Stemina

BIOMARKER DISCOVERY

from cell culture to patients • stemina.com

Elizabeth L.R. Donley, JD, MBA, MS
Chief Executive Officer
Gabriela Gebrin Cezar, DVM, PhD
Chief Scientific Officer

About Stemina Biomarker

History

- Founded in 2006
- Raised $2.6 million in seed funding 2007
- Raised $1 million angel funding 2009

Expertise

- Human Embryonic Stem Cell culture
- Identification of small molecules
- Metabolomic biomarkers of toxicity and disease

Products

- devTOX™ only hES cell-based birth defect screen; **NOW AVAILABLE**
- cardiomyocytes – **available Q1 2010**
- cancer stem cells – **available Q2 2010**

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The Stemina Advantage

Uses Human Embryonic Stem Cells to:

- Differentiate physiologically relevant efficacy/toxicity targets
- Recapitulate cell pathways revealing mechanisms & candidate biomarkers

Purpose:

- High throughput ID of human biomarkers
- Reduce compound attrition due to safety concerns
- Reduce *in vivo* animal testing

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Key Employees

Stemina
BIOMARKER DISCOVERY

PAUL R. WEST Ph.D; Director Bio-Analytical Chemistry
- *20 years mass spectrometry experience*
- *15 years at Abbott*

ALAN SMITH Ph.D; Senior Scientist Computational Biology
- *Developed metabolomics platform*
- *Dr. Cezar's post-doc*

APRIL M. WEIR MS*; Senior Scientist Cell Biology Biochemistry*
- *hES cell culture, experimental design*
- *Project management*

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devTOX assay™

Predicting Developmental Toxicity

Stemina's devTOX Assay

- Metabolomics + hES Cells
- Initially developed in a 6-well format
- Current efforts to transition assay to a high-throughput format (96-well)

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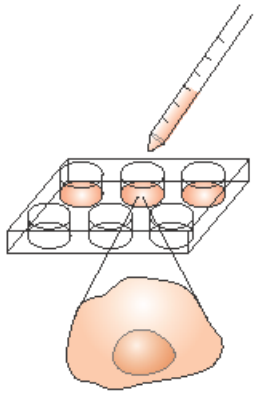
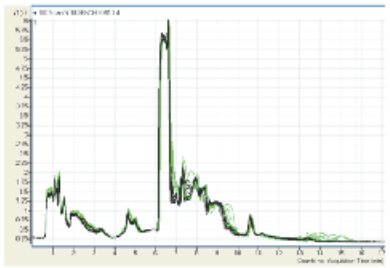
devTOX Methods



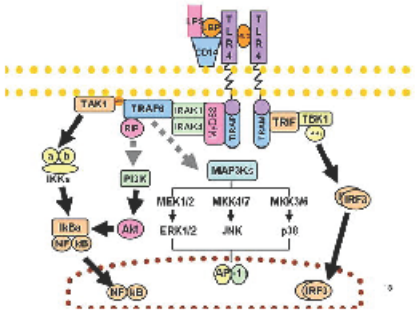
Dose hES cells with drugs of known teratogenicity

Metabolites are secreted in response to treatment



Metabolomic profiling

Identify Biomarkers and use for:
- toxicity assay
- diagnostic tools



Identify biochemical pathways to understand disease mechanisms and use for:
- disease models
- disease treatments

April M. Weir

1. Culture hES cells
2. Dose hES cells
3. Collect and quench spent medium
4. Prepare samples for MS
5. Perform MS on samples
6. Perform statistical analysis on MS features to identify biomarkers
7. Annotate and validate biomarkers
8. Track biomarkers to pathways

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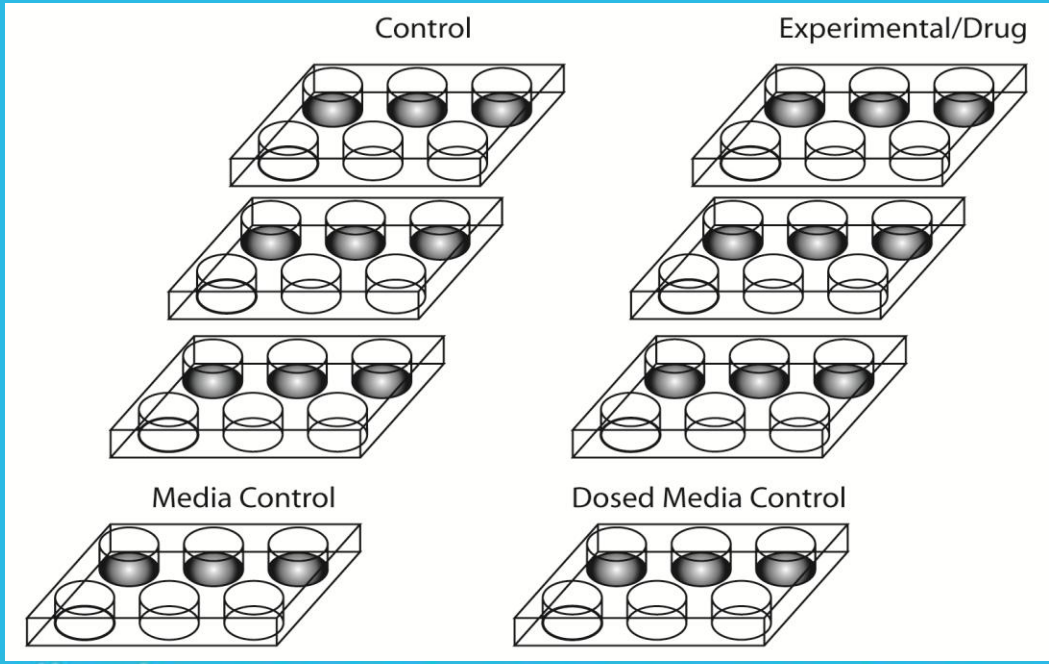
6 Well Experimental Design

devTOX assay™

- One cell line H9 and one compound per analysis
- 9 replicates, 3 wells per plate
- Control, dosed, media control and dosed media
- Dosed at published circulating dose



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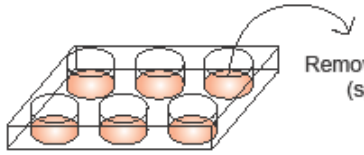
96-well vs 6 well Sample Preparation

devTOX assay™

96-well centrifugal device
• 10 KDa MWCO

In a clean, empty 96-well plate (Eppendorf, round bottom) add 133 uL of ACN and 200 uL of the spent media from each well of culture plate into corresponding wells of empty plate.
Remove contents (333uL) of quenched sample and add to washed Millipore Ultracel-10 filter plate
Place filter plate in the centrifuge at 4^{O}C for 200 minutes at 2000 x g
After centrifuging, throw away the filter plate and place collection plate in the Speed Vac to dry samples overnight.
After samples are dried, reconstitute sample in 70 uL of 50:50 ACN:0.1% Formic Acid.
Add the reconstituted sample to an Agilent pointed bottom 96-well plate analysis.

Single sample centrifugal device
• 3 KDa MWCO



	Quench Spent Medium	Exclude Proteins	Concentrate and Reconstitute
			
Material	15 mL Conical Tube	5KDa MWCO column 3KDa MWCO column :s and microfuge tubes	Sample vial & insert Cap & Septa
Solvent	Quenching Agent	Sterile Water	Mass Spec Solvent
Protocol	Collect spent medium and quench to stop metabolic activity.	Dilute sample in high molecular weight cut-off column, centrifuge, and discard column.	Dry sample and reconstitute with a solvent amenable to mass spectrometry.

