



WHERE
insights LEAD TO
answers

In Vivo Micronucleus and Comet Assays

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In Vivo Micronucleus Assay

Topics for Discussion

Micronucleus Assay

- Definitions
- Background Information
- Mechanism of Formation
- Exposure Methods
- Study Design
- Scoring
- Evaluation Criteria
- Sample Data

Standard Genetic Toxicology Testing Approach

Gene Mutation	Cytogenetic Damage	DNA Damage/Transformation
<i>In Vitro</i>	<i>In Vitro</i>	<i>In Vitro</i>
Bacterial Mutation (Ames) assay	Chromosome Aberrations	Cell Transformation (Bhas 42)
Mouse lymphoma (TK ^{+/-}) assay or CHO/HGPRT assay	Micronucleus (3D Skin micronucleus)	Cytotoxicity (e.g., Neutral Red Uptake, Cell Growth Inhibition)
<i>In Vivo</i>	<i>In Vivo</i>	<i>In Vivo</i>
Transgenic Rodent Mutation Assay (Big Blue)	Micronucleus	DNA Damage (Comet assay , 3D Skin Comet Assay)
<i>Pig-a</i> Assay	Chromosome Aberrations	DNA Repair (e.g., UDS assay)

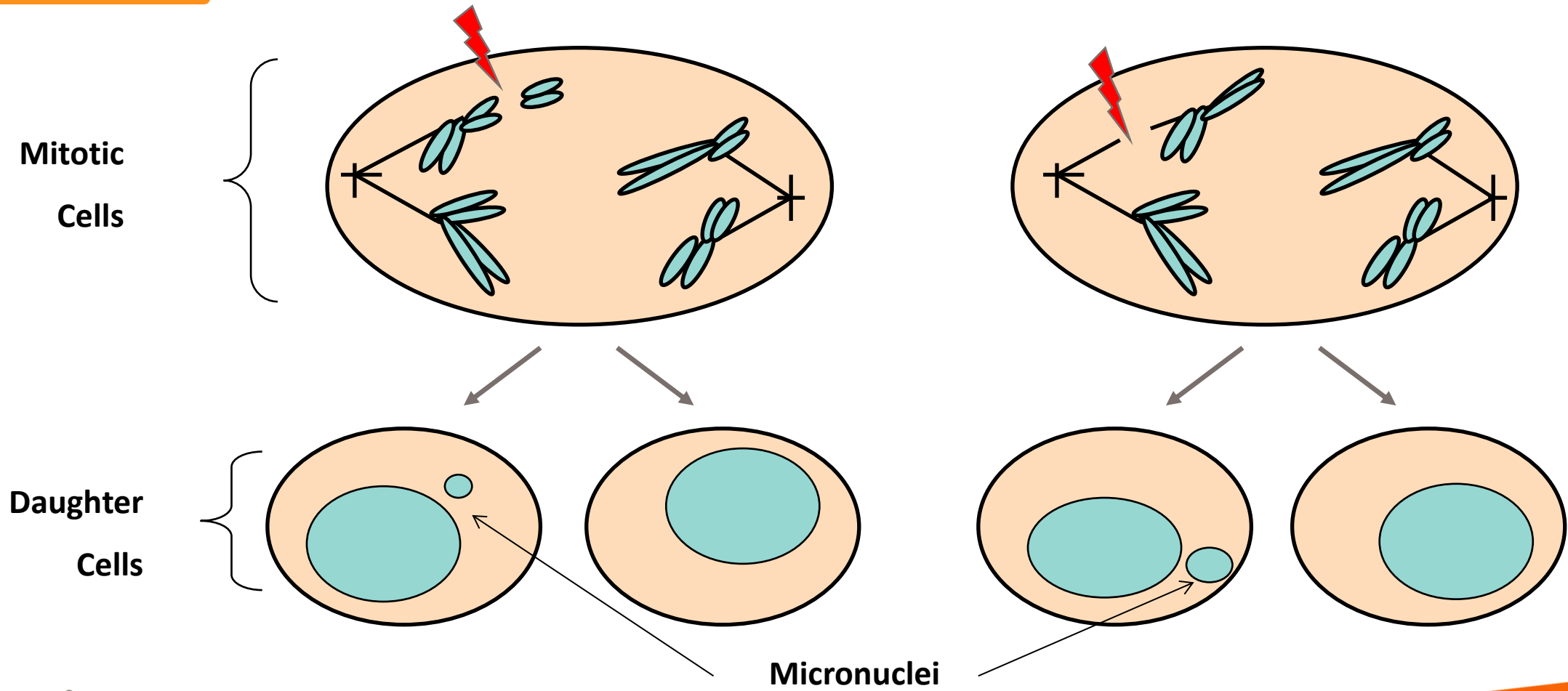
Definitions

- BM: bone marrow
- PB: peripheral blood
- Clastogen: an agent that induces chromosome breaks (structural damage)
- Aneugen: an agent that induces changes in chromosome number
- Micronucleus (MN): a membrane-bound body consisting of entire chromosomes or acentric chromosome fragments that lag behind at mitotic anaphase. At telophase, these chromosomes or chromosome fragments don't segregate properly and result in single or multiple micronuclei in the cytoplasm.
- PCE: polychromatic (immature) erythrocyte found in the bone marrow.
- RET: reticulocyte; an immature erythrocyte found in the peripheral blood
- MNPCE: micronucleated polychromatic erythrocyte
- MNRET: micronucleated reticulocyte

Background Information

- Initially described in 1973 by Heddle
- Used to assess the potential for DNA damage that may
 - > Alter chromosome structure
 - > Interfere with the mitotic apparatus causing changes in chromosome number
- Includes relevant factors that may influence genotoxic activity in vivo
 - > ADME (absorption, distribution, metabolism, excretion)
 - > DNA repair
 - > May detect some genotoxic agents 'missed' with in vitro systems
 - Requiring hematopoietic system
- Performed under OECD testing guideline 474 (Mammalian Erythrocyte Micronucleus Test)
 - > Most recent version adopted 29 July 2016 incorporating the peripheral blood methodology

Mechanism of Micronuclei Formation



Exposure Methods

- Test System

- > Rats (Wistar Han, Sprague Dawley)
- > Mouse (CD-1, ICR, C57BL6)

- Dose Administration

- > Oral gavage
- > Intravenous (slow bolus, bolus, infusion)
- > Subcutaneous
- > Intraperitoneal
- > Inhalation

- Dose Formulations

- > Freshly prepared unless stability has been demonstrated
 - Solids
 - dissolved or suspended in vehicle
 - Individual formulations prepared for each dose group
 - Liquids
 - Administered directly
 - Diluted in vehicle serially or individual formulations prepared for each dose group
- > Vehicle selection based on
 - Compatibility with test system
 - Route of administration
 - Test article solubility
 - Ability to prepare a uniform solution or workable suspension

Assay Design

Dose Range Finding Assay (DRF)

- Evaluation of test article toxicity used to determine a maximum tolerated dose (MTD)
 - > Doses selected from client data or recommendation
 - If no information is available, test up to limit dose of 200 mg/kg/day or maximum feasible dose (MFD)
 - > Dose animals to cover intended duration of micronucleus assay
 - Generally, one to four administrations
 - Observe animals for 24 to 72 hours
 - Time differs based on number of administrations
 - Assess mortality, clinical observations, and body weights
 - If requested, body temperatures and frequency of polychromatic erythrocytes (%PCE) can also be assessed.

