Newborn screening for severe combined immunodeficiency: breaking the bubble

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CHAPTER 2

An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program

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ABSTRACT

Newborn screening of severe combined immunodeficiency through the detection of T-cell receptor excision circles will provide the opportunity of treating before the occurrence of life-threatening infections. With the EnLite Neonatal TREC assay (PerkinElmer) and end-point PCR, 39 samples (3.0%) of 1295 heel prick cards of the Dutch newborn screening program required a retest after initial analysis. After retest, 21 samples (1.62%) gave TREC levels below cut-off. A significant reduction in TREC levels was observed in heel prick cards stored for three months (N = 33) and one year (N = 33). Preterm newborns (N = 155) showed significantly lower TREC levels and a higher retest-rate than full-term newborns. Peripheral blood spots of 22 confirmed SCID patients and 17 primary immunodeficiency patients showed undetectable or low TREC levels. These findings suggest that the EnLite Neonatal TREC assay is a suitable method for SCID screening in the Netherlands, thereby providing guidance in the decisions concerning implementation into the Dutch program.

INTRODUCTION

Severe combined immunodeficiency (SCID) comprises a group of heterogeneous genetic disorders of the immune system, characterized by the dysfunction of T-lymphocyte maturation and development. In addition to a lack of T-cell-mediated immunity, SCID patients can present with various subtypes of disrupted differentiation or function of B-lymphocytes and natural killer cells [1]. Newborns with SCID usually present with severe infections and failure to thrive during the first months of life. Affected children face a fatal outcome unless their immune system is replaced by hematopoietic stem cell transplantation (HSCT) or gene therapy [2]. Transplantation outcome improves significantly if children receive a transplant before the age of 3.5 months and prior to the occurrence of the first infection [3]. Moreover, it was shown that SCID babies diagnosed at birth due to positive family history have significantly improved overall survival and transplantation outcome compared to the firstborn affected family member [4]. Screening of SCID leading to early diagnosis has shown to be cost-effective in spite of a low incidence of the disease [5]. These findings imply that SCID is a suitable candidate for newborn screening.

Worldwide, many SCID screening pilots have been conducted and implementation of SCID in the newborn screening programs is discussed extensively [6-11]. In 2010, SCID was added to the Recommend Uniform Newborn Screening Panel of the United States resulting in the incorporation of SCID in the national screening programs of > 33 states [6]. A recent review of the Dutch Health Council also identified SCID as a suitable candidate for newborn screening. Consequently, in July 2015, the Dutch Ministry of Health adopted the advice of the Dutch Health Council to incorporate SCID in the Dutch newborn screening program.

Newborn SCID screening is based on the detection of T-cell receptor excision circles (TRECs) in dried blood spots using polymerase-chain reaction (PCR) techniques. TRECs are stable circular DNA fragments formed during the T-cell receptor rearrangement process, thereby serving as a biomarker for newly formed T-lymphocytes. Healthy newborns present with TRECs in large quantities, while SCID patients show low or undetectable TREC levels [12]. A TREC assay for newborn screening is now commercially available in the form of the EnLite™ Neonatal TREC kit (PerkinElmer, Turku, Finland). Confirmatory testing such as flow cytometry and gene sequencing should be performed after initial TREC screening to confirm the diagnosis of SCID and exclude other T-lymphocytopenia associated disorders.

In anticipation of the implementation of SCID screening into the Dutch newborn screening program, this study aims to obtain more experience with the available TREC assay. We demonstrated that the EnLite Neonatal TREC assay is a suitable method for newborn screening for SCID in the Netherlands, with applicability of the screening protocol for the Dutch screening laboratories as an important finding of this study.

