

WHERE
insights LEAD TO
answers

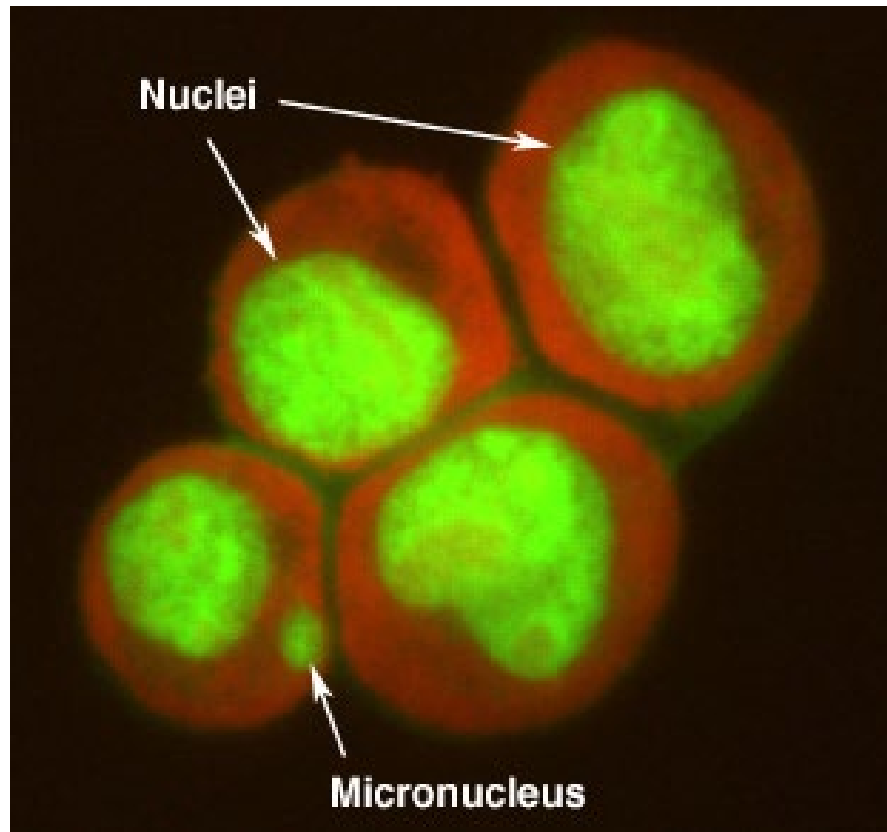
In Vitro Flow Cytometry Assays for Genetic Toxicology at Inotiv

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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 screening or 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

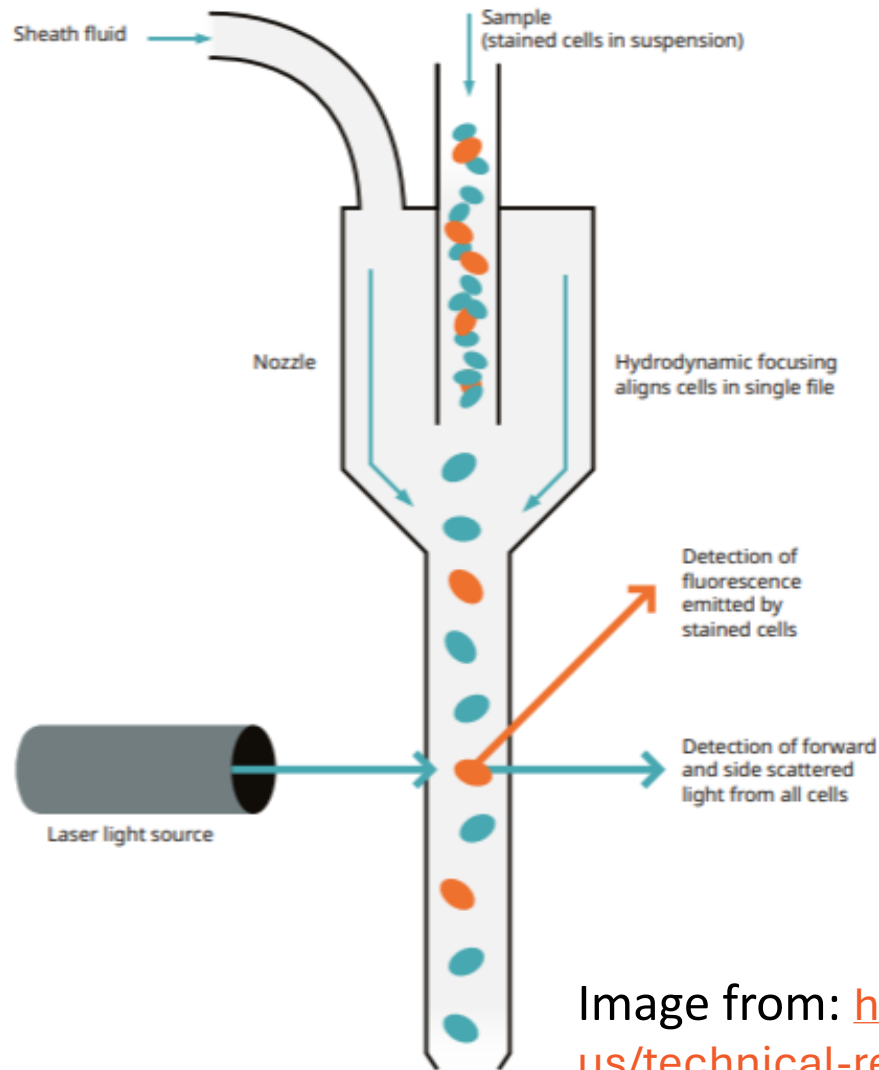
At Inotiv, 2 types of instruments are available for collecting data from genetic toxicology studies by flow cytometry.

