

WHERE
insights LEAD TO
answers

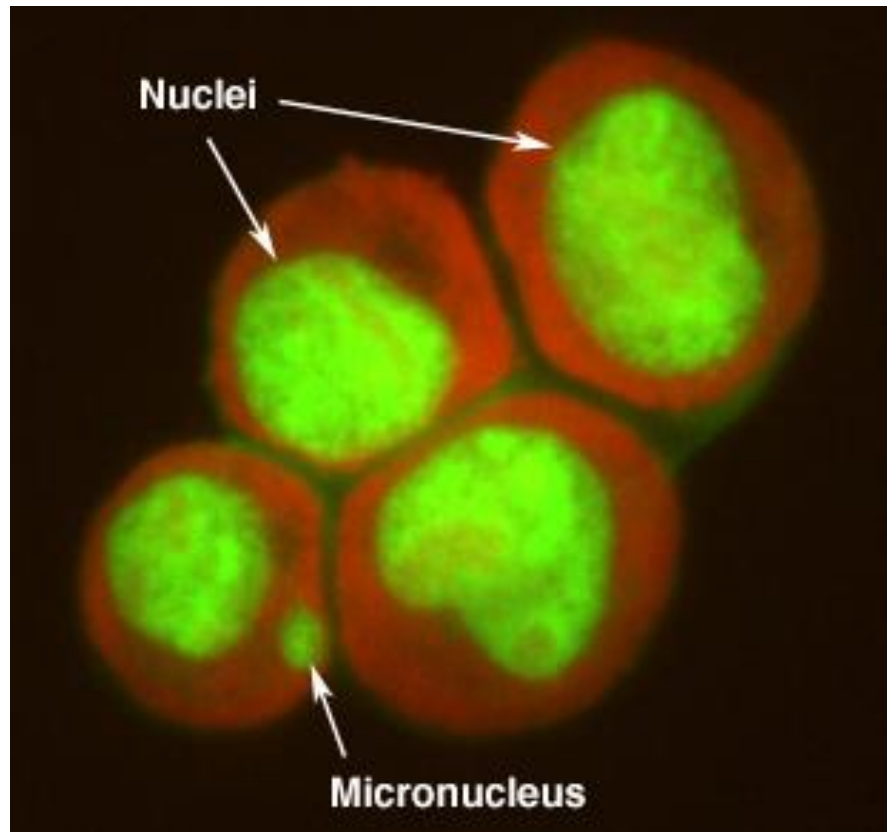
In Vitro Flow Cytometry Assays for Genetic Toxicology at Inotiv

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Outline of Presentation

- Brief review of micronucleus assay
- Flow cytometry basics
- Flow cytometry-based micronucleus assay - MicroFlow®
- Assay design
- MicroFlow data
- MultiFlow® as a mechanistic follow-up test
- MultiFlow data

Micronucleus Assay Review



- Detects DNA damage in the form of DNA fragments (clastogenic response) or whole chromosomes (aneugenic response) that don't migrate to the poles properly during mitosis.
- These show up in the cell cytoplasm as small pieces of stained nuclear material, or micronuclei, upon cell division.

Alternate Analysis Format – Flow Cytometry



Becton Dickinson FACSCanto II

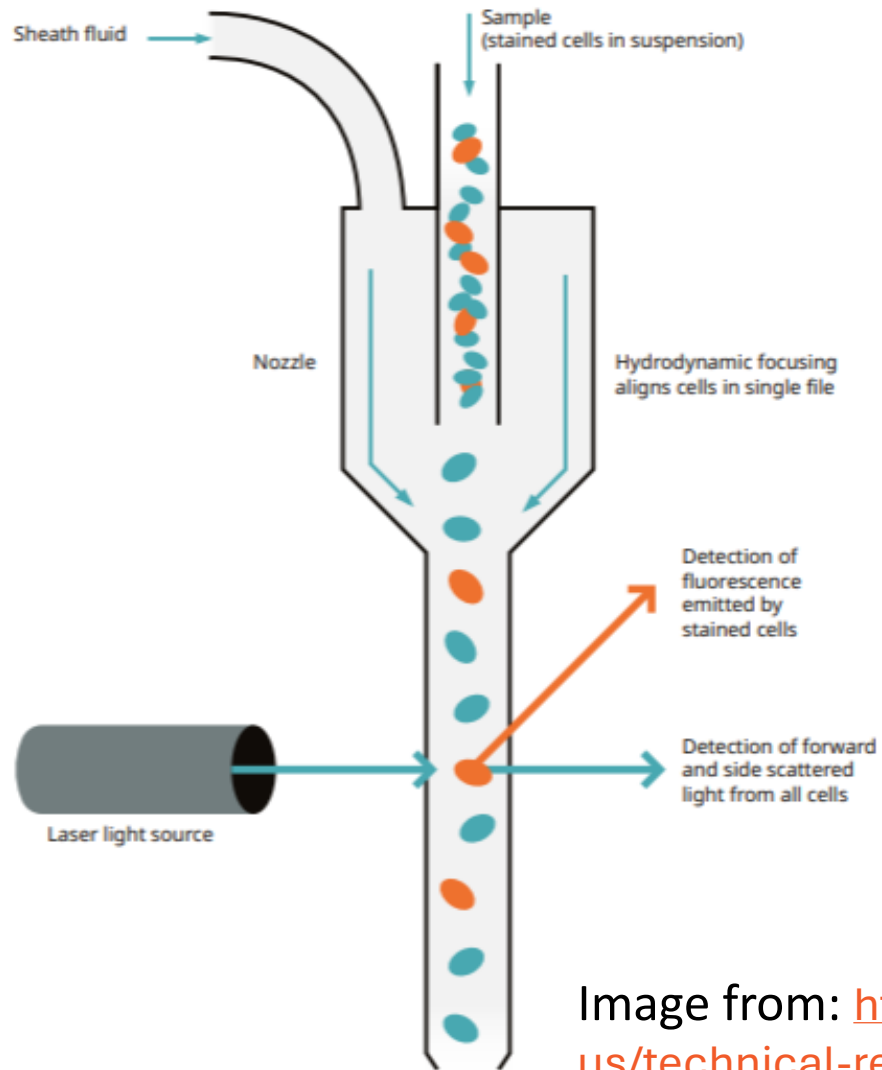
At Inotiv, 2 types of instruments are available for collecting data from genetic toxicology studies by flow cytometry.

