Newborn screening for spinal muscular atrophy

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Clinical Features of Spinal Muscular Atrophy (SMA)

- A neuromuscular disease resulting in the progressive degeneration of motor neurons
- Symptoms include loss of normal motor function and respiratory failure; can result in death
- 3 clinical types based on age of onset and severity

Type I: Birth – 6 mos.
Type II: 6 mos. – 2 years
Type III: 18 mos. – 3+ years

SMA is the leading genetic cause of death among infants

- The birth prevalence of SMA is approx. 1 in 10,000
- Type I (infantile-onset) is the most common form
- The majority of children with Type I SMA do not survive beyond 2 years without effective therapy
- FDA approved therapy exists
- SMA has been nominated for inclusion on the Recommended Uniform Screening Panel (RUSP)

Genetic characterization of SMA

- **Autosomal recessive inheritance**
- **Approximately 96% of SMA cases are caused by mutations in the *SMN1* gene**
  - ***SMN1* encodes for survival of motor neuron (SMN) protein**
- **Among the *SMN1* mutations, most involve the loss of *SMN1* exon 7 (on both chromosomes) by deletion or gene conversion**
  - **Loss of this gene region results in a non-functional SMN protein**
  - ***SMN2*, a paralog of *SMN1*, may moderate the disease severity**
    - *SMN2* can only produce 10% of the SMN protein produced by *SMN1*

Wirth B., *Human Mutation* (2000), 15:228-37

Several different molecular assays have been used to detect SMA

- Restriction Fragment Length Polymorphism (RFLP) test
- High Resolution Melting (HRM) analysis
- Multiplex Ligation-Dependent Probe Amplification (MLPA)
- Luminex Genotyping
- DNA sequencing
- Quantitative (qPCR)/ Real-time PCR (RT-PCR)

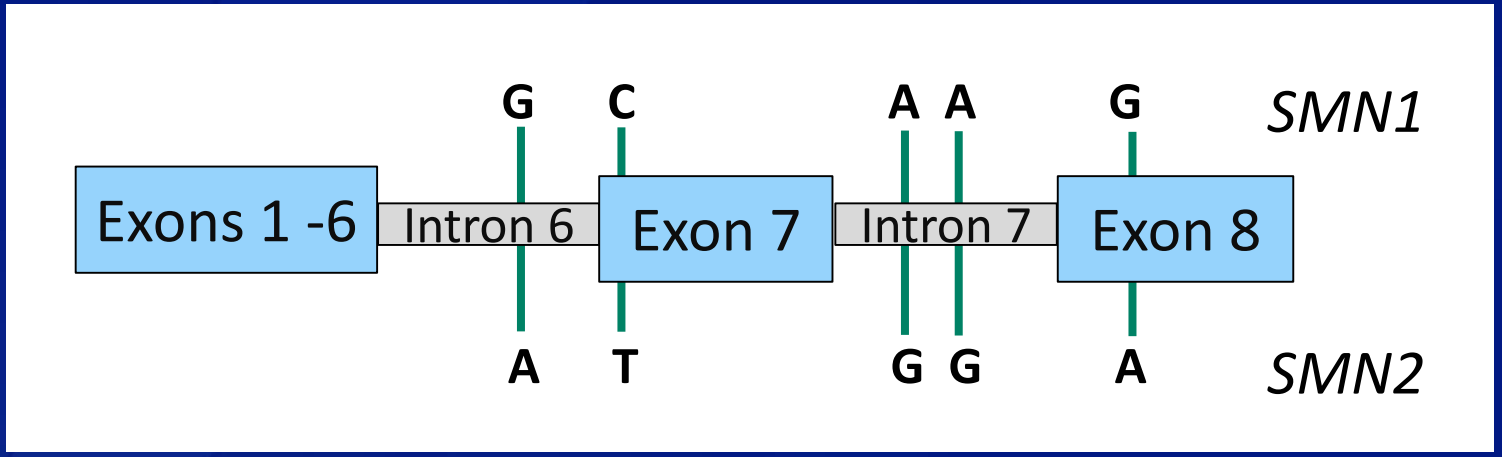
Real-time PCR is one method that can be used to screen newborns for SMA

- **Real-time PCR allows for high throughput screening**
- **Most state newborn screening labs are already using this method**
  - Labs are equipped with the necessary instrumentation
  - Staff is familiar with procedure
- **Reactions can be multiplexed**
  - Reduced cost to include a new assay
  - May not require added labor cost to run

What are some challenges associated with using real-time PCR to screen for SMA?

