NEWBORN SCREENING
Yesterday, Today, and Tomorrow

Edwin W. Naylor, Ph.D., M.P.H.

Medical University of South Carolina
and
Parabase Genomics, Inc
History of Phenylketonuria (PKU)

- 1953 – Bickel – Low phenylalanine diet.
- 1969 – Tada – BH4 cofactor defects.
- 1999 – Kure – BH4 responsive PKU
The PKU Card

3/16”punch

Fits in a standard business envelope
History of Mandatory Newborn Screening in Pennsylvania

- 1965 – Phenylketonuria
- 1975 – Congenital Hypothyroidism
- 1992 – Sickle Cell Disease
- 1993 – Maple Syrup Urine Disease
- 2000 – Galactosemia
- 2000 – Congenital Adrenal Hyperplasia
One of These Two Is Famous... And Living in Buffalo

The City's New Look
For nearly 20 years, BIA was used to test for a disease from a dried blood specimen.

- Method relies on growth of bacteria in presence of phenylalanine.
- Poor precision.
- False + of 1%

Many laboratories replaced with Fluorometric assays.

- Improvements noted
- False positive rates 0.1%
Early Expansion of Newborn Screening (1960-1980)

- Maple Syrup Urine Disease (BIA)
- Homocystinuria (BIA)
- Tyrosinemia (BIA)
- Galactosemia
  - E. Coli Phage
  - Fluormetric Assay (Beutler)
- Biotinidase Deficiency (Enzyme)

- Congenital Hypothyroidism (T4 and/or TSH)
  - Foley (1974)
  - Dussault (1973)

- Congenital Adrenal Hyperplasia (17-OH-P)
  - Pang (1977)

- Cystic Fibrosis (IRT)
  - Crossley & Elliott (1977)
History of Expanded/Supplemental Screening in Pennsylvania

- 1984 – Supplmental NBS at Magee
- 1989 – 2nd Tier DNA screen for CF
- 1992 – MS/MS screening introduced
- 1994 – Confirmatory DNA testing for other disorders (Gal, Bio, CAH)
- 1994 – Neo Gen Screening spun off from Magee
- 2003 – Neo Gen acquired by Pediatrix
Expanded Screening Technology (1993)

- DNA- for specific mutations (ex. CF)
- 2nd tier analysis
- Tandem Mass Spectrometry (MS/MS) - measures the weight of molecules in a specimen
METHODOLOGY

Automated Sample Preparation

- One 3/16th in. blood disk
- Butyl ester derivatization for enhance sensitivity, selectivity, and throughput.
- Batched microtiter plate automated sample handling and preparation.

Automated Sample Injection

- Two minute analysis time.
- Microtiter plate format.
- Small sample volume automated injection (10 µL sample loop)
- Low sample carry-over, versatile multiple platforms

High Throughput Electrospray MS/MS

- Low Flow Rate (<20 µL/min) for enhanced sensitivity.
- Robust, sensitive, low maintenance systems. 120,000 specimens analyzed per year per instrument.
- Comprehensive multi-function MS/MS panel
- Automated data and result processing.
Electrospray Ion Source

ABI / MDS-SCIEX API-300, 365 and 3000

Capillary

Electrode
Total Newborns Screened Using MS/MS

2,117,013

Through August 31\textsuperscript{th}, 2004
Total Disorders Detected Using MS/MS

705

Frequency: 1 positive case per 3,003 newborns
<table>
<thead>
<tr>
<th>Amino Acid Disorders</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylketonuria</td>
<td>255</td>
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<tr>
<td>Maple Syrup Urine Disease</td>
<td>55</td>
</tr>
<tr>
<td>Citrullinemia</td>
<td>13</td>
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<tr>
<td>Argininosuccinic Aciduria</td>
<td>5</td>
</tr>
<tr>
<td>Homocystinuria</td>
<td>3</td>
</tr>
<tr>
<td>Tyrosinemia (Type 1 &amp; 3)</td>
<td>2</td>
</tr>
<tr>
<td>Arginase Deficiency</td>
<td>1</td>
</tr>
</tbody>
</table>
Phenylketonuria (PKU)

