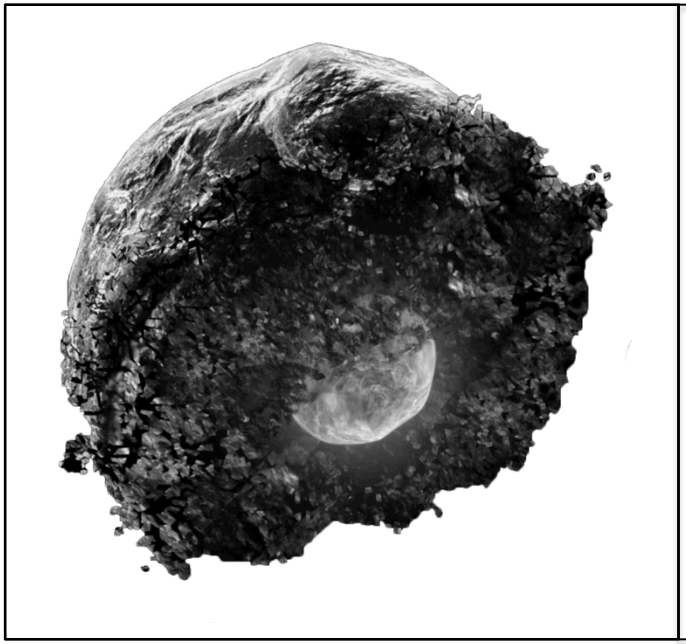
Application of a Multiplexed Flow Cytometric Assay and Machine Learning to Provide Genotoxic Mode of Action Information



Jeffrey C. Bemis, PhD
Director of Clinical Studies

Litron Laboratories

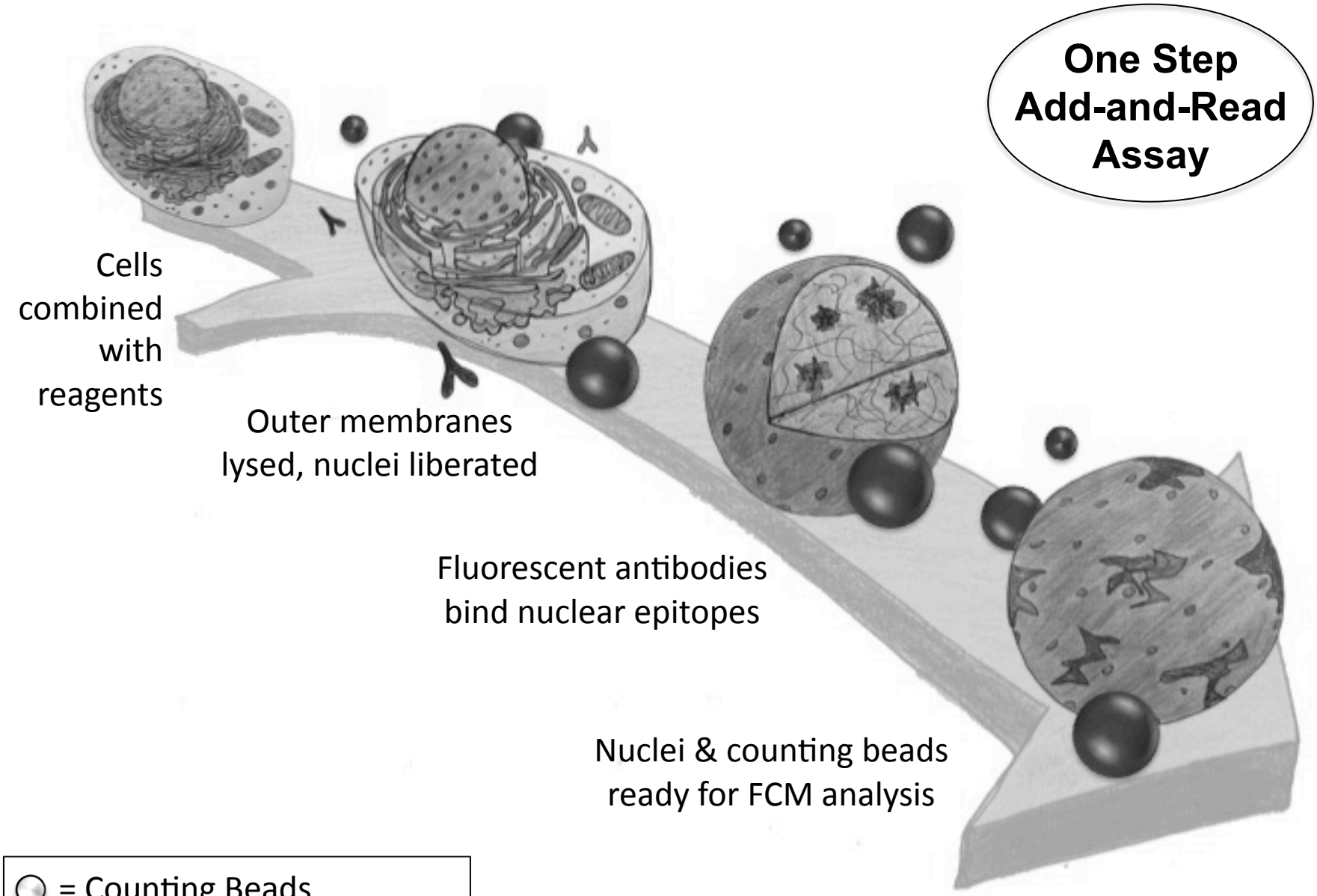
This presentation contains unpublished data – please do not distribute

Today's Theme

- More efficient, higher information content assays...
  - improve compound screening
  - complement regulatory-required assays
  - expand range of information

Coming soon!