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.

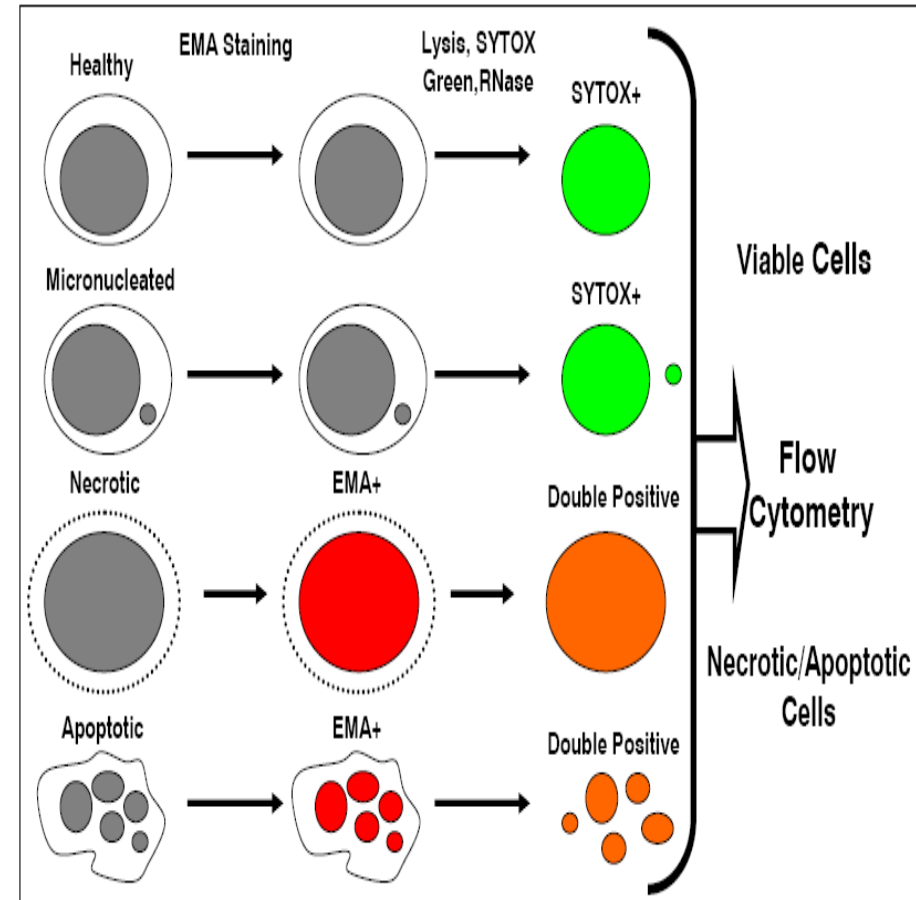
Image from: <https://www.abcam.com/en-us/technical-resources/guides/flow-cytometry-guide/what-is-flow-cytometry>

