Improving the performance of the in vivo comet assay and considerations for following up in vivo comet positive results

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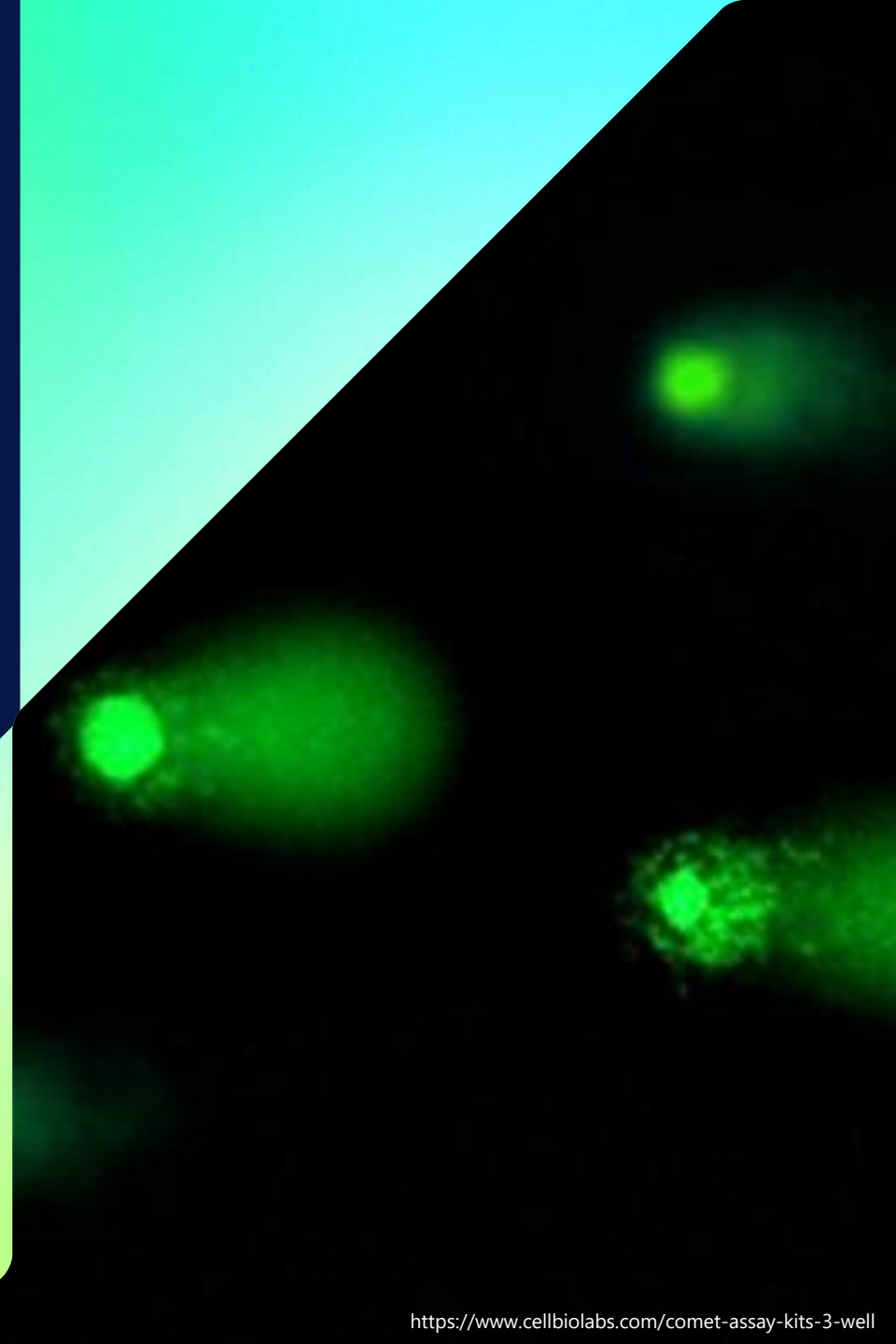
EFSA Stakeholders Workshop 3-4 November 2025



https://www.cellbiolabs.com/comet-assay-kits-3-well

Acknowledgements and Disclosures

Thanks to:

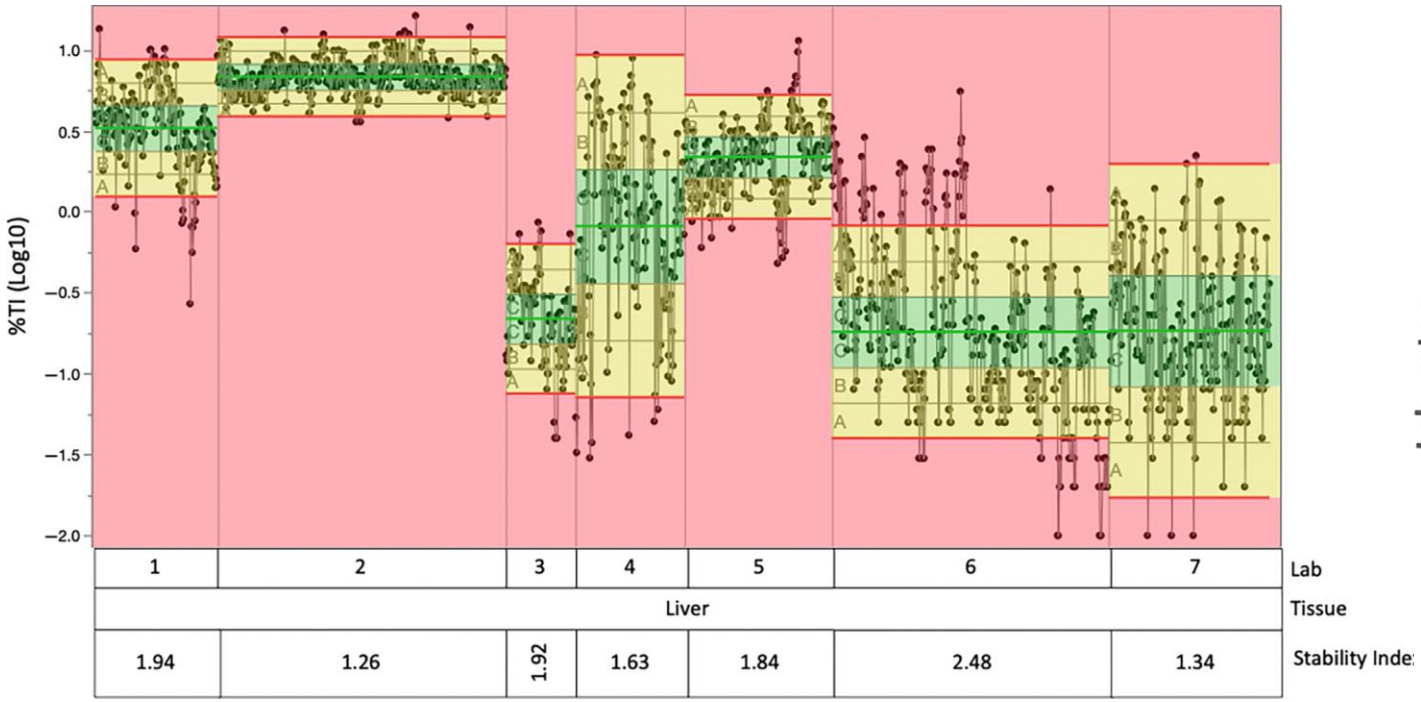
Members of the International Workshops on Genotoxicity Testing (IWGT) Statistics and In Vivo strategies working groups