Both instruments are capable of handling samples from 96-well culture plates and from single tubes; both have been validated for 21CFR Part11 compliance.



Milltenyi MACSQuant 10

Flow Cytometry



The flow cytometer captures several different parameters from the same population of cells as they flow past the detector in single file, including measurement of forward scatter and side scatter, which are used to determine size and granularity of the cell, as well as any fluorescent labels that have been applied to the cells.

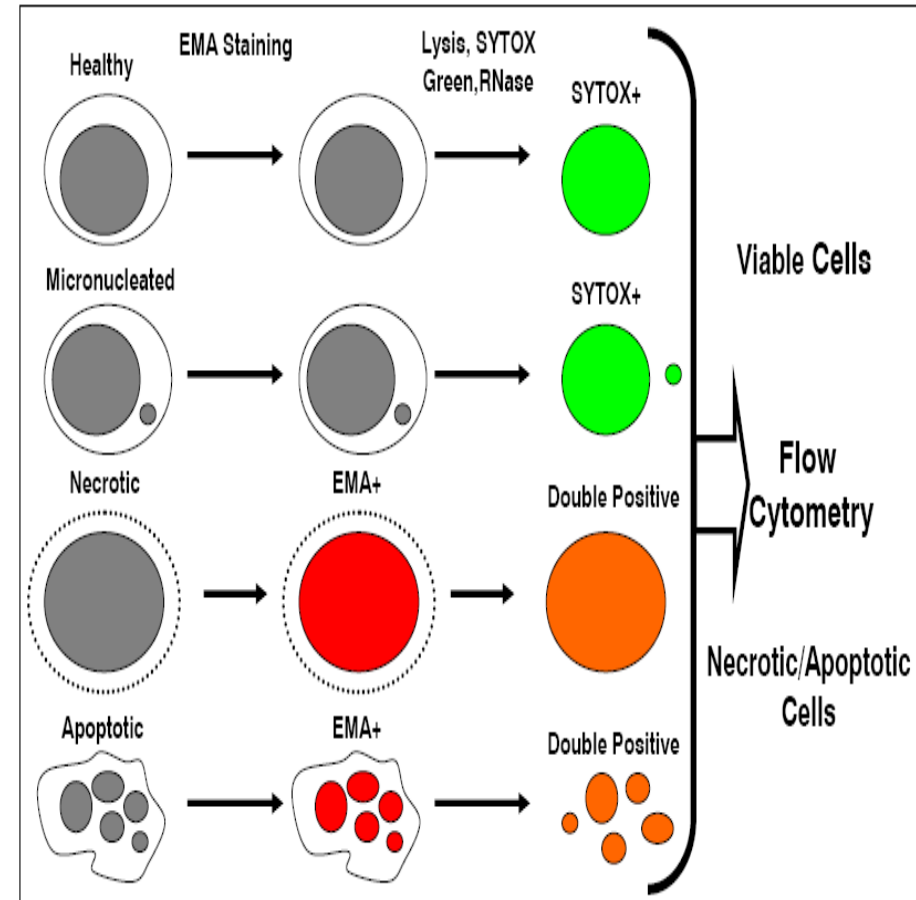
Image from: <https://www.abcam.com/en-us/technical-resources/guides/flow-cytometry-guide/what-is-flow-cytometry>

Analysis of Micronuclei Induction Using the MicroFlow In Vitro Kit

μ Flow[®] MN Assay (Litron Laboratories) : Sequential, 2-Dye Cytotoxicity and Micronucleus Assay in Human TK6 Cells

The In Vitro MicroFlow kit from Litron Laboratories (Rochester, NY) uses a dual staining approach to separate DNA fragments arising due to processes such as apoptosis and necrosis from fragments arising from fragments that are the result of true genotoxic processes.

DNA fragments with dual staining are gated out and only true micronuclei are counted.



From: **MicroFlow In Vitro Instruction Manual**, Litron Laboratories, Rochester, NY, Version 240418 (96-well) and Version 240319 (standard), www.litronlabs.com

Sample Plate Layout for 96-Well Assay Format

	In-vitro MN 27hr-S9; 4hr-S9; 4hr+S9											
	1	2	3	4	5	6	7	8	9	10	11	12
A			Dose 8	Dose 7	Dose 6	Dose 5	Dose 4	Dose 3	Dose 2	Dose 1		
B			Dose 8	Dose 7	Dose 6	Dose 5	Dose 4	Dose 3	Dose 2	Dose 1		
C			Dose 8	Dose 7	Dose 6	Dose 5	Dose 4	Dose 3	Dose 2	Dose 1		
D			Dose 8	Dose 7	Dose 6	Dose 5	Dose 4	Dose 3	Dose 2	Dose 1		
E		PC 1	PC 1	PC 1	PC 1							
F		PC 1	PC 1	PC 1	PC 1		DMSO	DMSO	DMSO	DMSO	DMSO	
G		PC 2	PC 2	PC 2	PC 2		DMSO	DMSO	DMSO	DMSO	DMSO	
H		PC 2	PC 2	PC 2	PC 2		DMSO	DMSO	DMSO	DMSO	DMSO	

