Newborn screening for severe combined immunodeficiency: evaluation of a commercial TREC based method in Victorian dried blood spots.

Original article

Dr Stephanie Richards [1,2]
Dr James Pitt [3,4]
Dr Sharon Choo [1,2]

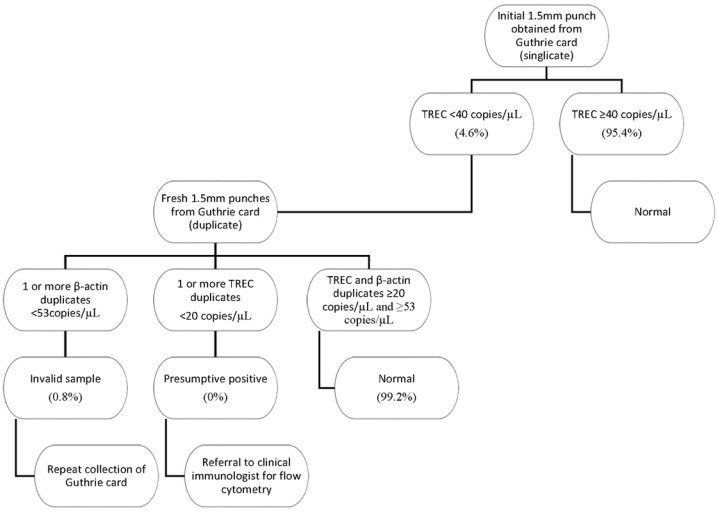
Corresponding author –
Dr Stephanie Richards
Immunology Laboratory – Laboratory Services
Royal Children's Hospital
50 Flemington Road, Parkville VICTORIA 3052
(03) 9345 5725
stephanie.richards@rch.org.au

[1] Immunology Laboratory, Laboratory Services, Royal Children's Hospital, Melbourne Australia
[2] Department of Allergy and Immunology, Royal Children's Hospital, Melbourne Australia
[3] Victorian Clinical Genetics Services, Murdoch Children's Research Institute, The Royal Children's Hospital, Melbourne Australia
[4] Department of Paediatrics, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jpc.13659

Acknowledgements

The authors would like to acknowledge the Victorian Clinical Genetics Service Newborn Screening Laboratory, in particular Nicholas Tzanakos, for assisting with access to prospective control and SCID/CDGS DBS and practical aspects of the assay performance. The authors would like to acknowledge Dr Paul Eckert's laboratory (Murdoch Children's Research Institute and Children's Cancer Centre, The Royal Children's Hospital, Parkville and Department of Paediatrics, University of Melbourne), in particular Dr Ray Bartolo, for assistance with and access to equipment for performance of the assay. We would also like to acknowledge PerkinElmer, Inc. the manufacturer of the EnLite™ Neonatal TREC kit for loan of the VICTOR™ EnLite plate reader and technical support throughout the duration of the study. Lastly, we acknowledge Dr Francis Lee from the CDC Newborn Screening Translational Research Initiative for the provision of quality control material and technical support during the study.



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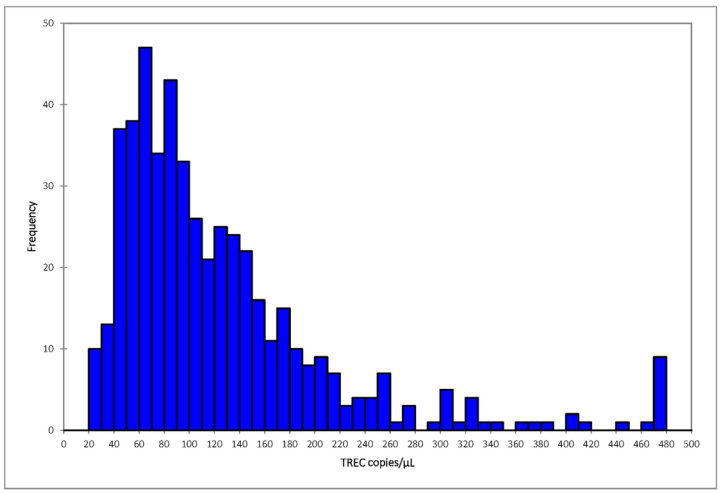

