UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Establishing an in-house real-time polymerase chain reaction assay to quantify T-cell receptor excision circles and kappa-deleting recombination excision circles for use in screening newborn babies for inborn errors of immunity

Submitted in fulfilment of the requirements for the degree of Masters in Science (Medical Immunology) in the Faculty of Health Sciences, University of Pretoria

By Shudufhadzo Singo

Student number: 18017411

Supervisor: Doctor Luyanda Kwofie

Co-supervisor: Doctor Catherine Worsley

Co-supervisor and Head of Department: Professor Pieter Meyer

2024

Declaration: Authorship

I, Shudufhadzo Sharon Singo declare that the dissertation I am submitting to obtain a Masters of Science in Medical Immunology in the Faculty of Health Sciences, University of Pretoria, is my original work and has not been submitted before for this degree at this institution or any other institution. I diligently adhered to the practice of properly referencing and acknowledging all the information derived from other sources that I integrated into my dissertation.

Signature: 

Date: 12 December 2024

Acknowledgements

I would like to express my gratitude to the subsequent individuals and organisations for significantly contributing to my research.

1. Doctor Luyanda Kwofie, my supervisor, for the support in facilitating this research. I am appreciative of her encouragement and support throughout the entirety of my studies. Additionally, I appreciate her input and patience as I conducted this research.

2. Doctor Catherine Worsley, my co-supervisor, for her support and guidance throughout my research. For reviewing my written work and offering perceptive feedback.

3. Professor Pieter Meyer, my co-supervisor, for his guidance and assistance throughout my research. Reviewing my written work and offering perceptive feedback. For providing assistance throughout the statistical analysis of my research.

4. I am grateful to the National Health Laboratory Service (NHLS) for allowing me to use their laboratory space and equipment required to conduct my research.

5. The National Research Foundation (NRF), for providing me with the financial support that enabled me to complete my research. None of the conclusions and recommendations presented in this research were influenced by the NRF; they are all the work of the author.

6. Siyakhula Study, for granting me permission to utilise stored preserved dried blood spot samples, which were used in the course of this research. In addition, for providing detailed demographic information and data related to the samples used in this research.

7. The Faculty of Health Sciences Masters Committee and the Faculty of Health Sciences Research Ethics Committee, for reviewing my research protocol and providing perspective feedback. For approving my research proposal.

8. I am grateful to the newborn babies and carers who participated in our study. This study could not have been possible without the samples contributed by these participants. All of the work completed in this study is due to their willingness to participate in the Siyakhula study.

9. My mother, for consistently encouraging and supporting me throughout my academic career. For believing in me and my aspirations. For her well-wishes regarding my prosperity and contentment.

10. My brothers, for their unwavering support and belief in my abilities. For their encouragement and cheering for my success. For making sure that I always had what I needed to succeed.

11. I would like to thank God for bringing me this far and guiding me through this journey. I am glad for God's presence in my life, which has enabled me to reach this milestone.

I am very grateful to all individuals whose assistance contributed to the accomplishment of my research. I am beyond grateful for all the assistance that has been provided for me.

Executive Summary

Inborn errors of immunity (IEI) are genetic disorders that hinder immune responses and are underdiagnosed, particularly in low-to-middle income countries. These disorders occur in one in every 500 live births. Early detection is crucial for preventing health complications. The need for early detection of IEI has led to the development of newborn screening (NBS) programmes; however, many countries lack routine NBS due to financial and technological challenges. The present study aimed to develop an in-house screening technique for T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) quantification using real-time polymerase chain reaction (PCR) to screen for IEI in newborn babies.

We developed an in-house real-time PCR technique to detect and quantify TREC and KREC in human immunodeficiency virus (HIV)-exposed uninfected (HEU) and HIV-unexposed uninfected (HUU) neonates, using dried blood spot (DBS) samples collected and preserved as part of the Siyakhula study cohort. Deoxyribonucleic acid (DNA) was extracted from DBS samples and TREC and KREC genes were simultaneously detected using a newly developed real-time PCR technique.

Results revealed that HUU infants had statistically significant higher TREC concentrations (*P*-value = 0.006) than HEU infants, however, no significant difference in KREC concentrations were noted between the two cohorts. Statistical significance was determined by a *P*-value <0.05. These results indicate reduced TREC levels in immunologically vulnerable babies (HEU).

The TREC and KREC assays showed significant potential as biomarkers for IEI screening. Importantly, the simultaneous detection and quantification of TREC/KREC is a cost-effective method for IEI screening.

Keywords:

Dried blood spot (DBS), Inborn errors of immunity (IEI), kappa-deleting recombination excision circles (KREC), Real-time polymerase chain reaction (real-time PCR), T-cell receptor excision circles (TREC), Primary immunodeficiency (PID), severe combined immunodeficiency (SCID), manual DNA extraction, automated DNA extraction, HIV-exposed uninfected (HEU).

Table of Contents

Declaration: Authorship i

Acknowledgements ii

Executive Summary iv

Acronyms and abbreviations ix

List of Symbols xiv

List of figures xv

List of tables xvi

Chapter 1: Literature Review 1

1.1. Introduction and historical background 1

1.2. Types of inborn errors of immunity 3

1.2.1. Primary immunodeficiency diseases 5

1.2.1.1. Combined immunodeficiency 5

1.2.1.1.1 Severe combined immunodeficiencies 5

1.2.1.1.2. DiGeorge Syndrome 6

1.2.1.2. Predominantly antibody deficiencies 6

1.2.1.2.1. Agammaglobulinemia 7

1.2.1.2.2. Common variable immune deficiency 8

1.2.1.3. Primary phagocytic disorders 8

1.2.1.4. Complement deficiencies 9

1.2.2. Primary immune regulatory disorders 9

1.3. Common treatments used in patients with inborn errors of immunity 10

1.4. Immune dysfunction in human immunodeficiency virus exposed uninfected newborn babies 11

1.5. T-cell receptor excision circles and Kappa-deleting recombination excision circles 12

1.5.1. T-cell receptor excision circles 13

1.5.2. Kappa-deleting recombination excision circle 15

1.6. Screening of inborn errors of immunity 15

1.7. Problem statement 17

1.8. Aims and objectives 18

1.8.1. Aim 18

1.8.2. Objectives 18

Chapter 2: Materials and methods 19

2.1. Study design 19

2.2. Study setting 19

2.3. Study population 19

2.4. Ethics 20

2.5. Study methodology 20

2.5.1. Sample collection and storage 20

2.5.2. Genomic deoxyribonucleic acid extraction methods 20

2.5.2.1. Dried blood spot processing for deoxyribonucleic acid extraction 20

2.5.2.2. Dried blood spot DNA extraction using Maxwell 16 blood DNA purification kit 21

2.5.2.3. Dried blood spot deoxyribonucleic acid extraction using the high pure polymerase chain reaction template preparation kit 21

2.5.3. Measuring the purity and concentration of deoxyribonucleic acid using a NanoDrop® 1000 ultraviolet-visible spectrophotometer 23

2.5.4. Real-time polymerase chain reaction 23

2.5.4.1. Optimization of primers 23

2.5.4.2. Standard curve construction 27

2.5.4.3. Quantification of T-cell receptor excision circles and kappa-deleting recombination excision circles 27

2.6. Statistics 29

Chapter 3: Results 30

3.1. Demographics 30

3.2. Dried blood spot deoxyribonucleic acid concentration and purity 32

3.2.1. Comparison of deoxyribonucleic acid extraction methods from dried blood spots 32

3.2.2. Deoxyribonucleic acid extraction results for newborns and their mothers (Siyakhula cohort) 39

3.3. Quantitative real-time polymerase chain reaction 40

3.3.1. Standard curve 40

3.3.2. Detection of T-cell receptor excision circles and kappa-deleting recombination excision circles in samples from newborn babies. 41

Chapter 4: Discussion and Conclusion 46

4.1. Discussion 46

4.1.1. Deoxyribonucleic acid extraction comparison 46

4.1.2. Quantification of T-cell receptor excision circles and kappa-deleting recombination excision circles 49

4.2. Conclusion 50

4.3. Strengths of the study 51

4.4. Limitations of the study 51

4.5. Recommendations 52

References 53

Appendices 69

Appendix A: Ethics approval letter 69

Appendix B: Reproduction of the formation of TREC diagram from another article permission slip 70

Appendix C: Demographic characteristics of the human immunodeficiency virus-exposed uninfected and human immunodeficiency virus-unexposed uninfected newborn cohorts: this represents the average value of all the neonatal hospital visits for week 0 and 24 months. .. 71

Appendix D: The demographic characteristics of the human immunodeficiency virus-exposed uninfected and human immunodeficiency virus-unexposed uninfected infants' mothers study population, which includes women living with human immunodeficiency and women living

without human immunodeficiency virus, represent the maternity visits week 36 of pregnancy and 24 months after birth. 75