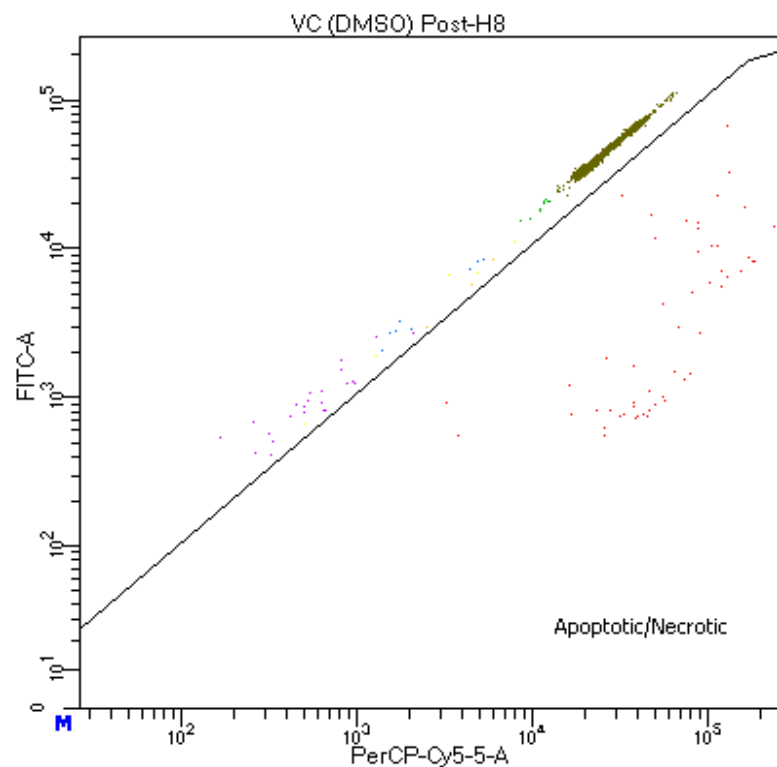
Key	TA - µg/mL
	VC: DMSO
	PC: 27hr-S9: VIN 0.75ng/mL, 1.5ng/mL; 4hr-S9: VIN 5ng/mL, 10ng/mL; 4hr+S9: CP 3µg/mL, 7.5µg/mL

Assay Design – OECD TG487

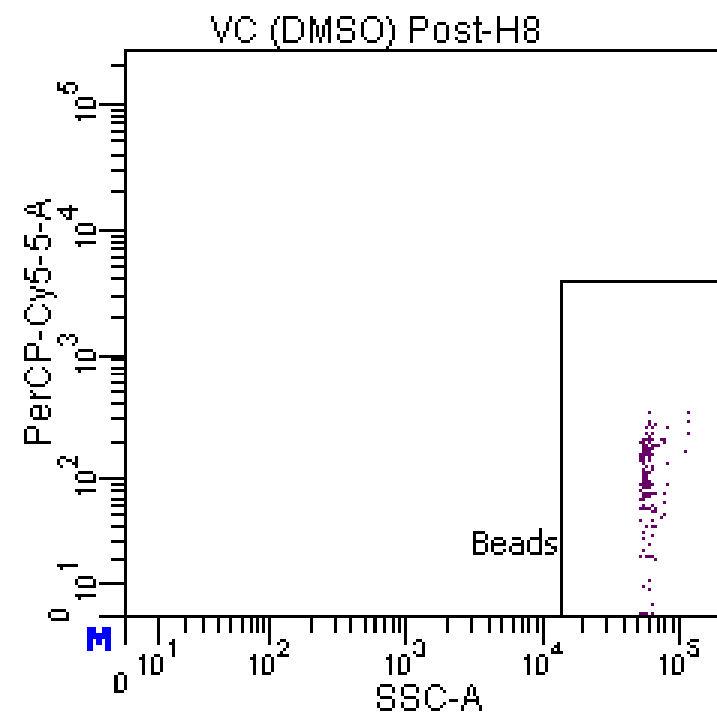
- Three test conditions evaluated:
 - > Short-term (3-6 hours) $\pm$ S9
 - > Continuous exposure (24-27 hours) –S9
- At least 3 exposures from each test condition analyzable
- Top analyzable exposure levels meet 1 of these requirements:
 - > 50-60% cytotoxicity
 - > Precipitate at end of exposure
 - > Use of limit dose
 - 2000 μ g/mL or 10 mM (whichever is lower) for chemicals
 - 500 μ g/mL or 1 mM (whichever is lower) for pharmaceuticals (ICH guidance)

