



WHERE
insights LEAD TO
answers



In Vitro Cytogenetic Assays

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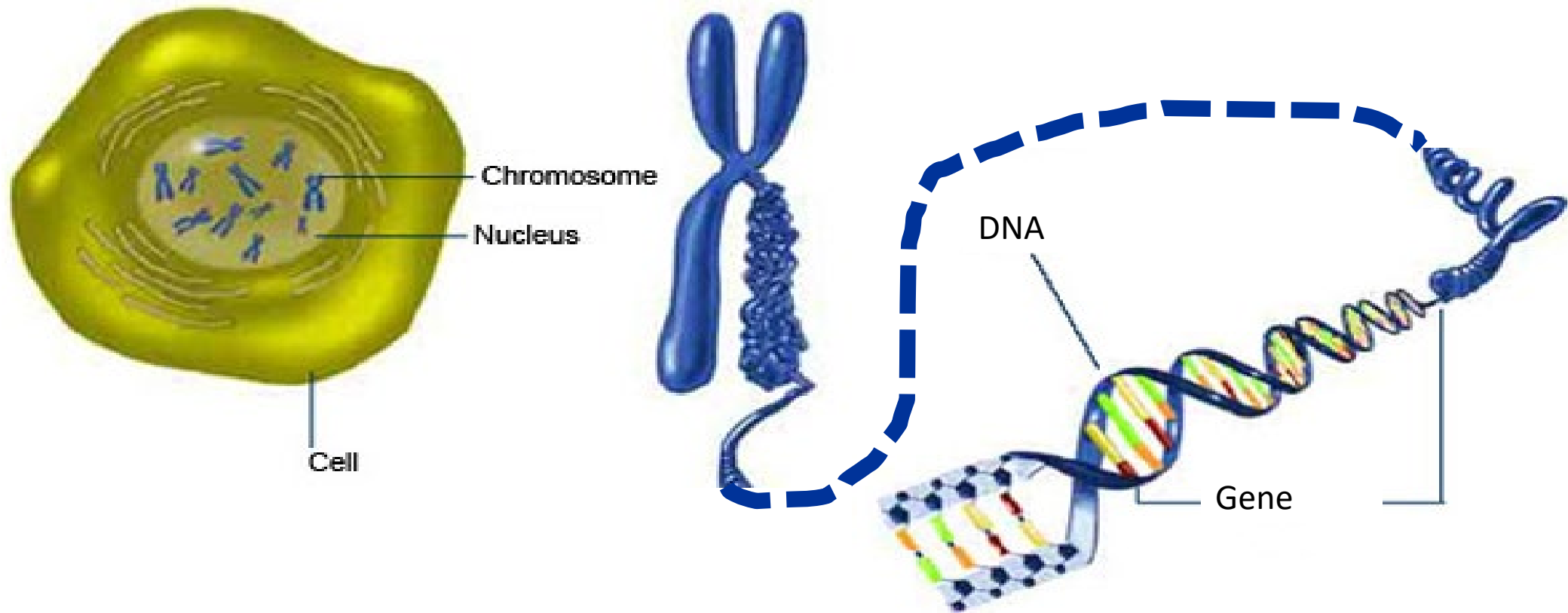
Principal Scientist, Genetic Toxicology

Topics for Discussion

- What is Cytogenetics assay
- Types of Cytogenetics assays
- Testing Guidelines
- Test System Theory
- Study Design
- Criteria for a Valid Test
- Evaluation Criteria
- Sample Data
- Clarifying Equivocal Responses
- You Make the Call
- Summary

Cytogenetic Assay

**Study of genetic damage
at chromosomal level**



Types of Cytogenetics Assay

- Chromosomal Aberration (CA) Assay
- Micronucleus (MN) Assay

Purpose of In Vitro Cytogenetic Test

In vitro Chromosome aberration test

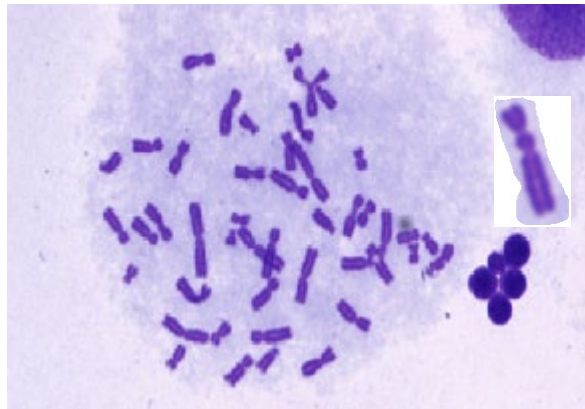
- The *in vitro* chromosomal aberration test is genotoxicity test detection of structural chromosomal aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid. Numerical aberration (Polyploidy and endoreduplication) could be detected this assay; however, this test is not designed to measure aneuploidy
- Worldwide regulatory acceptance

In vitro micronucleus test

- The *in vitro* micronucleus (IVMN) test is genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells. Micronuclei 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 IVMN test is an *in vitro* method that provides a comprehensive basis for investigating chromosome damaging effect induced by both Aneugens and Clastogens
- Worldwide regulatory acceptance

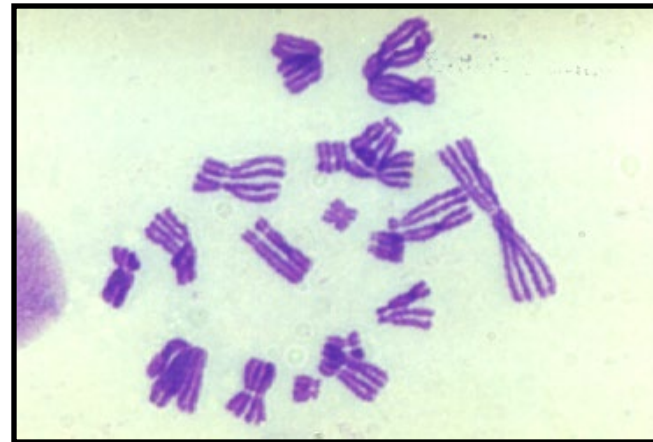
Chromosomal Aberration (CA) Assay (OECD 473 (Updated 2016))