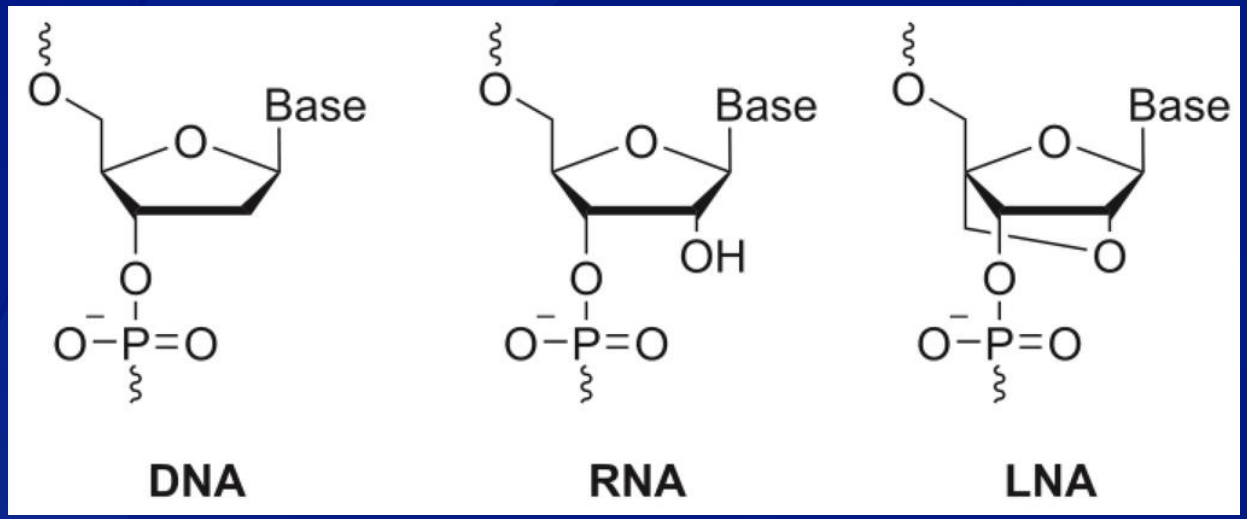
Challenge #1:

SMN1 has a paralog, the *SMN2* gene, which has nearly identical genomic sequence



- There are only 5 nucleotide differences between the two genes
- For real-time PCR, it is important to avoid detecting *SMN2* when trying to identify the loss of *SMN1*

LNA (locked nucleic acid) nucleotides can be used to distinguish single nucleotide differences



- LNAs can be incorporated into primers and probes to discern single nucleotide differences between *SMN1* and *SMN2*
- This would allow for discriminatory amplification and/or signal detection of *SMN1* only

Astakhova K., *Chemosensors* (2014), 2(3):196-206

Initial SMA assay developed at CDC

***SMN1* (*SMN2*) Intron 7 Sequence**

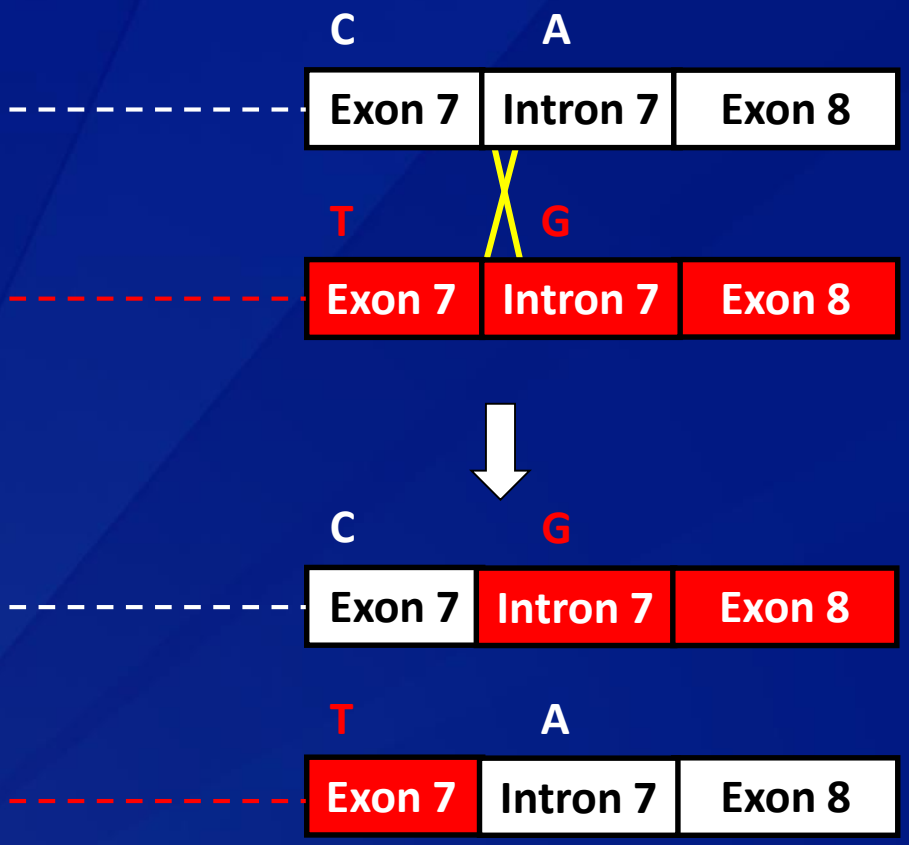
ttttgtaaaactttatggtttgtggaaaacaaatgttttgaacatttaaaa
agttcagatgttaA (G) aaagttgaaaggttaatgtaaaacaatcaatatta
aagaattttgatgccaaaactattagataaaaggttaatctacatccctact

