Highly specific and sensitive TaqMan SCID/SMA multiplex assay with rapid and optimized DBS workflow

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ABSTRACT

Intensive research in early detection of SCID/SMA is essential to discover prevention methods for infants' permanent disabilities or death. In addition to Severe Combined Immunodeficiencies (SCID), Spinal Muscular Atrophy (SMA) has been recently added to the US-RUSP list. SMA is a motor neuron disorder caused by mutation in the *SMN1* gene, whereas SCID constitutes a series of immune system functionality diseases exhibiting low levels of T-cell receptor excision circles (TREC). Since the implementation of a single test for both SMA and SCID is currently sought after globally, we developed a real-time SCID/SMA multiplex assay[1,2] that permits concomitant measurement of *SMN1*, *TREC*, and *RNaseP* reference. We designed the SMA assay to target exon 7 of *SMN1* gene and effectively eliminated non-specific detection of the highly similar *SMN2* gene. This high SMN1 target specificity of SMA assay limits both ambiguous calls and requirement for retesting. We also confirmed TREC target specificity and the SCID assay's high sensitivity with TREC copy number detection capability of as low as 9 copies per reaction. Additionally, we were able to substantially improve DBS sample preparation method and reduce the number of steps to a minimum. In conclusion, we have developed a highly specific, sensitive, and robust multiplex assay for SMA and SCID testing with a rapid and streamlined turnaround workflow to aid further research efforts.

MATERIALS AND METHODS

Sample preparation:

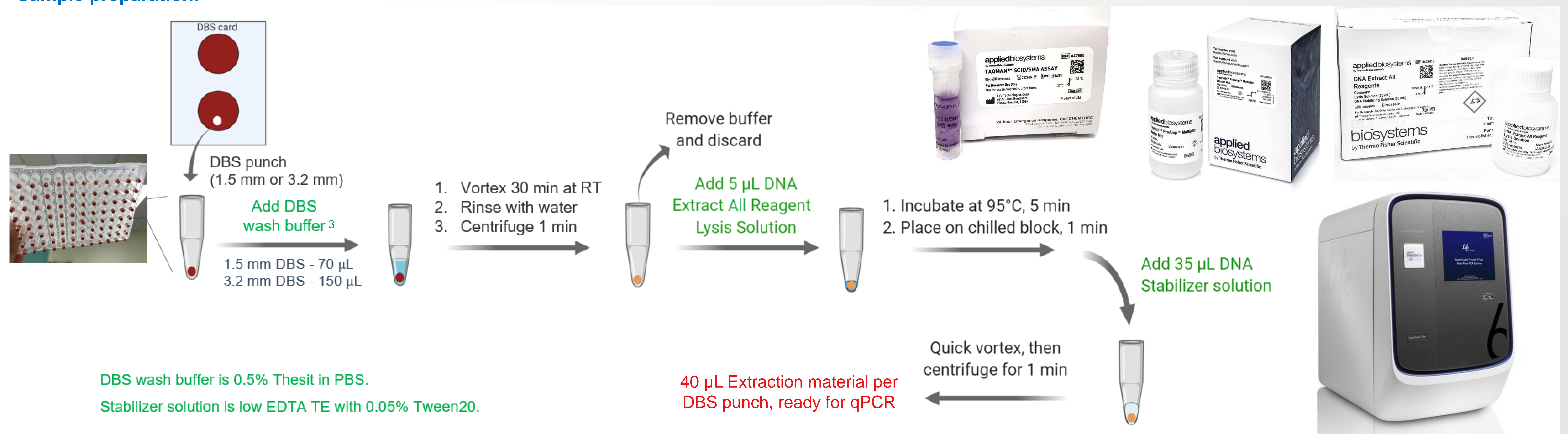

Figure 1. DBS workflow. DBS workflow duration is ~40 min total with ~35 min hands-off time. DBS punch-to-answer duration for 96-well plate format is < 2 hr.

qPCR reaction set up:

Component	Volume per reaction	
	96-well	384-well
2X TaqPath™ ProAmp™ Multiplex Master Mix	10.0 µL	7.5 µL
20X TaqMan™ Newborn Screening SCID/SMA Assay	1.0 µL	0.75 µL
Extraction material	9.0 µL	6.75 µL
Total PCR Reaction volume	20.0 µL	15.0 µL

Instrument and Cycling Condition:

Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System, 96-well (0.2 mL - 4485689, 0.1 mL - 4485699) and 384-well (4485691). Cycling condition: 95ºC 20s hold, follow by 40 cycles of 95ºC 1s, 60ºC 20s. Total cycling time is less than 40min.