METHODS

Study population

Anonymized (clinical follow-up of putative positive results was not an aim of this part of the study) fresh heel prick cards (N = 1295) from the Dutch Newborn Screening program of the regions Gelderland and Utrecht were used. Dried blood spots were collected between 72 and 168 hours after birth and analyzed in singlicate within five days after collection. All parents or representatives gave informed consent for the use of patient material for scientific research. The use of anonymized heel prick cards was approved by the Working group Scientific Research Newborn Screening of the Dutch screening organization. Heel prick cards from newborns who received a blood transfusion were excluded from the study. Secondly, singular analysis of heel prick cards stored for two weeks (N = 61), one month (N = 63), three months (N = 33) and one year (N = 33) at 4˚C was carried out, to evaluate the effects on samples that are in transport for prolonged periods of time or lifted from long term storage for e.g. confirmatory re-analysis of newborn samples or retrospective studies. Heel prick cards of 155 preterm newborns (birthweight ≤ 2500 g and gestational age ≤ 36.0 weeks) stored at 4 ˚C and not older than two months were included. Filter paper cards (PerkinElmer 226 paper, PerkinElmer, Shelton, USA) were spotted with peripheral blood from 22 patients with a clinical, genetically confirmed, SCID diagnosis, (affected genes: ADA N = 2, RAG1 N = 6, RAG2 N = 2, IL2Rg N = 4, JAK3 N = 2, XLF N = 2, Artemis N = 2, CD3E N = 2) and of 27 patients with a primary immunodeficiency (PID), potentially SCID, however not confirmed by genetic analysis. These cards were included in the analysis. Samples were obtained according to the rules of the Medical Ethical Committee of the Erasmus MC, Rotterdam. At the Erasmus MC, blood or bone marrow samples of these 49 patients clinically suspected of a potential immunodeficiency, were analyzed by flow cytometry. Based on the flow cytometric results, certain genes were selected and studied by PCR or Sanger sequencing techniques resulting in the SCID diagnosis above.

Reference samples were kindly provided by the Newborn Screening Translational Research Initiative at the Center for Disease Control and Prevention (CDC, Atlanta, Georgia) as part of the Model Performance Evaluation Survey (MPES, the MPES is an

international collaborative research project among newborn screening laboratories). The set consisted of nine TREC reference dried blood spots. Six specimens were created out of cord blood: two specimens with TREC levels close to the cut-off (S356 and L4), two specimens with medium TREC levels (Hi and S339), one specimen with TREC levels below average (L2) and one SCID-like specimen with low or no TREC levels (SCID 2). Two samples were created out of peripheral blood mononuclear cells (PBMC) depleted blood to which a known number of TREC copies was added (B-TREC Cal 3250 TREC copies/µl blood and B-TREC Cal 5 62.5 TREC copies/µl of blood). Lastly, one blood specimen (blood with buffy coat removed, named UnSat) with TREC and reference gene levels below the cut-off levels was included.

Calibration dried blood spot (DBS) samples (PerkinElmer) with TREC levels of 28 copies/µl (A), 167 copies/µl (B) and 578 copies/µl (C) were included in triplicate. Control spots (PerkinElmer) C1 (low TREC, low β-actin), C2 (no TREC, normal β-actin) and C3 (normal TREC, normal β-actin) were included in duplicate. Both calibration spots and control spots were prepared from porcine whole blood with a hematocrit level of 48–55% containing purified salmon-sperm, TREC and β-actin DNA. Cards with patient material were stored at room temperature according to standards of the Erasmus MC, while the original heel prick cards and CDC reference materials were stored at 4–7 ˚C in accordance with the screening laboratory procedures. Calibration- and control spots were stored at −30 ˚C to −16 ˚C as indicated in the kit instructions.

DNA elution from dried blood spot punches

The TREC assay was performed according to the EnLite Neonatal TREC kit instructions (Perkin Elmer). From each heel prick card single 1.5 mm discs were directly punched in a 96 wells plate (3410–0010, Bio-Rad, Veenendaal, the Netherlands) using a Wallac DBS puncher (1296-071, PerkinElmer). To prevent any static interference, plates were passed through an ionizing gate (Eltex Elektrostatik GmbH, Weil am Rhein, Germany). Blank reactions without sample material were carried out in triplicate to check for contamination. Elution buffer and reagent mixture were prepared according to the kit instructions (PerkinElmer) in a pre-PCR area. After punching, 10 µl of Elution buffer (ready for-use buffer with MgCl2) was added to each well of the PCR-plate. PCR-plates were sealed, centrifuged (500 × g, 60 s) and incubated for 45 min at 98 ˚C and 2 min at 4 ˚C in a Bio-Rad Thermal Cycler S1000 (Bio-Rad, Veenendaal, the Netherlands).

