Newborn Screening for Severe Combined Immunodeficiency (SCID) in the Laboratory

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What is SCID?

• A heterogeneous group of inherited disorders caused by single gene defects resulting in a combined immune deficiency

• Prevalence: ~ 1:50,000

• Over 20 different genetic forms: hundreds of mutation sites

• All have profound defects in T lymphocyte differentiation and function

• Some (not all) have defects in B cell and/or NK cell differentiation as well

• End result: patients cannot fight viral, bacterial, fungal or opportunistic infections
* No molecular defect in known SCID genes.
SCID Newborn Screening Implementation in the US

- Cumulative No. of States, District & Territories
- Percent of US newborns

# Newborn Screening Programs
- WI
- MA
- CA, NY
- MI
- CT, MS
- DE
- CO, FL, TX
- MN, OH
- UT
- WI,
- PA
- WA, OR
- WV, ME
- IL, RI
- DC, NH
- HI, MT
- HI
- IA, NM
- SC, SD
- AR
- PR
- TN, GA
- ID, ND
- MO
- NC

Newborn Screening Test for SCID

TREC Assay

measuring T Cell Receptor Excision Circles using DNA from dried blood spots collected routinely on all newborns

- TREC - extrachromosomal DNA produced during rearrangement of V-D-J regions in TCR gene – essential step for the production of T cells
- Any immune defect that affects T cell production or destruction will cause a decrease in TREC
- Phenotypic assay (not genotypic)

* T cell receptors are protein molecules on T cells surface, responsible for recognition of antigens
Formation of \( \delta \text{Rec-}\Psi \text{Ja} \) TREC during *Delta segment* deletion in rearrangement of T cell receptor gene

Chromosomal 14 germline TCR \( \alpha/\delta \) chain loci (all cells)

Chromosomal 14 TCR \( \alpha/\delta \) chain loci (T cells)

Extrachromosomal DNA \( \delta \text{Rec-}\Psi \text{Ja} \) TREC

Chromosomal 14 TCR \( \alpha \) chain locus

\[ \begin{align*}
\text{V} \alpha 1 & \quad \text{V} \alpha 2 & \quad \text{V} \alpha 70 & \quad \delta \text{Rec} & \quad \psi \text{Ja} & \quad \text{Ja} 1 & \quad \text{Ja} 2 & \quad \text{Ja} 3 & \quad \text{Ja} 61 & \quad \text{Ca} \\
\downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow \\
\text{V} \alpha & \quad \text{Ja} 1 & \quad \text{Ja} 2 & \quad \text{Ja} 3 & \quad \text{Ja} 61 & \quad \text{Ca} \\
\end{align*}\]

\( \text{V} \alpha - \text{Ja} 1 - \text{Ca} \) rearrangement to form \( \alpha \) chain exon
TREC Quantitative PCR Assay Platforms
Selected by US newborn screening laboratories

- Real time PCR with extracted DNA
- In-situ Real time PCR
- PE EnLite Neonatal TREC Kit

Number of Labs:

<table>
<thead>
<tr>
<th>Platform</th>
<th>Number of Labs</th>
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<tbody>
<tr>
<td>Real time PCR with extracted DNA</td>
<td>15</td>
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<tr>
<td>In-situ Real time PCR</td>
<td>10</td>
</tr>
<tr>
<td>PE EnLite Neonatal TREC Kit</td>
<td>8</td>
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Real time PCR TREC Assay
(with TaqMan probes)
**TCRD** TREC Sequence: 376 bp (out of 85Kb) flanking the δRec-ΨJa signal joint

```
AAAGAGGGCAGCCCTCTCTCCAAGGCAAAATGGGGCTCTGTGGGGAAAGAGGGGTGCCTCTGTC
AACAAAGGTGATGCCACATCCCTTTCAACCATGCTGACACCTTTTGTTTTTGTAAGGTGCCCACCT
CCTGTG^CAGGCTGATGCACTAGGCACCTCACCCCGTGCTTAACCCTGCGAGCTGGCCACGGGCC
TGTCTGCTCTTCATTACCGTTTCTCACGAGTTGCAATAAGTTCAAGCCCTCCATGTCACACTGTGTT
TTCCATCCTGGGAGTGTTCACAGCTATCCCAAGCCCCACGCTGACGATCACGGCCGCCAACAC
ACTCTGATGCCACGACAGACCACCGGAGAAATGTCAGACAAGATCGCCT
```

Blue – CDC forward primer, Green – CDC reverse primer binding site, Red – Taqman probe, ^ - signal joint position
Real-time PCR with TaqMan Probes

F Primer → Polymerization → Fluorophore → Probe → Quencher → R Primer

Probe cleavage
TREC Real-time PCR Amplification Profile

Amplification curves of samples with decreasing TREC content (L to R)

Cycle of Quantification (Cq) inversely correlates with template concentration
Number of PCR cycles required to reach fluorescent threshold is inversely proportional to the template copies

\[ y = -3.335x + 37.454 \]

\[ R^2 = 0.998 \]

Efficiency = 99.5%
Multiplex TREC/\textit{SMN1}/RNaseP Assay
In Newborn Screening for SCID and Spinal Muscular Atrophy (SMA)