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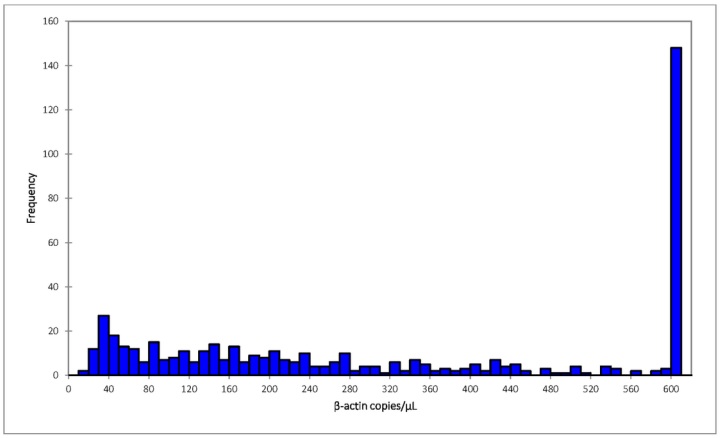

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Abstract

Aim: Severe combined immunodeficiency (SCID) is the most severe form of primary immunodeficiency, and is fatal in infancy if untreated. As early diagnosis is associated with improved outcomes, SCID is an ideal condition to consider for inclusion in a newborn screening program in Australia. In this feasibility study we evaluated the EnLite™ Neonatal TREC kit for detection of T-cell receptor excision circles (TREC) from newborn screening dried blood spots for the identification of known SCID patients in Victoria.

Methods: TREC copies/µL were measured retrospectively for 14 children diagnosed with SCID or complete DiGeorge syndrome (CDGS) from 2005-2015 at the Royal Children's Hospital, Melbourne. In addition, TREC copies/µL were measured for 501 prospective de-identified newborn screening cards.

Results: 11/14 known SCID or CDGS samples were correctly identified as presumptive positive samples with low or undetectable TREC on duplicate testing. The remaining 3 samples also had low or undetectable TREC on duplicate testing, but were considered invalid due to insufficient β-actin DNA amplification. Of the 501 prospective newborn screening samples, none were identified as presumptive positive samples on duplicate testing.

Conclusions: The EnLite™ Neonatal TREC kit correctly identified known SCID or CDGS patients as presumptive positive samples, and initial cut-offs for TREC and β-actin in the Victorian newborn screening population were determined. A larger pilot study is required to confirm these proposed cut-offs and to evaluate the cost and implementation of this screening program in Victoria, Australia. Overall, this study provides preliminary data to support the introduction of this assay to the newborn screening program in Victoria.

What is already known about this topic?

1. Severe combined immunodeficiency is an appropriate condition for consideration of inclusion in a newborn screening program in Australia as early, pre-symptomatic identification and treatment is associated with improved survival and health outcomes
2. Measurement of T cell receptor excision circles (TREC) has been shown to be a reliable screening test for SCID and has been implemented in >40 states in the United States of America and some European countries with success

What does this paper add?

1. A commercially available kit to measure TREC copies/µL, the EnLite™ Neontal TREC kit, correctly identified retrospective newborn screening dried blood spot samples from known

SCID or complete DiGeorge syndrome patients diagnosed at the Royal Children's Hospital, Melbourne from 2005-2015 as presumptive positive samples

2. From the measurement of TREC copies/μL in 501 de-identified prospective newborn screening dried blood spots, preliminary cut-offs for a pilot SCID screening program in Victoria have been proposed
3. This study provides preliminary data to support the introduction of measurement of TREC to the newborn screening program in Victoria

Key Words

DiGeorge syndrome, dried blood spot testing, newborn screening, severe combined immunodeficiency (SCID), T cell receptor excision circle (TREC)

Introduction

Severe combined immunodeficiency (SCID) is the most severe form of primary immunodeficiency in humans, and occurs as the result of mutations in genes that are required for the development and function of T lymphocytes [1]. As a consequence, children with SCID have a profound defect in both cell-mediated and humoral immunity, and if undiagnosed and untreated, SCID is inevitably fatal in early infancy and/or childhood [2, 3]. Haematopoietic stem cell transplant is a potentially curative treatment for children diagnosed with SCID, and in some cases, enzyme replacement and/or gene therapy may be considered [1, 3]. Early diagnosis and treatment of SCID is associated with improved outcomes, and newborn screening (NBS) for SCID enables identification of affected infants shortly after birth, thereby facilitating earlier definitive therapy [1].

