Newborn Screening for Severe Combined Immunodeficiency: An Improved Real-time PCR Assay

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Introduction Severe combined immunodeficiency (SCID) is a group of disorders caused by more than a dozen single gene defects. All known gene mutations cause a defect in the development of normal naïve T-cells, leading to combined cellular and humoral immunodeficiency. The Wisconsin Newborn Screening (NBS) program began statewide screening for SCID in January, 2008.[1] SCID was added to the Recommended Uniform Screening Panel in May, 2010.

Most NBS programs screen for SCID by assaying T-cell receptor excision circles (TRECs) using real-time quantitation PCR (RT-qPCR).[2] TRECs, generated during T cell development, are abundant in healthy infants' dried blood NBS specimens, and are very few or undetectable in the dried blood spots of patients with SCID.[3] In a RT-qPCR TREC assay protocol, a reference gene amplification is used as an isolated DNA quality and quantity indicator: screened specimens are deemed as unsatisfactory specimens when low TREC results are accompanied with a poor reference gene amplification. Commonly used reference genes are β-actin and RNase P. To date, the quantitation of TRECs by RT-qPCR appears to be compatible with the state NBS programs, but challenges remain, such as to obtain sufficient DNA from NBS so there are fewer reported unsatisfactory specimens, which require repeat NBS; to obtain easily accessible and reliable DNA copy number quantitation standards to achieve accuracy and consistency of the quantitation of TRECs. A simple yet efficient DNA isolation method and reliable DNA copy number quantitation standards containing both the target and reference gene would further improve SCID newborn screening assay performance.

Objective To develop and validate an improved method of quantitating TRECs from dried blood spots (DBS) on de-identified residual NBS cards using a novel DNA isolation method and novel gBlock constructed TREC/RPP30 DNA copy number quantitation standards.

Methods

The de-identified residual dried DBS underwent single wash with a laboratory developed DNA isolation buffer. This buffer has been patented by the Wisconsin Alumni Research Foundation and is now licensed and sold as part of Quantabio's Extracta DBS. The DNA isolation was achieved by incubating the washed specimens with 54 µL of the same buffer at 96ºC for 25 minutes. One ninth (6 µL) of the extract solution was used in a standard real-time PCR reaction with gBlock constructed TREC/RPP30 as quantitation standards. The validation process consisted of assay analytic performance (accuracy, linearity, precision, and reproducibility). The screening cutoff value was determined based on a TREC value distribution in a set of 6,018 de-identified residual dried blood NBS specimens, and assay clinical performance (sensitivity, specificity, and clinical validity) was also evaluated accordingly.

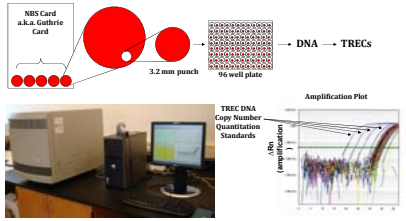

Figure 1: TREC Assay Workflow

TREC sequence
RPP30 sequence

TCCCTTTCAACCATGCTGACACCTCTGGTTTTTGTAAAGGTGCCCACTCCTGTGCACGGTGATGCATAGGCACC
TGCACCCCGTGCCTAAACCCTGCAGCTGGCACGGGCCCTGTCTGCTCTTCATTCACCGTTCTCACGTTTTTAGA
CTGGCGCGCGCGGACGGTCATGGGACTTCAGCATGGCGGTGTTTGCAGATTTGGACCTGCGAGCGGGTTCT
GACCTGAAGGCTCTGCGCGGACTTGTGGAGACAGCCGCTCACCTTGGCTATTCAGTTGTTGCTATC

Figure 2: TREC and RPP30 Sequence Information in the Constructed TREC/RPP30 DNA Copy Number Quantitation Standards gBlock

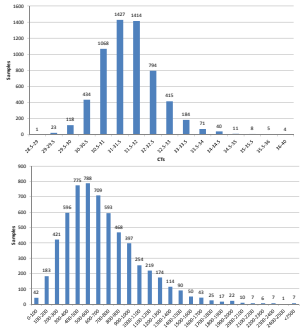

Figure 3: TREC Assay Ct and TREC Copy number Distributions in 6,018 tested de-identified Residual NBS specimens