Structural Aberrations

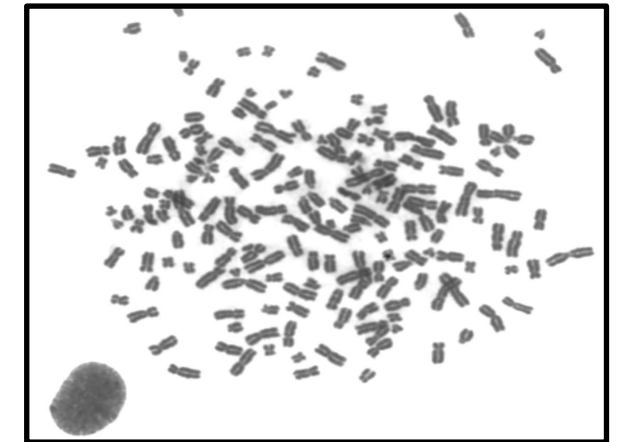


Dicentric
chromosome

Numerical Aberrations



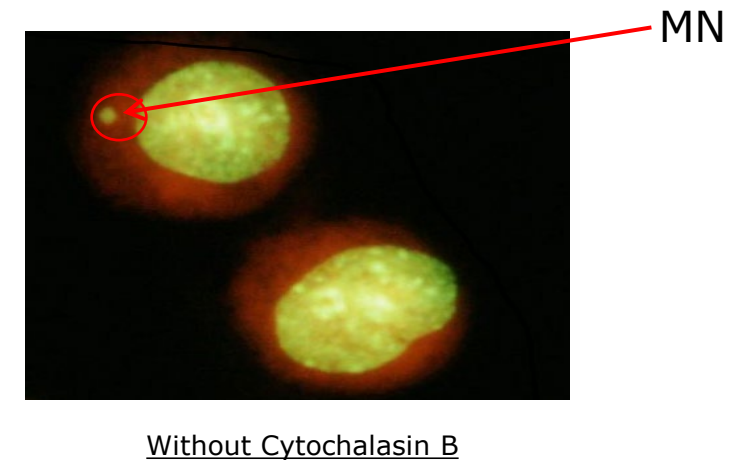
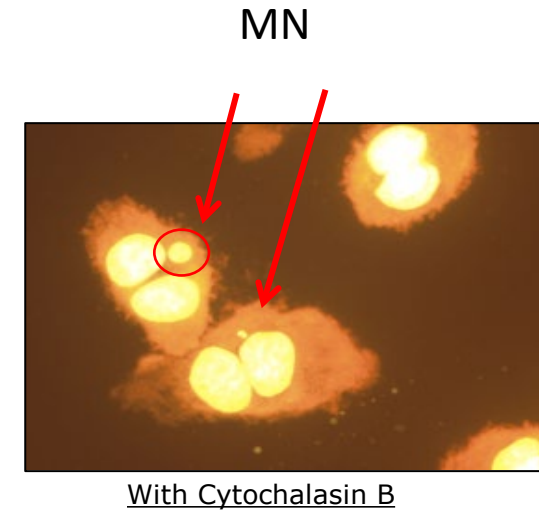
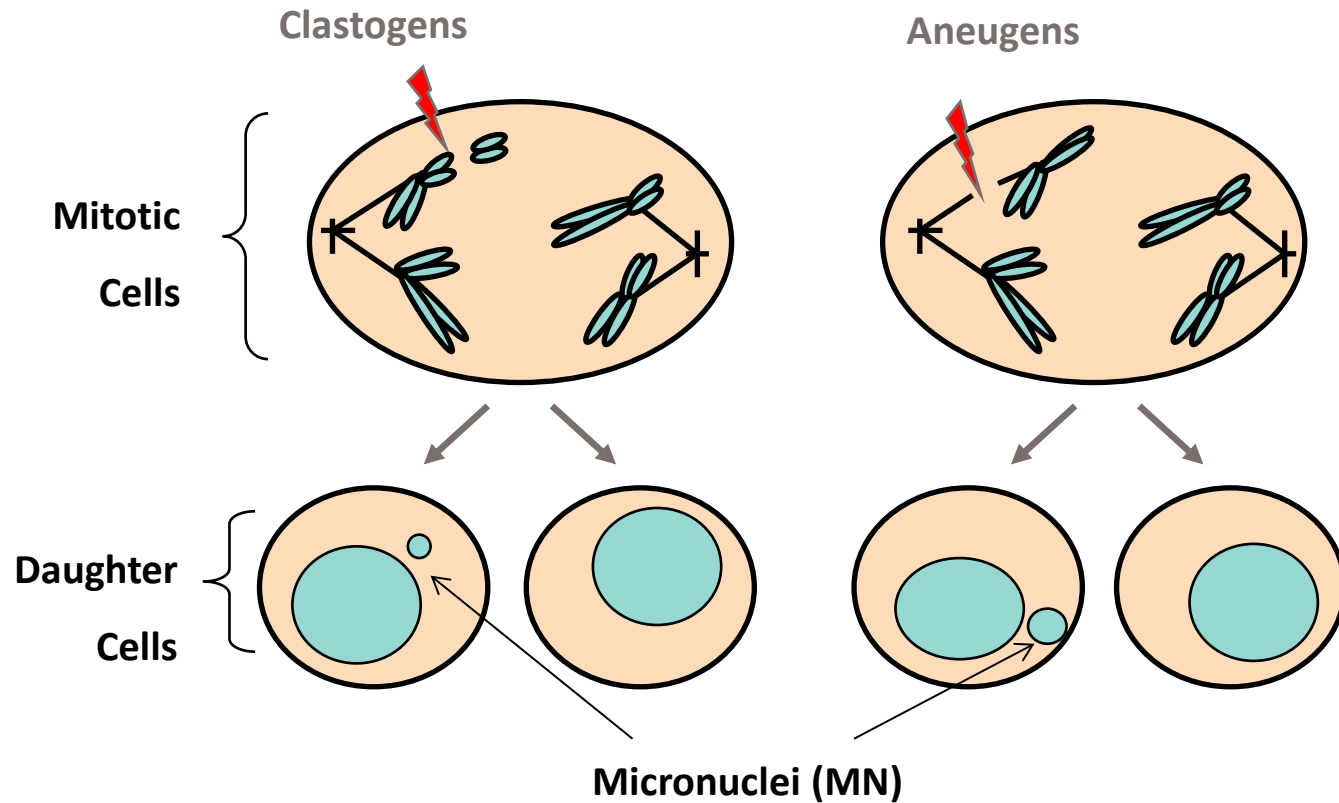
Endoreduplication



Polyploidy

Micronucleus (MN) Assay

(OECD 487, updated 2016, 2023)



Testing Guidelines

Chromosome Aberrations Assay (Guideline was first developed in 1983 and has been revised on several occasions since then)

OECD – 473 (Last Revision, 29 July 2016)

FDA: Redbook 2000: IV.C.1.b

EPA: OPPTS 870.5375

EU: B10

Micronucleus Assay (Guideline was first developed in 2010 and has been revised twice)

OECD – 487 (First revision- 29 July 2016, Last Revision – 4 July 2023)

