Newborn screening for severe combined immunodeficiency using a novel and simplified method to measure T-cell excision circles (TREC)

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ABSTRACT

The prognosis of children with severe combined immunodeficiency (SCID) depends on a presymptomatic diagnosis and early treatment before complications occur. We established and tested a simplified, practical and economic newborn screening method based on the quantification of T-cell receptor excision circles (TRECs) on dried blood spots (DBSs) through qPCR. Our method was validated by the analysis of 11 positive controls, which all showed an absence of TRECs, thus yielding a sensitivity of 100%. Further, we analyzed 6034 anonymized newborns of whom 6031 (99.95%) showed a normal TREC qPCR with a median of 600 estimated TREC copies/1.6 mm punch. The test showed a recall-rate of 0.05%.

We present a highly sensitive, specific and time- and cost-effective method of TREC quantification, which is suitable for SCID newborn screening. In comparison to established methods, our test requires only 25% of the input material, doesn’t require DNA purification and significantly reduces time and cost requirement.

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1. Introduction

SCID is a group of >20 disorders caused by different genetic defects [1]. The incidence has been calculated at approx. 1:58,000 live births [2]. All types of SCID have in common the lack of functional T-cells, leading to a combined cellular and humoral immunodeficiency. Without allogeneic hematopoietic stem cell transplantation (HSCT), children die in the first years of life because of severe infections. The only way to prevent death is early diagnosis followed by HSCT, before infections occur [3]. Indeed, studies have shown that the most important prognostic value for the primary outcome and the long-term survival rate is the clinical status of the patient (patients with active infection at the time of transplantation have a survival rate of 50%, whereas those with no infection 82–90%) [4–5].

Because of the value of early pre-symptomatic diagnosis, SCID fulfills all the criteria for a disease to be targeted by newborn screening and was recommended being added to the panel of NBS illnesses in 2011 [6]. While a method to screen neonates for SCID in a high throughput format in dried blood spots has not been available until recently, the development of a practicable test by Chan and Puck in 2005 has been a breakthrough [7].

Recently, a number of studies in the US and in Europe proved the importance and the validity of a SCID newborn screening [2,3].

Efforts have been made in several research laboratories in order to establish an optimal screening test combining good sensitivity and specificity at costs affordable in a massive-scale format [8].

Nevertheless, no agreement on the method and the panel of diseases to be screened has been achieved so far. The most promising method is the quantification of T-cell Receptor Excision Circles (TRECs) on dried blood spots (DBS) by real-time quantitative PCR (qPCR) [9]. TRECs are small episomal pieces of DNA which are generated in the thymus during the VDJ-T-cell receptor gene rearrangement and are therefore good markers for naïve T-cells [10].

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Several efforts have been made to implement methods for the early diagnosis of SCID [5,11–17]. We established and tested a robust method to detect SCID in newborns through the quantification of TREC by qPCR. Our method showed a very high sensitivity and specificity and compared favorably to existing methods in terms of time and costs, which plays an important role in the perspective of a population screening on large numbers.

2. Material and methods

2.1. Screened samples

DBS specimens were obtained from Guthrie cards collected by the Newborn Screening Laboratory of Heidelberg University in the period between October and December 2012. After completing the other routine screening tests, DBSs were de-identified and punched for the TREC assay.

After having obtained a written consent from the parents, anonymized DBS specimens of newborns and children with different types of T-cell deficiency were used as positive controls (Supplemental Table 1).

2.2. Ethical approval

This pilot study was approved by the ethics committee of the University of Heidelberg.

2.3. DNA extraction

A new protocol for DNA extraction was established for the purpose of this study. 1.6 mm punches were incubated in sealed 96-well plates with 13 μl lysis reagent (DNA Extract All Reagents Kit, Cat. Number 4402599, Applied Biosystems) for 3 min at 95 °C. Lysis was stopped by adding 13 μl of stabilizing reagent (DNA Extract All Reagents Kit, Cat. Number 4402599, Applied Biosystems).

2.4. qPCR

Real-time quantitative PCR for TREC quantification was performed in a total volume of 20 μl containing 10 μl Mastermix (SensiFAST Probe Hi-ROX Kit, Bioline), 2 μl TREC forward-primer 10 μM, 2 μl TREC reverse-primer 10 μM, 0.3 μl FAM-TAMRA-labeled TaqMan TREC probes 1:10 (Life Technologies), 0.8 μl BSA 1% and 2 μl DNA extract.