Appendix E: Deoxyribonucleic acid concentration and purity, comparison between the high pure polymerase chain reaction template preparation kit (manual extraction) and the Maxwell 16 blood deoxyribonucleic acid purification system (automated extraction). 78

Appendix F: Deoxyribonucleic acid concentration and purity for the human immunodeficiency exposed uninfected and unexposed uninfected newborns. 79

Appendix G: Deoxyribonucleic acid concentration and purity for the women living with human immunodeficiency virus and those living without human immunodeficiency cohorts. 81

Appendix H: Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination circles detection in newborn samples. . 83

Appendix I: Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination circles detection in samples of women living with human immunodeficiency virus and those living without human immunodeficiency virus. 87

Acronyms and abbreviations

A	Adenine
ADA	Adenosine deaminase
aHUS	Atypical haemolytic uremic syndrome
ANOVA	Analysis of variance
APGAR	Appearance, pulse, grimace, activity, and respiration
AR	Autosomal recessive
ART	Antiretroviral therapy
BCR	B-cell receptor
BLNK	B-cell linker
BTK	Bruton's tyrosine kinase
C	Cytosine
C1-INH	Complement component 1 esterase inhibitor
C1q	Complement component 1q
CD	Clusters of differentiation
CD4+	Clusters of differentiation 4
cDNA	Circular deoxyribonucleic acid
CGD	Chronic granulomatous disease
CH	Confoederatio helvetica
CHARGE	Coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, & ear abnormalities
CI	Confidence interval
CID	Combined immunodeficiency

CT	Threshold cycle
CV	Coefficient of variation
CVID	Common variable immunodeficiency
DBS	Dried blood spots
Del22q11	Chromosome 22q11.2 deletion syndrome
DGS	DiGeorge syndrome
DNA	Deoxyribonucleic acid
DS	Deletion syndrome
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FAM	Fluorescein amidites
FOXP3	Forkhead box P3
G	Guanine
HEU	HIV-exposed uninfected
HEX	Hexachlorofluorescein
HIgM	Hyper IgM syndromes
HIV	Human immunodeficiency virus
HSCT	Haematopoietic stem cell transplant
HUU	HIV-unexposed uninfected
IEI	Inborn errors of immunity
Ig	Immunoglobulin
IGHM	Immunoglobulin heavy constant mu
IGLL1	Immunoglobulin lambda like polypeptide 1
IGRT	Image-guided radiation therapy

IPEX	Immunodysregulation polyendrocrinopathy enteropathy X-linked syndrome
IQR	Interquartile range
IUIS	International Union of Immunological Societies
JAK3	Janus kinase 3
KREC	kappa-deleting excision circles
LAD	Leukocyte adhesion deficiency
LDL	Low detectable levels
MA	Massachusetts
MCP	Membrane cofactor protein
Min	Minute/Minutes
MS	Mass-Spectrometry
N/A	Not available
NBS	Newborn screening
NJ	New Jersey
NK	Natural killer cells
NTC	Non-template control
PAD	Predominantly antibody deficiency
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PID	Primary immunodeficiency disorders
PIDD	Primary immunodeficiency disorders
PIK3CD	Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit delta

PIK3R1	Phosphatidylinositol-4,5-Biphosphate 3-Kinase Catalytic Subunit Delta
PIRD	Primary immune regulatory disorders
PKU	Phenylketonuria
PMPs	Paramagnetic Particles
PMTCT	Prevention of mother to child transmission
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RSA	Republic of South Africa
RSD	Relative standard deviation
RTE	Recent thymic emigrants
RT-PCR	Real-time polymerase chain reaction
S	Seconds
SCID	Severe combined immunodeficiency
T	Thymine
T1DM	Type 1 diabetes mellitus
TAMRA	Tetramethylrhodamine
TCR	T-cell receptor
TCRAC	T-cell receptor alpha constant
TLR	Toll like receptor
TOP2B	Topoisomerase II beta
TREC	T-cell receptor excision circles
Tregs	Regulatory T-cells
TX	Texas
UK	United Kingdom

USA	United States of America
UV	Ultraviolet
VDJ	Variable, Diversity, Joining gene segments
WA	Washington
WAS	Wiskott-Aldrich syndrome
WI	Wisconsin
WLWH	Women living with human immunodeficiency virus
WLWOH	Women living without human immunodeficiency virus
XL	X-linked
XLA	X-linked agammaglobulinemia

List of Symbols

≤	Less than or equal to
>	Greater than
%	Percent
µL	Microliter
µM	Micromolar
C	Concentration
°C	Degree Celsius
cm	Centimetres
H_2O	Water
Kg	Kilograms
mL	Millilitre
mm	Millimetre
n	Sample size
ng/µL	Nanograms per microliters
nm	Nanometres
nM	Nanomolar
x g	Relative centrifugal force
α	Alpha
β	Beta
γ	Gamma
δ	Delta
R^2	Coefficient of determination
V	Volume

List of figures

Page

Figure 1: Flow diagram illustrating two main classifications of inborn errors of immunity and some of their specific subgroups. 4

Figure 2: Formation of TREC-coding joint. 14

Figure 3: Comparison of the 260/280 ratio (purity) for the automated DNA extraction method (Maxwell® 16 blood DNA purification kit) and the manual DNA extraction method (High purity PCR template preparation kit). 34

Figure 4: A Passing Bablok regression analysis to compare the automated and manual DNA extraction methods for extracted DBS DNA concentration and purity with a sample size of 15. 36

Figure 5: Multicomponent plots for TREC and KREC detection. 38

Figure 6: Standard curve for TREC and KREC circles. 40

Figure 7: A box plot illustrating the statistical significance of TREC quantity between HEU and HUU newborns. 43

Figure 8: Comparison of TREC and KREC quantitative real-time PCR results between WLWH and WLWOH. 45

List of tables

Page

Table 1. Primer and probe sequences. 24

Table 2. Polymerase chain reaction for T-cell receptor excision circle and kappa-deleting recombination excision circles assay. 25

Table 3. Polymerase chain reaction for T-cell receptor alpha constant assay (Reference control gene). 26

Table 4. Reagent setup for T-cell receptor excision circles, kappa-deleting recombination excision circles, and T-cell receptor alpha constant assays. 28

Table 5. Summary of the demographic features of the research group, comparing human immunodeficiency-exposed uninfected newborn babies with human immunodeficiency-unexposed uninfected newborn babies. 31

Table 6. Summary of the demographic features of the research group, comparing women living with human immunodeficiency virus with women living without human immunodeficiency virus. 32

Table 7. Deoxyribonucleic acid extraction results of the high-purity polymerase chain reaction template preparation kit (manual extraction) and the Maxwell® 16 blood deoxyribonucleic acid purification system (automated extraction). 33

Table 8. Statistical measures used for the assessment of intra- and inter-assay in DNA concentration. 35

Table 9. Deoxyribonucleic acid extraction results in the newborn babies' study population. 39

Table 10. Results of deoxyribonucleic acid extraction for the study cohort of infant mothers. 39

Table 11. The statistical measures of T-cell receptor excision circles and kappa-deleting recombination excision circles standard curves. 41

Table 12. Comparison of quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination excision circles between HIV-exposed uninfected and HIV-unexposed uninfected. 42

Table 13. Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination excision circles between Women living with HIV and Women living without HIV. 44

Chapter 1: Literature Review

1.1. Introduction and historical background

Inherent defects in the immune system, commonly known as inborn errors of immunity (IEI), are a diverse array of genetic disorders that are caused by harmful genetic mutations that hinder both the innate and adaptive immune responses.[1-4] The origin of IEI, previously known as primary immunodeficiencies (PID), was first documented in 1952, when Ogden Bruton recorded a young boy who experienced 19 episodes of pneumococcal infections, exhibited a deficiency in serum gamma globulins, and recovered following intramuscular infusion of gamma globulins.[5] Two years prior, Glanzmann and Riniker documented a newborn exhibiting severe lymphopenia and lymphoid tissue atrophy, who died to illnesses in infancy.[6] Following that, Hitzig documented more children with early-onset, life-threatening illnesses who were deficient in both gamma globulins and lymphocytes.[7] This illness, originally termed Swiss-type agammaglobulinemia to differentiate it from Bruton's agammaglobulinemia, is currently known as Severe Combined Immune Deficiency (SCID).[8] The combination of isolated agammaglobulinemia and SCID demonstrated the essential functions of humoral and cellular immunity in infection prevention, leading to the subsequent identification of T and B-cells a few years thereafter.[9]

