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


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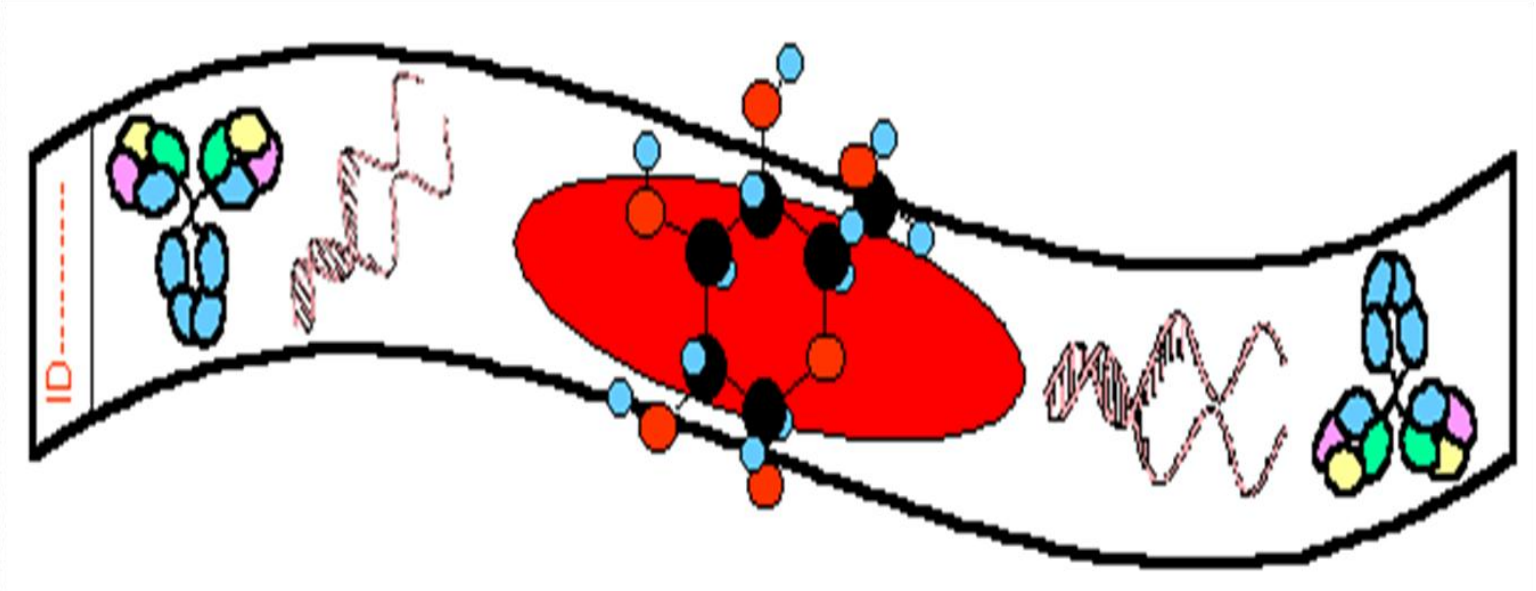
National Center for Environmental Health