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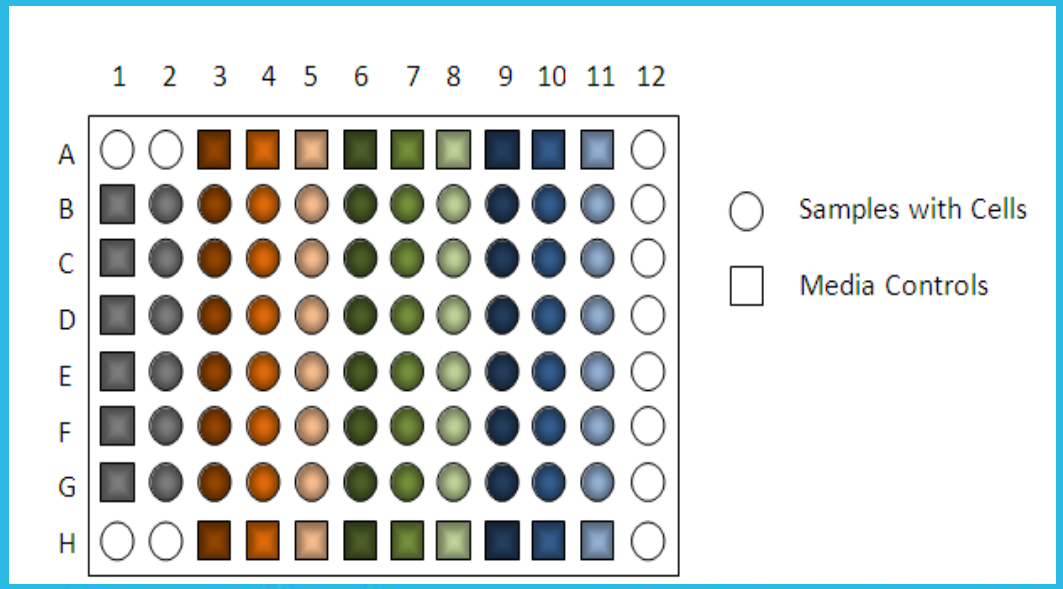
96 Well Experimental Design

- Three compounds per plate
- Dosed at circulating dose, 10x above, and 10x below
- Control, dosed, media control and dosed media
- 6 replicates per dose with 3 doses allow for visualization of fold changes over a broader range



Symbol	Samples	Sample Name
	Media Controls	ST003F-95-1B-G
	Controls	ST003F-95-2B-G
	VPA Dosed Media Control	ST003F-95-3A/3H
	Valproic Acid ([1.66 mg/mL])	ST003F-95-3B-G
	VPA Dosed Media Control	ST003F-95-4A/4H
	Valproic Acid ([0.166 mg/mL])	ST003F-95-4B-G
	VPA Dosed Media Control	ST003F-95-5A/5H
	Valproic Acid ([0.0166 mg/mL])	ST003F-95-5B-G
	Cytosine Arabinoside Dosed Media Control	ST003F-95-6A/6H
	Cytosine Arabinoside ([2.19 ug/mL])	ST003F-95-6B-G
	Cytosine Arabinoside Dosed Media Control	ST003F-95-7A/7H
	Cytosine Arabinoside ([0.219 ug/mL])	ST003F-95-7B-G
	Cytosine Arabinoside Dosed Media Control	ST003F-95-8A/8H
	Cytosine Arabinoside ([0.0219 ug/mL])	ST003F-95-8B-G
	Doxylamine Dosed Media Control	ST003F-95-9A/9H
	Doxylamine ([1.03 ug/mL])	ST003F-95-9B-G
	Doxylamine Dosed Media Control	ST003F-95-10A/10H
	Doxylamine ([0.103ug/mL])	ST003F-95-10B-G
	Doxylamine Dosed Media Control	ST003F-95-11A/11H
	Doxylamine ([0.0103 ug/mL])	ST003F-95-11B-G

6-well vs. 96-well Summary

devTOX assay

Feature	6-well	96-well
hES cell culture	Split 1:10	Count 250K cells/well
	2 day wait time prior to dosing	1 day wait time prior to dosing
	H9	H1, H7, H9
Post-Dose Analysis	(none)	Cell Viability
		Differentiation
Sample Preparation	3 KDa MWCO	10 KDa MWCO
	Column	96-well plate
Throughput	2 drugs in 1 week	54 drugs in 1 week

Sensitivity Is Not Compromised in 96-well Format

6 Well sample analysis- 3 x 10^6 cells/well in 2.5 ml media.
~54,000 cells/5ul injection on column.

96 Well sample analysis – 2.5 x 10^5 cells/well in 200 ul media.
~230,000 cells/5 ul injection on column.

4.25 fold increase in overall sensitivity

Mass Spectrometry Optimization for 96-well Format

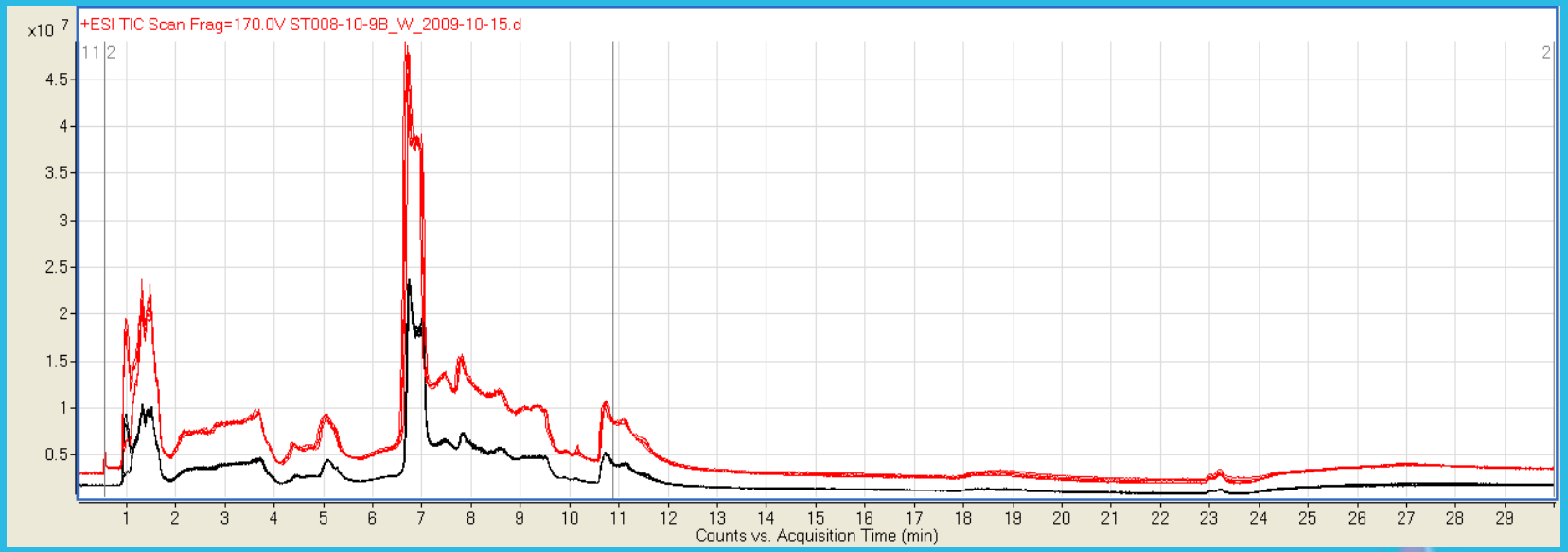
Several variables of the mass spectrometry were optimized

- 'Junk dump' – first 30 seconds of eluent discarded
- Injection Solvent
- Run Time

'Junk Dump'™

devTOX assay

Two-fold increase in sensitivity resulting from the first 30 seconds of LC eluent being diverted to waste



- **Red Chromatograms: First 30 seconds of the run diverted to waste**
- **Black Chromatograms: No diversion to waste**
- **All other method conditions are identical**