- The loss of *SMN1* intron 7 was detected using a LNA probe (in green)
  - LNA substitutions underlined
- The LNA probe was designed to selectively bind *SMN1* by discriminating between the mismatch nucleotides of *SMN1* and *SMN2*
  - *SMN1* nucleotide **(A)** and *SMN2* nucleotide **(G)**
- Forward and reverse primers (in yellow) will amplify both *SMN1* and *SMN2* sequences

Taylor, J. et al., *Clin. Chem*, (2015), 61 (2): 412-9

Challenge #2:

Recombination between *SMN1* and *SMN2* can result in a hybrid genotype



*Yin-Hsiu C. et al., *The Journal of Pediatrics* (2017); **Hahnen, E. et al., *Am. J. Hum. Genet.*, (1996), 59: 1057-1065

Revised SMA Assay – Part 1

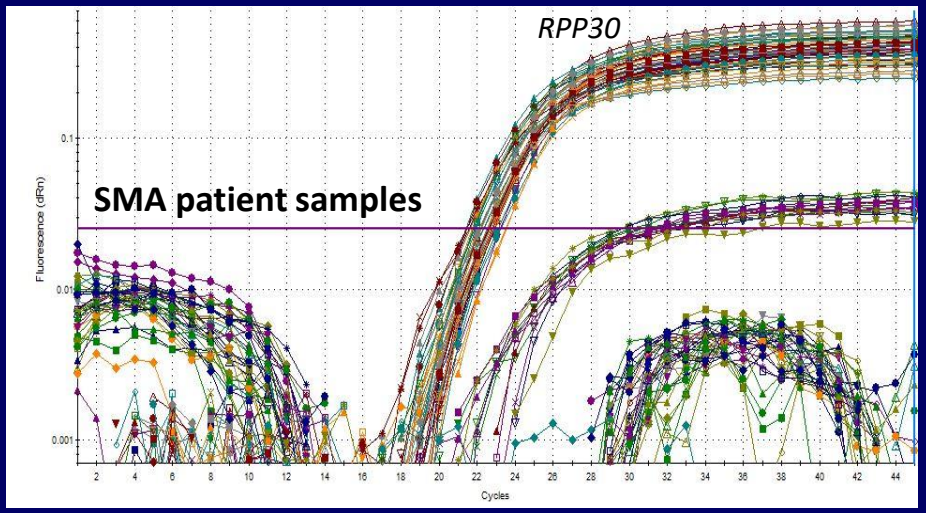
***SMN1* (*SMN2*) Exon 7 Sequence**

taagtaaaatgtcttgtgaaacaaaatgcttttaacatccataaagc
tatctatatatagctatctatG(A)tctatatagctatttttttaactt
cctttattttccttacaggggtttC(T)agacaaaatcaaaaagaaggaag
gtgctcacattccttaaattaaggagtaagtctgccagcattatgaaagt

- We modified the previous assay to target exon 7 and reduce the possibility of false positive or false negative results due to hybrid genotypes
  - The LNA probe was designed to selectively bind *SMN1* by discriminating between the mismatch nucleotides of *SMN1* **(C)** and *SMN2* **(T)**
  - Forward and reverse primers (in yellow) will amplify both *SMN1* and *SMN2* sequences