Maximum Tolerated Dose (MTD)

- Dose inducing some clinical signs of toxicity
 - > No mortality or moribundity
- Dose inducing a marked decrease in bone marrow PCEs
 - > Reduction in %PCE or %RET compared to control indicating an inhibition of erythropoiesis
 - Bone marrow: >80% PCE
 - Peripheral blood: >95% RET
- Limit dose
 - > 2000 mg/kg/day (<14 days)
 - > 1000 mg/kg/day (≥14 days)
- Maximum feasible dose (MFD)
 - > Highest dose level able to be administered based on solubility of test article and dose volume limitations.
- Safety multiples are not appropriate

Single Administration Micronucleus Assay

Single dose administration, two collections

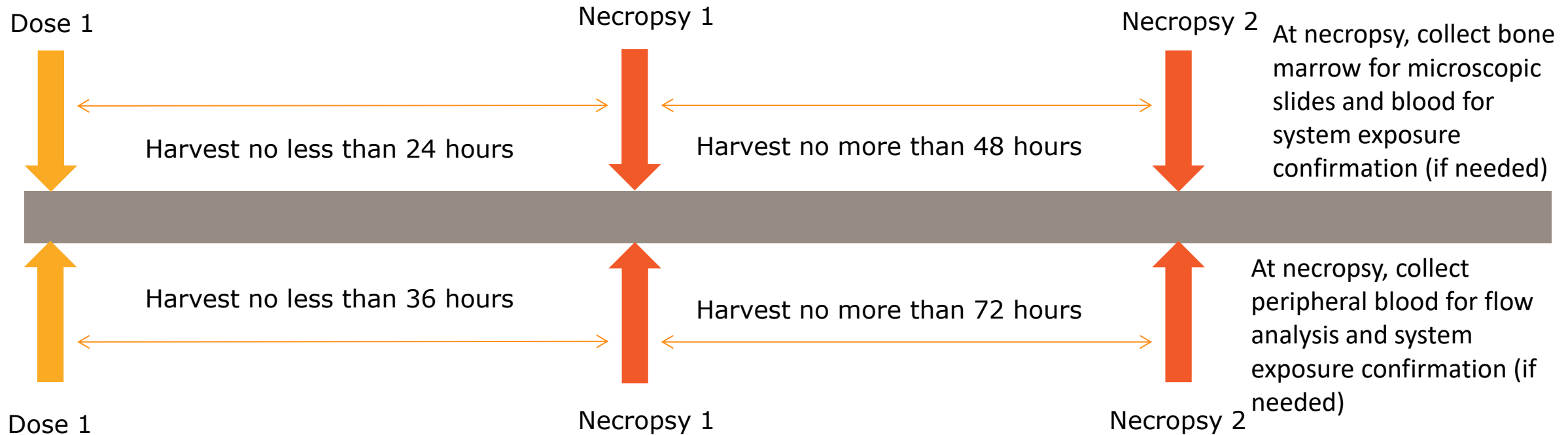
Group Number	Treatment	Number of Animals Dosed*		
		24 hours (BM)	48 hours (BM)	36 hours (PB)/ 72 hours (PB)
1	Vehicle Control	6	6	6
2	Test Article: Low Dose	6	--	6
3	Test Article: Mid Dose	6	--	6
4	Test Article: High Dose**	6	6	6
5	Positive Control	6	--	6
Total Animals			42	36

*At least 5 animals should be available for scoring from each group

** High dose should be limit dose (2000 mg/kg), defined maximum tolerated dose (MTD) or maximum feasible dose (MFD)

Systemic exposure should be confirmed – plasma collection and analysis

Single Administration Micronucleus Assay



Multiple Administration Micronucleus Assay Design

Two dose administrations, one collection

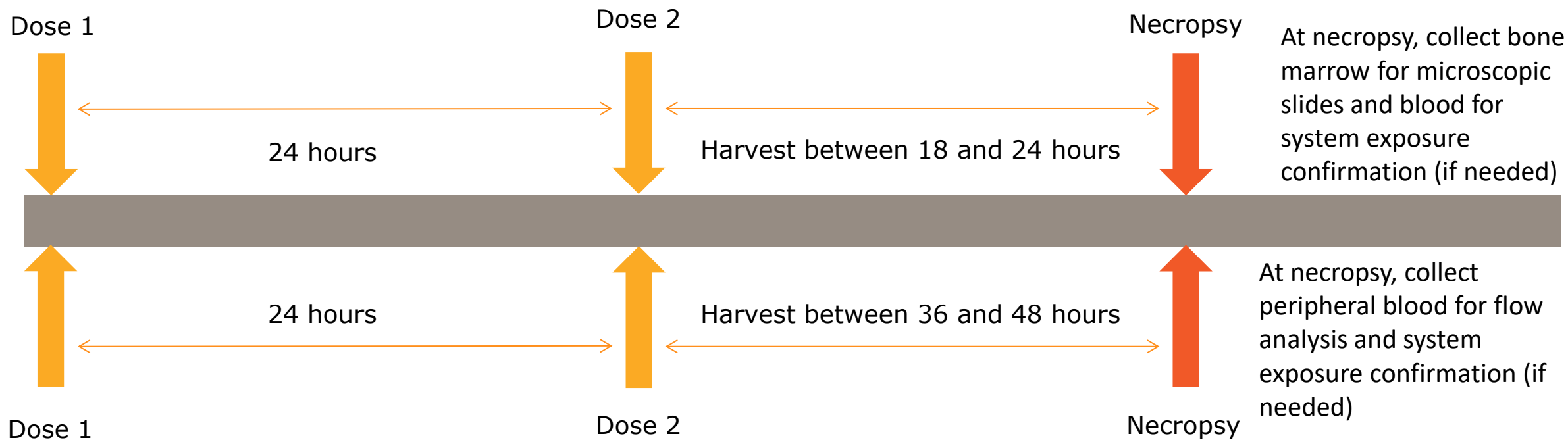
Group Number	Treatment	Number of Animals Dosed*
		24 hours (BM)/48 hours (PB)
1	Vehicle Control	6
2	Test Article: Low Dose	6
3	Test Article: Mid Dose	6
4	Test Article: High Dose**	6
5	Positive Control	6
Total Animals		30