PCR amplification and signal measurement

After elution, the seal was removed and 20 µl of reagent mixture was added to each well of the PCR-plate. Next, the plate was resealed, centrifuged briefly (1 min, 500g) and placed in the Thermal Cycler. Amplification reactions consisted of an initial

denaturation-cycle of 5 min at 98 ˚C and 37 cycles of 15 s at 98 ˚C, 1 min at 62.5 ˚C and 15 s at 72 ˚C. Following the amplification step, probe hybridization was allowed for 5 min at 95 ˚C, 60 min at 35 ˚C and 5 min at 23 ˚C. After completion of amplification and hybridization, the PCR plate was centrifuged for 2 min at 500g and was placed in the Victor EnLite fluorometer (model 1420-0220, PerkinElmer) to measure the fluorescence signal of TREC and β-actin. Calibration curves were created by the EnLite Workstation software, based on fluorescence counts measured at 615 nm, 665 nm, and 780 nm. The intensity of the fluorescence signal is directly related to the number of TREC/β-actin DNA copies/µl. For the corrected results, the TREC and β-actin levels of the samples were fitted against the ArcSinh transformed concentrations of the calibration spots by using unweighted linear regression. The control dried blood spots were required to have the correct number of TREC- and β-actin copies/µl as a quality control. The measurement of β-actin signal is only relevant if TREC levels of a sample are below cut-off in the initial analysis. The analysis should be repeated in duplicate from the same heel prick card if the β-actin signal is too low. Results were considered invalid or inconclusive in case one of the duplicate spots gave β-actin levels below a cut-off (40 copies/µl). After this retest in duplicate, the β-actin levels are interpreted to verify whether elution and amplification were sufficient. In this study, an experimental TREC cut-off level of 40 copies/µl was used to distinguish screen positive samples, based on advisory information from the kit insert of the manufacturer. The manufacturer advices to perform a large sample size pilot study to establish the preferred cut-off value based on the normal population distribution in order to establish a reasonable referral rate without any loss of cases.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS version 22.0 (SPSS Inc., Chicago, Illinois). Two-tailed statistical analysis was performed and $P < 0.05$ was considered statistically significant. The Mann-Whitney test and (un)paired t-test were used to determine the difference in TREC levels between fresh heel prick cards and stored heel prick cards and heel prick cards of preterm newborns and full-term newborns. Intra- and inter-assay variation was determined using logarithmically transformed data. All transplantations were performed according to European society for Blood and Marrow Transplantation guidelines. Blood samples were routinely obtained and analyzed after approval by the institutional review board (protocol P01.028). Informed consent was provided by the patient and/or a parent or guardian.

RESULTS

Calibration curves were generated using the TREC- and β-actin levels of the blank reactions and calibration dried blood spots A–C. The correlation coefficients of the calibration curve of 28 runs were typically above 0.9900 for TREC (range 0.9760–0.999) and 0.9880 for β-actin (range 0.9679–0.9999).

To determine the precision of the TREC assay, assay variation was estimated using the control samples (C1, C2, C3) included in each run. The control samples C1, C2 and C3 gave results in accordance with the pre-set targets with an intra-assay variation of the C1 control of 0.46 Ln copies/µl and an intra-assay variation of the C3 control of 0.45 Ln copies/µl. Average based inter-assay variation of the C1 control was 0.23 Ln copies/µl and 0.18 Ln copies/µl for the C3 control. The TREC median of the C2 control was 1.0 copy/µl while 94.6% of all measured values presented ≤ 5 copies/µl.

