Validation of a TREC (T cell receptor excision circles) newborn screening assay for the diagnosis of Severe Combine Immunodeficiency Syndrome (SCID)

Overview

Severe Combined Immunodeficiency (SCID) is a group of genetic disorders caused by several single-gene defects. Regardless of the specific defect, all SCID babies have a blockage of T-cell development, resulting in functional deficiencies in both T & B cells. T-cell counts approach zero in affected newborns. As a result of this deficiency, newborns lack a functional immune response to various infectious agents. This often leads to frequent hospitalization due to severe chronic infections. Death within the first year of life is common.[1]

Early diagnosis and treatment of SCID by haematopoietic stem-cell transplantation (HSCT) is crucial to prevent the demise of these newborns, and to help re-build a functional immune system. It has been demonstrated that HSCT is 95% effective when transplantation is administered within the first 3.5 months of life. If treatment is performed after this window has passed, long term survival rates drop to 60-70%.[2] This clearly exhibits the importance of early diagnosis and treatment. The true incidence of SCID is unknown; estimates range from 1/40,000 to 1/100,000.[3] There is currently no known link between SCID and any particular ethnicity.

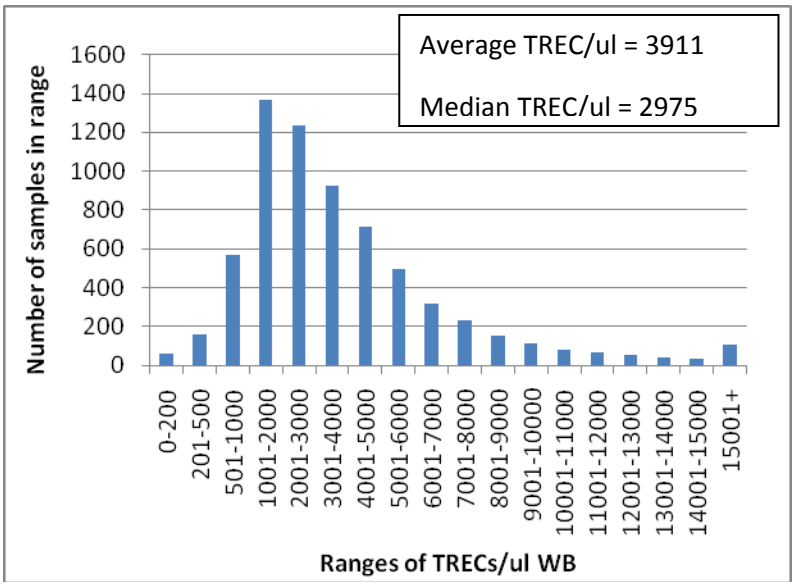
In January of 2010, the Secretary's Advisory Committee for Heritable Disorders in Newborns and Children unanimously agreed to recommend the addition of SCID to the uniform newborn screening panel.[4] However, immune function and T-cell counts are difficult to illicit directly from a dried blood spot which is the specimen type utilized by newborn screening programs. Other newborn screening programs have adopted an assay for a substitute marker to determine the presence or absence of mature T-cells.[5,6] During normal T-cell development in the thymus, a short section of DNA is excised from the T-cell receptor gene. The ends of this linear sequence are ligated together to form a circular DNA episome. This circular piece of DNA is deemed a T-cell receptor excision circle or TREC.[7] A lack or very low number of these TRECs is indicative of a severe T-cell lymphopenia such as SCID. It is the intent of the New York State Department of Health Newborn Screening Program to test for the quantity of TRECs in dried blood spots to identify those babies that have a deficiency of TRECs as has been shown for SCID. Other immune deficiencies characterized by an absence or depletion of T cells may also be detected by this assay.