Samples:

Umbilical cord blood samples to simulate normal newborn DBS sample, Geriatric blood samples to simulate SCID-like DBS sample, and **CDC Proficiency Test (PT) samples** (Umbilical cord blood, SMA-like, SMA Carrier-like), Blank DBS (fresh DBS, no blood), and nuclease-free water for NTC (No Template Control); Plasmids with TREC, SMN1, SMN2, and RNaseP target sequences as inserts.

Multiplex TaqMan™ Assay Fluorescent Label Assignment:

TaqMan™ SCID/SMA assay (A47927): SMN1- FAM, TREC- JUN™, RNaseP (genomic control)- VIC™. Primers and TaqMan® dual labeled probes were synthesized by Thermo Fisher and formulated into a 20X TaqMan™ Assay.

TaqMan™ SCID/SMA Plus assay (A48567): SMN1- FAM, KREC- ABY™, TREC- JUN™, RNaseP (genomic control)- VIC™. Primers and TaqMan™ dual labeled probes were synthesized by Thermo Fisher and formulated into a 20X TaqMan™ Assay.

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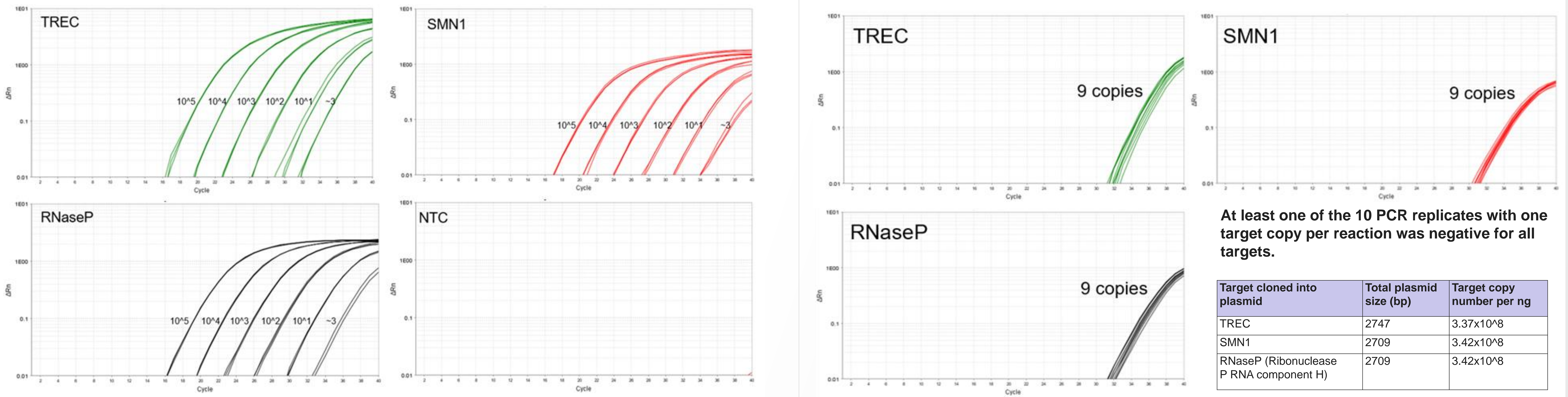
Results

TaqMan™ SCID/SMA assay

Limit of Detection



Target cloned into plasmid	Total plasmid size (bp)	Target copy number per ng
TREC	2747	3.37x10^8
SMN1	2709	3.42x10^8
RNaseP (Ribonuclease P RNA component H)	2709	3.42x10^8

Figure 2. Limit of Detection of TaqMan™ SCID/SMA assay is calculated to be 9 copies for all three targets including RNaseP genomic control. LOD screening was carried out with plasmids containing respective targets as inserts starting at ~3x10^5 copies per reaction in triplicate reactions. At 9 copies per reaction, all three targets showed positive amplification for 10 PCR replicates. (QS6 Flex, 0.1 mL platform)