The mean TREC level of 1295 anonymized Dutch heel prick cards was 111.8 copies/µl blood (median TREC: 96 copies/µl). With the TREC cut-off set at 40 copies/µl blood, 39 samples (3.0%) required a retest after the initial analysis (Figure 1). After retest, 21 samples (1.62%) gave TREC levels in either duplicate spot below cut-off and β-actin levels in both duplicates above the cut-off of 40 copies/µl. Only one sample of the 1295 dried blood spots gave β-actin below cut-off and would therefore require a second heel prick.

The TREC cut-off level based on the 2.5 percentile of the data analyzed with the EnLite Neonatal TREC assay was 39 copies/µl. Table 1 shows the decrease in total numbers of annual referrals when different TREC cut-off levels are applied.

To determine the effect of storage time on TREC levels in dried blood spots, a comparison between fresh and stored heel prick cards was performed. In a first experiment the median TREC levels of fresh heel prick cards was 84 copies/µl blood. After storage for two weeks it was 90 copies/µl blood. Moreover, the median TREC level of the fresh heel prick cards was 85 copies/µl with a median TREC level after storage for one month of 87 copies/µl. No significant difference in TREC levels was observed between fresh heel prick cards and heel prick cards stored for two weeks ($P = 0.86$) or one month ($P = 0.10$).

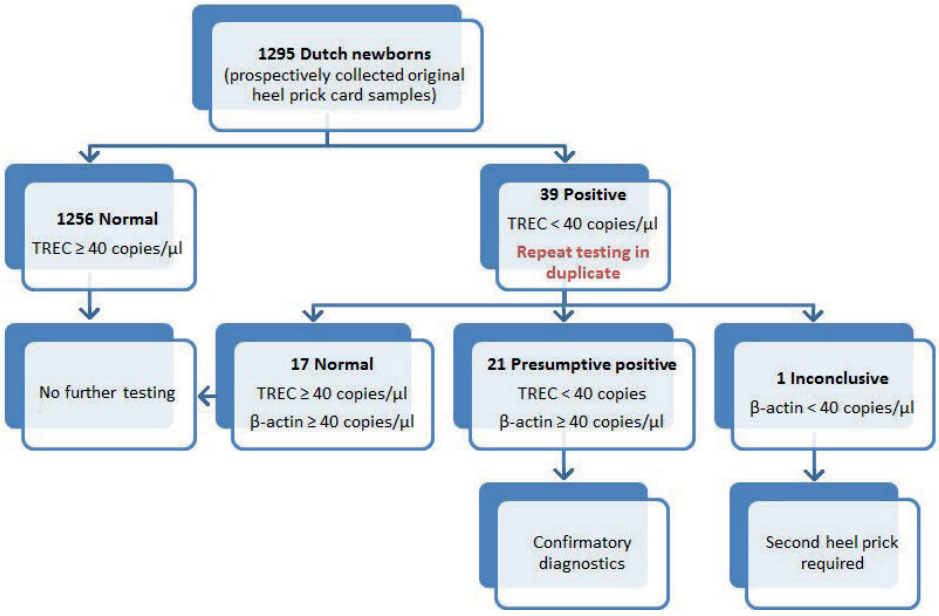

Figure 1. Flow chart of the EnLite Neonatal TREC assay, including results of 1295 fresh anonymized heel prick cards from Dutch newborns.

Table 1. Percentage of positives after initial testing, percentage of presumptive positives after second round testing and the total number of annual referrals with different TREC cut-off levels based on 175,181 births in 2014 [14].

	40 copies/µl	39 copies/µl	35 copies/µl	30 copies/µl	25 copies/µl	22 copies/µl
Percentage of positives after the initial test (%)	3.0	2.5	1.54	0.77	0.39	0.15
Percentage of presumptive positives/ referral rate (%)	1.62	1.31	0.69	0.54	0.23	0.08
Annual referrals (N)	2838	2295	1209	946	403	140

A second comparison was made between fresh heel prick cards and unpaired heel prick cards stored for 3 months and one year at 4–7 ˚C, respectively. Data in Figure 2 show a decrease in TREC levels of heel prick cards stored for 3 months and one year. The median TREC level of the fresh heel prick cards (N = 90) that were included in the same runs was 109.5 copies/µl. The median TREC level of the heel prick cards stored for three months (N = 33) was 69 copies/µl (P = 0.0008), and of cards stored for one year (N = 33) was 69 copies/µl as well (P < 0.0001).