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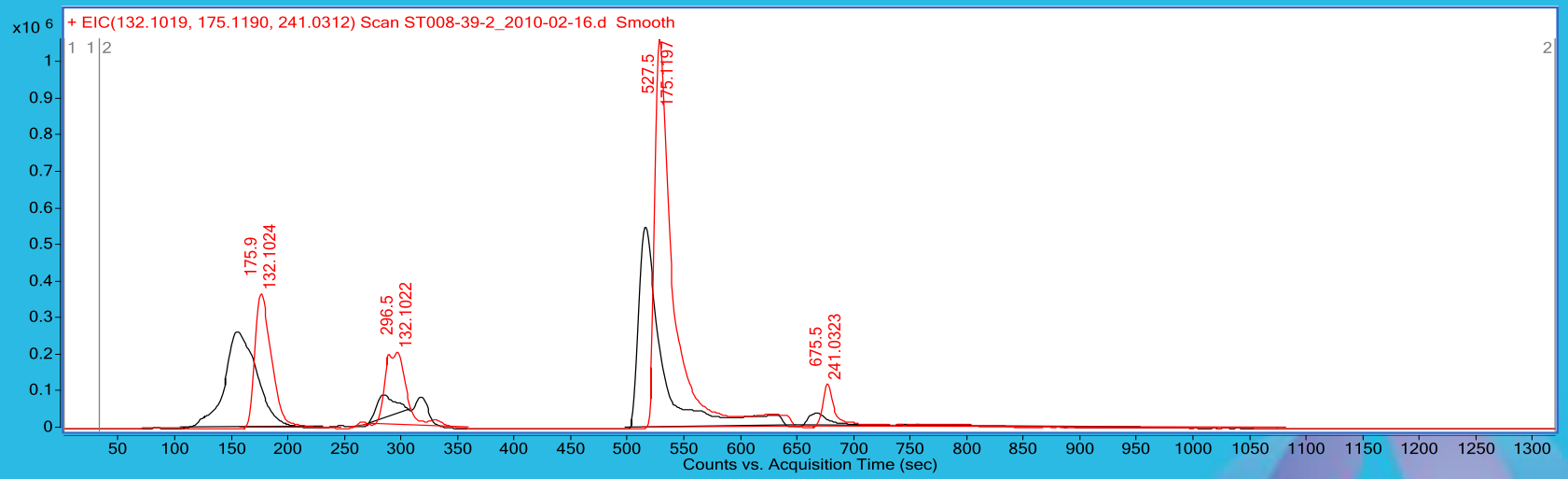
Changing the Injection Solvent Improves Spectrometry

By changing the injection solvent from 0.1% Formic acid to a 50:50 mix of acetonitrile and 0.1% Formic acid an improvement in peak shape, sensitivity, and sample solubility was noted. Also, the less hydrophilic components of the secretome may now be accessible due to the increase in organic solvent.



EIC QC Peaks 0.1% Formic vs 50:50 ACN: 0.1% Formic

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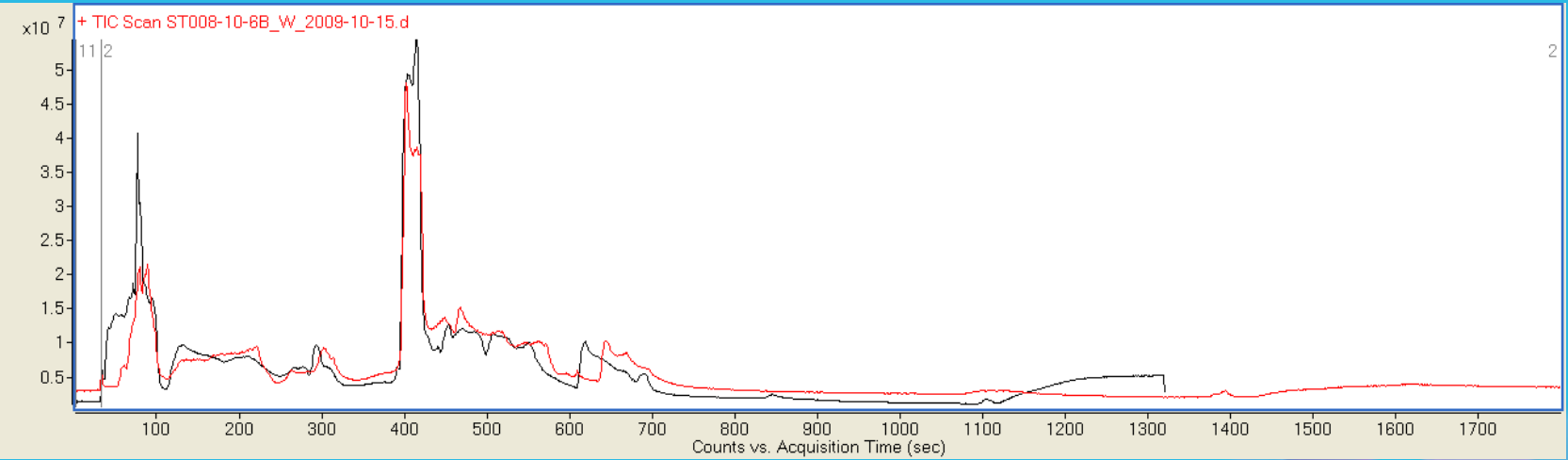
MS Run Time Is Shortened Without Altering the Chromatography

Modify the existing method by ending the gradient after the analytes of interest elute, and then doubling the flow rate during the equilibration step shortens the existing run time by 8 minutes while maintaining the 'history' of the chromatography.



TIC: Normal HILIC analysis vs shortened HILIC analysis

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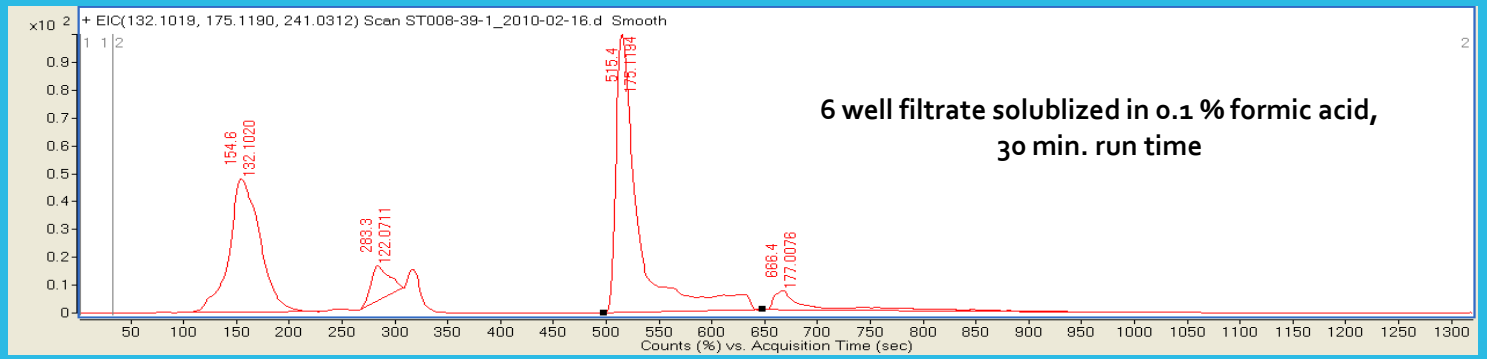
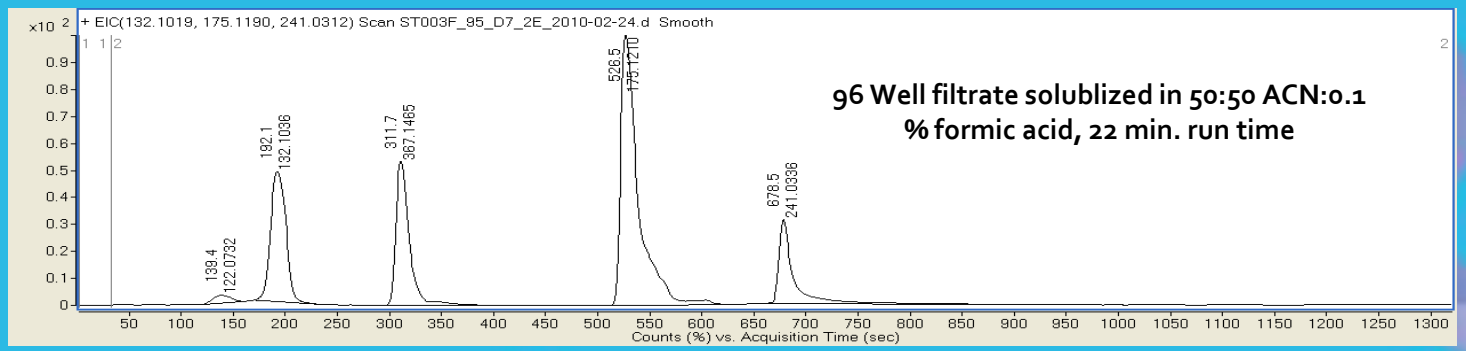
Sensitivity, Reproducibility, and Peak Symmetry is Optimized With Shorter Run Time and New Solvent

