J Clin Immunol (2014) 34:323–330
DOI 10.1007/s10875-014-0007-6

ORIGINAL RESEARCH

Screening of Neonatal UK Dried Blood Spots Using a Duplex TREC Screening Assay

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Received: 3 September 2013 / Accepted: 17 February 2014 / Published online: 28 February 2014
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Abstract
Purpose Severe Combined Immunodeficiency (SCID) is considered to be a paediatric emergency and unless identified promptly can be life-threatening. Frequently, infants are not diagnosed with SCID until they have become seriously ill with infection leading to treatment complications and a poorer prognosis. We aimed to test a newly available commercial duplex assay to measure T cell receptor excision circles (TRECs) to establish if this would be suitable for newborn screening for SCID in the UK.
Methods Over 5000 anonymous retrospective dried blood spots (DBS) were used alongside 18 confirmed SCID positive DBS with a newly available duplex assay to measure TRECs levels and control gene levels. We also included testing of premature babies and babies from neonatal intensive care units (NICU) as these have been shown to have high false positive rates in other TREC screening assays.
Results All 18 SCID DBS samples were successfully identified as SCID positives in the study. The number of presumptive positives detected was dependent on the TREC cut-off threshold settings. When analysed with five different TRECs cut-off values (20, 25, 30, 35 and 40 TREC copies/μl blood) the presumptive positive rate ranged from 0.04 to 1.00 % of samples tested. Premature infants and neonates from NICU did not show high presumed false positive rates in this assay.
Conclusions The study demonstrated that this duplex assay kit will identify all newborns with SCID as presumptive positives. The data also shows that with suitable TREC cut-off settings the number of presumptive positives from non-SCID newborns will be manageable in the context of a national screening service.

Keywords SCID · TRECs · newborn screening · Guthrie cards

Introduction

Severe combined immunodeficiencies (SCID) are a group of inherited diseases of the immune system characterised by profound abnormalities of T cell development. Infants with SCID require prompt clinical intervention to prevent severe life-threatening infection and studies show significantly improved survival in babies diagnosed at birth as a result of a previous family history [1]. Although SCID is genetically heterogeneous with over 18 different gene defects identified, all SCID patients have an abnormality of thymopoiesis [2]. A plasmid containing a T cell receptor excision circle (TREC) generated by Douek et al. [3] are an accurate biomarker of thymic output and a number of studies have shown significantly decreased TREC levels in SCID patients [4].

Recent studies have shown that TREC levels can be used as a newborn screening tool to identify babies born with SCID [4–8] and in the US newborn screening has been implemented

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on a state-by-state basis [9, 10]. However there is no consensus method for SCID screening and laboratories currently use different, non-standardised, assays for detection of TRECs in the newborn dried blood spots (DBS). [4–8].

There are a number of problems that must be overcome with screening for SCID in the UK using non-standardised assays including the complexity of the method, issues regarding leftover genomic DNA once screening is completed and a paucity of information regarding normal TREC levels in the population. In England alone there are 13 regional newborn screening laboratories and any new test would need to be introduced in a uniform manner across all sites to ensure standardised measurement. The new test would also need to be carried out directly on the DBS to avoid any contentious issues (including storage issues) arising from whole genomic DNA extractions. Finally, the assay needs to be tested on a sufficient number of DBS (normal and those from known SCID cases) to establish cut-offs for presumptive positives prior to any nationwide deployment.

To address these issues, we have carried out a study to test a newly developed commercial assay (EnLite Neonatal TREC kit, Perkin Elmer) for newborn screening for SCID on the UK population. We report that the use of this commercial standardised assay for SCID newborn screening would allow for identification of all newborns with SCID and also addresses the concerns raised above.

Methods

Normal Guthrie Card Samples

This study was approved by the NRES Ethics Committee East Midlands (reference 12/EM/0422). All blood spots were taken using Astron 226 filter paper (Perkin Elmer). Freshly taken dried blood spots from 5081 anonymised Guthrie cards in the regional newborn screening lab at Great Ormond Street Hospital, UK were used. Any Guthrie card samples from newborns who had received a blood transfusion <72 h prior to collection were omitted from the study. The following demographics were obtained from each Guthrie card prior to anonymisation; date of specimen, gestational age category at birth (>36 weeks, 36–32 weeks, <32 weeks), whether baby was on PICU/NICU at time of sampling. Numbers of patients within each gestational age category of the newborn at birth is shown in Table I and PICU/NICU status is given in Table II. The median time between sample collection and punching was 39 days.