TREC quantities from a dried blood spot are measured by a quantitative real-time PCR assay that utilizes TaqMan® Chemistry. Two targets of interest are amplified in each SCID reaction;

our TREC target and an RNase P amplification control target. The TREC TaqMan probe is labeled with a 5'-FAM reporter dye and the RNase P probe is labeled with a 5'-VIC reporter dye. Each probe has a 3' quencher dye. The assay is run and target quantities are ascertained by the amount of FAM & VIC signal generated on an Applied Biosystems 7900HT Fast Real-Time PCR system. The quantity is derived from the number of cycles it takes for the fluorescent signal to reach a given threshold. This cycle number is referred to as the threshold cycle or Ct. A standard curve of known TREC copy numbers is run in each assay to allow for the calculation of TRECs per µl of whole blood. The standard curve is generated by serial dilution of a plasmid containing TREC specific sequences.

Population screening

We have screened 6738 de-identified newborn bloodspots using quantitative real time PCR to quantitate the number of TRECs per µl of whole blood. The average TRECs/µl for all samples was 3911 and the median was 2975. The figure below shows the distribution of TREC values in these samples. We have initially set our cutoff point for a presumptive positive for SCID at **≤200 TRECs/µl**. We feel this is a conservative cutoff which will ensure that we don't miss any true positives, but will minimize the number of samples requiring repeat testing. We will re-evaluate this cutoff periodically as we increase the number of samples screened. In our sample of 6738 bloodspots, 62 had a calculated TREC value of ≤200 TRECs/µl of whole blood (0.92%). Of these, 15 (24%) did not amplify for RNase P suggesting insufficient DNA in the reaction. Of the 47 that had low TREC values on the initial screen and normal RNase P levels, 11 of these have been repeated and found to have normal TREC values. The remainder of the presumptive positives are currently being retested.



Testing of known SCID samples

There is a limited availability of samples from known SCID patients. We obtained eight (8) samples from the Wisconsin Newborn Screening Program that they found to have low TREC values using a method similar to ours. These patients were not confirmed to have SCID, but rather SCID-like syndromes and other immunologic deficiencies with T cell depletion. All eight of these samples gave TREC values below our cutoff of 200 TRECs/µl of whole blood.

SAMPLE ID#	RNase P Ct	TREC Ct	TRECs/ µl WB
WISCONSIN_LOW_TREC1	33.296795	ND	0.00
WISCONSIN_LOW_TREC2	35.257	37.00481	134.33
WISCONSIN_LOW_TREC3	36.83746	38.35576	61.67
WISCONSIN_LOW_TREC4	36.49628	39.043102	37.33
WISCONSIN_LOW_TREC5	37.402367	ND	0.00
WISCONSIN_LOW_TREC6	36.32565	ND	0.00
WISCONSIN_SCID_LIKE	35.179768	ND	0.00
WISCONSIN_SCID_LIKE	35.18	ND	0.00

We received nine (9) blinded specimens from Dr. Rebecca Buckley at Duke University. The diagnosis was revealed after testing had been completed. The results are shown in the table below. There were several specimens from individuals confirmed to have SCID and the mutation causing the disease was identified. All except one of the SCID samples gave a TREC/µl value below our normal cutoff. Most showed no detectable TRECs. One sample from an individual with X-linked SCID gave a normal TREC value. There is a concern that that sample may have been switched with another sample. The samples received from Duke were hand labeled and difficult to read. We have asked Dr. Buckley at Duke to try to get repeats on these specimens, but she has not yet responded. TREC values for adult blood samples have been inconsistent in this assay. The values tend to be low for adults, but can vary widely. We strongly suspect samples were switched. We also believe these were old specimens which may have affected DNA quality.