FDA and EPA: No update but accept the OECD recommendations

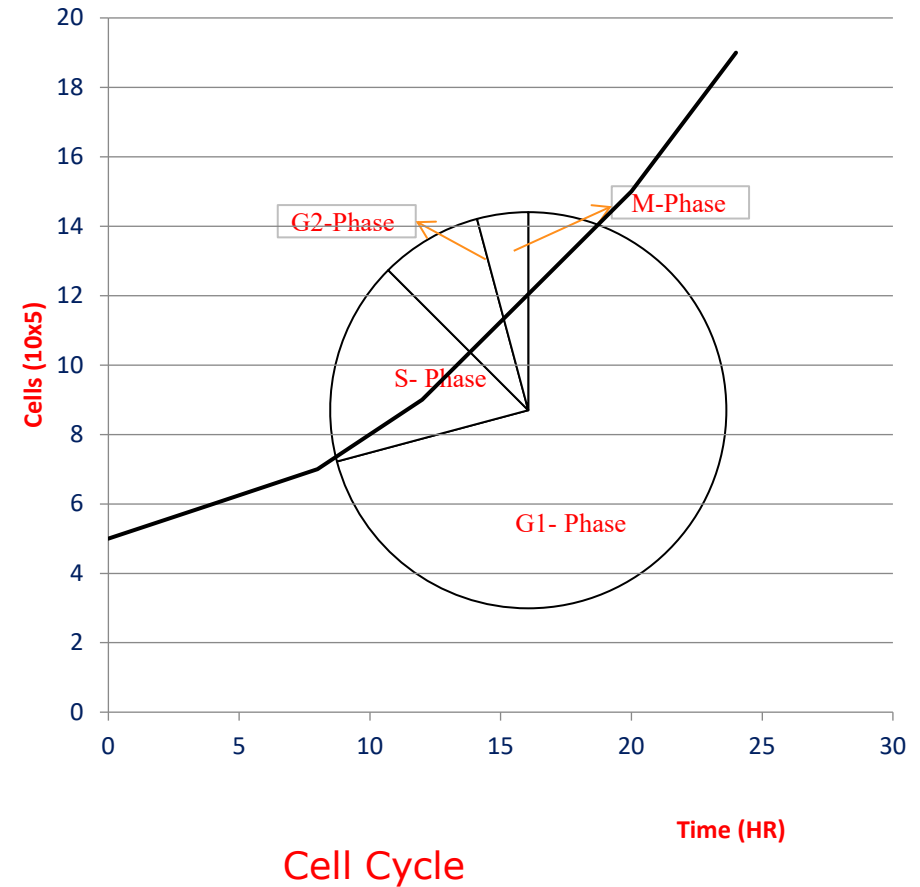
Test System

Primary Cells: Peripheral Blood Lymphocytes from Human or rodents (Rat/Mouse)

Cell Lines: Chinese Hamster Ovary (CHO), TK6 (human lymphoid cells), V79 (Hamster Lung cells)

Unsynchronized Cells preferred

Active Cell Division is crucial in this assay



Test System and Culture Method

Suspension cells



Primary cells: Human
Peripheral Blood cells

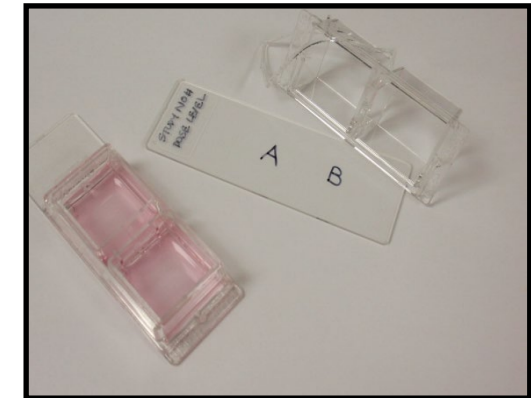
Cell lines: TK6

Adherent cells



Flask Culture

Cell lines: Chinese hamster Ovary (CHO), Chinese hamster
Lung (CHL or V79), A375 Cells, A549 Cells



In situ Culture

Solvent/Vehicle Determination

- A solubility test is performed to determine suitable solvent.
- Aqueous solvents (culture media, water, PBS, saline etc.) is preferred, used up to 10% v/v in culture.
- Organic solvents (DMSO, Ethanol, Acetone), used up to 1% v/v in culture.
- Exotic solvents (DMF, THF, Acetonitrile), untreated control should be used.
- Test article solution is preferred but workable suspension could be used. Workable suspension is defined as free-flowing formulation without adhering to the wall of container.

Study Design

Preliminary Cytotoxicity or Dose Range Finder Trial

- Single culture per dose level
- High Dose: Limit Dose
- 10 to 15 concentrations at half-log dosing interval
- 3 Treatment Conditions:
 - 3/4 hr $\pm$ S9 (short treatment), and
 - 20/24/27 hr without S9 (Continuous treatment)
- pH : Physiological range 7.0 ± 0.5
- Osmolality: <500 mOsmol
- Precipitate observation at the beginning and at the end of treatment (mostly unaided eye)

Basis for doses selection for definitive portion of assay

Definitive Trial

5-10 concentrations
Duplicate cultures/dose level

Positive Controls

(Chromosome aberration Test)

- Mitomycin C or MMC (without activation)
- Cyclophosphamide or CP (With S9 activation)

(Micronucleus Test)*

- Mitomycin C or MMC (without activation)
- Cyclophosphamide or CP (With S9 activation)
- Vinblastine or VB (without activation)

**Concurrent aneugenic and clastogenic positive control*

Experimental Design (Chromosome aberrations Test)

Peripheral blood lymphocytes = 0.6 mL

Complete culture medium (RPMI-1640 containing 15% heat inactivated fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg/mL streptomycin) and 1% phytohemagglutinin (PHA).



Cells are incubated under standard conditions ($37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air) for 44-48 hours.



±S9 4hr treatment followed by recovery hour 16 hrs).
-S9 continuous for 1.5 to 2 x of normal cell cycle length.
Appropriate positive controls (direct and indirect clastogenic), solvent control, untreated control etc.,



- 2 hr prior to harvest, colcemid
- Harvesting of cultures
- Slide Preparation
- Staining with Giemsa stain
- Evaluation of cytotoxicity (Mitotic Index) followed by Evaluation of Chromosome Aberrations

Verbal Results



Experimental Design (Micronucleus Test)

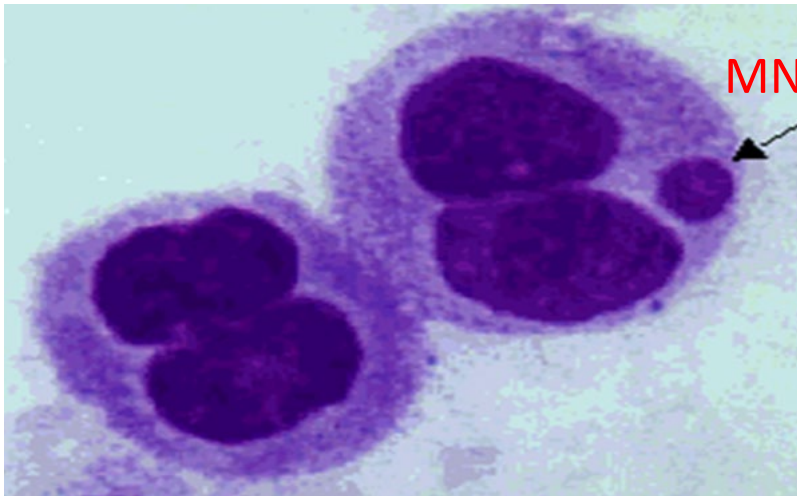
Culture initiation



±S9 3 to 6hr followed by recovery hour covering 1.5 to 2x of normal cell cycle length (24 to 27 hrs).
-S9 continuous for 1.5 to 2x of normal cell cycle length.
Appropriate positive controls (direct and indirect clastogenic, aneugenic), solvent control, untreated control etc.,



