State of the Art High-throughput Approaches to Genotoxicity: Flow Micronucleus, Ames II, GreenScreen and Comet

June 28, 2012 EPA Computational Toxicology Communities of Practice

Dr. Marilyn J. Aardema Chief Scientific Advisor, Toxicology

Dr. Leon Stankowski Principal Scientist/Program Consultant

Ms. Kamala Pant Principal Scientist

Helping to bring your products from discovery to market

BioReliance

Agenda 11am-12 pm

1. Introduction
   Marilyn Aardema 5 min
2. In Vitro Flow Micronucleus Assay - 96 well
   Leon Stankowski, 10 min
3. Ames II Assay
   Kamala Pant 10 min
4. GreenScreen Assay
   Kamala Pant 10 min
5. In Vitro Comet Assay - 96 well TK6 assay
   Kamala Pant 10 min
6. Questions/Discussion
   15 min

BioReliance®

Genetic Toxicology Testing in Product Development

Discovery/Prioritization

↓

Lead Optimization

↓

Structure activity relationship analyses useful in very early lead identification
High throughput early screening assays

Screening versions of standard assays to predict results of GLP assays

GLP ↓ Gate

GLP Gene Tox Battery

↓

Perform assays for regulatory submission according to regulatory guidelines

Follow-up assays to solve problems

Additional supplemental tests to investigate mechanism and to help characterize human risk

BioReliance

High Throughput Genotoxicity Assays

- Faster
- Cheaper
- Uses Less Chemical/Drug
- Non-GLP
- Predictive of GLP assay/endpoint
- Mechanistic Studies (large number of conc./replicates)
- Automation

Example of Use of Genotoxicity Screening Assays: EPA ToxCast™

Problem: Tens of thousands of poorly characterized environmental chemicals

Solution: ToxCast™– US EPA program intended to use:

- High throughput screening
- Genomics
- Computational chemistry and computational toxicology

To permit:

– Prediction of potential human toxicity

– Prioritization of limited testing resources

www.epa.gov/ncct/toxcast

BioReliance EPA ToxCast Award July 15, 2011

- Assays
  - In vitro flow MN
  - In vitro Comet
  - Ames II
  - GreenScreen
- 25 chemicals of known genotoxicity to evaluate the process/assays (April-June 2012)
  - In vitro flow MN
  - In vitro Comet
  - Ames II

6

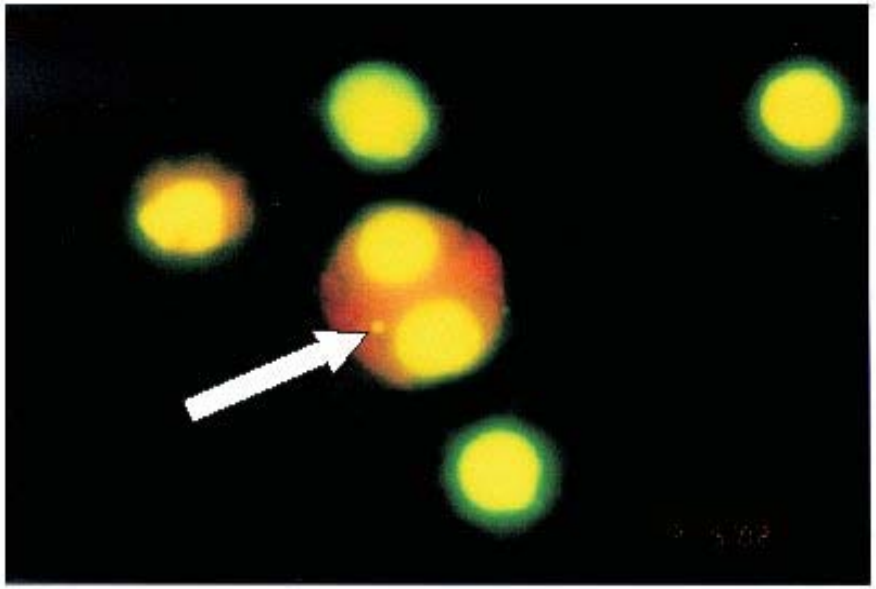
Agenda 11am-12 pm

1. Introduction
   Marilyn Aardema 5 min
2. In Vitro Flow Micronucleus Assay - 96 well
   Leon Stankowski, 10 min
3. Ames II Assay
   Kamala Pant 10 min
4. GreenScreen Assay
   Kamala Pant 10 min
5. In Vitro Comet Assay - 96 well TK6 assay
   Kamala Pant 10 min
6. Questions/Discussion
   15 min

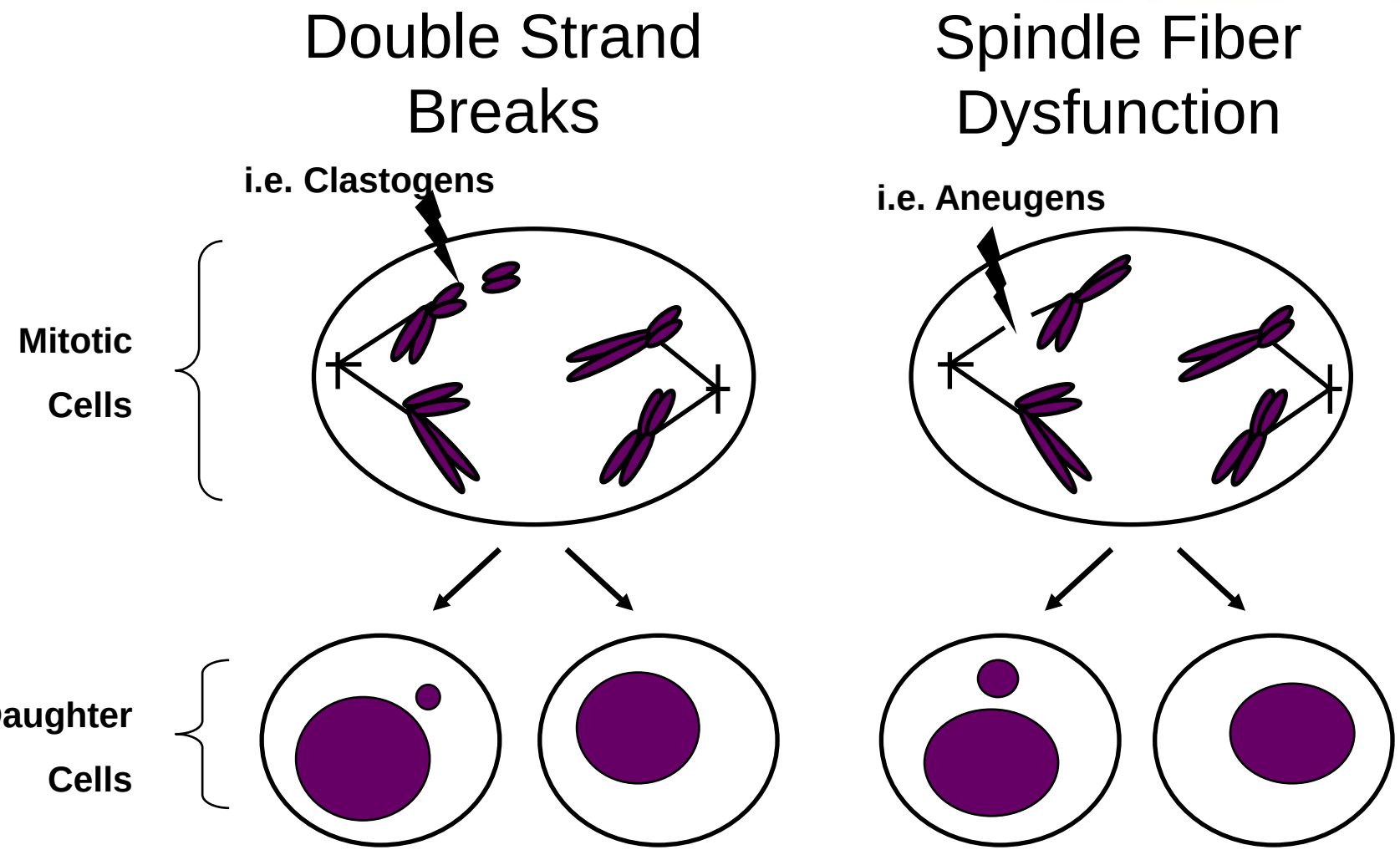
BioReliance®

In Vitro Micronucleus Assay
OECD TG 487 (approved July 2010)

- Alternative to chromosome aberration assay
- Comparatively easy to evaluate
- Experimental design nearly same, but typically use cytochalasin B (cytokinesis inhibitor)
- Nuclei divide in presence of cytochalasin B, but not cytoplasm (ensuring cell division)



