Molecular Technology in Newborn Screening: SCID and Beyond

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History of NBS Molecular Testing

- **1994**
  - Washington: hemoglobin second tier testing (Hb S, C, and E) by RELP
  - Wisconsin: *CFTR* mutation analysis for F508del

- **1998**
  - New England: 2 GALT mutations (Q & N) by RFLP

- **1999**
  - New England: MCADD (c.985A>G) by RFLP

- **2005**
  - Wisconsin: MSUD (p.Y438N) by Tetra-primer ARMS-PCR
History of NBS Molecular Testing

• 2006
  – New York: Krabbe disease (3 polymorphisms & 5 mutations) by DNA sequencing

• 2008
  – Wisconsin: TREC assay for SCID screening by Real-time PCR
    • 1st use of molecular test as a primary test for population screen

• 2010
  – 36 NBSPs in US use molecular testing for CF
Severe Combined Immunodeficiency (SCID)

Then...

Now...
Severe Combined Immunodeficiency (SCID)

- **Infections in first year of life**
  - recurrent, etiology bacterial, viral and fungal
  - persistent despite routine treatment
  - severe--including sepsis, meningitis
  - opportunistic pathogens, such as PCP (pneumonia)
- **Failure to thrive, chronic diarrhea**
- **T cells decreased or absent**
  - poor proliferation *in vitro* to mitogens
- **B cells absent or non-functional**
  - low Ig’s after maternal IgG wanes; no specific antibody responses
- **Fatal without immune reconstitution**
SCID Genetic Analysis

- X-linked SCID is most common form (males)
- Specific gene defect can be found in 80% of cases (15 genes known)
- Clinical applications:
  - Carrier and prenatal dx
  - Predict response to BMT
  - Gene therapy

Buckley Ann. Rev Imm 2004
Available Curative Treatment Modalities for SCID

• Bone Marrow Transplantation

• Gene Therapy (X-linked and adenosine deaminase deficiency SCID)
Does SCID fulfill NBS criteria?

- Prevalence of the disease (1:100,000 or greater)
  - SCID: 1:66,000 (conservative estimate)
- Can the disorder be detected by routine physical exam?
  - SCID: No, SCID baby appears normal at birth.
- Does the disorder have a short asymptomatic period after birth?
  - SCID: Yes, SCID baby can be protected by passive maternal immunity.
- Does the disease cause serious medical complications?
  - SCID: Yes, universally fatal within the first year of life
- Is there potential for successful treatment?
  - SCID: Yes, hematopoietic stem cell transplantation
- Is there a confirmatory test?
  - SCID: Yes, lymphocyte subpopulation analysis (flow cytometry)
- Does early intervention leads better outcome?
  - SCID: Yes!
- Is there a screening test?
  - SCID: Yes, measurement of TRECs using real-time qPCR
SCID: Benefits of Early Diagnosis

- 46 SCID infants with HSCT at than 3.5 months of age or less: 96%
- 113 SCID infants with HSCT at greater than 3.5 months of age: 66%

Screening for SCID in Newborns
Considerations

• Many genes
• Many mutations in each known gene
• Some genotypes still not known
TRECs are reduced in nearly ALL forms of SCID
T-cell Generation in Newborns

• Two mechanisms:
  – Thymic output
  – Postthymic T-cell proliferation

• Consequences:
  – Majority of T-cells are naïve T cells in newborns.
  – TREC s are diluted out, and 10% T cells contain TREC s in newborns.

Gent et al, Clinical Immunology. 2009; 133: 95–107
Generation of T cell receptor excision circles (TRECs) occur in >70% of all new (naïve) T cells and can be detected by PCR.
Overall Analysis Scheme

NBS Card (NSC) a.k.a. Guthrie Card
Dried blood spots (DBS)

3 mm punch

96 well plate
Extract DNA
Amplify TREC by real-time QPCR
Analyze

TREC plasmid calibrators

ΔRn (amplification)

ABI 7900HT Fast Real-Time PCR System
Multiplexing _384-well Plate
Number of Infants Screened

<table>
<thead>
<tr>
<th></th>
<th>Affected</th>
<th>Unaffected</th>
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<tbody>
<tr>
<td>Test Positive</td>
<td>TP</td>
<td>FP</td>
</tr>
<tr>
<td>Test Negative</td>
<td>FN</td>
<td>TN</td>
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Sensitivity = \[ \frac{TP}{TP + FN} \]  
Positive Predictive Value = \[ \frac{TP}{TP + FP} \]  
Specificity = \[ \frac{TN}{TN + FP} \]  

TREĆ Copy Numbers

Normal Population

True Negatives

Mean (Affected)

Mean (Normal)

Cutoff

Affected

True Positives

False positives

False negatives
SCID Testing Algorithm

- **Full term** < 40
  - Preterm < 25
    - TREC & Actin Assay on 2 additional punches
      - Full term: TREC < 30
        - Actin > 10,000
          - Screening Abnormal
      - Full term: TREC < 30
        - Actin < 10,000
          - Screening inconclusive
      - Preterm: TREC < 25
        - Actin > 10,000
          - Screening Abnormal
      - Preterm: TREC < 25
        - Actin < 10,000
          - Screening inconclusive