Example MicroFlow Data

EMA-Positive (NA Dye 1) Events

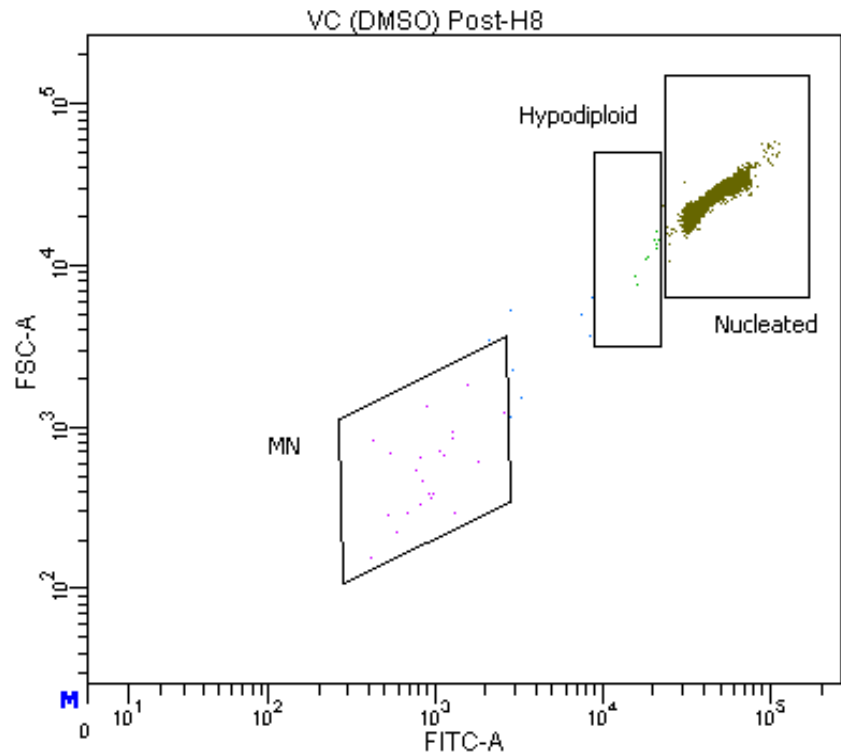


Bead Count

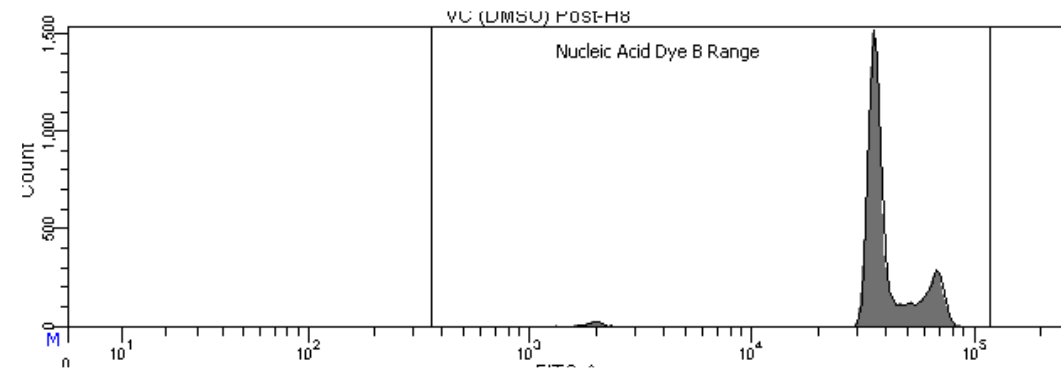


Example MicroFlow Data – Vehicle Control

Nuclei, Micronuclei, and Hypodiploid

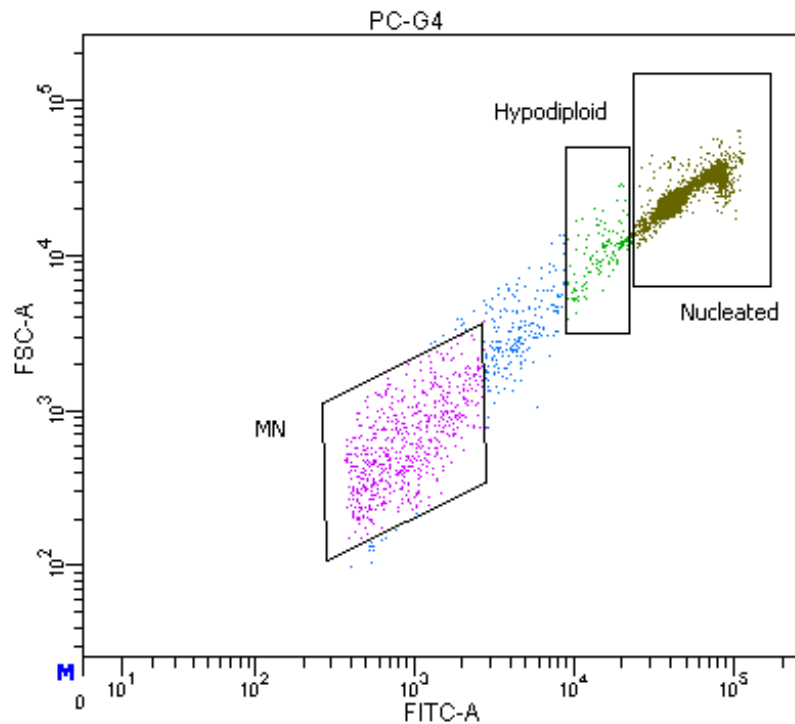


Cell Cycle

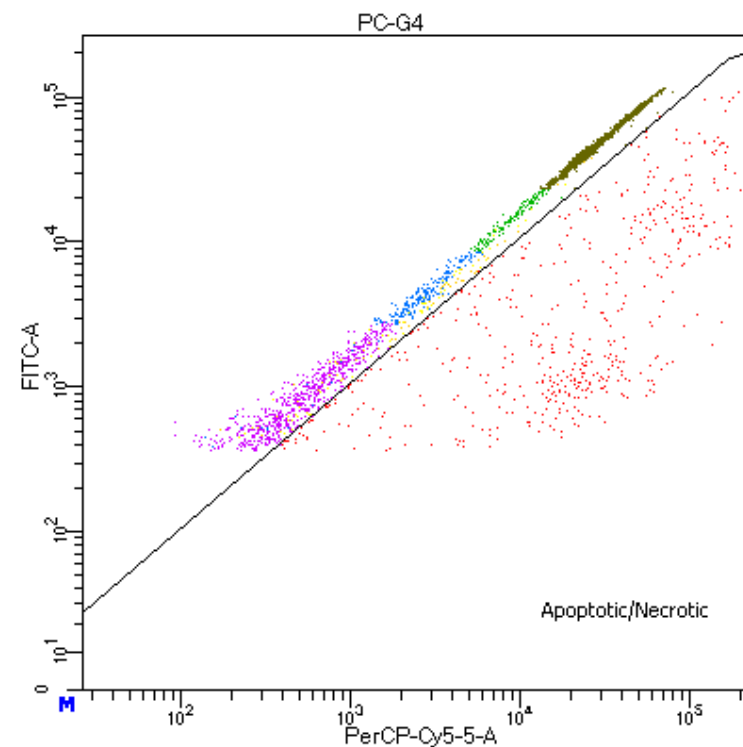


Example MicroFlow Data – Positive Control

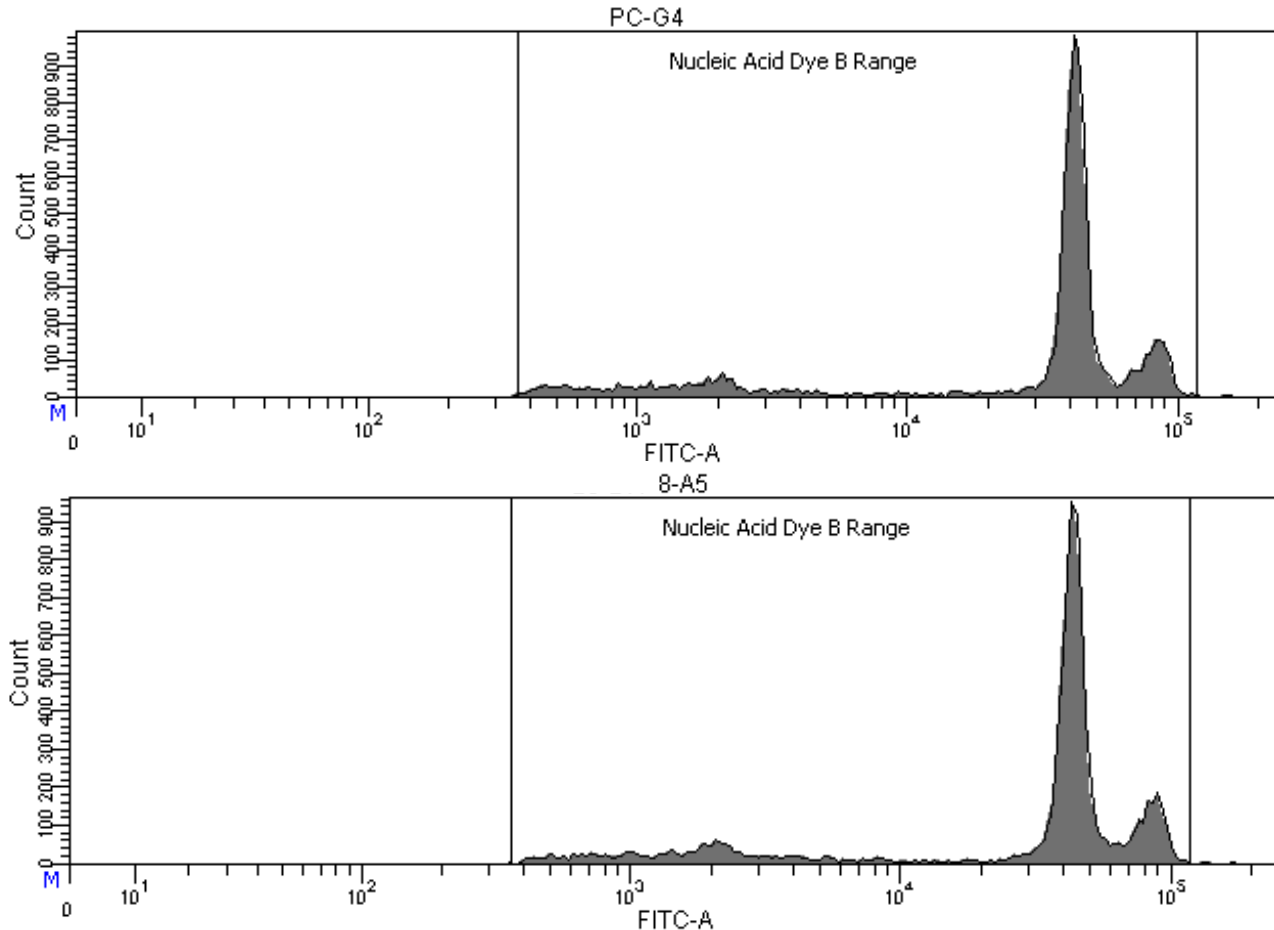
Nuclei, Micronuclei, and Hypodiploid



EMA-Positive (NA Dye 1) Events



Example MicroFlow Data – Aneugens



Cell cycle diagram
of PC - vinblastine

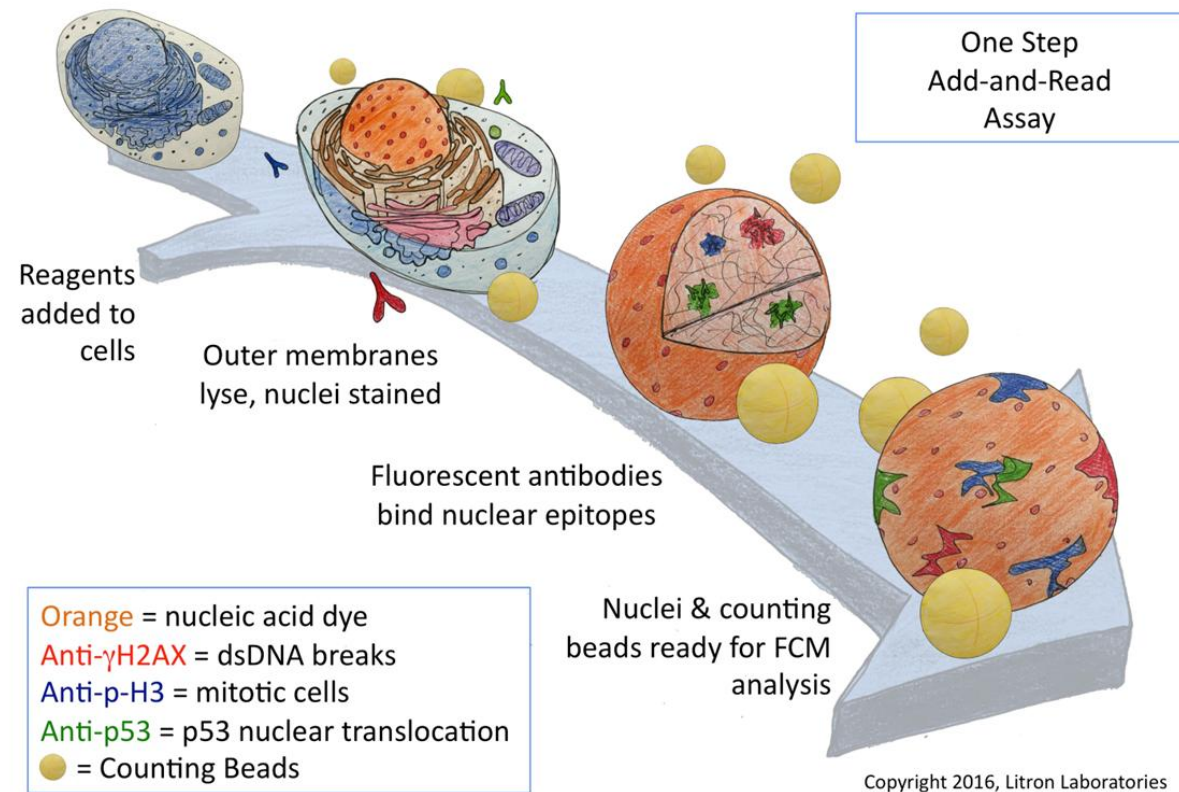
Cell cycle diagram of TA
that subsequently
flagged as aneugenic in
the MultiFlow assay

Example MicroFlow Data

Tabulated Data – Group Averages

Concentration (µg/mL)	Relative Survival (%)	# MN Events	# Nucleated Events	%MN	Fold %MN	%Apoptotic/ Necrotic	Fold %Apoptotic/ Necrotic
0 (Control)	100.00	28	5000	0.55	1.00	1.70	1.00