Harvesting of cultures
Slide Preparation
Staining and Evaluation of
Cytotoxicity



Giemsa Stain



Acridine Orange Stain



Evaluation of MN induction

Exogenous Metabolic Activation System

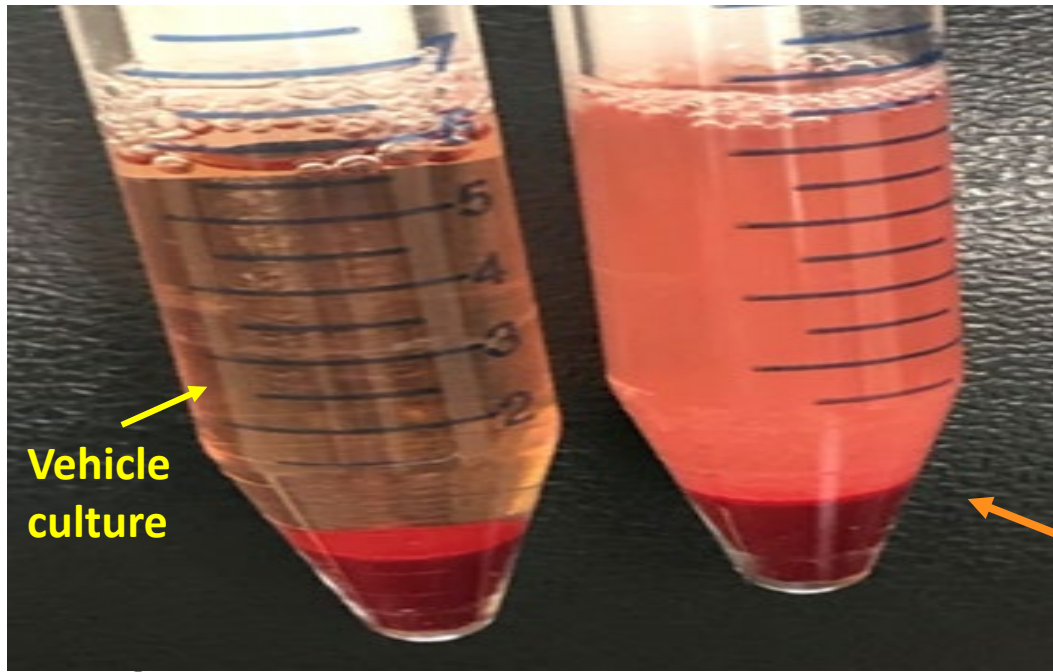
- Phenobarbital/beta-naphthoflavone-induced rat liver S9 homogenate
- Human liver pooled S9 homogenate; Syrian Hamster S9 Homogenates
- S9 fraction is supplemented with various combination of cofactor mix
- Primary Hepatocytes

Component	Final Concentration in Cultures*
NADP (sodium salt)	1 mM
Glucose-6-phosphate	1.25 mM
Potassium chloride	10.3 mM
Magnesium chloride	2.5 mM
Sodium phosphate (dibasic)	12.8 mM
S9 homogenate (PB/BNF)	15 µL/mL (1.5% v/v)

*S9 fraction typically ranges from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. Use of Aroclor 1254 induced S9 is gradually phasing out.

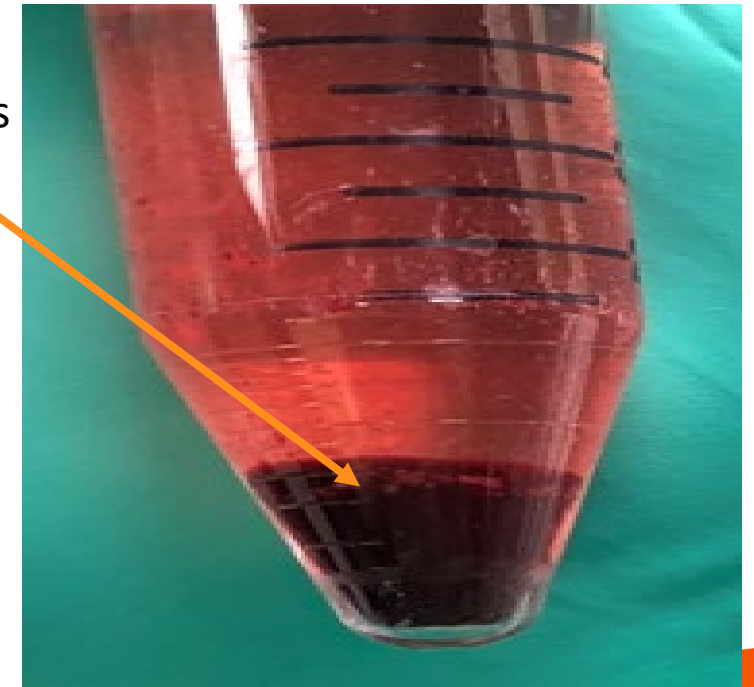
Factors to Consider *In Vitro* Genotoxicity Testing

- Osmolality: Blood - ~300 mOsmol /kg. Tissue Culture media - 280 to 300 mOsmol, up to 500 is safe in the *in vitro* genotoxicity assay.
- pH: Physiological pH range 7.0 ± 0.5 .
- Precipitation: Lowest precipitating concentration at the end of treatment.

