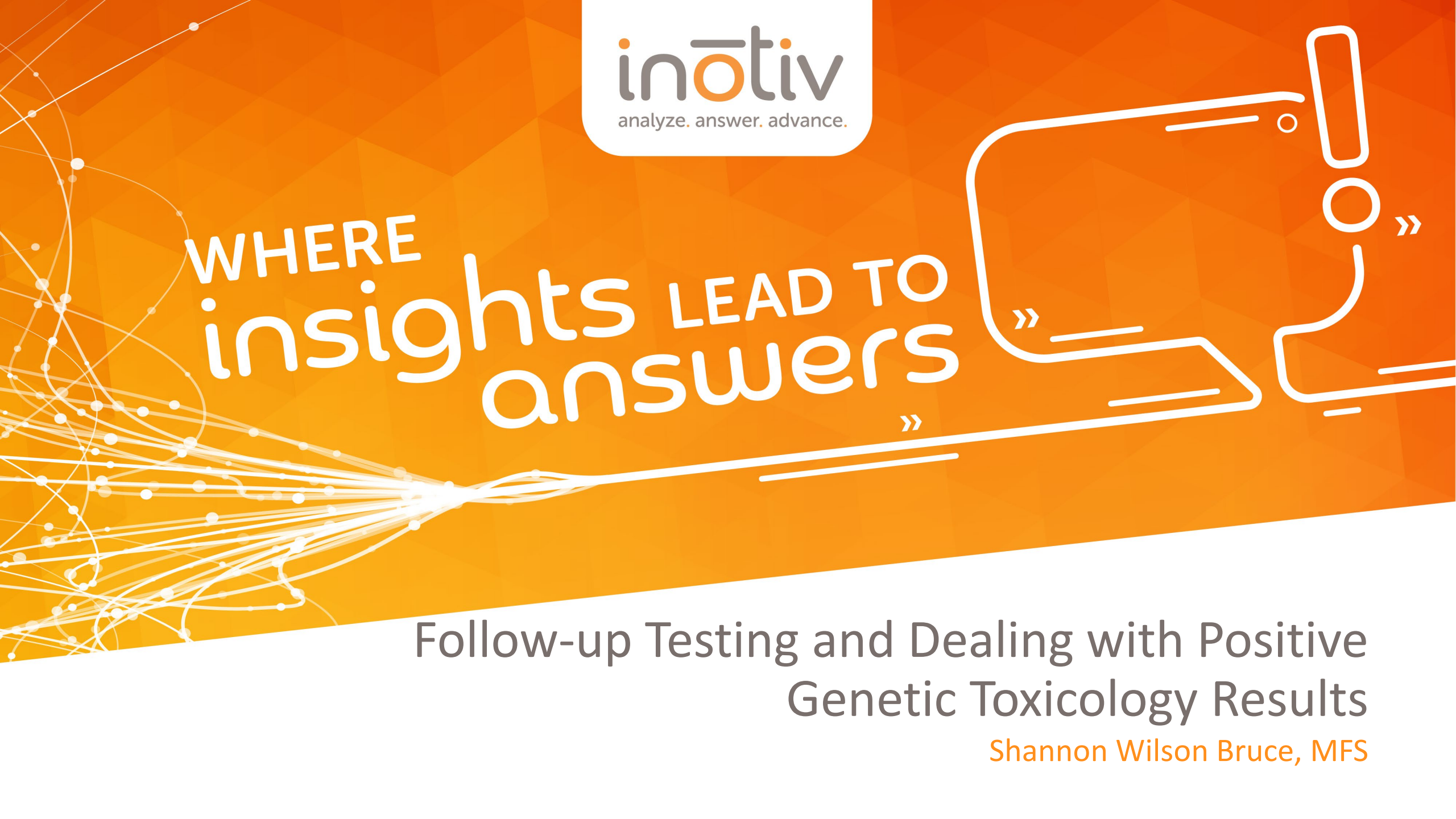




WHERE
insights LEAD TO
answers

Follow-up Testing and Dealing with Positive Genetic Toxicology Results

Shannon Wilson Bruce, MFS

International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidances for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

June 2012

S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals

May 2012

**M7(R2) Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk
Guidance for Industry¹**

July 2023

M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals

January 2010

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

The background is white with scattered light gray geometric elements: small circles, double chevrons (»), and parallel lines. An orange geometric pattern is visible in the bottom right corner.

Follow-up Testing for *in vitro* Positive Results

ICH (S2)R1 Guideline: Follow-up Testing to *in vitro* Tests

Ames negative; positive *in vitro* mammalian cell assay

Sufficient WoE to
indicate lack of relevance

One *in vivo* assay
(micronucleus)

Insufficient WoE to indicate
lack of relevance

Additional *in vitro* studies to
address reproducibility or
provide mechanistic information

Two *in vivo* assays, with
different tissues
(micronucleus & comet)