MultiFlow™ Method



MultiFlow™ Reagents

REAGENT	PURPOSE
Detergent	Liberates nuclei for analysis
Nucleic acid dye + RNase	Cell cycle and polyploidization
Latex microspheres at a known density	Facilitates absolute nuclei counts, a measure of cytotoxicity
Anti-phospho-H3-PE	Metaphase cell marker
Anti-γH2AX-Alexa 647	DNA double strand break marker
Anti-p53-FITC (human only)	Genotoxicity marker, specific to nuclear translocated target
Anti-Cleaved PARP	Apoptosis

* combination kits

single Ab kits ←

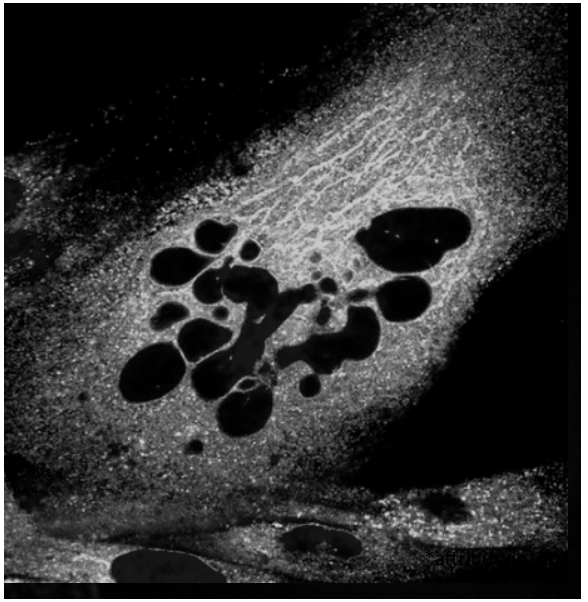
Case Study:

H2AX, Phospho-Histone H3, p53 kit - to determine genotoxic Mode of Action

Bryce SM, Bernacki DT, Bemis JC, Dertinger SD. Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach. Environ Mol Mutagen. 2016 Apr;57(3):171-89.

Definitions

- Clastogens
  - DNA double strand breaks
- Aneugens
  - malsegregation of chromosome(s)
  - polyploidy

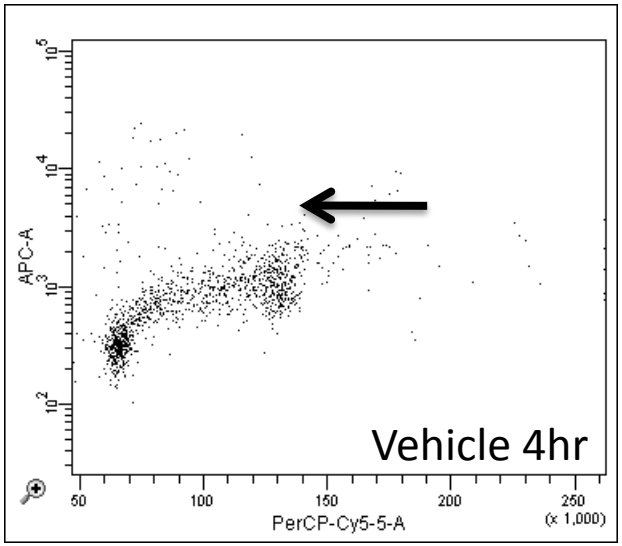


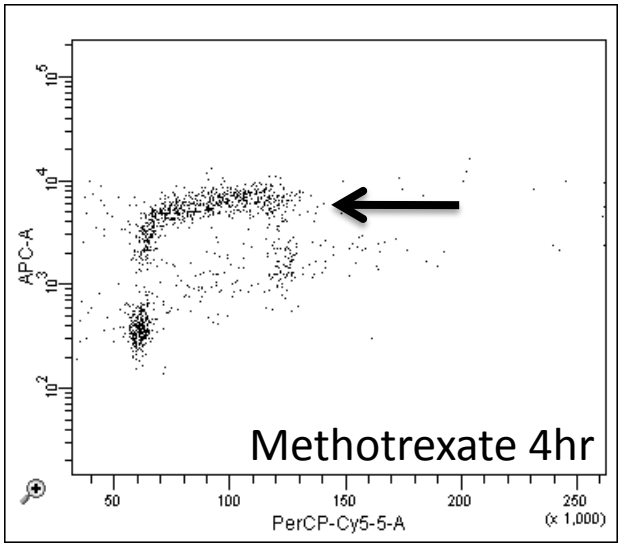
- Non-genotoxicants
  - may be cytotoxic
  - may damage DNA through secondary pathways (apoptosis, damage to lysosomes, etc)

*By Joerg Shoreer, Princeton University. http://www.princeton.edu/artofscience/gallery2006/view.php%3Fid=24.html