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	

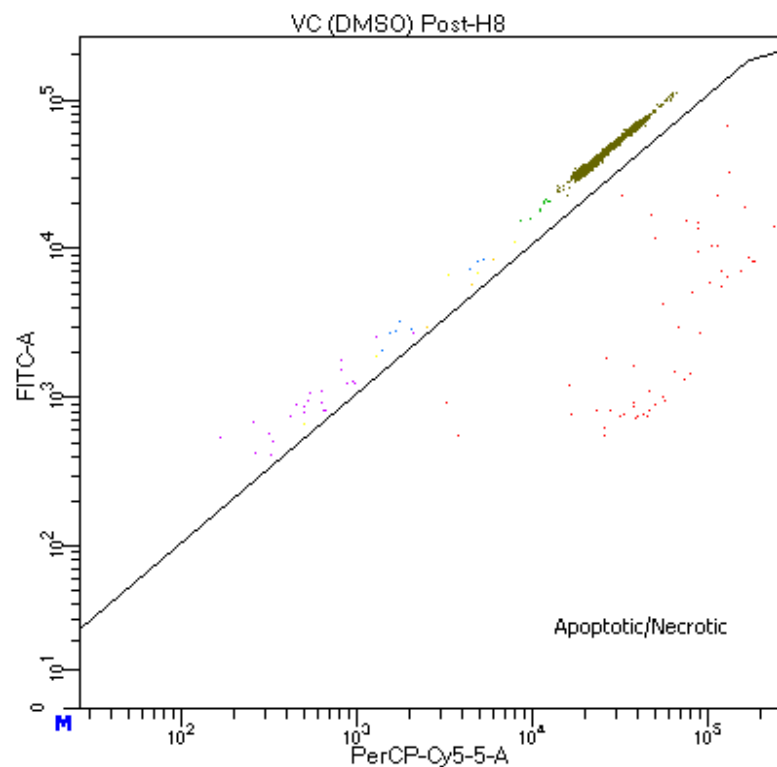
Key	TA - µg/mL
	VC: DMSO
	PC: 27hr-S9: VIN 0.75ng/mL, 1.5ng/mL; 4hr-S9: VIN 5ng/mL, 10ng/mL; 4hr+S9: CP 3µg/mL, 7.5µg/mL

Assay Design – OECD TG487

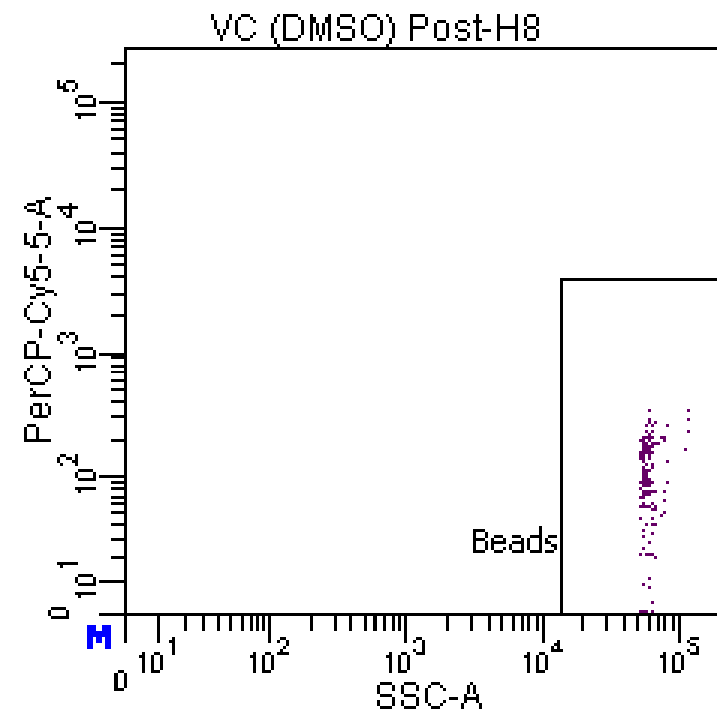
- Three test conditions evaluated:
 - > Short-term (3-6 hours) $\pm$ S9
 - > Continuous exposure (24-27 hours) –S9
- At least 3 exposures from each test condition analyzable
- Top analyzable exposure levels meet 1 of these requirements:
 - > 50-60% cytotoxicity
 - > Precipitate at end of exposure
 - > 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