*At least 5 animals should be available for scoring from each group

** High dose should be limit dose (2000 mg/kg), defined maximum tolerated dose (MTD) or maximum feasible dose (MFD)

Systemic exposure should be confirmed – plasma collection and analysis

Multiple Administration Micronucleus Assay Design



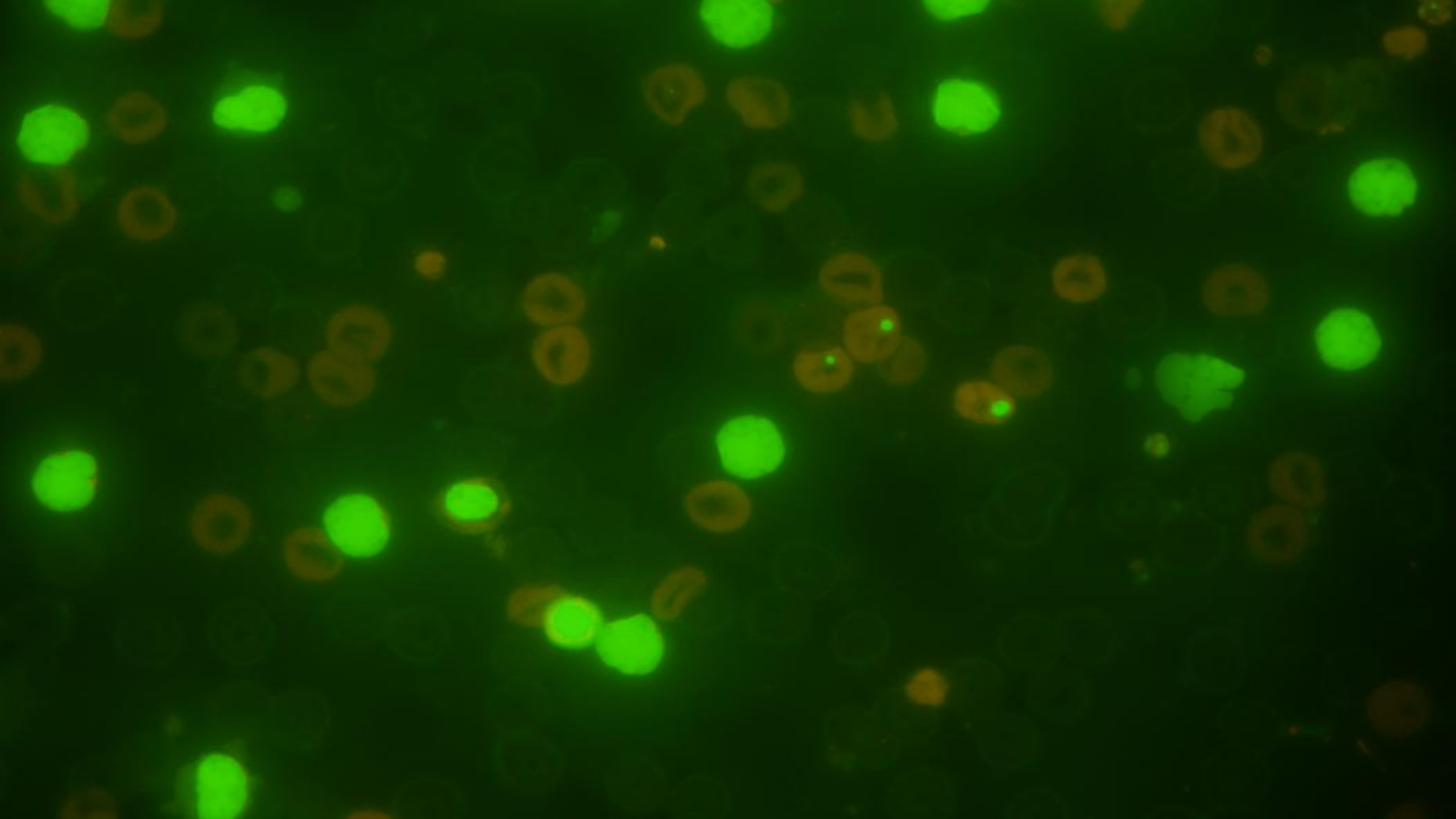
Scoring for Micronuclei

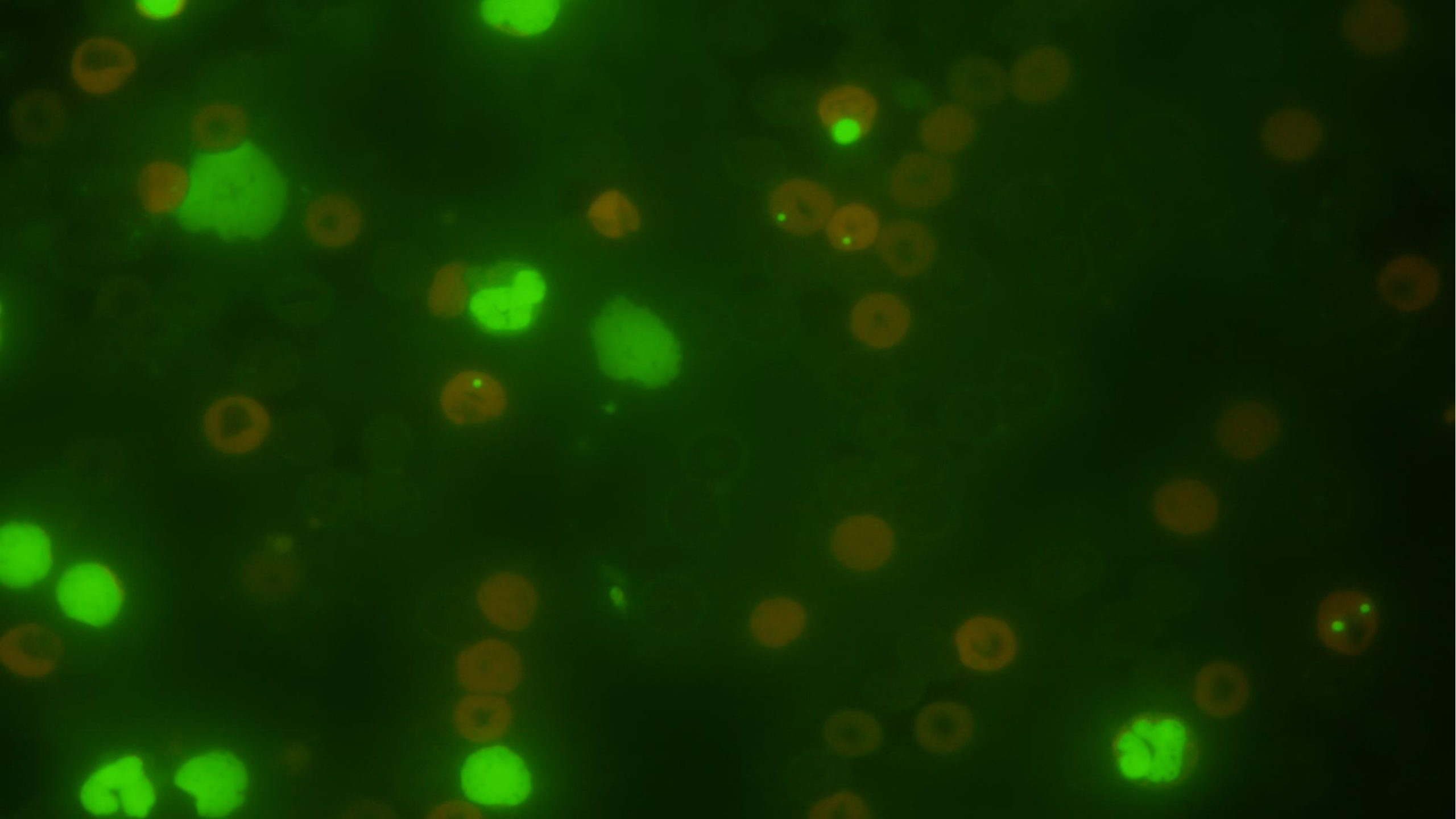
Bone Marrow Evaluation

- Slides stained with acridine orange
- Slides blind coded before submission for scoring
- Scored using fluorescence microscope
- Assess at least 500 total erythrocytes (polychromatic + normochromatic erythrocytes) for bone marrow toxicity
- Assess at least 4000 polychromatic erythrocytes for micronuclei