SAMPLE ID#	Diagnosis	RNaseP Ct	TREC Ct	TRECs/ ul WB
DUKE_A	Autosomal recessive SCID	31.558989	ND	0.00
DUKE_B	ADA deficient SCID	33.014534	40.325634	24.00
DUKE_C	Adult normal control	FAIL	FAIL	FAIL
DUKE_D	IL7R alpha deficient SCID	35.199734	ND	0.00
DUKE_E	Adult normal control	35.649094	37.341736	236.67
DUKE_F	ZAP70 deficient SCID	36.0488	ND	0.00
DUKE_G	X SCID	34.289658	34.70495	2383.33
DUKE_H	Adult normal control (same as E)	32.251995	ND	0.00
DUKE_I	X SCID	34.71556	ND	0.00

We tested eleven (11) bloodspots from the New York State newborn screening program for TRECs based on chart notes that indicated a possible immune deficiency. Several of these infants were diagnosed with DiGeorge Syndrome whose symptoms can vary widely, but in some cases may involve a depletion of T cells due to improper thymic development. One sample was diagnosed as having SCID, most consistent with Omenn's syndrome. One had a note that a cousin had died of SCID at 6 month of age. Three had symptoms of an immunodeficiency that had not yet been defined. All of the DiGeorge syndrome infants had lower TREC values, but only 3/6 had values below our cutoff of 200 TRECs/µl, two (2) of which demonstrated no detectable TRECs. This is not unexpected considering the variability of symptoms in DiGeorge syndrome. The Omenn Syndrome sample did not demonstrate any detectable TRECs. The uncharacterized immunodeficiencies all had normal TREC values. The results are shown in the table below.

SAMPLE ID#	diagnosis	RNase P Ct	TREC Ct	TRECs/µl WB
DK_CONTROL_#1	Omenn's syndrome	29.873442	ND	0.00
DK_CONTROL_#2	undefined immunodeficiency	29.080547	34.105007	2566.67
DK_CONTROL_#3	cousin died of SCID at 6 mos	30.538	32.85426	4016.67
DK_CONTROL_#4	undefined immunodeficiency	33.235653	35.62847	586.67
DK_CONTROL_#6	DiGeorge syndrome	32.34049	37.425304	168.33
DK_CONTROL_#8	DiGeorge syndrome	27.5687	ND	0.00
DK_CONTROL_#9	DiGeorge syndrome	28.393581	35.927555	341.67
DK_CONTROL_#10	undefined immunodeficiency	28.85822	35.704357	401.67
DK_CONTROL_#11	DiGeorge syndrome	29.61525	ND	0.00
DK_CONTROL_#12	DiGeorge syndrome	29.656	36.54786	220.00

DK_CONTROL_#13	DiGeorge syndrome	29.823633	38.025913	300.00

Intra-assay reproducibility

We tested seven (7) screen negative samples identified in our population screen and three (3) samples from infants with a confirmed diagnosis of SCID for intra-assay reproducibility. Each sample was run in quadruplicate on the same plate. The average TRECs/µl, the standard deviation (SD) and the coefficient of variation (CV) of the 4 replicates was calculated for each sample. There was very little variability in Ct values for RNase P or TREC between the 4 replicates of each sample. The results are shown in the tables below.

Sample ID	A_RNase P Ct	B_RNase P Ct	C_RNase P Ct	D_RNase P Ct	RNase P Ct_Avg	SD	CV
SCID_AUTOVAL5901	29.40	29.02	29.17	28.59	29.04	0.34	0.01
SCID_AUTOVAL5902	29.39	28.83	28.81	28.54	28.90	0.36	0.01
SCID_AUTOVAL5903	29.21	28.62	28.20	28.01	28.51	0.53	0.02
SCID_AUTOVAL5904	29.92	29.90	29.22	28.95	29.50	0.49	0.02
SCID_AUTOVAL5905	31.24	30.94	30.39	30.51	30.77	0.39	0.01
SCID_AUTOVAL5906	30.86	30.44	29.29	29.35	29.98	0.79	0.03
SCID_AUTOVAL5907	30.49	30.29	29.77	29.84	30.10	0.35	0.01
OMENN_#061771004	35.38	35.93	35.65	35.07	35.51	0.37	0.01
OMENN_DK#1	29.79	29.57	29.89	29.96	29.80	0.17	0.01
SCID+_041471815	35.55	35.98	35.80	37.29	36.15	0.78	0.02