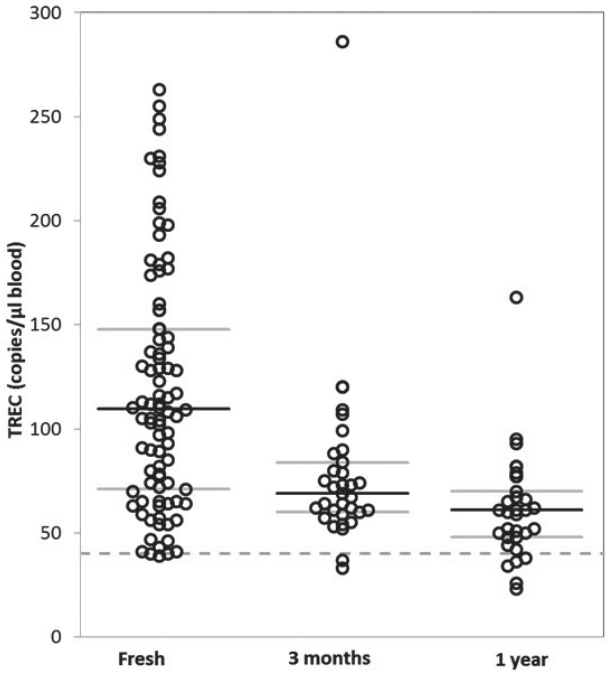

Figure 2. Results of the analysis of 90 fresh heel prick cards using the EnLite Neonatal TREC assay; 33 heel prick cards stored for three months and 33 heel prick cards stored for one year. Horizontal black lines show the median TREC levels, horizontal grey lines depict the standard deviation.

Samples of all 22 genetically confirmed SCID patients (with T lymphocytopenia and known underlying mutations) had low or absent TREC levels and far below the cut-off level (range 0–4 TREC copies/µl) (Figure 3). In addition, a series of 27 samples of PID patients without a defined genetic diagnosis were tested. In 14 patients, the TREC level was below 7 copies/µl. Twelve of these patients were clinically suspected for SCID and had strongly reduced T-cell numbers. Initial genetic testing did not reveal the identification of the genetic defect, but further genetic testing via whole exome sequencing is now considered. Two of the 14 samples with TREC < 7 copies/µl were from adult patients with common variable immunodeficiency (CVID). It is known that a part of CVID patients have reduced TREC and KREC levels [13]. The remaining 13 PID patients had TREC levels above 25.5 copies/µl; none them had T-cell lymphopenia. The clinical diagnosis varied between CVID (N = 5), hyper IgM syndrome (N = 2), suspicion for SCID (which was not confirmed after flowcytometric phenotyping) (N = 2), and unclassified PIDs (N = 4). The mean TREC level of confirmed SCID patients was 0.34 copies/µl blood (median: 0 copies/µl). The mean TREC level of PID patients with inconclusive diagnoses was 46 copies/µl blood (median: 6.3 copies/µl).