Known SCID Positive Guthrie Card Samples

Anonymised stored Guthrie card samples from 18 known retrospective SCID positive samples were obtained to run in the study (4 of these were from adenosine deaminase deficient SCID samples and were labelled as such for the study). Of the other cards 2 were from newborns with Gamma-chain SCID, 2 were from newborns with Omenn's SCID, 2 were from newborns with RAG deficient SCID, one from a newborn with PNP SCID and 7 from newborns with undefined SCID. Of this latter group 6 cards were from totally T cell lymphopaenic newborns (CD3 count <300 cells/μl) and one was from a newborn with T low B+NK+SCID (CD3 count 470 cells/μl). The age of the known positive samples ranged from 6 months to 4 years old.

Table I Gestational age category of the newborn at birth - normal samples

Gestational age category of the newborn at birth	No. samples	Percentage of all samples
<32 weeks	86	1.7
32–36 weeks	230	4.5
>36 weeks	4765	93.8

Centers for Disease Control and Prevention Samples

Control samples were kindly provided from the Newborn Screening Quality Assurance Program at the Center for Disease Control and Prevention (CDC) in the US. These comprised of five sets of DBS; blanks (leucocyte depleted cord blood), SCID (mononuclear cell depleted cord blood), two different sets of low cell count normal samples (low cell count cord blood) and a high cell count normal sample (high cell count cord blood). These samples were run at the end of the study as external control samples and were not included in the final analysis.

EnLite Neonatal TREC Assay-Punching

The assay was performed according to the EnLite Neonatal TREC kit instructions (Perkin Elmer). Single 1.5mm punches were taken from each DBS using a Panthera-Puncher 9 with a 1.5 mm punch head into EnLite 96-well PCR Plates, (both Perkin Elmer). To avoid the effect of static interference when

Table II Intensive care at the time of specimen collection - normal samples

Intensive care at the time of specimen collection	No. samples	Percentage of all samples
No	4689	92.3
Yes; NICU/PICU	77	1.5
Yes; CICU	0	0.0
Yes; unit unknown	315	6.2

punching, an ionizing gate (Eltex Elektrostatik) was used during the punching process. Triplicate 1.5mm punches were also taken into each plate from the EnLite Neonatal TREC kit calibrator samples (consisted of three DBS calibrators, A-C, with known quantities of TREC and ß-actin DNA). Triplicate blank reactions (no DBS) were also carried out on each plate. Three control samples provided with the kit were also run in duplicate. The controls consisted of low control (low TREC, low ß-actin), no TREC control (no TREC, normal ß-actin), and high control (normal TREC, normal ß-actin). Blank reactions were added to check for contamination, high control to prove that the amplification has been successful, no TREC control to prove that normal and no TREC samples can be discriminated, low control to prove that normal and low TREC and ß-actin can be discriminated.

EnLite Neonatal TREC Assay – Elution, PCR and Probe Hybridisation

Elution reagents from the kit were thawed and prepared according to the kit instructions, then 10 μl was added to each well of the PCR plate. A Bio-RAD S1000 Thermal Cycler and a Bio-RAD C1000 Thermal Cycler were used for all thermal cycling steps. Plates were sealed, briefly spun down and then incubated at 98 °C for 45 min, followed by 4 °C for 2 min. After elution the reagent mixture was made according to the kit instructions and 20 μl was added to each well of the PCR plate. The plate was re-sealed, spun down briefly and PCR amplification was carried out as per kit instructions. These were initial denaturation at 98 °C for 5 min followed by 37 cycles of 98 °C for 15 s, 62.5 °C for 1 min and 72 °C for 15 s. Directly after PCR amplification a probe hybridisation step was performed comprising 95 °C for 5 min, 35 °C for 1 h and 23 °C for 5 min.