It has been acknowledged that, depending on the unique characteristics of the disease, malignancy, autoimmunity, allergy, and autoinflammation, can frequently, and in certain instances, primarily, appear as clinical symptoms linked to monogenic immune abnormalities.[10] The term "inborn errors of immunity" has been introduced to encompass the extensive array of phenotypes linked to these illnesses.[11] Inborn errors of immunity occur in approximately one in every 500 live births and are genetic defects that are classified based on both the immunological system involved and clinical manifestations observed.[2-3] These disorders are predicted to develop in 1 in 1,000 to 1 in every 5,000 individuals.[12-13] They may develop at any stage of life, with particular diagnoses being more common at specific ages.[14] Inborn errors of immunity are underdiagnosed, and especially among countries with high rates of consanguinity and large populations, the number of recorded cases and reported prevalence is significantly lower than the estimated values.[15] These disorders may go undiagnosed for years, possibly due to milder, less specific symptoms, lack of awareness of IEI among clinicians, and lack of appropriate diagnostic assays.[3]

Delays in diagnosis lead to significant morbidities and deaths, which raises the financial burden for the management of the disorders.[15] These disorders negatively impact the quality of life of children,

limiting emotional, social, physical, and academic functioning.[16] A diagnosis of these disorders necessitates a comprehensive review of the patient's medical history, with a focus on infections and symptoms associated with immune dysfunction (such as autoimmunity and recurrent fevers), and a physical examination that looks for indicators of immune dysfunction and infection sequelae.[14] Based on the probable diagnosis, a laboratory assessment of the immune system can be conducted.[14] This assessment can include components of the complement pathway, immune cell frequency and functioning, and antibody quantity and function.[14]

Early detection and intervention for IEI may prevent individuals from experiencing severe and potentially fatal outcomes associated with infections or autoimmunity.[13] This has resulted in the development of newborn screening (NBS) for IEI.[17] Newborn screening can be done by quantifying T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC).[18] It has been proposed that the combined TREC and KREC assay may be used in the diagnosis of IEI and in the monitoring of immune reconstitution in adult patients.[19] Nonetheless, the TREC and KREC assay screening method is incapable of identifying numerous severe IEI and immune dysregulation conditions.[17] Sequencing may offer a viable approach for screening a broader spectrum of health disorders.[17] This has prompted the consideration of genomics-based newborn screening.[20-22] This methodology is, however, impractical from both logistical and financial standpoints within the framework of NBS.[23]

Genetic screening may be economically impractical; thus, a two-tiered strategy has been proposed to increase the number of IEI that can be detected by NBS.[17] An alternative screening method involving a global study could ascertain the effective application of NBS in reducing rates of mortality.[17]

Routine NBS is currently unavailable in many countries. Financial constraints, technological difficulties, insufficient understanding of the significance of early diagnosis of IEI, and the relegation of IEI among other health concerns contribute to the delays in the execution of NBS initiatives for IEI.[17] The primary reason for the delayed implementation of routine NBS for IEI in many countries is financial constraints, indicating the need for the development of innovative, cost-effective technologies for screening infants for various diseases, which is essential to address this issue.[17,24] As a result, the focus of the current study was to develop an in-house screening technique for TREC and KREC quantification using real-time polymerase chain reaction (PCR) with the goal of screening for IEI in newborn babies. This study was conducted on stored samples obtained from the Siyakhula

cohort of human immunodeficiency virus (HIV)-exposed uninfected (HEU) and HIV-unexposed uninfected (HUU) newborn babies. This larger study investigated differences between HEU and HUU newborn populations, as infants born HEU are recognised to have an elevated risk of infections during their initial months of life. The study included mothers of neonates to further assess the reliability of TREC and KREC quantification.

1.2. Types of inborn errors of immunity

The International Union of Immunological Societies (IUIS) currently classifies IEI based on 499 genetic defects and 485 diseases, and includes immunodeficiency, autoimmunity, and immune dysregulation.[1,4,25] These mutations cause abnormalities in immune cell functioning, including T-cells (cellular immune deficiencies), antibody production by B-cells (humoral immune deficiencies), impaired antigen presentation, defects in natural killer (NK) cell cytotoxicity, Toll-like receptor (TLR) activation, phagocytosis, macrophage activation, and complement activation.[2-3,26-27]

These include phenotypes linked to infection, malignancy, autoimmune disease, and inflammatory disorders.[1] Inborn errors of immunity can be classified into two categories: primary immunodeficiency diseases and primary immune regulatory disorders (Figure 1).[14] These are caused by different genetic mutations, and the most commonly occurring disorders are discussed below.

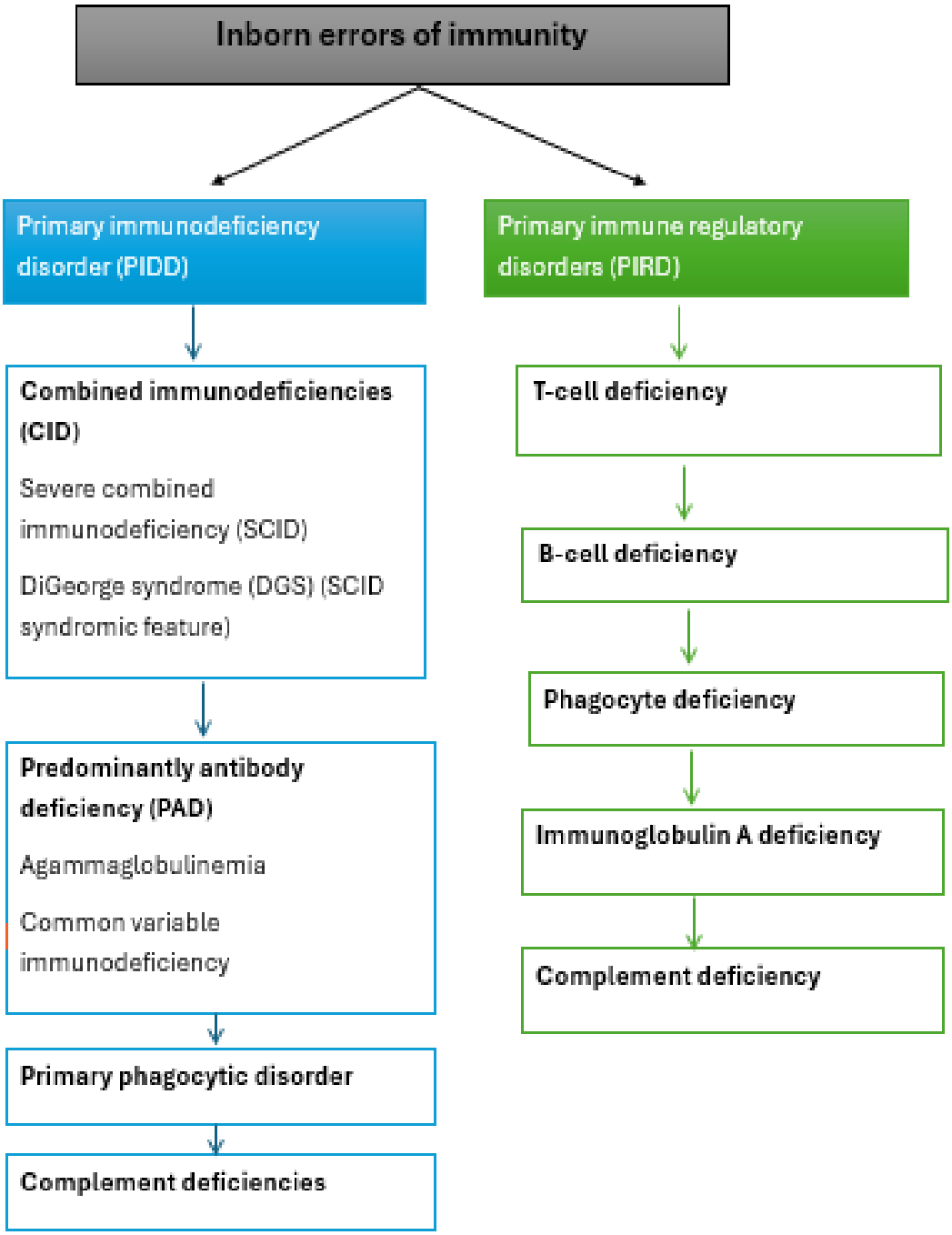

Figure 1: Flow diagram illustrating two main classifications of inborn errors of immunity and some of their specific subgroups.