Sample ID	A_TREC Ct	B_TREC Ct	C_TREC Ct	D_TREC Ct	TREC_Ct_Avg	SD	CV
SCID_AUTOVAL5901	33.82	34.13	33.94	33.77	33.92	0.16	0.00
SCID_AUTOVAL5902	34.54	34.52	34.45	34.62	34.53	0.07	0.00
SCID_AUTOVAL5903	34.11	34.59	34.01	34.46	34.29	0.27	0.01
SCID_AUTOVAL5904	35.77	35.21	35.42	34.46	35.21	0.55	0.02
SCID_AUTOVAL5905	35.47	35.69	35.72	35.48	35.59	0.13	0.00
SCID_AUTOVAL5906	33.63	33.89	33.86	33.78	33.79	0.12	0.00
SCID_AUTOVAL5907	34.14	34.34	34.02	34.08	34.15	0.14	0.00
OMENN_#061771004	ND	ND	ND	ND	ND		
OMENN_DK#1	ND	ND	ND	ND	ND		
SCID+_041471815	ND	ND	ND	ND	ND		

The letters A, B, C, and D represent the four replicates of each sample. There was no amplification of TREC for the three SCID samples, yet RNase P amplification was good in these samples indicating adequate DNA quantity.

Inter-assay reproducibility

Eighty-four (84) samples that screened negative in our population screen and four (4) confirmed SCID samples were tested for inter-assay reproducibility by running each sample in three (3) independent runs. Samples showed little variability between runs for RNase P and TREC Ct values. The average Ct value, standard deviation (SD) and the coefficient of variation (CV) across runs was calculated. The results are shown in the table below.

Sample ID	Avg. RNase P Ct value across 3 runs	SD	CV	Avg. TREC Ct value across 3 runs	SD	CV
SCID_AUTOVAL5901	27.63	0.59	0.02	32.40	0.51	0.02
SCID_AUTOVAL5902	27.67	0.61	0.02	33.33	0.34	0.01
SCID_AUTOVAL5903	27.17	0.40	0.01	33.08	0.34	0.01
SCID_AUTOVAL5904	28.53	0.86	0.03	33.74	0.88	0.03
SCID_AUTOVAL5905	29.95	0.61	0.02	34.75	0.79	0.02
SCID_AUTOVAL5906	28.34	0.53	0.02	32.27	0.39	0.01
SCID_AUTOVAL5907	29.21	1.03	0.04	32.91	0.71	0.02
SCID_AUTOVAL5908	26.95	0.95	0.04	32.75	0.52	0.02
SCID_AUTOVAL5909	27.87	0.66	0.02	32.02	0.59	0.02
SCID_AUTOVAL5910	28.05	0.66	0.02	32.77	0.66	0.02
SCID_AUTOVAL5911	28.16	0.46	0.02	33.38	0.41	0.01
SCID_AUTOVAL5912	29.29	0.73	0.02	32.82	0.58	0.02
SCID_AUTOVAL5913	29.38	0.67	0.02	32.87	0.73	0.02
SCID_AUTOVAL5914	30.08	0.51	0.02	34.02	0.31	0.01
SCID_AUTOVAL5915	28.01	0.65	0.02	33.36	0.96	0.03
SCID_AUTOVAL5916	27.10	0.58	0.02	33.57	1.03	0.03
SCID_AUTOVAL5917	27.93	0.76	0.03	33.83	0.80	0.02
SCID_AUTOVAL5918	27.21	0.89	0.03	32.98	0.55	0.02
SCID_AUTOVAL5919	28.28	0.73	0.03	33.21	0.22	0.01
SCID_AUTOVAL5920	28.50	0.65	0.02	33.11	0.65	0.02
SCID_AUTOVAL5921	28.18	0.55	0.02	33.93	0.32	0.01
SCID_AUTOVAL5922	28.00	0.60	0.02	32.69	0.59	0.02