Before optimization



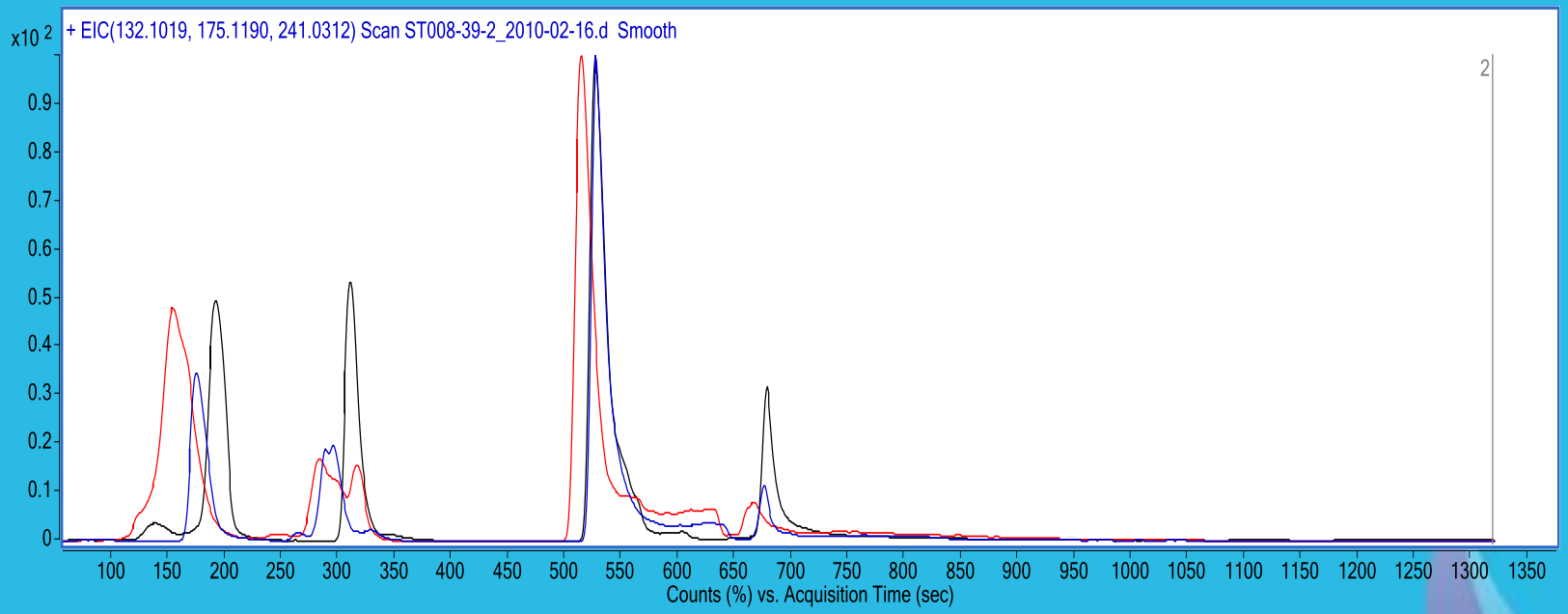
6 well filtrate solublized in 0.1 % formic acid, 30 min. run time

After optimization



96 Well filtrate solublized in 50:50 ACN:0.1 % formic acid, 22 min. run time

Complete Optimization Improves Overall MS Signal



All chromatograms have been normalized to the highest peak.

6 well filtration method with 0.1 % Formic injection solvent

6 well filtration method with 50:50 injection solvent

96 well filtration method with 50:50 injection solvent.

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From Cell Culture to Metabolites

