A multiplex assay for concurrent newborn screening of spinal muscular atrophy (SMA) and severe combined immunodeficiency (SCID)

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Adding new conditions to the newborn screening panel
Spinal Muscular Atrophy (SMA)

- Most common *lethal* autosomal recessive disorder in infants
- Birth prevalence: 1 in 6,000
- 1 in 40 people are heterozygous carriers
- Progressive muscle weakness resulting from degeneration of the anterior horn neurons
- Caused by absence of a fully functional gene that produces the survival motor neuron (SMN) protein
SMN protein encoded by two *SMN* genes

- Loci on chromosome 5q13
- Both genes contain 9 exon and 8 intron - 20 kb
- *SMN1* - telomeric location
  - main functional gene – encodes 38K SMN protein
  - gene deletion/conversion leads to SMA
- *SMN2* – centromeric location
  - differs from *SMN1* by only 5 nucleotides
  - SNP causes incorrect splicing → exclusion of exon 7 in mRNA
  - 10% efficiency in protein production
  - variable copy number
SMA subtypes

SMA Type 1:
- onset < 6 mos
- never able to sit unsupported
- generally do not live >2yr

SMA Type 2:
- onset 7 - 18 mos
- never able to walk
- reduced life span – adolescent or young adulthood

SMA Type 3:
- onset 3 - 17 yr of age
- life-long physical disabilities
- normal life span

In the absence of SMN1, more copies of SMN2 associated with milder phenotypes
### SMA Drug Pipeline 2014

<table>
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<th>Preclinical: discovery</th>
<th>Clinical development</th>
<th>FDA</th>
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<tr>
<td><strong>Identification</strong></td>
<td>Optimization</td>
<td>Safety &amp; Manufacture</td>
<td>Phase 1</td>
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<td>Trophos/olesoxime</td>
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<td>Indiana U/Small Molecules</td>
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<tr>
<td>Cytokinetics/Tirasemtiv</td>
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For optimal outcome, therapy should start soon after birth and before symptoms develop, which would require newborn screening for the genetic defect.
Our two major considerations in developing a newborn screening test for SMA:

1. Use real-time PCR platform:
   • already established in many newborn screening laboratories
   • proven throughput adequate for newborn screening

2. Multiplex within an existing assay
   • minimal additional labor and material costs
Major challenge for a SMA real-time PCR assay: discrimination between \textit{SMN1} and \textit{SMN2}

\textit{SMN1}

\texttt{ctgtaaaaacctttatggtttgtgaaaaacaaatgtttttgaaacattaacaagttcagatgttaAaaagttgaaggttaatgtaaaacaatcaatattaagaattttggatgccaaaacctaggataaaaggttaatctacatccctactagattaattctcataacttaactggttggtttAtgtggaagaaacataccttcacaataaaagagcttttaggatatgatgccattttatatcatagtaggcagaccagcagacgttttttttttatgatgatgggataacctaggcatactgcactgta}

cactctgacatatgaagtgtctcagtcagtttaactggttggttcacagaggacatggtttaactggaaattcgtcaa
gcctctggtttctaaattttctcattttgcaggaatgctgggcataag

\textit{SMN2}

c\texttt{ctgtaaaaacctttatggtttgtgaaaaacaaatgtttttgaaacatttttttaaaagttcagatgttaGaaagttgaaggttaatgtaaaacaatcaatattaagaattttggatgccaaaacctaggataaaaggttaatctacatccctactagattaattctcataacttaactggttggtttGtgtggaagaaacataccttcacaataaaagagcttttaggatatgatgccattttatatcatagtaggcagaccagcagacgttttttttttatgatgatgggataacctaggcatactgcactgta}

cactctgacatatgaagtgtctcagtcagtttaactggttggttcacagaggacatggtttaactggaaattcgtcaa
gcctctggtttctaaattttctcattttgcaggaatgctgggcataag
Locked Nucleic Acid (LNA) Nucleotide

A modified RNA nucleotide

The ribose moiety has an extra bridge connecting the 2' oxygen and 4' carbon

The bridge "locks" the ribose in the 3'-endo conformation

This significantly increases the melting temperature in an oligonucleotide duplex

Allows higher hybridization temperature → increased specificity of a probe
Locked Nucleic Acid (LNA) $SMN1$ Probe

**Probe sequence**

$5'$- C A A C T T T T T A A C A T C T -3'$

**Gene sequence**

$3'$- G T T G A A A A A T T G T A G A -5'$

Expected Probe Hybridization Profile
Temperature Gradient real-time PCR

60 degree Celsius

62 degree Celsius

63 degree Celsius

65 degree Celsius

Graphs showing the fluorescence units (RFU) over cycles for different temperatures.
SMN1 Real-Time PCR Amplification Curves from DNA extracted from reference cell lines

1 cycle: 20' at 95°C
45 cycles:
15'' at 95°C Denaturation
60'' at 65°C Annealing-Elongation

Threshold 0.025
Multiplex TREC/\textit{SMN1}/RNaseP Assay on Reference Materials

- **Normal Newborn**
- **SMA Infant**
- **SCID Positive Control**

Graphs showing the fluorescence (dRn) over cycles for different samples. The markers used are:
- TREC (FAM)
- \textit{SMN1} (Cy5)
- RNase P (HEX)
TRCE Cq (in Extracted Cord Blood DNA)

\[ y = 0.954x + 1.577 \]

TREC Cq (With SMN1 vs Without SMN1)
Mean Difference: 0.143 cycle

Adding SMN1 did not significantly affect the TREC Results
DBS *In Situ* Multiplexed Real-Time PCR Assay

Punch one 2.0 mm disc from each DBS specimen into PCR tubes

Wash with 125 µl of DNA wash buffer S2 (shake for 15 minutes at RT)

Discard S2 wash buffer
Add 15 µl of qPCR mastermix (complete with primers & probes for TREC, SMN1 and RNase P)

Run real-time PCR
45°C for 5 min, 95°C for 20 min
45 cycles of [ 95°C x 15 sec + 65°C x 1 min ]
## Multiplex Real-time PCR assay for SMA/RNaseP/TREC on 26 Blinded DBS Samples from SMA Patients and Carrier parents

<table>
<thead>
<tr>
<th>Sample</th>
<th>Donor Status</th>
<th>Age</th>
<th>SMN1 Cq</th>
<th>RNaseP Cq</th>
<th>TREC Cq</th>
<th>copies/µl</th>
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<tr>
<td>P-02</td>
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<td>4</td>
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<td>21.8</td>
<td>22.0</td>
<td>32.9</td>
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Discussion

• We have multiplexed the \textit{SMN1} target within the existing real-time PCR assay for TREC
• The assay can simultaneously screen DBS for SCID and SMA
• The modified assay requires minimal change to assay protocol and does not alter TREC results
• The inclusion of the SMA screening reagents only adds an extra three cents to the current TREC assay

For those labs wanting to do more:
• We have also developed a second tier assay based on droplet digital PCR, which can
  – Confirm the absence of \textit{SMN1} gene in the sample
  – Provide with precision the number of \textit{SMN2} gene, which can be valuable for prognosis and medical management
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