WoE supports the lack
of relevant genotoxicity

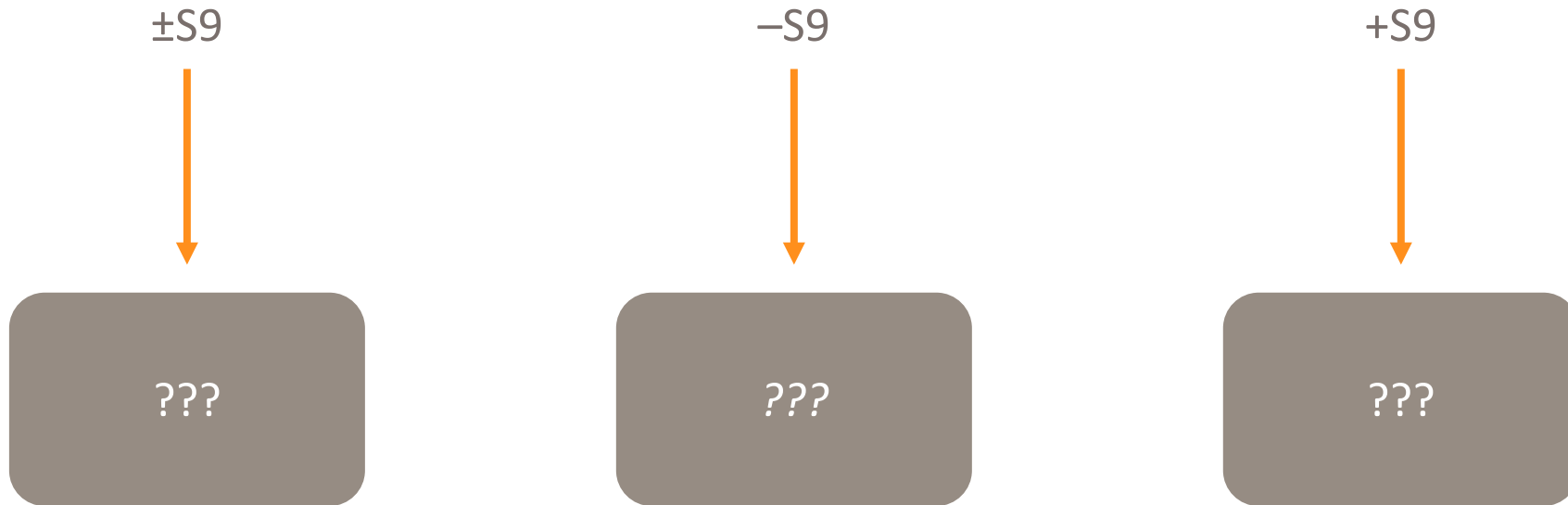
Insufficient WoE to
indicate lack of relevance

Weight of Evidence/Mode of Action

- *in silico* analysis of structural considerations
- Evidence for an indirect/threshold mechanism not relevant *in vivo*
 - > inhibition of DNA synthesis
 - > reactive oxygen species produced only at high concentrations
 - > nucleotide pool imbalance
 - > glutathione depletion
 - > pH or osmolality effects
- Known mechanism that indicates chromosome loss/aneuploidy
- MultiFlow® to assess clastogenic/aneugenic/non-genotoxic MOA
- FISH/CREST centromere staining experiments to evaluate chromosome loss

ICH (S2)R1 Guideline: Follow-up Testing to *in vitro* Tests

Ames positive; negative *in vitro* mammalian cell assay



ICH (S2)R1 Guidelines: Genetox Test Battery*

Option 1**	Option 2
A test for gene mutation in bacteria	A test for gene mutation in bacteria
An <i>in vitro</i> mammalian cell assay for chromosomal damage or gene mutation	
An <i>in vivo</i> test for genotoxicity (generally chromosomal damage in blood or bone marrow)**	Assessment of <i>in vivo</i> genotoxicity in two tissues (typically, micronucleus & comet)***

* Conducted according to OECD test guidelines

** Required for chemicals toxic to bacteria (e.g., antibiotics)

*** Can be combined with other genetox assays or integrated into repeat dose studies.

No requirement for
in vivo mutation test

Can Comet Substitute for TGR as a Follow Up to an Ames Positive?

Mutat Res Gen Tox En 839 (2019) 21–35



ELSEVIER

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

journal homepage: www.elsevier.com/locate/gentox

A comparison of transgenic rodent mutation and *in vivo* comet assay responses for 91 chemicals

David Kirkland^{a,*}, Dan D. Levy^b, Matthew J. LeBaron^c, Marilyn J. Aardema^d, Carol Beevers^e, Javed Bhalli^f, George R. Douglas^g, Patricia A. Escobar^h, Christopher S. Farabaughⁱ, Melanie Guerard^j, George E. Johnson^k, Rohan Kulkarni^f, Frank Le Curieux^l, Alexandra S. Long^g, Jasmin Lott^m, David P. Lovellⁿ, Mirjam Luijten^o, Francesco Marchetti^g, John J. Nicolette^p, Stefan Pfuhler^q, Daniel J. Robertsⁱ, Leon F. Stankowski Jr.ⁱ, Veronique Thybaud^r, Sandy K. Weiner^s, Andrew Williams^g, Kristine L. Witt^t, Robert Young^f

Yes!

No!