Genetic Toxicology Technical Committee (GTTC) of the Health and Environmental Sciences Institute (HESI) in vivo workgroup

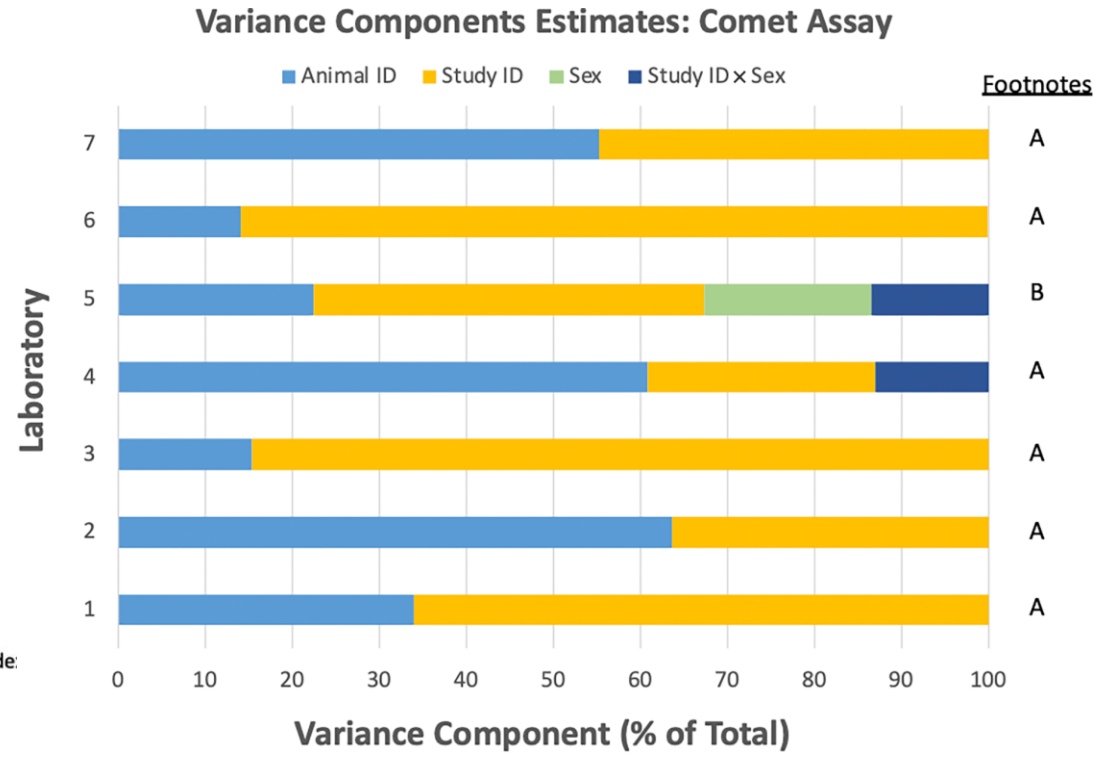
This presentation incorporates discussions and publications from the many contributors to these two groups. The views expressed are those of the contributors and do not necessarily reflect the statements, opinions, conclusions, or policies of the U.S. NIH-NIEHS or FDA, Health Canada, ECHA, ANSES, or Japan NIHS.

Are In Vivo Comet Historical Control Data Reflective of Biological Variation?

IWGT 2022 Statistics Work Group: Dertinger et al, 2023 (DOI: 10.1002/em.22541)



I-charts of % TI (log10 transformed) for rat liver negative HCD from 7 labs
Stability Index: closer to 1.0 = greater stability over time



A = confounding of effects detected, REML fit utilized
B = data unbalanced, REML fit was utilized.

Variance component estimates for %TI (log10 transformed) for rat liver negative HCD from same 7 labs.

Key IWGT 2022 In Vivo Work Group Comet Assay Conclusions

Beevers et al, 2023 (DOI: 10.1002/em.22578)

1. *Comparison of comet test results to laboratory historical control data (HCD) should not be used in data evaluation, unless it is demonstrated that the HCD distribution is stable and the predominant source of HCD variation is due to animal, not study, factors.*

Evaluation and Interpretation of Results

59. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if:

a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control,

b) the increase is dose-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations.

Potential for an increase in studies with data evaluation solely via statistical analysis.