1.2.1. Primary immunodeficiency diseases

Primary immunodeficiency disorders are a group of disorders characterised by recurrent and/or severe occurrence of infections.[14] These disorders can be classified into various groups, such as combined immunodeficiency (CID), predominantly antibody deficiencies (PADs), primary phagocytic disorders, and complement deficits, among others.[25]

1.2.1.1. Combined immunodeficiency

Combined immunodeficiencies refers to disorders that involve abnormalities in the quantity or functionality of lymphocytes, particularly T-cells, but can also impact B or NK cells.[14] These disorders are usually the most severe form of primary immunodeficiency disorders (PIDD) and can manifest as viral, bacterial, and fungal infections.[14] Various genetic conditions can result in this disorder, and SCID stands out from the other disorders due to its association with complete genetic variations that have full penetrance and fatal functional consequences.[28] This leads to a heightened vulnerability to potentially lethal infections.[28] Hypomorphic genetic variations result in a group of immunodeficiencies that are accompanied by less severe symptoms, known as "leaky SCID".[25] A study that was done on a cohort of patients in the Middle East in 2013 exhibited syndromic features linked to combination immunodeficiencies, resulting from compromised gene activity in non-immune cells. Examples of such syndromes include Bloom syndrome, DiGeorge syndrome (DGS) and, coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, & ear abnormalities (CHARGE).[29]

1.2.1.1.1 Severe combined immunodeficiencies

Severe combined immunodeficiency is a monogenic autosomal recessive (AR-SCID) or X-linked recessive (XLSCID) disorder.[30-34] Neonates with SCID are susceptible to severe and recurring infections that are often fatal in the first two years of life if not properly diagnosed and treated.[30-34] Severe combined immunodeficiency is characterised by severe immune system dysfunctions that result in the absence or dysfunction of T- and B-cells derived from the thymus gland and bone marrow, respectively.[26,32,35-36] This, in turn, impacts negatively on both cellular and humoral adaptive immunity.[26,32,35-36]

Several gene alterations have been linked to unusual clinical and immunological phenotypes of SCID.[36] All known gene mutations impair the development of normal naïve T-cells, which results in low numbers of T-cells and inactive B-cells.[35] It is estimated that up to 50% of infants living with SCID die due to not being diagnosed early enough for life-saving therapies to be implemented.[35]

Infants with SCID may appear to be healthy at birth with symptoms occurring between the second and third months of life.[30,32,35] Infectious complications are the most common feature of the disease, with opportunistic infections predominating.[30,32,35] Severe immunodeficiencies and other T-cell disorders may be attributed with syndromal features or congenital heart defects, including cartilage-hair hypoplasia, ataxia telangiectasia, or DiGeorge syndrome (22q11.2 deletion syndrome), the latter being a relatively common IEI characterised by thymus hypoplasia or aplasia which requires a thymus transplant rather than haematopoietic stem cell transplantation (HSCT).[34]

1.2.1.1.2. DiGeorge Syndrome

DiGeorge syndrome is a complex condition caused by embryopathy as a result of among other mutations, a deletion on the 22nd chromosome (del22q11).[37] Around 75% of individuals with DGS have immune system derangements, as well as hypoparathyroidism which is associated with T-cell lymphopenia.[38] Immune dysregulation causes autoimmune disease in 10% of patients, with juvenile rheumatoid arthritis, vitiligo, and Grave's disease being the most common.[39-40] This syndrome occurs in approximately one in 4,000 to 6,000 live births.[26,39] DiGeorge syndrome can be inherited in an autosomal dominant manner, but *de novo* microdeletions account for 90% of cases.[26,39]

Patients with DGS may have craniofacial, parathyroid, thymic, and cardiac outflow tract developmental abnormalities.[39] These individuals have a high incidence of both provoked and unprovoked seizures.[40]

1.2.1.2. Predominantly antibody deficiencies

Predominantly antibody deficiencies, are medical conditions characterised by aberrant B-cell or antibody count or functionality, leading to repeated sinopulmonary infections.[14] These are the most prevalent kinds of IEI globally, affecting approximately one in 600 individuals .[41-43] These individuals have deficient quantities of one or more immunoglobulin isotypes and insufficient synthesis of

antibodies specific to pathogens, resulting in the development of both infectious and non-infectious symptoms.[44-49]

Although infections are frequently observed in cases of PAD, it can be challenging to identify the specific pathogen.[50] This difficulty arises from the increased likelihood of obtaining false-negative results in serologic testing, which is caused by abnormalities in the generation of antibodies that target the pathogen.[50] Furthermore, patients undergoing image-guided radiation therapy (IGRT) may exhibit false-positive serology.[50] Hence, it is imperative to use blood or tissue cultures, immunofluorescence labelling, PCR testing, or amplicon sequencing for precise identification of the pathogen and to ensure a reliable diagnosis.[50] Primary antibody deficiencies are categorised into several clinical phenotypes. The primary clinical manifestations encompass agammaglobulinemia, common variable immune deficiencies (CVID), deficiencies in immunoglobulin (Ig) isotypes, specific antibody deficiency, and hyper-IgM phenotype (HIgM).[49]

1.2.1.2.1. Agammaglobulinemia

Agammaglobulinemia, a more severe form of PAD, primarily presents itself during early life.[49] Agammaglobulinemia is a condition that is characterised by a complete lack of the major serum immunoglobulin classes because of a significant decrease in B-lymphocytes in the peripheral blood.[49,51] B-cell lymphopenias that are severe in infancy may be caused by congenital immune defects like X-linked agammaglobulinemia (XLA).[34]

X-linked agammaglobulinemia is the most common consequence of this defect, accounting for 85% of all cases of B-cell lymphopenia.[52] In 2019, the estimated occurrence varied between 1 in every 200,000 live births and one in every 100,000 live births.[53] X-linked agammaglobulinemia is a result of deleterious mutations in the gene encoding Bruton's tyrosine kinase (*BTK*).[49] Carriers of this disorder are particularly prone to viral and bacterial infections.[52] Agammaglobulinemia symptoms appear between the ages of three and six months, as maternal immunoglobulin levels drop.[52] The autosomal recessive type of agammaglobulinemia results from abnormalities in B-cell formation or survival caused by mutations in the B-cell linker (*BLNK*), phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*), immunoglobulin lambda like polypeptide 1 (*IGLL1*), cluster of differentiation (CD)/B-cell antigen receptor complex-associated protein alpha chain and MB-1 membrane glycoprotein (CD79A), CD79B, Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit delta (*PIK3CD*),

topoisomerase II beta (*TOP2B*), or immunoglobulin heavy constant mu (*IGHM*) genes.[53-54] In the absence of a family history, the diagnosis of agammaglobulinemia is usually delayed.[34] Early detection of agammaglobulinemia carriers is critical because these children are prone to chronic and debilitating respiratory infections.[34]

1.2.1.2.2. Common variable immune deficiency

Common variable immune deficiency is the most common type of PAD, characterised by low levels of immunoglobulins (Ig) and hypogammaglobulinemia.[49] Most individuals with CVID exhibit a normal count of B-cells in circulation, indicating that functional abnormalities impact the latter phases of B-cell maturation.[49] These mutations can impact the signalling and activation of B-cells, the machinery responsible for isotype switching, and the processes of somatic hypermutation and cytokine signalling.[49] Similarly, the malfunctioning activation of B-cells by T-cells, caused by faulty signalling through the CD40-CD40 ligand (CD40L) interaction, is a significant factor in PAD.[55] Furthermore, alongside the recurring infections in CVID, there may also be additional clinical symptoms such as gastrointestinal disorders, allergies, lymphadenopathy, and autoimmune illnesses.[56-57] Autoimmunity is a frequent occurrence and manifests in 20-30% of people with CVID.[56-57] The clinical and genetic characteristics of CVID vary widely. While over thirty genes have been associated with CVID, the genetic cause remains uncertain in most cases, 2-10% of individuals have identifiable monogenic diseases[49]

1.2.1.3. Primary phagocytic disorders

Primary phagocytic diseases are defined by atypical quantities or functionality of phagocytes (such as neutrophils, dendritic cells, or macrophages), resulting in recurring skin infections and abscesses in internal organs.[14]

1.2.1.4. Complement deficiencies

Complement deficiencies occur when one or more components of the complement system are either missing or not functioning properly.[14] These conditions can manifest as an elevated susceptibility to pyogenic or Neisserial infections, a lupus-like disease, or atypical haemolytic uremic syndrome (aHUS).[25] Component deficits can arise either from genetic factors (primary) or from external causes (secondary).[58] The inheritance pattern for most cases is autosomal recessive, with the exception of properdin deficiency, X-linked, and factor B, complement component 1 esterase inhibitor (C1-INH), and membrane cofactor protein (MCP)/CD46 deficiency which are autosomal dominant.[58] Heterozygous carriers typically do not show any clinical symptoms.[58] Accurate identification of hereditary disorders can be achieved through a thorough examination of medical history and careful research of family lineage.[58] Secondary deficiencies arise due to inflammation-induced consumption of complement, auto-antibodies targeting complement component 1 (C1q) or C1 inhibitor, reduced synthesis, or elevated catabolism.[59] Complement deficiencies make up approximately one to six percent of all primary immunodeficiencies, although in specific national registries, this percentage may increase to 10%.[60-61]