Mutation Research - Genetic Toxicology and Environmental Mutagenesis 868-869 (2021) 503386



ELSEVIER

Contents lists available at ScienceDirect

Mutation Research - Genetic Toxicology
and Environmental Mutagenesis

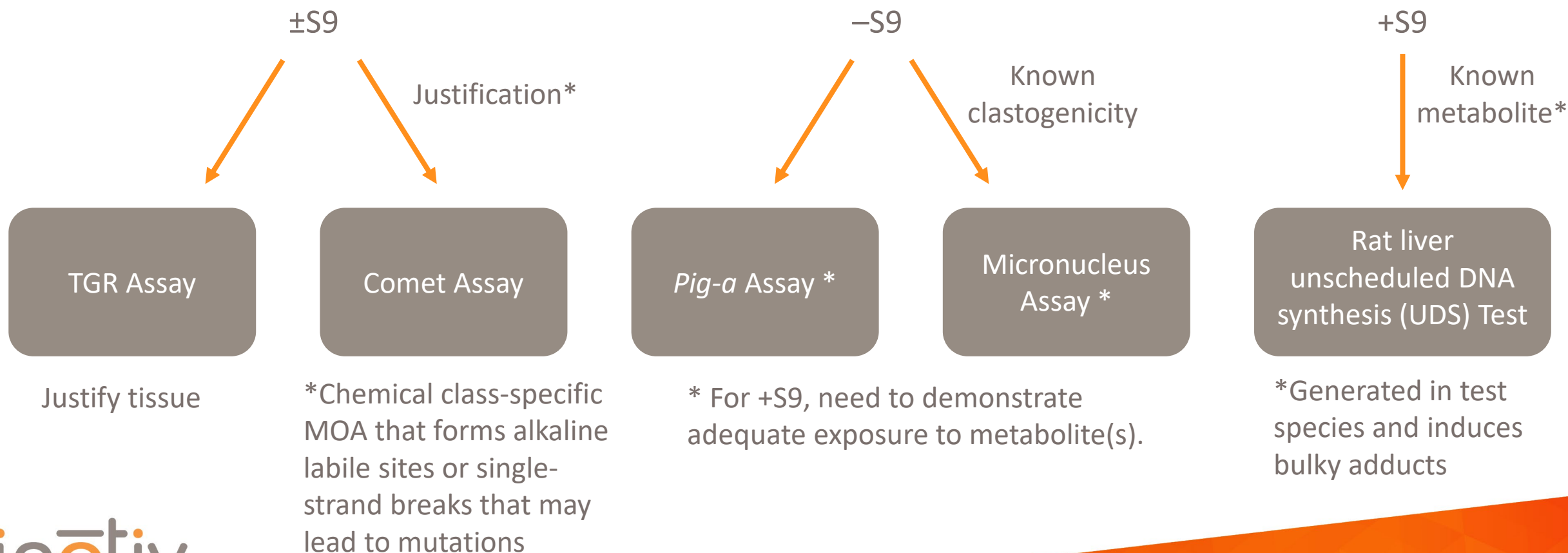
journal homepage: www.elsevier.com/locate/gentox

Appropriate *in vivo* follow-up assays to an *in vitro* bacterial reverse mutation (Ames) test positive investigational drug candidate (active pharmaceutical ingredient), drug-related metabolite, or drug-related impurity

Timothy W. Robison^{a,*}, Robert H. Heflich^b, Mugimane G. Manjanatha^b, Rosalie Elespuru^c, Aisar Atrakchi^d, Nan Mei^b, Wei Ding^e

ICH M7(R2) Guideline: Follow-up Testing for Ames Positive Impurities

Ames positive; negative *in vitro* mammalian cell assay



Critical Considerations for *in vivo* Genetox Assays

- Hazard identification, not risk assessment
- Is a dose range finding study necessary?
- Test single or both sex?
- Pharmaco-/toxicokinetics of test article absorption and metabolism
- Kinetics of expression/detection of genotoxic damage
- Selection of top dose
- Target tissue exposure
- Factors that can confound results interpretation

Selection of Top Dose

Study Design	OECD	ICH
Standalone acute study	• 2000 mg/kg limit dose (<14 days) • maximum tolerated dose (MTD) or maximum feasible dose (MFD) • suppression of bone marrow red blood cell production (micronucleus) – 20-50% of controls (microscopy) – statistical difference and up to 5% of controls (flow cytometry) • exposure plateau/saturation	• 2000 mg/kg limit dose • MTD • MFD • suppression of bone marrow red blood cell production (micronucleus)
Integrated into repeat dose toxicity study	• 1000 mg/kg/day limit dose (≥14 days) • suppression of bone marrow red blood cell production (<i>Pig-a</i>) – statistical difference and up to 20% of controls • assume same as acute study	• Option 1: toxicology study meets the criteria for an adequate study to support human clinical trials • Option 2 or follow-up: – 1000 mg/kg limit dose (≥14 days) – ≥50% of top dose that would be used for acute administration – MFD – exposure plateau/saturation – exposure accumulation