Table 1: Statistics Summary of TREC Assay Ct and TREC Copy number Distributions in 6018 tested de-identified Residual NBS specimens

STATS	TREC Ct value	TREC copy number value
N	6017	6018
Mean	31.54	694
Median	31.48	628
Std Dev	0.87	370
Percentile 95%	33.04	222
Percentile 90%	34.09	113
Percentile 99.3%	34.44	82
Percentile 99.9%	35.6	37
Cutoff	34.75	75
Cutoff Percentile	99.65%	99.65%
N<=Cutoff	21	21
%<=Cutoff	0.349%	0.349%
2SD	33.28	
3SD	34.15	
4SD	35.02	

Results and Conclusion

The assay showed satisfactory accuracy, linearity, precision, and reproducibility based on the results from a set of dried blood specimens with known TREC copy numbers.

Table 2: Assay Accuracy

Sample	CDC Assigned Results (TREC/µL)	Obtained Results (TREC/µL)	N	Comments
CDC_Cal_1	856	1077	76	Outliers from 1 extract (4 data points) removed (404 TREC/µL)
CDC_Cal_3	254	279	80	
CDC_Cal_5	55	56	71	Outliers from 2 extracts (8 data points) removed (396 TREC/µL and 214TREC/µL) One sample with failed PCR was removed
CDC_Cal_7	16	26	80	

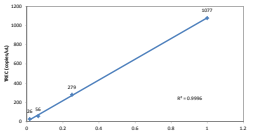

Figure 4: Assay Linearity

Table 3: Assay Precision and Reproducibility

ID	Day 1	Day 2	Day 3	Day 4	MEAN	STDEV	CV (%)
CDC1_856	931	962	1412	1003	1077	225	21
CDC1_C1	31.11	31.06	30.70	30.93	30.95	0.18	0.59
CDC3_254	262	315	287	252	279	28	10
CDC3_C1	32.97	32.65	32.97	33.07	32.91	0.18	0.56
CDC5_55	58	56	46	64	56	7	13
CDC5_C1	35.23	35.44	35.76	34.94	35.34	0.35	0.98
CDC7_16	27	27	20	27	26	3	13
CDC7_C1	36.41	36.30	36.70	36.08	36.37	0.26	0.71

The assay's sensitivity and specificity were also satisfactorily evidenced by 100% concordance with the expected results on a set of residual CDC proficiency test samples from 2012 to 2015. The assay correctly identified all confirmed SCID/severe T cell lymphopenia cases detected by the Wisconsin NBS program, using the original dried blood NBS specimens.

Table 4: Assay Results on Residual CDC Proficiency Testing Samples

Expected Clinical Assessment	N	Assay Concordant Results
No Follow-up Required	42	42
Follow-up Required; leukocyte-reduced specimen	14	14
Follow-up Required; SCID-like specimen	12	12

Table 5: Assay Results on Previously Identified SCID Screening Abnormal Samples

Clinical Assessment	Original TREC Assay Results (Copies/µL)			Newly Developed TREC Result (Copies/µL)
SCID	0	0	0	17
SCID	0	0	0	0
SCID	0	0	0	0
SCID	0	3	7	13
SCID	0	0	0	0
SCID	0	0	0	0
SCID	0	0	0	0
DGS (22q11)	0	0	0	0
DGS (22q11)	14	0	16	50
DGS (22q11)	17	45	34	56
DGS (22q11)	19	34	25	36
DGS (22q11)	10	31	17	26
DGS (22q11)	27	27	29	43
DGS (22q11)	8	11	4	13
DGS (22q11)	8	11	9	23

We have successfully validated an improved RT-qPCR to quantitate TRECs used in NBS for SCID and other severe T cell lymphopenias.

References

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2. Baker MW, Grossman WJ, Laessig RH, Hoffman GL, Brokopp CD, Kurtycz DF, Cogley MF, Litsheim TJ, Katcher ML, Routes JM. Development of a routine newborn screening protocol for severe combined immunodeficiency. *J Allergy Clin Immunol*. 2009;124(3):522-527.
3. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol*. 2005; 115:391-8.

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