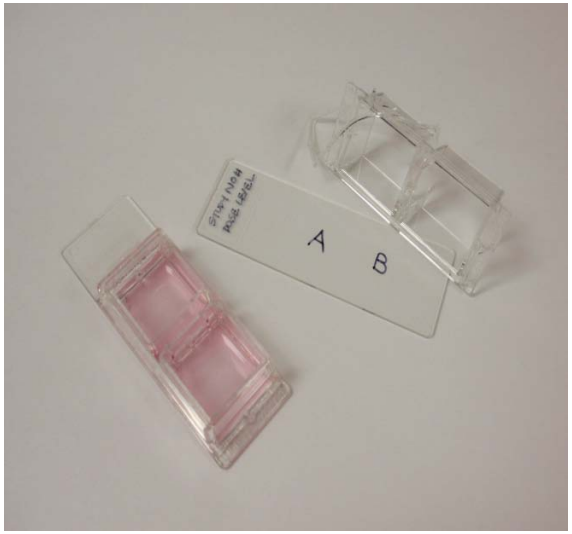
Mechanisms of Micronucleus Formation



BioReliance

In Vitro Micronucleus Assay OECD TG 487

In Situ



Monolayer

Suspension

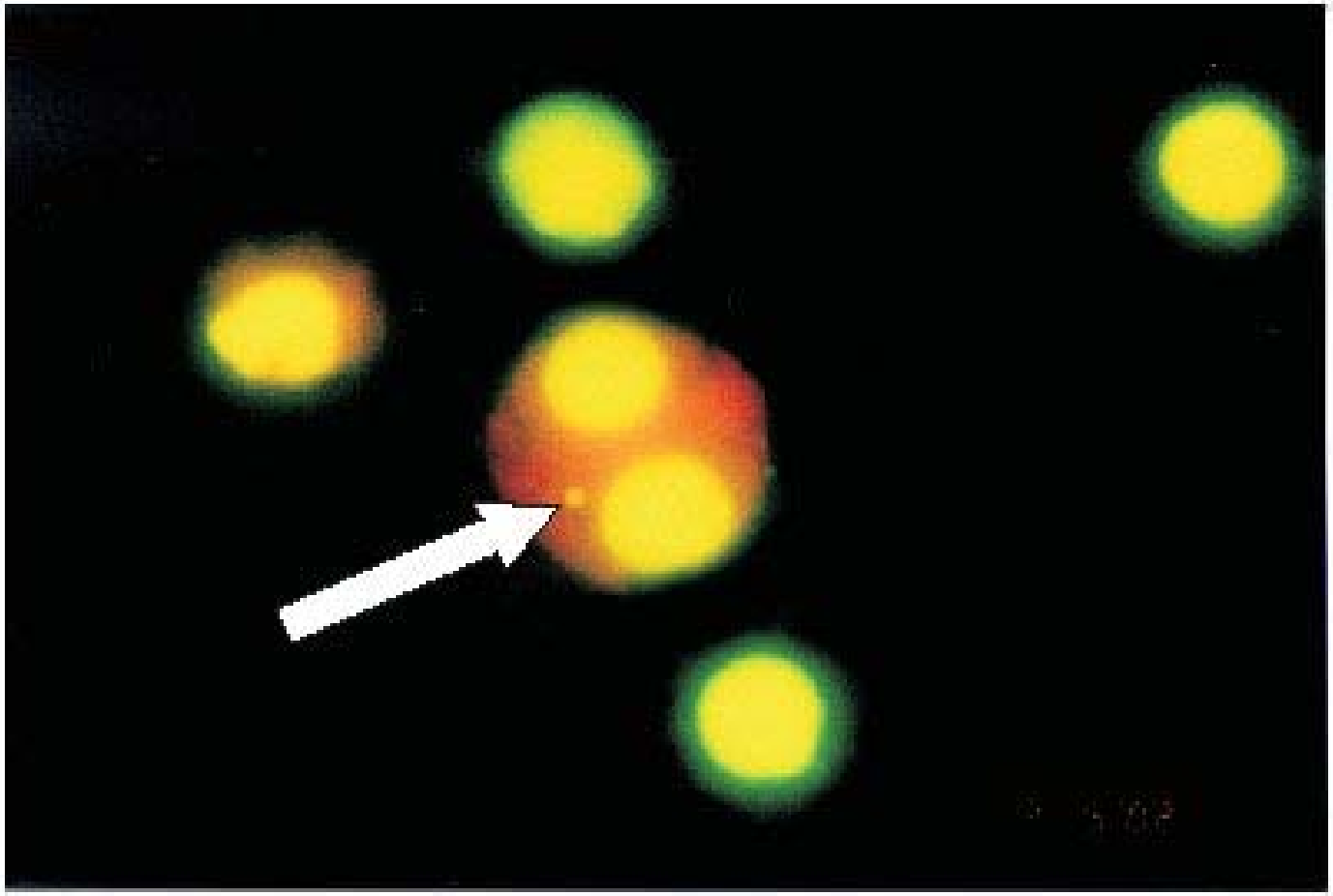
Cytotoxicity: Cytokinesis-Block Proliferation Index (CBPI) method

$$CBPI = \frac{1 \times \text{No. of Mononucleated cells} + 2 \times \text{No. of Binucleated cells} + 3 \times \text{No. of Multinucleated cells}}{\text{Total number of Cells}}$$

At least 1000 cells/dose level analyzed for CBPI, and 2000 binucleated cells/dose level for MN induction

BioReliance

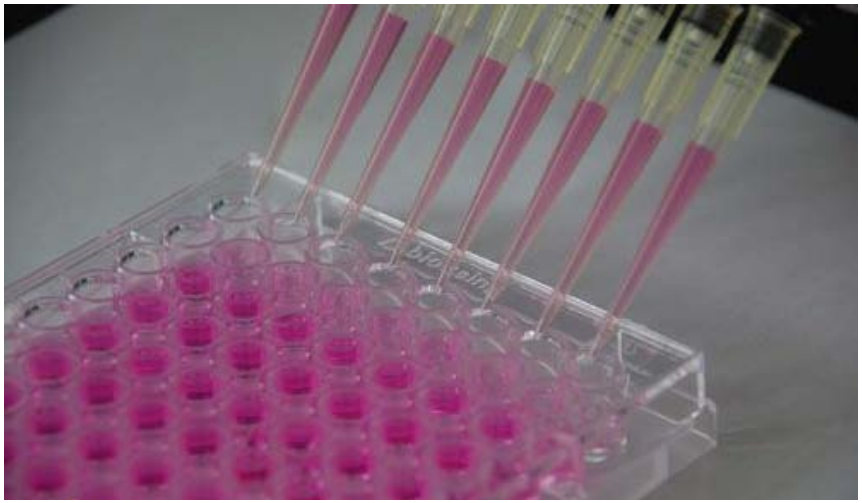
In Vitro Micronucleus Assay Manual Scoring



BioReliance®

ToxCast Experimental Design

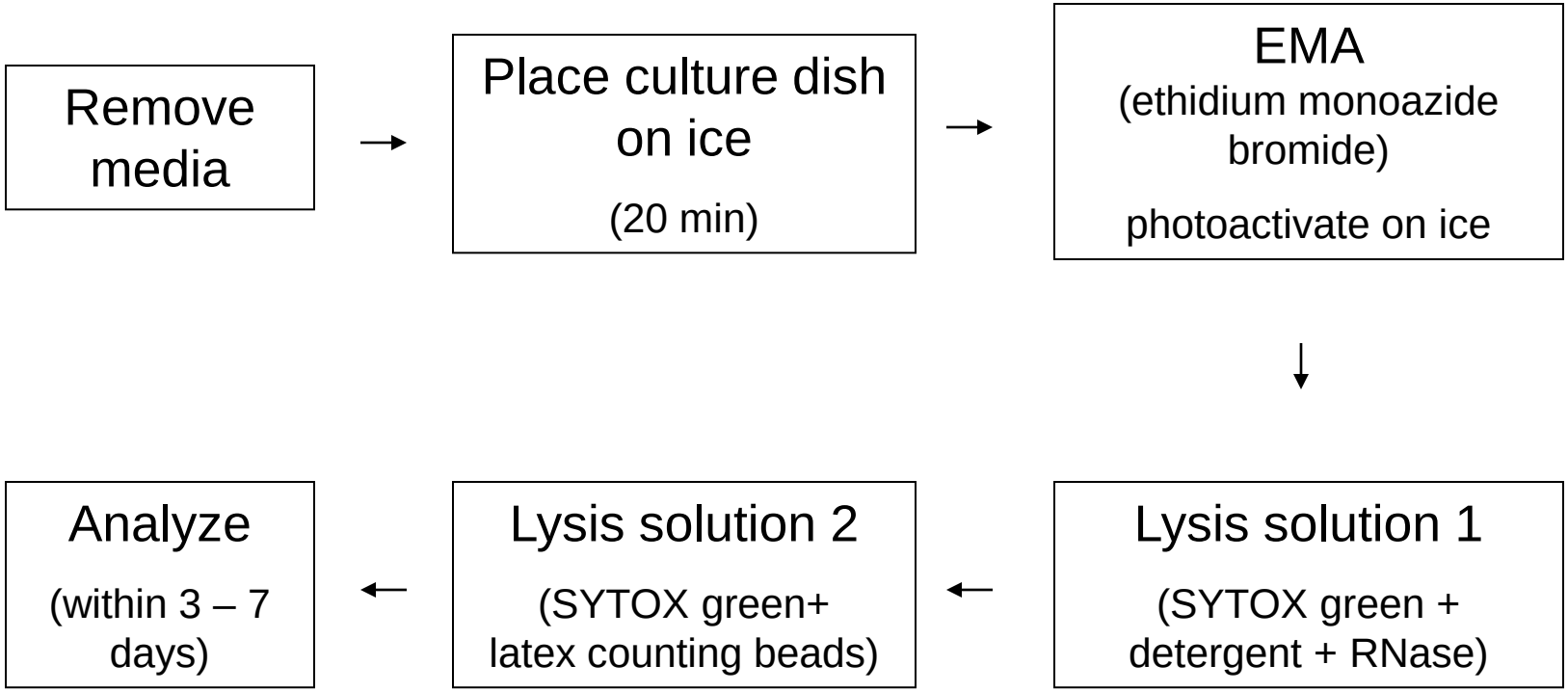
- 96-well format
- no cytochalasin B
  - test article @ 9 dose levels
  - 3 positive controls @ 2 dose levels
  - 1 vehicle control
  - four test articles per plate with or without metabolic activation (S9)
  - limit dose (~200 µM) or 40 – 60% relative survival
  - 5000 cells analyzed per well
  - all in duplicate cultures