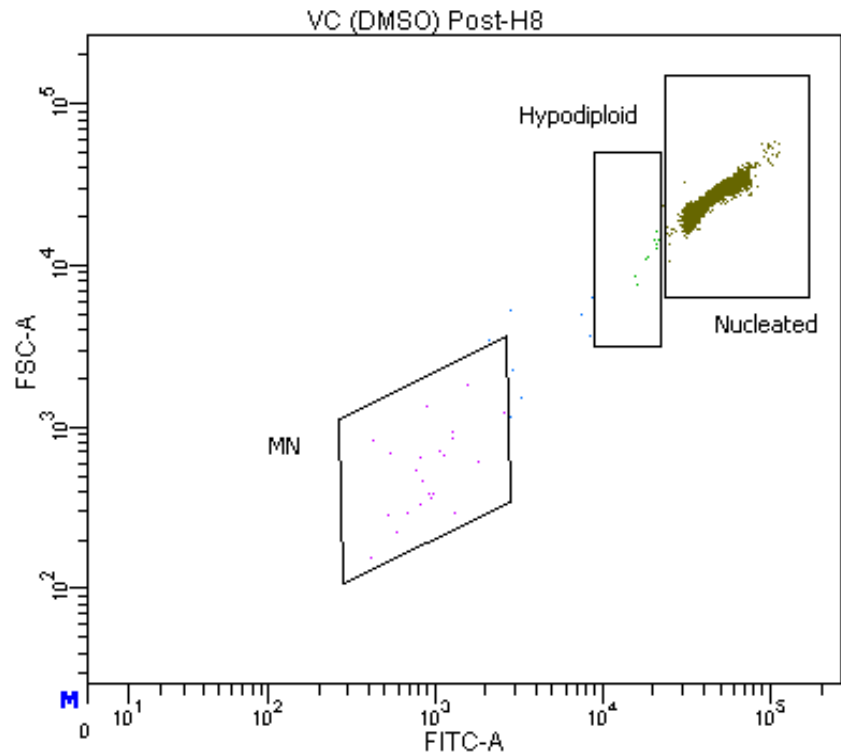


Bead Count

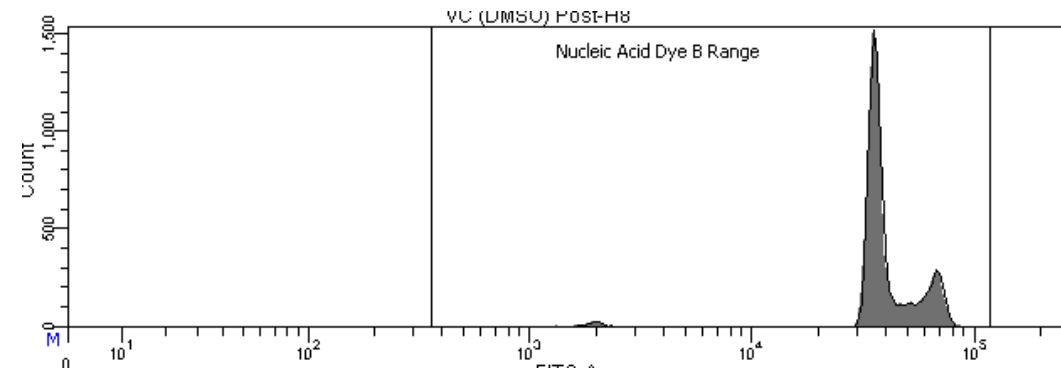


Example MicroFlow Data – Vehicle Control

Nuclei, Micronuclei, and Hypodiploid

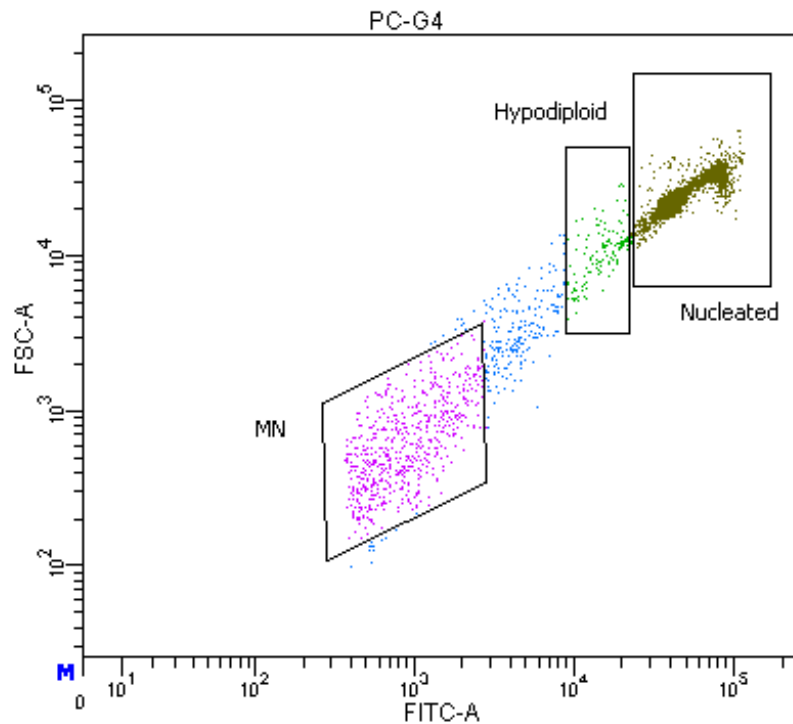


Cell Cycle

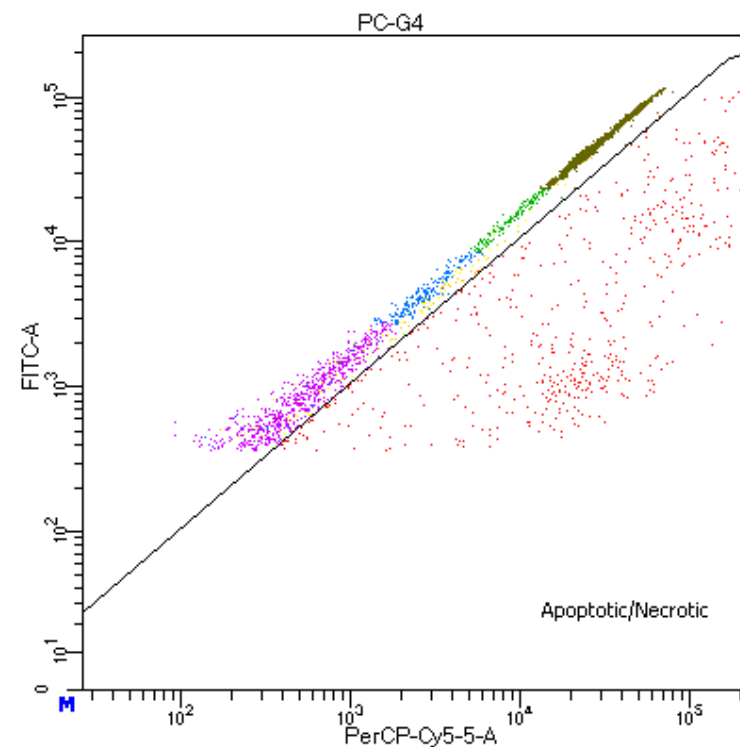


Example MicroFlow Data – Positive Control

Nuclei, Micronuclei, and Hypodiploid



EMA-Positive (NA Dye 1) Events



Example MicroFlow Data

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

Litron MultiFlow[®] Assay

- Flow cytometric assay utilizing TK6 human lymphoblastoid cell line
 - > Advantages
 - Human cell line
 - Cost effective and rapid
 - Detects clastogenic and aneugenic markers
 - GOOD FOR LEAD SELECTION
 - > Disadvantage
 - No OECD test guideline

Litron MultiFlow® Screening Assay

- Detects multiple biomarkers
 - > Translocation of p53 into the nucleus (DNA damage, cell stress)
 - > Phosphorylation of gamma-H2AX (DNA double strand breaks)
 - > Phosphorylation of histone H3 (mitotic cells)

