An Unbiased Metabolomics Approach to Create and Apply Targeted Biomarker Assays in Human Stem Cell Models

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Client Relations and Operations

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Stemina Biomarker Discovery

- **History**
  - Founded in 2006

- **Expertise**
  - Metabolomics: Study of endogenous metabolism
  - Human stem cells, cardiac cells and neural cells
  - Identification of small molecules associated with toxicity and disease

- **Development of Diagnostics and Predictive Toxicity Screens**
  - Human stem cell based assays
    - devTOX: prediction of developmental toxicity (service offering)
    - Cardiotoxicity screen: prediction of cardiac toxicity

Diagnosis blood test for Autism
What is devTOX?

• A suite of human pluripotent stem (hPS) cell based *in vitro* assays designed to predict the developmental toxicity potential of:
  – Pharmaceuticals
  – Industrial chemicals
  – Consumer product ingredients
  – Environmental chemicals

• Two assays offered
  – Metabolomics based: prediction and untargeted analysis for hypothesis generation and testing
  – Targeted assay: *measure dose response* using predictive biomarkers ornithine and cystine
Metabolomics to Targeted Attribute

<table>
<thead>
<tr>
<th>Attribute</th>
<th>devTOX™</th>
<th>devTOX™ QuickPredict™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes/No Teratogenicity Prediction</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Turn Around Time</td>
<td>~ 6 weeks</td>
<td>&lt; 2 weeks</td>
</tr>
<tr>
<td>Cost</td>
<td>$$$</td>
<td>$</td>
</tr>
<tr>
<td>Endpoint</td>
<td>Metabolomics high, med and low dose</td>
<td>Dose dependent change in specific biomarkers indicates teratogenicity potential (Tp)</td>
</tr>
<tr>
<td>Pathway Perturbation Data</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

We used tightly controlled metabolomics experiments over many replicates to identify a predictive set of biomarkers that enabled more rapid analysis.
Laboratory Workflow

- **Cell Culture**
  - Expand hPS cells on Matrigel in mTeSR1
  - Plate in 96-well assay plates

- **Exposure**
  - Expose to compounds (48-72 hr)
  - Collect media
  - Analyze viability

- **Sample Prep.**
  - Filter media samples to remove molecules > 10 KDa

- **LC/MS**
  - Analyze spent media using Agilent LC/MS
  - HILIC chromatography

- **Data Analysis**
  - **Discovery**: Many biomarkers
  - **quickPredict**: Two specific biomarkers
  - Relative Measurements
  - Reference treatment
Identification of Biomarkers

Metabonomics
- Distinct Metabolic signature

Model Creation
- Develop using a training set
- Evaluate in blinded test set

Mine & Refine
- Balance biomarker predictivity & detection

Develop & Evaluate Assay
- Structural confirmation
- Increase robustness & throughput
- Show reproducibility

Establishment and Assessment of a New Human Embryonic Stem Cell-Based Biomarker Assay for Developmental Toxicity Screening

Birth Defects Research Part B: Developmental and Reproductive Toxicology

PMID: 24123775
Agilent instrumentation

- High Resolution
  - 6224 TOF
  - 6500 series Q-TOF
- Triple Quadrupole
  - 6490
- 1290 Infinity LC systems
- Electrospray Ionization
How we use them:
Biomarker Discovery to Targeted Assays

Q-TOF and TOF
- Metabolomics
- Cast a wide net
- Detect many features
- Apply Bioinformatics tools

Q-TOF
- Structural Confirmation
- Predictive biomarkers

QQQ or TOF
- Develop targeted assays for practical use
Finding a Predictive Metabolic Signature

Untargeted Method (HRMS)

Column: Phenomenex HILIC; 100 x 3mm; 5um

Solvent gradient
A: 0.1% Formic Acid in Water
B: 0.1% Formic Acid in ACN

23 minutes per injection
2 injections per sample: ESI pos and neg

Agilent QTOF or TOF MS:

• Scan range: \( m/z \) 70-1600 amu @ 3 Hz
• ~ 5 ppm mass accuracy (MS)
• < 20 ppm mass accuracy (MS-MS)
• 2 GHz Extended dynamic range
• 5 orders of magnitude dynamic range
Targeting Biomarkers

From a Complex metabolic signature
23 min. analysis x 2

Represent signature & retain predictivity

To a Targeted Method (HRMS)

Column: Waters AcquityBEH Amide;
2.1 x 50 mm; 1.7 um

Solvent gradient
A: 0.1% Formic Acid in Water
B: 0.1% Formic Acid in ACN

6.5 minutes per injection
1 injection per sample: ESI pos

Ornithine (secreted)