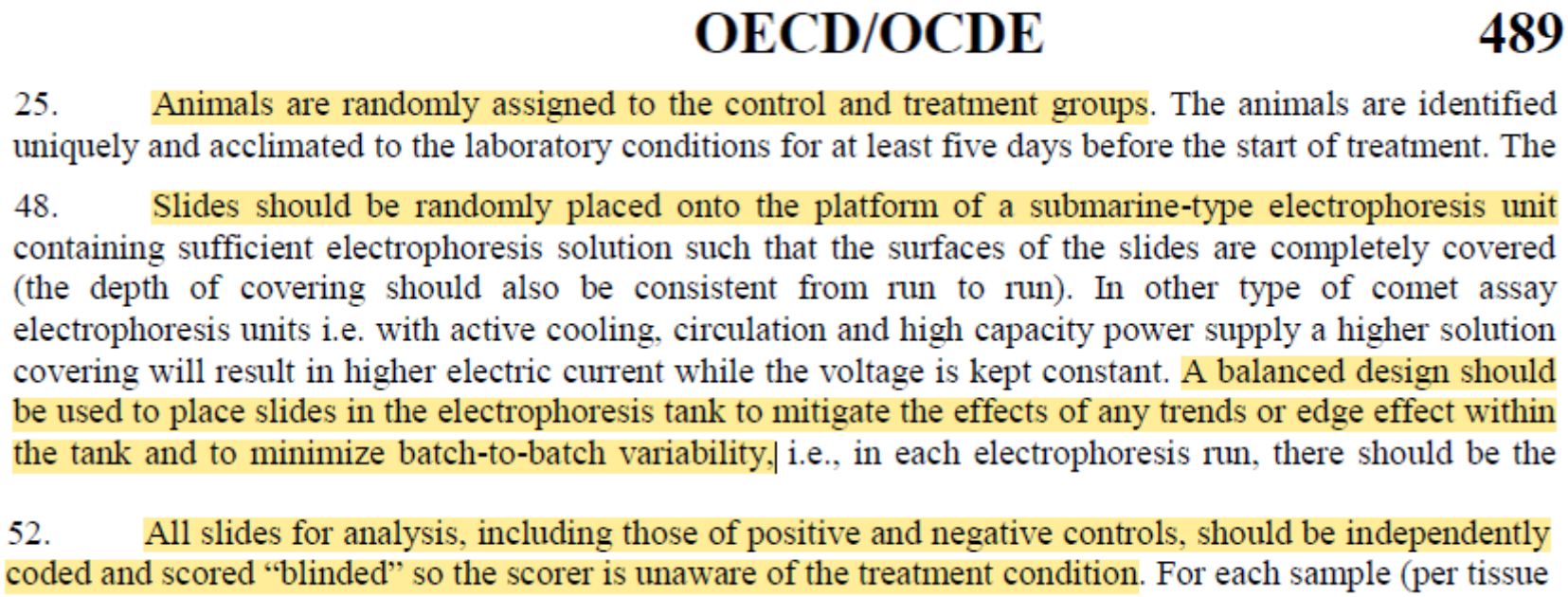
HOWEVER.....

2. *Because methodological differences in comet studies could result in variable data interpretations, more data are required before best practice recommendations can be made.*

Statistical interpretations are only reliable if the Comet methodologies used are robust and are clearly reported.

Potentially Confounding Factors: Implementation of Robust Block Designs

Randomised study designs are a critical assumption for statistical analysis. OECD TG489 includes several recommendations for randomization during the conduct of an in vivo comet assay



OECD/OCDE 489

25. Animals are randomly assigned to the control and treatment groups. The animals are identified uniquely and acclimated to the laboratory conditions for at least five days before the start of treatment. The

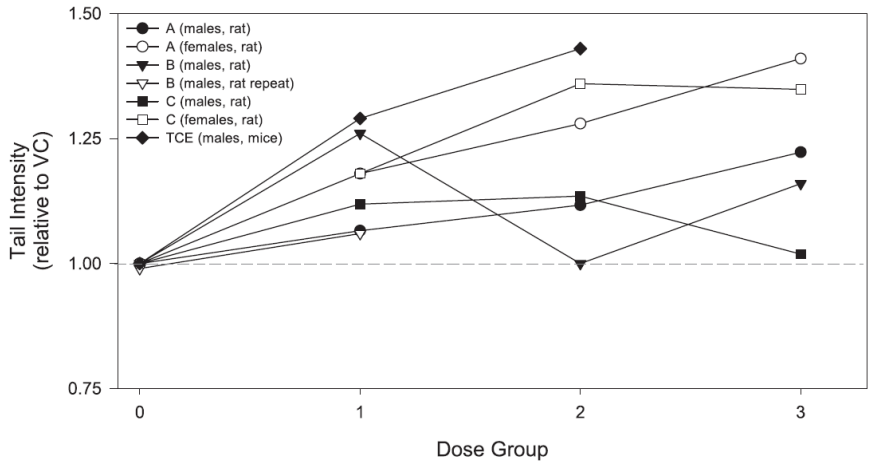
48. Slides should be randomly placed onto the platform of a submarine-type electrophoresis unit containing sufficient electrophoresis solution such that the surfaces of the slides are completely covered (the depth of covering should also be consistent from run to run). In other type of comet assay electrophoresis units i.e. with active cooling, circulation and high capacity power supply a higher solution covering will result in higher electric current while the voltage is kept constant. A balanced design should be used to place slides in the electrophoresis tank to mitigate the effects of any trends or edge effect within the tank and to minimize batch-to-batch variability, i.e., in each electrophoresis run, there should be the

52. All slides for analysis, including those of positive and negative controls, should be independently coded and scored "blinded" so the scorer is unaware of the treatment condition. For each sample (per tissue

In addition, **the order of animal dosing and necropsy** has also been shown to be critical

Tendency for monotonic increases in % tail intensity across studies

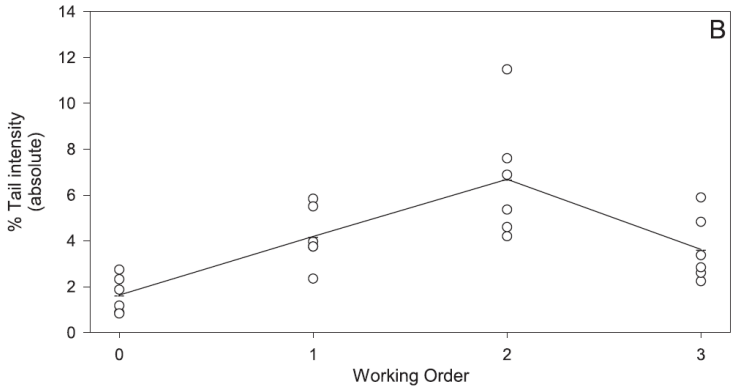
Struwe et al, 2011 Mutagenesis vol. 26 no. 3 pp. 473–474

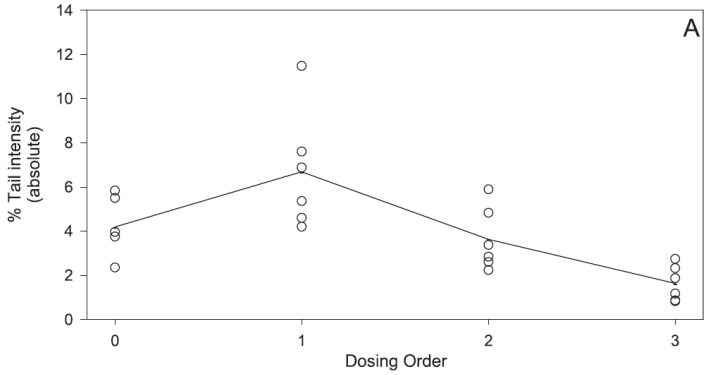


4 Studies conducted at the same CRO, all concluded negative by CRO and authors due to low magnitude of increase relative to control