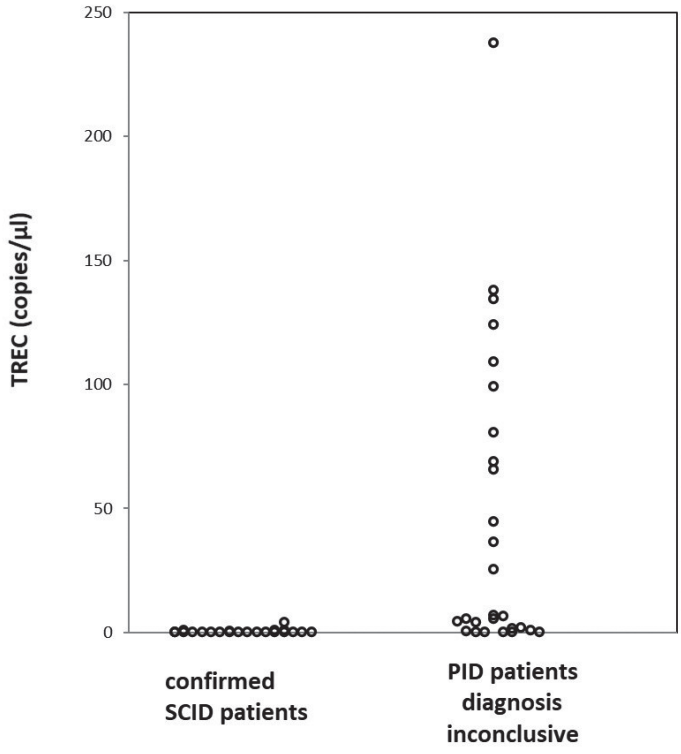

Figure 3. Results of the analysis of peripheral blood samples of 22 genetically confirmed SCID patients and 27 PID patients without genetic diagnosis using the EnLite Neonatal TREC assay.

Table 2 shows data of TREC analyses of samples of preterm newborns. The mean TREC level of 155 heel prick cards of preterm newborns was 64.9 copies/µl with a median of 55 copies/µl, compared to (112 copies/µl blood in full-term infants (median: 96 copies/µl, $P < 0.0001$)). Of the 155 samples, 45 specimens gave TREC levels below the cut-off level of 40 copies/µl resulting in a retest-rate of 29%. A 9.6 fold difference was observed compared to the retest-rate of full-term infants (3.0%).

The nine TREC reference DBS samples were analyzed in two different runs in duplicate. Eight out of nine specimens gave results within the preset categories. Four cord blood specimens showed normal results (TREC/β-actin levels ≥ cut-off) with medium TREC levels, TREC levels close to cut-off and TREC levels below average, respectively (L4, Hi, S339, L2). The SCID-like specimen (SCID2) gave a presumptive positive result with TREC levels close to zero and β-actin levels within the standard reference range. The PBMC-depleted blood samples gave TREC levels according to the added number of cells (B-TREC Cal 3250 TREC cells/µl and B-TREC Cal 5 62.5 TREC cells/µl). The blood

sample with the buffy coat removed (UnSat) gave TREC and β-actin levels below the cut-off levels (as expected). The result of only one cord blood specimen (S356) differed from the target values in two runs in duplicate. While this sample should have given TREC levels close to cut-off (40 copies/µl), the results showed TREC levels far above cut-off with a mean TREC level of 270 copies/µl.

Table 2. Descriptive statistics for TREC levels (copies/µl) of 155 preterm samples, categorized by gestational age.

Gestational age	Number of samples	Mean (TREC copies/µl)	Median (TREC copies/µl)
≤28 weeks	16	44.5	45.5
29–32 weeks	61	60.1	50.0
33–36 weeks	78	72.8	59.0

DISCUSSION

In this study, an evaluation of a TREC SCID screening assay was performed evaluating the applicability in the Dutch screening program. Of the 39 fresh heel prick specimens that gave TREC levels below the pre-set cut-off of 40 copies/µl blood, 21 samples presented presumptively positive, 17 samples gave normal results and one sample presented as inconclusive after re-analysis in duplicate, resulting in a referral-rate of 1.62%. In routine screening, these presumptively positive newborns would be referred for confirmatory diagnostics such as flow cytometry or gene sequencing. With 175,181 births in 2014 [14], a referral rate of 1.62% would result in annually about 2838 referrals for follow-up diagnostics, or 55 infants per week distributed among the five Dutch screening laboratories.

Results of experiments on the influence of storage times on TREC levels confirmed previous unpublished results by the manufacturer that indicate that TREC levels decrease with increasing storage time, but not for storage times up to one month at room temperature. As the Dutch newborn screening program receives heel prick cards of the Caribbean Netherlands, with shipping times up to two weeks, we thus expect that these samples will not yield compromised TREC levels due to prolonged storage in transportation.