BioReliance

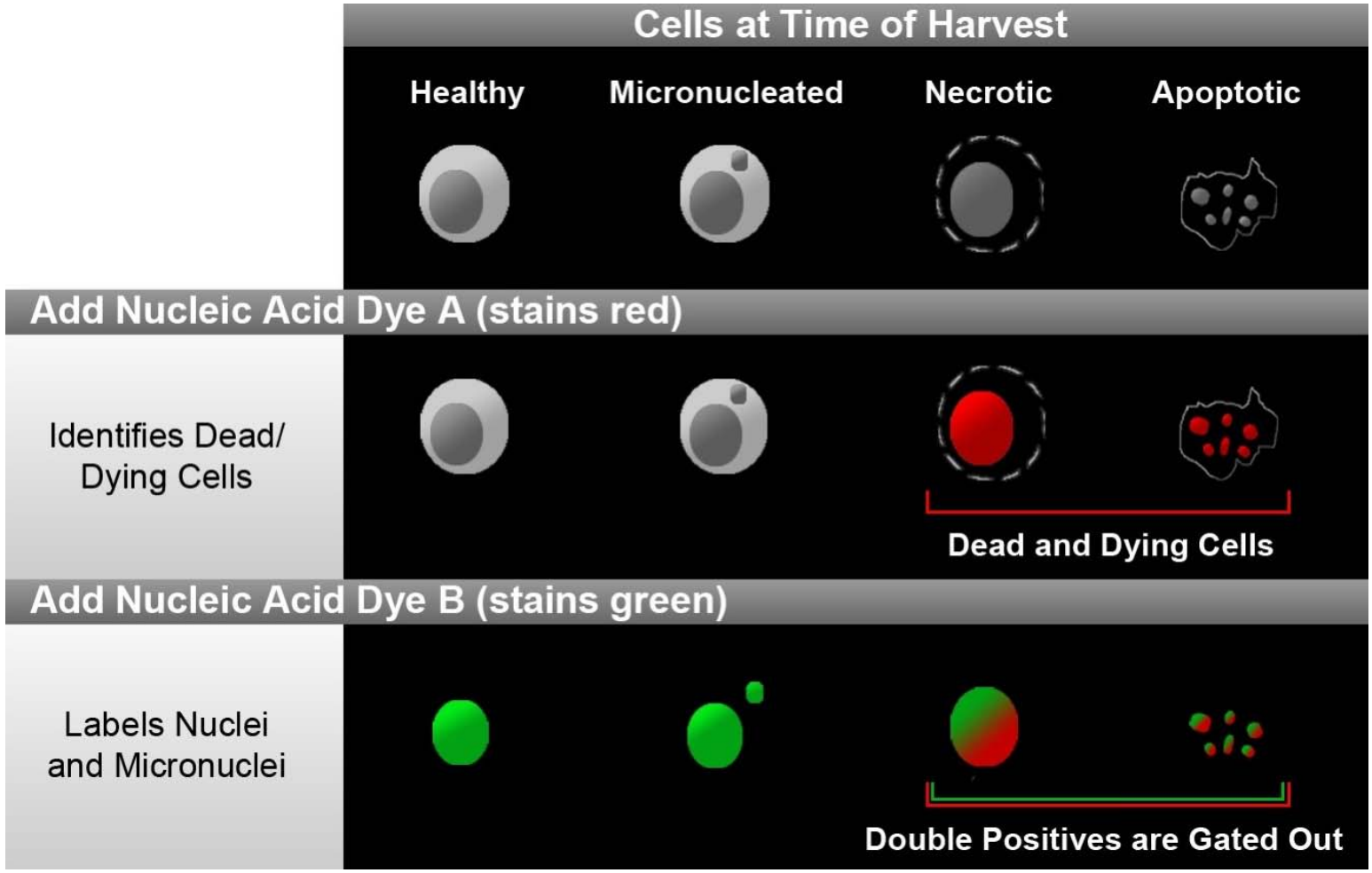
Experimental Design (ToxCast)

Treatment similar to typical but harvesting/scoring is different



BioReliance

Flow Cytometric Scoring

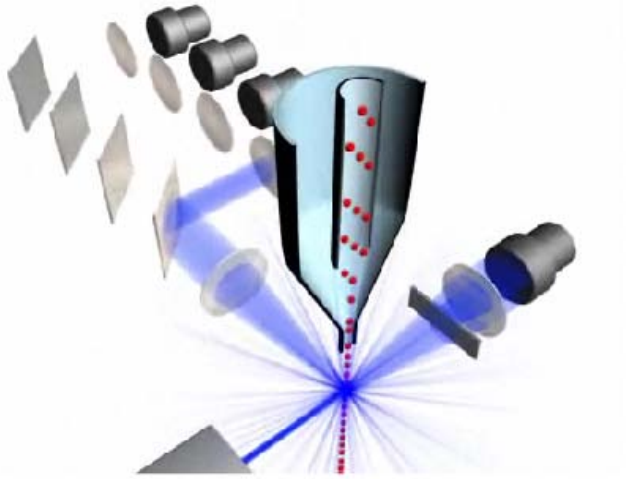


Courtesy of Litron Laboratories

BioReliance

Flow Cytometric Scoring

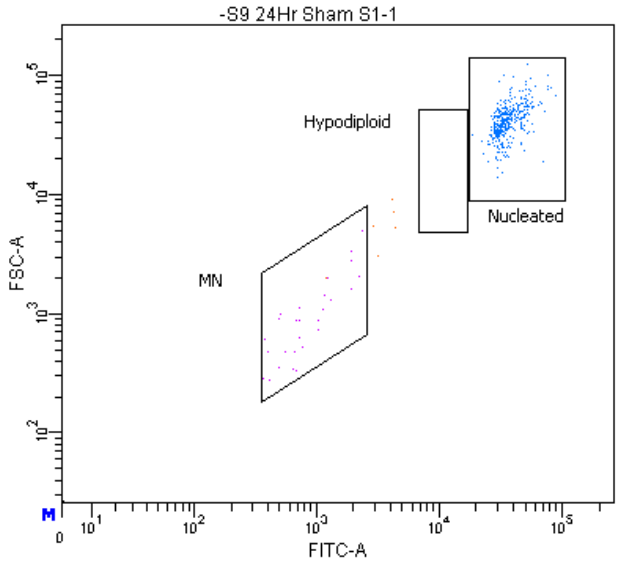
BD FACS Canto II



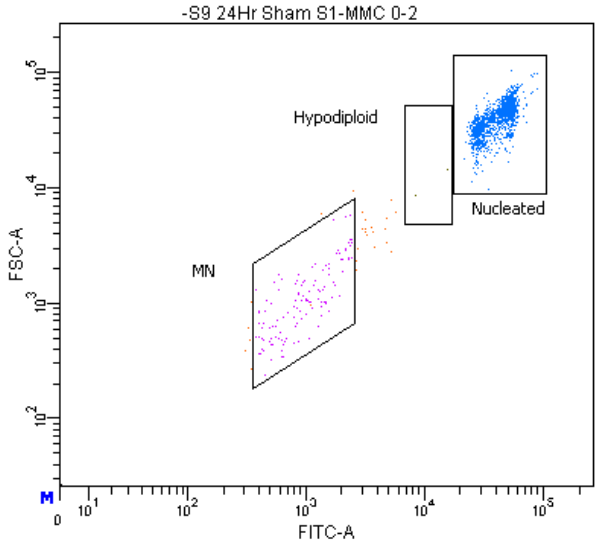
Flow Cell

BioReliance

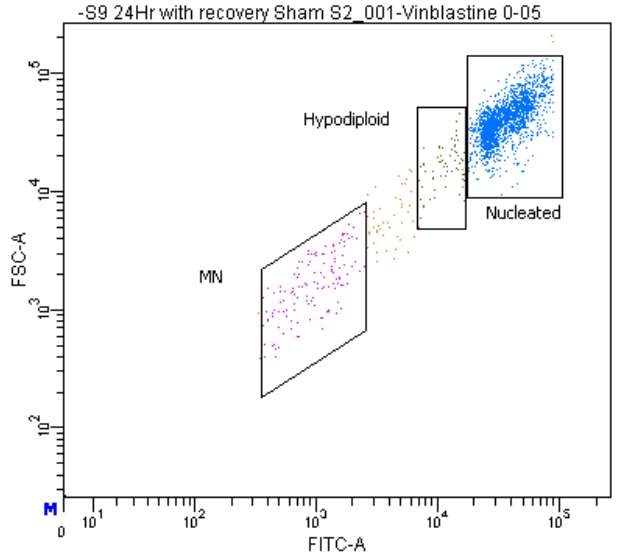
Results



Vehicle control



Mitomycin C
(Clastogen)



Vinblastine
(aneugen)

BioReliance

96-Well Flow MN Validation

“OECD 10” from TG 487

- **Clastogens not requiring S9**
  - araC
  - MMC
- **Clastogens requiring S9**
  - B(a)P
  - CP
- **Aneugens**
  - COL
  - VB
- **Negative substances**
  - DEHP
  - NAL
  - PYR
  - NaCl