Peripheral Blood Evaluation

- Blood fixed in ultra cold methanol for at least 72 hours before staining
- Fixed peripheral blood stained with anti-rat/mouse CD71 antibody, anti-rodent CD61 antibody, RNase solution, and propidium iodide
- Analyze on flow cytometer after calibration with malaria biostandard (488 nm excitation)
- Assess at least 10,000 reticulocytes for micronuclei





Criteria for a Valid Test

- %MNPCE or %MNRET should ideally be within the 95% control limits of the distribution of the historical negative control database.
 - > If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).
- The positive control must induce a significant response ($p < 0.05$) in %MNPCE or %MNRET as compared to the vehicle control.
- At least five animals per group available for analysis for one timepoint.

Evaluation Criteria

- Positive Response

- > At least one test material dose exhibits a statistically significant increase in micronuclei when compared to the concurrent vehicle control ($p \leq 0.05$), and
- > When multiple doses are examined at a particular sampling time, the increase is dose related ($p \leq 0.01$) when compared to the concurrent vehicle control, and
- > Results of the group mean of the individual animals in at least one group are outside the 95% control limit of the historical negative control data

- Negative response

- > No statistically significant increase in micronuclei at any dose levels when compared to the concurrent vehicle control ($p > 0.05$)
- > No dose-dependent increase in micronuclei as compared to the concurrent vehicle control ($p > 0.01$)

Bone Marrow Micronucleus – Sample Data

Treatment	Sex	Time (hours)	%PCE (mean ± SD)	Toxicity (%)	%MNPCE (mean ± SD)	Number of MNPCE/PCE Scored
Vehicle	M	24	50.5 ± 4.0	--	0.09 ± 0.05	22/24000
Test Material A						
500 mg/kg/day	M	24	46.3 ± 1.9	8	0.11 ± 0.03	26/24000
1000 mg/kg/day	M	24	46.6 ± 5.7	8	0.13 ± 0.05	31/24000
2000 mg/kg/day	M	24	45.8 ± 3.4	9	0.10 ± 0.02	24/24000
Cyclophosphamide monohydrate						
50 mg/kg/day	M	24	47.0 ± 3.0	7	1.95 ± 0.52**	468/24000

**Statistically significant increase compared to vehicle control (t-test, $p < 0.05$)

The background is a vibrant orange with a subtle, repeating geometric pattern of triangles and hexagons. Scattered across the surface are various white symbols: single and double chevrons (» and >>), horizontal lines of varying lengths, and small open circles. These elements are arranged in a way that suggests movement or data points. A white diagonal line runs from the bottom left towards the bottom right corner.

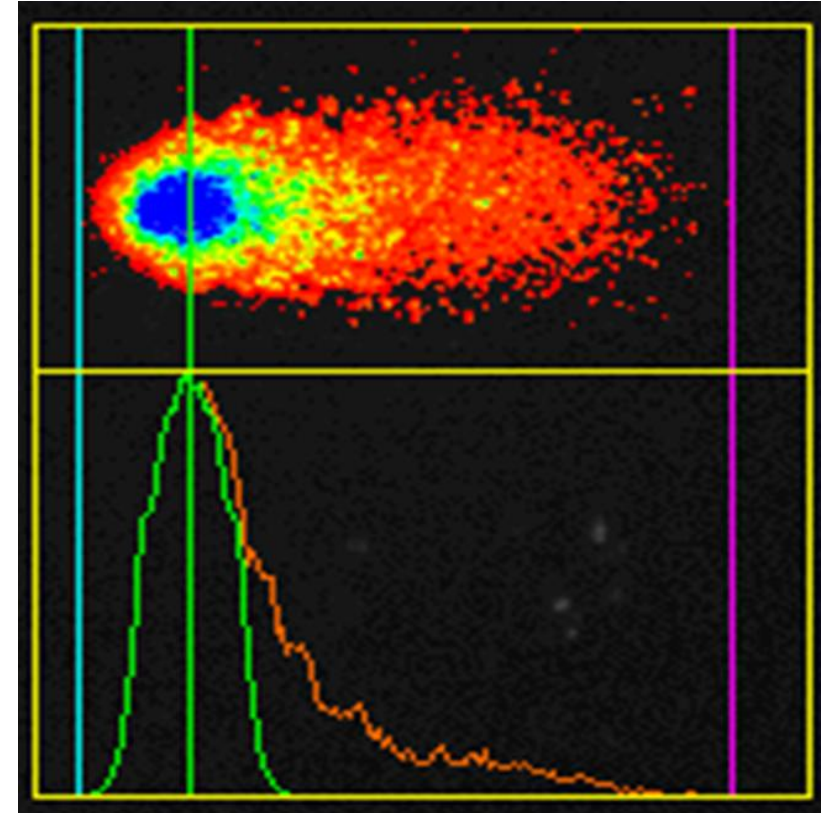
In Vivo Comet Assay

Topics for Discussion

- Comet Assay
 - > Background Information
 - > When to perform?
 - > Study Design
 - > Tissues and Techniques
 - > Scoring
 - > Evaluation Criteria
 - > Sample Data