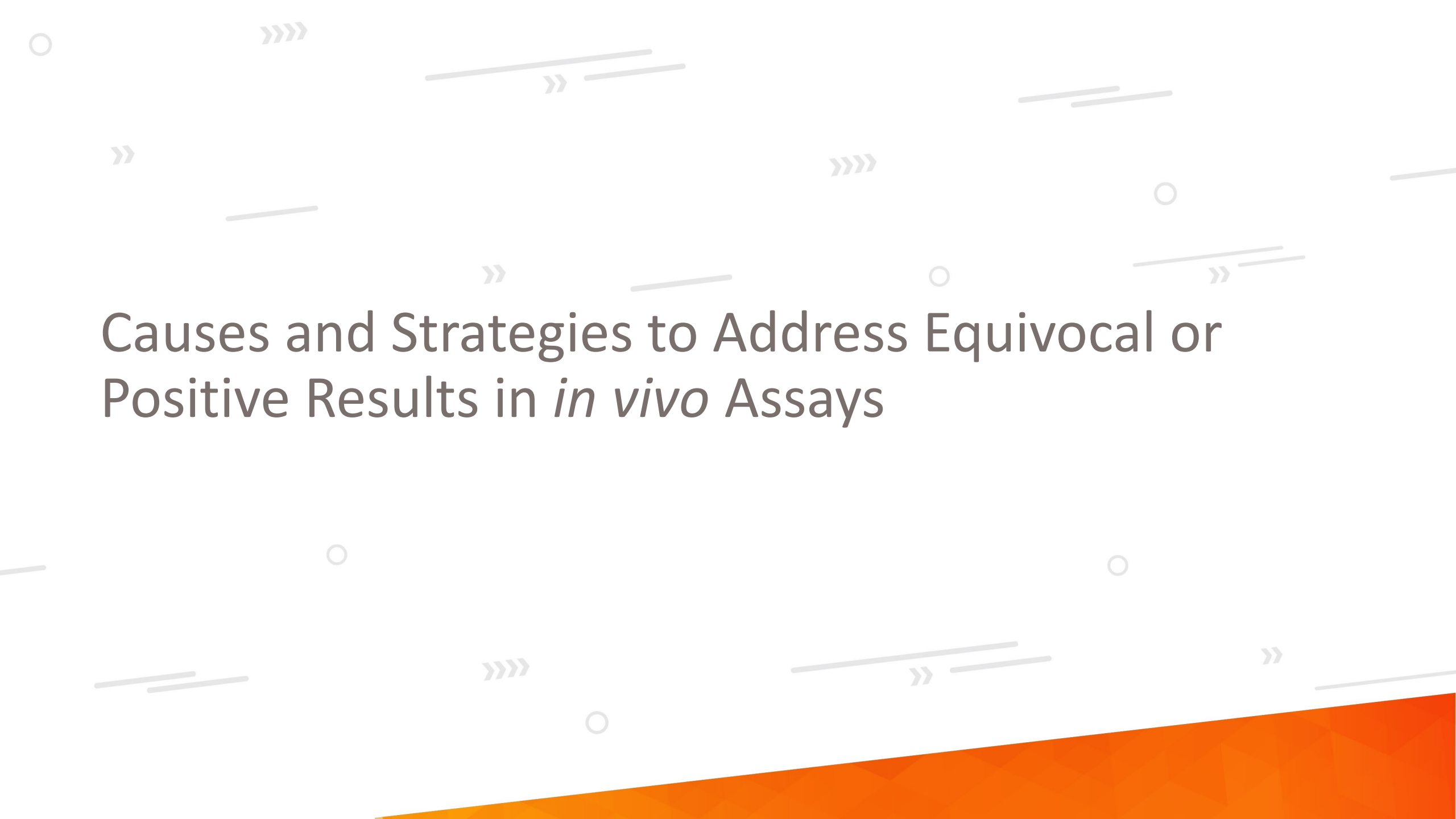
More stringent
criteria

Target Tissue Exposure

Situation	ICH Recommendation
<i>in vitro</i> test is negative	- Exposure assumed from results of previous absorption, distribution, metabolism and excretion (ADME) studies - Assessment of systemic exposure as below
<i>in vitro</i> test is positive or not done	- Cytotoxicity - MN (& <i>Pig-a</i>): significant change in the proportion of immature erythrocytes among total erythrocytes in the tissue used - chromosomal aberration: significant reduction in mitotic index - other assays: toxicity in the liver or tissue being assessed - Bioavailability - measurement of drug related material in blood or plasma - direct measurement of drug-related material in target tissue

Must demonstrate exposure in study animals

- If systemic exposure is similar to or lower than expected clinical exposure, consider alternative strategies:
- (i) different route of administration
 - (ii) different species with higher exposure
 - (iii) different tissue or assay

The background is white with scattered light gray geometric elements: small circles, double chevrons (»), and parallel horizontal lines. An orange geometric pattern is visible in the bottom right corner.

Causes and Strategies to Address Equivocal or Positive Results in *in vivo* Assays