96-Well Flow MN Validation

MMC

CP

DEHP

NaCl

+S9

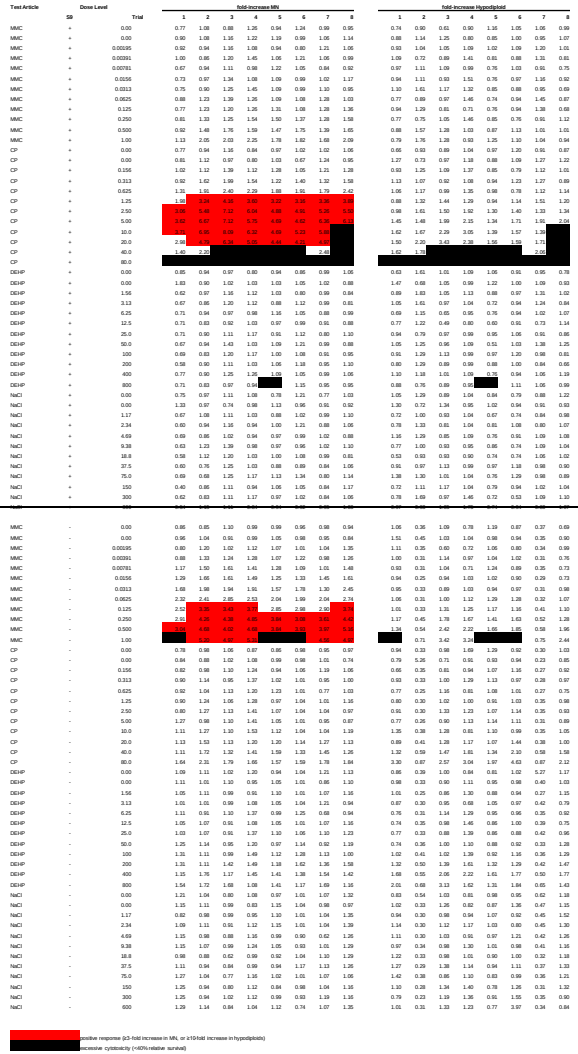
MMC

CP

DEHP

NaCl

–S9



BioReliance®

96-Well Flow MN Validation

Test Article	S9	Dose Level	Trial	fold-increase MN 1	2	3	4	5	6	7	8	fold-increase Hypodiploid 1	2	3	4	5	6	7	8
Vehicle	+	0.00		0.77	0.94	1.16	0.84	0.97	1.02	1.02	1.06	0.66	0.93	0.89	1.04	0.97	1.20	0.91	0.87
Vehicle	+	0.00		0.81	1.12	0.97	0.80	1.03	0.67	1.24	0.95	1.27	0.73	0.97	1.18	0.88	1.09	1.27	1.22
CP	+	0.156		1.02	1.12	1.39	1.12	1.28	1.05	1.21	1.28	0.93	1.25	1.09	1.37	0.85	0.79	1.12	1.01
CP	+	0.313		0.92	1.62	1.99	1.54	1.22	1.40	1.32	1.58	1.13	1.07	0.92	1.08	0.94	1.23	1.27	0.89
CP	+	0.625		1.31	1.91	2.40	2.29	1.88	1.91	1.79	2.42	1.06	1.17	0.99	1.35	0.98	0.78	1.12	1.14
CP	+	1.25		1.98	3.24	4.16	3.60	3.22	3.16	3.36	3.89	0.88	1.32	1.44	1.29	0.94	1.14	1.51	1.20
CP	+	2.50		3.06	5.48	7.12	6.04	4.88	4.91	5.26	5.50	0.98	1.61	1.50	1.92	1.30	1.40	1.33	1.34
CP	+	5.00		3.62	6.67	7.12	5.75	4.69	4.62	6.36	6.13	1.45	1.48	1.99	2.15	1.34	1.71	1.91	2.04
CP	+	10.0		3.71	6.95	8.09	6.32	4.69	5.23	5.88		1.62	1.67	2.29	3.05	1.39	1.57	1.39	
CP	+	20.0		2.98	4.79	6.34	5.05	4.44	4.21	4.97		1.50	2.20	3.43	2.38	1.56	1.59	1.71	
CP	+	40.0		1.40	2.20					2.48		1.62	1.78					2.06	
CP	+	80.0																	

positive response (≥3-fold increase in MN, or ≥10-fold increase in hypodiploidy)

excessive cytotoxicity (<40% relative survival)

BioReliance

96-Well Flow MN Validation

Test Article	S9	Dose Level	Trial	fold-increase MN 1	2	3	4	5	6	7	8	fold-increase Hypodiploid 1	2	3	4	5	6	7	8
Vehicle	-	0.00		0.86	0.85	1.10	0.99	0.99	0.96	0.98	0.94	1.06	0.36	1.09	0.78	1.19	0.87	0.37	0.69
Vehicle	-	0.00		0.96	1.04	0.91	0.99	1.05	0.98	0.95	0.84	1.51	0.45	1.03	1.04	0.98	0.94	0.35	0.90
MMC	-	0.00195		0.80	1.20	1.02	1.12	1.07	1.01	1.04	1.35	1.11	0.35	0.60	0.72	1.06	0.80	0.34	0.99
MMC	-	0.00391		0.88	1.33	1.24	1.28	1.07	1.22	0.98	1.26	1.00	0.31	1.14	0.97	1.04	1.02	0.31	0.76
MMC	-	0.00781		1.17	1.50	1.61	1.41	1.28	1.09	1.01	1.48	0.93	0.31	1.04	0.71	1.24	0.89	0.35	0.73
MMC	-	0.0156		1.29	1.66	1.61	1.49	1.25	1.33	1.45	1.61	0.94	0.25	0.94	1.03	1.02	0.90	0.29	0.73
MMC	-	0.0313		1.68	1.98	1.94	1.91	1.57	1.78	1.30	2.45	0.95	0.33	0.89	1.03	0.94	0.97	0.31	0.98
MMC	-	0.0625		2.32	2.41	2.85	2.53	2.04	1.99	2.04	2.74	1.06	0.31	1.00	1.12	1.29	1.28	0.32	1.07
MMC	-	0.125		2.52	3.35	3.43	3.77	2.85	2.98	2.90	3.74	1.01	0.33	1.31	1.25	1.17	1.16	0.41	1.10
MMC	-	0.250		2.91	4.26	4.38	4.85	3.84	3.08	3.61	4.42	1.17	0.45	1.78	1.67	1.41	1.63	0.52	1.28
MMC	-	0.500		3.04	4.68	4.02	4.68	3.84	3.93	3.97	5.16	1.34	0.54	2.42	2.22	1.66	1.85	0.58	1.96
MMC	-	1.00			5.20	4.97	5.31			4.56	4.97		0.71	3.42	3.24			0.75	2.44

positive response (≥3-fold increase in MN, or ≥10-fold increase in hypodiploidy)

excessive cytotoxicity (<40% relative survival)

BioReliance

96-Well Flow MN Validation

		%MN	%Hypo
-S9:	n	795	795
	average	1.76	0.73
	SD	0.54	0.28
	min	0.40	0.12
	max	4.20	2.10
	95% UCL	2.85	1.28
+S9:	n	801	801
	average	1.46	0.31
	SD	0.49	0.15
	min	0.50	0.08
	max	5.60	2.96
	95% UCL	2.44	0.61

Results

TX001580 -S9

Wells		Conc. (µM)	Nucleated #Events	Beads and P4 #Events	Nuclei to Bead	MN #Events	MN %Parent	Hypo #Events	Hypo %Parent	Relative Survival (%)	fold increase MN	fold increase Hypo
Vehicle		0.00	5000	230	21.9	44	0.85	14	0.27	100	1.00	1.00
A3	B3	0.781	5000	207	24.2	40	0.80	16	0.32	111	0.94	1.17
A4	B4	1.56	5000	223	22.5	41	0.80	15	0.29	103	0.94	1.06
A5	B5	3.13	5000	172	29.1	47	0.90	15	0.29	133	1.06	1.06
A6	B6	6.25	5000	193	26.0	44	0.85	17	0.34	119	1.00	1.24
A7	B7	12.5	5000	233	21.5	54	1.05	17	0.33	98	1.24	1.20
A8	B8	25.0	5000	199	25.2	46	0.90	14	0.27	115	1.06	0.99
A9	B9	50.0	5000	260	19.2	47	0.90	14	0.28	88	1.06	1.02
A10	B10	100	5000	265	18.9	43	0.85	18	0.35	86	1.00	1.28
A11	B11	200	5000	269	18.6	62	1.20	24	0.47	85	1.41	1.74

Non-toxic and negative

For Illustration Purposes

BioReliance

Results

TX011587 -S9

Wells		Conc. (µM)	Nucleated #Events	Beads and P4 #Events	Nuclei to Bead	MN #Events	MN %Parent	Hypo #Events	Hypo %Parent	Relative Survival (%)	fold increase MN	fold increase Hypo
Vehicle		0.00	5000	230	21.9	44	0.85	14	0.27	100	1.00	1.00
G3	H3	0.797	5000	214	23.4	52	1.00	15	0.29	107	1.18	1.06
G4	H4	1.59	5000	184	27.2	55	1.05	14	0.27	125	1.24	0.98
G5	H5	3.19	5000	212	23.6	50	1.00	16	0.31	108	1.18	1.13
G6	H6	6.38	5000	207	24.2	82	1.55	19	0.36	111	1.82	1.34
G7	H7	12.8	5000	213	23.5	77	1.50	24	0.46	108	1.76	1.70
G8	H8	25.5	5000	302	16.6	60	1.20	27	0.53	76	1.41	1.96
G9	H9	51.0	5000	324	15.5	52	1.00	25	0.49	71	1.18	1.82
G10	H10	102	5000	526	9.5	51	1.00	19	0.37	44	1.18	1.38
G11	H11	204	65	478	0.1	1	0.85	1	0.76	1	Cytotoxic	Cytotoxic

Cytotoxic and negative

For Illustration Purposes

BioReliance

Results

TX001542 -S9

Wells		Conc. (µM)	Nucleated #Events	Beads and P4 #Events	Nuclei to Bead	MN #Events	MN %Parent	Hypo #Events	Hypo %Parent	Relative Survival (%)	fold increase MN	fold increase Hypo
Vehicle		0.00	5000	230	21.9	44	0.85	14	0.27	100	1.00	1.00
C3	D3	0.916	5000	205	24.4	53	1.05	20	0.38	112	1.24	1.42
C4	D4	1.83	5000	174	28.8	44	0.85	19	0.38	132	1.00	1.39
C5	D5	3.66	5000	196	25.5	47	0.95	20	0.39	117	1.12	1.46
C6	D6	7.33	5000	156	32.2	52	1.05	27	0.53	147	1.24	1.96
C7	D7	14.7	5000	212	23.6	84	1.65	42	0.82	108	1.94	3.03
C8	D8	29.3	5000	251	19.9	127	2.35	150	2.84	91	2.76	10.51
C9	D9	58.6	5000	302	16.6	369	5.70	651	10.81	76	6.71	39.94
C10	D10	117	5000	539	9.3	101	1.95	32	0.62	42	2.29	2.30
C11	D11	234	29	497	0.1	20	36.75	2	3.00	0	Cytotoxic	Cytotoxic

Aneugenic signature

For Illustration Purposes

BioReliance

Results

TX003211 +S9

Wells		Conc. (µM)	Nucleated #Events	Beads and P4 #Events	Nuclei to Bead	MN #Events	MN %Parent	Hypo #Events	Hypo %Parent	Relative Survival (%)	fold increase MN	fold increase Hypo
Vehicle		0.00	4766	538	8.9	92	1.88	18	0.37	100	1.00	1.00
A3	B3	0.781	5000	532	9.4	113	2.15	19	0.36	106	1.15	0.98
A4	B4	1.56	5000	492	10.2	101	1.95	22	0.43	114	1.04	1.17
A5	B5	3.13	5000	471	10.6	120	2.30	20	0.39	119	1.23	1.06
A6	B6	6.25	5000	492	10.2	129	2.50	16	0.30	114	1.33	0.82
A7	B7	12.5	5000	578	8.7	170	3.25	20	0.39	97	1.73	1.05
A8	B8	25.0	5000	568	8.8	227	4.25	27	0.51	99	2.27	1.40
A9	B9	50.0	5000	577	8.7	412	7.45	39	0.71	97	3.97	1.92
A10	B10	100	5000	682	7.3	504	8.90	43	0.78	82	4.75	2.11
A11	B11	200	3561	919	3.9	402	9.85	49	1.21	43	5.25	3.28