EnLite Neonatal TREC Assay – Signal Measurement

After PCR and probe hybridisation the PCR plate was spun down for 2 min at 500×*g* and was transferred to a Victor EnLite fluorometer (Wallac Oy) for TREC and ß-actin signal measurement. The EnLite Workstation software version used in the study was 1.0 Release Candidate 2. Quality control procedures were carried out to ensure accuracy of the run. The blank wells were required to have median fluorescence detection levels below pre-set levels to ensure absence of contamination. The calibrators were required to have median fluorescence levels within pre-set acceptance limits and the EnLite workstation software prepared calibration curves for both TRECs and ß-actin using copies/μl values of the calibrators. The kit control samples were required to give the correct copies/μl results. Once these run quality checks had been performed the TREC and ß-actin results were interpreted for the anonymised DBS samples and the SCID positive DBS.

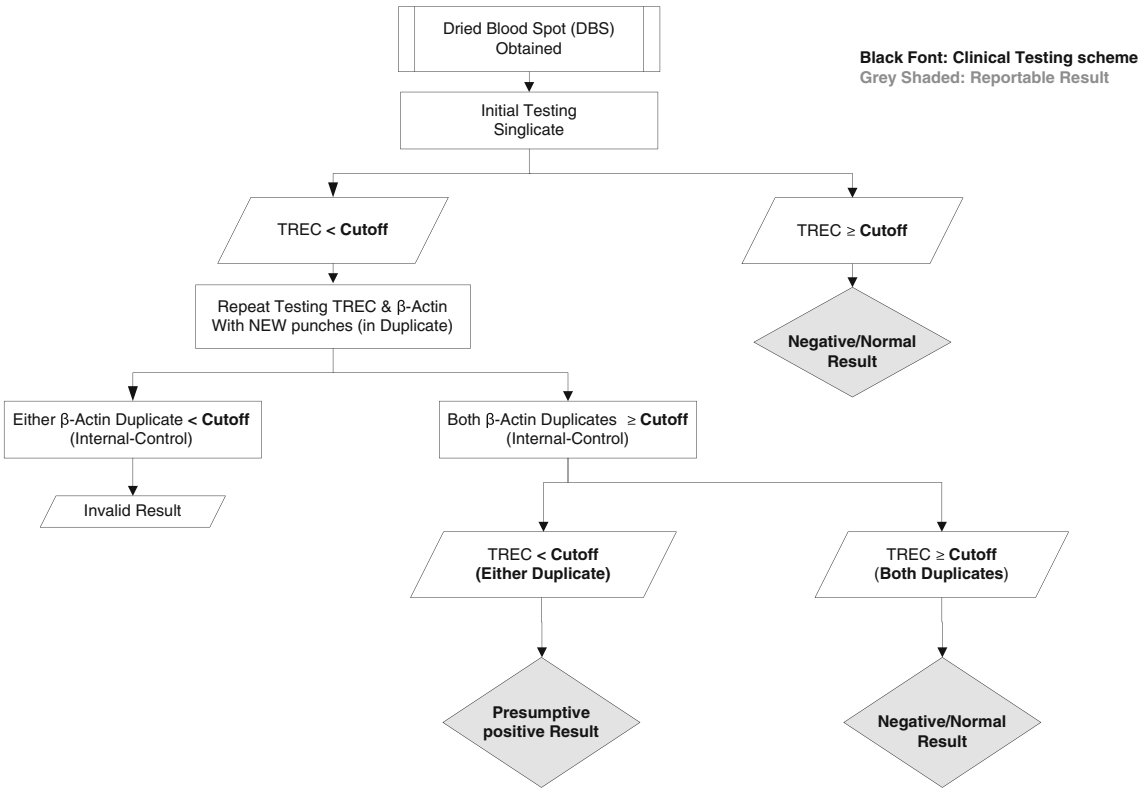

Fig. 1 Algorithm for determination of presumptive positives for SCID using the EnLite Neonatal TREC kit