Cystine (media)
## Targeted Assay Retains Predictivity

<table>
<thead>
<tr>
<th>Training Set Compounds</th>
<th>FDA Pregnancy Category</th>
<th>Humans</th>
<th>devTOX Discovery</th>
<th>devTOX quickPredict</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>A</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>A</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Levothyroxine</td>
<td>A</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Saccharin</td>
<td>A</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Thiamine</td>
<td>A</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>B</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Doxylamine</td>
<td>B</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>B</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
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<tr>
<td>Caffeine</td>
<td>C</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>C</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
</tr>
<tr>
<td>Retinol</td>
<td>C</td>
<td>NON</td>
<td>NON</td>
<td>NON</td>
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<tr>
<td>5-Fluorouracil</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>All-trans Retinoic Acid</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Busulfan</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Cytosine Arabinoside</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>D</td>
<td>TER</td>
<td>NON</td>
<td>NON</td>
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<tr>
<td>Hydroxyurea</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Valproic Acid</td>
<td>D</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
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<tr>
<td>Accutane</td>
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<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>X</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>X</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
<tr>
<td>Warfarin</td>
<td>X</td>
<td>TER</td>
<td>TER</td>
<td>TER</td>
</tr>
</tbody>
</table>
Benefits of Targeted Quantitative Analysis

• Move from a relative measurement to quantitative measurement
  – Reduce assay variability
  – Increase robustness
  – Speed data processing
    • Specific quantitation vs profiling
  – Ease lab-to-lab method transfer with partners
  – Enable assay validation
Overview: Quantitative Method Development

Targeted Metabolomics
- Predictive biomarkers
- Known MS/MS spectrum
- Stable labeled internal standards

Transition to QQQ
- Develop MRM method
- Qualifiers
- Optimize parameters via source infusion

Linearity & Sensitivity
- Response of labeled vs endogenous
- Matrix effects
- Target range

TOF vs QQQ
- Back to back analysis of plates
- Compare assay output

Production & validation
Impact of Internal Standards

Ornithine Variability: IS Normalization

CV reduced by 10%

Cystine Variability: IS Normalization

Curve fit impacted

CV reduced by 19%
Showed analyte concentrations within linear range

Cystine (media)

Ornithine (secreted)

R² = 0.999

R² = 0.998
Assay Transferability

• Compare QQQ and TOF methods
  • Relative measurements versus quantitative
  • Two cells lines used (hES and iPSC)
  • Test linearity on the TOF

• Test set 20 compounds run over 7 days
  • Same plate analyzed on TOF and QQQ
    – IS normalized
    – TOF relative (DMSO controls)
    – QQQ standard curve
  • Compare assay performance across methods
    – Values within 3 fold for predicted Tp considered similar
    – Biological variability accounted for with controls and QC parameters for cell viability
Similar Assay Performance

TOF Teratogenicity Potential (µM)

QQQ Teratogenicity Potential (µM)

0.001 0.01 0.1 1 10 100 1000

0.001
0.01
0.1
1
10
100
1000

R^2 = 0.9332
p-value = < 0.0001
Platforms Show Similar Assay Performance

Dose Response
iPS cells
IS normalized

Diphenhydramine: Cystine

Diphenhydramine: Ornithine

www.stemina.com  4/9/2014  18
Similar Assay Performance

<table>
<thead>
<tr>
<th>IS Normalized</th>
<th>TOF1</th>
<th>QQQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teratogenicity Potential (µM)</td>
<td>1.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Diphenhydramine**

- **Viability**
- **o/c Ratio (TOF)**
- **o/c Ratio (QQQ)**

iPS cells
Similar Assay Performance

<table>
<thead>
<tr>
<th>IS Normalized</th>
<th>TOF1</th>
<th>QQQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teratogenicity</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Potential (μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Carbamazepine

- Viability
- o/c Ratio (TOF)
- o/c Ratio (QQQ)

hES cells
Similar Method Performance

Can we move to a quantitative method on the TOF for added capacity?

Ornithine Curve TOF

\[ y = 74568x - 51280 \]
\[ R^2 = 0.9994 \]

Cystine Curve TOF

\[ y = 115025x - 40061 \]
\[ R^2 = 0.9966 \]
Summary & Conclusions

**Targeted**
- From dozens of predictive features down to just two analytes
- Optimized methods for ornithine and cystine
- One mode (ESI positive)
- Incorporated stable labeled internal standards

**Fast**
- Reduced run time from 23 min to 6.5 min
- Eight fold increase in LC/MS throughput
- Three fold reduction in assay cycle time

**Quantitative**
- Target analytes are within linear range
- Internal standards significantly reduce CV
- TOF and QQQ perform similarly across target range
• Evolve from relative quantitation to concentration measurements
• Speed sample preparation
• Redefine QQQ method if needed to expand the chemical space
• Fit for purpose validation
Establishment and Assessment of a New Human Embryonic Stem Cell-Based Biomarker Assay for Developmental Toxicity Screening

J.A. Palmer, A.M. Smith, L.A. Egnash, K.R. Conard, P.R. West, R.E. Burrier, E.L.R. Donley, and F.R. Kirchner

Birth Defects Research Part B: Developmental and Reproductive Toxicology Volume 98, Issue 4, pages 343–363, August 2013
Predicting Human Developmental Toxicity of Pharmaceuticals Using Human Embryonic Stem Cells and Metabolomics

P. West, A. Weir, A. Smith, E.L.R. Donley and G. Cezar

Toxicology and Applied Pharmacology. Volume 247, Issue 1, 15 August 2010
Pages 18-27.

Identifying Developmental Toxicity Pathways for a Subset of ToxCast Chemicals Using Human Embryonic Stem Cells and Metabolomics


Toxicology and Applied Pharmacology. Volume 257, Issue 1, 15 November 2011
Pages 111-21
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