Division of Laboratory Sciences

# Stretching The Blood Spot

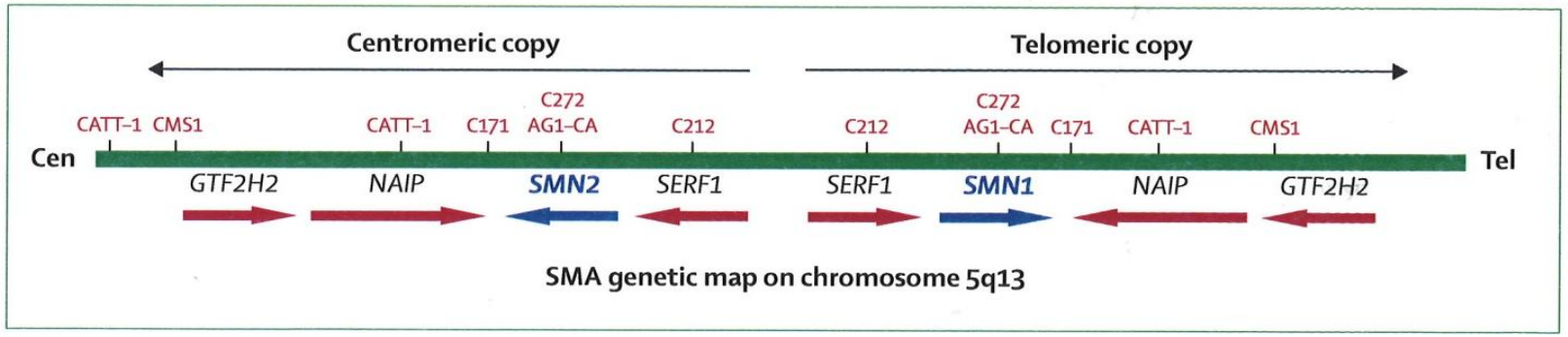



**Adding new conditions to the newborn screening panel**

# 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 suvival motor neuron (SMN) protein

# SMN protein encoded by two *SMN* genes



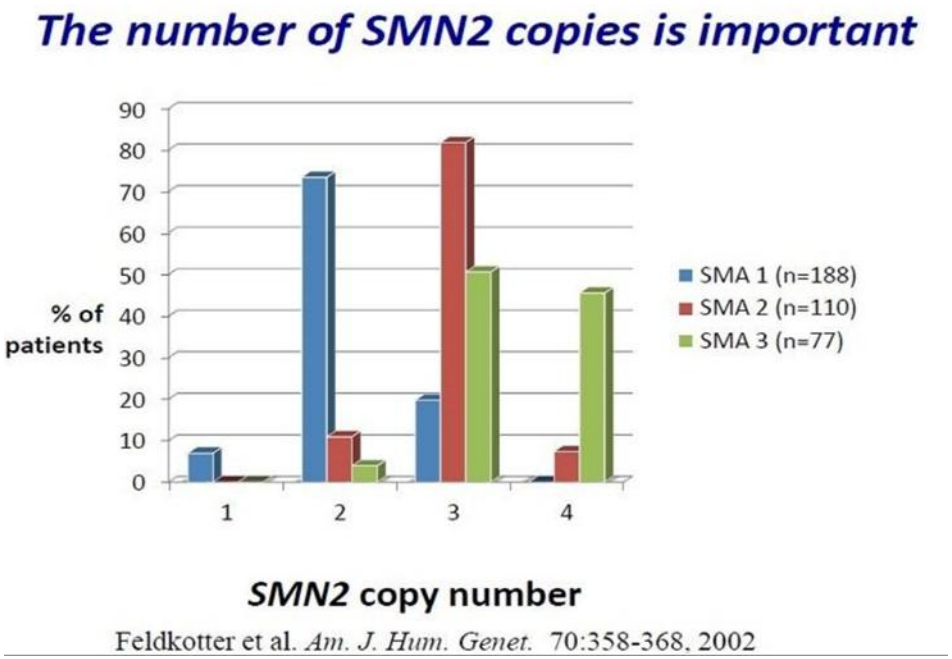

- **Loci on chromosome 5q13**
- **Both genes contain 9 exon and 8 intron - 20 kb**
- ***SMN1* - telomeric location**
  - **main functional gene – encodes 38K SMN protein**
  - **gene deletion/conversion leads to SMA**
- ***SMN2* – centromeric location**
  - **differs from *SMN1* by only 5 nucleotides**
  - **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**

2003-12 NINDS Spinal Muscular Atrophy Project to expedite therapeutics development

# SMA Drug Pipeline 2014


| | Identification | Optimization | Safety & Manufacture | Phase 1 | 2 | 3 |
|---|---|---|---|---|---|---|
| Trophos/olesoxime | ---------- | ---------- | ---------- | ---------- | ---------- | ----------> |
| ISIS/BIOGEN/ASO | ---------- | ---------- | ---------- | ---------- | ---------- | ----------> |
| Pfizer/Quinazoline | ---------- | ---------- | ---------- | ---------- | ----------> | |
| AVEXIS/NW/Genetherarpy | ---------- | ---------- | ---------- | ---------- | ----------> | |
| PTC/Roche/Small molecular | ---------- | ---------- | ---------- | ---------- | ----------> | |
| CA Stem Cell/Motor neuron | ---------- | ---------- | ---------- | hold | | |
| Parateck/Tetracyline | ---------- | ---------- | ----------> | | | |
| NINDS/INDOprofen | ---------- | ---------- | ----------> | | | |
| Genzyme/CNS gene therapy | ---------- | ---------- | ----------> | | | |
| Novartis/Small molecule | ---------- | ---------- | ----------> | | | |
| CAIBR/Small Molecules | ---------- | ----------> | | | | |
| Indiana U/Small Molecules | ---------- | ----------> | | | | |
| OSA/Um/Morpholinos ASO | ---------- | ----------> | | | | |
| Harvard/Small Molecules | ----------> | | | | | |
| Cytokinetics/Tirasemtiv | ----------> | | | | | |