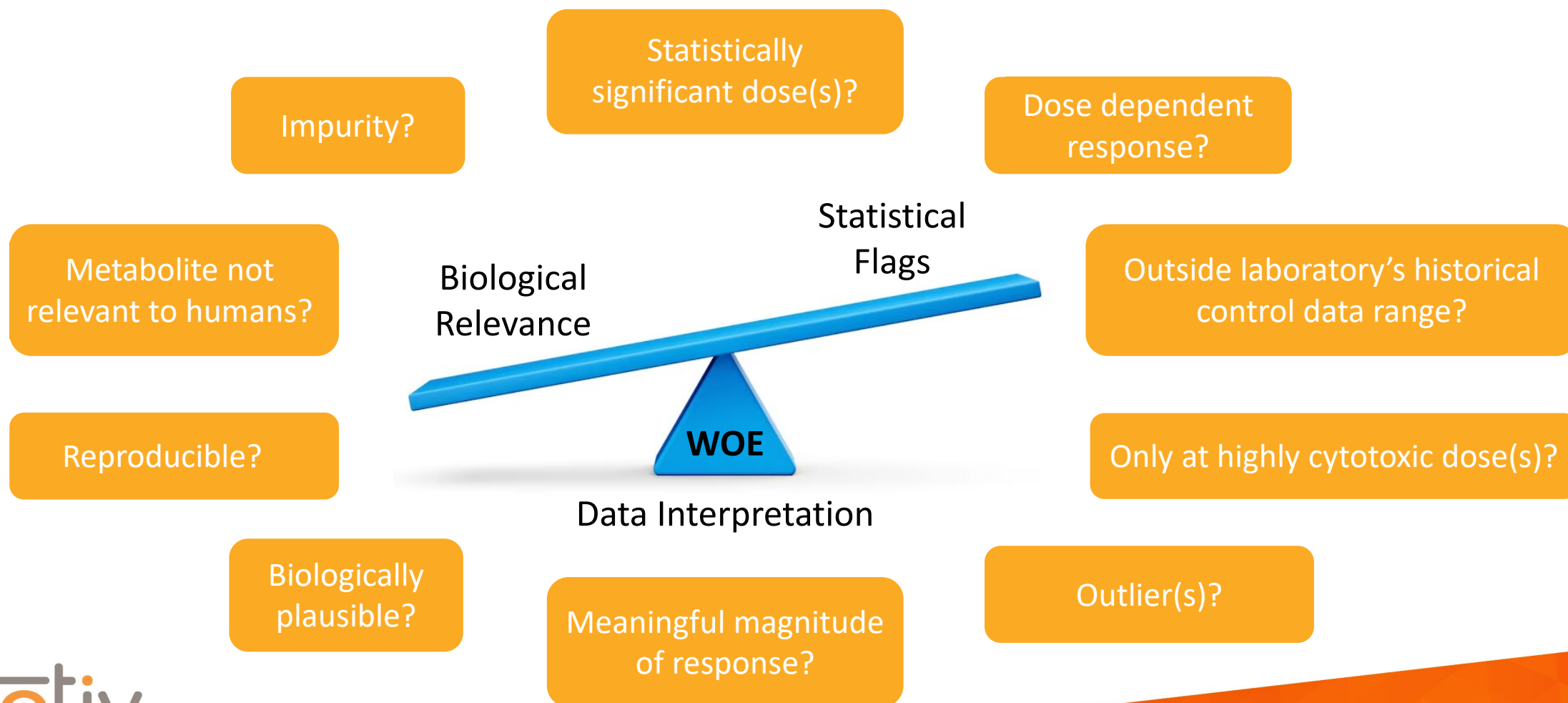
in vivo Genetox Assays: Results Interpretation

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- A. None of the treatment groups exhibits a statistically significant increase compared with the concurrent negative control,
- B. There is no dose-related increase at any sampling time when evaluated by an appropriate trend test,
- C. All results are inside the distribution of the laboratory's historical negative control data, and
- D. Exposure of the target tissue to the test substance(s) occurred.

➤ Otherwise, result is equivocal or positive.

Assessing Equivocal/Positive Results in Genetox Assays



Physiological Effects Can Lead to Misleading Positive Results

- Disturbance in erythropoiesis (e.g., regenerative anemia; extramedullary hematopoiesis) → ↑ micronuclei
 - Hyper- or hypothermia → ↑ micronuclei
 - Stress → ↑ micronuclei
 - Changes in spleen function (reduced clearance of micronucleated cells in blood) → ↑ micronuclei
 - Indirect, toxicity related effects → ↑ DNA strand breaks
 - Clonal expansion of spontaneous jackpot mutation → ↑ mutation frequency
-
- It is important to consider all available toxicological and hematological findings when evaluating genotoxicity data.
 - Indirect effects related to toxicological changes may have a safety margin and may not be clinically relevant.

Follow-up Testing for Equivocal or Unexpected Positive *in vivo* Results

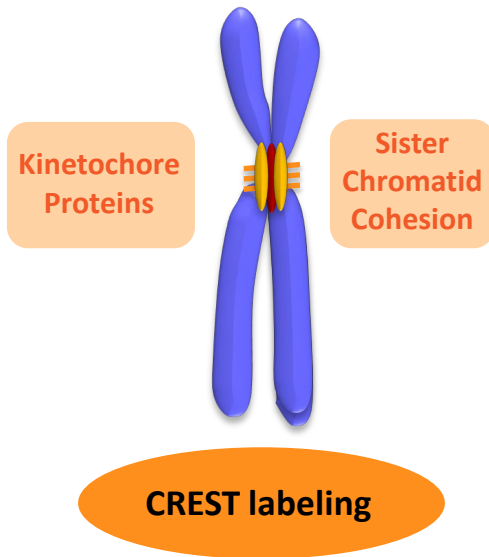
- Score more cells (or more animals if available)
- Evaluate backup samples
- Assess for confounding cytotoxicity or physiological response
 - > histopathology (comet)
 - > clinical chemistry (comet)
 - > hyper-/hypothermia (MN)
 - > changes in spleen function (reduced clearance of micronucleated cells in blood)
 - > pre-existing jackpot mutations (*Pig-a*)
- Repeat study to resolve an equivocal or assess reproducibility of test outcome
- Investigate mechanism of action to inform relevance of positive result
 - > *in vitro* MultiFlow® or FISH/CREST labeling
 - > CREST staining of micronuclei in bone marrow erythrocytes
 - > transcriptomics (e.g., RNA-Seq; targeted RT-PCR; biomarkers)

The background is white with scattered light gray geometric elements: circles, double chevrons (»), and parallel lines. An orange gradient bar with a low-poly pattern is at the bottom right.