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Secreted Metabolites

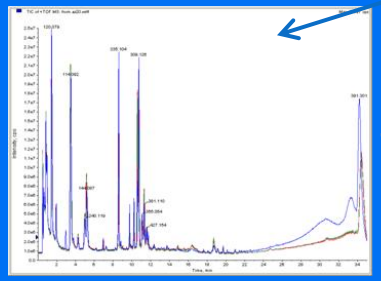
Culture Medium

Small Molecule Preparation

Extracted Metabolites < 10000 Da

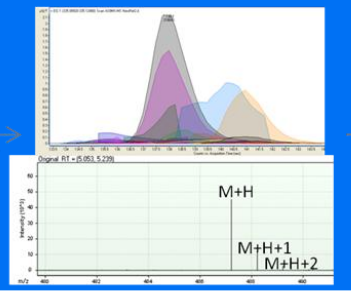
LC-MS analysis

HILIC chromatography
High Mass Accuracy QTOF

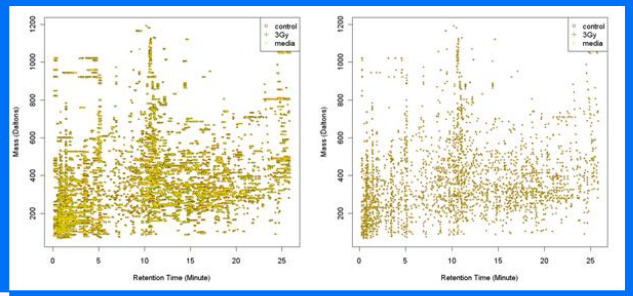


Data Acquisition

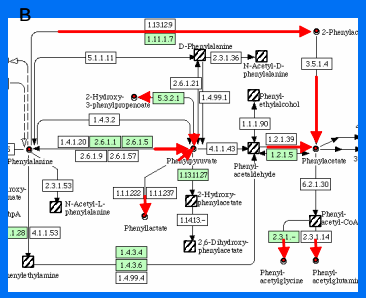
Centroid
Deisotope



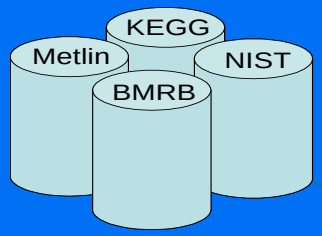
Generate mass features



Data Binning and Alignment



Functional Annotation



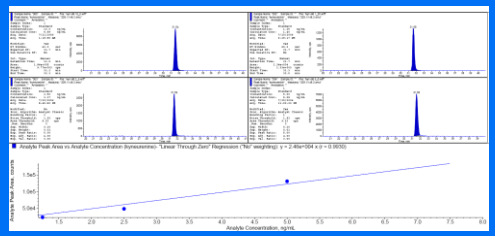
Bioinformatics

Chemoinformatics

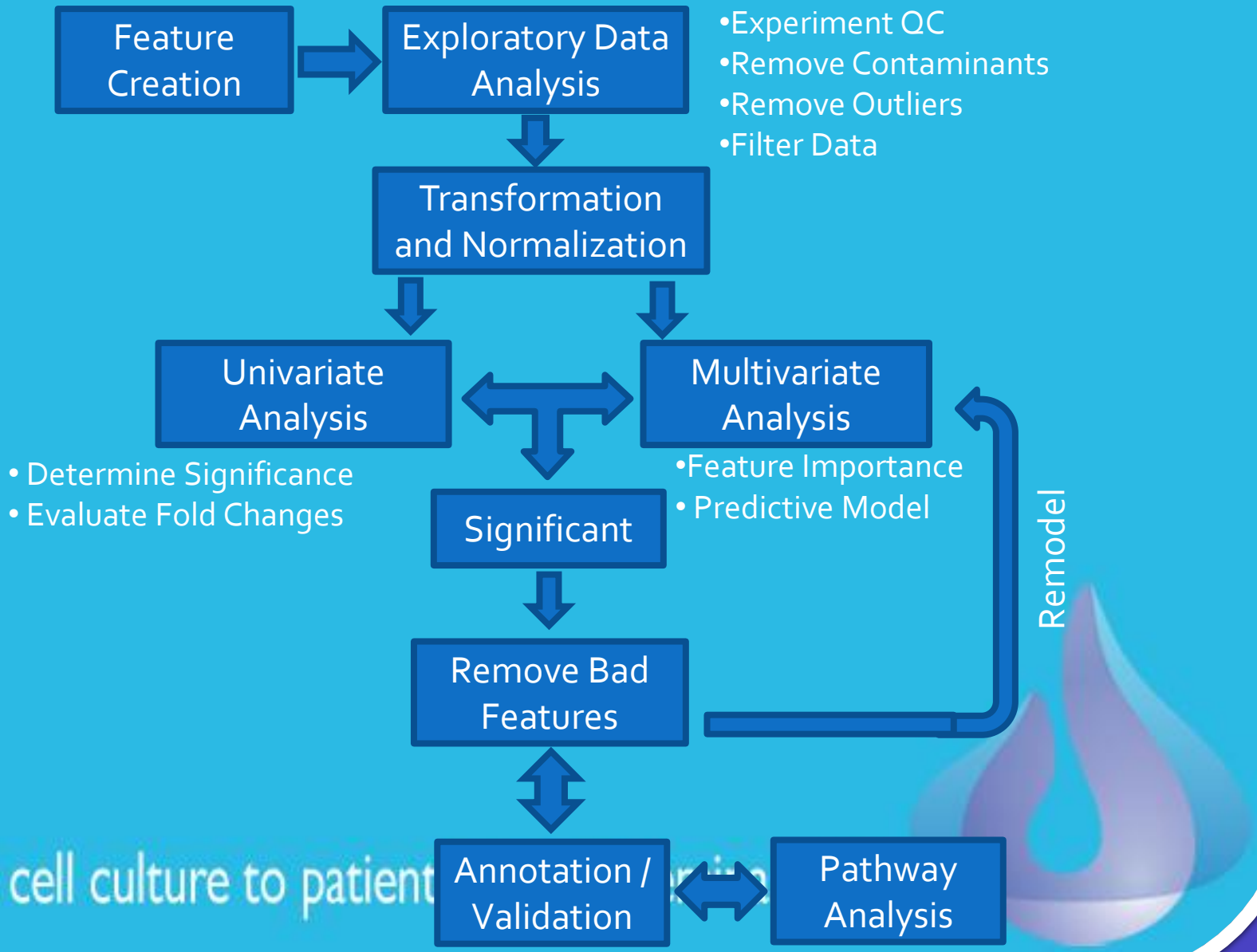
Feature Annotation

Statistical Analysis

$\text{Log2}(\text{Abundance}_{tb}) = \text{Treatment}_t + \text{Error}_t$



General Metabolomics Data Analysis Process

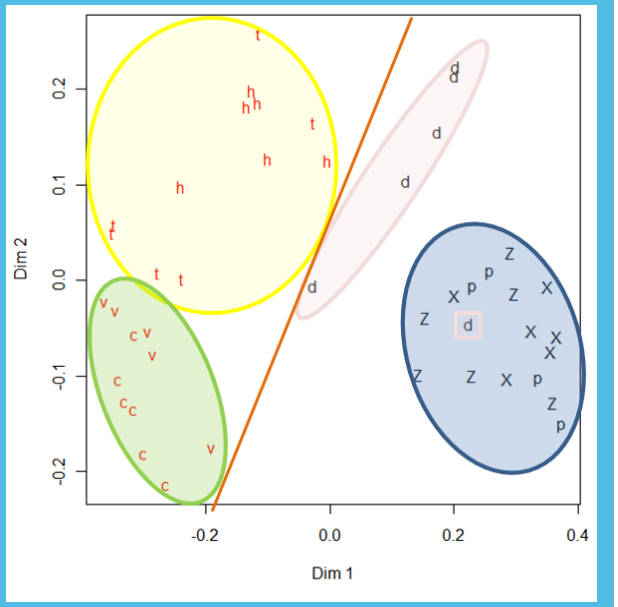


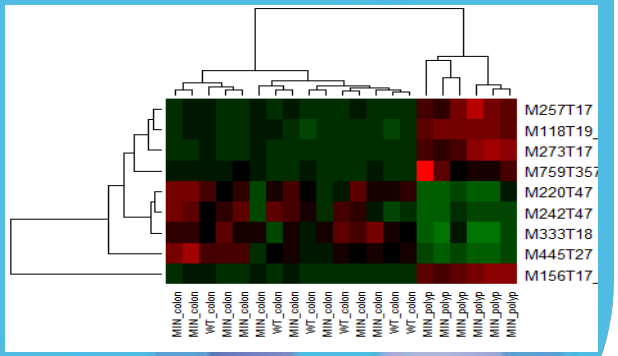
from cell culture to patient

Global Metabolite Profiling

devTOX assay

- Measure Metabolic Changes Related to Sample
  - Not concerned with individual metabolites
  - Measuring pull metabolites have on sample grouping
  - Metabolites are scored by importance
- Chemometrics Analysis
  - Multivariate Statistical Methods
  - Clustering
  - Discriminate Analysis
  - Machine Learning Methods
    - Random Forest, Support Vector Machines
  - Identification of metabolites by VIP scores
  - Predictive Modeling
- Informatics
  - Mass Feature Annotation
    - MS-MS and spectral pattern matching
  - Pathway Placement and Enrichment





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Stemina's Data Management Process

Goal:
Make the Complex Simple

Laboratory

Data Analysis Pipeline

Samples And Prep

LC/MS Analysis

Create molecular features

Biomarker Discovery

Annotation and Identification

Validation

Sample and Project Metadata

Sample Preparation Status

Instrument work lists

Open Source

Agilent Systems

File Conversion

LC/MS raw data

XCMS Molecular Feature Extraction

MassHunter Molecular Feature Extraction

Automated EIC s

R Statistical Analysis

Data Visualization

Database Searching

Statistical Analysis

Data Visualization

Database Searching

Pathway Analysis

KEGG

Metlin

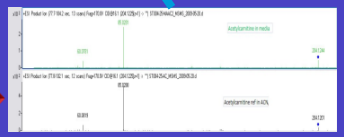
HMDB

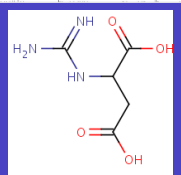
BMRB

Databases

LIMS

- Access to all data
- Inventory Management
- Control of processes
- Sample Tracking
- LC-MS Quality Control
- LC-MS File Tracking
- Chemometrics
- Feature Annotation
- Functional Analysis
- Interpretation of Results
- Validation
- Interpretation of mass spectra





Stemina Classification	Drug	ECVAM Classification	FDA Classification
Non-Teratogens	Ascorbic Acid	Non-Teratogens	A
	Isoniazid		C
	Penicillin G		B
	Saccharin		A
	Folic Acid		A
	Levothyroxine		A
	Retinol (blind 1)		A
	Doxylamine (blind 2)		A
	Thiamine (blind 8)		A
	Aspirin	Weak/Moderate Teratogens	C
	Caffeine		B
	Diphenhydramine		B
	Indomethacin*		B
Teratogens	Dexamethasone *		C
	Diphenylhydantoin		D
	Methotrexate		X
	5-Fluorouracil	Strong Teratogens	D
	Busulfan		D
	Cytosine Arabinoside		D
	Hydroxyurea		D
	Retinoic Acid		X
	Thalidomide		X
	Valproic Acid		D
	Amiodarone (blind 3)		D
	Rifampicin (blind 4)		C
	Carbamazepine (blind 5)		C
	Accutane (blind 6)		X
	Cyclophosphamide (blind 7)		D

Iterative modeling process to continually refine feature set as new test data is added to the training set.