NTL 102, full scan

mass / charge

Phenylketonuria

P/T ratio

gly

val

leu

met

phe

tyr

phe

met

val

leu

gln

pro

ala

mass / charge

gly

asp

val

leu

gln

pro

ala

mass / charge

gly

asp
MS/MS and PKU Detection

- Measure both Phenylalanine and Tyrosine
- Obtain a “Molar” Ratio for Diagnosis.
- Higher Precision and accuracy enables detection of PKU less than 24 hours.
- False + rate <0.01%
Acylcarnitine Profile (Normal)
Fatty Acid Oxidation Disorders

- MCAD Deficiency 156
- VLCAD Deficiency 33
- LCHAD/TFP Deficiencies 18
- SCAD Deficiency 12
- CPT II / Translocase Deficiencies 15
- MADD Deficiencies 11
MCAD Deficiency

Homozygous A985G
### MCAD Deficiency

#### 2nd-Tier DNA Molecular Results

<table>
<thead>
<tr>
<th>Combination</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>A985G / A985G</td>
<td>138</td>
</tr>
<tr>
<td>A985G / T199C</td>
<td>19</td>
</tr>
<tr>
<td>A985G / Other</td>
<td>49</td>
</tr>
<tr>
<td>Other / Other</td>
<td>5</td>
</tr>
<tr>
<td>Other / ?</td>
<td>4</td>
</tr>
<tr>
<td>? / ?</td>
<td>10</td>
</tr>
</tbody>
</table>
The Light Cycler
MCAD Example
Organic Acidurias

- Methylmalonic Aciduria: 31
- Propionic Aciduria: 28
- Glutaric Aciduria-Type 1: 27
- Isovaleric Aciduria: 19
- 3-Methylcrotonyl Glycinuria: 17
- HMG CoA Lyase Deficiency: 2
- β-Ketothiolase Deficiency: 1
- Multiple CoA Carboxylase Deficiency: 1
Propionic Acidemia

mass / charge

% Intensity

280 300 320 340 360 380 400 420 440 460 480

C3

C2

d3C2
d3C3

C3

C2

d3C2
d3C3

C3

C2

d3C2
d3C3

C16

d9C14
d3C16

C18:1
PROPIonic ACIDEMIA
Second Tier Molecular Results

6 Del14Ins12 / ? (US & Canada)
2 Del14Ins12 / Del14Ins12 (Chile)
2 E168K / E168K (Chile)
1 E168K / ? (Chile)
6 N536D / N536D (Amish)
32 ? / ?
Glutaric Acidemia
Why MS can make a difference.
### Glutaric Aciduria – Type 1

#### 2nd Tier DNA Molecular Results

**Newborns (n=30)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>A421V / A421V</td>
<td>23</td>
<td>Amish</td>
</tr>
<tr>
<td>A421V / R313W</td>
<td>1</td>
<td>Amish</td>
</tr>
<tr>
<td>A421V / R88C</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>R227P / G390R</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Wt (2) / wt (2)</td>
<td>3</td>
<td>Unknown</td>
</tr>
<tr>
<td>? / ?</td>
<td>1</td>
<td>Arab</td>
</tr>
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</table>
## Glutaric Aciduria – Type 1

### 2nd Tier DNA Molecular Results

#### Older High Risk Cases

<table>
<thead>
<tr>
<th>Combination</th>
<th>Count</th>
<th>Family Status</th>
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<tbody>
<tr>
<td>A421V / A421V</td>
<td>11</td>
<td>(Amish)</td>
</tr>
<tr>
<td>A421V / R88S</td>
<td>2</td>
<td>(Unknown)</td>
</tr>
<tr>
<td>A421V / V400M</td>
<td>1</td>
<td>(Unknown)</td>
</tr>
<tr>
<td>A421V / 219delC</td>
<td>1</td>
<td>(Unknown)</td>
</tr>
<tr>
<td>A421V / A349T</td>
<td>1</td>
<td>(Amish)</td>
</tr>
<tr>
<td>A421V / R294Q</td>
<td>1</td>
<td>(Unknown)</td>
</tr>
<tr>
<td>A421V / M191T</td>
<td>1</td>
<td>(Unknown)</td>
</tr>
<tr>
<td>A421V / wt (2)</td>
<td>1</td>
<td>(Amish)</td>
</tr>
</tbody>
</table>
Organic Acidemias

3-Methyl-Crotonyl-CoA Carboxylase Deficiency
(3-Methylcrotonylglycinuria)