SCID_AUTOVAL5923	28.39	0.55	0.02	33.36	0.62	0.02
SCID_AUTOVAL5924	27.15	0.51	0.02	32.69	0.46	0.01
SCID_AUTOVAL5925	27.49	0.54	0.02	31.95	0.80	0.02
SCID_AUTOVAL5926	27.80	0.64	0.02	33.08	0.82	0.02
SCID_AUTOVAL5927	28.71	0.46	0.02	35.94	0.80	0.02
SCID_AUTOVAL5928	27.74	0.51	0.02	31.22	0.60	0.02
SCID_AUTOVAL5929	28.31	0.73	0.03	33.68	0.47	0.01
SCID_AUTOVAL5930	27.77	0.59	0.02	33.72	0.46	0.01
SCID_AUTOVAL5931	27.45	0.46	0.02	34.19	0.11	0.00
SCID_AUTOVAL5932	27.85	0.45	0.02	33.10	0.12	0.00
SCID_AUTOVAL5933	28.26	0.82	0.03	36.46	0.43	0.01
SCID_AUTOVAL5934	28.65	0.58	0.02	32.97	0.63	0.02
SCID_AUTOVAL5935	28.17	0.44	0.02	33.88	1.03	0.03
SCID_AUTOVAL5936	27.36	0.64	0.02	32.36	0.34	0.01
SCID_AUTOVAL5937	29.33	0.79	0.03	33.02	0.67	0.02
SCID_AUTOVAL5938	26.89	0.47	0.02	32.84	0.63	0.02
SCID_AUTOVAL5939	28.40	0.41	0.01	33.95	0.37	0.01
SCID_AUTOVAL5940	26.25	0.73	0.03	33.76	0.53	0.02
SCID_AUTOVAL5941	28.37	0.92	0.03	33.58	0.48	0.01
SCID_AUTOVAL5942	28.01	0.92	0.03	33.27	0.49	0.01
SCID_AUTOVAL5943	27.89	0.61	0.02	33.52	1.14	0.03
SCID_AUTOVAL5944	27.00	0.36	0.01	32.11	0.44	0.01
SCID_AUTOVAL5945	28.48	0.54	0.02	33.72	0.56	0.02
SCID_AUTOVAL5946	28.72	0.77	0.03	34.51	0.30	0.01
SCID_AUTOVAL5947	29.48	0.67	0.02	33.75	0.64	0.02
SCID_AUTOVAL5948	27.81	0.72	0.03	33.22	0.80	0.02
SCID_AUTOVAL5949	27.56	0.74	0.03	32.39	0.37	0.01
SCID_AUTOVAL5950	27.29	0.44	0.02	33.91	0.29	0.01
SCID_AUTOVAL5951	28.92	0.65	0.02	33.89	0.66	0.02
SCID_AUTOVAL5952	28.17	0.62	0.02	34.53	0.34	0.01
SCID_AUTOVAL5953	27.83	0.51	0.02	32.68	0.56	0.02
SCID_AUTOVAL5954	28.10	0.68	0.02	34.02	0.23	0.01
SCID_AUTOVAL5955	26.94	0.55	0.02	33.51	0.33	0.01
SCID_AUTOVAL5956	27.46	0.74	0.03	34.04	0.26	0.01
SCID_AUTOVAL5957	27.39	0.61	0.02	32.09	0.42	0.01
SCID_AUTOVAL5958	28.50	0.43	0.02	33.32	0.52	0.02

