECD 473), modified for medical devices, or
    - an in vitro mouse lymphoma tk+/- assay using L 5178Y (tk+/-) cells (see OECD 490), modified for
    - medical devices, including detection of small (slow growing) and large colonies, or
    - an in vitro mammalian cell micronucleus test for chromosomal damage and aneugenicity (see OECD
    - 487), modified for medical devices

*If the medical device is known to contain nanomaterials testing according to OECD 471 is not recommended

- All tests shall be performed in the presence and absence of metabolic activation. Metabolic activation is achieved by addition of a co-factor-supplemented post mitochondrial fraction (S9)

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GENE MUTATIONS IN BACTERIA (AMES TEST)

- Principle – OECD 471
- Ames test uses several strains of bacteria (Salmonella, E.coli) that carry a particular mutation.
- Point mutations are made in the histidine (Salmonella typhimurium) or the tryptophan (Escherichia coli) operon, rendering the bacteria incapable of producing the corresponding amino acid.
- These mutations result in his- or trp- organisms that cannot grow unless histidine or tryptophan is supplied.
- Culturing His- Salmonella in a media containing mutagenic chemicals, causes mutation in the histidine or tryptophan encoding gene, such that they regain the ability to synthesize histidine (His+) or tryptophan (trp+). This is the reverse mutation.
- These reverted bacteria will then grow in histidine- or tryptophan-deficient media, respectively.

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AMES TEST – STUDY DESIGN

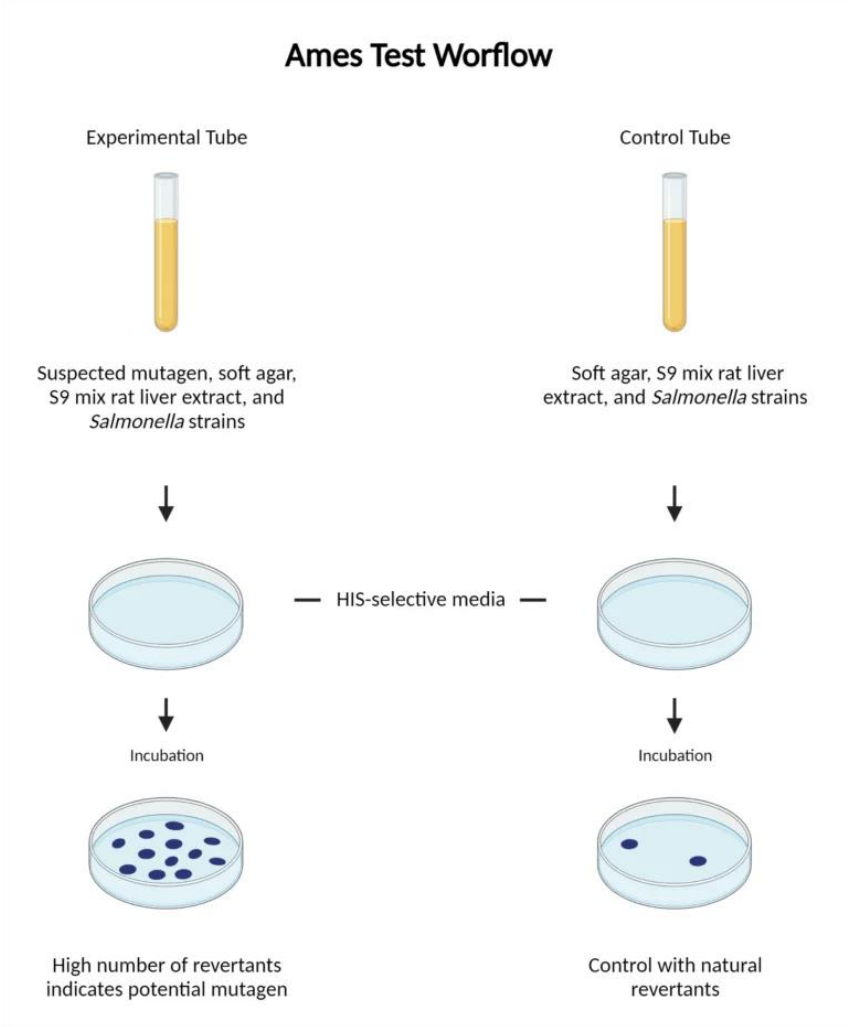
To cover a wide range of mutational events, five tester strains are included in the Ames mutagenicity test:

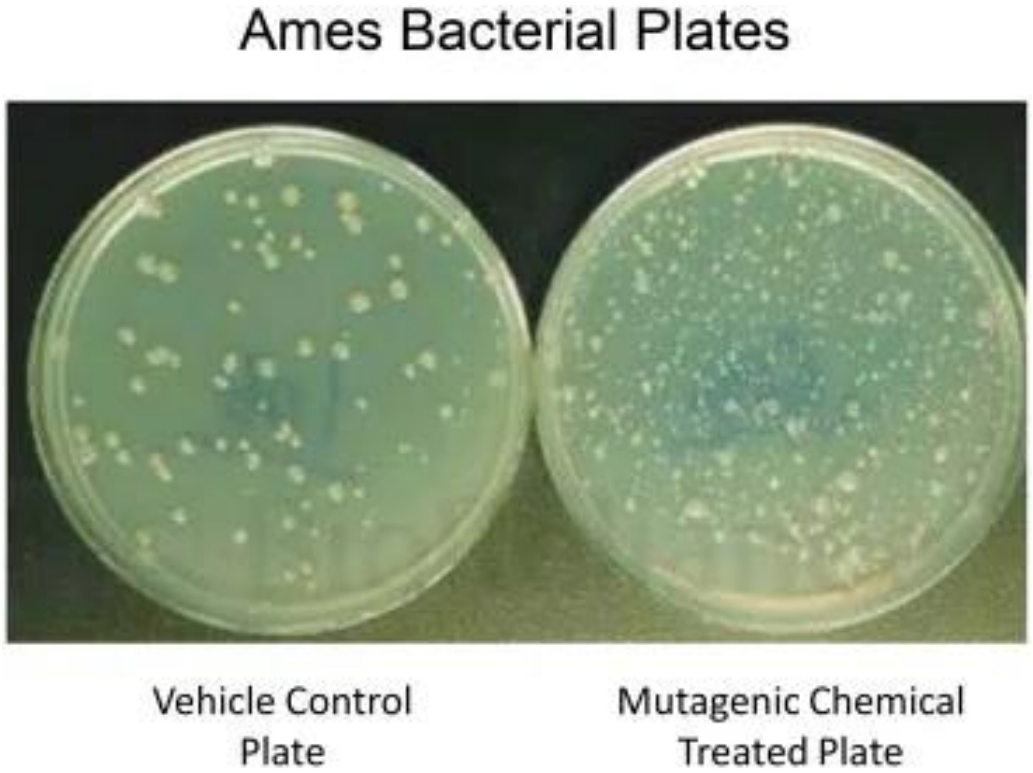
Strain	Detects
TA98	frameshift
TA100 **TA1535**	base substitution base substitution
TA1537, TA97, or TA97a	frameshift
TA102, WP2 uvrA, or WP2 uvrA (pKM101)	base substitution, x-linker (TA102 only)

- TA*** strains are Salmonella typhimurium, WP2*** strains are E. coli
- Initially single dose (100ul or 5mg/plate or neat extract)
- If cytotoxic, repeat test with 5 dose levels
- Triplicate plates
- Positive and negative controls
- +/- metabolic activation (S9 mix)
- Total 210 plates for 5 dose study

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GENE MUTATIONS IN BACTERIA (AMES TEST)





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MAMMALIAN CELL TESTS

- an in vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells (see OECD 473), modified for medical devices, **or**
- an in vitro mouse lymphoma tk+/- assay using L5178Y (tk+/-) cells (see OECD 490), modified for medical devices, including detection of small (slow growing) and large colonies, **or**
- an in vitro mammalian cell micronucleus test for chromosomal damage and aneugenicity (see OECD 487), modified for medical devices.