1.2.2. Primary immune regulatory disorders

Primary immune regulatory disorders are a category of IEI characterised by an excessive inflammation, frequent autoimmunity affecting multiple tissues, abnormal growth of lymphocytes, severe allergic reactions, and an increased risk of developing cancer.[14] Primary immune regulatory disorders occur due to mutations in genes that are involved in regulating the inflammatory or immune response.[14] These disorders have emerged as a novel classification of diagnoses characterised by immune dysfunction as the major feature and immunodeficiency as the secondary feature.[14] These illnesses arise due to a disruption in the immunological tolerance pathways, which can impact diverse aspects of the immune system and contribute to the development of various disease processes and the regulation of autoimmunity.[62]

These diseases are linked to mutations in different genes, with the main disorder being immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused through mutations in the Forkhead Box P3 (*FOXP3*) gene, which result in a deficiency in the production of CD4+CD25+ regulatory T-cells (Tregs), leading to a Treg defect.[25,63-65] Primary immune

regulatory disorders constitute a diagnostic and therapeutic dilemma for physicians because they can occur in multiple organs and might be seen by different medical specialties.[66] Autoimmunity can be either antibody- or cell-mediated, and it can specifically target different types of cells in the body.[66] This can result in various conditions such as cytopenia (low blood cell counts) by attacking haematopoietic cells, type 1 diabetes mellitus (T1DM) or thyroiditis by affecting endocrine organs, dermatitis, vitiligo, alopecia or arthritis by impacting the skin and connective tissue, hepatitis or glomerulonephritis by damaging visceral organs, and uveitis or inflammatory bowel disease by affecting the epithelial barrier.[66] Each of these occurrences can be common: immune thrombocytopenia purpura affects between 1.9 and 6.4 out of every 100,000 children yearly, whereas T1DM has an annual incidence rate of 22.9 per 100,000.[67-68] Early detection of possible primary immune regulatory disorders (PIRD) is challenging unless there is a development of multi-organ autoimmunity or the presence of additional factors such as a positive family history or severe and recurring infections.[66] Food or drug allergies, rhinitis, and asthma, can also occur.[66] Dysregulated lymphoid cell regulation might potentially combine with faulty viral management, specifically with the Epstein-Barr virus (EBV), leading to an increased susceptibility to lymphoproliferation and the formation of lymphoma.[66]

1.3. Common treatments used in patients with inborn errors of immunity

Early diagnosis is crucial for selecting appropriate therapy, such as the potential use of antibiotic prophylaxis for various types of IEI.[12,69] The selection of an antibiotic should be determined by the diagnosis, the patient's infection history, and established guidelines.[14] In cases where guidelines are not available, expert expertise and consensus should be relied upon.[14]

Immunoglobulin replacement is a fundamental aspect of treatment for PADs.[14] Primary immunoregulatory disorders are commonly controlled through the use of immunomodulatory agents.[14] Throughout historical events, immunomodulatory drugs have exerted significant impacts on the immune system.[14] Haematopoietic stem cell transplantation is a highly effective treatment for a wide range of CIDs affecting the ability of immune cells to engulf and destroy pathogens (phagocytic abnormalities), and PIRD.[14] Typically, patients who receive HSCT at a younger age tend to have more favourable outcomes.[14] This is especially crucial for patients diagnosed with SCID who are dependent on bone marrow transplantation or gene therapy in order to live past the age of one year.[14] The science and technology enabling safe and efficient gene therapy for primary

immunodeficiency diseases have significantly progressed during the past three decades.[14] Gene therapy is currently accessible for certain subtypes of SCID: Wiskott-Aldrich syndrome (WAS), leukocyte adhesion deficiency (LAD), and chronic granulomatous disease (CGD), either through clinical or research means.[12,70] Investigations are also being conducted on editing the endogenous gene for some subtypes of SCID, hyper IgM syndromes (HIGM), CGD, IPEX, and other related conditions.[70]

1.4. Immune dysfunction in human immunodeficiency virus exposed uninfected newborn babies

Despite not achieving the global goal of eliminating paediatric HIV by 2015, significant advancements have been made in reducing the number of newborn babies infected with HIV through the broadening of prevention of mother-to-child transmission (PMTCT) programmes.[71] However, the transfer of HIV from mother to child still occurs, especially in sub-Saharan Africa, where 90% of new infections take place.[71] As the coverage of PMTCT programmes increases, the number of children infected with HIV is decreasing[71-72] resulting in the number of infants who are exposed to HIV but remain uninfected increasing to over 1 million between the years 2009 and 2014.[71-72] Exposure to HIV and or antiretroviral therapy (ART), during gestation could significantly impact birth outcomes, growth, and development.[71] In developing nations, HEU newborn babies may have a higher likelihood of being born with small for their gestational age or having a lower birthweight when compared to HUU newborn babies.[71] Human immunodeficiency virus-exposed uninfected newborn babies with low birthweight have a significantly increased risk of mortality, ranging from 2.5 to 12 times higher than their HUU counterparts.[73-75] Among HEU newborn babies, the risk of neonatal mortality is at least six times higher for those born prematurely, with low birthweight, or who were too tiny for gestational age.[76] In addition, these newborn babies are reported to have at least twice the risk of mortality before one year of age.[76]

Babies born to mothers living with HIV are at a greater risk of immune dysfunction due to exposure to HIV as well as to ART, irrespective of whether the newborn babies are HIV-infected or not.[77] The HEU newborn babies are commonly more susceptible to frequent respiratory tract infections, possibly as a result of lower concentrations of maternal antibodies being transferred from mothers living with HIV to their babies.[77-78] To prevent HIV transmission through breast milk, babies are often formula-fed. This, however, limits the transfer of immunoglobulin A (IgA), present in breast milk, to the

newborn which increases their susceptibility to infection.[77] There are notable differences in T-cell populations of HEU newborns compared to HUU infants including elevated frequencies of activated T-cells in early life, expanded memory T-cell populations, and increased numbers of immature T-cells suggesting disrupted T-cell development.[79]The present study compared the levels of TREC and KREC in HEU newborn babies with those of HUU newborn babies.

Inborn errors of immunity have historically been diagnosed using complete blood counts and serological tests including immunoglobulin levels, vaccine titres, and complement levels. However, these tests are often insufficient for an accurate diagnosis due to the heterogeneity of the symptoms and cell types involved. Recent advancements in the understanding of the immune system have led to the development of novel immunologic assays such as the TREC/KREC assay and flow cytometry to aid in the diagnosis of IEI.[80]

1.5. T-cell receptor excision circles and Kappa-deleting recombination excision circles

T-cell receptor excision circles and kappa-deleting recombination excision circles are markers of appropriate lymphocyte synthesis, and various IEI are associated with low levels of these markers.[2] Specific mutations influence the TREC levels differently.[2] Most individuals with IEI have low levels of TREC and KREC, however, some IEI like *Janus kinase 3* (*JAK3*)-deficiency disorder have normal levels of KREC.[2] The levels of TREC in individuals living with HIV correlate with variables such as CD4+ T-cell count, age, and plasma HIV ribonucleic acid (RNA) levels of the individual.[81]

1.5.1. T-cell receptor excision circles

T-cell receptor excision circles are small extra chromosomal fragments of DNA that are produced in naïve T-cells in the thymus during the variable, diverse, joining gene segments (V[D]J)-T-cell receptor gene rearrangement process that is responsible for the diversity of the alpha beta (αβ) and gamma delta (γδ) T-cell receptor (TCR) repertoires (Figure 2).[82-83] The TCR repertoire is diverse with gene recombination events resulting in a large number of TREC. This rearrangement occurs approximately 69% of the time in all δ recombination events, making detection possible. As a result, TREC can be used to identify T-cells that have recently left the thymus.[82,84]

Thymic output monitoring is significant in developing suitable diagnostic and treatment strategies for individuals with IEI, as well as for monitoring the reconstitution of the immune system after treatment with chemotherapy, post-haematopoietic stem cell or thymus transplantation.[32,84-85] Levels of TREC are initially very low in patients living with HIV compared to uninfected age-matched controls, however, normal levels of TREC are restored in these individuals following initiation of ART.[32,84-85] Levels of TREC are crucially reduced in individuals with CD4+ T-cell counts less than 200 cells/microlitre (μL).[81]

Levels of TREC are usually higher during childhood and decrease with age.[34] T-cell receptor excision circles are not detectable in patients with congenital thymus absence.[34,85] Infant screening for TREC levels is widely used globally, offering a good combination of sensitivity and specificity while remaining cost-effective.[3]