Real-time quantitative PCR for β-actin quantification was also performed in a total volume of 20 μl containing 10 μl Mastermix (SensiFAST Probe Hi-ROX Kit, Bioline), 0.7 μl 1:3 VIC-labeled Beta Actin TaqMan Gene Expression Assay (Nr Hs03023880, Order Nr 4,448,484, Life Technologies) containing β-actin primers and probes and 2 μl DNA extract.

The DNA sequences of primers and probes are listed in Table 1. The qPCR reactions were carried out on a StepOnePlus real-time PCR System (Applied Biosystems) in 96-well plates and underwent 1 cycle of 2 min at 50 °C, 1 cycle of 10 min at 95 °C and 45 cycles of 30 s at 95 °C and 30 s at 60 °C.

A fixed quantification cycle (Cq) was set for data collection and amplification analysis during the exponential phase of the PCR amplification. Calibration curves for TREC quantification were obtained by using a 10-fold serially diluted TREC plasmid. β-actin was used as a semi quantitative internal control to assess successful DNA extraction in patients whose TREC copy number did not achieve the fixed cutoff (see Results section).

2.5. Statistical analysis

ROC curves were generated in order to fix an appropriate cutoff for TREC-absolute value and TREC-Cq value.

3. Results

We established and tested a new high performance assay for SCID screening based on a qPCR assay for the quantification of TREC. A very fast DNA extraction phase requiring only few minutes was followed by the qPCR analysis. Calibration curves for TREC quantification were obtained by serially diluting a plasmid coding for the TREC sequence (Fig. 1a–b). β-actin served as semi quantitative internal control. The method was highly reproducible and quantitative over a range from 10 to 100,000 copies per punch and showed some advantages in comparison to other existing methods [8].

3.1. Establishing TREC cutoff value in a newborn screening population

We collected Guthrie cards of 6046 consecutive unselected samples after the standard NBS program had been completed. Because of the low frequency of SCID (<1:60,000) these samples were expected not to include SCID patients. Twelve samples had to be excluded as they contained too little material. Fig. 1 shows an example of the results obtained from the qPCR TREC assay.

We thus analyzed 6034 samples, in which we found a distribution of TREC copy number from 0.1–5109 copies/punch with an average of 814 copies/punch and a median of 600 copies/punch; the 1st percentile laid by 243 copies/punch and the 99th percentile by 3067 copies/punch. Through statistical analysis based on ROC-curves (Fig. 2) we analyzed the distribution of the results and calculated an appropriate cutoff for the interpretation of the results. Within the positive controls we observed a single TREC-value of 94 copies/punch possibly due to sample contamination, so that the cutoff was fixed at 95 TREC copies per 1.6-mm DBS punch.

Based on the cutoff-value, we established an algorithm for the sample analysis (Fig. 3). The quantification of simply TREC copy number was used as a first tier. For samples that did not pass the first tier, a second tier included actin as an internal control. Only samples that failed the second tier would be recalled in a non-anonymized setting.

Fig. 4 illustrates the distribution of TREC values in our study population in comparison to the positive controls. 5964 (98.8%) of 6034 tested infants had an initial TREC value above the cutoff. 70 infants had an initial TREC value below the cutoff, which led us to perform a second TREC analysis including an internal control with beta actin to distinguish between real TREC-negative results and “DNA amplification failure” (retest-rate 1.2%). The majority of these samples (59 of 70; 85%) passed the second tier with a TREC copy number above the cutoff and clearly detectable actin (Cq Value < 30). For 11 newborns (15%) the analysis of a second punch from the same blood sample was needed, because of “DNA amplification failure”, which identified TRECs above the cutoff in 8. Therefore, in 3 of 6034 samples no TRECs above the cutoff could be detected. These newborns must therefore be suspected to have T-cell deficiency and would have been recalled for further investigations in a non-anonymized setting (recall-rate 0.05%; Fig. 3).

<table>
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<th>Table 1</th>
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<tr>
<td>Sequences of TREC primers and probes [11]. Sequences of β-actin primers and probe according to the Gene expression Assay Nr Hs03023880, Order Nr 4448484, Life Technologies.</td>
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<td>Name</td>
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<tr>
<td>TREC forward primer</td>
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<td>TREC reverse primer</td>
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