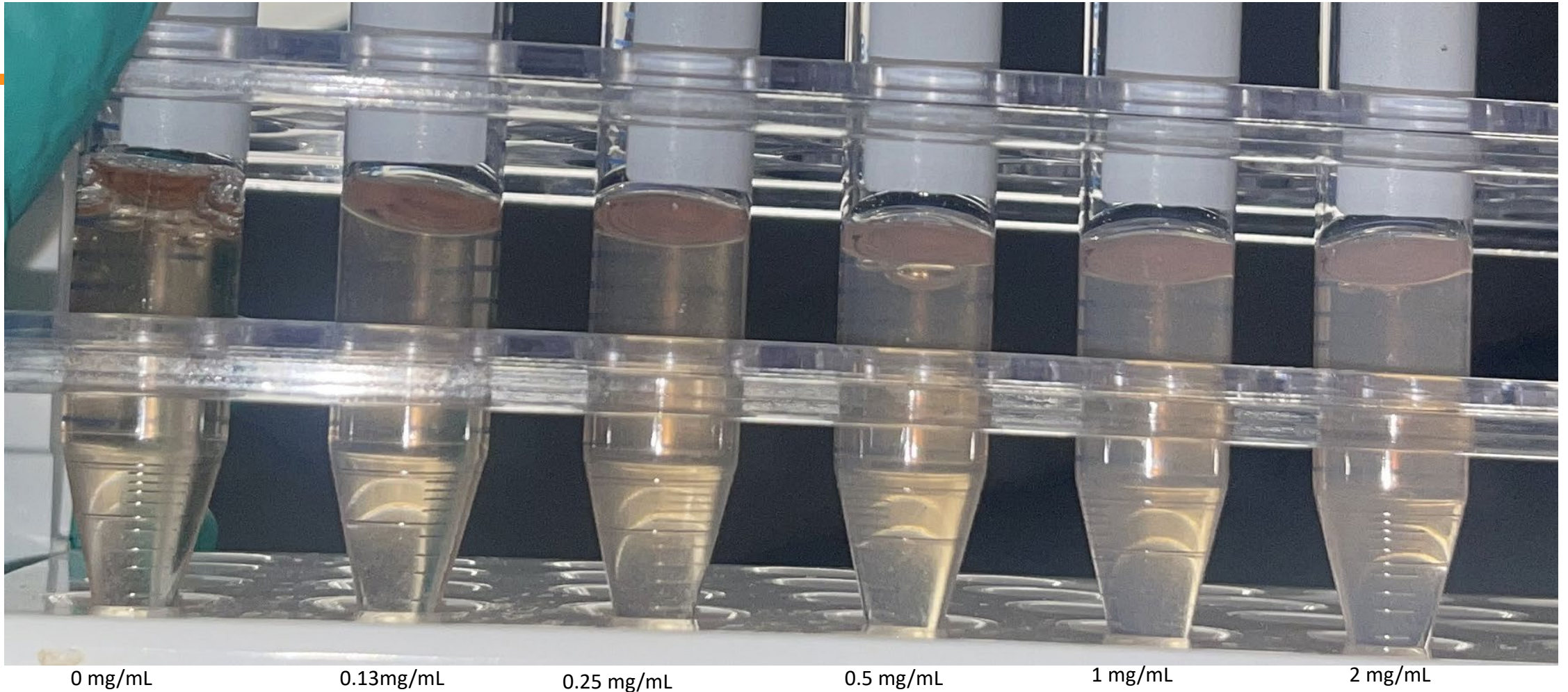


White TA particles trapped in pellet

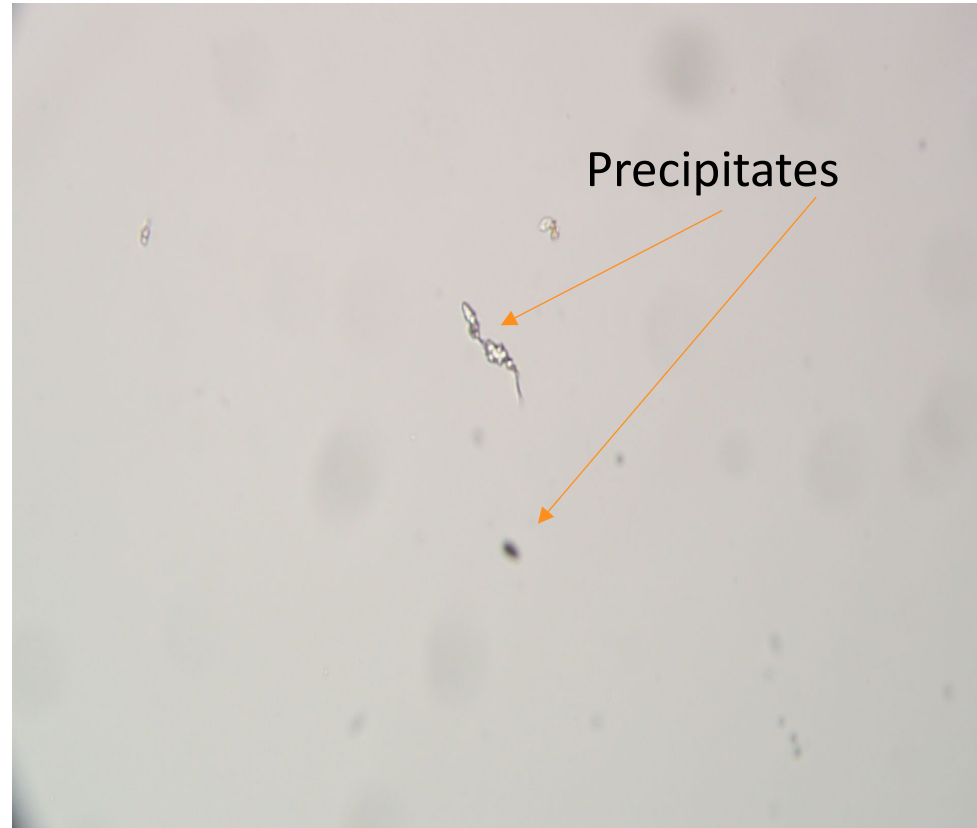
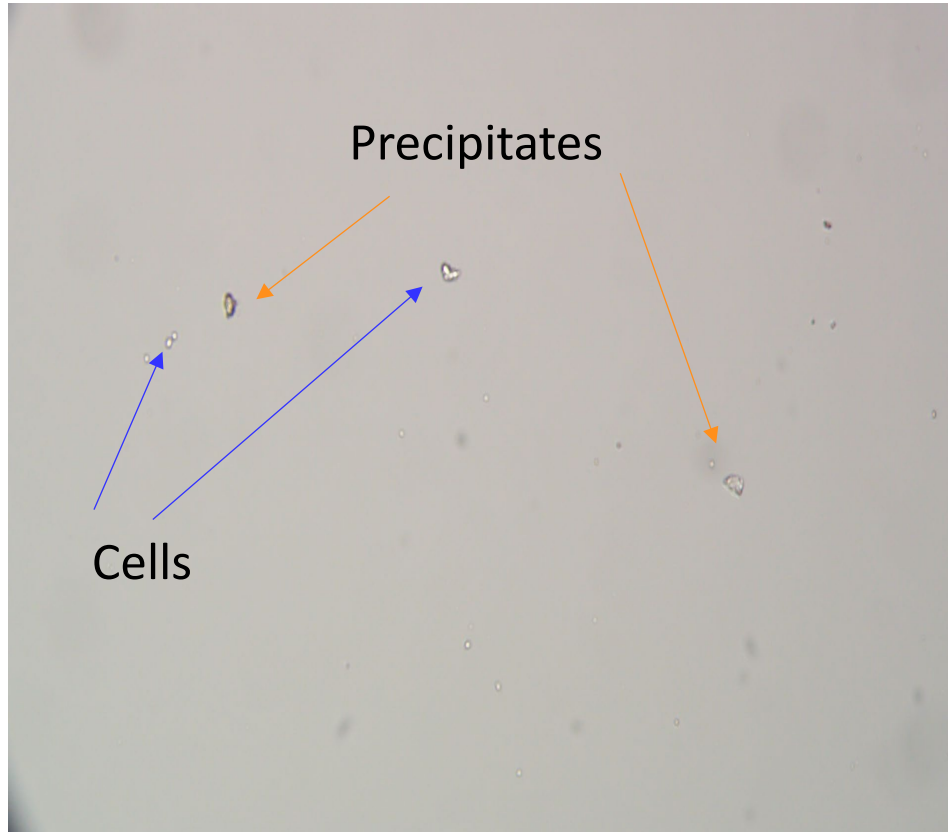
Extra layer of test article over pellet. Cloudiness is mix of partial RBC lysis and test article