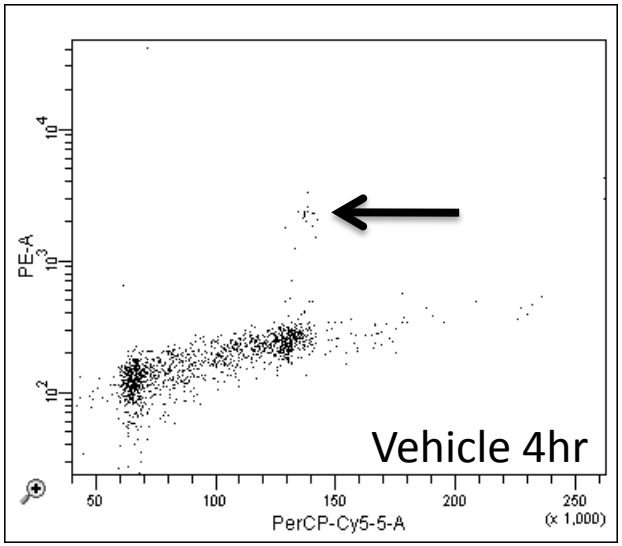
Example flow plots

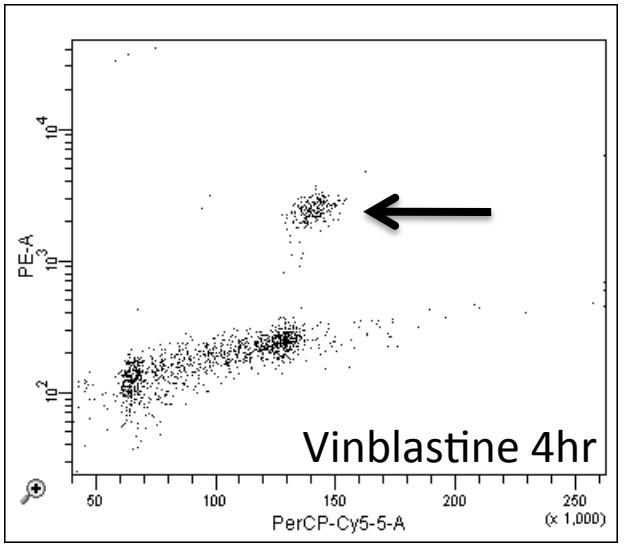
γH2AX





Phospho-histone H3

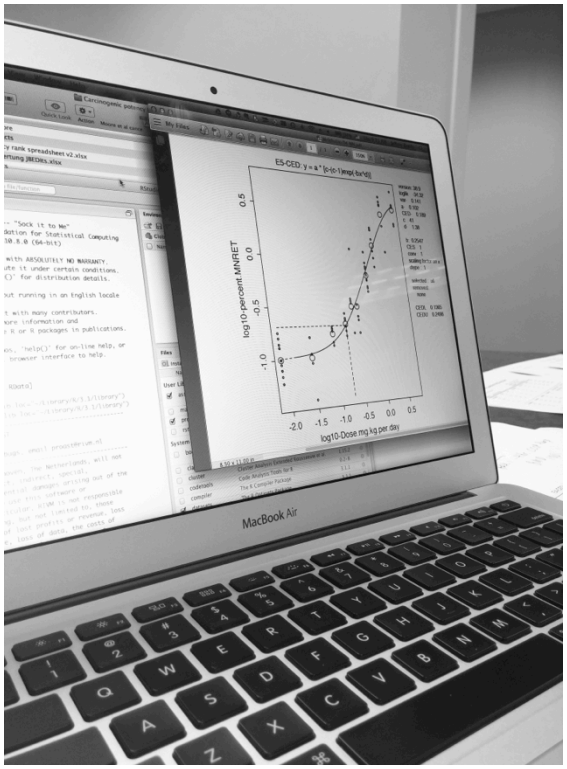




Experimental Design

- 79 chemicals from ECVAM published list and expert consensus
  - 14 aneugens, 27 clastogens, and 38 non-genotoxicants
- Treated TK6 cells in 96 well plates; solvent usually DMSO; top concentration 1 mM
  - extreme cytotoxicity or precipitation dictated some top conc.
- Up to 20 closely spaced concentrations (Sqr2), no replicate wells
- Concurrent vehicle and positive controls
- 24 hrs continuous exposure
- Study an early and late time point: 4 & 24 hr