Results

Initial Singlicate Test Results

The algorithm used for determination of SCID positives is shown in Fig. 1. The TREC cut-off was also initially set at 40 copies/μl. The TRECs cut-off was set higher than we would expect to use routinely in a UK NBS laboratory so that we could collect excess re-test data from the subsequent duplicate punches to allow for post study analysis with a variety of different cut-offs. This permitted us to establish the most suitable TRECs cut-off for the UK population. The overall descriptive statistical results for TREC copies/μl, first round results are given in Table III. One of the normal samples gave a result of 0 TREC copies/μl. However this sample also had 0 ß-actin copies/μl indicating that a poor quality punch was taken from this card during singlicate testing leading to DNA amplification failure. As expected the mean, median and maximum TREC copies/μl are lower in the SCID positive group than in the normal DBS. With the initial TRECs cut-off set at 40 copies/μl there were a total of 209 samples (4.1 %), including all the SCID positive samples, requiring a repeat test according to the algorithm in Fig. 1. The mean, median and ranges for TRECs levels in the <32 weeks, 32–36 weeks and >36 weeks gestational category are shown in Table IV. The mean, median and ranges for TRECs levels in the non-intensive care group, the NICU/PICU group and the intensive care (unit unknown) group are given in Table V. The mean and median level for preterm babies (<36 weeks) and for neonatal intensive care or paediatric intensive care unit (NICU or PICU) babies was no different from the normal samples. There were 51 samples in which ß-actin values were <35 copies/μl (0.01 %) while TRECs levels remained above the cut-off threshold.

Repeat Duplicate Test Results

Samples with TRECs levels <40 copies/μl were selected for re-testing. These 209 samples were repeat tested on duplicate punches from the same DBS used for the initial punch and from this all of the SCID positive samples, including all ADA SCID samples and a SCID with low but not absent T cells, were identified as presumptive positives. Singlicate TRECs and duplicate TRECs and β actin results for each individual SCID positive sample are given in Table VI.

The normal sample with 0 TREC copies/μl and 0 ß-actin copies/μl from singlicate testing had levels well above the TRECs cut-off after subsequent duplicate testing (74 and 88 TRECs copies/μl) proving the initial singlicate testing punch was of poor quality. Fifty one (1 %) of the anonymised normal DBS were identified as presumptive positives upon re-testing using the initial TRECs cut-off of 40 copies/μl. Histograms showing the distribution of results of TRECs for all samples are shown in Fig. 2. Only one sample of the 5081 DBS tested gave an invalid test result (using 35 copies/μl ß-actin). This would be considered a DNA amplification failure and would require a second heel prick blood spot. If extrapolated to the UK population (~700,000 newborns per year), this would mean an extra 138 repeat heel pricks.

As already stated a TRECs cut-off set at 40 copies/μl would give 1 % presumptive positives. Since this was an anonymised study, we could not perform confirmatory flow cytometric testing on presumptive positive samples and so we cannot rule out that any of these may be from genuine SCID positive babies. With an approximate number of live UK births at 700,000 per annum this would equate to 7000 presumptive positives per year. This would clearly be an unfeasible number of presumed false positives and place undue burden on the downstream referral centres. To determine suitable TRECs cut-offs we re-evaluated the data with cut-offs at 35, 30, 25 and 20 copies/μl (Fig. 3 and Table VII). These data show that at a TREC cut-off level of 20 copies, we would expect 0.04 % presumptive positives after duplicate testing which would lead to 280 referrals for confirmatory testing from the total newborn population. This is comparable

Table III Descriptive statistical results for TRECs copies/μl, first round results

Outcome	No. samples	Mean	Median	Min.	Max.
Normal	5081	119	101	0	1160
ADA SCID	4	3	2	0	6
SCID	14	4	2	0	15
Total	5099	119	100	0	1160

Table IV Descriptive statistics for TREC copies/μl - normal samples by gestational age

Gestational age category of the newborn at birth	No. samples	Mean	Median	Min.	Max.
<32 weeks	86	110	89	22	415
32–36 weeks	230	115	91	16	714
>36 weeks	4765	120	101	0	1160

Table V Descriptive statistics for TREC copies/μl - normal samples, by intensive care unit

Intensive care at the time of specimen collection	No. samples	Mean	Median	Min	Max
No	4689	121	102	0	1160
Yes; NICU/PICU	77	98	75	18	367
Yes; unit unknown	315	105	87	11	442

Table VI All singlicate TRECs and duplicate TRECs and β actin results for known SCID positive samples

SCID DBS number	TRECs singlicate result (copies/μl)	TRECs duplicate results (copies/μl)	β actin duplicate results (copies/μl)
1	1	0/0	35/61
2	6	0/0	710/1160
3	0	2/0	24300/14100
4	5	4/1	237/137
5	0	7/4	2160/1660
6	9	1/0	322/694
7	2	0/0	7930/1990
8	15	1/1	8390/13600
9	2	1/1	317/279
10	0	0/2	356/322
11	0	0/0	700/480
12	11	0/0	1190/967
13	1	0/0	2510/1800
14	0	0/0	1560/1740
15	8	0/0	42/70
16	0	0/0	227/900
17	4	9/1	17300/5320
18	1	0/0	33200/498000

with the 0.02 % presumptive positives in California [10] and the 0.03 % full-term (0.045 % pre-term) presumptive positives in Wisconsin [11]. In our study one quarter (n=70) of the presumptive positives would be from the <36 week population. The other 210 would come from the normal term population. A TREC cut-off at 20 copies/μl would still detect all SCID samples included in the study (Fig. 3). Once a population-wide study is carried out in the UK the precise number of presumptive positives will be established.