**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 *SMN1* and *SMN2*

***SMN* Intron/Exon 7**

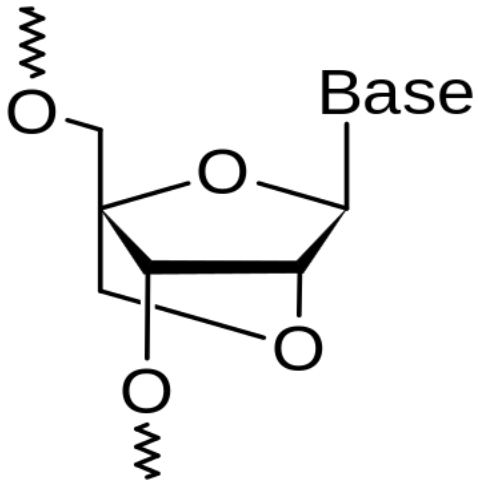
***SMN1***

ctgtaaaactttatggtttgtggaaaacaaatgttttgaacatttaaaaagttcagatgttaAaaagttgaaag
gttaatgtaaaacaatcaatattaaagaattttgatgccaaaactattagataaaaggttaatctacatccctact
agaattctcatacttaactggttggttAtgtggaagaaacatactttcacaataaagagctttaggatatgatgcc
attttatatcactagtaggcagaccagcagacttttttttattgtgatatgggataacctaggcatactgcactgta
cactctgacatatgaagtgctctagtcaagtttaactggtgtccacagaggacatggtttaactggaattcgtcaa
gcctctggttctaatttctcatttgcaggaaatgctggcatag

***SMN2***

ctgtaaaactttatggtttgtggaaaacaaatgttttgaacatttaaaaagttcagatgttaGaaagttgaaag
gttaatgtaaaacaatcaatattaaagaattttgatgccaaaactattagataaaaggttaatctacatccctact
agaattctcatacttaactggttggttGtgtggaagaaacatactttcacaataaagagctttaggatatgatgcc
attttatatcactagtaggcagaccagcagacttttttttattgtgatatgggataacctaggcatactgcactgta
cactctgacatatgaagtgctctagtcaagtttaactggtgtccacagaggacatggtttaactggaattcgtcaa
gcctctggttctaatttctcatttgcaggaaatgctggcatag

# 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**

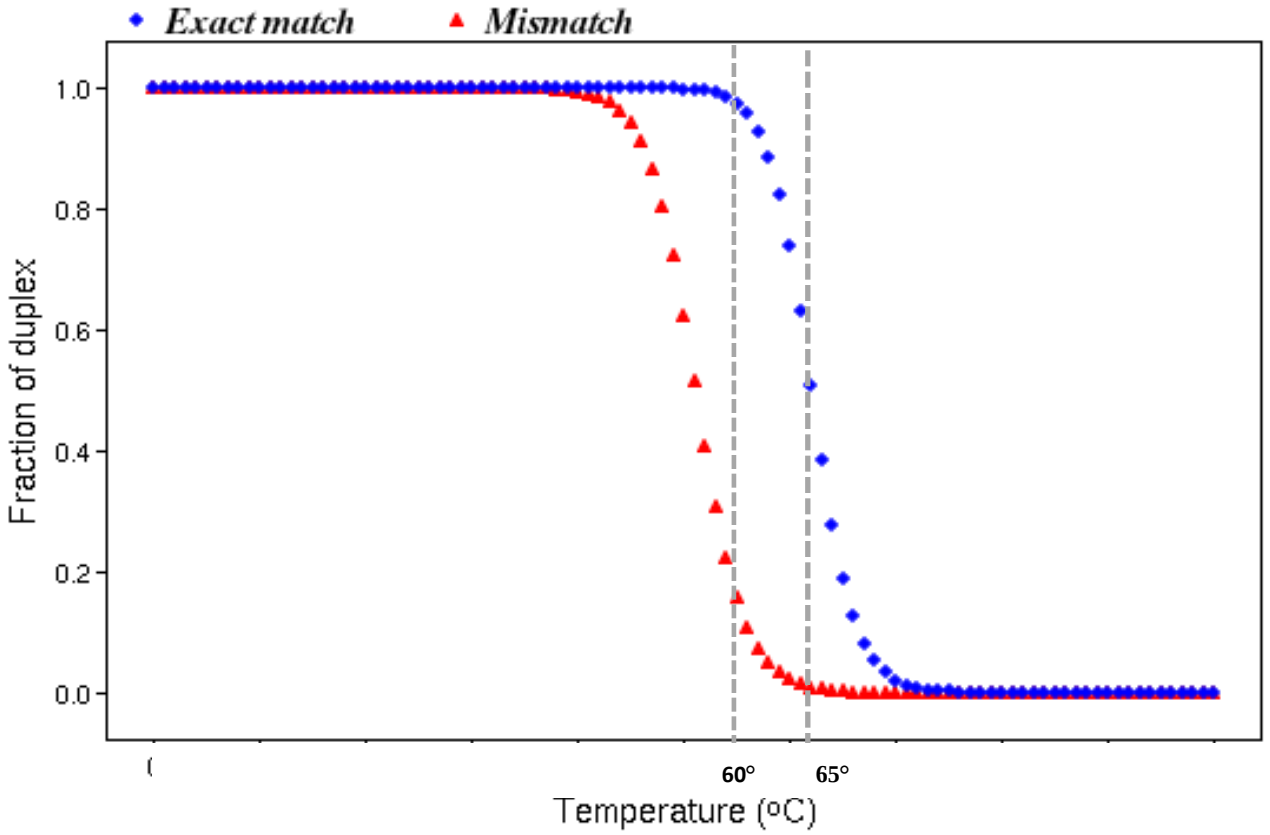
**This significantly increases the melting temperature in an oligonucleotide duplex**