Maranda, B. et al., *Clin. Biochem.*, (2012), 45(1-2): 88-91

Assay gives *non-specific* amplification some of the time when testing samples derived from SMA patients



Non-specific signal from *SMN2*

No signal

- The LNA probe designed to recognize *SMN1* only can bind the *SMN2* amplicon, producing non-specific signal in SMA patient samples

Revised SMA Assay – Part 2

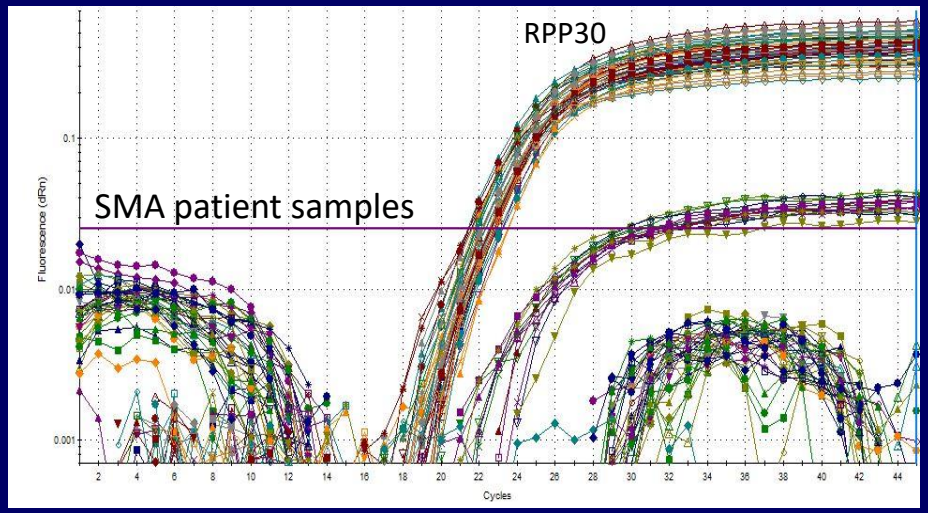
***SMN1* (*SMN2*) Exon 7 Sequence**

taagtaaaatgtcttgtgaaacaaaatgcttttaacatccatataaagc
tatctatatatagctatctatG(A)tctatatagctatttttttaactt
cctttattttccttacaggggtttC(T)agacaaaatcaaaaaagaaggaag
gtgctcacattccttaaattaaggagtaagtctgccagcattatgaaagt
gaatcttacttttgtaaaactttatggtttgtggaaaacaaatgttttg
aacatttaaaaagttcagatgttaA(G)aaagttgaaaggttaatgtaaa
acaatcaatattaaagaattttgatgccaaaactattagataaaaggtta

- We replaced the original, reverse primer with an *SMN1*-specific LNA primer (in blue) to eliminate *SMN2* amplification

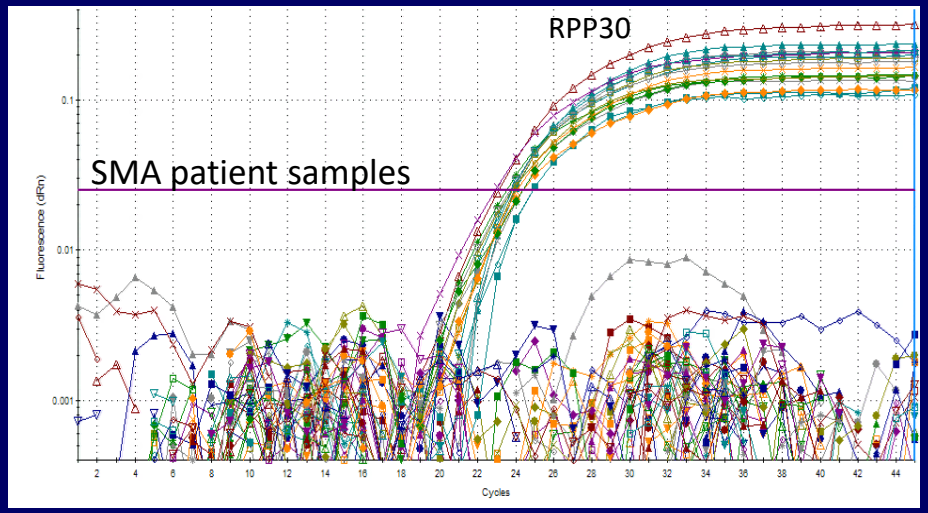
Assay specificity improves by adding LNA primer