Overview



Select Chemical Training Set & Exp. Design

ID Candidate Biomarkers

- Wald statistic
- McFadden's R^2
- ROC

Select Biomarkers *via* Univariate L-R

Optimize FCM Multiplexed Assay

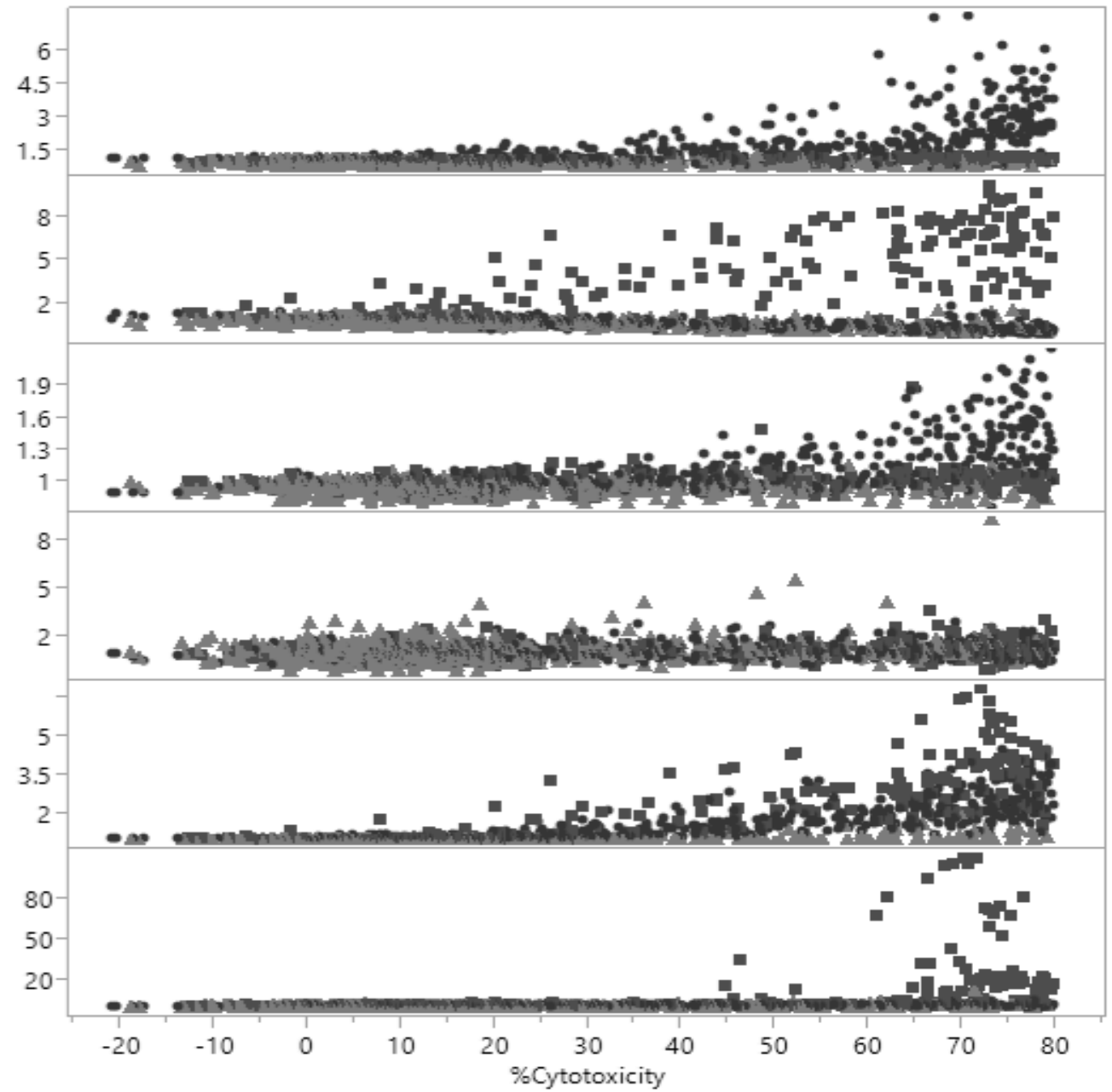
- Wald statistic
- McFadden's R^2
- ROC

Build Multinomial L-R Model (Forward Selection)

Cross Validation

Interlab Validation

○ Clastogens □ Aneugens △ Non-Genotoxicants



Valuable for Class Discrimination?

YES

YES

YES/NO

NO

YES

YES

Univariate L-R Analyses

Based on Wald statistic, McFadden's R^2 and ROC values

- 4 and 24 hr γH2AX are predictive of class
- 4 and 24 hr p-H3 are predictive of class
- 24 hr p53 is predictive of class
- 24 hr polyploidy is predictive of class
- Note: why provisionally 4 hrs when both 4 & 24 hr are predictive of class? There may be theoretical advantages to studying early effects that are not confounded by secondary processes