Centers for Disease Control and Prevention Sample Results

The five sets of CDC samples tested gave the expected results. Blank samples (leucocyte depleted cord blood) gave invalid results (TREC and ß-actin levels lower than cut-offs). SCID samples (mononuclear cell depleted cord blood) gave presumptive positive results (TREC levels below cut-off, ß-actin above cut-off). The two different sets of low cell count normal samples (low cell count cord blood) and the high cell count normal sample (high cell count cord blood) gave normal results (both TRECs and ß-actin levels above cut-offs).

Intra- and Inter-Assay Variation

Variation of the assay was estimated by calculating precision using the quality control results (C1, C2 and C3) collected during the study. As the variation of the kit is not normally distributed, the mean values and standard deviations (SDs) were calculated on data with logarithmic transformation (Ln copies/μl). The mean values in the untransformed scale (copies/μl) were back-transformed from the logarithmic mean, and therefore the means in the untransformed scale are geometric means.

Intra- and inter-assay variation was then modelled with ANOVA (Table VIII) and these results were comparable with the precision information provided by the manufacturer

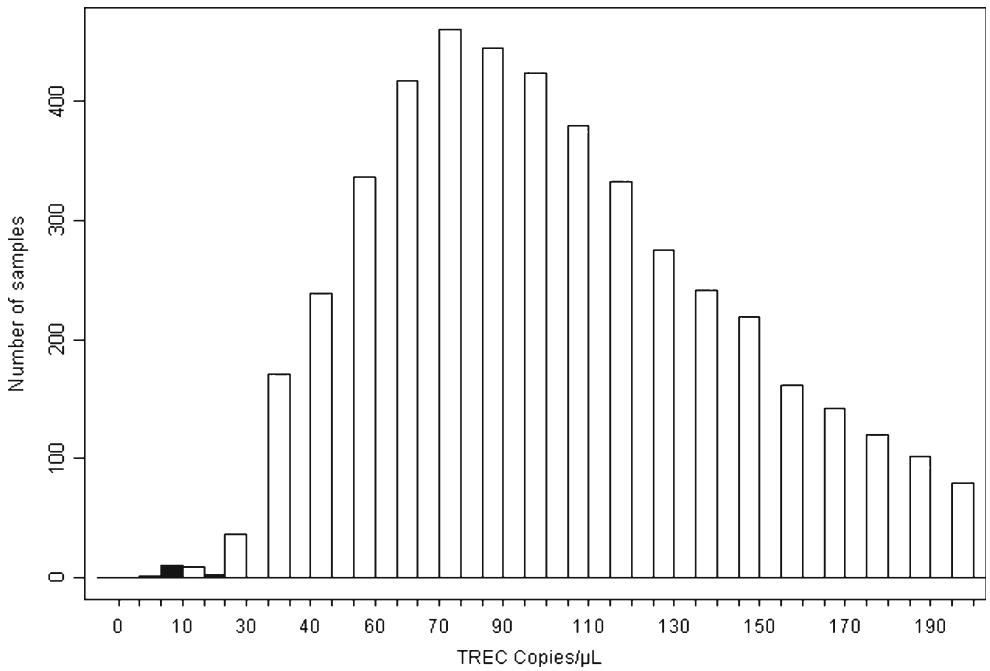

Fig. 2 Histograms showing the distribution of results for TRECs for all samples. SCID positive samples are highlighted by *filled bars*