Clastogenic signature

For Illustration Purposes

BioReliance

Results

TX000721		-S9 Conc.	Nucleated	Beads and	Nuclei to	MN	MN	Hypo	Hypo	Relative	fold increase	
Wells		(µM)	#Events	P4 #Events	Bead	#Events	%Parent	#Events	%Parent	Survival (%)	MN	Hypo
Vehicle		0.00	5000	152	33.2	123	2.36	31	0.61	100	1.00	1.00
C3	D3	0.781	5000	161	31.2	107	2.05	32	0.61	94	0.87	1.01
C4	D4	1.56	5000	147	34.0	115	2.20	40	0.77	102	0.93	1.27
C5	D5	3.13	5000	167	30.0	105	2.00	32	0.61	90	0.85	1.01
C6	D6	6.25	5000	143	35.1	124	2.40	36	0.70	106	1.02	1.15
C7	D7	12.5	5000	162	30.9	131	2.50	30	0.58	93	1.06	0.96
C8	D8	25.0	5000	182	27.5	195	3.70	41	0.78	83	1.57	1.29
C9	D9	50	1744	304	5.7	1227	36.80	142	4.56	17	Cytotoxic	Cytotoxic
C10	D10	100	10	300	0.0	4	25.45	1	3.70	0	Cytotoxic	Cytotoxic
C11	D11	200	1	328	0.0	1	50.00	1	33.33	0	Cytotoxic	Cytotoxic

Inconclusive

For Illustration Purposes

BioReliance

Agenda 11am - 12 pm

1. Introduction
   Marilyn Aardema 5 min
2. In Vitro Flow Micronucleus Assay - 96 well
   Leon Stankowski, 10 min
3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min
6. Questions/Discussion
   15 min

Rationale for using these Screening Assays

Ames II assay is liquid based Ames assay and can be automated.

- Treatment in 24-well plates and dispense and growth in 384-well plates, well suited for high throughput screening.

GreenScreen Assay is performed in 96-well plates and depending on the number of concentrations tested either 4 or 12 test articles per 96-well plate can be tested. Thus making this also a high throughput assay.

In Vitro Comet Assay – although the assay can be performed in the 96-well plate format, it is not really a high throughput screening assay since a huge number of slides have to be scored.

Rationale for Screening Assays

Non-GLP Assays can be used at early stages in drug discovery to select chemical candidates for further development.

Early screening assay advantages include:

- Low cost
- Rapid turn-around time
- Require minimal amounts of test articles
- Can be highly predictive

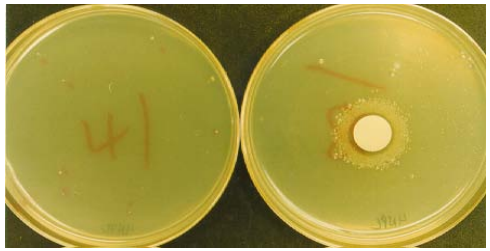
Customized design based on available sample

- Caution: should mimic GLP study as closely as possible to provide as good a correlation with the GLP study as possible

Screening
Genetic Toxicology Assays

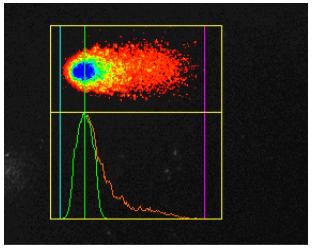
- Miniaturized “standard assays”

  - Bacterial Mutation Assays
    - Ames II



  - “New”” assays using engineered cells or “upstream” signals

In-Vitro COMET Assay



- GreenScreen Assay

BioReliance

Topics for Discussion

- Screening assays
  - Ames II
  - GreenScreen HC assay with and without activation
  - In-Vitro Comet assay
- Review protocols
  - Design and methodology
- Advantages and Limitations of each assay (if any)

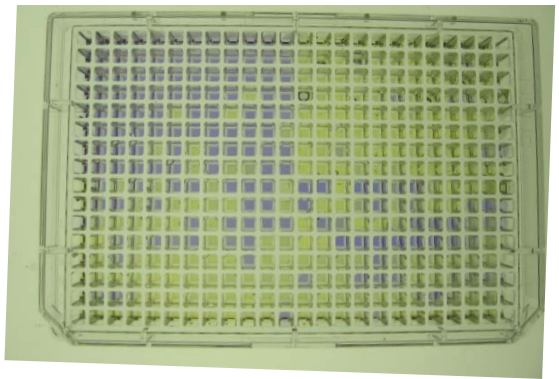
Agenda 11am - 12 pm

1. Introduction
   Marilyn Aardema 5 min
2. In Vitro Flow Micronucleus Assay - 96 well
   Leon Stankowski, 10 min
3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min
6. Questions/Discussion
   15 min

BioReliance

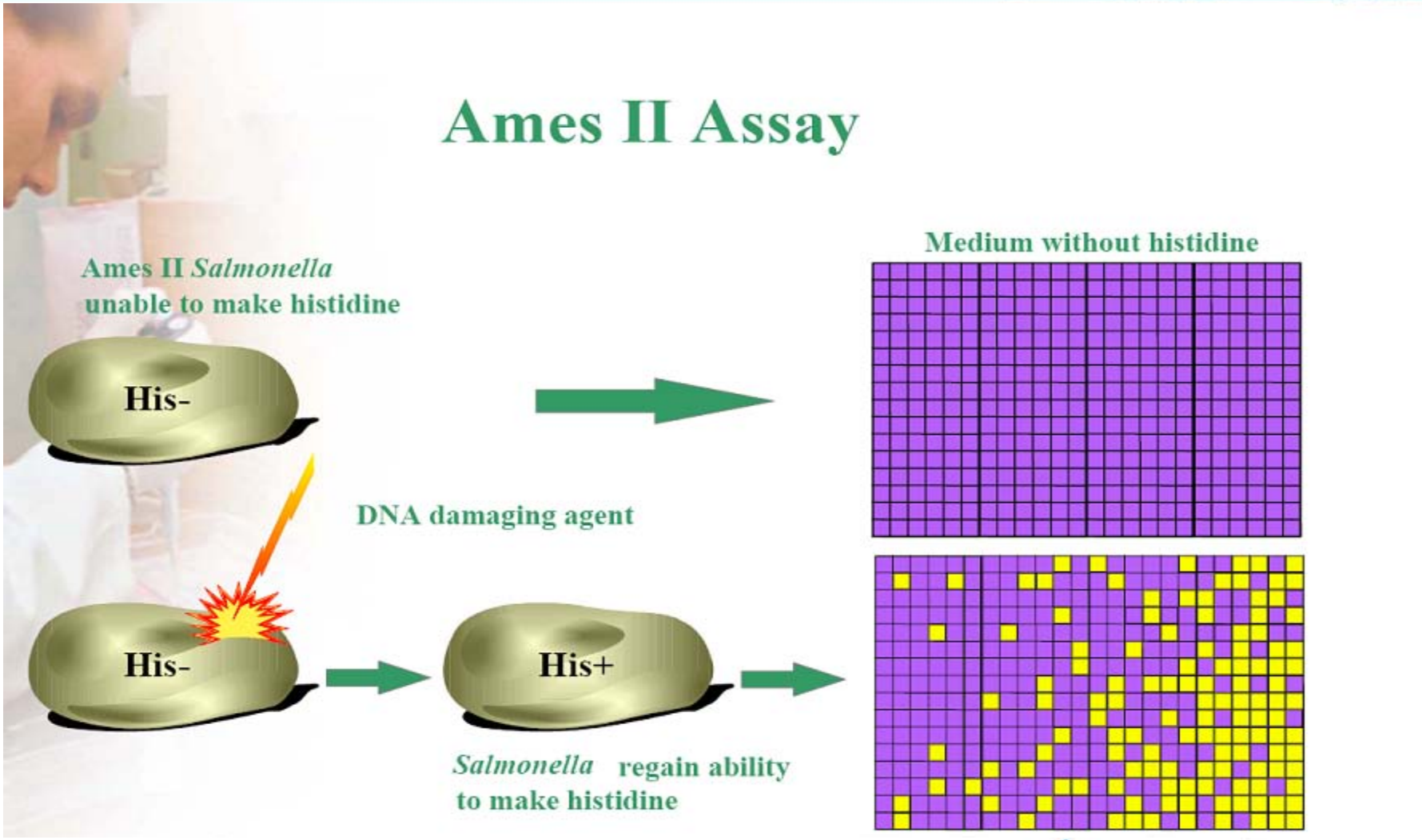
Ames II™ Assay

- Ames II™ – Screening version of the Ames assay
- Strains engineered for base-pair mutagens (TA7001 to TA7006)
- Standard TA-98 strain for frameshift mutagens
- University of California Berkley/Xenometrix technology licensed to BioReliance
  - Exclusive license in US and Japan
- Automated plating system can be used
- 2 to 5 mg test article needed