A reliable screening test is available for SCID and the validity of this test has been demonstrated following the introduction of SCID screening in the United States (US) in 2008 [4-6]. This test quantifies T cell receptor excision circles (TREC), which are pieces of DNA excised from the T cell receptor (TCR) gene during the development of mature T lymphocytes within the thymus [7, 8]. As all infants with SCID or complete DiGeorge syndrome (CDGS) have impaired T lymphocyte development, they have significantly reduced numbers of thymically derived T cells and therefore very low or absent TREC, compared to the normal population [3, 8]. TREC are stable in peripheral blood and can be quantified by polymerase chain reaction (PCR) of DNA isolated from the dried blood spots (DBS, also known as Guthrie cards) routinely collected for NBS [1, 3, 7]. A commercial neonatal TREC kit (EnLite™ Neontal TREC kit) is now available, facilitating the introduction of this test into routine diagnostic laboratories. A recent study from the United Kingdom (UK) found this to be a reliable method for

identification of SCID patients and several states in the US currently use this kit for SCID screening[9]. NBS for SCID is currently not performed in Australia. This study evaluated the use of the only commercially available TREC assay for identification of known SCID or CDGS patients in Victoria.

Materials and Methods

This study was approved by the Royal Children's Hospital Human Research Ethics Committee (HREC Reference No. 34190). For the retrospective arm of the study we analysed stored DBS samples from 12 children diagnosed with SCID and 2 children diagnosed with CDGS over the last ten years at the Royal Children's Hospital, Melbourne. For the prospective arm of the study 501 de-identified NBS DBS were analysed. Parental written consent for de-identified research was obtained at the time of DBS collection. The date of collection of the NBS sample, gestation of the infant (<32 weeks, 32-36 weeks and >36 weeks) and admission to a neonatal or paediatric intensive care unit were recorded for each sample. If an infant received a blood transfusion in the 72 hours preceding collection of the NBS sample or gestational age was not specified, the DBS was excluded from the study.

A single 1.5mm DBS punch was run in singlicate using the EnLite™ Neonatal TREC kit, as per the manufacturer's instructions. On first tier testing, if a TREC result was <40 copies/μL, duplicate punches from the same DBS were collected and re-tested. Based on previous studies using the EnLite™ Neonatal TREC kit, a TREC cut-off of <20 copies/μL for either duplicate on re-testing was used as the determination of a presumptive positive result [9] (Figure 1). Following our observation that samples stored for >30 days at room temperature had lower TREC results on repeat testing, any DBS that required repeat testing were placed at 4°C with desiccant until repeat testing was performed, which was performed within seven days of DBS sample collection. Similar results were identified by the kit manufacturer, PerkinElmer, in pre-licensing studies. β-actin was also measured on all samples as an internal control for DNA amplification. If the TREC result on singlicate testing was ≥40 copies/μL, the β-actin result was not acted upon. On duplicate testing, as per the manufacturer's recommendations, samples with <53 copies/μL of β-actin for either duplicate were considered invalid. Using the EnLite™ Neonatal TREC kit, the upper limit of detection for TREC was 474 copies/μL and for β-actin was 608 copies/μL. The storage time for the SCID/CDGS DBS samples ranged from seven months to eleven years. To attempt to control for the large variation in the age of the known SCID/CDGS DBS samples, five de-identified control samples collected on the same day as the known SCID/CDGS samples were run alongside, with β-actin copies/μL only recorded. As this was a quality control measure, it was considered unethical to record TREC copies/μL in these samples.

Quality control NBS DBS were obtained from the Newborn Screening Translational Research Initiative at the Center for Disease Control and Prevention (CDC) in the US. These samples included two samples with known TREC values, five samples designated as normal samples with variable TREC values, one SCID-like specimen and one sample with TREC and beta-actin values below reference range. These QC materials were run with each assay.