The cut-off level based on the 2.5 percentile of the data analyzed with the EnLite Neonatal TREC assay was 39 copies/µl. With this new cut-off level the retest-rate would drop to 2.5%. Table 1 shows the decrease in total numbers of annual referrals when different TREC cut-off levels are applied. A high referral rate could result in an excessive workload

for downstream referral centers. Therefore, lowering the TREC cut-off level is worth consideration. Since SCID patients present in most cases with very low or undetectable TREC levels, lowering the TREC cut-off would most likely not result in an increase of false-negative results. At a TREC cut-off level of 22 copies/µl the number of presumptive positives/referral rate would be 0.08%, which would result in an annual 143 referrals for follow-up diagnostics. These numbers are comparable with the referral rates of pilot studies in the UK (0.04%), California (0.02%) and Wisconsin (0.03%) [10]. The exact number of presumptively positive samples and the exact referral rate can only be established once a pilot is carried out in the Netherlands with a considerably larger sample size (e.g. 15–30.000). The inconclusive sample showed β-actin levels below cut-off without agreement between duplicates in three analyses. In the regular screening program, a second heel prick should be requested if samples present inconclusive after being analyzed in duplicate. With approximately 178,000 samples being analyzed annually, this would result in 137 second heel pricks each year. If the second heel prick shows inconclusive results as well, flow cytometry should be performed.

Consistent with previous studies [15-17], NBS samples of Dutch preterm newborns had low TREC levels and a higher retest-rate than samples of newborns with a full-term pregnancy. There are several options in which the SCID screening algorithm could be adjusted based on these findings. A first possibility would be to request a second heel prick of all preterm newborns that present presumptively positive after the second analysis. This second heel prick could be taken immediately or after the preterm newborn has reached the adjusted gestational age of 37 weeks (in accordance with the screening algorithm of the state of Delaware [6]). Previous research showed an increase and normalization of the concentration of T-cells as the age of the preterm newborn advanced [18]. In other states, such as Connecticut and New York, the cut-off level for preterm newborns was lowered in order to reduce the retest-rate [6]. Based on our data, the 2.5 percentile-based cut-off level for preterm newborns would be < 16 copies/µl. Finally, the same screening algorithm and cut-off levels for both full-term as well as preterm samples could be used (comparable to the algorithm used in the state of Michigan). In Michigan, a cut-off level was chosen at which newborns with a birth weight ≤ 2500 g showed a five-fold higher rate of false positive screening results compared to newborns with a birth weight > 2500 g. To prevent delayed diagnosis of preterm newborns with SCID, this balance between the number of false-positive and false-negative results was deemed acceptable [19].

In all 22 samples of confirmed SCID patients, the EnLite Neonatal TREC assay showed absent TREC levels or levels far below cut-off, indicating the applicability of the assay for detection of newborns with SCID. Of the 27 PID patients without a genetic diagnosis, 14 samples gave TREC levels below the cut-off level of 7 copies/µl. Twelve of these patients were clinically suspected for SCID and had strongly reduced T-cell numbers, however, Initial genetic testing did not reveal the identification of a genetic defect. Further genetic testing via whole exome sequencing is now considered. The other two samples were from CIVD patients with reduced T-cell numbers [13]. Previous studies suggested that TREC analysis of dried blood spots would be unable to detected newborns with an adenosine deaminase deficient (ADA) type SCID [20]. In this study, two patients with a mutation in the ADA gene (c.956_960delAAGAG and c.302G > A) were both detected with EnLite Neonatal TREC assay, confirming that although delayed-onset ADA deficiency might not be detected by TREC quantification, ADA-deficient newborns with a T-cell deficient phenotype will be identified using a TREC assay.

In conclusion, the first results with TREC assay imply that EnLite Neonatal TREC assay is a suitable method for newborn screening for SCID in the Netherlands. The introduction of SCID in the Dutch screening program is already sanctioned by the Dutch minister of Public Health, Welfare, and Sports [21]. With the findings of the current study, the first advisory information concerning the TREC assay for SCID screening is provided. With this knowledge, a first step is made in the integration of SCID screening in the Dutch screening program.

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