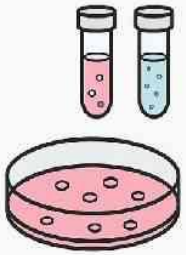
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IN VITRO CYTOGENETICS (CHROMOSOMAL ABERRATION ASSAY)

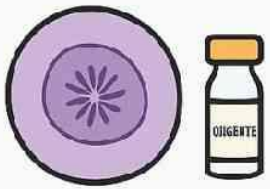
- The chromosomal aberration assay is an assessment of clastogenicity (large scale genetic damage) in cell lines such as
  - Chinese Hamster Ovary (CHO) cells or
  - primary cell cultures, including human or other mammalian peripheral blood lymphocytes
- Detects
  - Chromatid-type aberrations: Damage affecting a single chromatid (the most common type seen with chemical mutagens), including chromatid breaks, gaps, and exchanges.
  - Chromosome-type aberrations: Damage involving both sister chromatids equally, including whole chromosome breaks, dicentrics, and rings (typically induced by ionizing radiation)

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IN VITRO CYTOGENETICS









Experimental Preparation

Select and culture a suitable mammalian cell line until it reaches the logarithmic growth phase. Prepare the test substances at varying concentrations.

Cell Treatment

Administer the test substances to the experimental cell groups. For groups requiring metabolic activation, add the S9 mixture concurrently. Following a 3-6 hour treatment for the S9-activated group, wash the cells with a fresh culture medium; continue to culture the non-S9 and control groups without interruption.

Mitotic Arrest

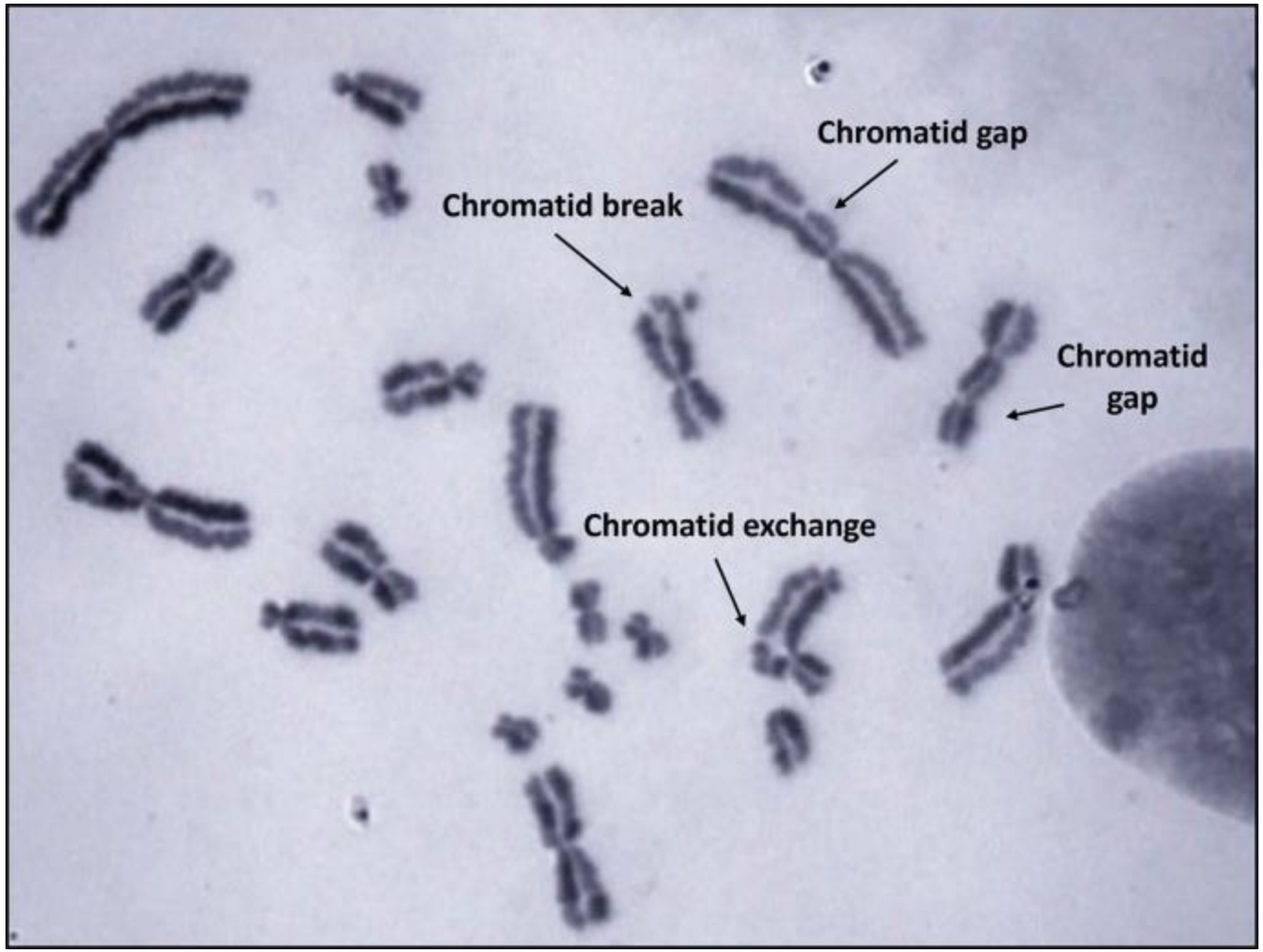
Introduce colchicine 2-4 hours prior to the conclusion of treatment to arrest cells in metaphase, preventing progression to anaphase.