Results

Fourteen known SCID and CDGS patients were tested in singlicate using the EnLite™ Neonatal TREC kit. All samples had TREC results <40 copies/µL on initial testing and were then re-tested in duplicate. Eleven of these samples were correctly classified as presumptive positives, including one case of ADA-deficiency SCID, with an initial TREC result of 25 copies/µL and duplicate TREC results of 14 copies/µL and 25 copies/µL (Table 1). Given that one duplicate result for this patient was less than the positive cut-off of 20 copies/µL, this result was interpreted as a presumptive positive and would have prompted further confirmatory testing. Three of the known SCID/CDGS patients had undetectable TREC levels on both singlicate and duplicate testing, but were classified as invalid results due to low β-actin duplicates using the manufacturer's cut-off of 53 copies/µL. Two of these three patients had a normal white cell count at diagnosis, suggesting no correlation between white cell count and β-actin <53 copies/µL. Given that the age of the DBS of the known SCID/CDGS patients varied from seven months to eleven years at the time of testing, an additional five storage matched control cards were tested alongside each known SCID/CDGS patient, and the β-actin results only recorded. The range of β-actin copies/µL for these retrospective control samples is shown in Table 1. In total, 10 (14%) of the retrospective control samples had an invalid β-actin result (<53 copies/µL).

A total of 501 de-identified prospective control DBS were tested in this study, using the same testing strategy as outlined previously (Figure 1). The distribution of the control TREC and β-actin results are shown in Figures 2 and 3. The mean TREC value for all samples was 124 copies/µL (median 99 copies/µL). Only one sample was collected from an infant <32 weeks gestation (47 TREC copies/µL) which, using the defined testing algorithm, would have been reported as normal. Mean TREC values were similar in infants born between 32-36 weeks gestation and infants born >36 weeks gestation (Table 2). The TREC copies/µL of the CDC quality control material were within expected targets set by NSQAP.

Of the 501 prospective control samples tested, 23 (4.6%) had an initial TREC result <40 copies/µL on singlicate testing. Of these, using a cut-off of <20 copies/µL on duplicate testing, none of the control

samples were identified as a presumptive positive. Given the small number of samples prospectively tested in this study, we did not expect to identify a presumptive positive sample. In other studies, a cut-off of 20 TREC copies/μL has been reported to result in a presumptive positive rate of 0.04% [9]. Using an alternative cut-off of 25 TREC copies/μL, as proposed by van der Speck *et al,* one sample would have been identified as a presumptive positive, which would correspond to a presumptive positive rate of 0.2% in our population[10]. The annual birth rate in Victoria in 2014 was 74 224 infants, and assuming a presumptive positive rate of 0.2%, this can be extrapolated to approximately 148 infants requiring confirmatory testing with flow cytometry in a twelve month period (Table 3)[11]. Of the 23 samples that required re-testing in duplicate, 4 (0.8% of total samples) were classified as invalid on duplicate testing as one or more of the β-actin duplicates were <53 copies/μL, suggesting possible DNA amplification failure. However the TREC results for these samples on duplicate testing were ≥40 copies/μL. There were 64 results (12.8%) with an initial β-actin result of <53 copies/μL, of which 4 had a TREC result <40 copies/μL and were retested in duplicate. The remaining 60 samples had TREC levels ≥40 copies/μL on singlicate testing, and therefore the β-actin result was not acted upon.

Discussion

In 2005, Chan and Puck described the utility of DBS for the detection of TREC by quantitative PCR as a method of population-based screening for SCID[12]. Since 2008, screening for SCID has been implemented in more than 40 states in the US, with several pilot studies performed in Europe and the UK [9, 10, 13-16]. To date, more than 100 cases of SCID have been identified by NBS worldwide[10, 16]. In all published studies, screening programs measuring TREC have correctly identified infants with typical and atypical SCID and no cases of SCID were not detected by NBS and later identified [13]. In additional to typical SCID, non-SCID immunodeficiencies associated with marked T cell lymphopenia may be detected by NBS with TREC, including partial DiGeorge syndrome, CHARGE syndrome and ataxia telangiectasia [13, 17, 18]. One potential limitation of NBS for SCID has been the reported inability to detect cases of SCID due to combined immunodeficiencies associated with gene defects affecting T cell development later than the T cell receptor recombination, or associated with impaired T cell signalling, survival or proliferation, such as ADA deficiency, ZAP-70 and MHC Class II deficiency [13, 19, 20]. In this study, the 14 known SCID/CDGS cases had low or undetectable TREC levels on singlicate testing, and 11/14 were correctly identified as presumptive positives on duplicate testing, including one case of ADA SCID. The remaining three cases of SCID had β-actin values below the 53 copies/μL cut-off on duplicate testing, resulting in classification of these samples as invalid. In routine practice, this would have prompted repeat collection of the DBS and/or further testing in these infants. In our