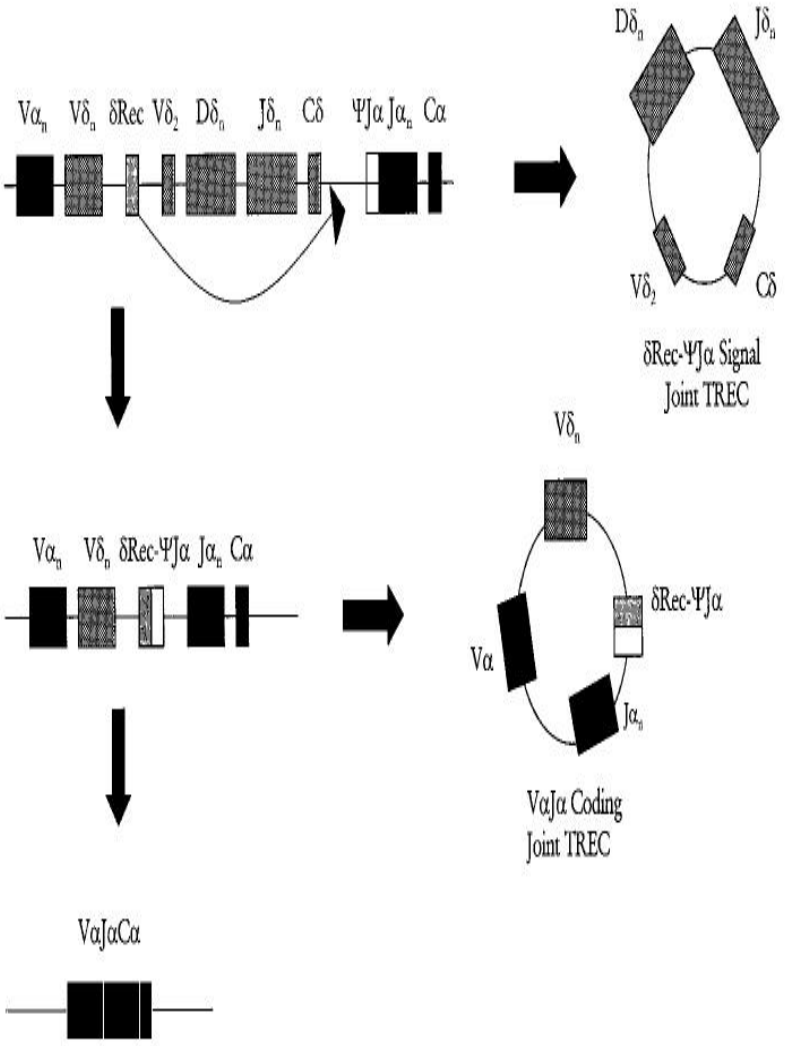

Figure 2: Formation of TREC-coding joint. The *TCRα* gene locus contains the d gene locus, which is excised before *TCRα* gene recombination. Recombination of *δRec* and *cJα* produces a chromosomal product and an extrachromosomal signal TREC (*δRec–cJα* signal joint TREC). Subsequent recombination activity results in the formation of the recombined chromosomal *VαJαCα* gene segment and a second TREC known as the coding joint TREC (*VαJα* coding joint TREC)[86] (Reproduced with permission, provided in Appendix B).

1.5.2. Kappa-deleting recombination excision circle

Kappa-deleting recombination excision circles are episomal deoxyribonucleic acid (DNA) fragments generated as by-products of B-cell receptor gene rearrangement during B-lymphocyte maturation.[3,27] These episomal DNA fragments do not replicate during mitosis and thus exhibit a dilution pattern that allows the quantitative estimation of cell replication.[3,27] Thus, regardless of the molecular aetiology of the IEI, patients with severe IEI who have B-cell lymphopenia have very low levels of KREC.[3,27]

The measurement of KREC improves diagnostic accuracy for specific SCID phenotypes such as adenosine deaminase (ADA)-SCID and allows for the identification of other IEI such as inherited agammaglobulinemia.[51] The detection of KREC provides a sensitive marker of newly formed B-cells, and improves the likelihood of identifying other forms of CID/SCID related to decreased B-lymphocyte numbers in newborn screening (NBS) and late-onset ADA-SCID.[36] The present study, in part, focuses on establishing an in-house method to detect different levels of KREC in HEU and HUU newborn babies.

1.6. Screening of inborn errors of immunity

Since the 1930s, when phenylketonuria (PKU) was first described, and the subsequent development of a laboratory test in the 1960s for detecting newborn babies with PKU, there have been notable technological advancements.[87-90] These advancements have allowed for the identification of a greater number and variety of treatable conditions through NBS programmes.[87-88] Historically, NBS tests have mostly focused on utilizing a tandem mass-spectrometric (MS/MS) method, which has proven to be successful in detecting several diseases such as congenital hypothyroidism, inborn errors of metabolism, and congenital adrenal hyperplasia.[88] The expansion of NBS programmes has been facilitated by technological advancements, allowing for the inclusion of a broader array of diseases.[88,91] For instance, IEI such as SCID can now be detected using DNA-based technological advances, specifically through the detection of TREC and KREC using quantitative PCR.[88,91]

The key goal of NBS is the prompt diagnosis of treatable and catastrophic types of IEI characterised by significantly decreased T- and B-cell counts, with the goal of reducing morbidity and mortality.[15,18,92] The launch of universal NBS for SCID in the United States of America (USA) has indicated an incidence of SCID at roughly 1:65,000 in 2019, which is considerably greater than

previously estimated.[93] The NBS programme promotes the early detection of infants with IEI, facilitating treatments to prevent infections and prompt referral for HSCT, when/if required.[11] The significance of this is emphasised by evidence indicating that younger age and infection status at the time of transplantation are critical determinants of enhanced outcomes following HSCT for SCID.[18,94]

The NBS programme assists physicians in diagnosing IEI early and administering prompt therapeutic interventions, hence preserving patient lives.[17] Consequently, there is an imperative necessity to enhance NBS for IEI by rising from the challenges associated with it being implemented.[17] This can solely be accomplished through the worldwide exchange of experiences, technologies, and resources.[17]

This may attract the attention of health authorities to the beneficial effects of NBS.[17] The growing awareness of IEI disorder management is apparent by an increase of referral centres globally; yet, the existence of national registries remains a concern.[17] Findings of a study done by Chong-Neto *et al*. in Brazil (2024) indicate that national registries for IEI exist in 110 (53.4%) nations, predominantly located in North America and Europe.[17] Despite the expected rise in the prevalence of IEI in the Middle East and North Africa due to a high rate of proximity with an increased incidence of autosomal recessive diseases there are very few registries in this region.[95] The disease burden is underestimated in middle- and low-income nations for various reasons, including the absence of registries.[96]

The NBS programmes in western countries have facilitated the early detection of SCID, proving to be cost-effective and advantageous for the prognosis of the patient.[97] The launching of NBS has resulted in a decline of the birth prevalence of SCID from 1:100,000 to 1:58,000 from 2014 to 2019 in the USA, and a threefold decrease in the average cost of early bone marrow transplantation compared to late transplantation.[18,98-101]

Severe combined immune deficiency was the first IEI identified by population screening and is the first NBS test based on molecular testing using dried blood spots (DBS).[99,102-103] This screening has made it possible to assess the impact of early therapies on the overall outcomes of afflicted newborns as well as the population prevalence of this illness.[99] The research in creating and implementing molecular testing for additional clinically relevant IEI has grown due to the success of SCID NBS.[99] The importance of early diagnosis and rapid therapy initiation to lower the incidence of morbidity and mortality is supported by developments in clinical care and novel medicines for

several IEI.[99] The presence of TREC and KREC biomarkers are not routinely tested for in the state health sector in South Africa but have been used as part of the standard clinical assessment of patients living with IEI in the private sector. Levels of TREC have been successfully utilised for NBS for SCID and are reported to be very low or undetectable in all individuals with SCID.[2]

This is required for the quick and efficient initiation of therapy as well as to prevent adverse effects and death.[26] This screening test is capable of diagnosing T-cell lymphopenia.[104] Promptly referring patients with a positive screen to an immunologist is crucial for conducting diagnostic and immunologic evaluations.[105] Newborn babies with IEI are vulnerable to life-threatening infections of various origins, including those caused by live vaccines.[26] T- and B-cell lymphopenia testing with NBS procedures will enable early diagnosis of IEI, significantly improving the lives of affected children.[26]