BioReliance®

Ames II™ Mutagenicity Assay



BioReliance®

Ames II™ Mutagenicity Assay

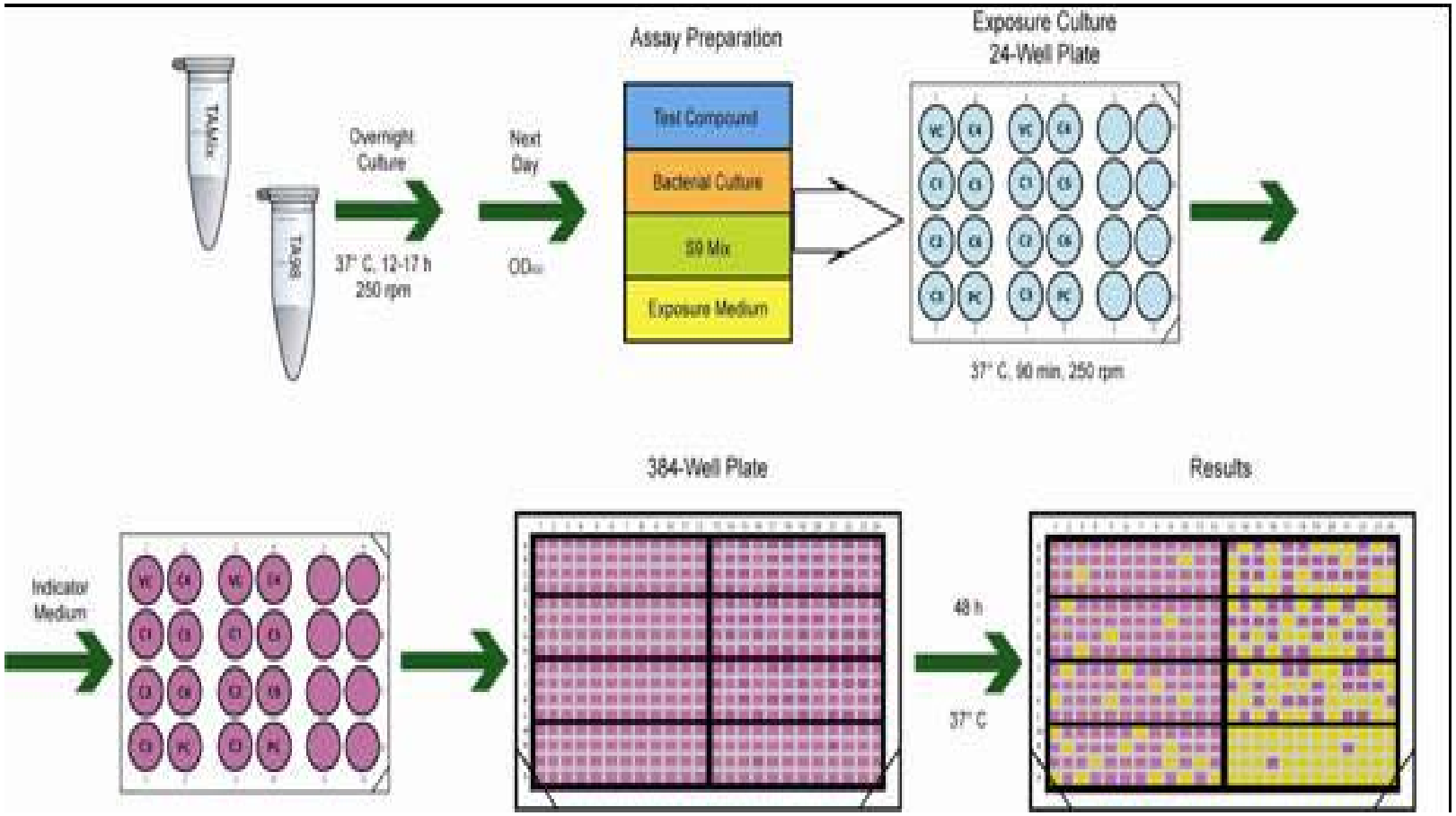
Genotype of the Ames II Tester Strains

Six Tester Strains

A•T → G•C TA7001	• *transition* mutation • *Gly* 153 (GG T) mutated to *Asp* 153 (GAT)
T•A → A•T TA7002	• *transversion* mutation • *Lys* 217 (AA A) mutated to *Ile* 217 (ATA)
T•A → G•C TA7003	• *transversion* mutation • *Gly* 153 (GG T) mutated to *Val* 153 (GTT)
G•C → A•T TA7004	• *transition* mutation • *Asp* 169 (GAG) mutated to *Gly* 169 (GGG)
C•G → A•T TA7005	• *transversion* mutation • *Asp* 169 (GAG) mutated to *Ala* 169 (GCG)
C•G → G•C TA7006	• *transversion* mutation • *Gly* 163 (GGA) mutated to *Arg* 163 (CGA)

BioReliance®

Ames II Assay Method



BioReliance®

Comparison of Ames & Ames II Tests

- Ames tests – testing using traditional Ames strains
  - GLP or ISO Ames test (testing for TA98,100,1535, 1537,102 or E.coli)
- Ames II tests –testing using both traditional and Ames II strains
  - Ames II individual tests: testing for TAMIX and TA98
  - Concordance with the regular Ames assay – 88%
  - Eight concentrations – highest ~ 200 µg/mL were tested in triplicate.

Ames II Assay Evaluation Criteria

- At least a two fold increase in the number of positive wells in two test article concentrations.
- The increase must be higher than the vehicle control historical range and dose dependence.
- Positive control – at least 25 positive wells or more.
- Negative control – within historical range.
- Limitation – toxicity measurement

Agenda 11am - 12 pm

1. Introduction

   Marilyn Aardema 5 min

2. In Vitro Flow Micronucleus Assay - 96 well

   Leon Stankowski, 10 min

3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min

6. Questions/Discussion

   15 min

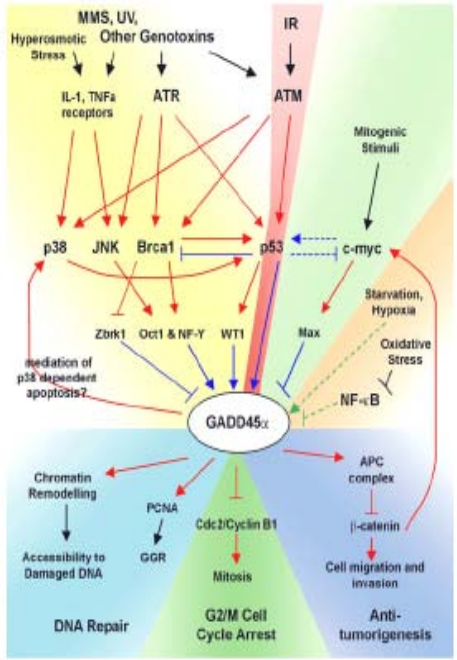
BioReliance

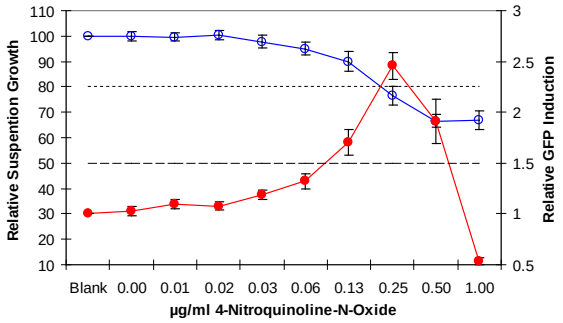
GreenScreen Human Cell Assay

GreenScreen Assay in Human TK6 Cells

Cell signal associated with DNA damage processing

- Uses p53 proficient cells
- With and without S9
- Population-wide response to “upstream” signal of DNA damage processing
- GADD45 target gene and Green Fluorescent Protein report gene
- 1 to 4 mg test article with and without S9



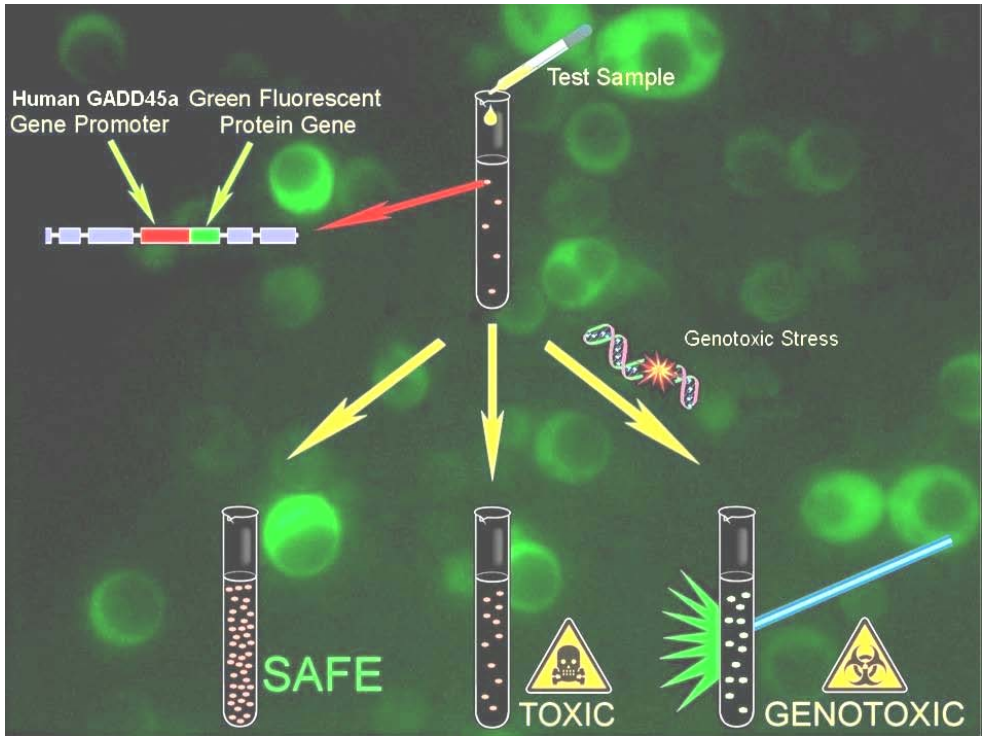


BioReliance®

GreenScreen Human Cell

Assay principle:

DNA damage increases cell fluorescence



Mix

Wait

Measure

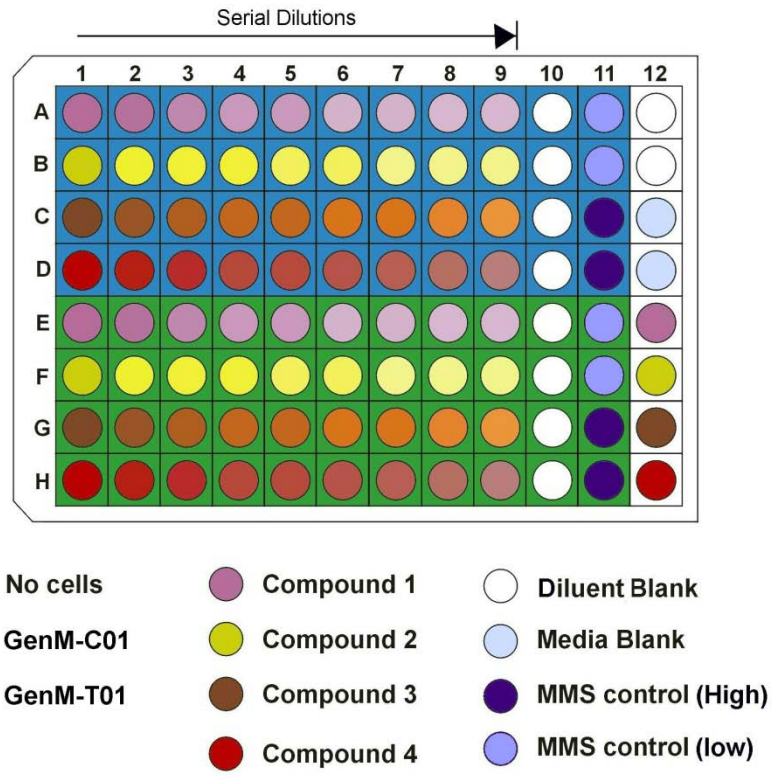
Slide courtesy of Gentronix, LTD.

BioReliance

GreenScreen HC Cell Lines

- GreenScreen cells constructed using TK6 cell line
- GADD45a gene (Growth Arrest and DNA Damage)
- Two cell constructs used in study
- GenM-T01 – fully functional plasmid with GADD45a and GFP genes
  - Functional GFP expressed following DNA damage
- GenM-C01 – plasmid missing 4 base pairs from start on GFP gene
  - Nonfunctional GFP produced
  - Control for cytotoxicity and non-specific autoflorescence
- Cells also contain hygromycin B resistance gene
  - Plasmids maintained in TK6 by adding hygromycin B to media

GreenScreen HC Without S9 Assay Overview



- 4 compounds per plate
- 9 two-fold dilutions
- internal positive controls
- control & test strain

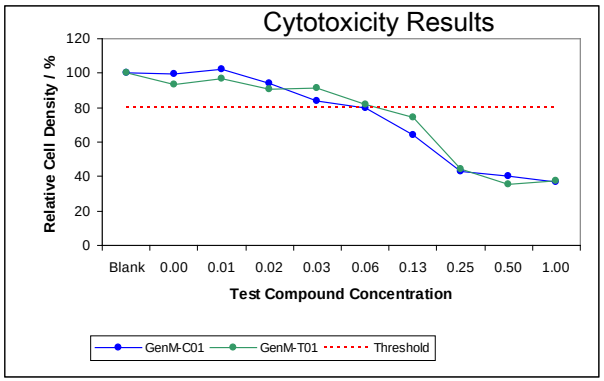
- plate set up in 20/30 minutes
- automated data collection
- results in 24/48 hours

- 1mg required to test up to 1000 µg/ml
- 10 µl of 10mg/ml stock

Slide courtesy of Gentronix, LTD.

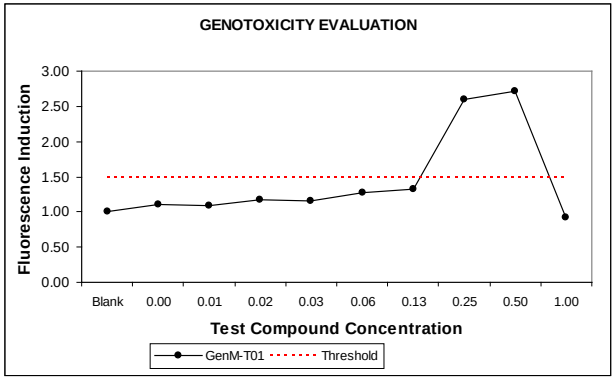
BioReliance

4-Nitroquinoline N-oxide (4-NQO)



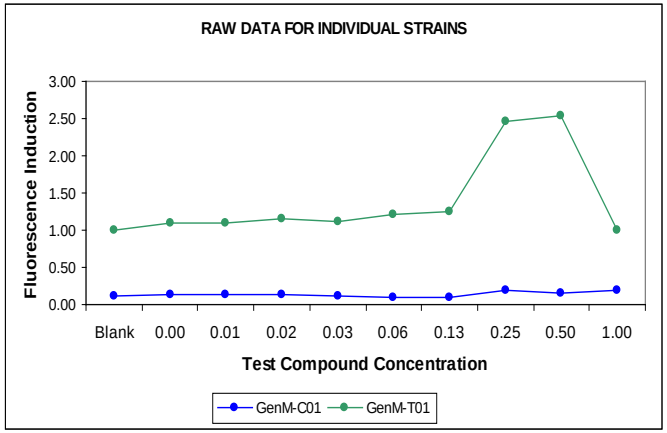
CYTOTOXICITY RESULT		
	STRONG POSITIVE	
LEC	0.13	ug/ml

CYTOTOXIC CONTROLS			
CELL LINE	CELL DENSITY		RESULT
	HIGH	*LOW*	
GenM-C01	35.3	67.5	PASS



GENOTOXICITY RESULT		
	STRONG POSITIVE	
LEC	0.25	ug/ml

GENOTOXIC CONTROLS			
CELL LINE	GFP INDUCTION		RESULT
	HIGH	*LOW*	
GenM-T01	2.87	1.36	PASS

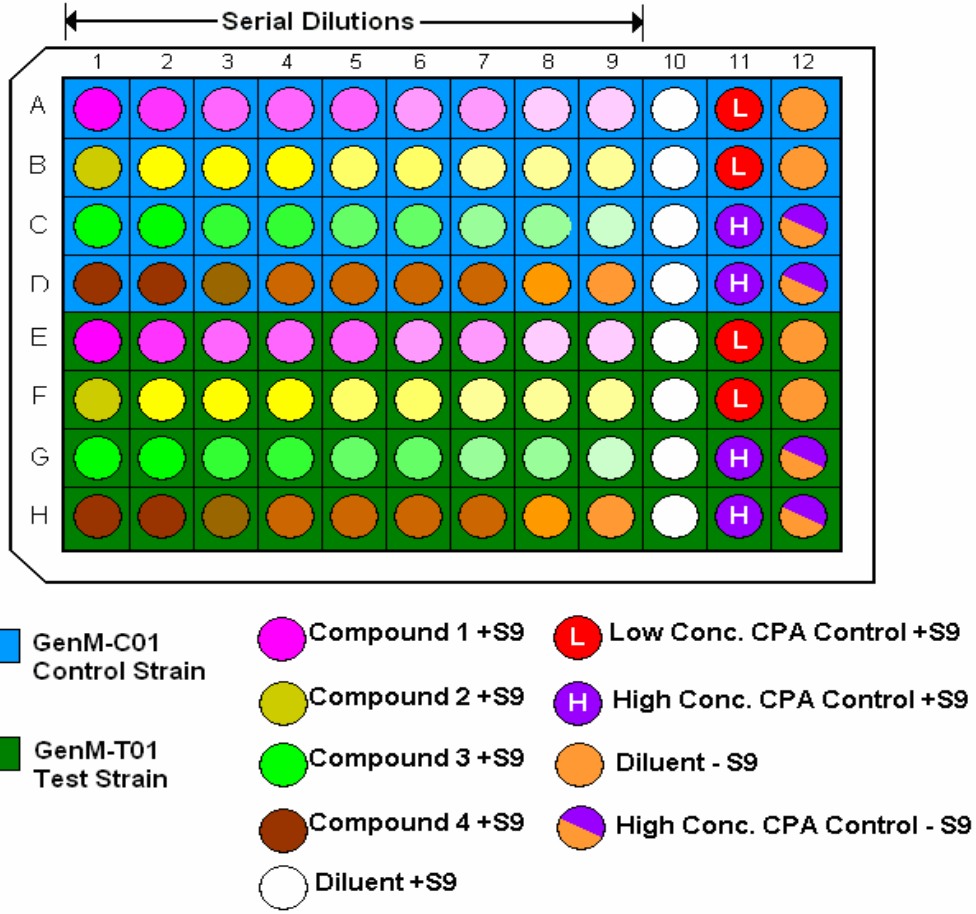


BioReliance

GreenScreen Assay with Metabolic Activation

- GreenScreen assay is performed with S9 activation.
- Same type of setup as without activation in 96-well plates.
- Test article treatment performed with S9 for only three hours.
- Cells centrifuged, washed and refed with media and grown for 48 hours.
- Stained with Propidium Iodide.
- Sample from the microplate on the flow cytometer.
- Read with the **GreenScreen HC S9 acquisition and analysis flow cytometry template**, collecting 10,000 events per well.

GreenScreen + S9 Overview



4 compounds per plate
9 two-fold dilutions
internal positive controls
control & test strain

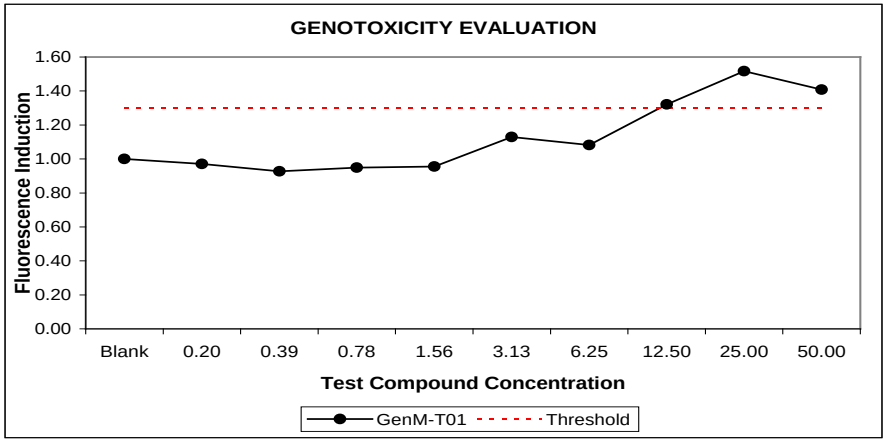
Treatment time – 3hours
automated data collection
results in 48 hours
Data by Flow Cytometry.