FISH/CREST Labeling to Assess Mode of Action

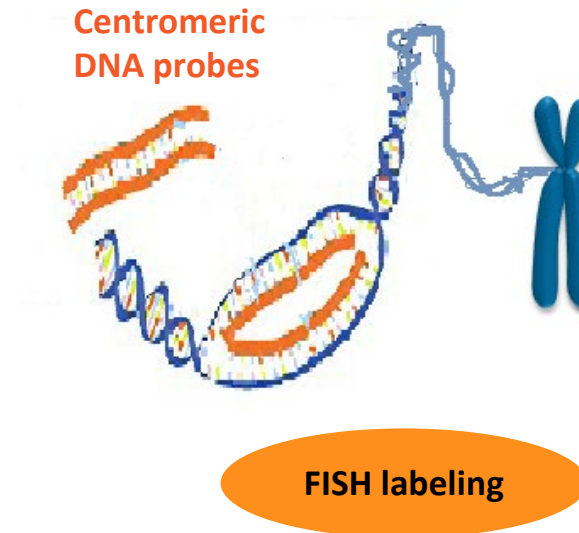
CREST and FISH Labeling - Molecular Targets

Calcinosis, Raynaud's phenomenon,
Esophageal motility abnormalities,
Sclerodactyly and Telangiectasia



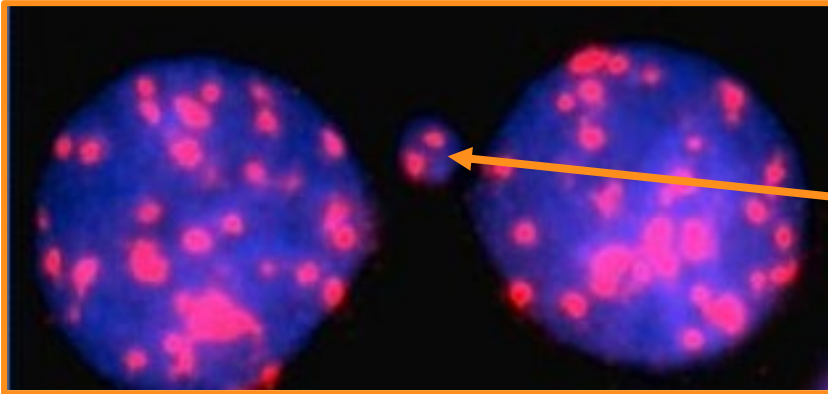
- Protein-based technique
- Primary antibody binds to kinetochore
- Fluorochrome tagged secondary antibody binds to primary antibody

Fluorescence In Situ Hybridization



- DNA-based technique
- Chromosome specific centromeric DNA tagged with fluorochrome

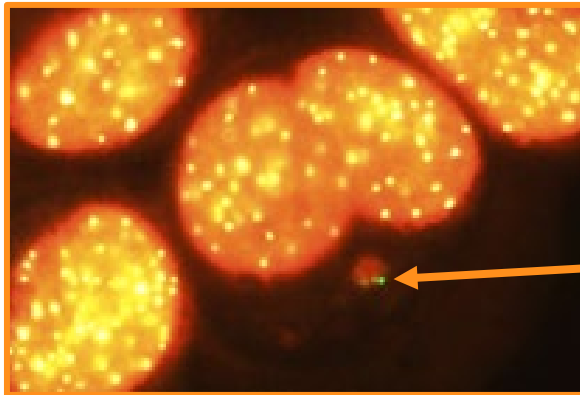
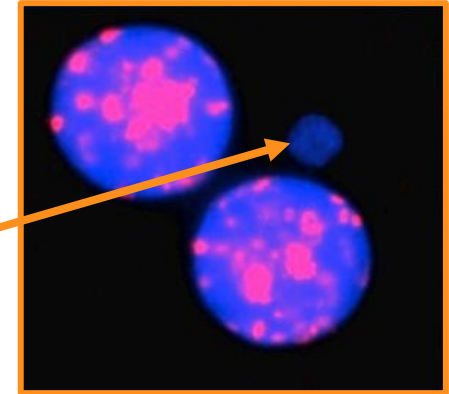
Microscopic Appearance in Cultured Cells