Result Analysis

Post-slide preparation, examine the chromosomal aberrations under a microscope and compute the aberration rate. Compare these findings with the control group to evaluate whether the test substance induces chromosomal aberrations.

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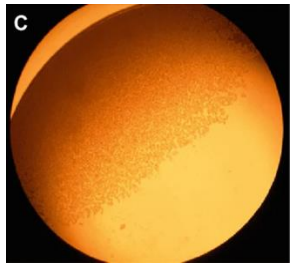
IN VITRO CYTOGENETICS



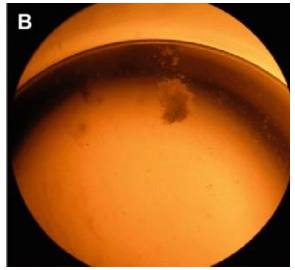
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MOUSE LYMPHOMA ASSAY

- The mouse lymphoma L5178Y tk+/− assay (MLA) is capable of identifying both gene mutation and clastogenicity.
- L5178Y clone 3.7.2C is heterozygous at the thymidine kinase locus (tk+/−)
- Forward mutation to tk−/− is identified by growth in the presence of the toxic base analogue trifluorothymidine (TFT)
- Colonies of TK-/- mutants can be either:
  - Large colonies (point mutations) – mutagens



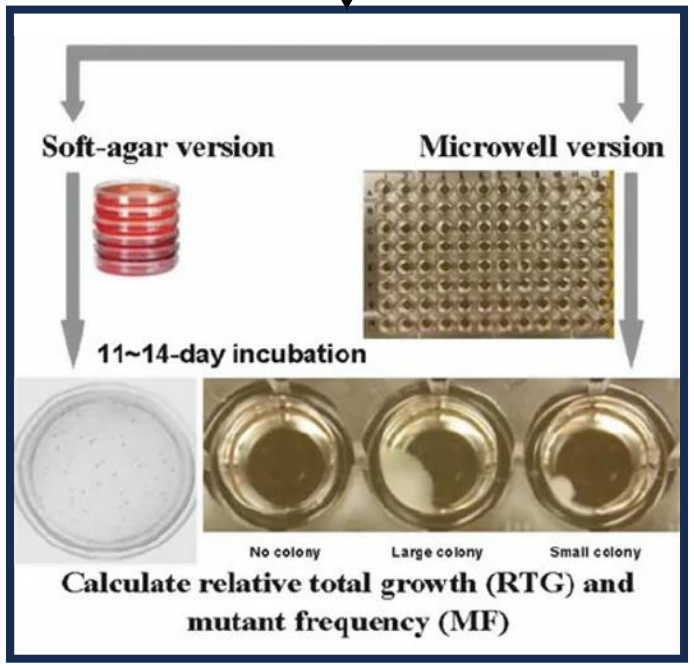
  - Small colonies (structural/chromosomal damage) – clastogens



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MOUSE LYMPHOMA ASSAY





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IN VITRO MICRONUCLEUS

- The in vitro micronucleus test detects micronuclei (MN) in the cytoplasm of interphase cells.
- MN may originate from acentric chromosome fragments (i.e. lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division.
- The MNvit test provides a basis for investigating chromosome-damaging potential in vitro that detects both aneugens and clastogens.
- For detection of MN, it is necessary that cells have undergone cell division during or after exposure to the test chemical.
- MN represents damage that has been transmitted to daughter cells, whereas chromosome aberrations scored in metaphase cells may not be transmitted.
- OECD 487 allows the use of protocols with and without the actin polymerization inhibitor cytochalasin B (cytoB).
- The addition of cytoB prior to mitosis results in cells that are binucleate and therefore allows for the identification of cells that have completed cell division.