Authors postulated methodological bias confounding the results

Performed a 5th study: altered the working order for animal dosing and necropsy



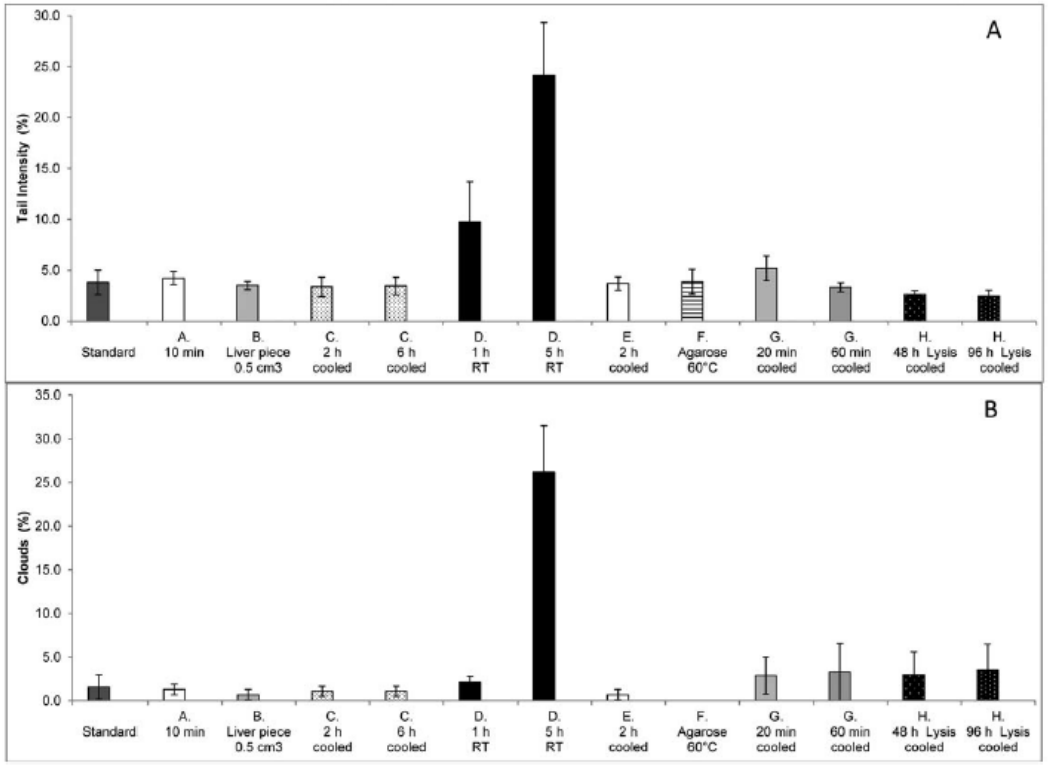


"…experimental bias is inadvertently introduced when keeping a fixed workup order starting with the control animals and proceeding in a dose-wise fashion…"

Group ID	Panel A	Panel B
0	Vehicle control	High dose group
1	Low dose group	Vehicle control
2	Intermediate dose group	Low dose group
3	High dose group	Intermediate dose group

Potentially Confounding Factors: Critical Methodology Factors

OECD TG489 acknowledges critical variables exist and considered parameters for such needed to be more precisely defined



Guerard et al, 2014: Environmental and Molecular Mutagenesis 55:114-121

OECD/OCDE **489**

methodologies and confirm acceptable low ranges of % tail DNA in target tissues of vehicle treated animals, and that positive responses can still be detected. In the literature, the freezing of tissues has been described using different methods. However, currently there is no agreement on how to best freeze and thaw tissues, and how to assess whether a potentially altered response may affect the sensitivity of the test.

6. Recent work demonstrates that the list of critical variables is expected to continue to become shorter and the parameters for critical variables more precisely defined (Guerard et al., 2014).

Key to changed condition

A	Time from necropsy to sample on ice increased from 2 min to 10 min
B	Size of tissue increased
C	Time sample on ice prior to processing increased from 1 h to 2 or 6 h
D	Tissue held at RT for 1 or 5 h
E	Cell suspension stored on ice for 2 h (standard processed immediately)
F	Temperature of agarose increased from 37°C to 60°C
G	Gel time for slides increased from 5 min to 20 or 60 min
H	Lysis time increased from <24 h to 48 or 96 h

TIME and TEMPERATURE identified as critical variables

New HESI GTTC Project

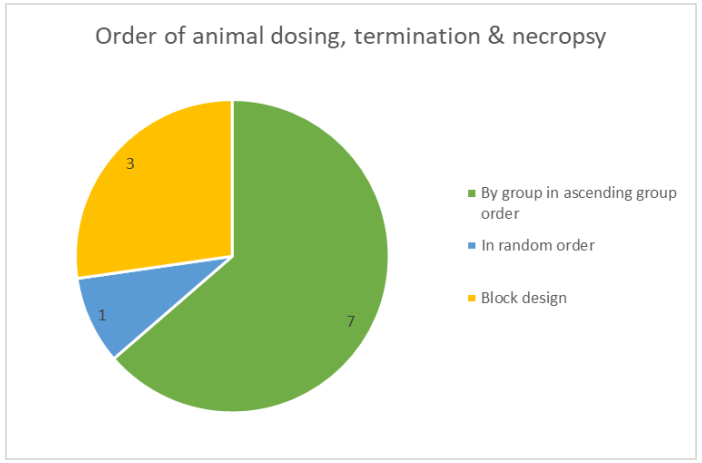
- Expand IWGT collation & evaluation of HCD for comet assay
- Examine contribution of nuisance factors on background data
- Extensive Excel Spreadsheet survey to capture lab methodologies
- Requested detailed HCD for number crunching – IN PROGRESS
- Critical parameters that may confound interpretation of comet data

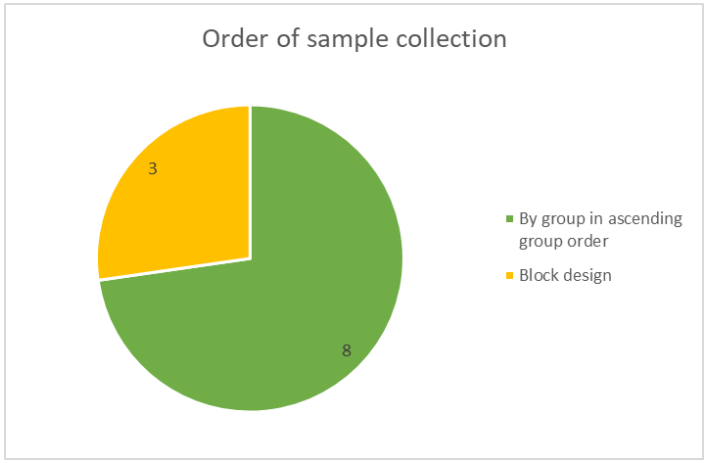
	B
	Parameter
	Animal ID
	Order of animal dosing, euthanasia & necropsy
	Number of necropsy days per study
	Order of sample collection
	Time of animal euthanasia
	Time of sample collection per individual animal
	Time from euthanasia to necropsy
	Time from necropsy to start of cell suspension preparation or frozen tissue storage
	Time from necropsy to tissue held on ice