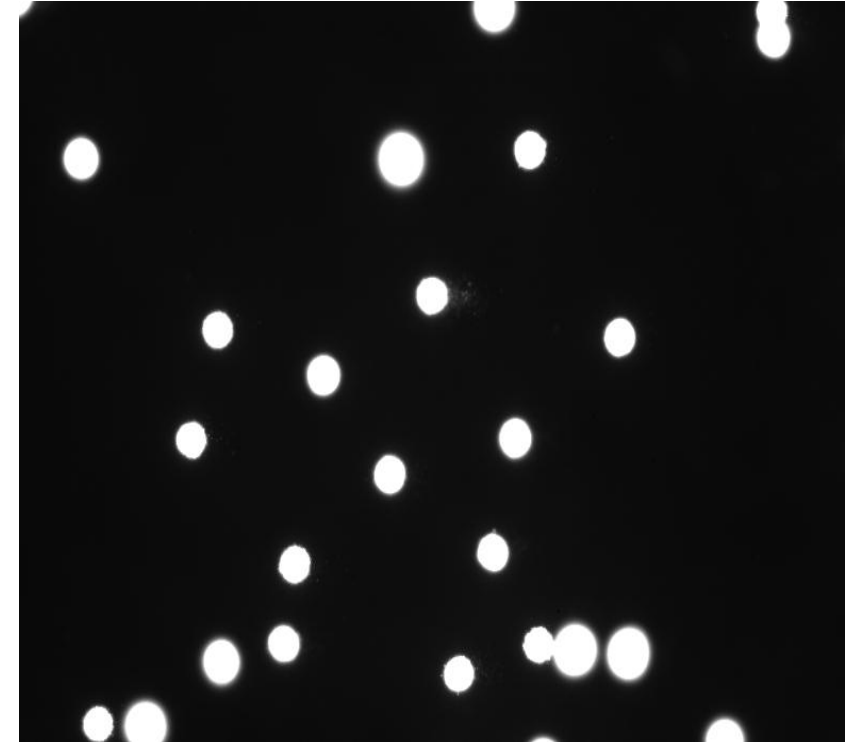
Comet Assay Background

- Single Cell Gel Electrophoresis (Comet) Assay
 - > Microgel electrophoretic technique that detects DNA damage in individual cells
 - > *In vivo* and *in vitro* applications
- Under alkaline conditions ($\text{pH} > 13$), the assay can detect
 - > DNA single strand breaks
 - > DNA double strand breaks
 - > DNA single strand breaks as a results for alkali-labile sites
 - > Nucleotide excision repair sites
- The level of DNA damage is directly correlated to the length and the amount of fragmented DNA that migrates outside of the cell nucleus (comet tail)
- Performed under OECD testing guideline 489 (*In Vivo* Mammalian Alkaline Comet Assay)
 - > Most recent version adopted 29 July 2016



When to Perform?

- As a second *in vivo* test
 - > Standalone assay
 - > In combination with the *in vivo* micronucleus assay (ICH S2(R) Option 2)
 - Acute dosing
 - Integrated into longer term toxicity studies (e.g., 14-, 28-, or 90-day dosing)
- To further evaluate *in vitro* positive results
- To assess DNA damaging potential
- To further evaluate an *in vivo* positive genotoxicity test
- To evaluate a tissue-specific genotoxic activity
 - > Can perform with any tissue in which a single cell suspension can be obtained.
 - > Does not require cell proliferation
 - > Can perform on site of first/direct contact
 - > Incorporates xenobiotic metabolism



Standard Genetic Toxicology Testing Approach

Gene Mutation	Cytogenetic Damage	DNA Damage/Transformation
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Mouse lymphoma (TK ^{+/-}) assay or CHO/HGPRT assay	Micronucleus (3D Skin micronucleus)	Cytotoxicity (e.g., Neutral Red Uptake, Cell Growth Inhibition)
<i>In Vivo</i>	<i>In Vivo</i>	<i>In Vivo</i>
Transgenic Rodent Mutation Assay (Big Blue)	Micronucleus	DNA Damage (Comet assay , 3D Skin Comet Assay)
<i>Pig-a</i> Assay	Chromosome Aberrations	DNA Repair (e.g., UDS assay)

When to Perform?

Revised - ICHS2R1		
Option 1*		Option 2
Ames (no repeat if clear positive or negative)		Ames (no repeat if clear positive or negative)
In vitro mammalian cell assay: (1 mM top concentration) Chromosome aberrations, OR: <i>tk</i> mutations in mouse lymphoma cells OR: <i>in vitro</i> micronucleus assay		No <i>in vitro</i> mammalian cell assays
<i>In vivo</i> micronucleus or chromosome aberration assay (rat/mouse, blood/bone marrow)** before phase 1		<i>In vivo</i> gene tox with two tissues**: 1) Micronuclei (not chrom ab) 2) Typically DNA breakage in liver 3) Prefer both endpoints in 1 study
before phase 2		Micronuclei analysis can be evaluated by slide or flow cytometry

Comet Assay Design

Two dose administrations, one collection

Group Number	Treatment	Number of Animals Dosed*
		2 to 6 hours
1	Vehicle Control	6
2	Test Article: Low Dose	6
3	Test Article: Mid Dose	6
4	Test Article: High Dose**	6
5	Positive Control	6
Total Animals		30

*At least 5 animals should be available for scoring from each group

** High dose should be limit dose (2000 mg/kg), defined maximum tolerated dose (MTD) or maximum feasible dose (MFD)

Systemic exposure should be confirmed – plasma collection and analysis

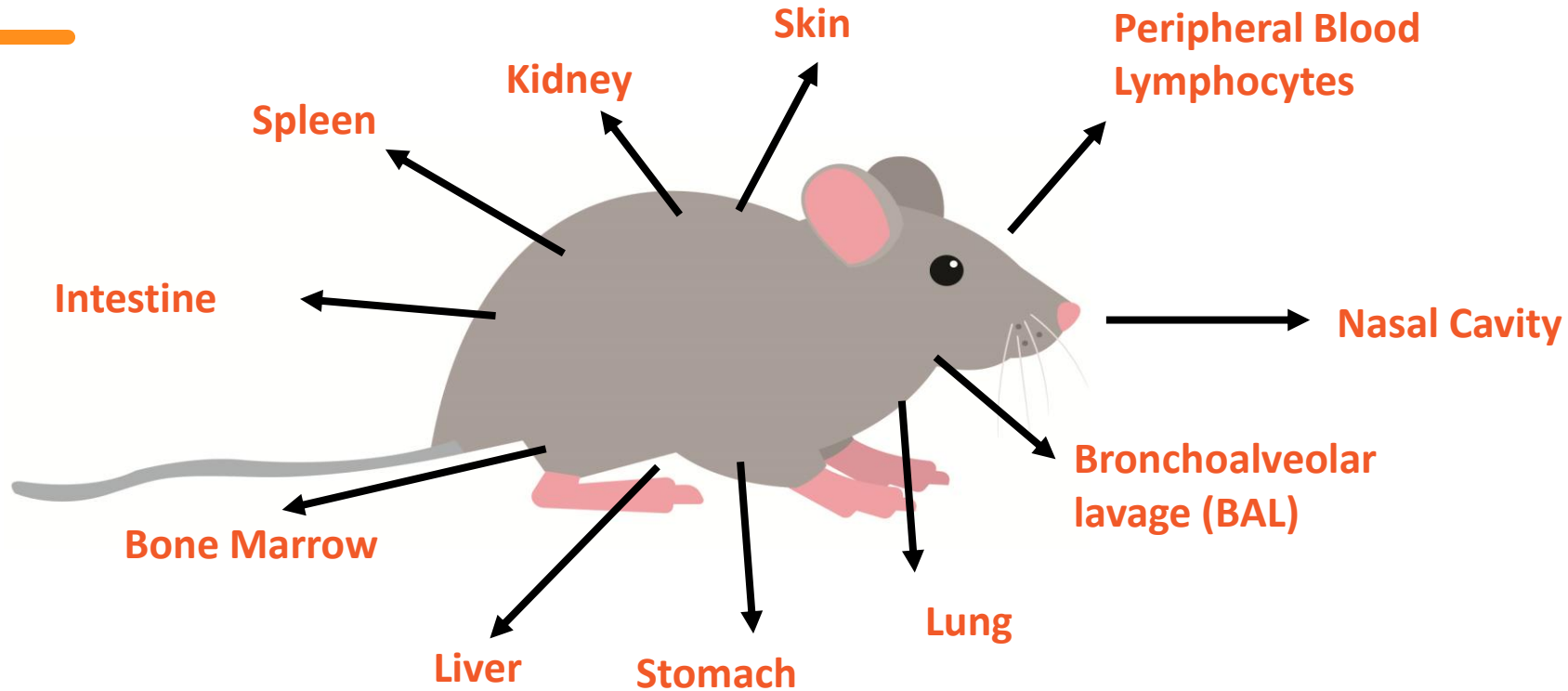
Tissue collection for possible histological assessment