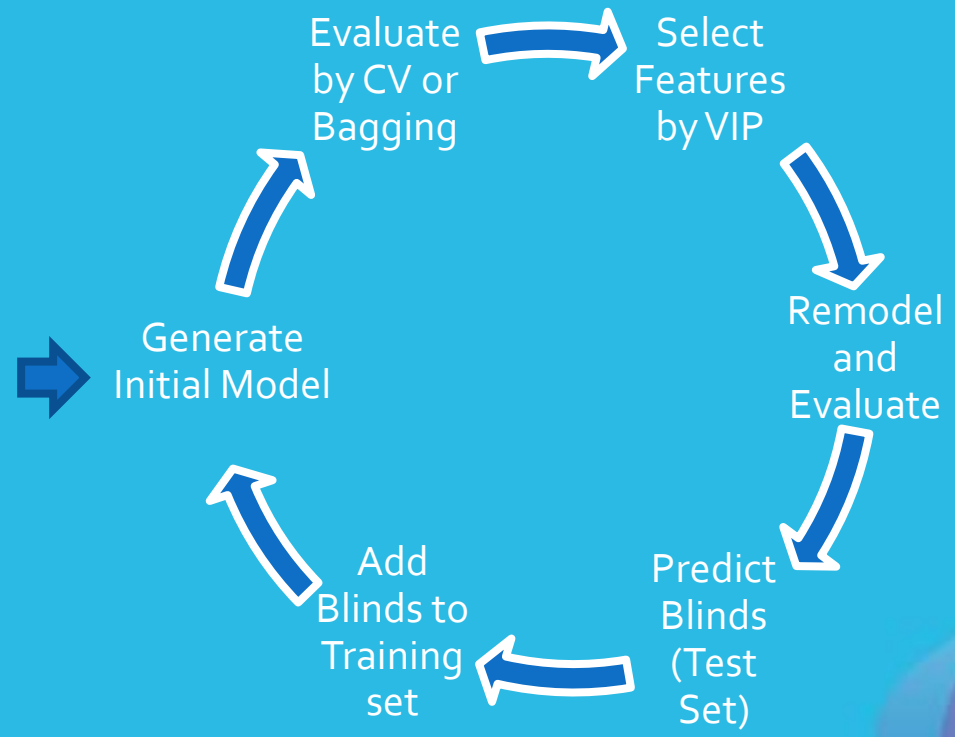
Merge features of Blinds and Known's

Select Features present in 75% of Experiments

Remove RT with high % contaminants

Combine ESI modes

Code Drugs based on observed human teratogenicity



Teratogenicity Model was 87.5% Predictive Under Experimental Conditions

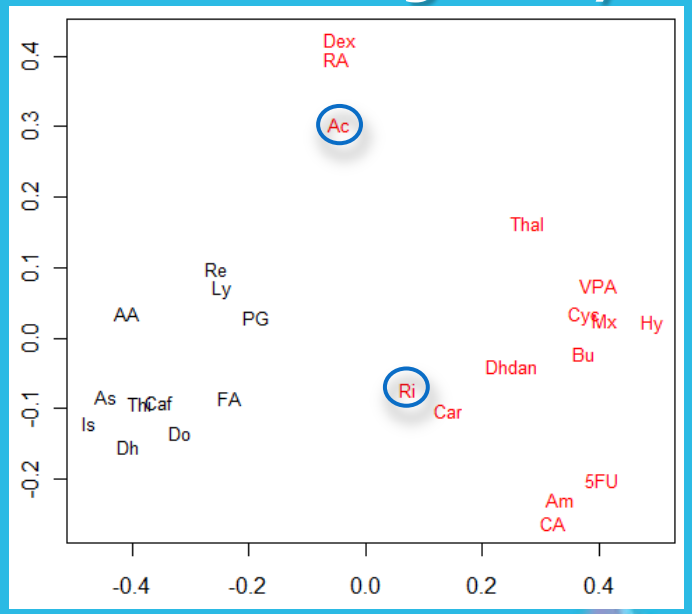
Blinded Trt Experiments	Actual	Predicted
Blind 1 (Retinol)	Non	Non
Blind 2 (Doxylamine)	Non	Non
Blind 3 (Amiodarone)	Ter	Ter
Blind 4 (Rifampicin)	Ter	Ter
Blind 5 (Carbamazepine)	Ter	Ter
Blind 6 (Accutane)	Ter	Non
Blind 7 (Cyclophosphamide)	Ter	Ter
Blind 8 (Thiamine)	Non	Non

- Features selected in training set used to predict "unknowns"
- 7/8 predicted correctly

Further Progress on Bioinformatics: Predictive Modeling of Teratogenicity

Model trained using 20 drugs of known teratogenicity

Non-Teratogens	Teratogens
Ascorbic Acid	Fluorouracil
Doxylamine	Busulfan
Levothyroxine	Cytosine Arabinoside
Penicillin G	Retinoic Acid
Retinol	Thalidomide
Isoniazid	Valproate
Folic Acid	Rifampicin
Thiame	Amiodarone
	Hydroxyurea
	Cyclophosphomide
	Carbamazepine
	Accutane



- Predictive model developed using Random Forest and feature selection
- Current model 87.5% predictive of blinded test set

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Fold Change Ratios Are Indicators of Teratogenicity

Arginine and Dimethylarginine

- EICs for these compounds were integrated
- Fold change of the resulting areas for controls vs. dosed were compared
- **Non-teratogens show smaller fold change ratios (between 0.9 and 1.1)**
- **Teratogens show larger fold change ratios (<0.9 and >1.1)**
- **No false negatives for teratogenicity**
- **Only ascorbic acid and caffeine are false positives.**

Fold Change Ratios of Dimethylarginine:Arginine Are Indicators of Teratogenicity

Stemina Classification	Compound	Arg fold change / ADMA fold change	Prediction
Non-Teratogens	Ascorbic Acid	1.28	Ter
	Aspirin	1.07	Non
	Caffeine	1.33	Ter
	Doxylamine (Blind 2)	0.97	Non
	Isoniazid	0.94	Non
	Levothyroxine	1.03	Non
	Penicillin G	0.96	Non
	Folic Acid	1.08	Non
	Retinol (Blind 1)	1.03	Non
	Thiamine (Blind 8)	1.00	Non
Teratogens	5-Fluorouracil	43.93	Ter
	Methotrexate	2.54	Ter
	Accutane (Blind 6)	0.55	Ter
	Amiodarone (Blind 3)	1.64	Ter
	Busulfan	1.12	Ter
	Carbamazepine (Blind 5)	1.12	Ter
	Cyclophosphamide (Blind 7)	1.56	Ter
	Cytosine Arabinoside	67.01	Ter
	Hydroxyurea	2.52	Ter
	Retinoic Acid	0.48	Ter
	Rifampicin (Blind 4)	0.81	Ter
	Thalidomide	0.85	Ter
	Valproic Acid	2.11	Ter

- **Non-teratogens show smaller fold change ratios (between 0.9 and 1.1)**
- **Teratogens show larger fold change ratios (<0.9 and >1.1)**