Maternal (11)
Isolated (4)
<table>
<thead>
<tr>
<th>Homozygote</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>df508 / df508</td>
<td>df508</td>
<td>df508</td>
<td>225</td>
</tr>
<tr>
<td>df508 / G551D</td>
<td>df508</td>
<td>G551D</td>
<td>12</td>
</tr>
<tr>
<td>df508 / G542X</td>
<td>df508</td>
<td>G542X</td>
<td>12</td>
</tr>
<tr>
<td>df508 / N1303K</td>
<td>df508</td>
<td>N1303K</td>
<td>8</td>
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<tr>
<td>df508 / W1282X</td>
<td>df508</td>
<td>W1282X</td>
<td>6</td>
</tr>
<tr>
<td>df508 / 621+1 G-&gt;T</td>
<td>df508</td>
<td>621+1 G-&gt;T</td>
<td>5</td>
</tr>
<tr>
<td>df508 / R1162X</td>
<td>df508</td>
<td>R1162X</td>
<td>5</td>
</tr>
<tr>
<td>df508 / 2183del AA-</td>
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<td>2183del AA-</td>
<td>5</td>
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<tr>
<td>df508 / E60X</td>
<td>df508</td>
<td>E60X</td>
<td>4</td>
</tr>
<tr>
<td>df508 / other</td>
<td>df508</td>
<td>other</td>
<td>31</td>
</tr>
</tbody>
</table>
Cystic Fibrosis (Con’t)

- **df508 / R117H**
  - Poly T (7,9) 15
  - Poly T (7) 4
  - Poly T (5,9) 2
  - Poly T (5) 1
  - Poly T (?) 7

- **df508 / df508C**

- **df508 / I148T**

29

6

4
Galactosemia
Mutations Detected by 2nd-Tier Testing

- Q188R
- S135L
- K285N
- L195P
- Exon 10
- N314D (Duarte)
## Galactosemia (Classical)  
### 2nd-Tier DNA Molecular Results

<table>
<thead>
<tr>
<th>Counts</th>
<th>Allele Combination</th>
<th>Counts</th>
<th>Allele Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>Q188R / Q188R</td>
<td>3</td>
<td>Q188R / Exon 10</td>
</tr>
<tr>
<td>3</td>
<td>Q188R / K285N</td>
<td>3</td>
<td>S135L / Exon 10</td>
</tr>
<tr>
<td>2</td>
<td>Q188R / L195P</td>
<td>7</td>
<td>Q188R / ?</td>
</tr>
<tr>
<td>1</td>
<td>Q188R / S135L</td>
<td>2</td>
<td>S135L / ?</td>
</tr>
<tr>
<td>2</td>
<td>K285N / K285N</td>
<td>1</td>
<td>L195P / ?</td>
</tr>
<tr>
<td>2</td>
<td>S135L / S135L</td>
<td>17</td>
<td>? /?</td>
</tr>
<tr>
<td>1</td>
<td>S135L / K285N</td>
<td>37</td>
<td>wt (5) / wt (5)</td>
</tr>
<tr>
<td>5</td>
<td>Exon 10 / Exon 10</td>
<td></td>
<td>(? Kin &amp; Epi)</td>
</tr>
</tbody>
</table>
Biotinidase Deficiency
Mutations Detected by 2nd-Tier Testing

- Q456H
- D444H:A171T
- D444H:Q456H
- D444H
- R538C
- G98:d7i3
- R157H
- D252G
- D444H:F403V
- D444H:Q456H
# Biotinidase Deficiency (Complete)

## 2nd-Tier DNA Molecular Results

<table>
<thead>
<tr>
<th>Combination</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>G98:d7i3 / G98:d7i3</td>
<td>4</td>
</tr>
<tr>
<td>Q456H / Q456H</td>
<td>2</td>
</tr>
<tr>
<td>D444H:A171T / D444H:A171T</td>
<td>2</td>
</tr>
<tr>
<td>D444H:A171T / R538C</td>
<td>1</td>
</tr>
<tr>
<td>D444H:A171T / Q456H</td>
<td>1</td>
</tr>
<tr>
<td>D444H:A171T / R157H</td>
<td>1</td>
</tr>
<tr>
<td>Q456H / D252G</td>
<td>1</td>
</tr>
<tr>
<td>Q456H / R157H</td>
<td>1</td>
</tr>
<tr>
<td>R538C / R157H</td>
<td>1</td>
</tr>
<tr>
<td>D444H:F403V / D444H:F403V</td>
<td>1</td>
</tr>
<tr>
<td>Q456H / ?</td>
<td>4</td>
</tr>
<tr>
<td>G98:d7i3 / ?</td>
<td>3</td>
</tr>
<tr>
<td>D444H:A171T / ?</td>
<td>1</td>
</tr>
<tr>
<td>R157H / ?</td>
<td>1</td>
</tr>
<tr>
<td>? / ? or wt / wt</td>
<td>2</td>
</tr>
</tbody>
</table>
Sickle Cell Disease
Mutations Detected by 2nd-Tier Testing