Precipitation test in cell free medium



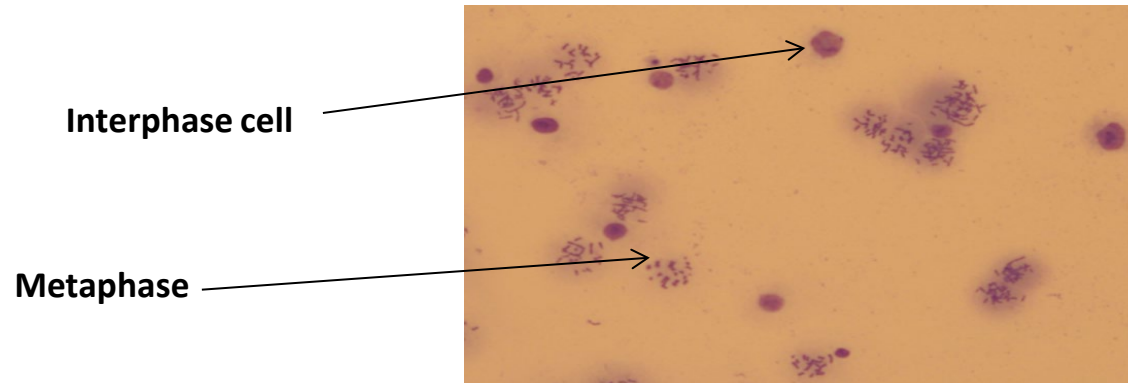
Precipitate Under Phase Contrast Microscope



Magnification =400x

Cytotoxicity Evaluation (Chromosome aberration test)

500 Cells = Metaphases + interphase nuclei



$$\text{Mitotic index (\%)} = \frac{\text{Total No. of metaphases}}{\text{Total cells scored (metaphase + interphase)}} \times 100$$

$$\text{Cytotoxicity} = \% \text{ Mitotic Index of Test Article} - \% \text{ Mitotic Index of Solvent}$$

Cytotoxicity Evaluation (Micronucleus test)

Micronucleus assay could be conducted with and without cytochalasin B (Cyto B).

When Cytochalasin B is not used

- Relative Increase in Cell Counts (RICC)
- Relative Population Doubling (RPD)

$$PD = \log\left(\frac{\text{Final cell number}}{\text{Initial cell number}}\right) \div \log 2$$

$$RPD = \frac{\text{No. of PDs in treated culture}}{\text{No. of PDs in control culture}} \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - RPD$$

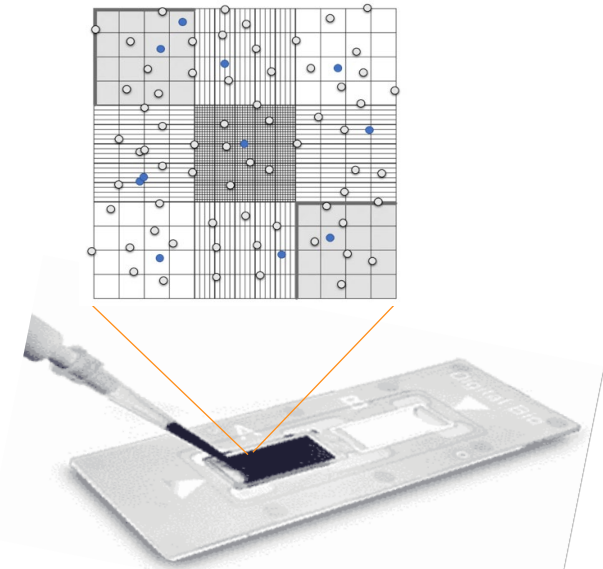
$$RICC = \frac{(\text{Final cell count})_T - (\text{Initial cell count})_T}{(\text{Final cell count})_C - (\text{Initial cell count})_C} \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - RICC$$

PD = Population doubling
RPD = Relative Population Doubling

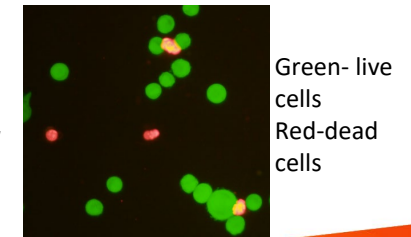


Beckman Coulter's counter



Hemocytometer

- *Ideal for cell lines*
- *Viable cell counts is recommended*



RICC = Relative increase in cell count

Cytotoxicity Evaluation

When cytochalasin B is used

- Cytokinesis-Block Proliferation Index (CBPI)
- Replication Index (RI)
- Viable cell counts not required

$$\text{CBPI} = \frac{((1 \times 1\text{N cells}) + (2 \times 2\text{N cells}) + (3 \times 3\text{N}/4\text{N}))}{\text{Total number of cells scored}}$$

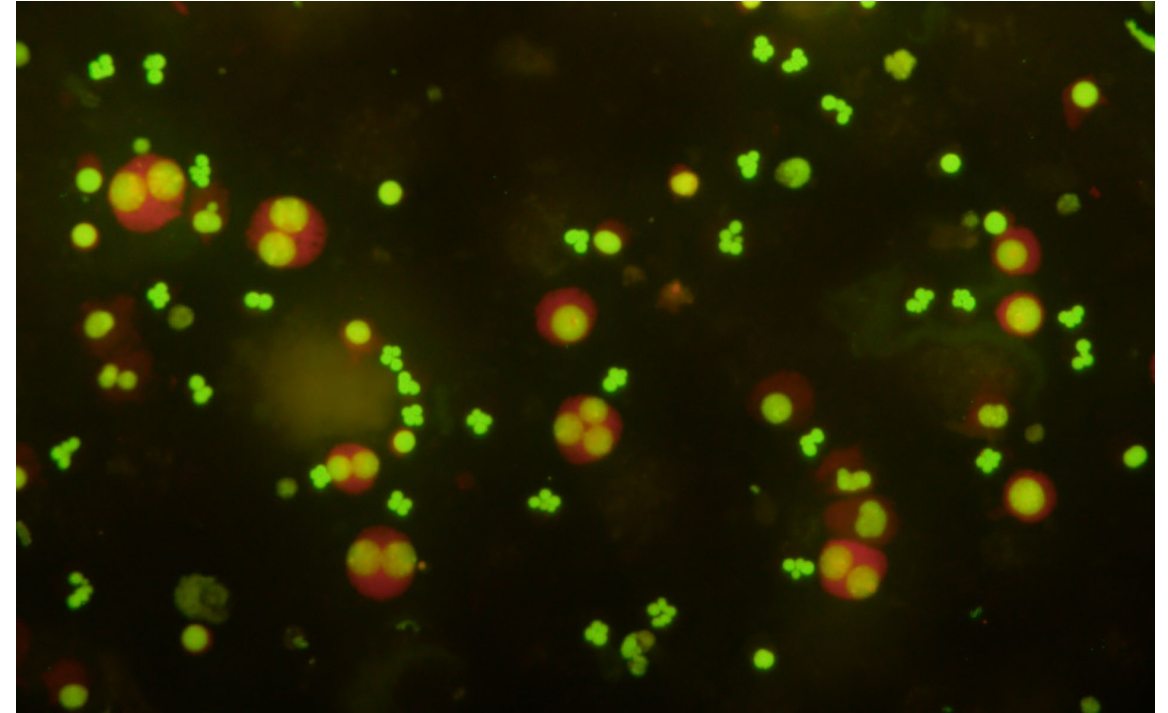
$$\% \text{ Cytotoxicity} = 100 - 100 \{(\text{CBPI}_T - 1) \div (\text{CBPI}_C - 1)\}$$

$$\text{RI} = \frac{(\text{No. of } 2\text{N cells} + 2(\text{No. of } 3\text{N}/4\text{N})_T) / (\text{Total cells scored})_T}{(\text{No. of } 2\text{N cells} + 2(\text{No. of } 3\text{N}/4\text{N})_C) / (\text{Total cells scored})_C}$$