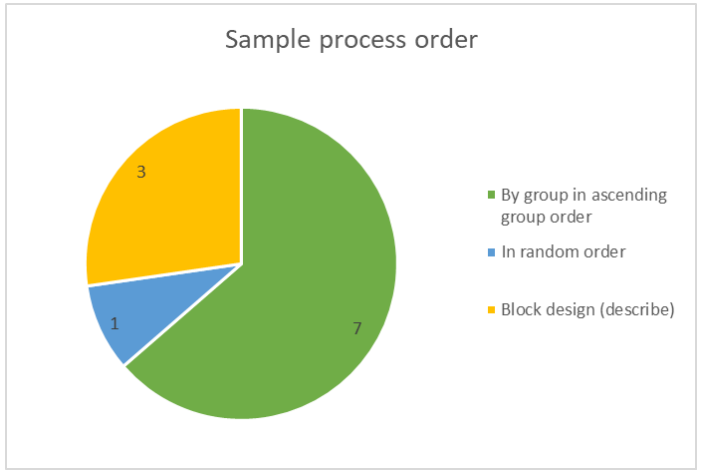
Dosing/necropsy order	**Data recording**
By group in ascending group order	Recorded per assay/animal/slide
In random order	Mandated by SOP
By group but not in ascending group order	Recorded per assay/animal/slide AND mandated by SOP
Block design (describe)	Not defined
Other	Not captured, can be calculated from data
	Other

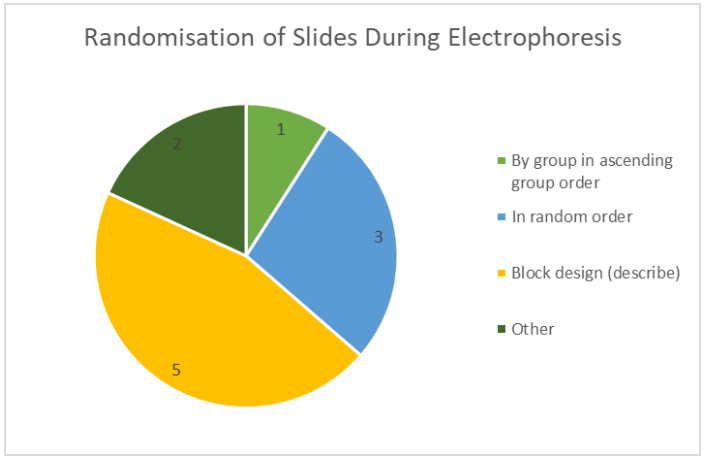
Potentially Confounding Factors: HESI GTTC Survey results

Implementation of Robust Block Designs





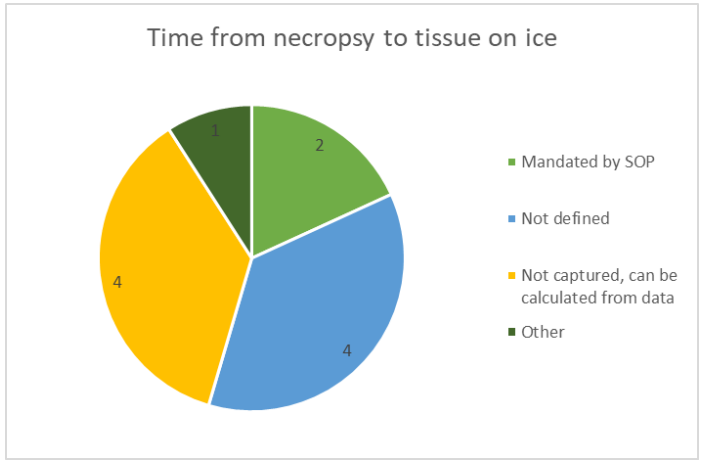


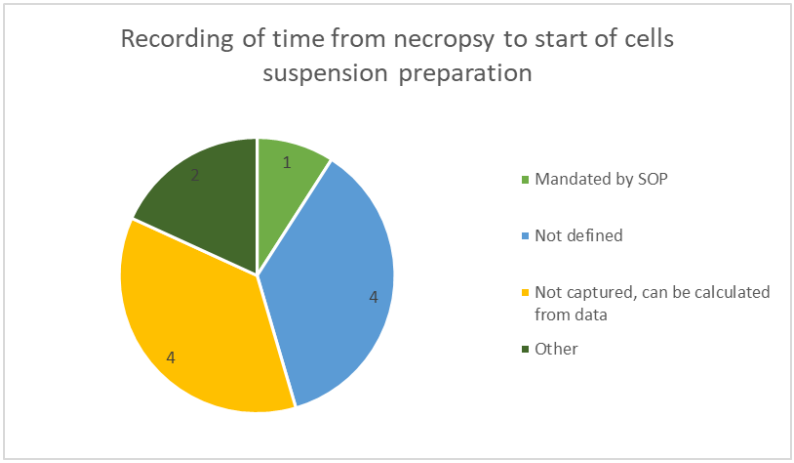


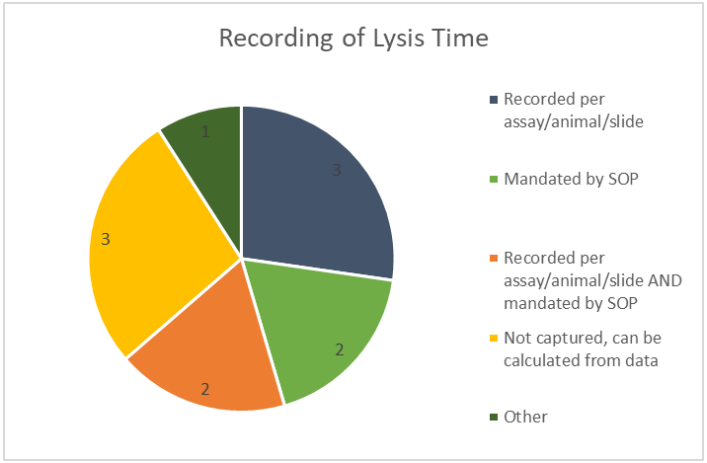
65-73%
(7-8 out of 11) laboratories process comet animals and samples in **strict dose group order**