Dose 1	110.21	27	5000	0.54	0.98	1.30	0.76
Dose 2	109.10	35	5000	0.71	1.28	1.40	0.82
Dose 3	95.61	90	5000	1.81	3.27	2.27	1.33
Dose 4	93.46	111	5000	2.22	4.02	2.43	1.43
Dose 5	82.11	315	5000	6.31	11.41	4.67	2.75
Dose 6	62.92	556	5000	11.11	20.11	7.17	4.22
Pos. Control	68.07	566	5000	11.31	20.47	7.33	4.31

MultiFlow DNA Damage Assay



MultiFlow Assay Procedure

- TK6 cells diluted to approximately $2 \times 10^5/\text{mL}$ and plated at $198 \mu\text{L}/\text{well}$ in 96-well plates
- Chemicals prepared in the vehicle- 20 concentrations with a square root of 2 concentration spacing. Treatments added at $2 \mu\text{L}$ for a final treatment volume of 1%. 4 chemicals plus positive and negative controls on a single plate.
- Cells incubated at 37°C and 6% CO_2 . Cells sampled at 4 and 24 hours post-treatment.
- At each time point, cell aliquot ($25 \mu\text{L}$) and MultiFlow kit reagent ($50 \mu\text{L}$) containing lysing agent, fluorescently-labeled antibodies, RNase, propidium iodide, and counting beads mixed and incubated at room temperature for 30 minutes.

MultiFlow Assay Procedure

- Data collected on a FACS Canto™II flow cytometer equipped with a BD™ High Throughput Sampler (HTS).
- FCS files transferred to Litron for analysis. Data converted to readable format and first tier data QC performed using FlowJo® software. Fold-change values relative to co-plated vehicle controls generated and data filtered by cytotoxicity ($\geq 20\%$ relative nuclei counts at 24 hours) are evaluated using an ensemble of 3 machine learning algorithms (logistic regression, random forest, artificial neural network) to generate clastogen and aneugen MOA probability scores. Fold-change values are also compared to Global Evaluation Factors which are biomarker-specific cutoff values previously determined to indicate a significant response in the biomarker.

