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**Review Article**

# Why Should TREC and KREC Quantification Assay Be Concerned about Screen of Newborns in Developing Countries?

**Amir Ghasemi**

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**Abstract**

Primary immunodeficiencies contain a set of several different diseases. Giving the fact that their clinical outcome ranges from mild to potentially life-threatening, detecting the patients with these diseases in the neonatal period is considered as the main goal of efforts that are currently being made. It has been reported that T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) are circular DNA segments produced in T and B cells during their maturation in the thymus and bone marrow, respectively. Fortunately, excision circles can be reliably quantified using real-time quantitative PCR techniques.

The TREC and KREC assays, as introduced in the newborn screening program (NBS), allow early identification of the disease and may result in discovering new genetic defects including Severe combined immunodeficiencies (SCID), primary agammaglobulinaemias (such as X-linked agammaglobulinaemia), and inherited haemophagocytic syndromes (such as familial haemophagocytic lymphohistiocytosis). Regarding, the cost-effectiveness, survival of children, and success in improving the life quality of children involved in newborn screenings for severe combined immunodeficiency has been demonstrated.

Here, we discuss about TREC and KREC assay, their applications, and also assessment of the cost effectiveness of an established program for newborn screening, in terms of TRECs and KRECs quantification in Iran.

**Keywords** TREC, KREC, Newborns screening.

*** Corresponding author:** Amir Ghasemi

**E-mail:** amghasemi@ufl.edu

1. Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran; Department of Infectious Disease and Immunology, College of Veterinary Medicine, University of Florida, FL, USA

**Generation of TRECs and KRECs**

The primary locations for generating new T- and B-cell from undifferentiated hematopoietic ancestors, are thymus and bone marrow (BM), respectively. An extremely various lymphocyte range, which was produced during lymphocytes development, can let the generated cells to react to a broad variety of antigenic exposure (1-3).

The generation of T cells expressing a functional T cell receptor (TCR) is a feature of T cell development. various domains of TCR genes are assembled during T cell development following rearrangement of variable (V), diversity (D), and joining (J) gene fragments by a process called as V(D)J recombination (4).

After differentiating B cells from precursors in bone marrow, and before they come out in peripheral blood and secondary lymphoid organs (5) B cells underwent V(D)J rearrangement of their Ig heavy and Ig light chain loci, to create a unique B cell antigen receptor (6). There is not any first exon in the gene complexes encoding the T- and B-cell receptor components. In order to form a functional first exon, rearrangements of V (D) J introduced in the genome to couple one of each segment together, are necessary. The rearrangement of the TR alpha/beta genes causes the removal of delta-coding segments situated in the TRA locus between the TRAV and TRAJ genes, and also the excision of these fragments are mandatory to generate the TRA chain. The ligation of signal ends of the excised segment results in forming a circular DNA termed TR excision circles (TRECs) carrying a signal joint (SJ) (7, 8). During B cell maturation, immunoglobulin k-deleting recombination excision circles (KRECs) are produced by k-deleting recombination allelic exclusion and also by isotypic exclusion of the λ chain (9, 10). Signal joint (sj) KRECs were cut out from genomic DNA, whereas Coding joint (cj) KRECs exist in the chromosome. Because sjKRECs were not replicated through cell division, their levels reduced; whereas cjKREC levels stayed similar to that of after B-cell division (9, 11).

**The TRECs and KRECs Quantification Assay**

Quantification of TRECs and KRECs are now considered to monitor naive T and B cells emigrating from the thymus and BM, respectively. The presence of signal joint segment within both TRECs and KRECs without similarity gives this possibility that both of them are detected by PCR. Currently, a TaqMan-based real-time quantitative (RQ-) PCR is used in order to detect TRECs and KRECs. Taking advantage of introduction of the TRECs and KRECs quantification assay method, to study the thymic output (8, 12-15) and replication history of B cells (16), attempts have recently been made to establish a Newborn screening for T and B cells. Upon cell division, they were not replicated and diluted in the young cells; therefore, they are stable (8, 17). TREC measurement has accurately been obtained by performing quantification compared to a control gene such as receptor 5, chemokine (C-C motif ),

albumin, or TRA constant (TRAC) gene (14, 18, 19). Furthermore, The quantity of TRECs has been stated either as absolute number of TREC molecules per µg of DNA within PBMC or as T lymphocytes (19-21) or per $10^6$ cells (22, 23) which are expressed TRECs per ml of blood (24, 25).