Assay Revision Part 1



Non-specific signal from *SMN2*

Assay Revision Part 2



No signal from *SMN2*

Technical concern:

Assay did not perform as expected in all environments

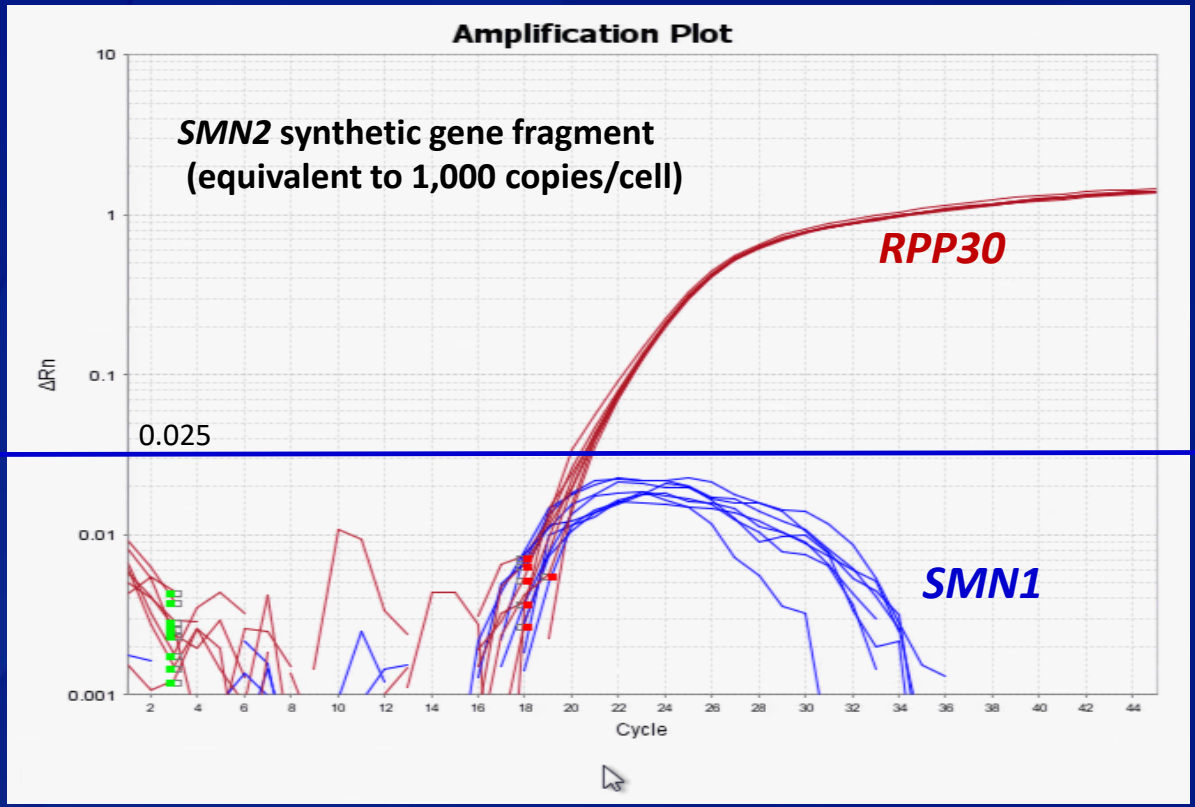
- Possible reasons for reduced assay efficiency:
  - Sensitive to DNA extraction method
  - Sensitive to type of Taqman master mix
  - Sensitive to temperature fluctuations > 1 degree Celsius
- Further method improvement was needed

LNA probe was redesigned to make the assay more robust

- **Factors important in the design of LNA probe for mismatch discrimination:**
  - Length of the probe
    - short (10-12 nucleotides)
  - Location of mismatch in the probe
    - center position within probe
  - Modification pattern
    - LNA substitution in triplet at site of mismatch
  - Identity of the mismatch
    - pyrimidine (C or T) at mismatch site within probe (discrimination is poor for G-T mismatches)