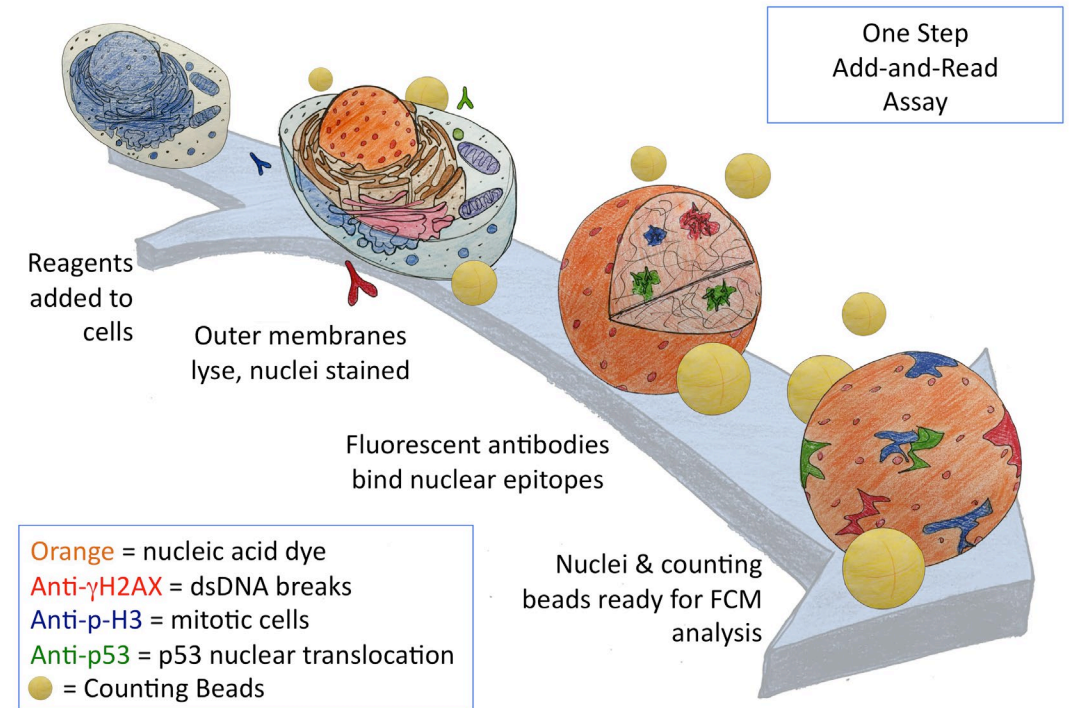
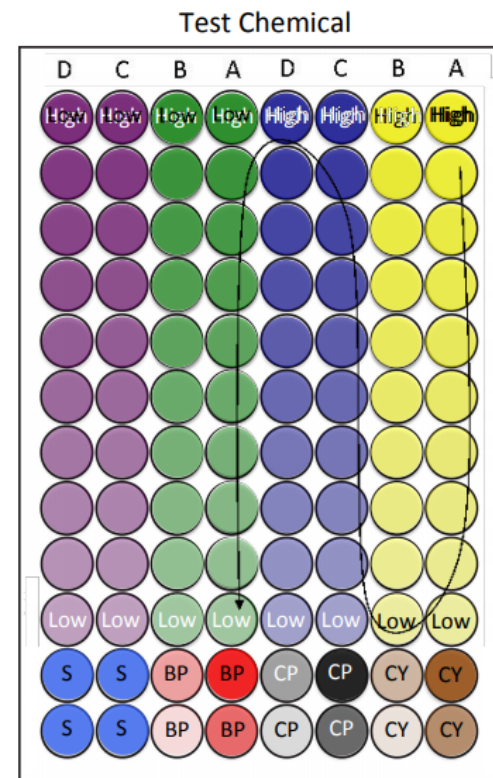
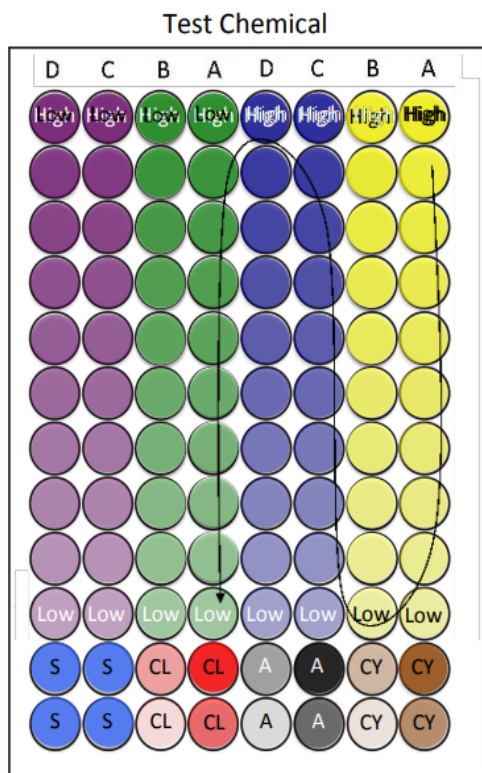


