Molecular Assessment of Dried Blood Spot Quality during Development of a Novel Automated in situ TREC qPCR Assay for SCID Screening

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Assay Background

On-card/ in situ format adapted from CDC – DBS punched directly into 96-well PCR plate for processing and amplification
Automated wash of DBS to reduce PCR inhibitors
Automated addition of PCR master mix
Lab prepared DBS calibrators and TREC plasmid added for QA-QC

For more detail, visit our poster “A Novel, Automated Cost Effective in situ TREC qPCR Assay for Newborn SCID Screening”.
## Assay Optimization

<table>
<thead>
<tr>
<th>DNA Extraction/DBS Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separate Extraction Step</td>
</tr>
</tbody>
</table>

### Current PHL Methods
- LifeTech Sample to SNP
- Qiagen Generation
- CDC on-card/in situ Method
- epMotion 5075 LH automated DBS wash
  - novel buffer

### PCR Amplification
- Master Mix*
- Primers
- Probes
- Fluorophores
- RNP kits

### Cycling Parameters
- Standard vs Fast Ramp
- Extension Times
- Pre-PCR 95°C Incubation Time
Early in situ assay development experiments suggested minimal DBS washing needed; however, these experiments were performed with laboratory prepared cord-blood derived calibrators.

Subsequent experiments with newborn DBS revealed significant differences between newborn DBS and lab prepared calibrators which directly impacted DNA extraction/DBS wash procedures.
DNA Recovery and Access Comparison

96-well PCR plate
On-card/in situ assay

Elution 1
Microcentrifuge tube
50 ul PCR water
95°C for 15 mins

5 ul from 50 ul elution

96-well PCR plate
On-card/in situ assay

Elution A
TREC copies

Elution 2
Microcentrifuge tube
50 ul PCR water
95°C for 15 mins

5 ul from 50 ul elution

96-well PCR plate
On-card/in situ assay

Elution B
TREC copies

A copies + B copies + C copies = total elution copies

On-card/in situ assay C
TREC copies

On-card/in situ assay
## DNA Recovery and Access Comparison

### Newborns

<table>
<thead>
<tr>
<th>Newborns</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>in situ</td>
<td>75</td>
<td>109</td>
<td>44</td>
<td>62</td>
<td>115</td>
<td>109</td>
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<td>276</td>
<td>337</td>
<td>264</td>
<td>279</td>
<td>294</td>
<td>217</td>
<td>269</td>
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<tr>
<td>percent in situ access</td>
<td>27.16%</td>
<td>32.18%</td>
<td>16.57%</td>
<td>22.06%</td>
<td>39.27%</td>
<td>50.51%</td>
<td>18.17%</td>
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</table>

### Lab Prepared Calibrators

<table>
<thead>
<tr>
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<th>4</th>
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<tr>
<td>in situ</td>
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<td>63</td>
<td>36</td>
<td>21</td>
<td>11</td>
<td>10</td>
<td>9</td>
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<tr>
<td>eluates</td>
<td>305</td>
<td>151</td>
<td>62</td>
<td>63</td>
<td>14</td>
<td>29</td>
<td>12</td>
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<tr>
<td>percent in situ access</td>
<td>41.17%</td>
<td>41.71%</td>
<td>58.68%</td>
<td>33.26%</td>
<td>76.15%</td>
<td>34.48%</td>
<td>69.62%</td>
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### Average Recovery

<table>
<thead>
<tr>
<th>Newborns</th>
<th>Lab Prepared Calibrators</th>
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<tbody>
<tr>
<td>Average recovery</td>
<td>29.42%</td>
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</table>
Intra-DBS TREC Variation

Multiple 2mm punches sampled from 16 newborn DBS to determine potential variation in TREC amount across the DBS.
### Intra-DBS TREC Variation

**Intra-spot test**

<table>
<thead>
<tr>
<th>Baby</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tr>
<td>Punches</td>
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<td>11</td>
<td>10</td>
<td>13</td>
<td>14</td>
<td>11</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>15</td>
<td>10</td>
<td>15</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Median</td>
<td>104</td>
<td>126</td>
<td>75</td>
<td>15</td>
<td>55</td>
<td>45</td>
<td>18</td>
<td>16</td>
<td>119</td>
<td>49</td>
<td>153</td>
<td>25</td>
<td>20</td>
<td>62</td>
<td>12</td>
<td>69</td>
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<tr>
<td>Std. Dev.</td>
<td>80</td>
<td>44</td>
<td>29</td>
<td>4</td>
<td>12</td>
<td>14</td>
<td>7</td>
<td>9</td>
<td>27</td>
<td>26</td>
<td>94</td>
<td>13</td>
<td>13</td>
<td>46</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>
DNA Carryover during DBS Punching

Hand punch used to punch blank Guthrie card after newborn samples without bleaching

Hand punch used to punch blank Guthrie card after newborn samples with bleaching
DNA Carryover

Amplification Plot

- Baby
- Blank 2
- Blank 1

Legend:
- A
- B
- C
- D
- E
- F
- G
- H
• Low level RNP contamination of blank punches occurs when using a multi-puncher. The Ct values range from ~31 to 44. TREC contamination was observed in ~5% of the blank punches, with a Cq value of range from ~36 to 44.
• SCID screens which use separate DNA extraction step may not detect DNA carryover.