- **Initial TREC assay**

- **Full term ≥ 40**
  - Preterm ≥ 25
    - Normal

Abnormal

inconclusive
**SCID Reporting Algorithm**

- **Full term screening Abnormal**
  - Call out results to clinical consultants
  - Confirmatory Dx
  - Work up
  - Call out results to PCP

- **Full term screening inconclusive**
  - Written report and request a repeating NBS specimen

- **Preterm screening inconclusive**
  - Written Report and recommending repeat NBS following NICU procedure

- **Preterm screening Abn.**
Confirmatory testing

• Flow cytometry
  lymphocyte subset enumeration for T, B and NK cell quantitation

• Lymphocyte (T and/or B) proliferation tests

• Quantitative immunoglobulin assessment (IgG, IgA, IgM and IgE)

• HIV testing (to rule out secondary causes of T-cell lymphopenia)

• Genetics testing

• Others: enzymes, Fluorescence in situ hybridization (FISH)
Special Considerations

• TREC copy numbers
  – Measurement units
  – DNA extraction
  – Calibrators

• TREC assay platform
  – Multiplexing vs. single target
  – 384-well vs. 96-well

• Automation

• QA/QC issues

• Premature Newborns
### Wisconsin Experience
*(January 1, 2008–December 31, 2012)*

<table>
<thead>
<tr>
<th>Infants Screened:</th>
<th>340,037</th>
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<tbody>
<tr>
<td>- Premature (&lt; 37 wks)</td>
<td>30,664</td>
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<tr>
<td>- Full term</td>
<td>309,373</td>
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<table>
<thead>
<tr>
<th>Abnormal results:</th>
<th>246</th>
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<tbody>
<tr>
<td>- Premature (&lt;37 wks)</td>
<td>147 (0.04%)</td>
</tr>
<tr>
<td>- Full term</td>
<td>99 (0.03%)</td>
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<table>
<thead>
<tr>
<th>Inconclusive Results:</th>
<th>472</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Premature (&lt;37 wks)</td>
<td>382 (0.11%)</td>
</tr>
<tr>
<td>- Full term</td>
<td>90 (0.03%)</td>
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**Total number of flow cytometry referral: 108**
Wisconsin Experience
(January 1, 2008–December 31, 2012)

Severe T cell Lymphopenia Cases

- Rac 2 mutation
- ADA SCID
- T-B-NK+ SCID
- T-B+NK+ (3)
- RAG 1 SCID
Wisconsin Experience
(January 1, 2008–December 31, 2012)

Other T cell Lymphopenia Cases

• Chromosomal abnormalities
  ▪ 22q11.2 deletion (11)
  ▪ Trisomy 21

• Syndromes with T cell impairment
  ▪ Jacobsen syndrome
  ▪ Tar syndrome
  ▪ Ectrodactyly ectodermic dysplasia
  ▪ Ataxia Telangiectasia

• Idopathic T-cell lymphopenia
Improving NBS for CF to Reduce Screening false positives using Next Generation sequencing Technology

IRT

Limited CFTR mutations panel

No Mut. One Mut. Two Mut.

Screening Normal CFTR2 147* Screening Positive

One Mut. Two Mut.

Screening Normal ?? Screening Positive

*Disease-causing mutations and mutations with varying consequences. (Sosnay et al, Nature Genetics, 2013)
Specific Aims

1. Establish a method of simultaneously detecting 162 CFTR mutations/gene variants using dried blood spot routine newborn screening specimens to create IRT/DNA/DNA CF screening opportunity.

2. Demonstrate that the three-tier IRT/DNA/DNA CF screening protocol would significantly reduce false positive screening results caused by identification of CF heterozygote carrier infants.

3. Demonstrate that it is cost effective to implement the three-tier IRT/DNA/DNA CF screening protocol into routine NBS for CF.
MiSeqDx Cystic Fibrosis System

• 162 CFTR mutations/variants (IUO version*)
  – 127 single nucleotide mutations/variants
  – 32 insertion/deletion mutations
  – 2 large deletions
  – PolyTG/PolyT region

*Product is currently under FDA review.
Cystic Fibrosis System Workflow

- Isolate DNA
- Prepare Libraries
- Pool Libraries
- Sequence on MiSeqDx
- Review Data
Sequencing Library Generation

CF variant-specific probes hybridize to flanking regions of interest in unfragmented gDNA.

- Hybridization
- Extension/Ligation
- PCR Primers Anneal
- PCR amplification

- PCR adds indices and sequencing primers
- Uniquely tagged library ready for cluster generation and sequencing
Genotyping-by-sequencing

Key Features:
1. Robust and logical work flow
2. 46 + 2 samples multiplexing platform
3. Dual indexing identification
4. Immediate result w/o additional informatics requirements

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