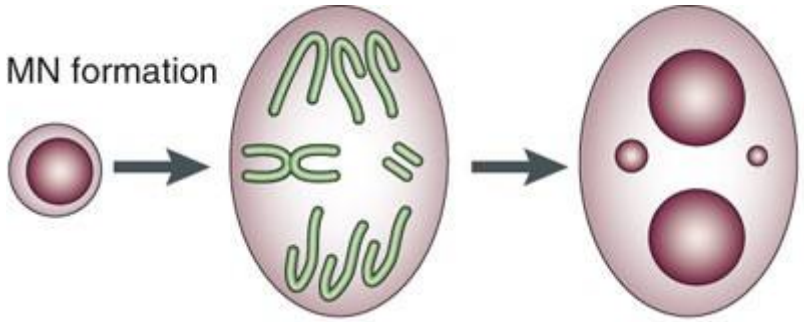
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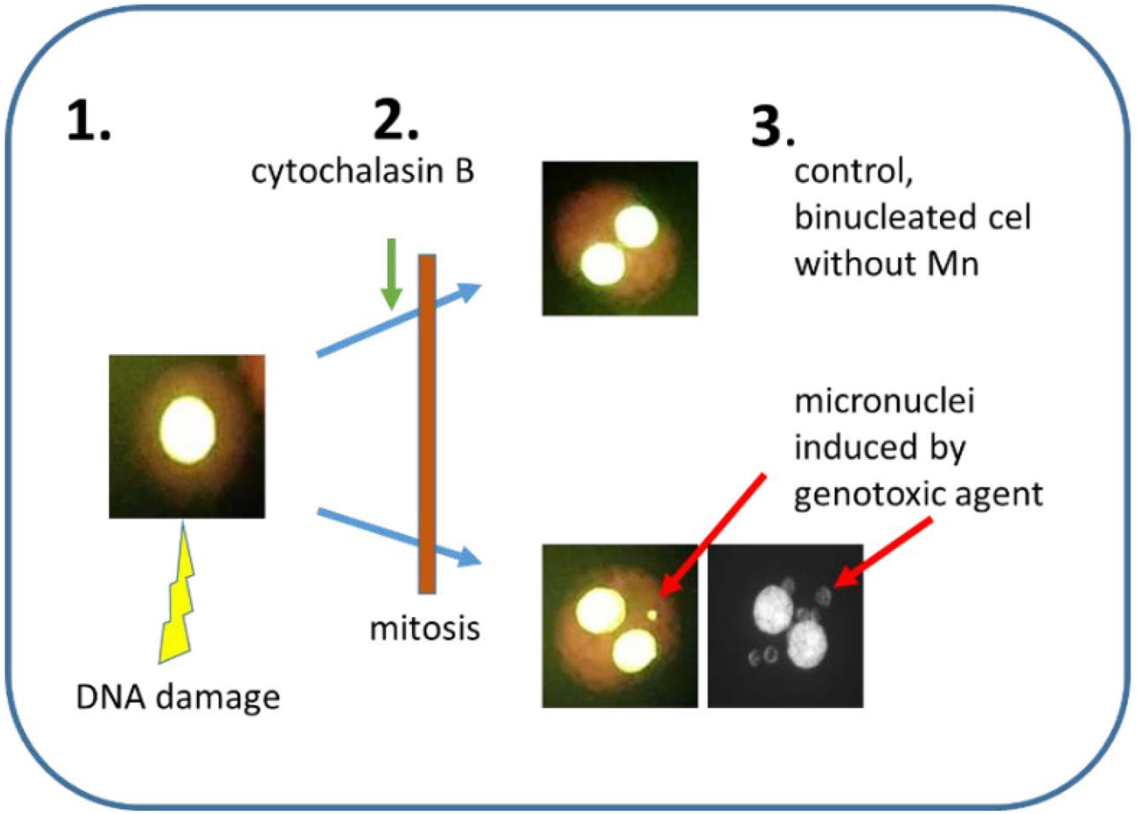
IN VITRO MICRONUCLEUS