prospective study, any samples with borderline TREC values between 20-40 copies/µL on duplicate testing were not characterised further with flow cytometry, as the management of patients with an equivocal or indeterminate TREC result is still not well defined [1]. It is possible that these samples may have had other causes of T cell lymphopenia, such as those described above, but it was considered unethical to re-identify these samples as part of this feasibility study. If NBS using TREC was introduced in Victoria, any samples with a duplicate TREC result less than the proposed cut-off would require prompt referral to a clinical immunologist, and further investigation with flow cytometry, including lymphocyte subsets and naïve T cell measurement.

The diagnostic algorithm and TREC cut-off value both for initial singlicate and subsequent duplicate testing varies considerably among centres in the US, UK and Europe[10,13, 16.] Here we have demonstrated that an initial cut-off of 40 TREC copies/µL would result in 4.6% of the population being re-tested in duplicate, which is comparable to 3.76% of samples in a UK study using the same initial cut-off [9]. Of these samples, the presumptive positive rate on duplicate testing using this cut-off would be 1.6% (8 of 501 samples), again similar to the presumptive positive rate reported in the UK study, which would be an unacceptable number of samples requiring re-testing [9]. A re-test TREC cut-off of 20 copies/µL gave a presumptive positive rate of 0% in our prospective study, while correctly identifying all SCID/CDGS patients in our retrospective study. A recent systematic review suggested that a TREC cut-off of 25 copies/µL may be an appropriate initial cut-off for implementation of routine NBS for SCID [10]. Applying this cut-off to our population would have detected one subject, increasing the presumptive positive rate to 0.2% (95% CI 0.005 – 1.1%), which is higher than that reported in most studies to date. This may reflect the relatively small number of patients in this study, and larger studies will be required to determine the optimal cut-off for TREC in the Victorian NBS population. It has been demonstrated in some studies that premature infants have lower TREC values [8,10,16]. There were insufficient numbers of premature infants in this study to confirm this finding, but consideration of the testing strategy for premature infants would need to be considered in a larger evaluation study, including timing of repeat DBS collection and TREC measurement.

The rate of invalid samples on duplicate testing (ie. samples with β-actin levels less than the manufacturer's cut-off of 53 copies/µL) was relatively high (0.8%) in our population. In addition, three of the known SCID/CDGS patients were classified as invalid on duplicate testing using this cut-off. In routine practice, this would require DBS recollection and repeat testing of these infants, which in the Victorian population, would lead to recollection for 594 infants. This would be a significant burden on the Victorian Newborn Screening Laboratory. In the population tested in this study, the

2.5th centile for β-actin levels was 27 copies/μL. Adopting this cut-off for an invalid result, none of the repeat prospective control samples would have been invalid. Adams *et al* used a β-actin cut-off of 35 copies/μL, and using this cut-off, 2 of the repeated samples would have required recollection, which would reduce the recollection number to 297 samples in a twelve-month period[9]. This high proportion of invalid results may reflect several factors, one of which is an inappropriately high β-actin cut-off for our population. It should be noted that in general, DNA amplification from known SCID/CDGS patients and retrospective age-matched control samples, as evidenced by β-actin amplification, was relatively preserved, with the majority of samples β-actin values ≥53 copies/μL. The rate of an invalid result when considering β-actin in isolation (result <53 copies/μL) was comparable between retrospective controls (14%) and prospective controls (12.8%), suggesting that the number of invalid β-actin results was independent of the age of the sample.

In the preliminary stages of this study, we identified that NBS samples more than one month of age on duplicate testing had lower TREC levels. However, in only 1 of 6 samples would this have affected the interpretation of the TREC result, from a normal to a presumptive positive result. Although DNA is considered to be stable in DBS for at least one month, humidity is known to affect TREC values, even within the first seven days after collection. Given this, collection and storage conditions, including temperature and humidity, will be an important practical consideration in the implementation of NBS using the EnLite^{TM} Neonatal TREC kit. As per the kit manufacturer's instructions, we elected to analyse samples that were ≤7 days from collection and stored any samples that required repeat testing at 4°C with desiccant until repeat testing could be performed. In routine practice, it would be anticipated that DBS would be tested within seven days of collection. It was beyond the scope of this study to perform repeat TREC measurements over several weeks for samples with initial normal TREC results to determine the time required between collection and subsequent reduction in TREC copies/μL, but this would be an important consideration in future studies. TREC results for the retrospective age-matched controls were not available, so the impact of storage of NBS cards on TREC levels was not ascertained in this study.