Multiple targets can be measured in a single real-time PCR test
End-point PCR TREC Assay

(PE EnLite Assay)
PE EnLite Neonatal TREC Assay

1. PUNCHING
- 1.5 mm DBS disc
- Into PCR-plates directly

2. DISPENSING & SEALING
- 10 µL of elution buffer
- Sealing
- Spin the plates
- DNA elution 45 min at 98°C
- 2 min at 4°C

3. DISPENSING & SEALING
- Unsealing
- 20 µL of PCR mix
- Sealing

4. AMPLIFICATION & HYBRIDIZATION
- Amplification and hybridization
- In thermocycler
- PCR 37 cycles: -1 hr 40 min
- 35°C x 1 hr
- 24°C x 5 min

5. MEASUREMENT
- TREC and β-actin
- Two signals from each well
- Time-resolve Fluorometer
Analysis of data

EnLite Assay

Copy number from standard curve

Real time PCR Assay

1. Cq
2. Copy number
3. Multiple of median

Cutoff value determination can be based on any of above
Cq: Cycle of Quantification

- The number of PCR cycles at which an amplification curve meets a predefined threshold of fluorescence
- Inversely proportional to TREC copies in sample

**Advantages**
- Direct read out from machine software
- Does not require standard curve
- Comparatively consistent reproducibility for same sample

**Limitations**
- Unfamiliar to most non-molecular biologists
- No ‘normalization’ mechanism to compensate for reagent and instrument variations
- Requires
  - Titrating each new reagent lot and adjusting concentration
  - Calibrating real time PCR instruments at regular intervals
  - QC at several TREC levels within each run – meeting predetermined acceptable Cq range for each QC result
TREC copy number

- Converting Cq into copy number through a standard curve

**Advantages**
- Easy to explain results
- Normalized results

**Limitations**
- No universal standard calibrator available
- Plasmid calibrators may vary in reactivity (circular or linearized)
  - Alternative: synthetic ds DNA gene fragment (gBlock, geneStrands)
- Plasmid calibrators do not undergo DBS DNA extraction process
  - Alternative: cell-based calibrator (B-TREC)
- Standard curve necessarily includes very low level of TREC; large difference in statistical variance at top and bottom levels of standard curve challenges validity of linear regression model and causes inconsistent slope
Problem of using simple linear regression model near limit of detection:

Significant variance difference over the range covered by curve → inconsistent slope and intercept
Multiple of Median (MoM)

- A measure of how far an individual test result deviates from the population median
- Expressed as a ratio (TREC copies in sample / Median TREC)

Advantages:
- Easy to understand
- Normalized results
- Does not require standard curve – can be calculated from Cq

Limitations:
- Needs a reasonable number of samples to obtain reliable population median (usually not a problem with NBS 1st assays)
Calculating MoM from Cq: formula derivation

MoM = TREC copies in sample / Median TREC copies for population

PCR doubles the copies with each cycle. If a sample contains \( S \) copies of TREC initially, after \( n \) cycles the level of TREC will be \( S \times 2 \times 2 \times 2 \ldots \times n \), or \( S \times 2^n \)

By definition, Cq is the number of PCR cycles when the level of amplification product (TREC) reached a certain pre-defined threshold. If the threshold is reached at cycle number Cq\(_S\) for sample S, the number of TREC copies at threshold is

\[ S \times 2^{Cq_S} \]

Similarly, for a sample containing population median \( M \), the amount of TREC copies at threshold, which is reached at cycle number Cq\(_m\), is

\[ M \times 2^{Cq_m} \]

Since the threshold for Cq determination is the same for both samples

\[ S \times 2^{Cq_S} = M \times 2^{Cq_m} \]

or

\[ S / M = 2^{Cq_m-Cq_S} = 2^{(Cq_m - Cq_S)} \]

MoM = \[ 2^{(Cq_m - Cq_S)} \]

where Cq\(_m\) and Cq\(_s\) represent median Cq and sample Cq respectively.
**MoM calculation**

**Example 1**

The Cq for sample A is 32, and the median Cq for population is 29

MoM for sample A = \(2^{(29-32)} = 2^{-3} = \frac{1}{2^3} = \frac{1}{8} = 0.125\)

Sample A contains 12.5% of the population median in TREC

**Example 2**

The Cq for sample B is 28.5, and the median Cq is 29

MoM for sample B = \(2^{(29-28.5)} = 2^{0.5} = 1.41\)

Sample B contains 141% of the population median in TREC

Note: In a previous survey, it was found that the cutoff value in copy numbers used by majority of labs to be around 10–15 % of their population median values, which corresponds to 0.10 – 0.15 MoM.
Reporting Newborn Screening Results for SCID

1. Categorical:
   a. Within reference range (Normal)
   b. Below reference range (TREC, follow-up required)
   c. Inconclusive (internal reference gene, repeat sample required)

2. Quantitative: copy number of TREC or Cq
August, 2017  Newborn Screening Status for SCID – US States and Territories

Screening for SCID
(91% of all US newborns)

At planning & procurement stage

At assay validation stage

Others
Thank you for your attention!

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