Screening TREC and KREC is essential in detecting IEI and would identify at-risk infants before serious and life-threatening infections occur, further reducing morbidity and mortality associated with IEI.[106] The vast majority of newborn babies with severe T- and B-cell lymphopenias, including those who have severe IEI, have no IEI family history and are asymptomatic during their first few weeks of life.[27] The primary objective of NBS is to identify pre-symptomatic newborn babies with possible serious or fatal disorders that can otherwise be treated successfully.[35]

1.7. Problem statement

A delay in diagnosing IEI is associated with less favourable results associated with therapy and management of the disorder.[107] The objective of NBS with TREC and KREC, which is presently being used in several countries, is to improve the prognosis of IEI by preventing delays in diagnosis.[15] The early detection of IEI is still difficult because older children and adults might have less severe and less focused symptoms, a lack of knowledge of IEI among medical professionals, and the unavailability of critical diagnostic tools like flow cytometry in hospital laboratories.[3] Newborn babies with these disorders seem normal at birth due to symptoms often not manifesting at birth. Most newborns with IEI perish from their condition before they are diagnosed, and those who survive have permanent health issues. Thus, it is essential to both permit patient diagnosis at institutions with limited resources and provide more affordable alternatives like real-time PCR as ultimately it is more cost-effective, available in most laboratories, and does not need specialised training such as flow cytometry.[3] There were 94,024 cases of IEI identified worldwide in 2018, a 21.8% rise from

2013.[15] Different regions have reported different rates of IEI prevalence; the USA has the highest rate, followed by Europe, Latin America, the Middle East, Asia, and Africa.[15] This may reflect increased screening for IEI in more developed countries. Thus, early identification is essential for IEI patients to increase the likelihood of proper care, which will greatly lower the risk of complications and enhance quality of life.[3] Screening for IEI may result in a considerable decrease in morbidity and mortality rates through early detection and care. In addition to implementing universal IEI routine NBS, this would call for the creation of guidelines and agreements for the reporting and interpretation of IEI NBS data as well as the adoption of consistent follow-up procedures.

1.8. Aims and objectives

1.8.1. Aim

The aim of this study was to develop an in-house real-time PCR method to detect and quantify TREC and KREC in newborn babies recruited as part of the larger Siyakhula cohort (HEU and HUU newborn babies).

1.8.2. Objectives

1.8.2.1. To establish an in-house method for simultaneous quantification of TREC and KREC using published primers and probes. This was achieved using real-time quantitative PCR with the T-cell receptor alpha constant (*TCRAC)* gene as the reference control gene.

1.8.2.2. To validate the extraction of DBS DNA using the Maxwell®16 DNA purification kit. DNA extracted from DBSs using the Maxwell®16 DNA purification kit was compared to DBS DNA extracted using a validated, high-purity PCR template preparation protocol. These techniques were compared by measuring the DNA concentration and sample purity using a NanoDrop spectrophotometer.

1.8.2.3. To measure TREC and KREC levels using a newly validated in-house real-time PCR method in HEU and HUU newborn babies to detect possible differences between the two groups.

Chapter 2: Materials and methods

2.1. Study design

This retrospective, laboratory-based longitudinal study aimed to develop an in-house real-time polymerase chain reaction (PCR) method to detect and quantify T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) in newborn babies. The in-house method of quantifying TREC and KREC was used for the analysis of the Siyakhula newborn babies' cohort (human immunodeficiency virus (HIV)-exposed uninfected (HEU) and HIV-unexposed uninfected newborn babies).

2.2. Study setting

Samples from the Siyakhula cohort (ethics reference number: 294/2017) were collected from antenatal clinics situated in Southwest Tshwane, South Africa (RSA). Samples were transported to the Department of Immunology at the University of Pretoria, Pretoria, RSA for processing and storage.

2.3. Study population

This study was conducted using samples collected from the larger Siyakhula study cohort. Pregnant mothers living with HIV and those living without HIV were recruited during prenatal check-ups as part of the Siyakhula study. The study also included paired newborn babies, and the newborn babies' samples were collected during the check-up visits to the clinic. A total of 62 newborn babies dried blood spot (DBS) samples were used in the current study: 32 HEU and 30 HUU newborn babies. In order to further evaluate the reliability of the recently developed in-house real-time PCR method, the study included mothers of newborns. The cohort of this study was expanded to include 21 women living with HIV (WLWH) and 24 women living without HIV (WLWOH). A total of 107 newborns and their mothers made up the study's cohort. Given that DBS samples were unavailable for some newborn mothers, the total sample size of the mothers does not match the sample size of the infants. The Siyakhula study included only women with singleton pregnancies. The mothers of the newborn babies provided written informed consent on behalf of themselves and their infants for their blood samples to be used for immunological profiling. The exclusion criteria for the Siyakhula study included inability to obtain informed consent, maternal hypertension, diabetes, tuberculosis,

multiple pregnancies, and foetuses with chromosomal or structural abnormalities. The samples used in this study were obtained with permission from the Siyakhula umbrella study.

2.4. Ethics

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria, Pretoria, RSA (ethics reference number: 573/2023). The study adhered to the ethical principles of the Declaration of Helsinki, 2013 version. The ethics approval letter is provided in Appendix A.

2.5. Study methodology

2.5.1. Sample collection and storage

This study used previously stored DBS samples from the Siyakhula study cohort. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes from HEU newborn babies (babies born to mothers living with HIV), HUU newborn babies (babies born to mothers living without HIV) and their mothers. The blood samples were used to make DBS on Whatman 903™ filter cards (Bio-Sciences Corporation, Waltham, MA, USA). A volume of 50 microliters (µL) was added to each DBS spot, and the filter cards were dried overnight at room temperature in a class II biosafety cabinet (Esco Lifesciences Group, Singapore). Each filter card was stored with desiccant silica gel (Merck, Modderfontein, RSA) in a low-gas-permeable zipper bag (Trans Africa Medicals, Edenvale, RSA) at room temperature.

2.5.2. Genomic deoxyribonucleic acid extraction methods

2.5.2.1. Dried blood spot processing for deoxyribonucleic acid extraction

Filter papers containing DBS were prepared for deoxyribonucleic acid (DNA) extraction by first cutting each DBS spot (6 millimetres [mm] circle) into small pieces using a pair of scissors that were cleaned between each sample. Small fragments of each DBS spot were placed in a sterilised 1.5 millilitres (mL) microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA, USA). This was followed by the addition of 200 µL of tissue lysis buffer along with 40 µL of reconstituted Proteinase K (Refer to section 2.4.2.3 for the reconstitution procedure of Proteinase K) and incubated at +55 degree Celsius (°C) for 1 hour (DBS older than a month were incubated for two hours) in an AccuBlock™ digital dry bath (Labnet International Inc., Edison, NJ, USA). Tissue lysis buffer and Proteinase K were included

in the high-purity PCR template preparation kit (Roche Life Sciences, Basel, CH). The microcentrifuge tubes were then vortexed (Infitek Ltd., Spokane, WA, USA) halfway through the incubation period to thoroughly mix the filter paper and the tissue lysis buffer Proteinase K mixture for the blood to be soaked out of the filter paper. Genomic DNA was extracted from the processed DBS samples immediately after incubation.

2.5.2.2. Dried blood spot DNA extraction using Maxwell 16 blood DNA purification kit

The Maxwell®16 cartridge (Promega Corporation, Madison, WI, USA) has seven wells: the first well contains lysis buffer, the second well contains MagneSil™ paramagnetic particles (PMPs), the third well contains a lysis buffer, and the fourth to seventh wells are filled with wash buffer. The last well was located on the ridge of the cartridge. To begin the process of DNA extraction, the cartridge was placed on a cartridge holder with the ridge side facing the numbered side of the cartridge holder. The seal covering the cartridge was removed and a plunger was placed in the last well. Processed DBS samples were added to the first well of the cartridge, and 100 µL elution buffer was added to the elution tube. The elution tubes and cartridges were placed in a Maxwell®16 automated instrument (Promega Corporation, Madison, WI, USA). The Maxwell®16 automated instrument was switched on and the protocol was followed as recommended by the manufacturer. As soon as the Maxwell®16 instrument had completed the DNA extraction, the elution tubes were removed from the instrument to avoid evaporation of the eluted DNA and placed on a magnetic device for 2-3 minutes (min) before transferring the purified DNA into a sterilised 1.5 mL microcentrifuge tube for storage. The cartridge and elution tubes were discarded. The purified DNA was stored at -20 °C (CamLab Ltd., Cambridge, UK) until use.

The DNA extracted with the high-purity PCR template preparation kit and Maxwell® 16 blood DNA purification kit was compared to determine the purity and concentration of the DNA for each method.