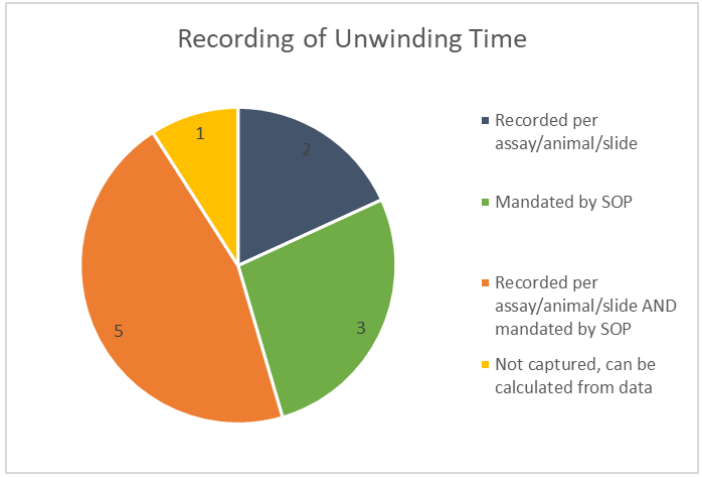
Potentially Confounding Factors: HESI GTTC Survey results

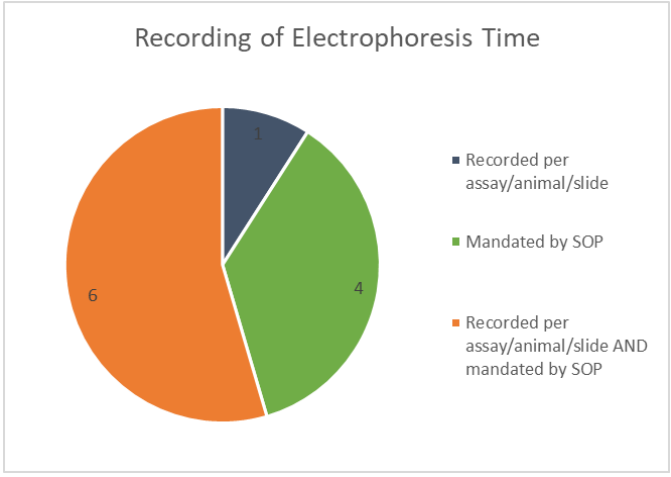
Control of Critical Variables: Time and Temperature







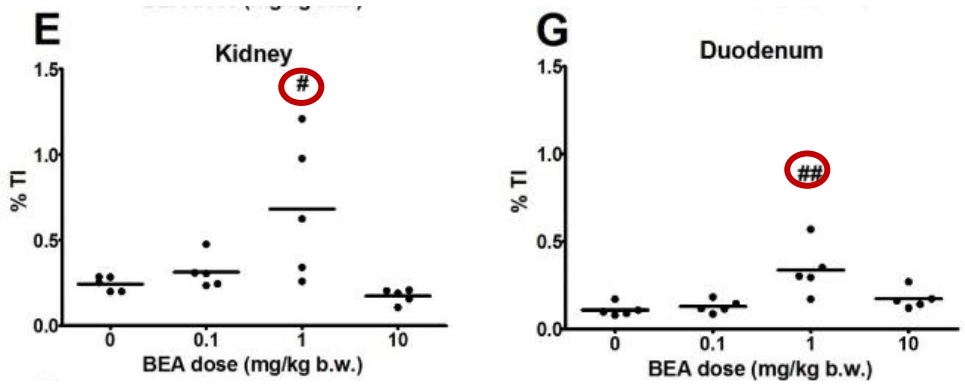




≤55%
(6 out of 11) laboratories **not recording critical parameters** on a per sample basis

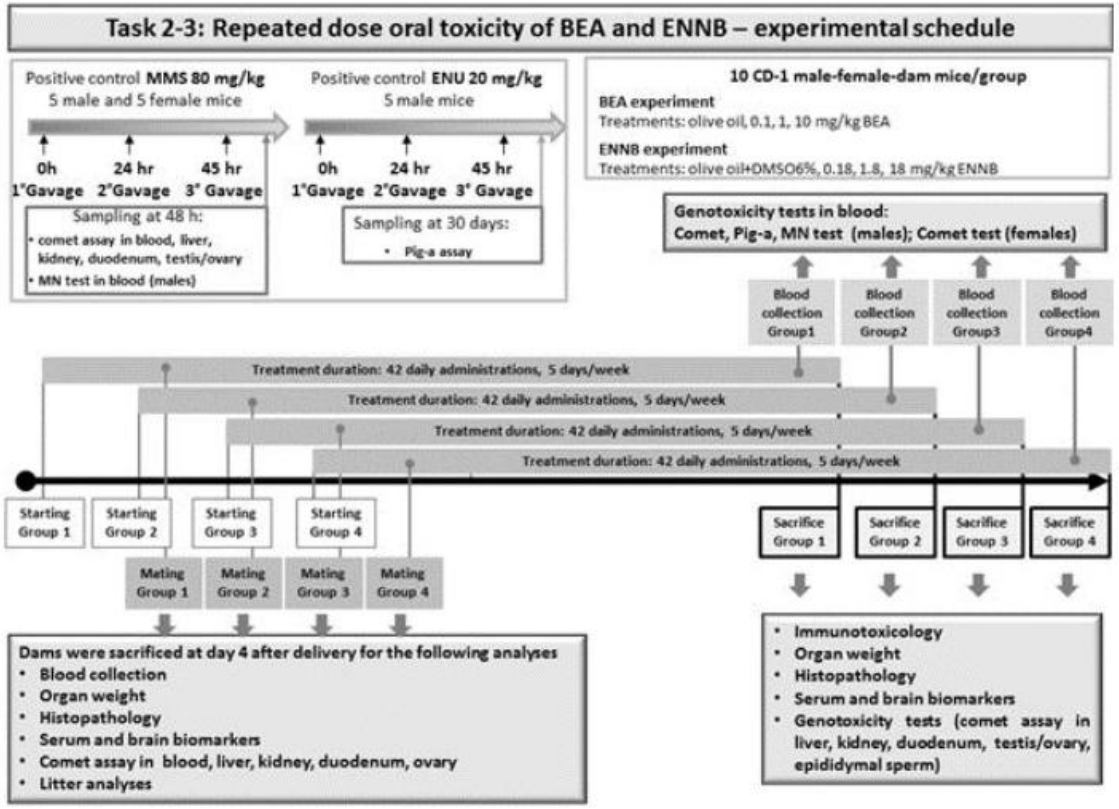
Methodology Can Significantly Impact Data Outcomes: Example 1