Sample Plate Layout for Single Test Article

	Multiflow -S9 plate map - single TA											
	1	2	3	4	5	6	7	8	9	10	11	12
A											DMSO	DMSO
B											DMSO	DMSO
C											Carb 50	Carb 12.5
D	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8	Dose 9	Dose 10	Carb 100	Carb 25
E	Dose 11	Dose 12	Dose 13	Dose 14	Dose 15	Dose 16	Dose 17	Dose 18	Dose 19	Dose 20	MMS 125	MMS 31.3
F											MMS 250	MMS 62.5
G											DMSO	DMSO
H											DMSO	DMSO
	Multiflow +S9 plate map - single TA											
	1	2	3	4	5	6	7	8	9	10	11	12
A											DMSO	DMSO
B											DMSO	DMSO
C											CP 50	CP 12.5
D	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8	Dose 9	Dose 10	CP 100	CP 25
E	Dose 11	Dose 12	Dose 13	Dose 14	Dose 15	Dose 16	Dose 17	Dose 18	Dose 19	Dose 20	BaP 50	BaP 12.5
F											BaP 100	BaP 25
G											DMSO	DMSO
H											DMSO	DMSO

PC doses are in μM

Successive TA concentrations differ by a factor of $\text{Sqr}(2)$, that is 1.4142-fold; in this way, every other concentration differs by a factor of 2

MultiFlow Biomarkers

- **γH2AX**
 - > detects DNA double strand breaks
- **Phospho-histone H3**
 - > serves as a mitotic marker
- **P53**
 - > represents a DNA-damage response marker
- **Polyploidy**
 - > Propidium iodide + RNase identifies nuclei with 8n and greater DNA content
- **Cleaved PARP**
 - > represents a marker of apoptosis

Clastogenic biomarkers

- 4hr γH2AX, 4hr p53, 24hr γH2AX, 24hr p53

Aneugenic biomarkers

- 4hr p-H3, 24hr p-H3, 24hr p53, 24hr polyploidy

Criteria for a Valid Assay

- Plates must pass criteria for average bead count and bead fluorescence and solvent and positive controls must meet certain requirements for response
- In addition, each well must also pass criteria for bead counts and fluorescence.
- Wells may also be excluded from analysis for other reasons:
 - > Wells from excessively cytotoxic exposures (relative nuclei counts $\leq 20\%$)
 - > Wells with precipitate
 - > Wells with distorted or aberrant fluorescence patterns caused by debris or other factors

MultiFlow Data Analysis

Machine Learning Models

- Clastogen or aneugen signatures are demonstrated by clastogen or aneugen probability scores, respectively, of
 - > $\geq 80\%$ from 2 consecutive concentrations or
 - > $\geq 90\%$ from 1 concentration
- For the TA to be considered as having an aneugenic or clastogenic signature, an ensemble approach is used, where 2 of the 3 models must agree

Global Evaluation Factors

- Clastogen signatures are demonstrated by fold increases in 2 consecutive concentrations that meet or exceed cutoffs for at least 2 of the following clastogenic responses:
 - > ≥ 1.45 Fold 24 Hour nuclear p53
 - > ≥ 1.40 Fold 4 Hour nuclear p53
 - > ≥ 2.11 Fold 24 Hour γ H2AX
 - > ≥ 1.51 Fold 4 Hour γ H2AX
- Similarly, aneugen signatures must meet or exceed these cutoffs:
 - > ≥ 1.45 Fold 24 Hour nuclear p53
 - > ≥ 5.86 Fold 24 Hour polyploidy
 - > ≥ 1.52 Fold 24 Hour phospho-histone H3
 - > ≥ 1.71 Fold 4 Hour phospho-histone H3

Example of a Clastogenic Signature by ML

Chemical and Top Concentration		% RNC	Clastogen Model			Aneugen Model		
			Random Forest	Neural Net	Linear Regression	Random Forest	Neural Net	Linear Regression
Etoposide 1uM	0.499849	21.27	86.68	0.00	99.88	11.37	0.00	0.00
Etoposide 1uM	0.353393243	26.71	87.01	5.70	99.10	11.36	0.00	0.00
Etoposide 1uM	0.249849023	32.87	84.69	7.94	95.82	13.68	0.00	0.00
Etoposide 1uM	0.176643259	37.82	84.02	63.68	81.73	14.00	0.00	0.00
Etoposide 1uM	0.124886784	39.52	81.52	98.16	49.99	15.47	0.00	0.00
Etoposide 1uM	0.088294956	54.85	62.83	6.94	31.43	28.56	0.00	0.05
Etoposide 1uM	0.062424534	59.91	61.49	0.13	11.06	28.34	0.00	0.10
Etoposide 1uM	0.044134146	62.03	73.03	3.22	6.67	14.37	0.06	1.95
Etoposide 1uM	0.031202841	79.88	54.05	0.14	4.11	23.24	5.79	9.73
Etoposide 1uM	0.022060409	80.32	65.77	42.46	6.42	14.43	5.09	15.97
Etoposide 1uM	0.015596709	78.77	43.59	0.21	1.24	22.88	8.55	25.37
Etoposide 1uM	0.011026873	76.94	59.13	0.83	1.22	22.17	7.91	36.62
Etoposide 1uM	0.007795999	82.43	28.46	0.20	0.30	30.93	17.00	44.41
Etoposide 1uM	0.005511772	84.92	19.84	1.52	0.14	28.35	54.47	39.26
Etoposide 1uM	0.003896822	88.74	13.23	0.47	0.03	24.53	50.84	19.02
Etoposide 1uM	0.002755053	82.58	21.40	0.50	0.03	27.04	56.82	45.75
Etoposide 1uM	0.001947823	85.24	21.00	0.33	0.03	31.13	23.08	48.16
Etoposide 1uM	0.001377111	86.97	21.30	0.16	0.02	31.91	52.91	55.75