**Allows higher hybrization temperature**
**→ increased specificity of a probe**

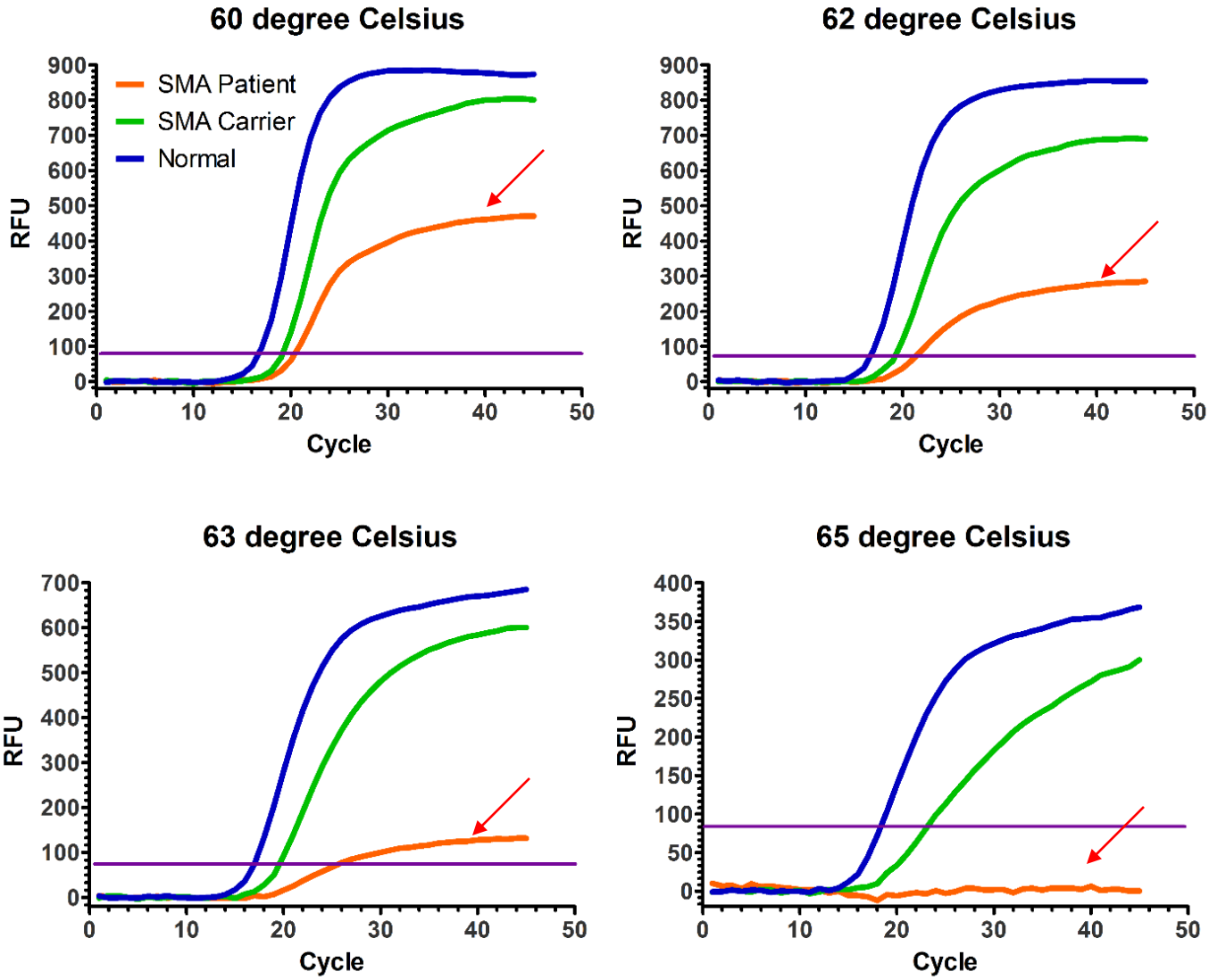
# Locked Nucleic Acid (LNA) *SMN1* Probe