Assay performance

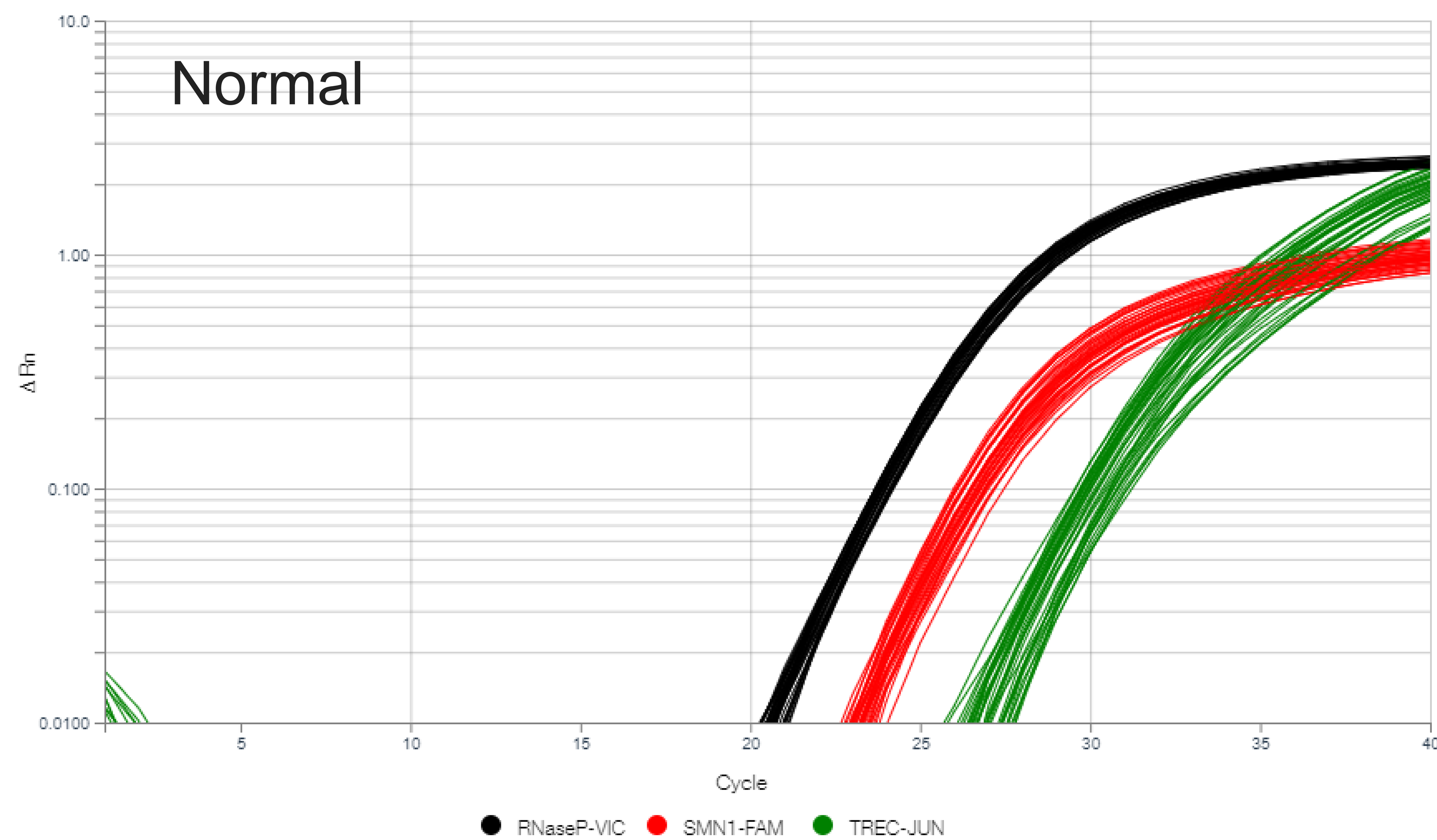


Figure 3. Amplification plot showing all three targets including RNaseP genomic control (**black lines**) amplified in a normal sample. Ten independent extractions were performed, and qPCR reactions were run in quadruplicates. Normal samples are used to establish a cut off Ct. (3.2 mm DBS punch size; QS6 Flex, 0.2 mL platform)