Centromeric DNA probes labeled with Cy3; counterstain DAPI

C^+ Micronucleus

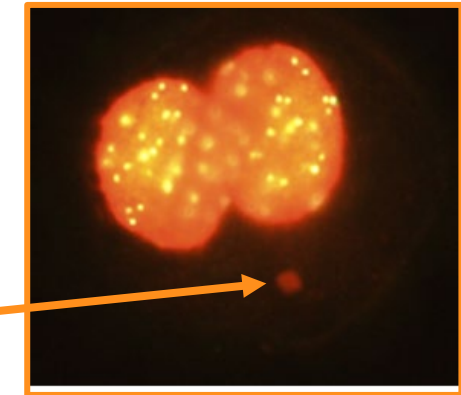
C^- Micronucleus



Kinetochore antibody labeled with FITC; counterstain propidium iodide

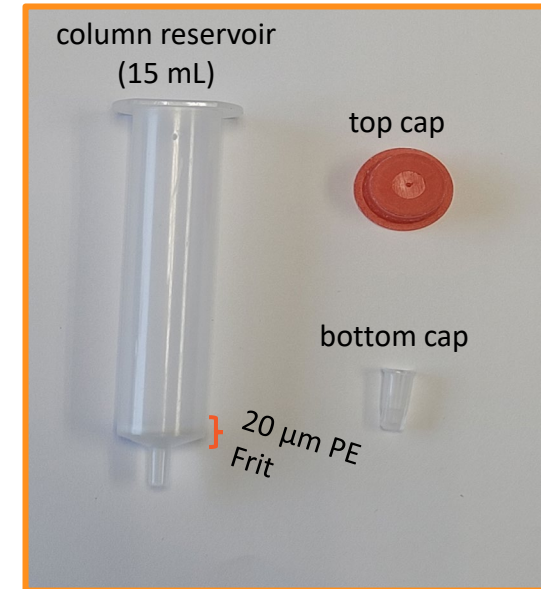
K^+ Micronucleus

K^- Micronucleus



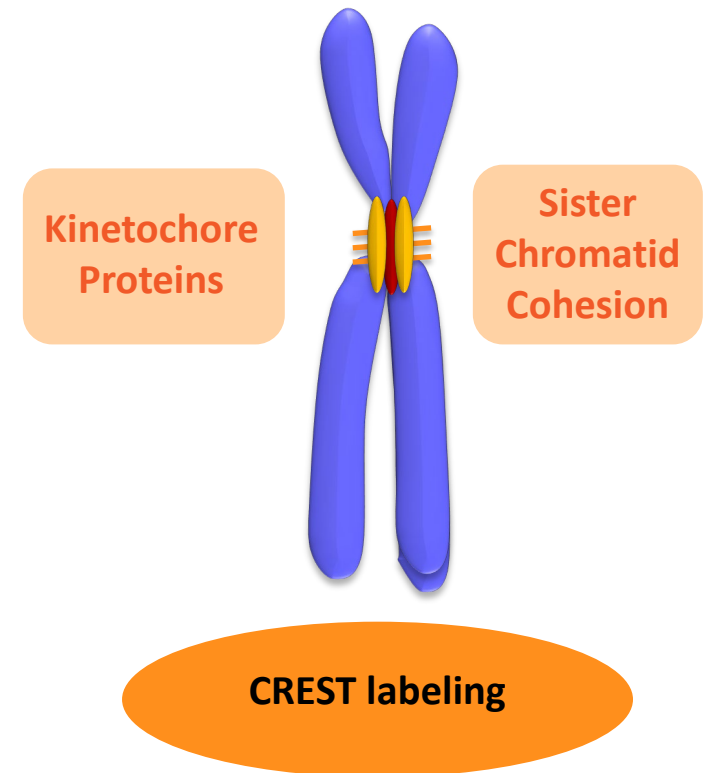
CREST Staining in Bone Marrow Erythrocytes

- Used to characterize mechanism leading to elevated micronucleus frequency
 - > CREST antibody stains kinetochores in micronuclei in bone marrow erythrocytes
 - chromosome loss (kinetochore positive [K^+ MN])
 - chromosome breakage (kinetochore negative [K^- MN])
 - > Can be incorporated in both bone marrow and peripheral blood micronucleus assay dosing regimens
- Bone marrow enriched for red blood cells using cellulose column method to remove nucleated cells
- Slides dried, fixed in cold methanol, dried, and stored frozen (-20°C) under nitrogen, protected from light, with desiccant



Selecting Doses for CREST Evaluation

- Base selection on concurrent or previously performed micronucleus assay results.
- Confirmation of previous results is recommended, especially when strain or test article lot has changed.
- Use dose(s) or animals with ≥ 2 -fold increase in micronuclei compared to the concurrent vehicle control.