SCID_AUTOVAL5959	28.61	0.70	0.02	35.47	0.65	0.02
SCID_AUTOVAL5960	28.67	0.49	0.02	32.91	0.55	0.02
SCID_AUTOVAL5961	28.15	0.71	0.03	32.17	0.45	0.01
SCID_AUTOVAL5962	27.90	0.62	0.02	33.48	0.79	0.02
SCID_AUTOVAL5963	26.64	0.67	0.03	31.32	0.65	0.02
SCID_AUTOVAL5964	28.14	0.40	0.01	33.83	0.59	0.02
SCID_AUTOVAL5965	27.18	0.58	0.02	33.52	0.76	0.02
SCID_AUTOVAL5966	28.00	0.89	0.03	31.98	0.95	0.03
SCID_AUTOVAL5967	28.66	0.63	0.02	33.02	0.92	0.03
SCID_AUTOVAL5968	28.16	0.98	0.03	32.83	0.35	0.01
SCID_AUTOVAL5969	27.98	0.68	0.02	32.46	0.42	0.01
SCID_AUTOVAL5970	27.60	0.50	0.02	33.21	0.60	0.02
SCID_AUTOVAL5971	27.93	0.44	0.02	32.46	0.45	0.01
SCID_AUTOVAL5972	27.73	0.51	0.02	32.31	0.50	0.02
SCID_AUTOVAL5973	27.10	0.59	0.02	33.00	0.70	0.02
SCID_AUTOVAL5974	28.47	0.49	0.02	33.80	0.67	0.02
SCID_AUTOVAL5975	28.02	0.49	0.02	32.78	0.50	0.02
SCID_AUTOVAL5976	28.31	0.62	0.02	32.09	0.43	0.01
SCID_AUTOVAL5977	27.85	0.55	0.02	32.31	0.69	0.02
SCID_AUTOVAL5978	27.46	0.67	0.02	32.33	0.64	0.02
SCID_AUTOVAL5979	27.23	0.56	0.02	32.99	0.58	0.02
SCID_AUTOVAL5980	27.42	0.38	0.01	33.40	0.40	0.01
SCID_AUTOVAL5981	26.46	0.52	0.02	32.51	0.49	0.01
SCID_AUTOVAL5982	27.70	0.35	0.01	34.84	0.91	0.03
SCID_AUTOVAL5983	28.58	0.74	0.03	34.31	0.40	0.01
SCID_AUTOVAL5984	26.12	0.46	0.02	33.15	0.67	0.02
Wisconsin_SCID+	34.67	0.36	0.01	ND		
NY_Omenn+_061771004	33.90	1.61	0.05	ND		
NY_Omenn+_DK1	28.62	0.31	0.01	ND		
NY_SCID+_041471815	33.29	0.09	0.00	ND		

The SCID samples consistently showed no detectable level of TRECs, yet good amplification of the RNase P control gene.

Conclusion

We have shown that we are able to quantitate the number of TRECs per µl of whole blood using a quantitative real-time PCR assay and a standard curve of known TREC values generated from a plasmid containing specific TREC sequences. We can control for adequate amplification in each sample by monitoring the co-amplification of a control gene, RNase P. There was a wide range of values obtained for TRECs/µl of whole blood in a population screen of over 6000 presumed normal newborns. We have initially set the cutoff for normal as >200 TRECs/µl. In our population screen, this resulted in less than 1% of samples flagged as presumptive positives to be retested for SCID. About 25% of these samples also had RNase P levels outside the normal range which probably indicates insufficient DNA in the reaction. Fifteen (15) of the 62 presumptive positives (24%) have been repeated; 11 were found to be normal when repeated and the other 4 still had low RNase P levels suggesting a poor DNA extraction. The remaining presumptive positives are currently being repeated.

Our analysis of specimens from individuals known to be diagnosed with SCID has shown that the majority of those samples have no detectable TRECs. There was one sample that we received from Duke that was obtained from a SCID patient according to their records, but gave a normal TREC value in our assay. We believe this to be a sample mix-up due to unclearly labeled tubes. We are requesting repeats of these specimens to analyze. We have shown that other immunodeficiencies in addition to SCID may be detected by this assay. DiGeorge syndrome, for example, sometimes demonstrated low or undetectable TREC levels in our assay. This disease can cause a wide spectrum of symptoms involving various organ systems. If the thymus and T cell development is affected, so-called “full DiGeorge syndrome”, we would expect to observe that in our TREC assay. We did not have any clinical data on the samples from individuals diagnosed with DiGeorge syndrome so we are unable to correlate TREC values with clinical presentation of the disease.

Our intra-assay and inter-assay reproducibility studies showed good consistency within runs and across runs for RNase P and TREC Ct values for each sample. This supports the accuracy of the automated set-up of DNA extraction and PCR reactions.

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