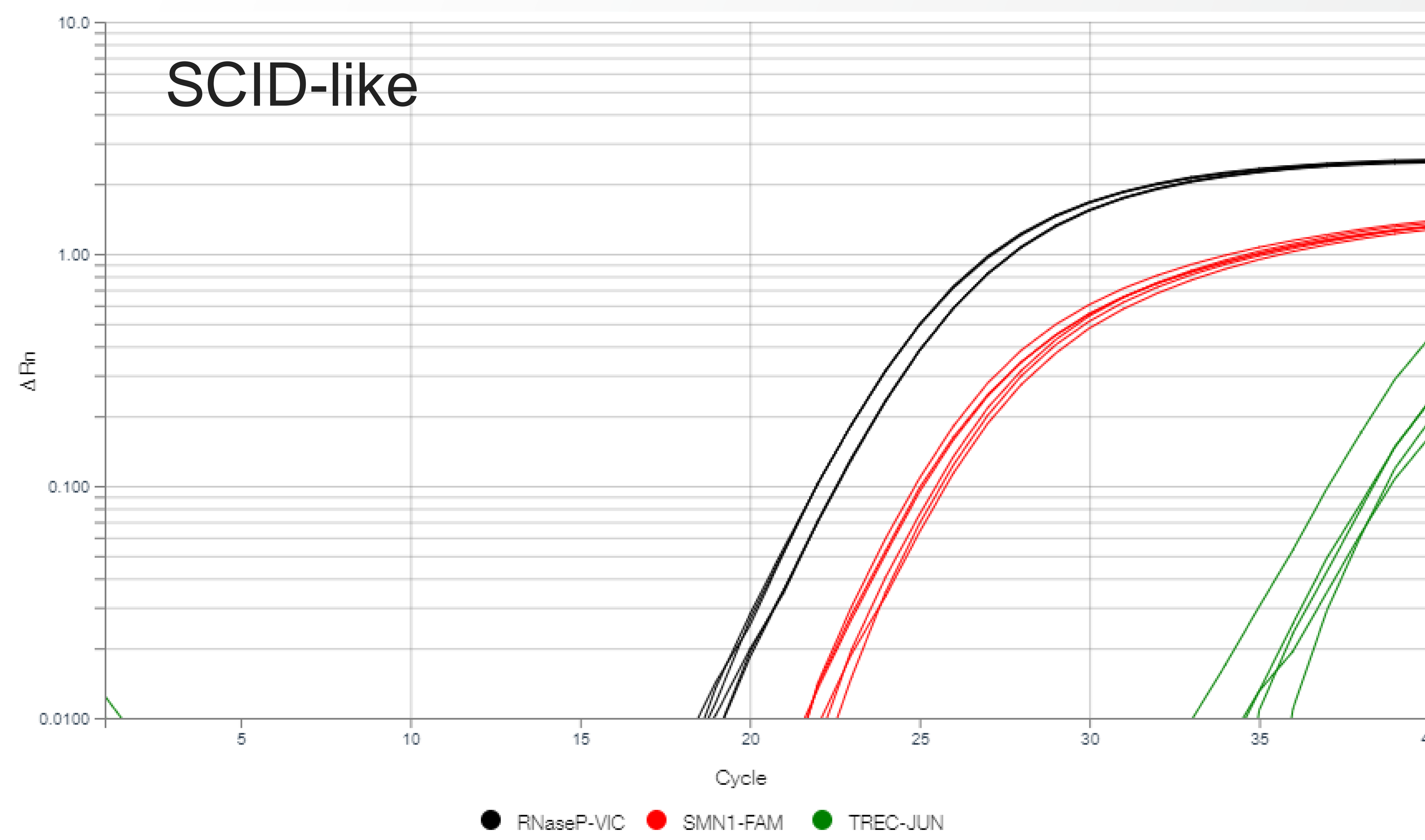


Figure 4. Amplification plot showing low T-cell receptor excision circles (TREC) amplification (**green lines**) compare to other targets in SCID-like samples from two donors (healthy adult aged > 65 yrs). TREC is a biomarker for T-cell development. Low TREC level can indicate severe combined immunodeficiency (SCID). qPCR reactions were run in triplicates per sample. (3.2 mm DBS punch size; QS6 Flex, 0.2 mL platform)