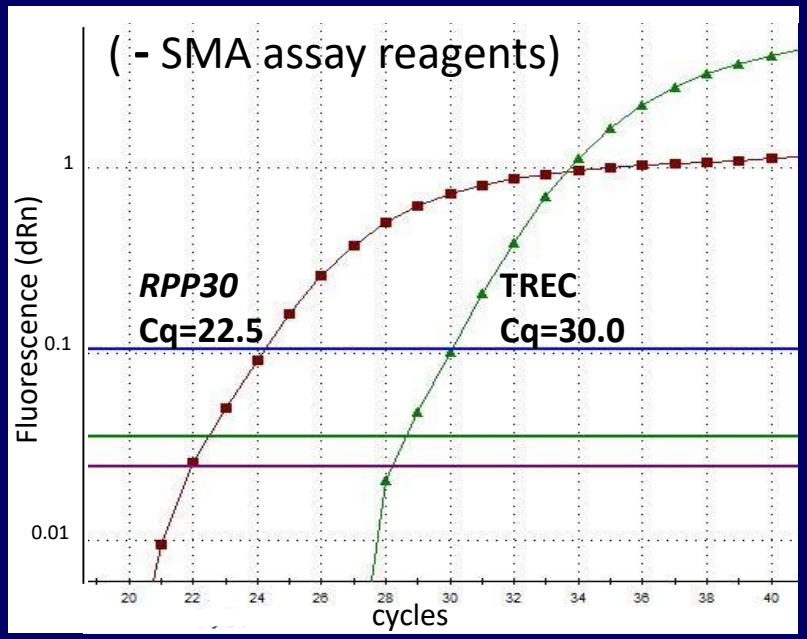
You, Y. et al., *Nucleic Acids Research*, (2006), 34(8)

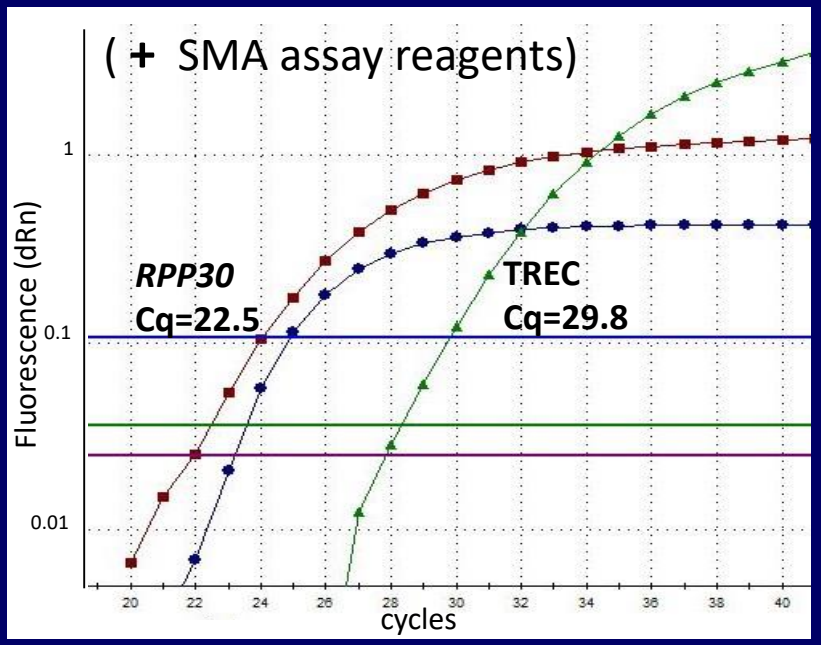
The Current Assay utilizes an *SMN1*-specific LNA probe with forward strand sequence



- We do not observe any non-specific signal in *SMN1* null samples even when challenged with an excess of *SMN2* sequence

This assay can also be multiplexed with primers and probes for RNase P (*RPP30*) and TREC