Sickle Cell Disease
- Hb S A173T
- Hb C G172A
- Hb E G232A

β-Thalassemia
- A (-29) G
- C (-88) T
- IVS 1
Detection of Hemoglobinopathies using Rapid Cycle PCR and Analysis of FRET Probes

Sickle Cell Disease 2nd-Tier DNA Molecular Testing
Benefits of 2nd Tier Light Cycler Screening

- Useful if common mutations are present
- Improves Sensitivity and Specificity
- Permits Genotype / Phenotype correlations
- Lays foundation for future primary DNA screening programs
Workflow for 2nd Tier Next Generation Sequencing Newborn Screening

Sample Isolation 2h
DNA Capture & Sequencing 92h
Raw Data Management 10h
Analysis & Interpretation 1h+

8 samples, 105 Hrs, <$10,000 = Real Neonatal Genomics!
Workflow for 2\textsuperscript{nd} Tier Next Generation Sequencing Newborn Screening

- High M.Wt. DNA
- PCR Amplifiable and NGS ready
- Processing Time $\sim$ 2 hrs
- Amenable to automation

Sample Isolation

Yield 400ng per Spot

More yield and DNA purity than alternate workflow
Workflow for 2\textsuperscript{nd} Tier Newborn Screening

**DNA Capture* & Sequencing**

* Room for improvement
** Process reduced from 19* days to 92 hrs

- Library Prep \(\sim 5 \text{ hrs}\)
- Capture \(\sim 66 \text{ hrs}\)
- HiSeq 2500 PE 75 \(\sim 21 \text{ hrs}\)
- Fast turnaround & High quality data

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S9</th>
<th>S10</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL READS (Mln)</td>
<td>92</td>
<td>68</td>
<td>77</td>
<td>75</td>
<td>90</td>
<td>85</td>
<td>96</td>
<td>49</td>
</tr>
<tr>
<td>MAPPED READS (Mln)</td>
<td>89</td>
<td>58</td>
<td>72</td>
<td>72</td>
<td>86</td>
<td>79</td>
<td>91</td>
<td>37</td>
</tr>
<tr>
<td>% READS MAPPED</td>
<td>96</td>
<td>86</td>
<td>95</td>
<td>96</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>76</td>
</tr>
<tr>
<td>READS ON TARGET (Mln)</td>
<td>69</td>
<td>38</td>
<td>56</td>
<td>56</td>
<td>66</td>
<td>61</td>
<td>71</td>
<td>21</td>
</tr>
<tr>
<td>% READS ON TARGET</td>
<td>74</td>
<td>56</td>
<td>73</td>
<td>75</td>
<td>73</td>
<td>73</td>
<td>74</td>
<td>42</td>
</tr>
</tbody>
</table>

Data generated on Nimblegen Exome and HiSeq2500
Variant calling GATK2 (Broad Institute, MA) by CFI and Real Time Genomics
2 Known (MSUD and PA) & 8 blinded samples were provided by Clinic for Special Children, PA (S1 - S10)
*Saunders et al., (2012) Rapid Whole Genome Sequencing for Genetic Disease Diagnosis in NICUs
## Detecting Known Cases

Identifying causal variants using population frequency and disease category as filters

<table>
<thead>
<tr>
<th>Type</th>
<th>Sample</th>
<th>Disease</th>
<th>PI+PD Hom.Re 126 Gene Panel</th>
<th>126 GP,Comm on Hom., Read &gt;4 MAF&lt;5% Gene Reads</th>
<th>Transcript Variant</th>
<th>Protein Variant</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amish*</td>
<td>whole blood</td>
<td>Propionic Acidemia</td>
<td>11</td>
<td>2</td>
<td>PCCB 18</td>
<td>c.1606A&gt;G</td>
<td>p.Asn536Asp Hom.</td>
</tr>
<tr>
<td>Amish*</td>
<td>DNA</td>
<td>Propionic Acidemia</td>
<td>15</td>
<td>2</td>
<td>PCCB 5</td>
<td>c.1606A&gt;G</td>
<td>p.Asn536Asp Hom.</td>
</tr>
</tbody>
</table>