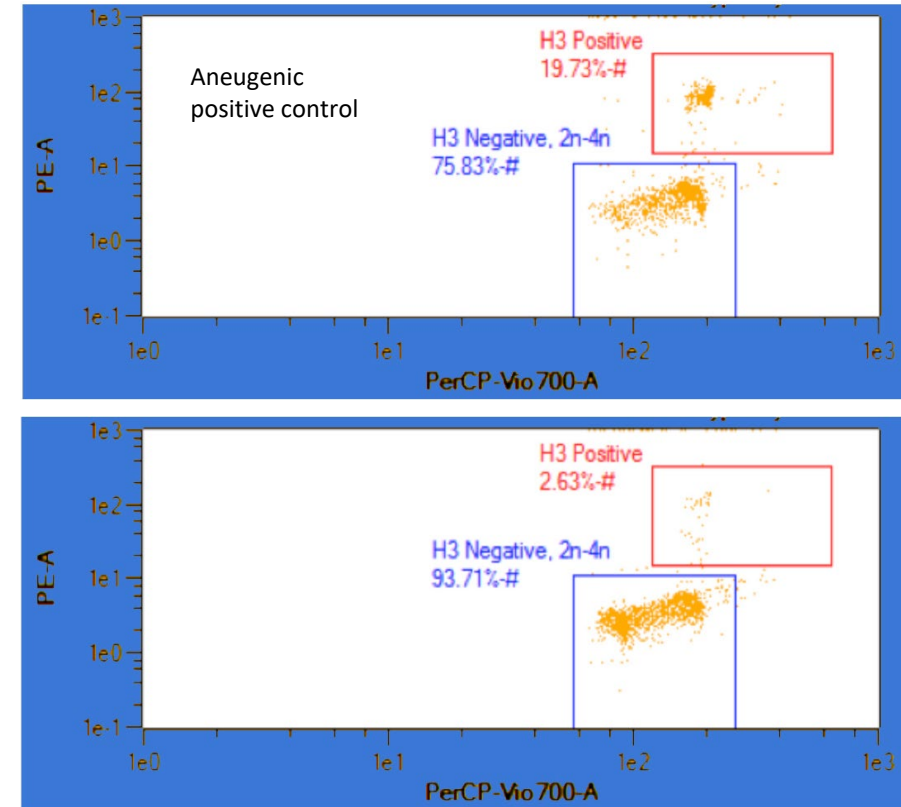
Illustration: Litron Labs

Assay Design



Data Processing and Analysis

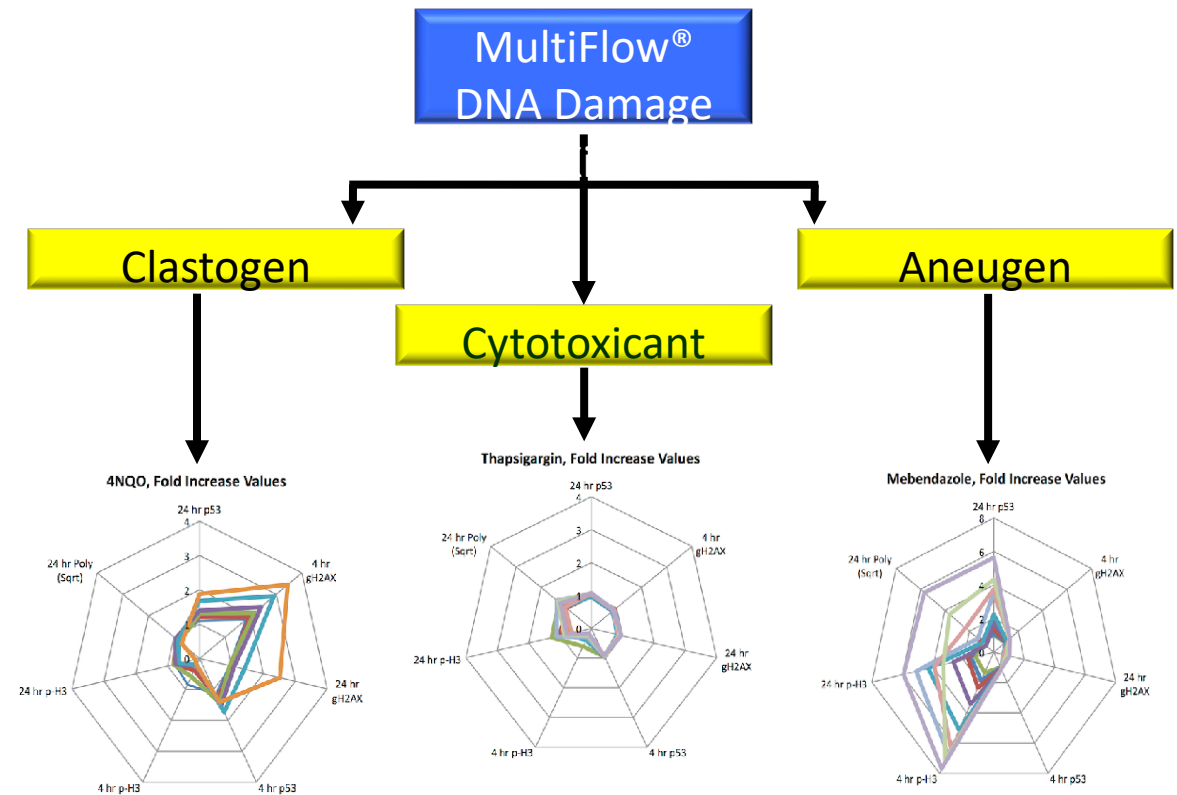
- Data sent to Litron for QC and analysis
- QC parameters include:
 - > Flow cytometer settings and gate positions
 - > Bead counts and fluorescence quality of entire plate
 - > Assessment of vehicle and positive controls
 - > Cytotoxic dose levels excluded from analysis



Litron Labs

Machine Learning used to predict MoA

- Biomarker responses at each dose level analyzed through three machine learning models: Multinomial logistic regression (LR), artificial neural network (ANN), and random forest (RF).
 - > Models are lab specific and based on initial testing and training sets of compounds with known MOAs
- Probability scores assigned:
 - > Genotoxic with clastogenic MOA
 - > Genotoxic with aneugenic MOA
 - > Non-genotoxic



Analysis using Global Evaluation Factors

- Threshold factors for biomarkers/time points developed at Litron compared to responses at each dose level:
 - For clastogens (-S9)
 - ≥ 1.51 Fold 4 Hour γ H2AX
 - ≥ 2.11 Fold 24 Hour γ H2AX
 - ≥ 1.40 Fold 4 Hour nuclear p53
 - ≥ 1.45 Fold 24 Hour nuclear p53
 - For clastogens (+S9)
 - ≥ 1.44 Fold 4 Hour γ H2AX
 - ≥ 1.31 Fold 24 Hour γ H2AX
 - ≥ 1.23 Fold 4 Hour nuclear p53
 - ≥ 1.12 Fold 24 Hour nuclear p53
 - For aneugens (-S9)
 - ≥ 1.71 Fold 4 Hour phospho-histone H3
 - ≥ 1.52 Fold 24 Hour phospho-histone H3
 - ≥ 5.86 Fold 24 Hour polyploidy
 - ≥ 1.45 Fold 24 Hour nuclear p53

A test article can be evaluated as genotoxic with an aneugenic or clastogenic MOA if either Machine Learning Models or GEFs predict it.

Validation of MultiFlow

5-Fluorouracil
Methyl methanesulfonate
Stauvudine
AMG-900
Carbendazim
Griseofulvin
Crizotinib
Vinblastine
Brefeldin A
CCCP
Cycloheximide
Tunicamycin

Etoposide
Hydroxyurea
Methotrexate
MNNG
Mebendazole
Nocodozole
Tozasertib
ZM-447439
Clofibrate
Hexachloroethane
Imatinib mesylate
Phenanthrene

Clastogens

Aneugens

Non-genotoxics

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

Thank you!



Questions?