In conclusion, the EnLite^{TM} Neonatal TREC kit was able to identify known SCID/CDGS patients in our population as presumptive positives, with no false-negative results. Initial arbitrary TREC and β-actin cut-offs of 25 copies/μL and 27 copies/μL respectively, derived through analysis of 501 routine NBS DBS using the EnLite^{TM} Neonatal TREC kit may be appropriate for SCID screening in the Victoria newborn screening population. The introduction of a PCR based method would require change in the current infrastructure in NBS laboratories, including additional training and instrumentation, and

an alternative 1.5mm punch head for use with the EnLite™ Neonatal TREC kit. Although these considerations will be important in the implementation of routine SCID screening, this study is the first evaluation of a commercially available kit for measurement of TREC in Australia, and provides preliminary data to support the introduction of this assay to the newborn screening program in Victoria.

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Table 1: TREC copies/µL of known SCID and CDGS patients

SCID Sample (storage in years)	Molecular diagnosis	Singlicate TREC copies/µL	Duplicate TREC copies/µL	Beta-actin duplicate copies/µL	Beta-actin retrospective control range copies/ µL	Result interpretation
1 (<1)	AK2 mutation (reticular dysgenesis)	0	0/0	61/47	23->608	Invalid
2 (3)	RAG-1 mutation	0	0/0	>608/>608	68-542	Presumptive positive
3 (4)	PNP deficiency	0	0/2	217/326	22-306	Presumptive positive
4 (4)	γ_c mutation	0	0/0	187/105	49->608	Presumptive positive
5 (4)	RAG-1 mutation	0	0/0	103/71	<16->608	Presumptive positive
6 (5)	RAG-1 mutation	0	0/0	26/31	79-480	Invalid
7 (5)	Molecularly undefined	0	0/0	492/>608	24-413	Presumptive positive
8 (5)	γ_c mutation	0	0/0	184/65	190->608	Presumptive

						positive
9 (6)	Complete DiGeorge syndrome	0	0/1	213/142	19->608	Presumptive positive
10 (8)	JAK3 mutation	0	0/0	99/163	26-555	Presumptive positive
11 (9)	γ_c mutation	0	0/0	137/88	24-110	Presumptive positive
12 (9)	DNA-ligase IV mutation	0	0/0	142/>608	161->608	Presumptive positive
13 (10)	ADA deficiency	25	14/25	121/92	17->608	Presumptive positive
14 (11)	Complete DiGeorge syndrome	0	0/0	38/27	73->608	Invalid

Table 2: TREC copies/μL by gestational age

Gestational age	Number of samples	Mean TREC copies/μL	Median TREC copies/μL	Range TREC copies/μL
<32 weeks	1	47	47	N/A
32-36 weeks	10	154	139	57-328
>36 weeks	490	123	99	20-474

Table 3: Estimation of effect of TREC copies/µL and β-actin copies/µL cut-offs on number of samples requiring confirmatory flow cytometry or recollection following duplicate testing in a 12-month period

Proposed cutoffs	**B-actin = 53**		**B-actin = 27**	
	Recollection (invalid)	Flow cytometry	Recollection (invalid)	Flow cytometry
TREC = 25	594 (0.8%)	148 (0.2%)	0	148 (0.2%)
TREC = 20	594 (0.8%)	0	0	0

Figure legends

Figure 1: Testing algorithm used in evaluation of the EnLite™ Neonatal TREC kit

Figure 2: TREC values (copies/µL) in prospective control population

Figure 3: β-actin values (copies/µL) in prospective control population

Author/s:
Richards, S;Pitt, J;Choo, S

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Date:
2018-01

Citation:
Richards, S., Pitt, J. & Choo, S. (2018). Newborn screening for severe combined immunodeficiency: Evaluation of a commercial T-cell receptor excision circle-based method in Victorian dried blood spots. JOURNAL OF PAEDIATRICS AND CHILD HEALTH, 54 (1), pp.14-19. https://doi.org/10.1111/jpc.13659.

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