CREST Staining

Stain with centromere positive control serum against kinetochore, 2 hours

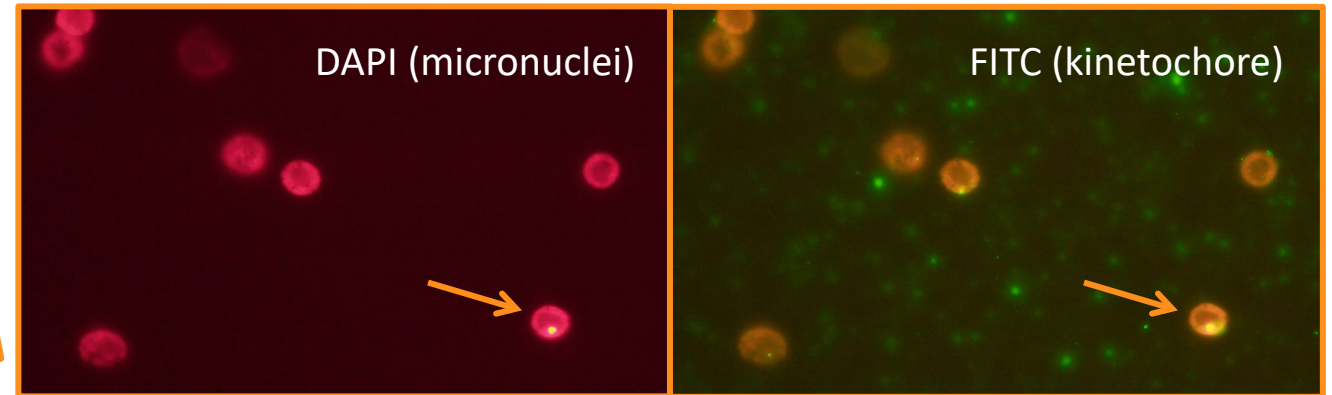
Wash, 10 min

Stain with FITC-conjugated secondary antibody, 2 hours

Wash, 10 min

Stain with propidium iodide or DAPI

Score ≥ 100 micronuclei per dose level by microscopy

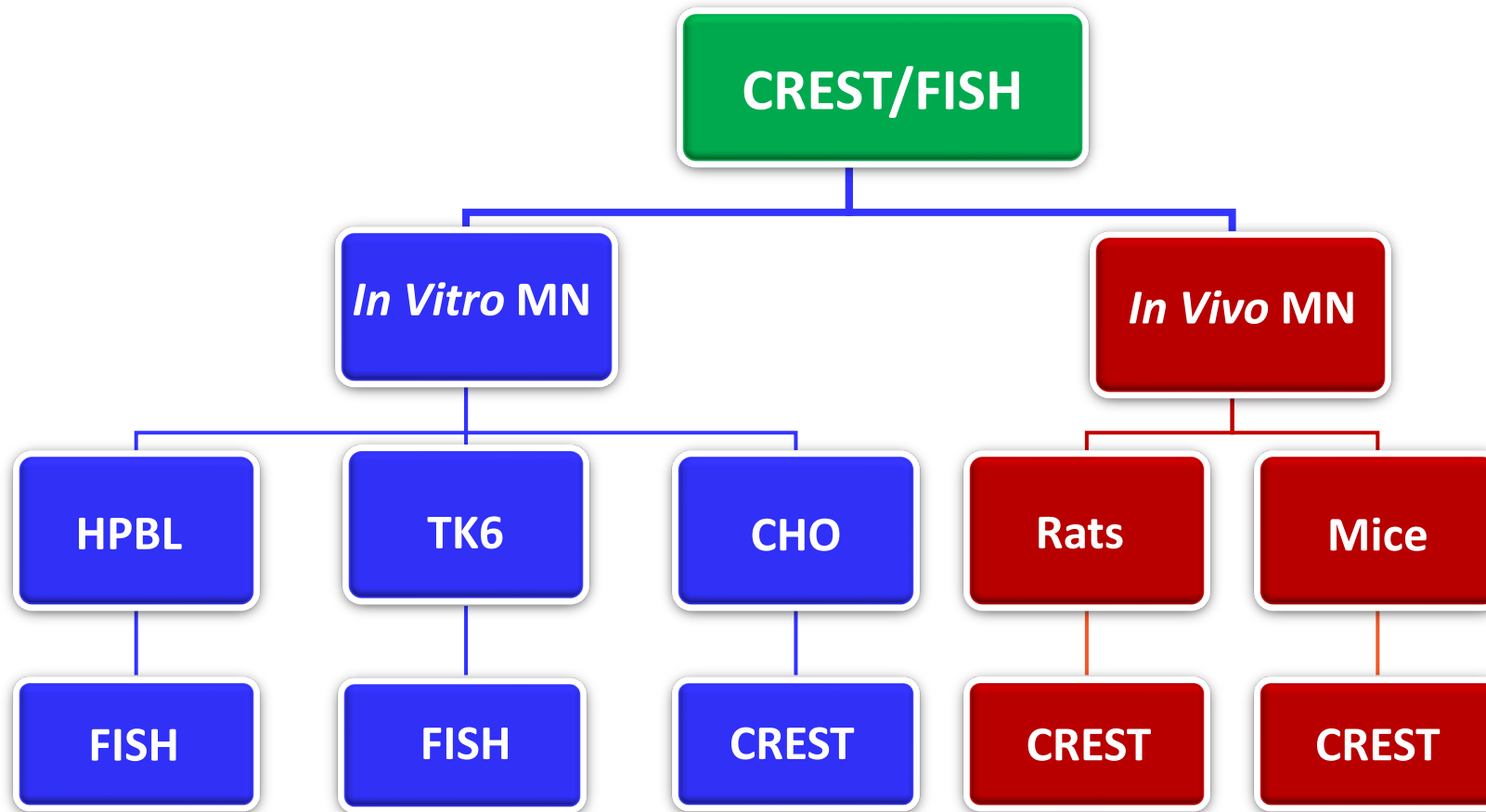


Evaluation Criteria:

- clastogen signal: $\leq 30\%$ K⁺ MN
- aneugen signal: $\geq 65\%$ K⁺ MN
- mixed clastogen/aneugen signal: 30% – 65% K⁺ MN
- aneugen and clastogen positive control slides included to confirm appropriate staining

Aneugenic MOA may permit estimation of a threshold in hazard/risk assessment.

CREST/FISH Labeling Services at Inotiv



HPBL – primary cells from healthy human donors

TK6 Cells – Human lymphoid cell line; functional *p53* gene

CHO Cells – Chinese Hamster Ovary cell line; impaired *p53* gene

9601 Medical Center Dr, Rockville

Explore our Genetic Toxicology Services