- A variety of common cell lines, such as CHO or primary cell cultures including human or other mammalian peripheral blood lymphocytes, may be used, with cell culture extracts chosen as recommended for the selected cell line.
- After exposure, washing and expression, the cultures are harvested, stained and dropped onto slides.
- Micronucleus frequencies shall be analysed in at least 2000 binucleate cells per test concentration and control.
- Flow cytometric methods allow the assessment of many more cells.

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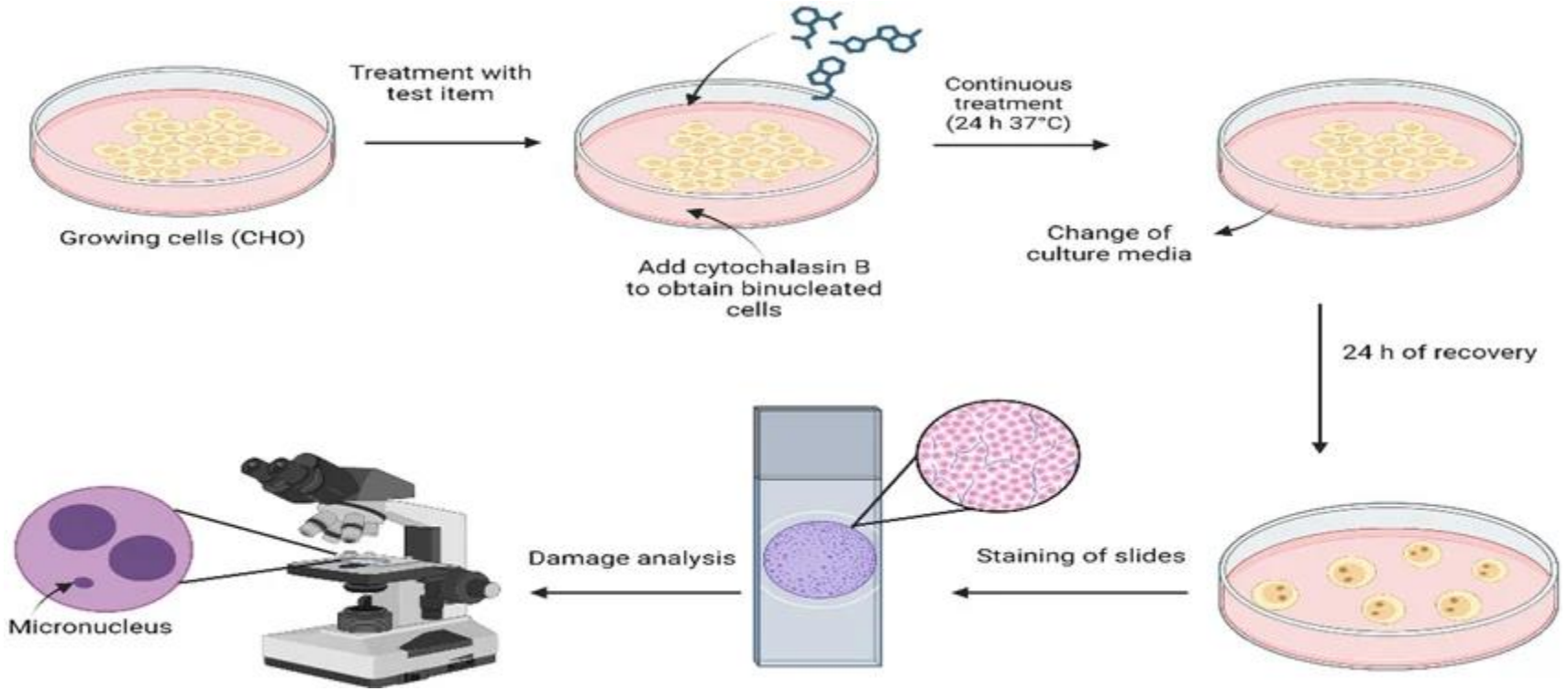
IN VITRO MICRONUCLEUS