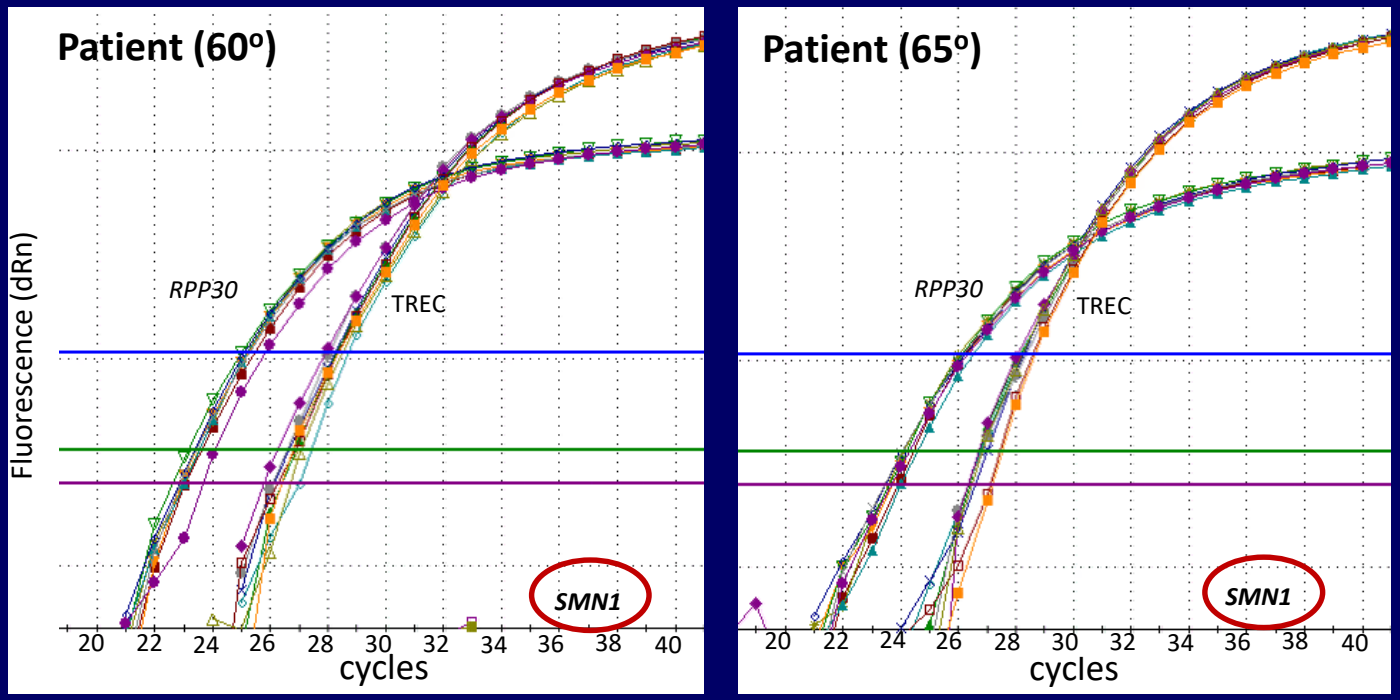
TREC

RPP30

SMN1

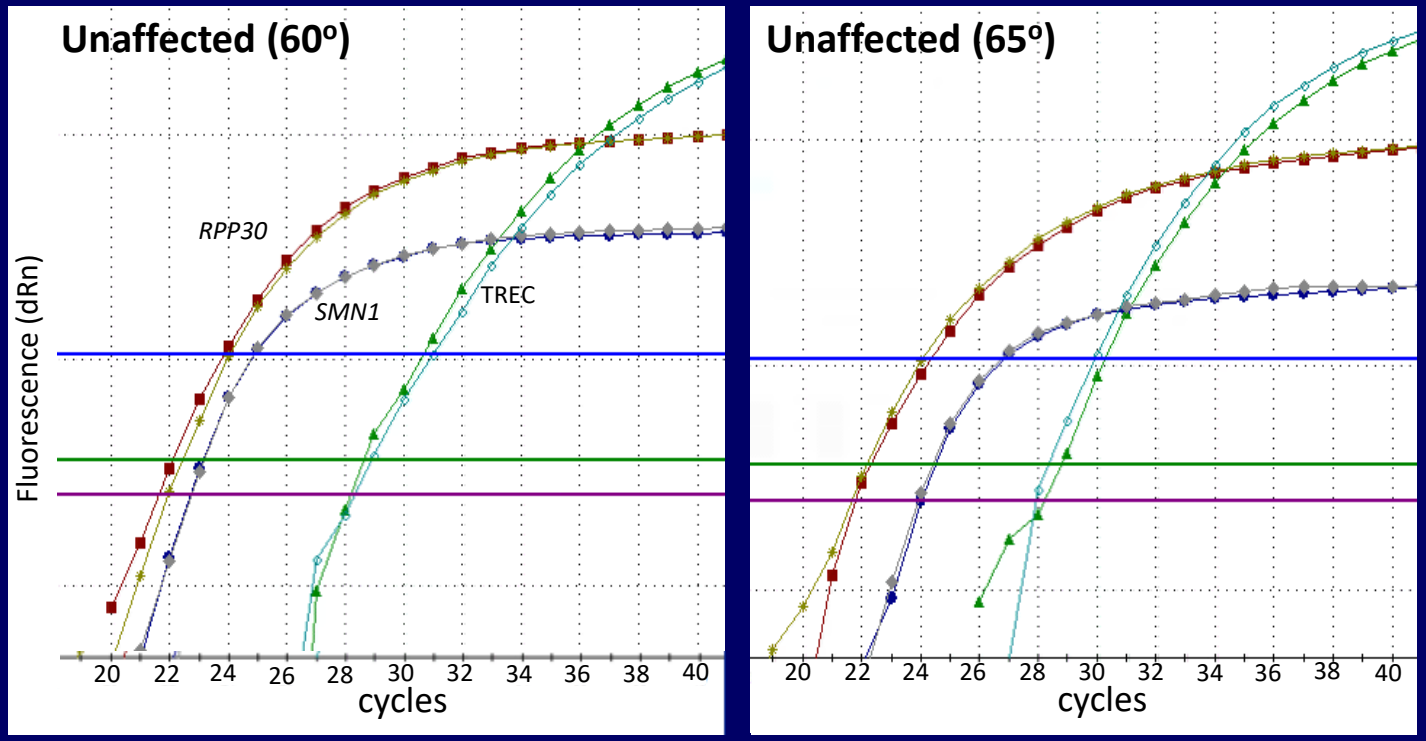
- Cq values for RNase P and TREC are unaffected by the addition of reagents for SMA