Comet Assay Design



At necropsy, collect tissue(s) for Comet assay and histopathology assessment (if needed)

Comet Assay Tissues



Rat: Liver, Kidney, Stomach, Duodenum, Jejunum, Colon, Peripheral Blood, Uterus, Skin, Nasal Cavity, Bone Marrow, Spleen, Lung, Thymus, and Bronchoalveolar lavage (BAL) cells

Mouse: Liver, Kidney, Stomach, Duodenum, Colon, Peripheral Blood, Bone Marrow, Spleen, and Testes

Comet Slide Preparation Techniques

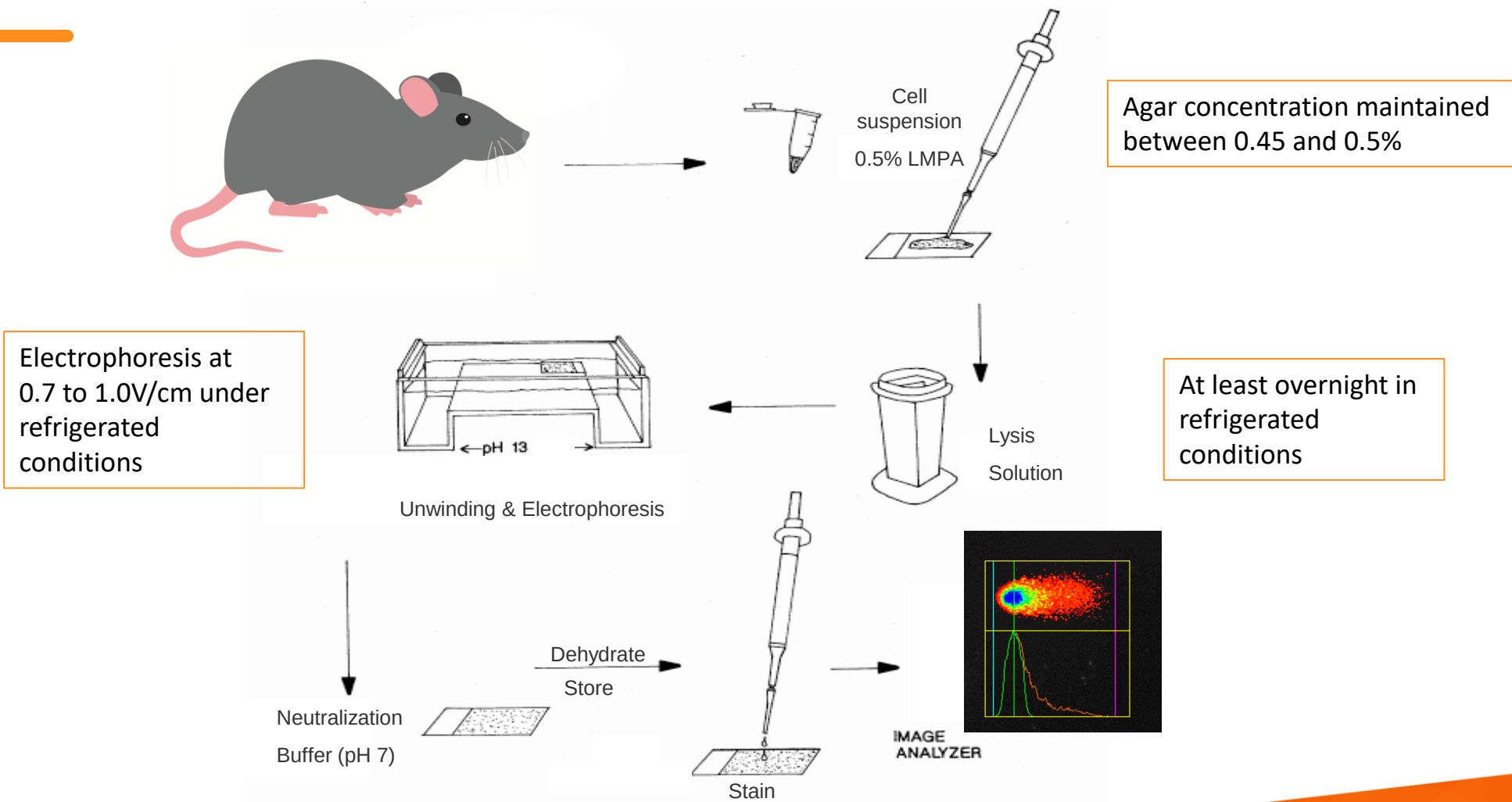
Fresh Tissue Method (RKV)

- Single cell suspension for each tissue is prepared on day of necropsy.
- Slides are prepared for each tissue on day of necropsy.
- Requires electrophoresis to be performed within 4 days of slide preparation.

Frozen Tissue Method (RTP)

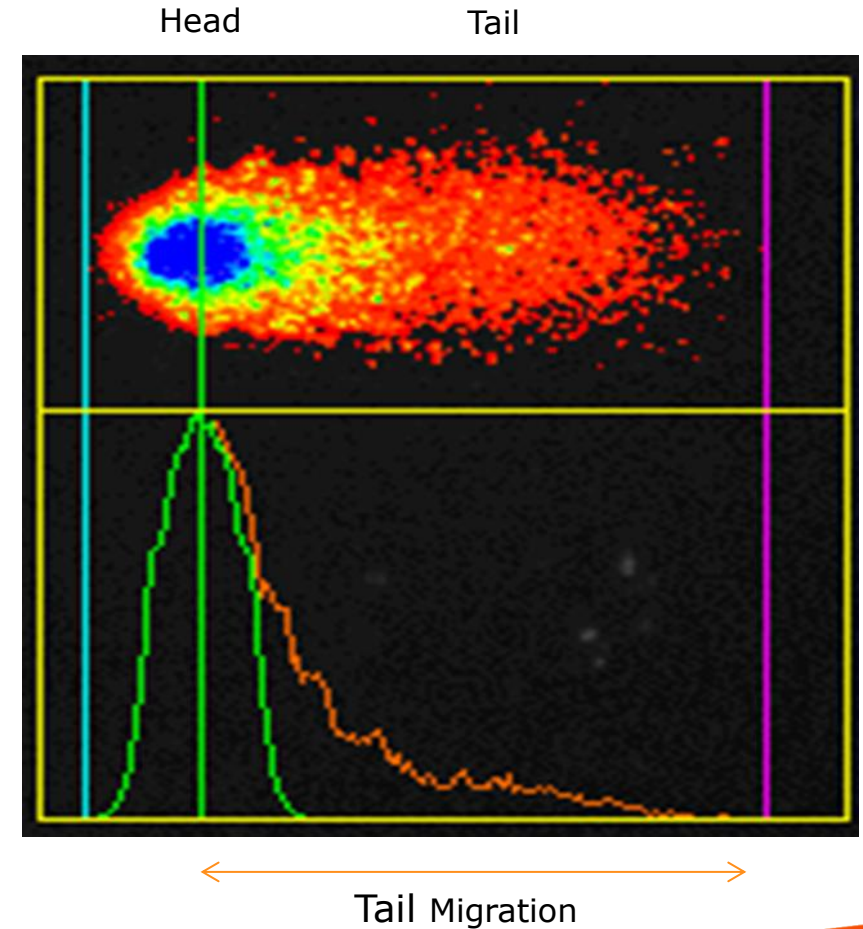
- Single cell suspension or tissue pieces flash frozen on day of necropsy.
- Slides prepared from frozen cell suspension or tissue pieces at a later date.
- Electrophoresis performed after overnight lysis.