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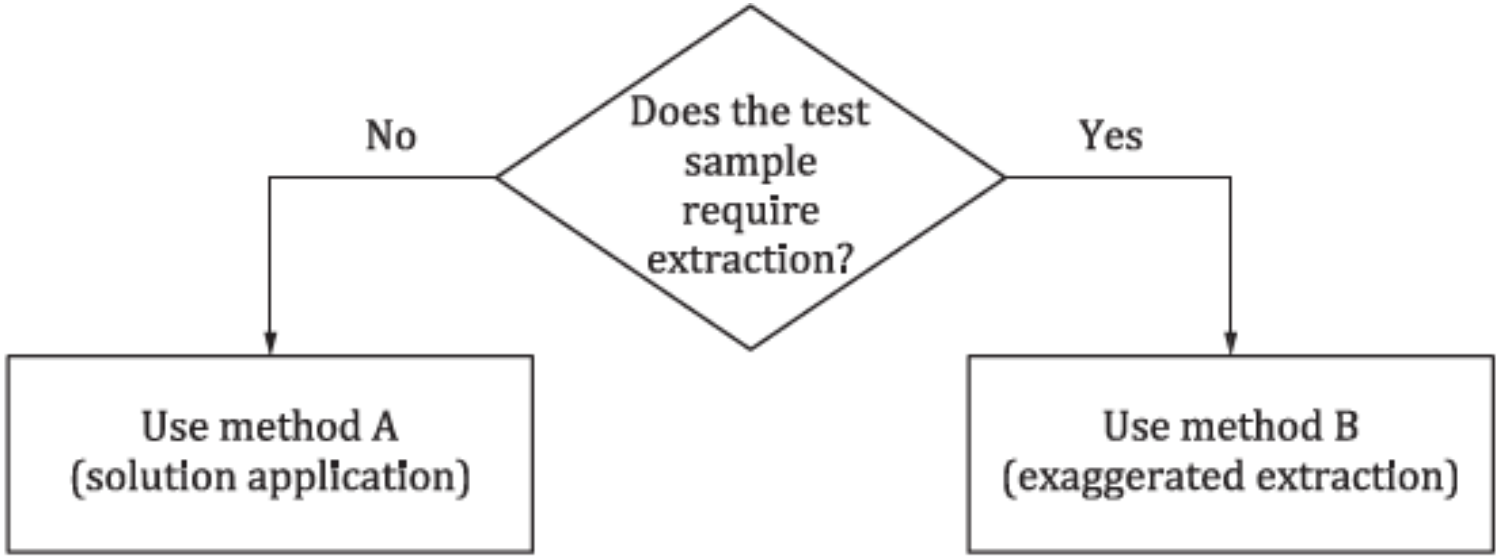
IN VITRO MICRONUCLEUS



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TEST SAMPLE PREPARATION – ISO 10993-3, ANNEX A

- The test sample preparation should follow one of the two proposed methods
  - When the test sample requires extraction, Method B shall be used.
  - Otherwise, Method A should be used.



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TEST SAMPLE PREPARATION – METHOD A

- The test sample is either dissolved or suspended (or partially dissolved) in the solvent.
- The final volume of test sample preparation added to the test system varies with the compatibility of the solvent in the test system.
- Solutions should be used immediately after preparation.
- Solution pH shall not be adjusted unless a rationale is provided.
- The solution shall not routinely be processed by filtration, centrifugation or other methods to remove suspended particulates.
- If cytotoxicity of the solution is expected, preliminary dose range assays can be useful to achieve the proper dose selection.
- Dosing at a single, maximum level is acceptable if cytotoxicity is not expected.