## Validation of False Positive/False Negatives by comparing different Methods

<table>
<thead>
<tr>
<th>CRADD Sample, Broad**</th>
<th>Exome Variants</th>
<th>Panel (in silico)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYNONYMOUS</td>
<td>11219</td>
<td>81</td>
</tr>
<tr>
<td>MISSENSE</td>
<td>10518</td>
<td>65</td>
</tr>
<tr>
<td>NONSENSE</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>SMALL INDELS</td>
<td>889</td>
<td>4</td>
</tr>
<tr>
<td>INTRON, UTRs</td>
<td>26083</td>
<td>232</td>
</tr>
<tr>
<td>SPLICE SITE</td>
<td>160</td>
<td>1</td>
</tr>
</tbody>
</table>

Data generated on Nimblegen Exome; variant calling GATK (Broad Institute, MA); Omicia (Emeryville, CA)

*Samples provided by Clinic for Special Children

** Puffenberger et al., 2013, PLoS ONE 7(1): e28936; Agilent Exome using Broad Pipeline
## Results of 8 Blinded and 2 Known Samples

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gender</th>
<th>Gene/Protein</th>
<th>Change 1</th>
<th>Change 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylketonuria</td>
<td>A/M</td>
<td>PAH</td>
<td>782 G&gt;A</td>
<td>R261Q</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>280-282 del ATC</td>
<td>I95 del</td>
</tr>
<tr>
<td>MSUD</td>
<td>M</td>
<td>BCKDHA</td>
<td>1312 T&gt;A</td>
<td>Y438N</td>
</tr>
<tr>
<td>MCAD</td>
<td>M</td>
<td>ACADM</td>
<td>985 A&gt;G</td>
<td>K329E</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>A/M</td>
<td>CFTR</td>
<td>1522-4 del TTT</td>
<td>Δf508</td>
</tr>
<tr>
<td>SCID</td>
<td>M</td>
<td>IL7R</td>
<td>2 T&gt;G</td>
<td>M1R</td>
</tr>
<tr>
<td>11-β-Hydroxylase</td>
<td>A</td>
<td>CYP11B1</td>
<td>1343 G&gt;A</td>
<td>R448H</td>
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<tr>
<td></td>
<td></td>
<td>ADA</td>
<td>646 G&gt;A</td>
<td>G216R</td>
</tr>
<tr>
<td>Galactosemia</td>
<td>A</td>
<td>GALT</td>
<td>563 A&gt;G</td>
<td>Q188R</td>
</tr>
<tr>
<td>Biotinidase</td>
<td>M</td>
<td>BTD</td>
<td>1459 T&gt;C</td>
<td>W487R</td>
</tr>
<tr>
<td>Homocystinuria</td>
<td>A</td>
<td>MTHFR</td>
<td>1129 C&gt;T</td>
<td>R377C</td>
</tr>
<tr>
<td>Propionic Acidemia B</td>
<td>A/M</td>
<td>PCCB</td>
<td>1606 A&gt;G</td>
<td>N536D</td>
</tr>
</tbody>
</table>
Summary: Targeted Next Generation Sequencing

- Can be used for 2nd-tier newborn screening
- Can be used for high risk and NICU screen
- Causal variants identified rapidly
- Lower cost than WES or WGS (<$1,000)
- Faster than WES or WGS (10 run in 100 h)
- >500 genes per panel
- Lower exon drop out rate (<2%)
Acknowledgements

- **Parabase Genomics (Sample Isolation)**
  - Andy Bhattachrjee, Ph.D.
  - Tanya Sokolsky, Ph.D.

- **Yale University (DNA Sequencing)**
  - John Overton, Ph.D.

- **Clinical Future, Inc (Raw Data Management)**
  - Alexander Zaranek, Ph.D.

- **RealTime Genomics, Inc (Raw Data Management)**
  - Francisco De-LaVega, D.Sc.
  - Brian Hilbush, Ph.D.

- **Omicia, Inc (Analysis and Interpretation)**
  - Martin Reese, Ph.D.

- **Clinic for Special Children (DNA Specimens)**
  - Holmes Morton, M.D.
  - Kevin Strauss, M.D.
  - Erick Puffenberger, Ph.D.