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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** *SMN1*<br>**C** *SMN2* | A | T | T | G | T | A | G | A | -5' |

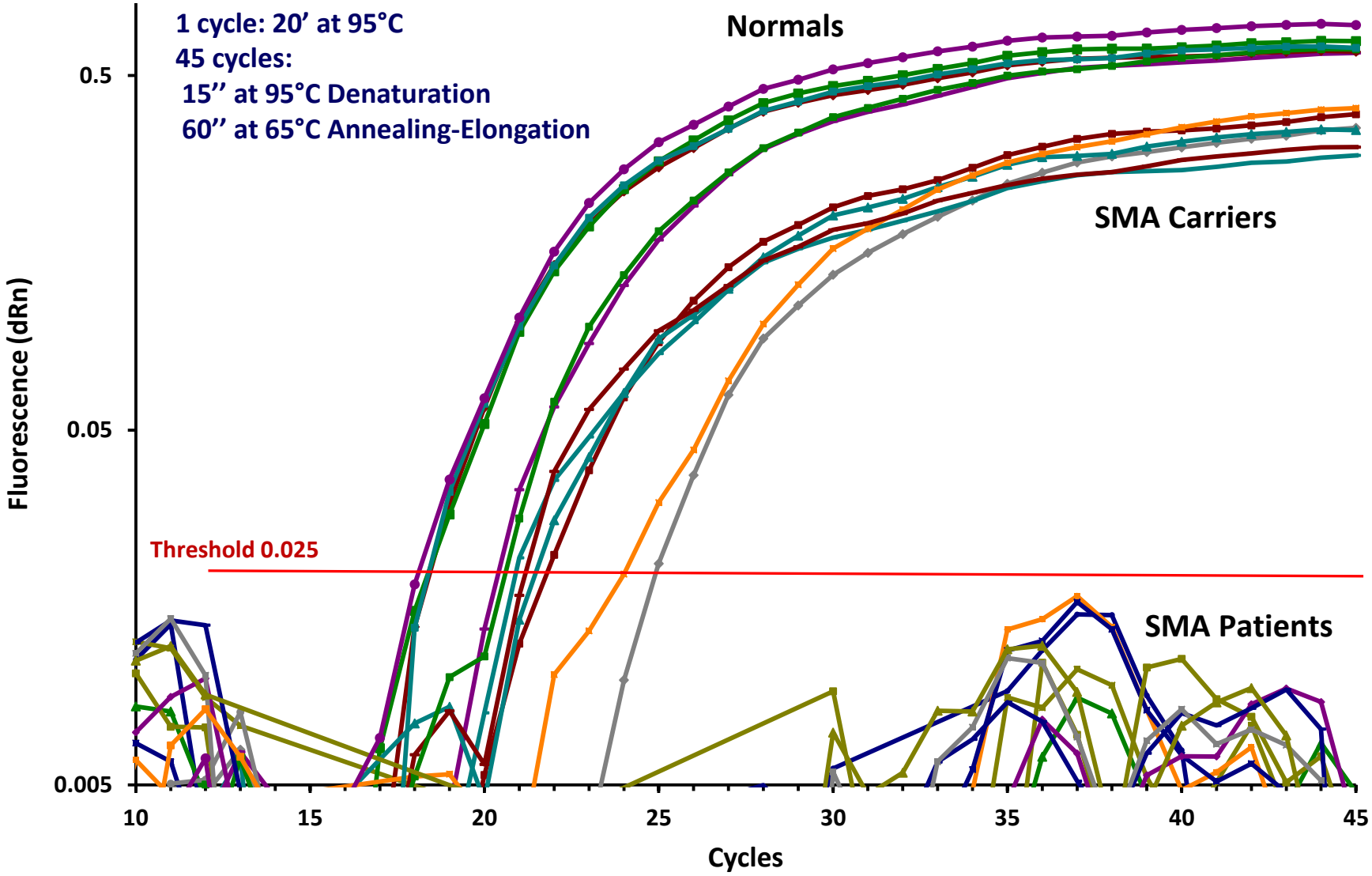
## Expected Probe Hybridization Profile



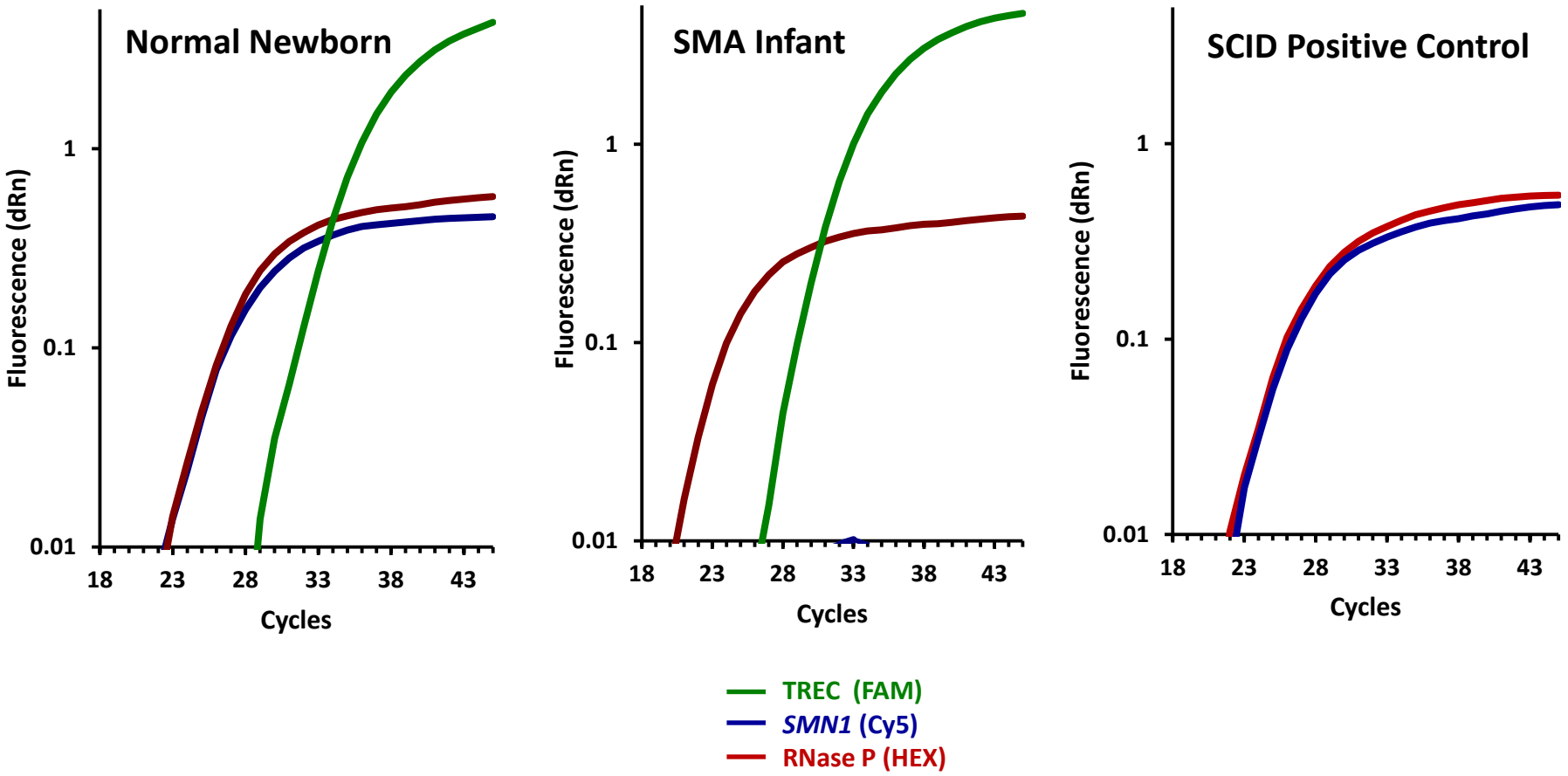