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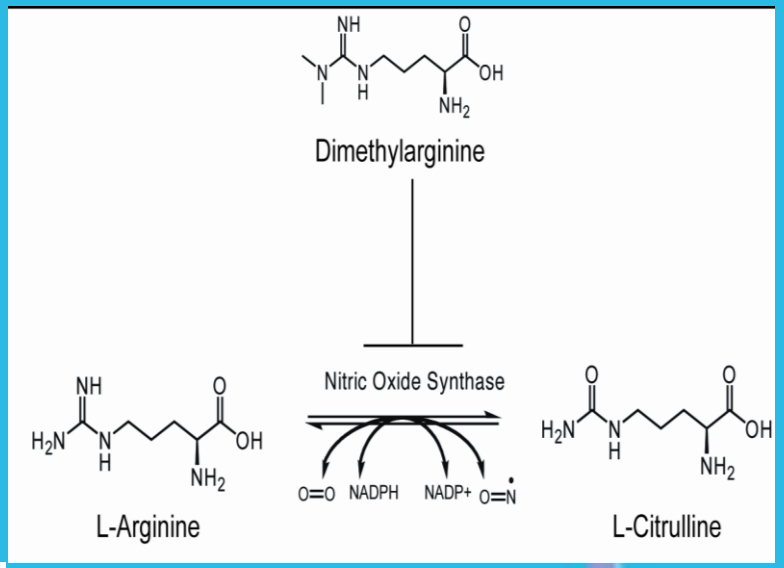
Metabolite Endpoints of Teratogenicity Pertain to the NOS Pathway

devTOX assay™

Unraveling Mechanism

The biologically active molecule nitric oxide (NO) is formed by the conversion of arginine to dimethhylarginine, with the release of NO. Asymmetric dimethylarginine inhibits this process. NO has multiple cellular molecular targets. It influences the activity of transcription factors, modulates upstream signaling cascades, mRNA stability and translation, and processes the primary gene products. In the brain, many processes are linked to NO.



Journal of Neurochemistry, 2006, **96**, 247–253 doi:10.1111/j.1471-4159.2005.03542.

Neural tube closure depends on nitric oxide synthase activity

Amir Nachmany, Veronica Gold, Asaf Tsur, Dan Arad and Miguel Weil

Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

High levels of nitric oxide (NO) block the process of NT closure in the chick embryo

Dimethyalrginine's Abundance Varies With Teratogen Concentrations

6-well (circulating dose):

Dosed (red)

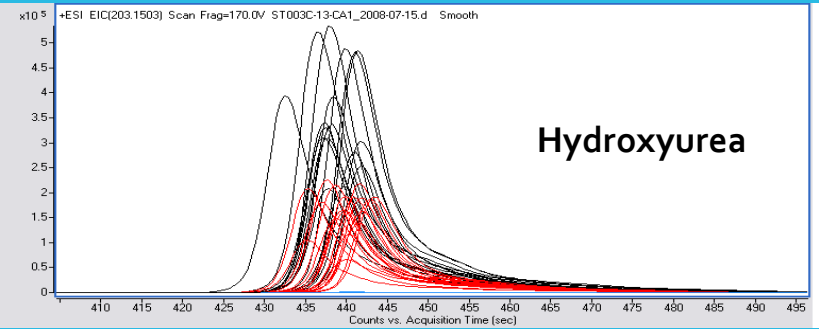
Controls (black)

96-well (3 dose levels):

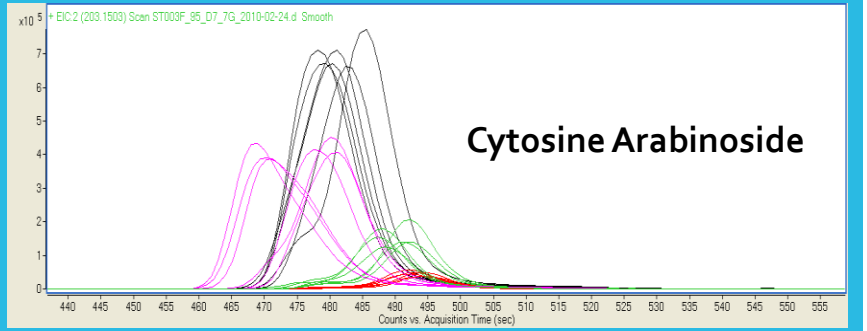
10x above circulating dose (red)

Circulating dose (green)

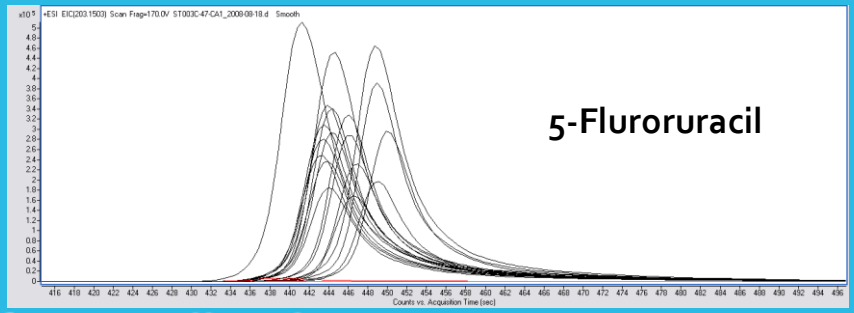
10x below circulating dose (pink)