Clastogen probability scores
≥80% in at least 2 consecutive
concentrations

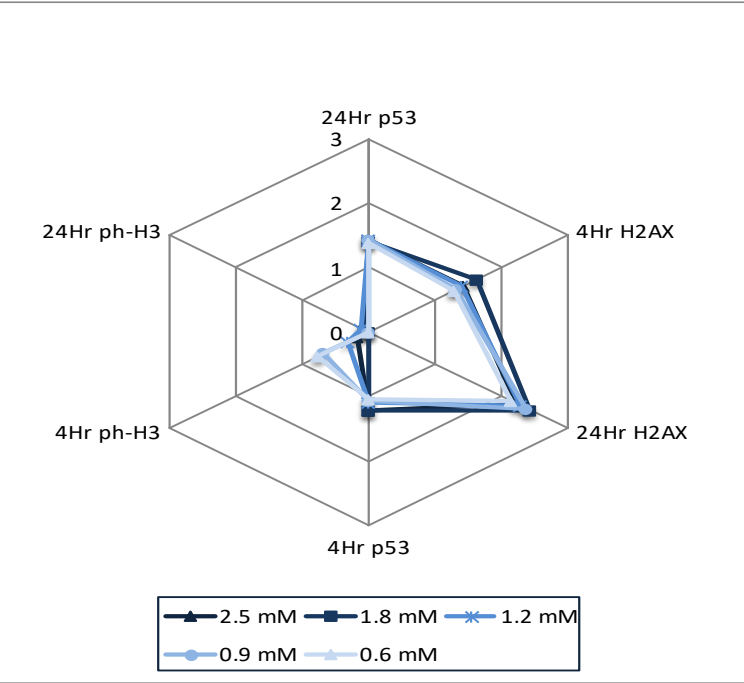
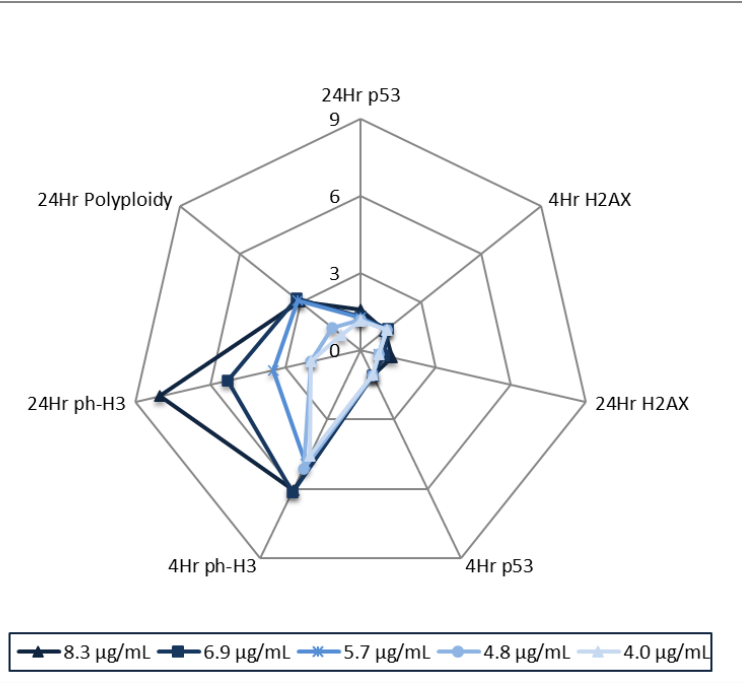
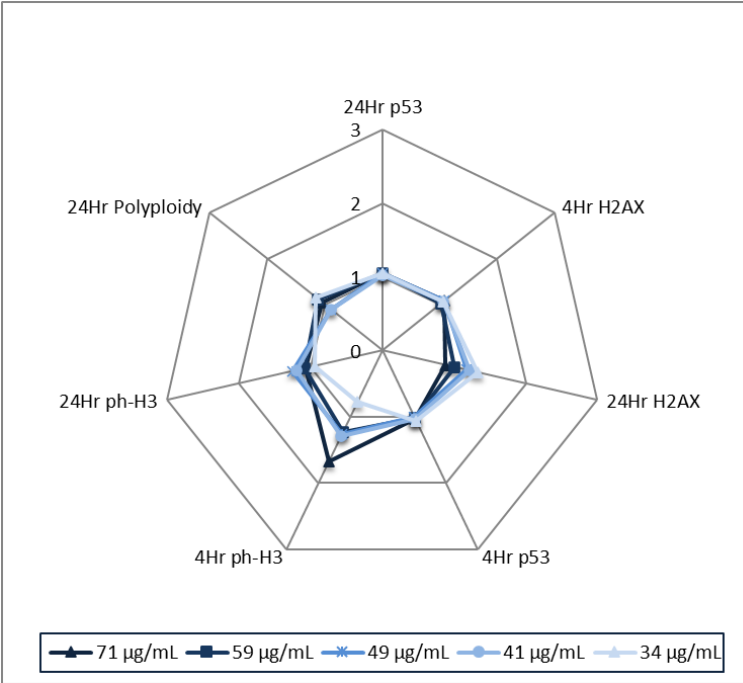
and/or

clastogen probability scores
≥90% in at least 1 concentration

in 2 of the 3 algorithms

= clastogenic response

Radial Plots of a Non-Genotoxicant, an Aneugen, and a Clastogen



Evidence of Genotoxicity?	Genotoxic Mode of Action
No	N/A

Evidence of Genotoxicity?	Genotoxic Mode of Action
Yes	Aneugen

Evidence of Genotoxicity?	Genotoxic Mode of Action
Yes	Clastogen

Explore our Genetic Toxicology Services