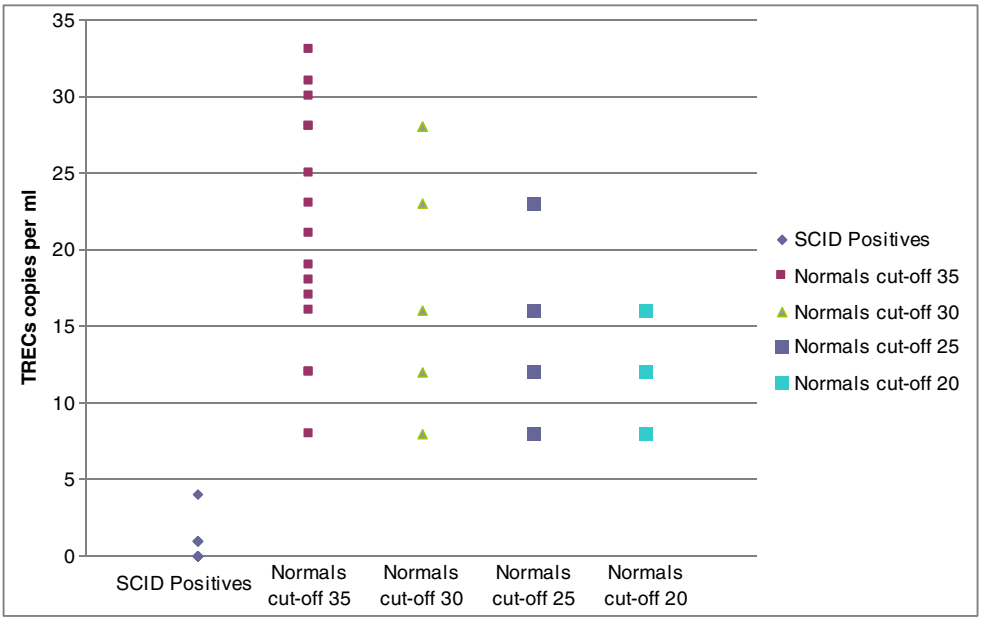

Fig. 3 *Dot plot* showing the numbers of presumptive positives after second round testing with different TRECs cut-off levels

(included in the kit instructions for use). We therefore concluded that the assay performed as intended by the manufacturer during the study period.

Discussion

Newborn screening for SCID through TREC analysis of DNA extracted from DBS has now been shown to be effective method for identifying SCID at birth. Screening programmes are now in operation in a number of states in the USA and, in over 2,000,000 babies screened, no cases of SCID have been undiagnosed demonstrating the sensitivity of the assay [10, 11]. However, different states have used different TREC assays with different algorithms for screening, a scenario that would not be considered for a national screening programme in the UK. To determine whether a standardised commercial assay (EnLite Neonatal TREC Kit) would have utility, we undertook a pilot study of over 5000 prospective DBS samples from the UK population to determine the feasibility of the assay, establish the TREC range in the UK and propose TREC cut-offs for any potential screening programme and test the ability of the assay to detect SCID in retrospective known DBS samples.

In this study no repeat runs were required due to assay failure and only one DBS gave an invalid result which would equate to 138 repeat DBS being taken per annum in the UK, based on there being approximately 700,000 live births per year in the UK [12]. This percentage (0.019 %) compares favourably with the percentage of repeat heel pricks required in the California study (0.08) and suggests that this assay is clearly robust enough to give results from DBS of varying quality without requiring excess numbers of repeat DBS being taken. The CDC control TREC samples gave the predicted results for all sample categories, illustrating that the kit performs to external quality assessment standards.

The EnLite Neonatal TREC Kit successfully detected 18 out of 18 SCID positive samples. The highest TRECs value seen in any of the SCID positive samples was 15 copies/μl

Table VII Percentage positives after first round testing and percentage presumptive positives after second round testing with different TRECs copies cut-offs and the effect of retesting all premature babies (<36 weeks gestation) on the predicted number of UK referrals

	40 copies/μl	35 copies/μl	30 copies/μl	25 copies/μl	20 copies/μl
Percentage positives after first round testing	3.76	2.03	0.77	0.35	0.20
Percentage presumptive positives	1.00	0.30	0.12	0.08	0.04
Total number of referrals per annum (including samples from babies <36 weeks gestation)	7000	2100	840	560	280
Percentage of presumptive positive samples from babies <36 weeks gestation	21.50	20.00	33.30	25.0	25.0
Total number of referrals per annum (excluding samples from babies <36 weeks gestation)	5495	1680	554	420	210