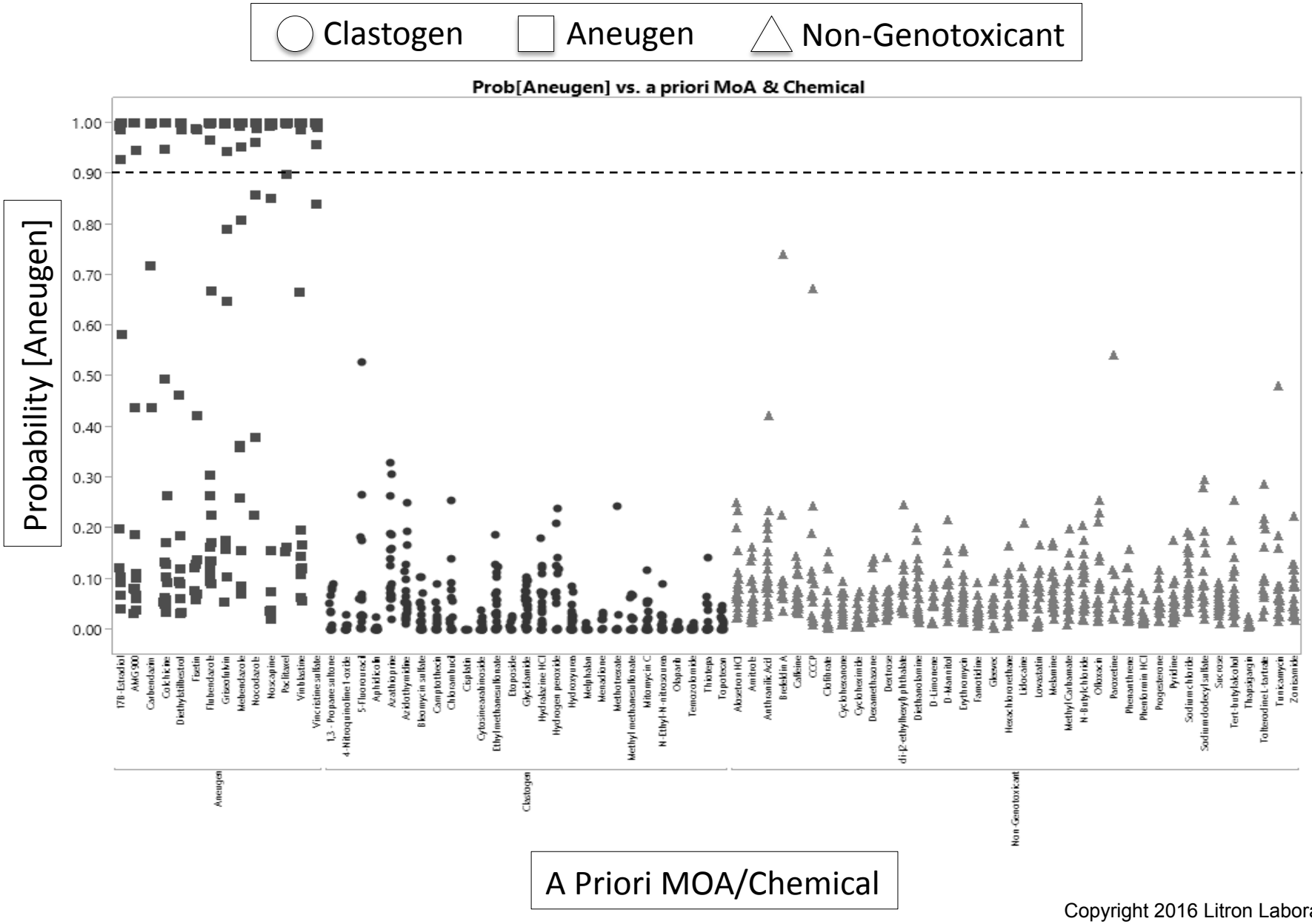
Summary Data

Endpoints	Wald	Pseudo R^2	ROC
γH2AX (4hr)	< 0.0001	0.2442	≥ 0.6464
p-H3 (4hr)	< 0.0001	0.2854	≥ 0.7572
p53 (24hr)	< 0.0001	0.3021	≥ 0.7181
Polyploidy (24hr)	< 0.0001	0.0960	≥ 0.5264
All 4 endpoints	≤ 0.0020	0.5502	≥ 0.8944
All 4 weighted on cytotoxicity	< 0.0001	0.7459	≥ 0.9596

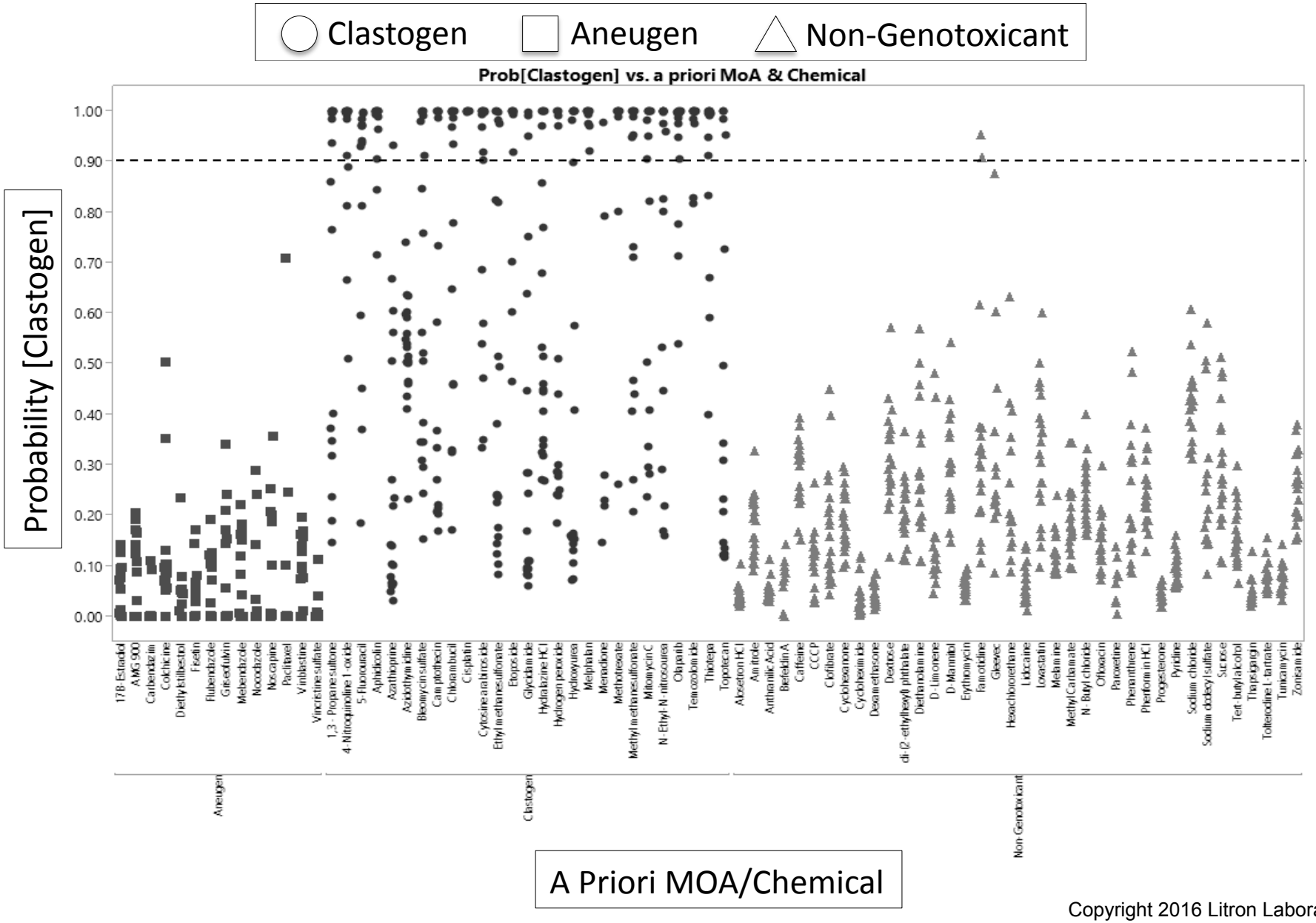
Synthesizing L-R Model Output

- Since L-R model is using data from every conc. the output consists of multiple probability scores—one for each conc.
- For a clastogen or aneugen call we require two consecutive concentrations to show ≥ 90% probability
- Non-genotoxicant call = no concentration showing ≥ 90% probability clastogen or aneugen
- Equivocal = only one concentration showing ≥ 90% probability of clastogen or aneugen

79 Chemical L-R Model (Aneugen Probability vs Chemical)



79 Chemical L-R Model (Clastogen Probability vs Chemical)



Performance of 4-Factor Model

- 79 chemicals, 75 correct, 2 equivocal
- 14/14 aneugens correctly classified
- 24/27 clastogens correctly classified
  - Misidentified AZT; azathioprine & menadione equivocal
- 37/38 non-genotoxicants correctly classified
  - Misidentified famotidine
- If we consider equivocal calls to be misclassifications, then concordance with *a priori* classification is 94.9%