Comet Assay Workflow

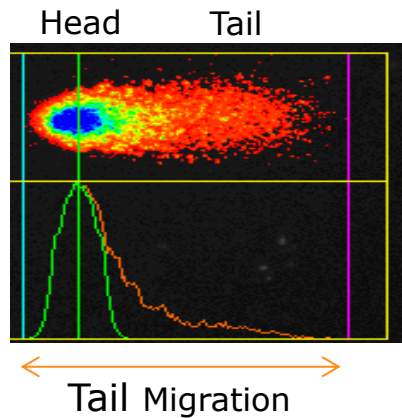


Comet Assay Scoring

- Slides are stained with SYBR™ Gold and assessed using a fluorescence microscope connected to an image analysis system
- Up to 150 cells are evaluated for:
 - > % tail intensity (also called % tail DNA)
 - > tail moment (also called Olive tail moment)
 - > tail migration
 - > Number of clouds (severely damaged cells often associated with cytotoxicity)



Comet Assay Scoring



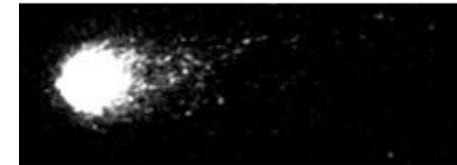
Level of DNA damage is correlated to the length and amount of fragmented DNA that migrates outside the cell nucleus (comet tail)

- %tail DNA (intensity)
 - > Amount of DNA in the tail
- Tail Moment
 - > Product of the distance between the center of the head mass, the center of the tail mass (tail length) and the amount of DNA in the tail
 - > Also known as Olive Tail Moment
- Tail Migration
 - > DNA migration length from the edge of the head to the smallest detectable fragment of the tail.

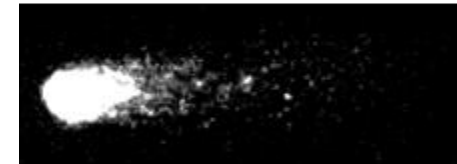
No damage



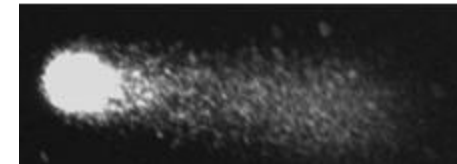
Low damage



Medium damage



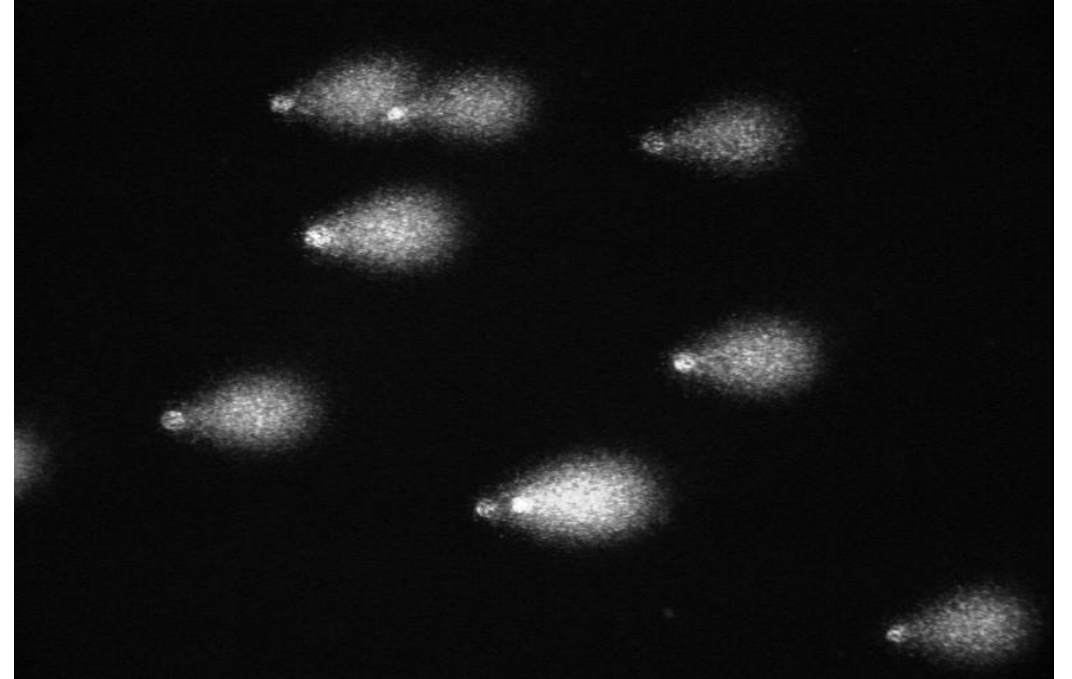
High damage



Comet Assay Scoring—Clouds

“Clouds” are

- A morphological indication of highly damaged cells
- Almost the entire cell's DNA is contained in the tail of the comet
- The comet head is reduced in size, almost non-existent
- Often associated with severe genotoxicity or cytotoxicity (such as cell necrosis, apoptosis, or degeneration)



Limited data available so far indicates that cytotoxicity may not generate increases or decreases in DNA migration in the *in vivo* Comet assay, but more definitive correlation between cytotoxicity and DNA migration is under investigation.

Criteria for a Valid Test

- %tail DNA should ideally be within the 95% control limits of the distribution of the historical negative control database.
 - > If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).
- The positive control must induce a significant response ($p < 0.05$) in %tail DNA as compared to the vehicle control.
- At least five animals per group available for analysis for one timepoint.