Laser Cutting Eliminates Nucleic Acid Cross-Contamination in Dried-Blood-Spot Processing

Sean C. Murphy,* Glenda Daza,† Ming Chang,* and Robert Coombs‡
Department of Laboratory Medicine, University of Washington Medical Center, Seattle, Washington, USA,* and Center for AIDS Research, Harborview Medical Center, Seattle, Washington, USA‡

Dried blood spots (DBS) are useful for molecular assays but are prone to false positives from cross-contamination. In our malaria DBS assay, cross-contamination was encountered despite cleaning techniques suitable for HIV-1. We therefore developed a contact-free laser cutting system that effectively eliminated cross-contamination during DBS processing.

# Newborn Data Overview

<table>
<thead>
<tr>
<th></th>
<th>TREC Cq</th>
<th>RNP Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALL NEWBORN</td>
<td>PRE-TERM</td>
</tr>
<tr>
<td>Number of values</td>
<td>4644</td>
<td>537</td>
</tr>
<tr>
<td>Minimum</td>
<td>27.68</td>
<td>27.80</td>
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<tr>
<td>Median</td>
<td>31.13</td>
<td>31.56</td>
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<tr>
<td>Maximum</td>
<td>38.45</td>
<td>36.90</td>
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<tr>
<td>Std. Deviation</td>
<td>1.065</td>
<td>1.249</td>
</tr>
</tbody>
</table>

![Graphs of TREC Cq and RNP Cq](image)

### Current Blinded Pre-Pilot Results

- **TREC below cut-off**: 39 of 4644, 0.84%
- **TREC below cut-off with valid RNP**: 4 of 4644, 0.09%
Model Performance Evaluation Surveys

Representative Newborn Amp Plot

CDC MPES#35 Amplification Plot

★ Survey sample identified by our assay as needing clinical follow-up*

Graph provided by Francis Lee, Centers for Disease Control and Prevention
### Primary Immunodeficiency Patient Samples

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>TREC Cqs</th>
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</thead>
<tbody>
<tr>
<td>SCID</td>
<td>2</td>
<td>UNDET, UNDET</td>
</tr>
<tr>
<td>Omenn Syndrome</td>
<td>2</td>
<td>UNDET, UNDET</td>
</tr>
<tr>
<td>22q11 del</td>
<td>4</td>
<td>39, 38.5, 37, UNDET</td>
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<tr>
<td>hypogammaglobulinemia</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

DBS from babies with known T Cell lymphopenia or SCID
Critical Parameters

Critical Findings and Parameters

- **epMotion Instrument settings must be customized:** Program available from SHL upon request
- Manufacturer’s instrument-to-instrument variation had to be overcome by calibrating the instruments to perform identically. There were slight variations in volumes pipetted and X-Y-Z parameters.
- Customized dispense and aspiration parameters were needed to remove all buffer from the wells.
- It is necessary to optimize wash well depth, volume, speed, incubation time, number times mixed during each wash step, and number of washes.

**PCR on ViiA7**

- A 2mm spot, not 3mm, must be used for *in situ* PCR in 96-well Fast plate.
- Use separate threshold settings for RNP and TREC appropriate for qPCR.
- Of the Master Mixes tested, Fast Advanced had the best performance for efficiency and background.
- Volume of master mix is critical for *in situ* PCR.
Key Features

Automated and easily adapted to laboratories with limited molecular capacity

- Low hands-on time reduced throughput time compared to manual methods
- No storage issues for extracted nucleic acid
- Streamlined workflow – no heating, shaking, centrifugation
- Use of a novel buffer found to be more effective and substantially less expensive than Generation DNA Purification Solution and Generation DNA Elution Solution (Qiagen), Limited disposables (only one PCR plate)
- Low cost (reagents and consumables)
SCID in situ Assay Portability

<table>
<thead>
<tr>
<th></th>
<th>Coralville</th>
<th>Ankeny</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TREC Cq</strong></td>
<td></td>
<td></td>
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<tr>
<td>Minimum</td>
<td>27.68</td>
<td>27.78</td>
</tr>
<tr>
<td>Median</td>
<td>31.24</td>
<td>31.11</td>
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<tr>
<td>Maximum</td>
<td>34.86</td>
<td>35.06</td>
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<tr>
<td>Std. Deviation</td>
<td>1.137</td>
<td>1.084</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Coralville</th>
<th>Ankeny</th>
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</thead>
<tbody>
<tr>
<td><strong>RNP Cq</strong></td>
<td></td>
<td></td>
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<tr>
<td>Minimum</td>
<td>21.05</td>
<td>20.99</td>
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<tr>
<td>Median</td>
<td>24.07</td>
<td>23.84</td>
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<tr>
<td>Maximum</td>
<td>27.4</td>
<td>26.85</td>
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<tr>
<td>Std. Deviation</td>
<td>0.7698</td>
<td>0.8113</td>
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</table>
• Screen positive criteria based on TREC quantity are highly variable (3.9 vs 40 vs 250).

• SCID TREC assays are based on the absence of detection using a molecular method which has the potential to detect as low as 1 copy. However, reliable detection of 1 copy is based on Poisson distribution.

• Therefore, any attempt to quantify as the copy number approaches 0 results in additional noise due to Poisson distribution.
• The in situ protocol was adapted from a procedure developed by the Centers for Disease Control and Prevention.
• Scott D. Rose Ph.D., Integrated DNA Technologies, designed the modified, dual quenched TREC probe.
• The TREC plasmid used in the standard curve was graciously provided by Jackie Gerstel-Thompson and Ann Comeau, New England Newborn Screening Program, University of Massachusetts Medical School.
• TREC plasmid was transformed and purified at the Roy J. Carver Center for Genomics (CCG), University of Iowa.
• Funding was provided by the Iowa Department of Public Health