Cross-Validation: Leave-One-Out

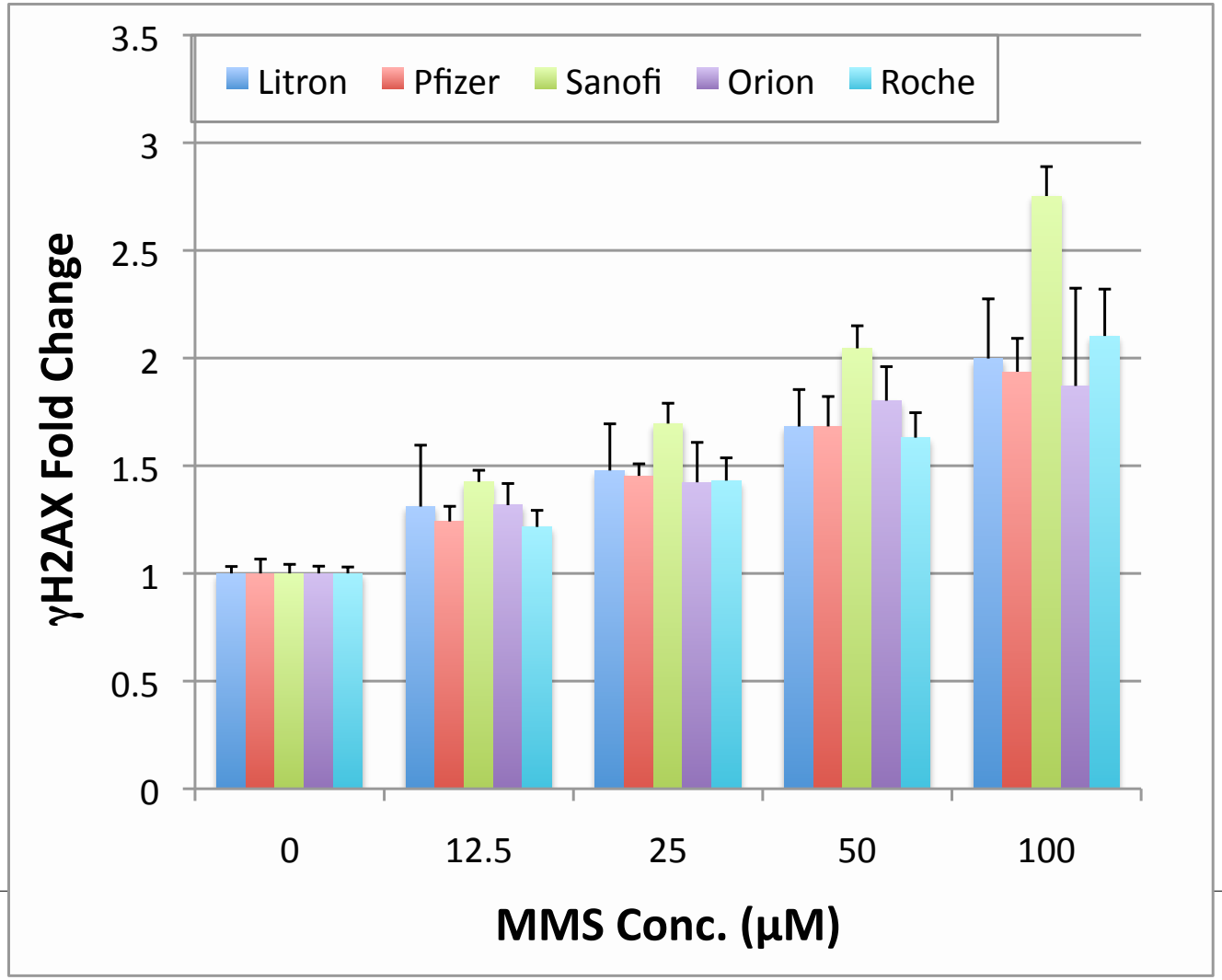
- Model generated with each chemical in turn left out of training set; make class prediction for hold-out
- 79 chemicals, 72 correct, 2 equivocal
- If we consider equivocal calls to be misclassifications, then concordance with *a priori* classification is 91.1%
- High concordance for L-O-O suggests model is not “overfitting” data and that it will be “generalizable” (meaning applicable to chemicals outside of training set)

Interlaboratory Transferability

- Six external collaborating labs received kit-formatted reagents and instructions
- TK6 cells, continuous exposure
- First question—do reference agents elicit expected responses?

Interlab Transferability: γH2AX Example

(only showing labs with n ≥ 4)