T= Test Culture

C = Control Culture

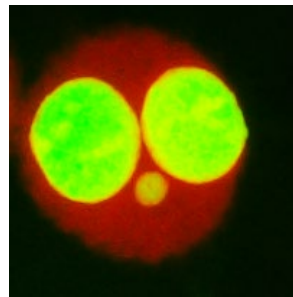
$$\% \text{ Cytotoxicity} = 100 - \text{RI}$$



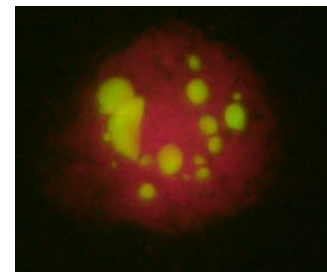
- A CBPI or RI ≥ 1.4 of the vehicle control at harvest
- Ideal for Primary cells, e.g., HPBLs

Slide Scoring for Micronucleus Evaluation

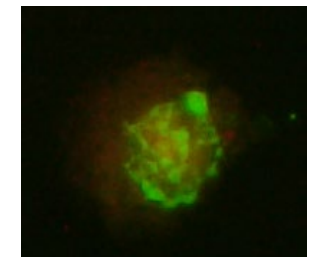
- In order to avoid scorers bias, all slides should be coded by person not involved in scoring.
- Without cytochalasin B experiment, 2000 mononucleated cells, 1000 cell per culture if replicates.
- When cytochalasin B is used, only binucleated cells with one or more micronuclei should be evaluated.
The binucleated cells must meet the following criteria:
 - the micronucleus should have the same staining characteristics as the main nucleus.
 - the micronuclei should be separate from the main nuclei or just touching (no bridges).
 - the micronuclei should be of regular shape and approximately 1/3 or less than the diameter of the main nucleus.
- Apoptotic and necrotic cells may be scored separately



MN-BN cells



Apoptotic Cell



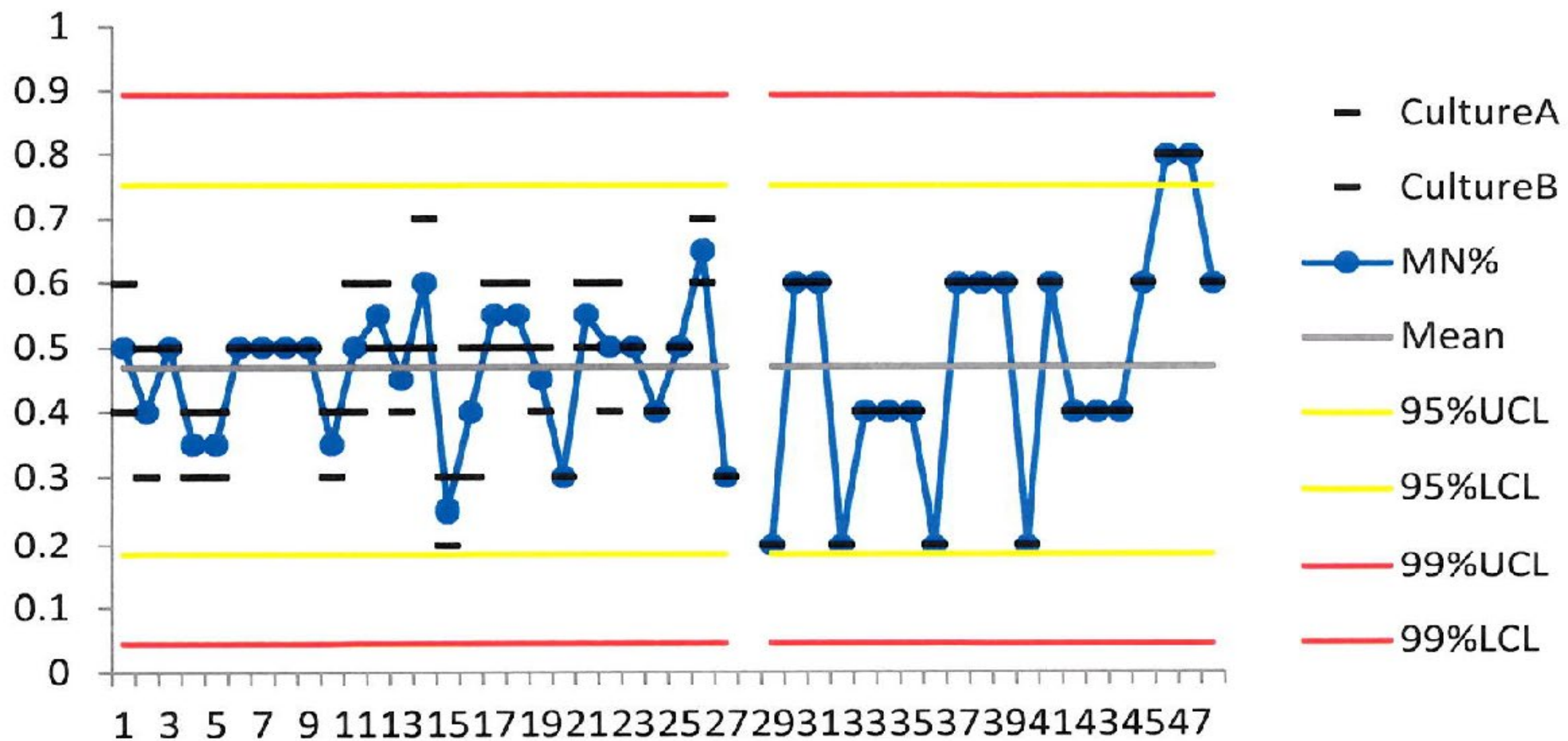
Necrotic Cell

Laboratory Proficiency & Historical Control Data

- A performing lab should conduct series of experiments with and without metabolic activation using reference test compounds with different mode of action.
- A range of concentrations of each reference compound should be evaluated to demonstrate sensitivity, reproducibility and concentration responsiveness of the test system.
- Evaluation Methods: Microscopy or Flow Cytometer or Image Analyzer.
- Laboratory should establish their own Historical control data using at least 10 valid independent assays. Historical control data should be periodically updated to reflect the current laboratory practice.
- Laboratory should also establish range and distribution of positive controls and solvent controls and untreated controls.
- Laboratories should use quality control methods, such as, control charts (e.g. C-charts or X-bar charts), to identify variability in positive and negative control data to show that the methodology is 'under control' in their laboratory.