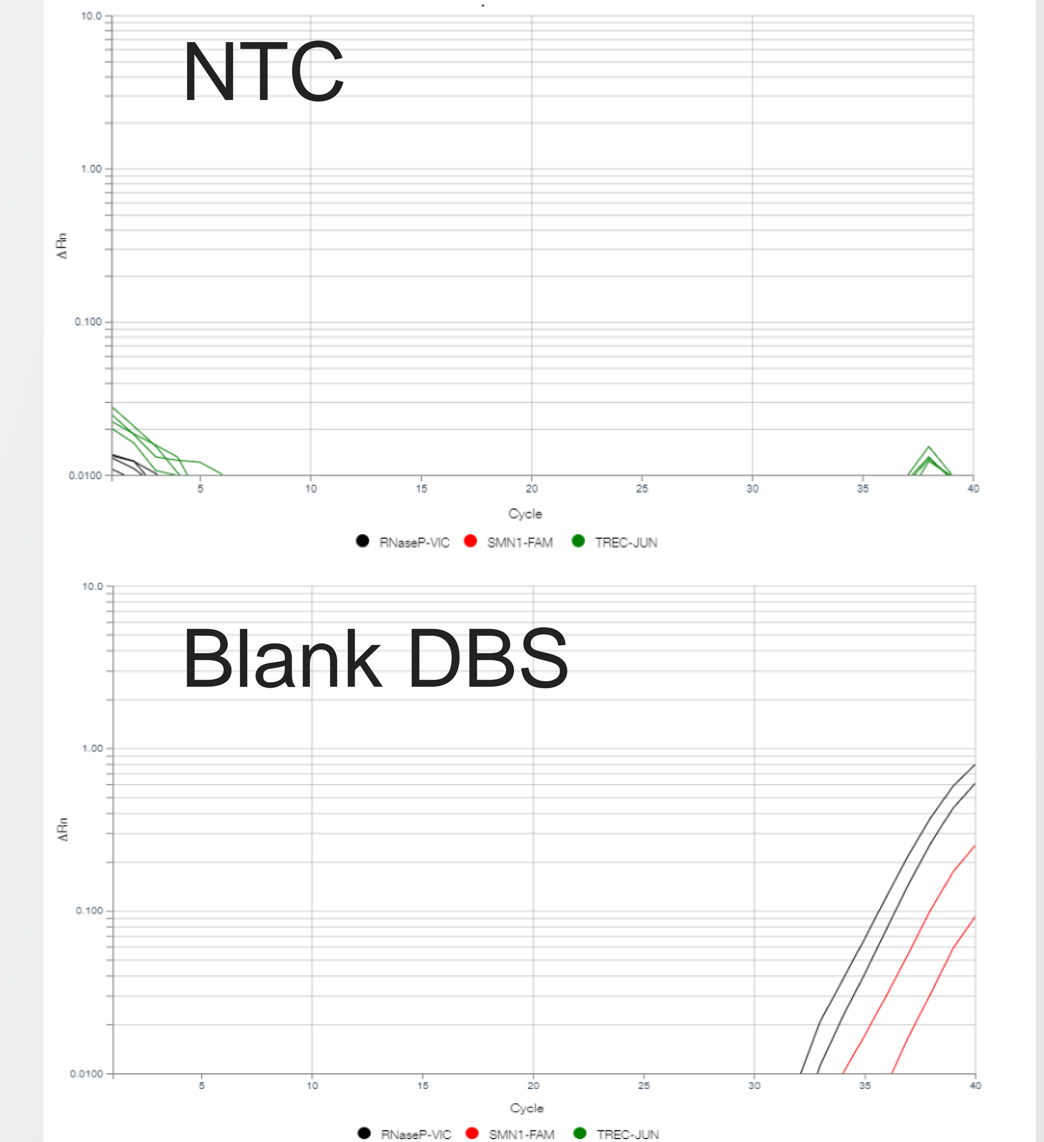


Figure 5. Amplification plot showing no target amplification in NTC wells (top) and Blank DBS samples (bottom). qPCR reactions were run in quadruplicates. (QS6 Flex, 0.2 mL platform)

Results

TaqMan™ SCID/SMA assay

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Assay performance: CDC PT Samples

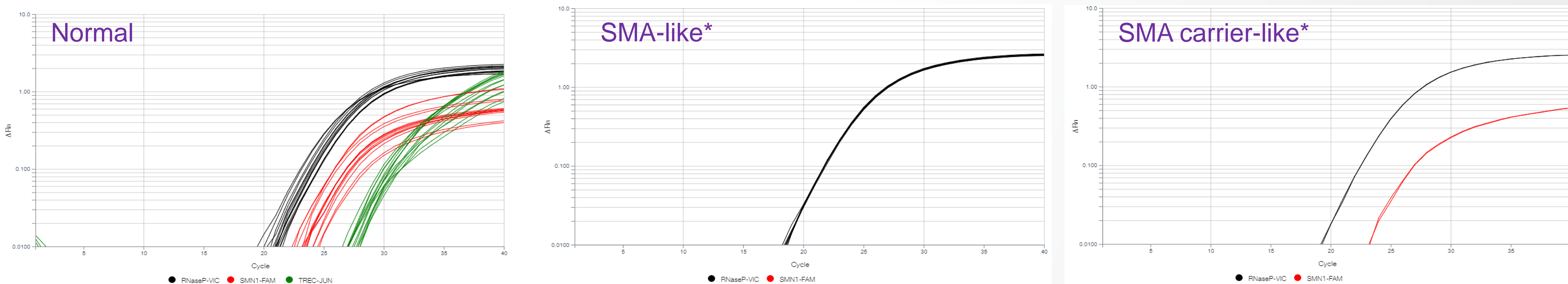

Figure 6. *Normal*: Amplification plot showing all three targets including RNaseP genomic control (**black lines**) amplified in seven unique normal PT samples (2 qPCR reactions/sample). *SMA carrier-like*: Amplification plot showing RNaseP and SMN1 target (**red lines**) in a SMA carrier-like PT sample (contains 1 functional SMN1 copy). (2 qPCR reactions/sample). *SMA-like:* Amplification plot showing RNaseP genomic control amplified in a SMA-like PT sample and "undetermined" Ct for SMN1 target (4 qPCR reactions/sample). *Note** - SMA carrier-like and SMA-like PT samples were prepared by adding transduced B-cells from patients to leukocyte-depleted RBC and serum to simulate newborn blood, hence these samples do not contain TREC molecules. (3.2 mm DBS punch size; QS6 Flex, 0.2 mL platform).