Evaluation Criteria

- **Positive Response**

- > At least one test material dose exhibits a statistically significant increase in %tail DNA when compared to the concurrent vehicle control ($p \leq 0.05$), and
- > When multiple doses are examined at a particular sampling time, the increase is dose related ($p \leq 0.01$) when compared to the concurrent vehicle control, and
- > Results of the group mean of the individual animals in at least one group are outside the 95% control limit of the historical negative control data

- **Negative response**

- > No statistically significant increase in %tail DNA at any dose levels when compared to the concurrent vehicle control ($p > 0.05$)
- > No dose-dependent increase in %tail DNA as compared to the concurrent vehicle control ($p > 0.01$)

Liver Comet Assay – Sample Data

Test item	Dose (mg/kg/day)	No. animals	Total no. of cells	% Tail Intensity		Olive Tail Moment		% Hedgehogs	
				Mean	SD	Mean	SD	Mean	SD
Vehicle	0	6	900	0.07	0.03	0.01	0.00	1.32	0.98
Test Material B	500	6	900	0.10	0.06	0.01	0.01	1.10	0.76
Test Material B	1000	6	900	0.12	0.06	0.01	0.01	0.62	0.17
Test Material B	2000	6	900	0.15	0.05	0.01	0.01	1.33	0.94
Ethyl methanesulfo nate	150	6	900	10.47**	2.92	1.82	0.62	3.03	1.28

**Statistically significant increase compared to vehicle control (t-test, $p < 0.05$)

'Mobile' Comet

- Personnel and equipment travel to another location to perform comet assay on fresh tissues.
 - > Tissue(s) processed from animal through electrophoresis
 - > Dehydrated slides are shipped to Inotiv for scoring and evaluation
- Used to add a genotoxicity endpoint onto a study currently in progress (e.g., 29-day repeat dose study or 90-day toxicity assay)
- Also used for uncommon routes of administration (e.g., nose-only or whole-body inhalation)



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

Combined *In Vivo* Micronucleus/Comet Assay

Integrated Micronucleus/Comet Assay

Three/four/fifteen/twenty-nine dose administrations, one collection

Group Number	Treatment	Number of Animals Dosed*
		2 to 6 hours – Comet Maximum 24 hours - Micronucleus
1	Vehicle Control	6
2	Test Article: Low Dose	6
3	Test Article: Mid Dose	6
4	Test Article: High Dose**	6
5	Positive Control	6
Total Animals		30

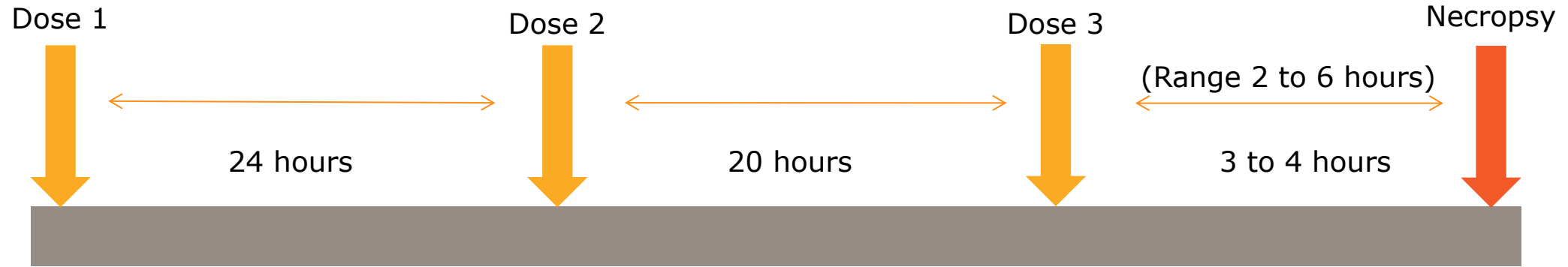
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** High dose should be limit dose (2000 mg/kg), defined maximum tolerated dose (MTD) or maximum feasible dose (MFD)

Systemic exposure should be confirmed – plasma collection and analysis

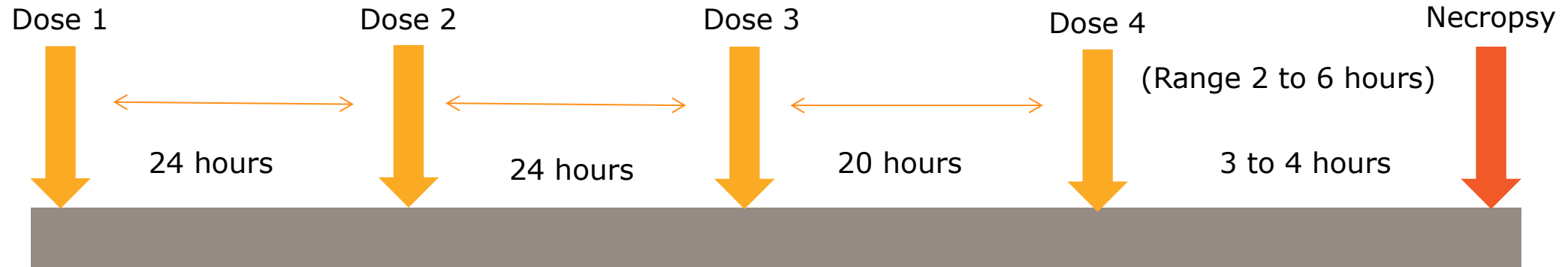
Tissue collection for possible histological assessment

Bone Marrow Micronucleus/Comet Assay



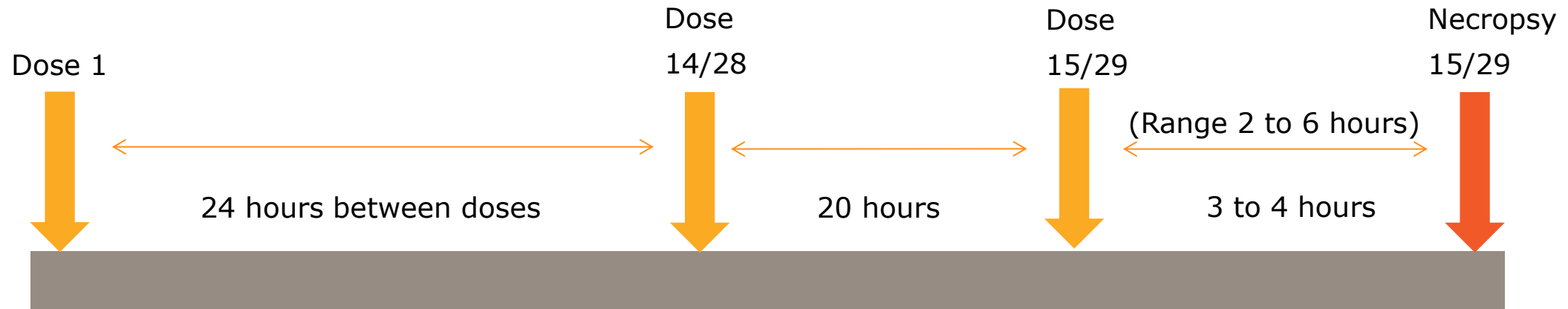
At necropsy, collect tissue(s) for Comet assay, **bone marrow for microscopic** micronucleus assay (maximum collection time is 24 hours post second dose), and tissue for histopathology assessment (if needed)

Peripheral Blood Micronucleus/Comet Assay



At necropsy, collect tissue(s) for Comet assay, **peripheral blood for flow cytometric** micronucleus assay (maximum collection time is 48 hours post second dose), and tissue for histopathology assessment (if needed)

Integrated Micronucleus/Comet Assay



At necropsy, collect tissue(s) for Comet assay, **peripheral blood for flow cytometric** micronucleus assay or **bone marrow for microscopic** Micronucleus assay, and tissue for histopathology assessment (if needed)

Study Timeline

- For GLP compliant studies
 - > Timing provided is for the endpoint only. Main study report will have longer timelines to allow incorporation of the endpoint reports into package.
 - > Verbal results in 6 to 8 weeks from necropsy
 - > Audited draft report 2 weeks from verbal results
- Results timing may change based on number of animals, dose levels, or organs.

Explore our Genetic Toxicology Services