Maranghia et al 2018. EFSA supporting publication 2018:EN-1406. 183 pp



BEA

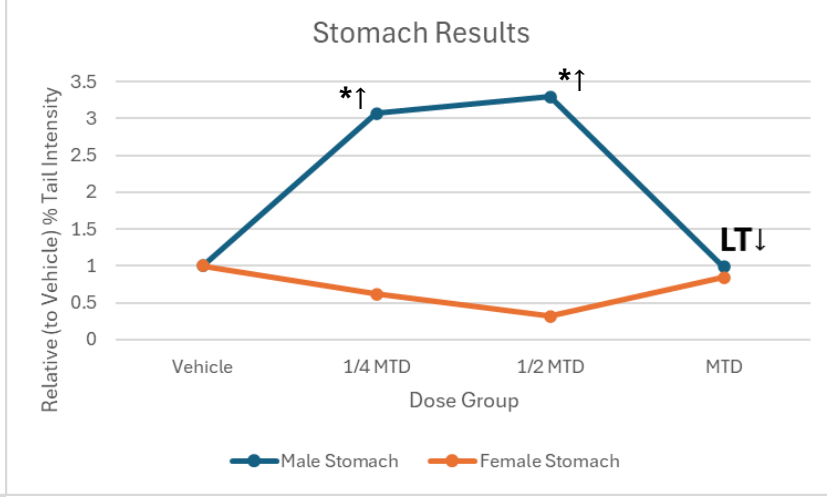
All the genotoxicity endpoints analyzed in the acute and repeated dose studies yielded negative results with the exception of Comet assay in duodenum and kidney but without a dose-related effect. Concluding, BEA has a low genotoxic potential; further investigations may be necessary under a cautionary approach.

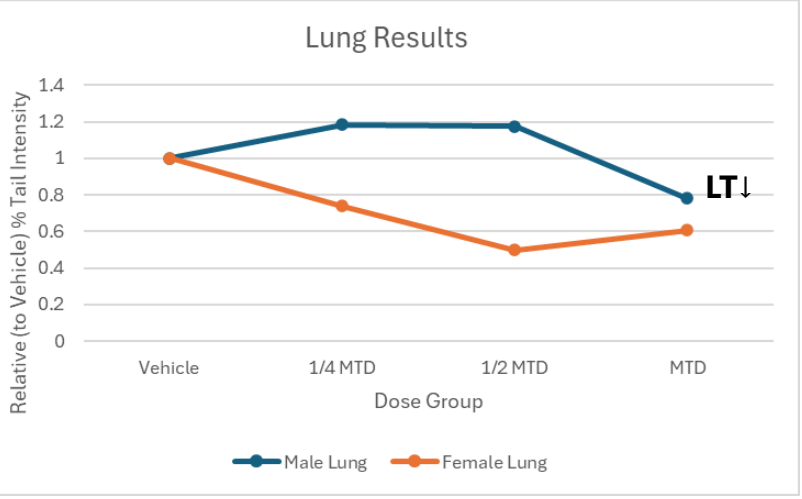


- No HCD presented, analysis solely on basis of statistics.
- Day-to-day variation for this laboratory is not reported
- No discussion or justification for comparisons of treated groups with vehicle control
- Maranghia et al conducted a wide range of in vitro and in vivo studies and EFSA CONTAM conclusion was favourable, **may not have been so easy to reach this conclusion with a more limited use of animals**

Methodology Can Significantly Impact Data Outcomes: Example 2

Consultancy Example: GLP study at a CRO





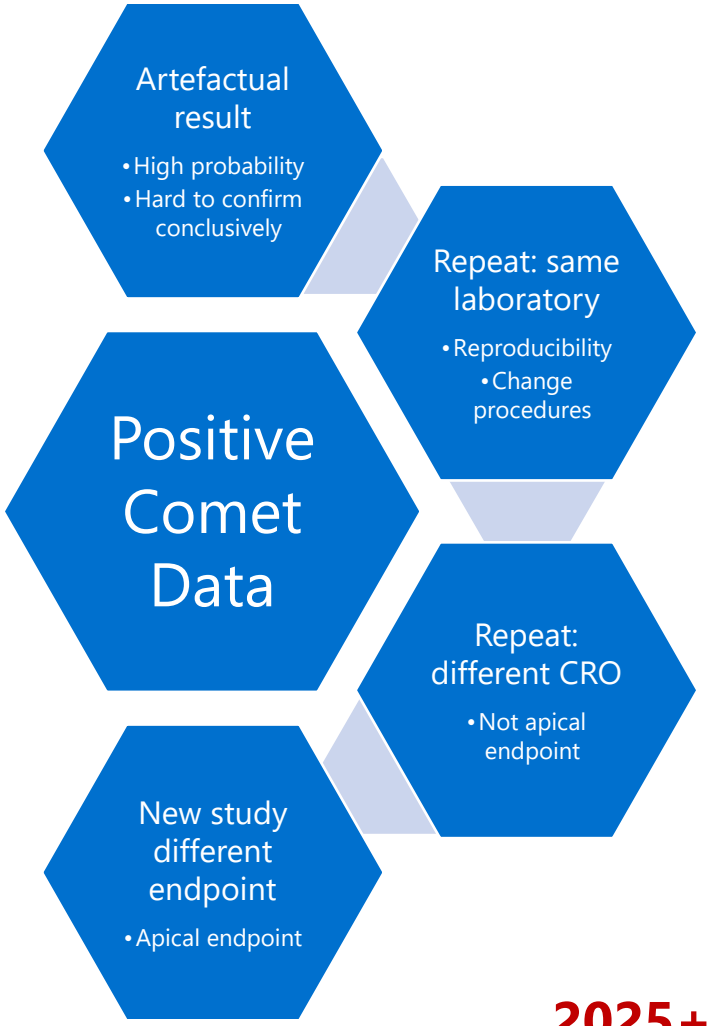
"Because of time-limiting and technical reasons, the comet assay was performed on three consecutive days."