Table VIII Control Precision descriptives for the three control samples (C1, C2 and C3) run in duplicate on every plate. As the variation of the kit is not normally distributed, the mean values and SDs have been calculated on data with logarithmic transformation (Ln copies/μl). The mean values in the untransformed scale (copies/μL) are backtransformed from the logarithmic mean, and therefore the means in the untransformed scale are geometric means

Sample	*n*	TREC mean (Ln copies/μl)	TREC geometric mean (copies/μl)	Intra assay SD (Ln copies/μl)	Inter assay SD (Ln copies/μl)	Total SD (Ln copies/μl)
C1	146	5.02	152	0.60	0.00	0.60
C2	146	0.66	2	0.78	0.40	0.88
C3	146	6.27	528	0.41	0.28	0.50

which was considerably lower than the initial TRECs cut-off value (40 copies/μl). This study is in keeping with previous studies and the larger prospective screening programmes in being able to detect all SCID samples regardless of underlying genetic diagnosis. In addition there was successful positive detection in our single SCID positive case in which there was some residual T cell development. In this study we had no SCID positive samples with maternal engraftment but other studies have shown that SCID positive samples with maternal engraftment would be detected using a TRECs-based assay [13]. Concerns that the TREC assays would not detect ADA deficiency with a SCID phenotype were not borne out by this study. Together with other data, this suggests that although delayed onset ADA deficiency (which presents later with a combined immunodeficiency) may not always be detected by TREC assays [14], those ADA deficient patients with a SCID phenotype are detected. The TREC assay detects significantly abnormal thymic development and as such is an exquisitely sensitive marker for this disease.

Since we could be confident that the assay would allow us to detect all SCID samples the next factor for us to consider was the presumptive positive rate. As shown in Fig. 3 and Table VII. A cut-off of 20 TREC copies/μl would be able to detect all SCIDs but would result in a referral rate of 0.04 % (n=280) per year. This is equivalent to 23 referrals per month which would be distributed amongst 14 paediatric immunology centres in the UK, although some centres would see a proportionately larger number because of the population distribution. Nevertheless, this would not place an undue burden on the immunology workforce or on the finances required for extra lymphocyte profiling. A cut-off at 20 copies/μl could therefore be proposed as starting cut-off for any potential national programme. Comparison with data from screening pilots carried out in the US show that our suggested cut-offs would lead to a comparable percentage of presumptive positives (0.02 % Wisconsin, 0.02 % Massachusetts, 0.08 % Navajo Nation, 0.16 % New York, 0.01 % California, 0.03 % Puerto Rico, 0.03 % Louisana). [15].

It has previously been shown that premature births can result in a lower TREC levels when measured from DBS [16, 17] so one possibility with screening is to routinely re-sample premature babies (<36 weeks gestation) in order to avoid this as a complicating factor. Our data appears to show no effect of gestational age on TREC levels using this method of analysis with mean TREC copies of 110, 115 and 120 in the <32 weeks, 32–36 weeks and >36 weeks gestational age category respectively (Table V). Despite this we have included the effect of routinely retesting premature babies on the referral rates and have included this information in Table VII.

At present we do not have defined costs for this assay and clearly a full health economic assessment would need to be carried out prior to any onset of newborn screening for SCID in the UK. However, the EnLite Neonatal TREC kit is easy to use and does not require laboratory staff to have any extensive previous molecular biology experience. The kit also contains the necessary quality controls and assay calibrators. This is important if the assay is to be carried out with complete uniformity at numerous regional NBS laboratories. The assay works on Guthrie cards collected within the existing UK framework and thus there is no reason for extra DBS material to be taken. The use of 1.5 mm sized punches differs from the more commonly used 3.2 mm punches used in UK NBS laboratories. This would require existing NBS laboratories to have an extra puncher or punch head and would also require the use of an ionizing gate to counter the increased static effects. Otherwise, there is no difference in actual hands-on time required to punch a plate of 1.5 mm versus 3.2 mm spots. The assay is carried out directly on the DBS punch and there is no leftover genomic DNA following an assay run, so the issue of genomic DNA storage becomes redundant. For these reasons, and since the assay clearly identifies SCID positive samples with a reasonable presumed false positive rate, we believe it would be a suitable method to use for newborn screening for SCID in the UK.

Acknowledgments The authors would like to acknowledge the assistance from Perkin Elmer in supporting this study. Perkin-Elmer supplied reagents free-of-charge and assisted with study design and data analysis.

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