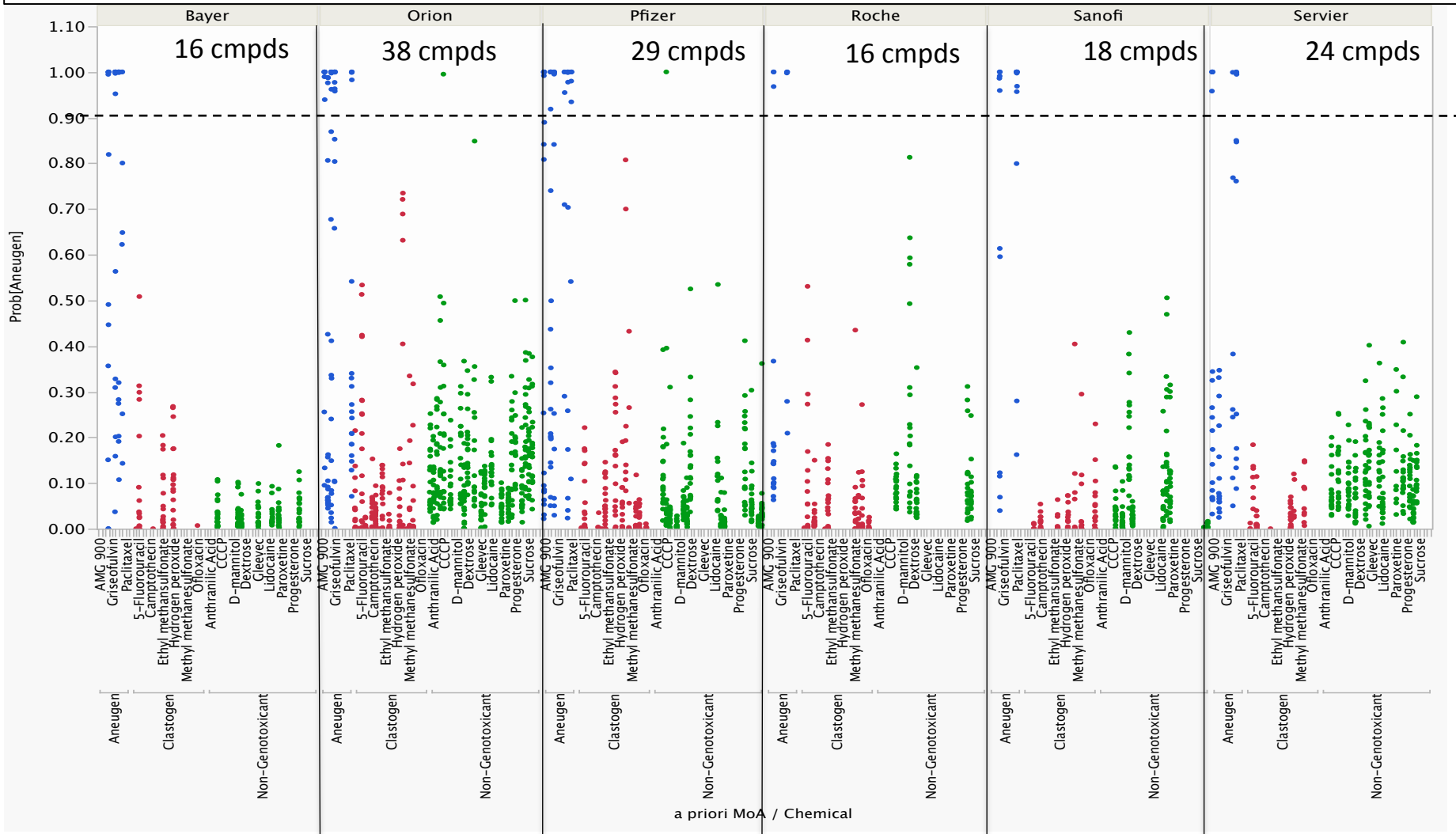


Interlaboratory Transferability

- Six external collaborating labs received kit-formatted reagents and instructions
- TK6 cells, continuous exposure
- First question—do reference agents elicit expected responses?
- Second question—do data from external labs work with reference lab model?
    - Collaborators chose from a list of compounds previously studied at Litron
    - Raw data returned to Litron and evaluated by MoA algorithm