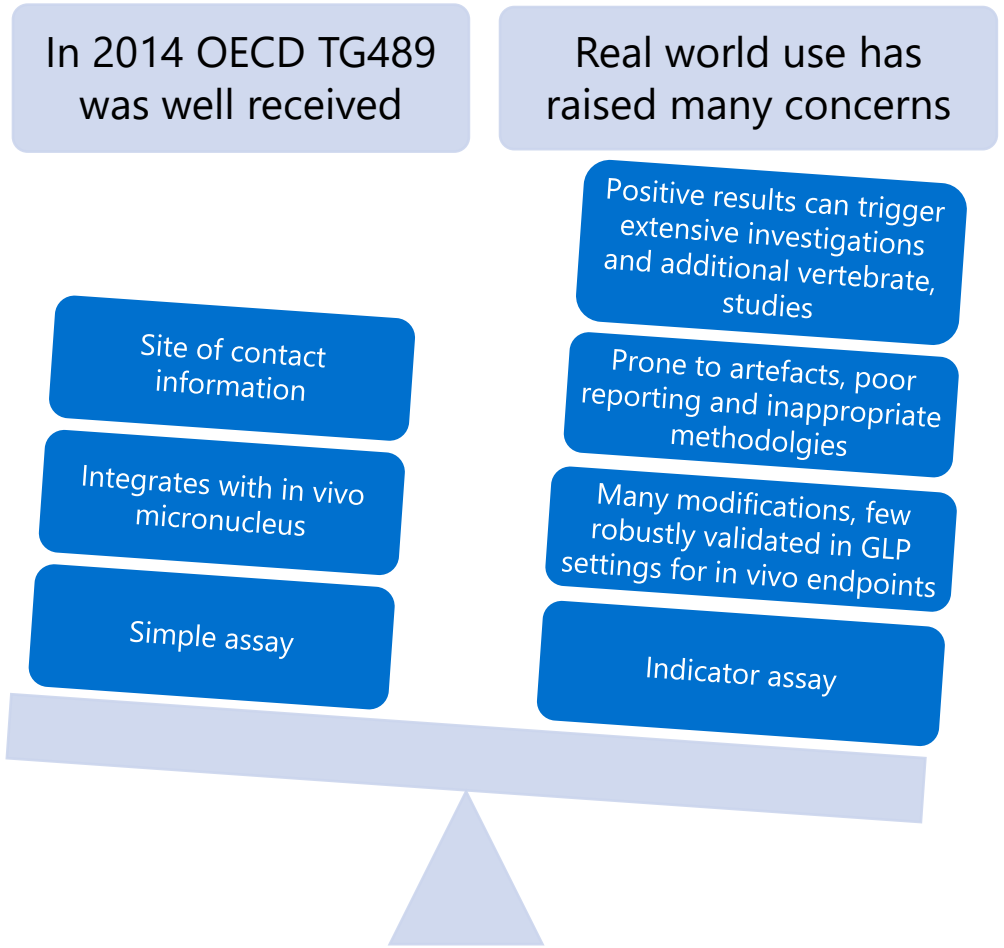
Group	Day of study		
	Day 1	Day 2	Day 3
Untreated	Sample		
Positive	Dose & Sample		
Vehicle		Dose	Dose & Sample
¼ MTD	Dose	Dose & Sample	
½ MTD	Dose	Dose & Sample	
MTD		Dose	Dose & Sample

Biological relevance of the data is **uninterpretable**

- Several data points were outside 95% control limits of very limited HCD (N=3)
- Only statistically reliable comparisons would be between groups sampled and processed on the same day
- Triggered a repeat study – **additional animal use and resources**
- **Increased regulatory concern regarding genotoxicity of substance**

Is the In Vivo Comet Assay Sufficiently Reliable Across Laboratories to Have a Default Role in a Regulatory Test Battery?

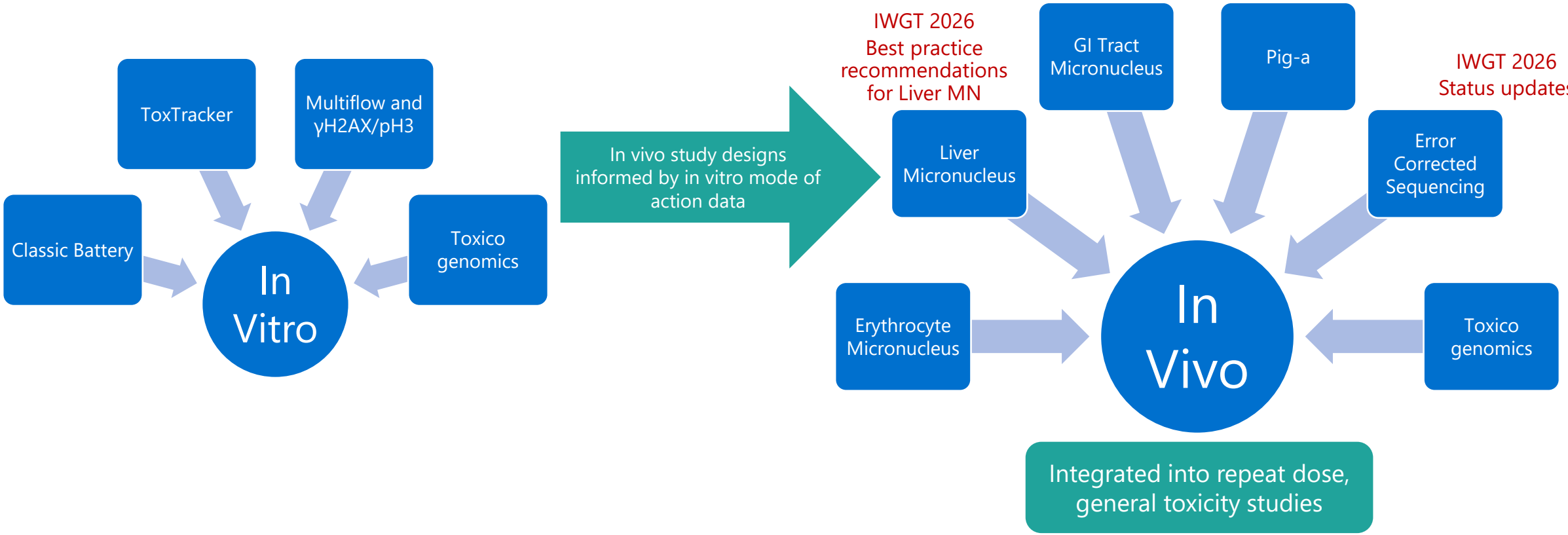




2025+ More informative approaches are now available

An Opportunity for NAMS and 3R-Friendly Approaches to In Vivo Genotoxicity Assessments

New assays and developments in existing assays provide an opportunity for a wider range of apical endpoints to be integrated into repeat dose toxicity studies, with study designs informed by in vitro mode of action data



Thank You For Your Attention