Example of C-Chart

Vehicle MN% -S9 Short Treatment



Assay Acceptability Criteria or Valid Test

- The concurrent negative control should be in the line of historical negative control data expected to fall within 95% control limit of historical control range. Slightly out of range data is accepted as long as there was no experimental error.
- Concurrent positive control should induce a statistically significant increase in MN frequencies compared to the concurrent negative control and response should be in the line of laboratory's historical positive control data.
- Cell proliferation index of the solvent control must be ≥ 1.4
- All experimental conditions ($\pm$ S9 short term and $-$ S9 continuous) were tested unless one resulted in positive for genotoxicity
- Adequate number of cells (e.g., 2000/dose levels with microscopy; and concentrations are analyzed, at least 3 concentrations.
- The criteria for the selection of top concentration are consistent with OECD testing guideline ($55 \pm 5\%$) or Lowest precipitating concentration or Limit dose level.
 - Chemicals: 2000 $\mu\text{g/mL}$ or 10 mM (whichever is lower)
 - Pharmaceuticals: 500 $\mu\text{g/mL}$ or 1 mM (whichever is lower)

Data Presentation and Statistical Test

- Irrespective of the number of damages, cell is considered as unit. (i.e., cell with 1 or 2 or 3 or 4 MN are considered one micronucleated cell). Similarly for the chromosome aberrations. Percent (%) aberrant cells at each dose level is determined.
- Each dose level is compared with solvent control using Fisher exact test is used for statistical significance ($p \leq 0.05$)
- Cochran-Armitage trend test for dose response ($p \leq 0.01$)

Evaluation and interpretation of results

- Once Criteria of Valid test are met, the test compound is considered positive if:
 - > at least one of the test concentrations exhibits a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and
 - > the increase is concentration-related ($p \leq 0.01$), and
 - > results are outside the 95% control limit of the historical negative control data.
- A test article will be considered to have induced a clear negative response if none of the criteria for a positive response were met.
- If the response is neither clearly positive nor clearly negative, in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations.
- In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal.

Dose Range Finder Trial (CA Test): Chemicals

TREATMENT ($\mu\text{g/mL}$)	MITOTIC INDEX	CYTOTOXICITY
Solvent	11.5	0
0.2	11	4
0.6	10.5	9
2	9.2	20
6	7.2	37
20	7.4	36
60	4.2	63
200	2.8	76
600	1.6	86
2000	0.6	95

Definitive Trial (CA Test): Chemicals

TREATMENT (µg/mL)	MITOTIC INDEX	CYTOTOXICITY
Solvent	14.2	0
1.0	13.8	3
2.5	12.4	2
5	10.2	10
➡ 10	12.2	9
➡ 20	9.8	25
➡ 40	6.8	52
60	5.8	59
80	4.8	66
100	3.2	77

Dose Range Finder Trial (CA test): Pharmaceutical

TREATMENT (µg/mL)		MITOTIC INDEX	CYTOTOXICITY
Solvent		11.5	0
0.05		10.2	11
0.15		11.5	0
0.5		9.8	15
1.5		10.4	10
5		10.2	11
15	P	9.8	15
50	P	8.8	23
150	P	8.2	29
500	P	9.2	20

P = Precipitate

Definitive Trial (CA assay): Pharmaceuticals

TREATMENT (µg/mL)		MITOTIC INDEX	CYTOTOXICITY
Solvent		15.2	0
1		14.8	3
2.5		12.8	16
5		13.8	19
10	P	11.8	22
15	P	12.6	17
20	P	11.2	26

P=Precipitate

Negative Result (CA assay): Data from 4-hour treatment

Treatment μg/mL	S9 Activation	Cytotoxicity	Treatment Time (hr)	Cells with Aberrations	
				Numerical (%)	Structural (%)
DMSO	-	0	4	0.5	0.5
50	-	11	4	0	0
100	-	23	4	0.5	0.5
300	-	52	4	0.0	1.0
MMC, 0.2	-	38	4	0	14.5**
DMSO	+	0	4	1.5	1.0
50	+	5	4	1.0	0.5
100	+	28	4	0	0.0
300	+	56	4	0	0.5
CP, (10)	+	42	4	1.5	12.5**

*p<0.05; **P<0.01; 95% Control Limit of Historical Solvent Control Range- (0 to 1.5%)

Positive Result (CA assay): Data from 4-hour treatment

Treatment µg/mL	S9 Activation	Cytotoxicity	Treatment Time (hr)	Cells with Aberrations	
				Numerical (%)	Structural (%)
DMSO	-	0	4	1.0	1
25	-	12	4	0.5	4.5*
50	-	34	4	1.5	11.5**
100	-	56	4	1.5	17.5**
MMC, 0.2	-	42	4	0.5	15.5**
DMSO	+	0	4	0.5	0.5
10	+	8	4	0	4.2*
25	+	24	4	1.5	11.5**
35	+	52	4	0	11.0**
CP (10)	+	44	4	0	37.0**

*p<0.05; **P<0.01; 95% Control Limit of Historical Solvent Control Range- (0 to 1.5%)

Equivocal Result (CA assay): Example 1

Treatment µg/mL	S9 Activation	Treatment Time	Cytotoxicity	Cells with Aberrations	
				Numerical	Structural
				(%)	(%)
DMSO	-	4	0	1.5	1
50	-	4	12	2.5	0.5
100	-	4	34	3.5	1.5
150	-	4	58	3	5.0**
MMC, 0.2	-	4	42	2	20**

**P<0.01; 95% Control Limit of Historical control range- (0 to 1.5%)

Equivocal response needs to be clarified with additional testing

Clarification of Equivocal Result (CA assay):

Repeat of Definitive Trial with closely spaced dose levels

Treatment µg/mL	S9 Activation	Treatment Time	Cytotoxicity	Cells with Aberrations	
				Numerical (%)	Structural (%)
DMSO	-	4	0		1
50	-	4	8		0.5
75	-	4	15		0
100	-	4	20		1
110	-	4	22		1.5
120	-	4	30		0
130	-	4	42		0
140	-	4	51		2
145	-	4	58		5**
150	-	4	65		7.5**
MMC, 0.2	-	4	42		20**

**P<0.01; 95% Control Limit of Historical control range- (0 to 1.5%)

You Make the Call

Data Set One

Treatment µg/mL	S9 Activation	Treatment Time	Cells with Aberrations	
			Numerical (%)	Structural (%)
Solvent (DMSO)	-	4	1.5	1.0
10	-	4	1.5	0.5
50	-	4	2.5	1.5
100	-	4	3.5	2.0
150	-	4	2.0	2.5
PC: MMC, 0.2	-	4	2.0	20**

**P<0.01; 95% Control Limit of Historical control range- (0 to 1.5%)

Solvent Control 95% Control Limit of Historical
data (0 -1.5%)

✓

Positive Control Significant than Solvent
control

✓

TA Concentrations 3 dose level analyzed
Not Significant,

✓

CALL – Negative

You Make the Call

Data Set Two

Treatment µg/mL	S9 Activation	Treatment Time	Cells with Aberrations	
			Numerical (%)	Structural (%)
Solvent (DMSO)	-	4	1.5	1.8
60	-	4	2.0	2.5
120	-	4	3.5	3.6
130	-	4	2.5	4.3
140	-	4	3.0	4.0
150	-	4	2.0	6**
PC: MMC, 0.2	-	4	1.0	18**

**P<0.01; 95% Control Limit of Historical control range- (0 to 1.5%)

Solvent Control 95% Control Limit of Historical data (0 -1.5%)

✓

CALL – Invalid Test

Positive Control Significant than Solvent control

✓

TA Concentrations 3 dose level analyzed
One concentration
Significant,

✓

Explore our Genetic Toxicology Services