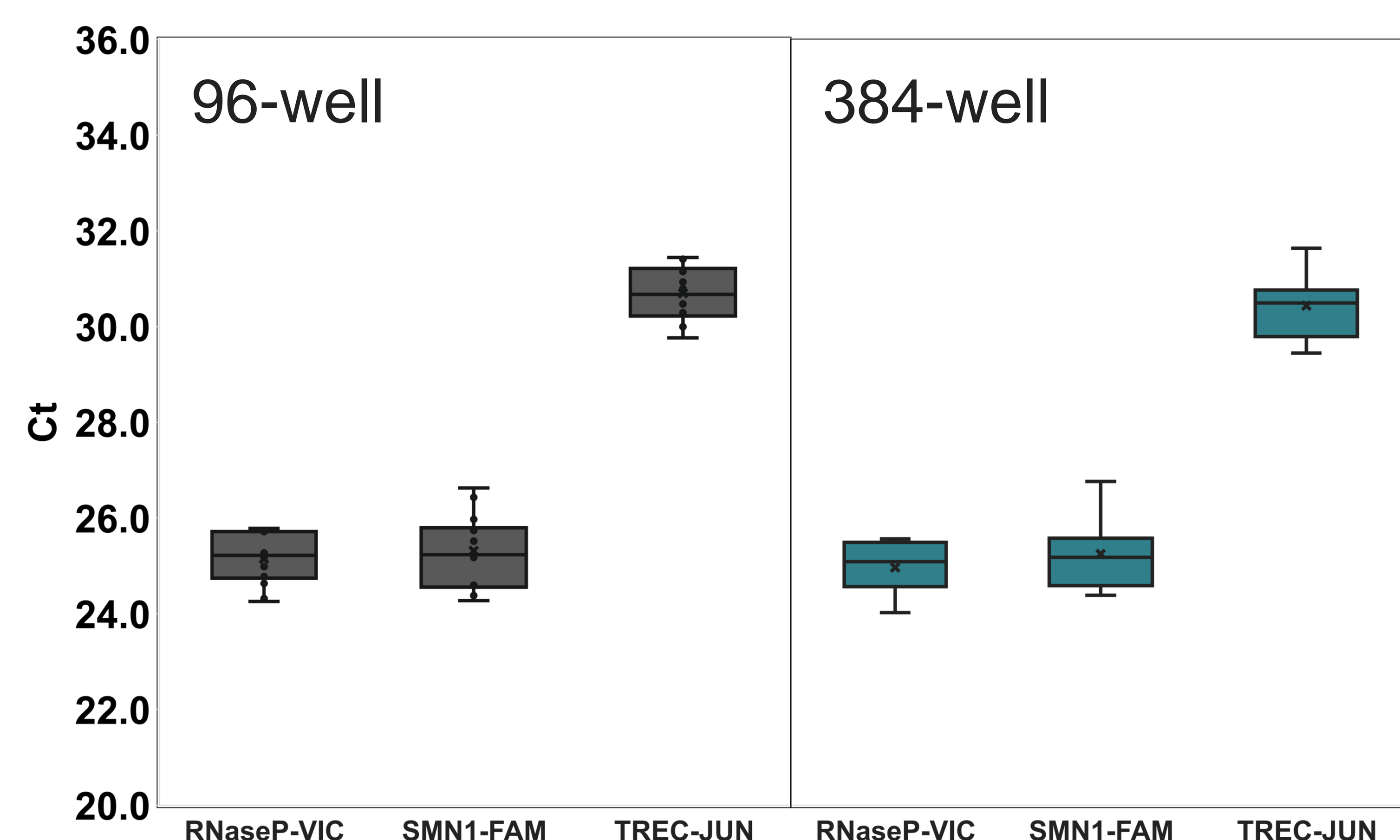

Figure 7. Box plot of Ct values of the three amplification targets for seven unique CDC PT normal samples shown in Figure 6 on QS6 Flex 96-well, 0.2 mL platform (left) and the same samples run on QS6 Flex, 384-well platform (right). Target threshold: 0.20 (RNaseP), 0.04 (SMN1), and 0.10 (TREC).