# Temperature Gradient real-time PCR



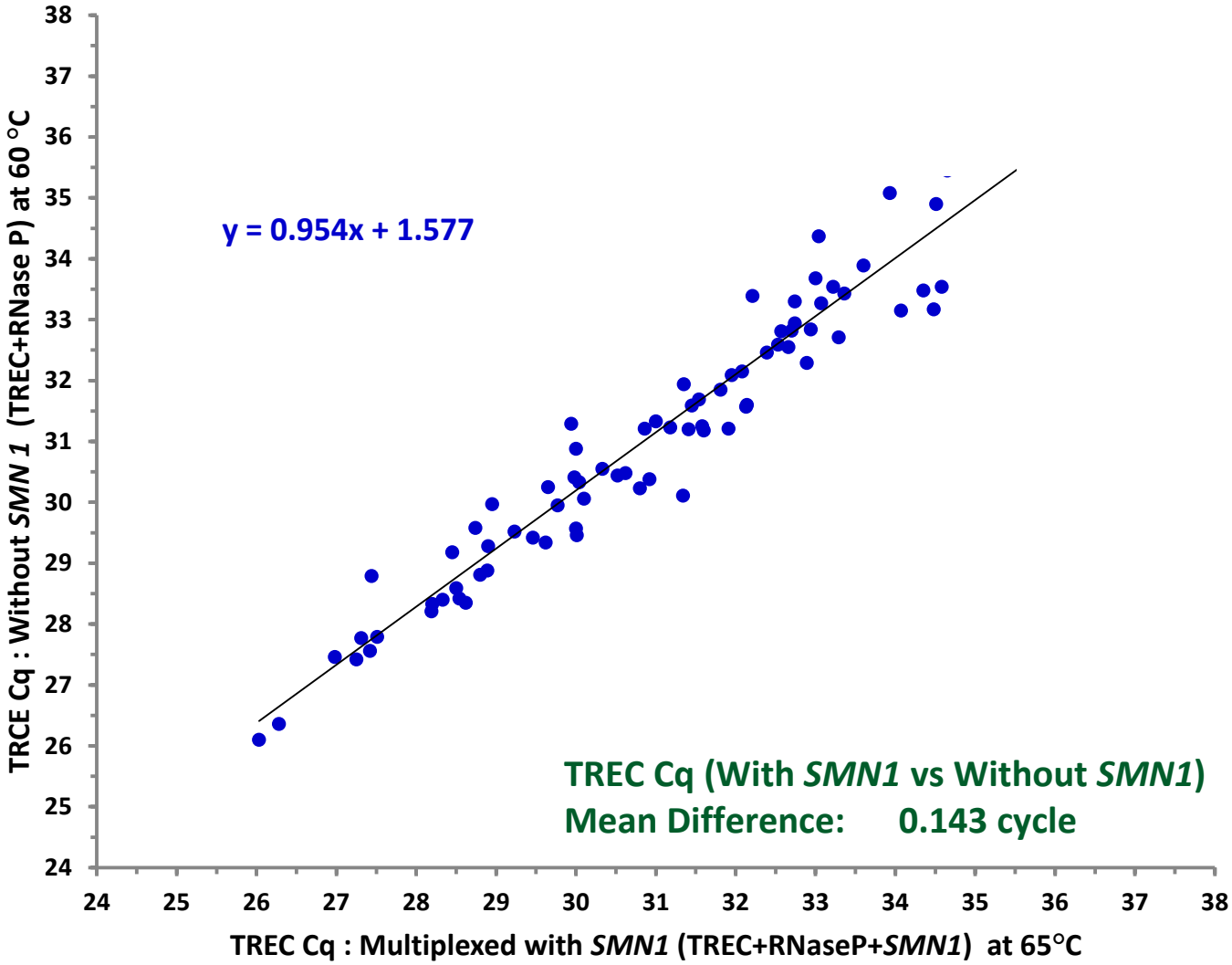
# *SMN1* Real-Time PCR Amplification Curves from DNA extracted from reference cell lines