As mentioned earlier, cjKREC levels remained similar to that of after B-cell division, whereas sjKREC levels decreased. It has been shown that by creation of a cell line that contains one coding joint and one signal joint per genome, it is possible to calculate KRECs level of samples using PCR (16). When DNA quantities of both the cell line and the samples are measured by PCR, the average number of cell divisions of the sample can be calculated as follows:

$$(CT_{\text{signal joint}} - CT_{\text{coding joint}})_{\text{sample}} - (CT_{\text{signal joint}} - CT_{\text{coding joint}})_{\text{cell line}}$$

regarding, Borte et al. has modified the KREC and TREC assay by setting up a triplex quantitative real-time PCR, in which by measuring simultaneous TRECs, KRECs and β-actin (ACTB) allows the quantification of newly produced T and B lymphocytes (26). ACTB amplification was used to assess the success of extraction of DNA from the Guthrie cards. The main advantage of the combined assay can be considered to be that the variability related to direct DNA quantification is eliminated using a unique standard curve obtained by diluting the triple-insert plasmid, which contains TREC, KREC, and ACTB fragments in a 1:1:1 ratio (26). In addition, it has been shown that TRAC is used to control both the quality and quantity of genomic DNA in the sample (27-30). Furthermore, the simultaneous quantification of the three targets in the same reaction would reduce the laboratory costs. In order to calculate TRECs or KRECs per $10^6$, PBMC DNA was extracted from PBMC isolated from heparinized blood using specific primers and probes, and for TRECs, KRECs, and control fragment the following calculation can be used:

$$\frac{mean\ of\ TRECs\ or\ KRECs\ quantified}{mean\ Control\ fragment\ quantified/2} \times 10^6$$

The obtained results were expressed either as copies/$10^6$ PBMC or as copies/milliliter.

***TRECs or KRECs as immunological markers***

TRECs and KRECs are circular DNA segments generated in T and B cells during their maturation in the thymus and bone marrow. These circularized DNA elements stayed in the cells, have no capability of replicating, and are diluted during cell division; consequently, they are considered as markers for new lymphocyte output (8, 9, 11, 23, 28, 30-34). Simultaneous real-time quantitative PCR for detection of TRECs and KRECs, is considered to be a reliable technique offering a novel method in the handling of T- and B-cell deficiency-related diseases (26, 29). In primary immunodeficiencies (PIDs), when this quantification of TRECs and KRECs are combined with other diagnostic techniques such as flow cytometric analysis of T- and B-cell

subpopulations, the measurement of the TRECs and KRECs would improve characterization of the diseases, the identification of patients' subgroups, and the monitoring of stem cell transplantation and enzyme replacement therapy (27, 30, 35-40). The TREC and KREC assays are introduced in the newborn screening program and allowing us to diagnose PIDs (11, 41) . In addition, the measurement of TREC and KREC levels can be also used as a substitute marker of lymphocyte output in acquired immunodeficiencies. In untreated HIV-infected patients, the low number of TRECs has been frequently documented, and therefore, the number of TRECs has been shown to increase following antiretroviral therapy (19). Furthermore, it has been shown that the number of TREC was lower in compared to control healthy group, that were measured using TREC/KREC quantification assay in HIV-infected patients who need antiretroviral therapy. However, the KREC number was significantly high, and was similar to the level found in control healthy groups (42).