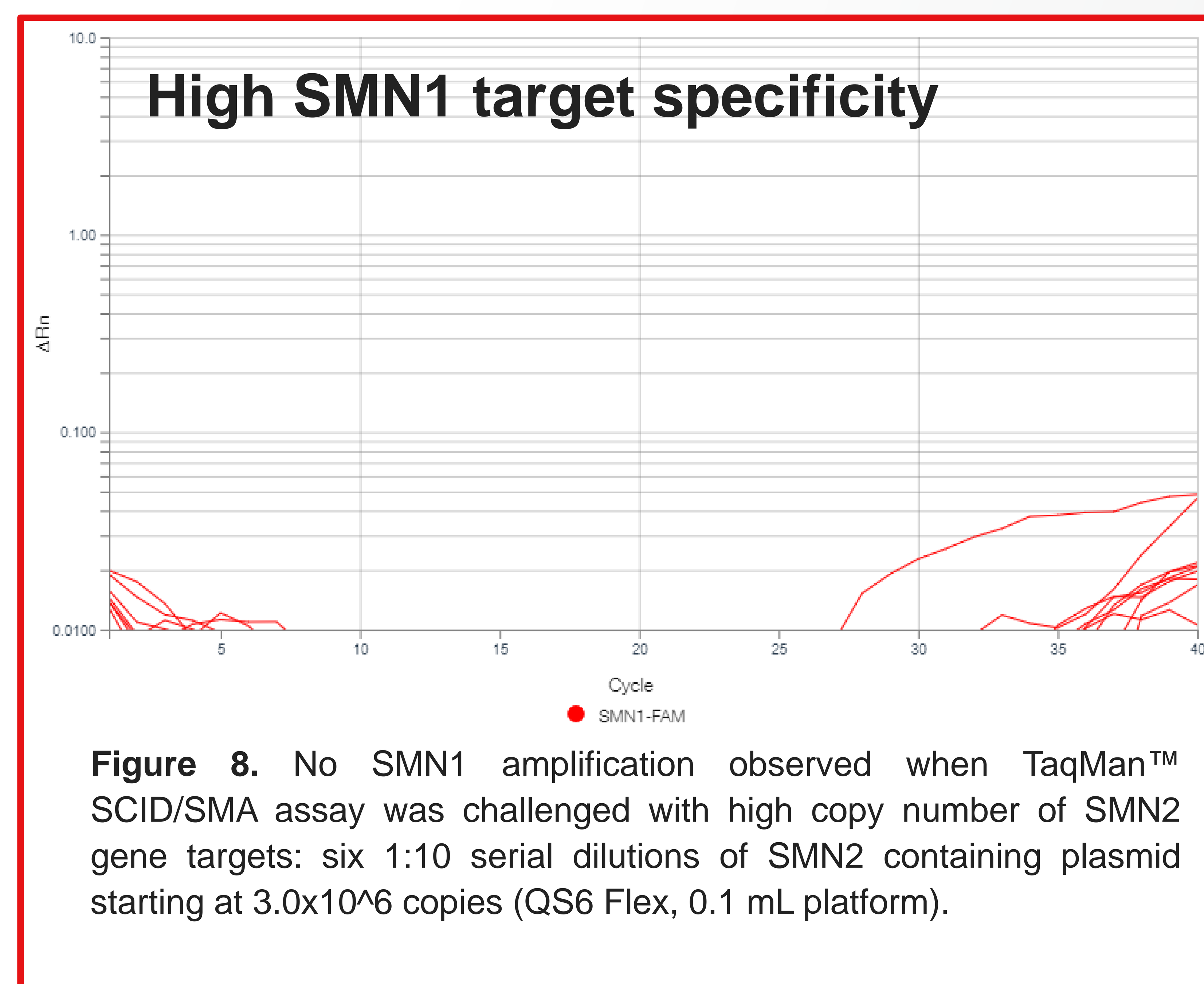

Figure 8. No SMN1 amplification observed when TaqMan™ SCID/SMA assay was challenged with high copy number of SMN2 gene targets: six 1:10 serial dilutions of SMN2 containing plasmid starting at 3.0x10^6 copies (QS6 Flex, 0.1 mL platform).