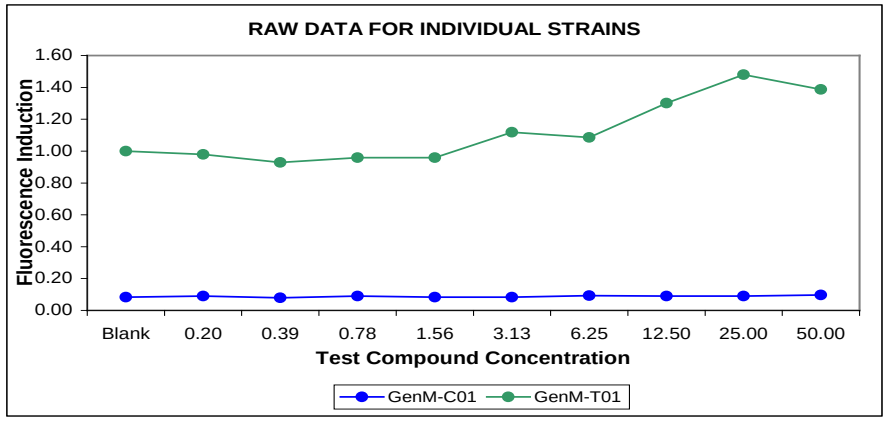
1mg required to test
up to 1000 µg/ml
10 µl of 10mg/ml stock

BioReliance

GreenScreen Assay +S9 Positive Control Data (CPA)

GENOTOXICITY RESULTS





GENOTOXICITY RESULT		
	POSITIVE	
LEC	12.50	ug/ml

GENOTOXIC CONTROLS	
RESULT	PASS

NOTES:

BioReliance

Evaluation Criteria

- **With and Without S9**
  - Assay should give a pass reading according to the software provided by Gentronix. Parameters checked – media contamination, optimum cell growth, auto-fluorescent or colorful test articles
  - Cytotoxicity – less than or equal to 80% relative cell survival (cytotoxic), 50% or less RCS (very cytotoxic)
  - Fold increase in GFP –
  - 1.5 or greater (genotoxic without metabolic activation),
  - 1.3 or greater (genotoxic with metabolic activation)
  - Limitation – solubility of test article, precipitating doses interfere with readings.

Agenda 11am - 12 pm

1. Introduction

   Marilyn Aardema 5 min

2. In Vitro Flow Micronucleus Assay - 96 well

   Leon Stankowski, 10 min

3. Genetic Toxicology Screening Assays
   a) Ames II Assay
      Kamala Pant 10 min
   b) GreenScreen Assay
      Kamala Pant 10 min
   c) In Vitro Comet Assay - 96 well TK6 assay
      Kamala Pant 10 min

6. Questions/Discussion

   15 min

BioReliance

In Vitro Comet Screening Assay

Single Cell Gel Electrophoresis to detect DNA damage

Why do in vitro Comet Assay?

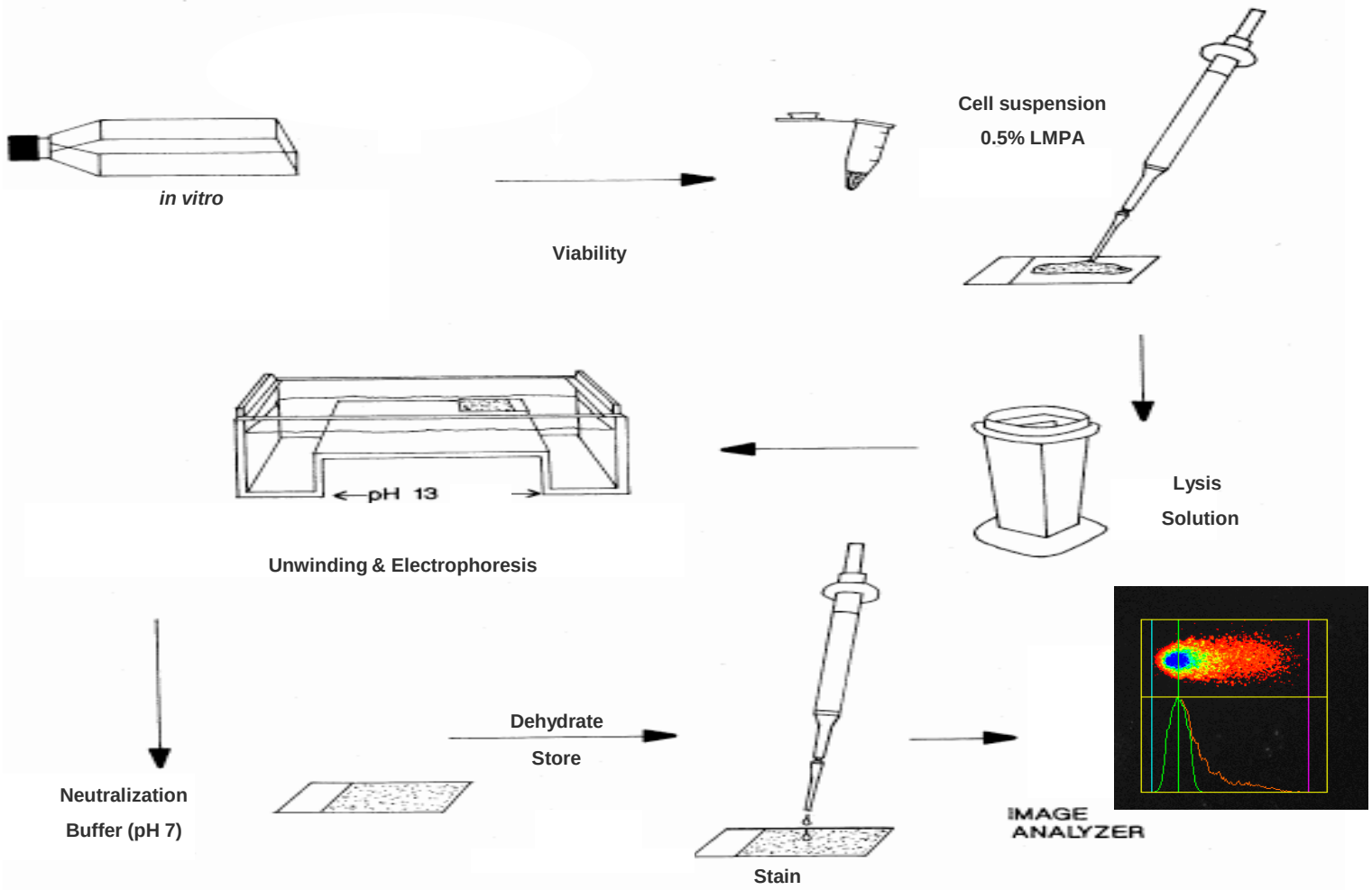
1. Mechanism of action
2. Predictive of genotoxicity
3. Could be an alternative to *in vitro* clastogenicity assay.
4. Could be used as a test for early drug candidate selection.
5. Clinical trial (if a DNA damaging drug is going through clinical trial – patient blood/bone marrow monitoring)

In Vitro Comet Screening Assay

Test System (cell lines, human blood, primary cells etc.) for ToxCast –TK6 cells

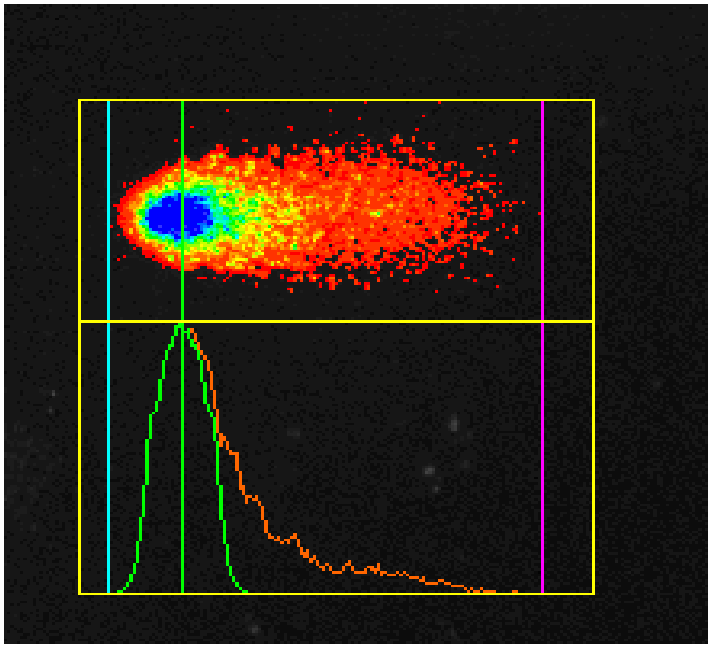
- Five concentrations of test article (no pre-tox), stating at ~ 200 µM highest dose.
- Positive (2AA and MMS, with and without activation respectively) and vehicle controls
- 4-hour treatment, with and without metabolic activation
- 96-well plate format
- Toxicity measured by number of clouds on the slides. Have scored up to 90% clouds.

In Vitro Comet Assay Methodology



BioReliance

Parameters of DNA Damage



Tail Length

DNA migration length from center of the head to smallest detectable fragment

% Tail DNA (Intensity)

Amount of DNA fragments in the tail

Olive Tail Moment

[(% Tail intensity) X (tail length)]

Tail Migration

DNA migration length from the edge of the head to smallest detectable fragment

In Vitro Comet Assay Evaluation

- Positive control – significant increase in DNA damage
- Negative/vehicle control within historical vehicle control range
- Highest dose scored up to 90% clouds
- Statistical significance in doses to evaluate positive response
- Limitation – so far toxicity measurements and statistical analysis are not well defined.

Thank You

Any Questions?

BioReliance®