### *Newborn screening*

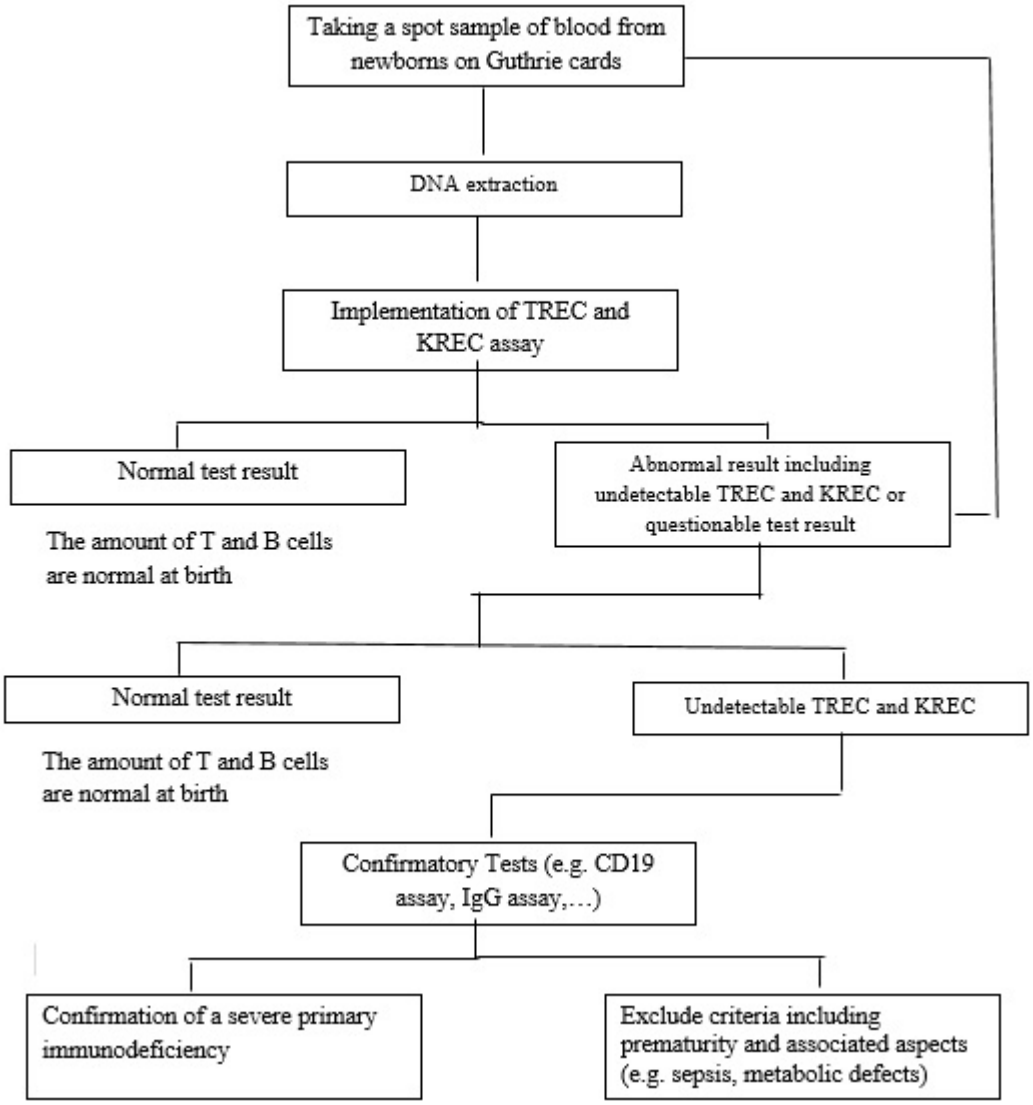
In addition to severe combined immunodeficiency (SCID) as a disorder that must be included in each newborn screening (NBS) programs on a population-based scale, the other severe PIDs must also be considered for NBS (17). The rationale reasons to diagnose the other PIDs as well as SCID in NBS, are mainly the severity of clinical outcome of the disease following the hidden stage, the existence of a therapeutic consensus at the best curative nature, a useful prognosis if patients are early diagnosed and treated, and the cost-effectiveness of NBS in consideration of the prevalence and follow-up costs of late-diagnosed patients. In this regard, the familial haemophagocytic lympho istiocytosis and Bruton's disease (X-linked agammaglobulinaemia; XLA) have already been suggested as screening candidates (26, 43-45). Fortunately, simultaneous detection of TRECs and KRECs has been developed, and can enable us to diagnose PIDs patients regardless of the genetic cause of the disease (29). In addition, this test was improved to be performed using blood from dried spots that was highly sensitive and specific for PIDs and was also cost-effective (26). In this regard, several pilot studies of NBS for SCID, integrated with plans for its definitive diagnosis and management, have been established in some US states. All infants diagnosed with SCID have taken transplantation or enzyme therapy, and fortunately, at the end no death was reported (45-51).

### **Therapy monitoring of primary and acquired immunodeficiencies by Quantification of TRECs and KRECs**

The use of TRECs and KRECs quantification assay are being expended. although It has been shown that innate immunity properly shows its function during one month after transplantation (52), generation of T cells can be significantly postponed and this matter predisposes human stem-cell transplantation (HSCT) recipients to prone to

infections and relapse of malignancies (53, 54). Therefore, frequently combined quantification of absolute TREC and KREC counts is considered as a suitable marker for monitoring early T- and B-cell neogenesis in adult patients treated by HSCT (27). Also, TRECs and KRECs were measured in patients with other PIDs (SCID, X-linked hyper IgM, Wiskott-Aldrich syndrome and familial hemophagocytic lymphohistiocytosis). In order to evaluate the thymic output in HIV-infected, the TREC assay was also used broadly in the patients treated with antiviral therapy (21, 55, 56). It has been shown that by passing one year from the therapy of HIV-infected patients, the number of KRECs was not modified, while a significant reduction has been observed in new B-cell emigrant from the BM after 6 years treatment (42).