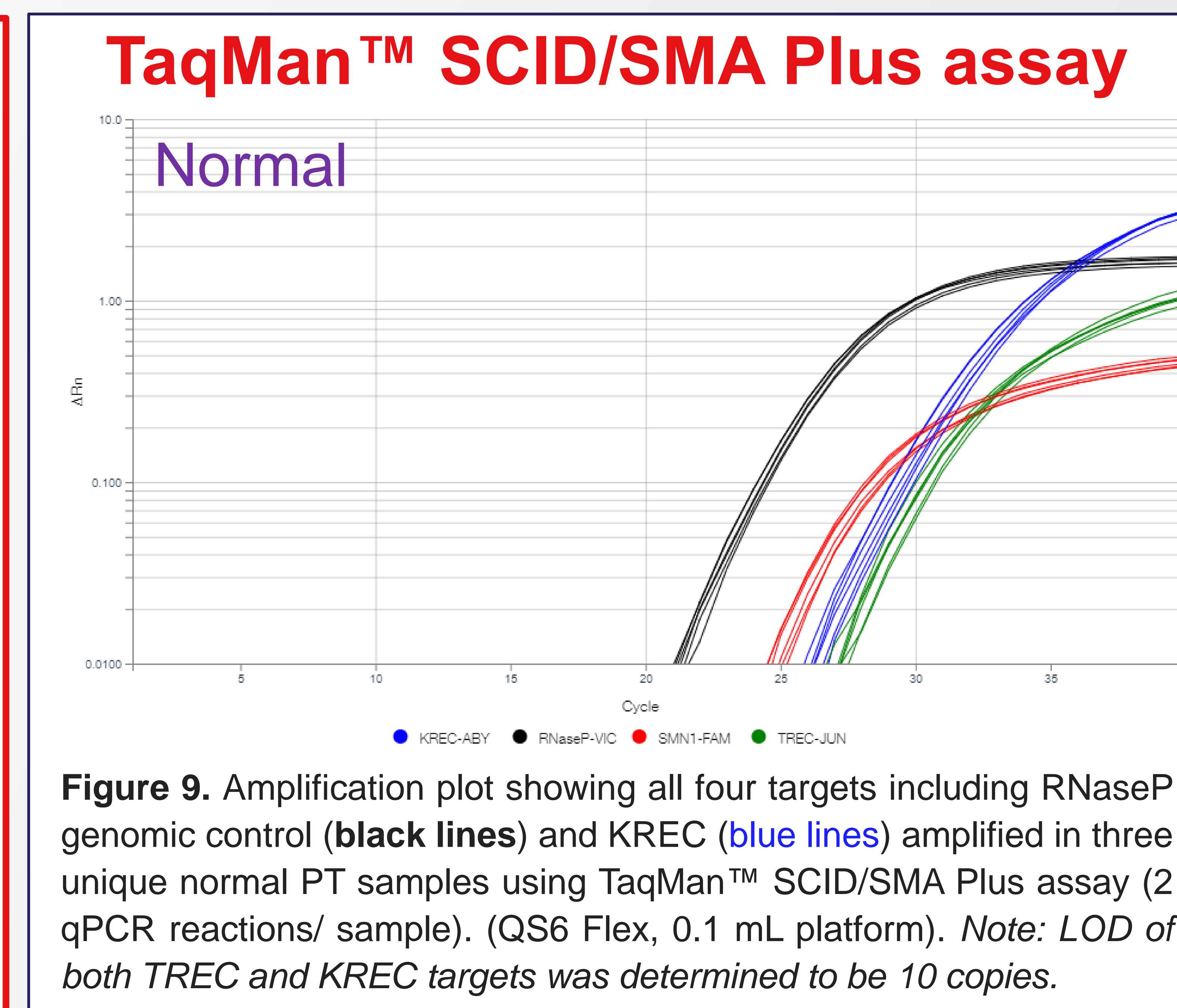

Figure 9. Amplification plot showing all four targets including RNaseP genomic control (**black lines**) and KREC (blue lines) amplified in three unique normal PT samples using TaqMan™ SCID/SMA Plus assay (2 qPCR reactions/ sample). (QS6 Flex, 0.1 mL platform). *Note: LOD of both TREC and KREC targets was determined to be 10 copies.*

CONCLUSION

We have a robust and consistent multiplex assay for SCID and SMA. Quantitative PCR instrument are prevalent in molecular biology laboratories, thus less capital investment hurdle to overcome. With < 2 hr turn around time from punch-to-answer, a DBS workflow with only 4 liquid dispensing steps at room temperature is amendable for automation of assay setup to further decrease hands on time and increase throughput.

References **1.** Feng et. al., 2017 *Genetics in Medicine.* DOI: 10.1038/gim.2016.215 **2.** Baker et al., 2009 *J Allergy Clin Immunol* DOI: 10.1016/j.jaci.2009.04.007 **3.** Czibere et al., 2019 *European Journal of Human Genetics* DOI: 10.1038/s41431-019-0476-4

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