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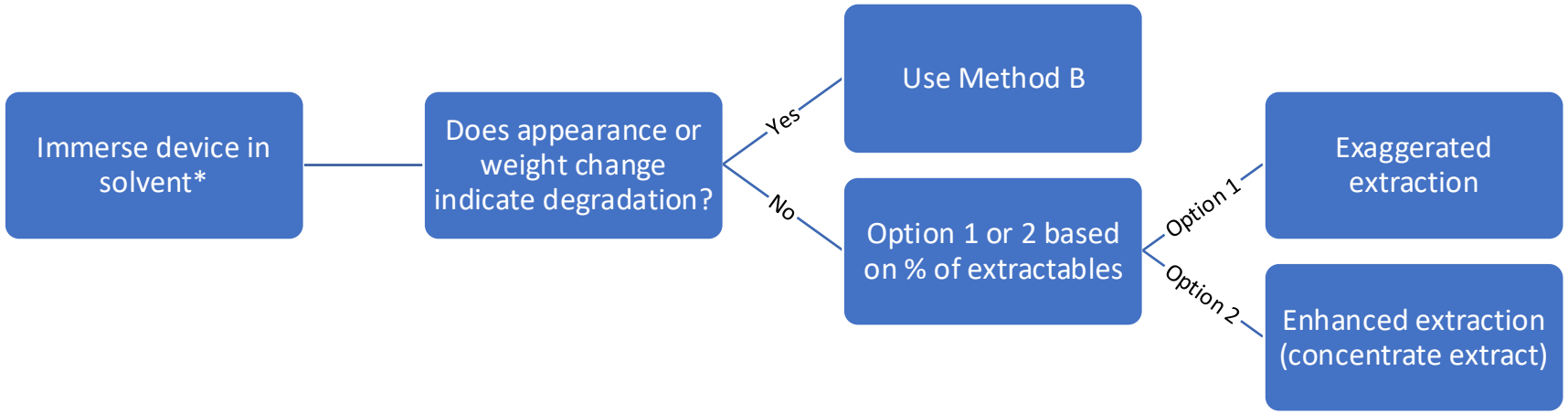
TEST SAMPLE PREPARATION – METHOD B

- The test sample shall be extracted in a vehicle compatible with the test system.
- The recommended polar vehicles are
  - saline (for the bacterial assays)
  - cell culture medium without serum (for the mammalian in vitro assays).
- The recommended non-polar vehicle is DMSO. Other non-polar vehicles such as undiluted PEG 400 and ethanol may be used.
- Extracts should be used within 24 h after preparation.
- Extract pH shall not be adjusted unless a rationale is provided.
- The extract shall not routinely be processed by filtration, centrifugation or other methods to remove suspended particulates.

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TEST SAMPLE PREPARATION – METHOD C

- Some authorities prefer Method C while others do not accept Method C



*Two or more solvents are tested to determine which solvent extracts the higher percentage of extractables from the test sample. Common solvents are methanol and acetone

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IN VIVO GENOTOXICITY TESTING – ISO 10993-3

- In vivo genotoxicity tests are not part of the recommended genotoxicity test battery because they are relatively insensitive and not likely to detect genotoxicity at the concentrations of substances generally found in medical devices or in medical device extracts.
- These concentrations can be insufficient to meet the limit dose required by the test methods.
- However, under certain circumstances, an in vivo test can be useful. These circumstances can include:
  - assessment of nanomaterial or other particulates suspected of sequestration in particular organs or tissues, or
  - when additional relevant factors [e.g. toxicokinetics, organ-specific toxicity] need to be considered.
- **In vivo tests are valid only with data demonstrating the test sample reached the target cells, organs, or tissues.**

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IN VIVO GENOTOXICITY TESTING

- Mutational analysis in target organs of rats or mice can be determined using one of several rodent transgenic mutation assays (Big Blue or Mutamouse);
- Chromosomal damage can be assessed in
  - the erythrocyte micronucleus assay,
  - the in vivo comet assay, or
  - the in vivo chromosomal aberration assay

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FOLLOW-UP EVALUATION

- If the in vitro tests are negative, the test sample can be considered non-genotoxic and further testing is not necessary.
- Equivocal results can require repeating tests with a modified protocol such as adjusting dilution of the test sample or extract.
- When any single in vitro test is positive or equivocal, then the following options shall be considered:
  - Identification of genotoxic constituents by chemical characterization (information gathering or analytical chemistry testing) or other testing, with appropriate steps taken to eliminate or manage any identified risks.
  - If confounding factors (e.g. osmolality, pH, cytotoxicity) are suspected to have caused the positive response or equivocal in an initial genotoxicity test, the dosing or test conditions may be modified with a justification to address confounding factors.
  - Presume that a genotoxicity risk exists and manage the risk in accordance with ISO 14971.

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