**A guideline for newborn screening of primary immunodeficiency diseases**



**Common variable immunodeficiency (CVID) classification by TRECs and KRECs quantification assay**

CVID is the most prevalent primary immunodeficiency associated with antibody deficiency and other various clinical manifestations at all ages (57). It was thought that CVID is a disease primarily caused by defect in B-cell function; whereas it has recently been reported that $CD4^{+}$T-cell numbers decreased in patients with CVID (36, 58). In this regard, it was excellently shown that the low levels of TREC and KREC are useful markers largely showing the survival rate in patients with CVID (40) Moreover, it has been proposed that TREC and KREC can be used as markers to differentiate CVID from combined immunodeficiency (CID), and also to evaluate the clinical consequences of each patient with CVID (40). In addition, the quantification TREC and KREC levels for classification would enable us to make decision on selecting a better treatment plan for each CVID patient (30, 40).

**Limitations of TREC and KREC quantification assay**

A large number of unexpected patients can be identified by TREC quantification assay, which has no criteria for SCID like DiGeorge and Down syndrome that are known to result in T-cell lymphopenia (49, 59). When maturation of T cells and B cells stops at early steps, TRECs and KRECs are not generated and are diluted after division of cell. Therefore, since they are only carried out by old thymic emigrant cells, and going to be vanished after cell death, caution is necessary in interpreting their number in the clinical assessment. Although expressing of the TRECs or KRECs number per ml of blood may overcome the issue of peripheral dilution (24, 60) this assay alone cannot still be considered as a direct clinical marker of immune disease. In addition, it has been also shown that number of TRECs is low in premature infants, regardless of not having immunodeficiency (61). Therefore, it is important to emphasize that the identification of SCID and agammaglobulinaemia at birth by TREC and KREC quantification assay, must be followed by other appropriate tests in order to confirm diseases.

PIDs were firstly thought to be rare, happening only in infants and young children, and to be related with severe clinical symptoms. However, by rising knowledge about PIDs, it was turned out that they are much more common than the first thought. The general frequency of PIDs has been estimated to be about 1:10,000 individuals (6). Based on the second report of the national registry in Iran, fifty percent of patients with PIDs are suffering from SCID and Predominantly

antibody deficiencies (62). By considering the birth rate of 1.82% and this fact that Iran's population reaches about 75 million by 2011(http://en.wikipedia.org/wiki/Demographics_of_Iran) , it is convincible to imagine that we would have about 62 newborns with SCID and Predominantly antibody deficiencies (PAD)

making high demands on the effectiveness and availability of screening tests. Unfortunately, these newborns would not be diagnosed until they became infected and be referred to medical centers. There is possibility that they die before their diseases being detected. Also, It was shown that there is a significant difference by considering the costs of transplantation for infants with SCID under 3.5 months of age (approx. 1 million dollars) and the costs for those with the ages over 3.5 months (more than 4 million dollars), as well as the cost of treating one baby with SCID that is not diagnosed until one of them has a serious infection can easily exceed 2 million dollars (51, 63).

Clearly, using a reliable screening test for detection of these disorders by low cost test would help improving the quality of life of patients and reducing their treatment expenses. In this regard, it was reported that a SCID screening test using TREC assay on dried blood spots, costs approximately 5 US Dollars that would be considered as cost-effective (64). However, our evaluation showed that the cost approximate can be 5 dollars per each newborn by considering TREC and KREC screening implementation on dried blood spots, equipment and Laboratory places, personnel and administration, education of staffs, interpretation, reporting, retesting of diagnosed cases and false-positive, and quality control for laboratory equipment.

## Conclusions

During the past few years, Newborn screening programs have been considered to find some serious disorders in the early days of newborns life, which help improving the quality of life of patients as well as reducing the cost of treatments. Excision circle assays are still one of the most promising candidate for NBS protocols including simultaneous quantification of TRECs and KRECs, and could support early diagnosis, classification of patient with CVID, and assessment of both T- and B-cell in patients receiving stem cell replacement therapy. In addition, the cost effectiveness and technically feasible reported in this review adding new information to the recent published data [8], and indicated that the quantification of TRECs and KRECs can be very useful in the NBS to find infants with primary immunodeficiencies.

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