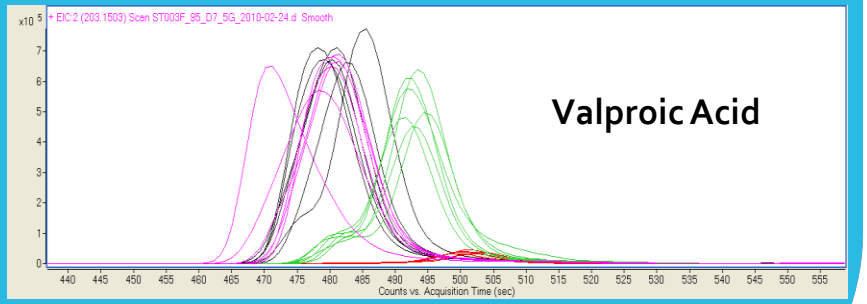
Hydroxyurea



Cytosine Arabinoside



5-Fluroruracil



Valproic Acid

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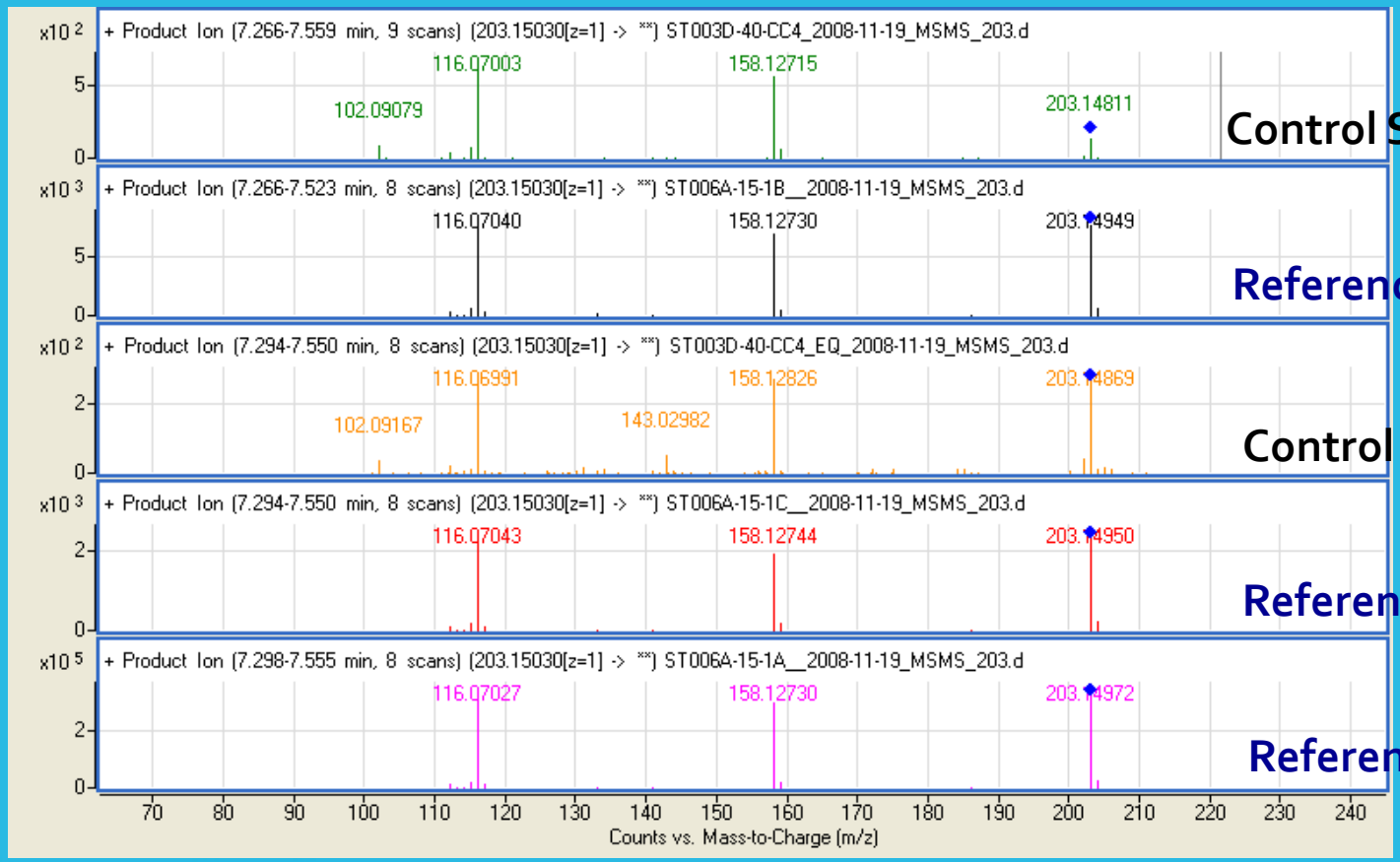
Validation of Dimethylarginine

MS/MS Fragmentation – A Good Match



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Metabolite Endpoints of Teratogenicity Pertain to the GABA Pathway

Clinical aspects of the disorders of GABA metabolism in children

Phillip L. Pearl[a] and K. Michael Gibson[b]

Purpose of review

There has been increased recognition of the pediatric neurotransmitter disorders. This review focuses on the clinical disorders of GABA metabolism.

Recent findings

The known clinical disorders of GABA metabolism are pyridoxine dependent epilepsy, GABA-transaminase deficiency, SSADH deficiency, and homocarnosinosis. Pyridoxine dependent epilepsy is diagnosed clinically but potentially more common presentations, with later and atypical features, widen the spectrum. No gene locus has been confirmed; the pathophysiology may involve alterations in PLP transport, binding to GAD, or other PLP-dependent pathways. SSADH deficiency is associated with developmental delay, prominent language deficits, hypotonia, ataxia, hyporeflexia, and seizures. Increased detection is reported when specific ion monitoring is used for GHB on urine organic acids. The most consistent MRI abnormality is increased signal in the globus pallidus. MR spectroscopy has demonstrated the first example of increased endogenous GABA in human brain parenchyma in this disorder. GABA-transaminase deficiency and homocarnosinosis appear to be very rare but require CSF for detection, thus allowing for the possibility that these entities, as in the other pediatric neurotransmitter disorders, are underrecognized.

Abbreviations

CSF	cerebrospinal fluid
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase

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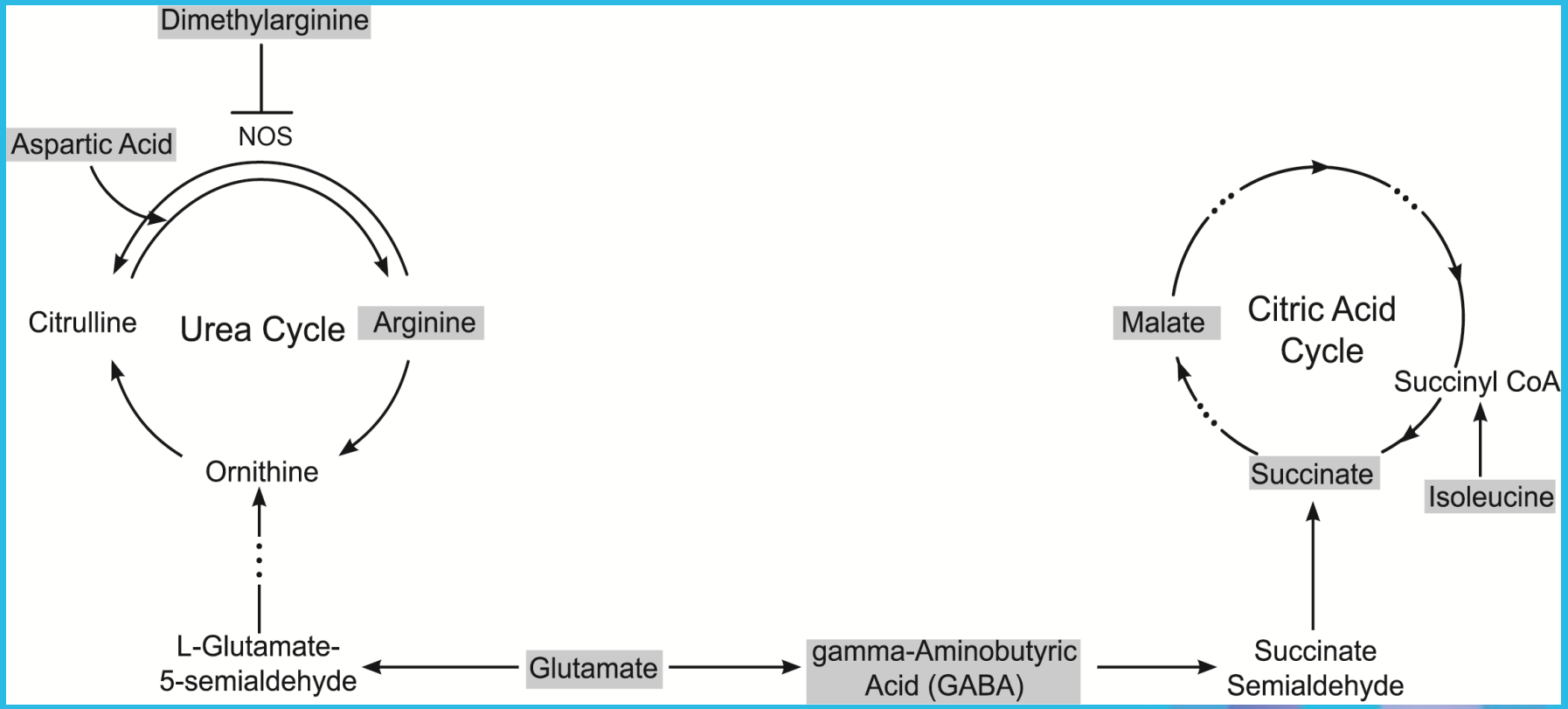
IS THERE MORE TO GABA THAN SYNAPTIC INHIBITION?

David F. Owens and Arnold R. Kriegstein‡*

In the mature brain, GABA (γ-aminobutyric acid) functions primarily as an inhibitory neurotransmitter. But it can also act as a trophic factor during nervous system development to influence events such as proliferation, migration, differentiation, synapse maturation and cell death. GABA mediates these processes by the activation of traditional ionotropic and metabotropic receptors, and probably by both synaptic and non-synaptic mechanisms. However, the functional properties of GABA receptor signalling in the immature brain are significantly different from, and in some ways opposite to, those found in the adult brain. The unique features of the early-appearing GABA signalling systems might help to explain how GABA acts as a developmental signal.

Owens DF and Kriegstein AR. Nature 3, 715-726 (2002).

Additional Biomarkers and Pathways of Developmental Toxicity



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