# Multiplex TREC/*SMN1*/RNaseP Assay
## on Reference Materials

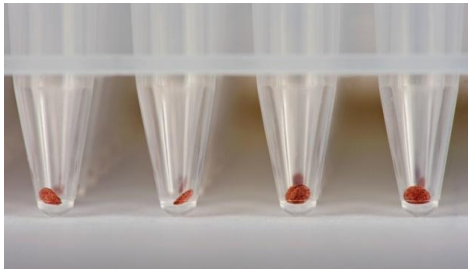


# TREC Cq (in Extracted Cord Blood DNA)

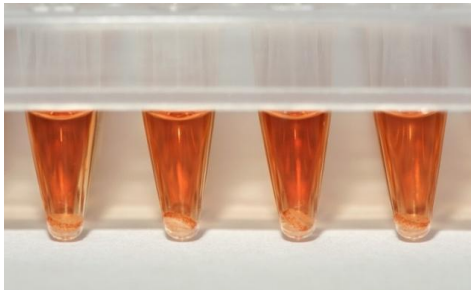



**Adding *SMN1* did not significantly affect the TREC Results**

# 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)**

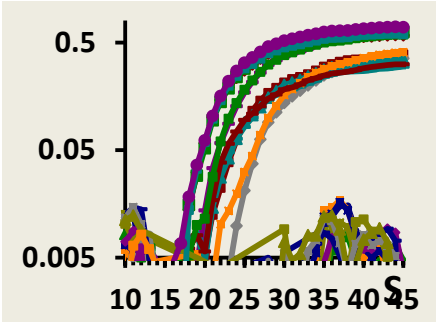
**Run real-time PCR**
**45°C for 5 min, 95°C for 20 min**
**45 cycles of [ 95°C x 15 sec + 65°C x 1 min ]**

## Multiplex Real-time PCR assay for SMA/RNaseP/TREC on 26 Blinded DBS Samples from SMA Patients and Carrier parents

| Sample | Donor Status | Age | SMN1 Cq | RNaseP Cq | TREC | |
|---|---|---|---|---|---|---|
| | | | | | Cq | copies/µl |
| P-02 | affected | 4 | No Ct | 23.8 | 30.2 | 163 |
| P-04 | affected | 2 | No Ct | 24.2 | 31.7 | 58 |
| *P-05* | affected | 50 | *No Ct* | 25.1 | 34.6 | *8* |
| P-07 | affected | 3 | No Ct | 23.5 | 29.6 | 246 |
| P-10 | affected | 1 | No Ct | 24.5 | 30.0 | 187 |
| P-15 | affected | 22 | No Ct | 23.7 | 30.4 | 142 |
| P-18 | affected | 13 | No Ct | 23.2 | 31.3 | 77 |
| P-22 | affected | 3 | No Ct | 23.2 | 29.5 | 263 |
| P-23 | affected | 1 | No Ct | 21.6 | 28.7 | 455 |
| P-25 | affected | 4 | No Ct | 22.6 | 28.5 | 522 |
| P-26 | affected | 2 | No Ct | 22.5 | 29.1 | 346 |
| P-01 | parent | 45 | 24.2 | 23.3 | 34.3 | 10 |
| P-03 | parent | 33 | 26.9 | 25.0 | 34.3 | 10 |
| P-06 | parent | 34 | 25.6 | 24.8 | 33.9 | 13 |
| P-08 | parent | 29 | 25.0 | 23.8 | 34.0 | 12 |
| P-09 | parent | 32 | 24.2 | 23.2 | 34.4 | 9 |
| P-11 | parent | 43 | 23.3 | 22.2 | 34.8 | 7 |
| P-12 | parent | 43 | 22.7 | 21.9 | 33.0 | 24 |
| P-13 | parent | 41 | 23.0 | 22.4 | 35.0 | 6 |
| P-14 | parent | 57 | 25.5 | 24.9 | 34.4 | 9 |
| P-16 | parent | 48 | 22.7 | 22.3 | 35.3 | 5 |
| P-17 | parent | 48 | 22.5 | 22.8 | 34.4 | 9 |
| P-19 | parent | 44 | 25.8 | 25.1 | 36.7 | 2 |
| P-20 | parent | 35 | 21.1 | 21.1 | 31.4 | 71 |
| P-21 | parent | 33 | 22.6 | 22.6 | No Ct | 0 |
| P-24 | parent | 25 | 21.8 | 22.0 | 32.9 | 26 |

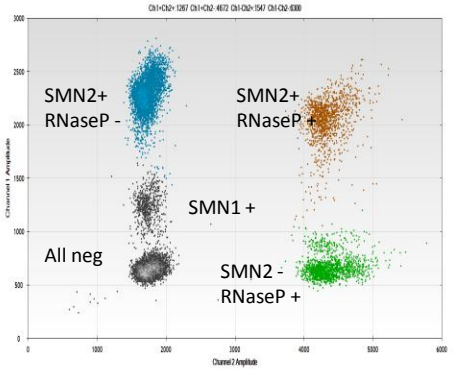
# Discussion

- We have multiplexed the *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 *SMN1* gene in the sample
  - Provide with precision the number of *SMN2* gene, which can be valuable for prognosis and medical management



# Acknowledgements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