Interlab Transferability: Aneugenicity Predictions

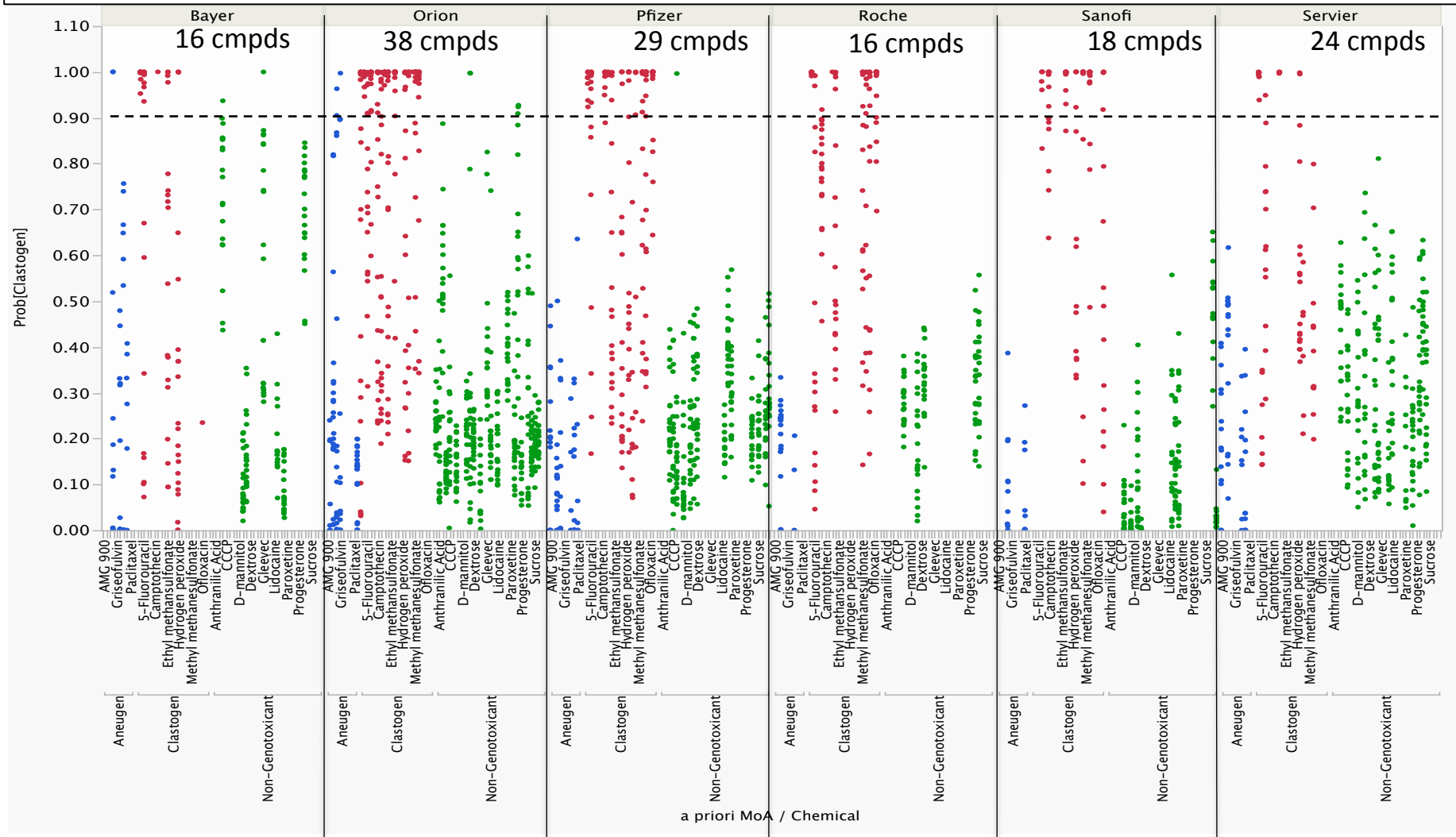
Aneugens Clastogens Non-Genotoxicants



Bayer	Orion	Pfizer	Roche	Sanofi	Servier
4/4 Aneugens	5/5 Aneugens	6/6 Aneugens	2/2 Aneugens	2/2 Aneugens	3/4 Aneugens

Interlab Transferability: Clastogenicity Predictions

Aneugens Clastogens Non-Genotoxicants



Bayer	Orion	Pfizer	Roche	Sanofi	Servier
5/5 Clastogens	13/13 Clastogens	12/12 Clastogens	8/9 Clastogens	7/7 Clastogens	5/7 Clastogens

Interlab Transferability: Summary

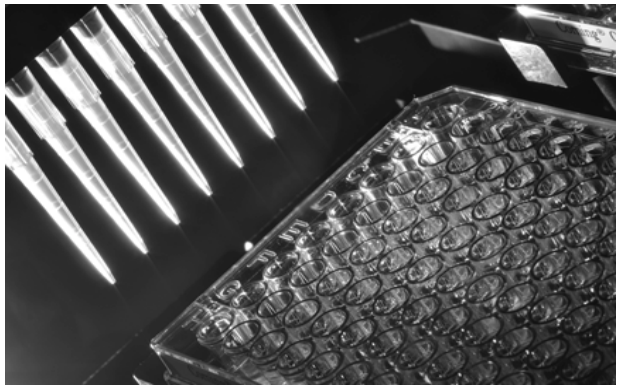
Lab	Total	Aneugens	Clastogens	Non-Genotoxicants	A Priori
Pfizer	29	6/6	12/12	9/11	93%
Sanofi	18	2/2	7/7	9/9	100%
Roche	16	2/2	8/9	5/5	93%
Orion	38	5/5	13/13	17/20	92%
Bayer	16	4/4	5/5	5/7	88%
Servier	24	3/4	5/7	13/13	88%

Final steps:

- collect and analyze all data
- explore methods of comparison
- publish/present outcomes

Conclusions

- Multiplexed genetox screening
  - reduced to a simple, one-step procedure
  - robust signals provides MoA classification
- Interlab transferability study
  - 6 external labs show excellent performance
- Future efforts
  - additional endpoints
  - expanded chemical sets
  - inter-lab studies



Acknowledgements

- Steve Bryce, Derek Bernacki, Svetlana Avlasevich, and Steve Dertinger

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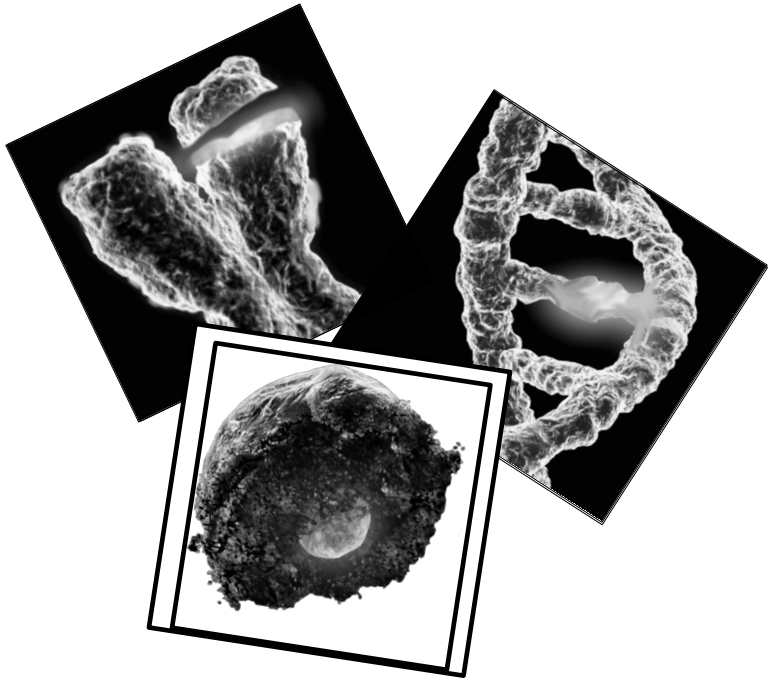
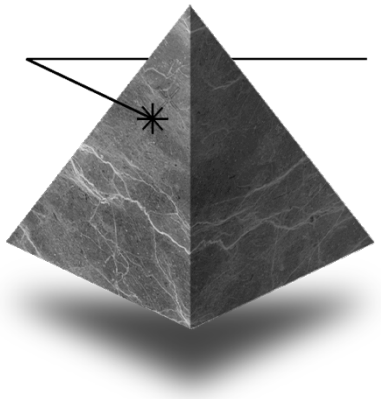


Thank you!

- Questions – jbemis@litronlabs.com



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Quality counts at the speed of light