The Current SMA Assay works at a range of temperatures from 60-65 degrees Celsius



- Patient samples are SMA test positive (no *SMN1* signal) at temperatures ranging from 60-65 degrees Celsius
- Don't need to worry about variations in instrument temperature affecting the results

The Current SMA Assay works at a range of temperatures from 60-65 degrees Celsius



- There is no observed effect of temperature on the Cq values for RNase P and TREC
- May not need to change the temperature of current TREC assay
- *SMN1* amplification in not negatively affected

SMA patients are correctly identified from dried blood spots when using the current assay

	Assay Results		Clinical Category
Sample Number	Cq- SMN1 Exon 7	SMN1 Result	SMA Status
1	24.69	Present	Unaffected/ Carrier
2	No Cq	Absent	Affected
3	26.43	Present	Unaffected/ Carrier
4	No Cq	Absent	Affected
5	No Cq	Absent	Affected
6	25.67	Present	Unaffected/ Carrier
7	No Cq	Absent	Affected
8	24.28	Present	Unaffected/ Carrier
9	24.23	Present	Unaffected/ Carrier
10	No Cq	Absent	Affected
11	24.15	Present	Unaffected/ Carrier
12	25.19	Present	Unaffected/ Carrier
13	25.21	Present	Unaffected/ Carrier
14	28.15	Present	Unaffected/ Carrier
15	No Cq	Absent	Affected
16	24.49	Present	Unaffected/ Carrier
17	24.78	Present	Unaffected/ Carrier
18	No Cq	Absent	Affected
19	26.31	Present	Unaffected/ Carrier
20	23.81	Present	Unaffected/ Carrier
21	22.99	Present	Unaffected/ Carrier
22	No Cq	Absent	Affected
23	No Cq	Absent	Affected
24	22.32	Present	Unaffected/ Carrier
25	No Cq	Absent	Affected
26	No Cq	Absent	Affected
27	23.35	Present	Unaffected/ Non-Carrier
28	23.46	Present	Unaffected/ Non-Carrier

Key Features of the Current SMA assay

Assay Design Elements

- Targets Exon 7 and *not* Intron 7
- Forward Strand LNA Probe provides robust specificity (no background signal from *SMN2)*

Assay Characteristics

- Multiplex capable: can be used with TREC assay by adding only a few extra reagents; lower cost
- Sensitive: identified 100% of SMA patients with loss of *SMN1* exon 7
- Flexible: (1) can be used at temperatures ranging from 60^{o}C -65^{o}C , (2) works using “in situ” method and with DNA extracted from dried blood spots

Additional Key Points

- Both the LNA primer and forward strand (FS) probe improve specificity in detecting loss of *SMN1* at exon 7
- Current Assay using FS probe is comparatively more robust and cost effective
- Use of a PCR clamp to suppress *SMN2* amplification has also been developed, which can add an additional layer of specificity in a second tier assay for samples that are inconclusive
- Droplet digital PCR can be used to determine copy number of *SMN1* and *SMN2*

CDC can provide consultation and technical support to labs interested in screening for SMA

- Pre assay development consultation
  - Providing sequence for SMA assay primers and probe
  - Integrating SMA into current TREC assay
- Reference materials for assay development and validation
- Individual training at CDC
  - Performing real-time PCR assay
  - Preparation of QC materials

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Thank you for your attention!

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