2.5.2.3. Dried blood spot deoxyribonucleic acid extraction using the high pure polymerase chain reaction template preparation kit

A high-purity PCR template preparation kit (Roche Life Sciences, Basel, CH) was used to extract DNA from the DBS samples. The kit contained a tissue lysis buffer, binding buffer, Proteinase K, inhibitor removal buffer, wash buffer, elution buffer, high-purity filter tubes, and collection tubes. Proteinase

K solution was stored at -20 °C, and the inhibitor removal buffer and wash buffer were stored at room temperature (22 °C). Proteinase K, inhibitor removal buffer, and wash buffer were prepared prior to use. Proteinase K solution was prepared by dissolving Proteinase K in 4.5 mL of double distilled water and the solution was aliquoted into microcentrifuge tubes. The inhibitor removal buffer was prepared by adding 20 mL of absolute ethanol (Fisher Scientific, Leicestershire, UK). The wash buffer was prepared by adding 80 mL of absolute ethanol to the wash buffer concentrate. The elution buffer was pre-warmed to 70 °C using an AccuBlock™ digital dry bath (Labnet International Inc., Edison, NJ, USA) (the elution buffer was kept in the heating block until required). To begin the process of manual DNA purification, 200 µL binding buffer was added to the processed DBS samples. Microcentrifuge tubes containing the processed DBS and 200 µL of binding buffer were mixed immediately and incubated at 70 °C in an AccuBlock™ digital dry bath (Labnet International Inc., Edison, NJ, USA) for 10 min. After the incubation step, the samples were removed and 100µL of isopropanol (ReAgent Chemical Services Ltd, Runcorn, UK) were added. The tubes were mixed by vortexing (Infitek Ltd., Spokane, WA, USA). The filter tube (spin column) was assembled into a collection tube and the sample was added to the upper reservoir of the filter tube. The assembled filter tubes (containing the sample) and collection tubes were centrifuged at 8000 relative centrifugal force (x g) (Merck Chemicals (Pty) Ltd, Germiston, RSA) for 1 min. The flow-through and collection tubes were discarded, a new collection tube was assembled with the filter tube, and 500 µL of inhibitor removal buffer were added to the upper reservoir of the filter tube. The assembled collection and filter tubes were centrifuged at 8000 × g for 1 min. The flow-through and collection tubes were discarded. A new collection tube was assembled with a filter tube and 500 µL of wash buffer was added to the upper reservoir of the filter tube. The assembled tubes were centrifuged for 1 min at 8000 x g. The collection tube and flow-through were discarded, and the wash step was repeated as described above. After the second wash step, only the flow-through liquid was discarded, and the collection tube was retained and combined with the filter tube again. The assembled collection tube and filter tube were centrifuged at 20 000 x g for 10 seconds (s) to remove the residual wash buffer. The collection tube was discarded, and the filter tube was assembled into a sterile 1.5 mL microcentrifuge tube. Pre-warmed elution buffer (100 µL) was added to the filter tubes. The assembled 1.5 mL microcentrifuge tube and filter tubes were centrifuged at 8000 × g for 1 min. The filter tube was then discarded. The 1.5 mL microcentrifuge tube containing the purified DNA, was stored at -20 °C (CamLab Ltd., Cambridge, UK) until use.

2.5.3. Measuring the purity and concentration of deoxyribonucleic acid using a NanoDrop® 1000 ultraviolet-visible spectrophotometer

The concentration and purity of DBS DNA extracted using the Maxwell®16 blood DNA purification kit and the high-purity PCR template preparation kit were compared. This comparison was performed to assess the accuracy and effectiveness of DBS DNA extraction using a Maxwell®16 blood DNA purification kit. The purity (A260/280) and concentration of the extracted DNA (2 µL) were measured in triplicate using a NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.5.4. Real-time polymerase chain reaction

2.5.4.1. Optimization of primers

Primers are short single-stranded DNA fragments that are used in PCR as a starting point for DNA synthesis. Probes are single-stranded sequences of DNA or ribonucleic acid (RNA) used to identify specific sequences of DNA or RNA. Primers and probes used for the real-time PCR assay in the current study were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The primers were purchased in highly concentrated, lyophilised form and diluted. The primers and probes used in the present study are listed in Table 1. Primer and probe sequences were acquired from the delivery receipt of Thermo Fisher Scientific's oligos, and the sequences were also referenced to Sottini *et al.*[108]

Table 1. Primer and probe sequences.

TREC	Forward	5'-CAC ATC CCT TTC AAC CAT GCT-3'
	Reverse	5'-TGC AGG TGC CTA TGC ATC A-3'
	Probe	5'-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3'
KREC	Forward	5'-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3'
	Reverse	5'-AGG AGC CAG CTC TTA CCC TAG AGT-3'
	Probe	5'-HEX-TCT GCA CGG GCA GCA GGT TGG-TAMRA-3'
TCRAC	Forward	5'-TGG CCT AAC CCT GAT CCT CTT-3'
	Reverse	5'-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3'
	Probe	5'-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3'

Abbreviations: A=Adenine; C=Cytosine; FAM=Fluorescein amidites; G=Guanine; HEX=Hexachlorofluorescein; KREC=kappa-deleting recombination excision circles; T=Thymine; TAMRA=Tetramethylrhodamine; TREC=T-cell receptor excision circles; TCRAC=T-cell alpha constant.

The lyophilised primers were resuspended in nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). A vortex was used to gently mix the primer and water (Infitek Ltd., Spokane, WA, USA). The concentration of the dissolved primers was 100 micromolar (µM). The 100 µM primer solution was diluted to a 10 µM stock solution by adding 10µL of 100µM concentrated primer was added to a microcentrifuge tube, 90 µL of nuclease-free water was then added to make a 10 µM stock solution, and the mixture was mixed by gentle vortexing. The primers were stored at -20 °C until use. The primer stock solution was further diluted to prepare a primer working solution that was used in the PCR reaction (diluted to a final concentration of 900 nM for primers and 250 nM for the probes). To determine the amount required for each primer and probe in a PCR reaction, the initial concentration (C), final concentration of primers and probes, and total volume (V) of each PCR reaction were used to calculate the amount (volume in µL) of primers and probes, as shown in Tables 2 and 3.

Table 2. Polymerase chain reaction for T-cell receptor excision circle and kappa-deleting recombination excision circles assay.

PCR component	**Initial concentration**	**Final concentration**	**Volume (µL) per reaction**	**Total volume**	
TREC forward	100 µM	900 nM = 0.9 µM	0.225 µL	0.225 µL × 107 samples	24.08 µL
TREC reverse	100 µM	900 nM = 0.9 µM	0.225 µL	0.225 µL × 107 samples	24.08 µL
TREC probe	10 µM	200 nM = 0.2 µM	0.500 µL	0.500 µL × 107 samples	53.50 µL
KREC forward	100 µM	900 nM = 0.9 µM	0.225 µL	0.225 µL × 107 samples	24.08 µL
KREC reverse	100 µM	900 nM = 0.9 µM	0.225 µL	0.225 µL × 107 samples	24.08 µL
KREC probe	10 µM	200 nM = 0.2 µM	0.500 µL	0.500 µL × 107 samples	53.50 µL
2x TaqmMan multiplex master mix	------	------	12.500 µL	12.500 µL × 107 samples	1,337.50 µL
Sterile distilled water (H_2O)	------	------	2.000 µL	2.000 µL x 107 samples	214.00 µL
DNA/Nuclease-free water	------	------	5.000 µL	------	------
Total	------	------	**25.000 µL**	------	

Abbreviations:/symbols TREC=T-cell receptor excision circles; KREC=kappa-deleting recombination excision circles; H_2O=Water; DNA=Deoxyribonucleic acid; PCR=Polymerase chain reaction; µM=Micromolar; nM=Nanomolar; µL=Microliter.

Table 3. Polymerase chain reaction for T-cell receptor alpha constant assay (reference control gene).

PCR component	**Initial concentration**	**Final concentration**	**Volume (µL) per reaction**	**Total volume**	
TCRAC forward	10 µM	900 nM = 0.9 µM	2.25 µL	2.25 µL ×107 samples	240.75 µL
TCRAC reverse	10 µM	900 nM = 0.9 µM	2.25 µL	2.25 µL × 107 samples	240.75 µL
TCRAC probe	10 µM	200 nM = 0.2 µM	0.50 µL	0.5 µL × 107 samples	53.5 µL
2x TaqmMan multiplex master mix	------	------	12.50 µL	12.5 µL × 107 samples	1,337.5 µL
Sterile distilled water (H_2O)	------	------	4.75 µL	4.75 µL × 107 samples	508.25 µL
DNA/Nuclease-free water	------	------	5.00 µL	------	------
Total	------	------	**25.00 µL**	------	

Abbreviations/symbols: PCR=Polymerase chain reaction; *TCRAC*= T-cell receptor alpha constant; µM=Micromolar; nM=Nanomolar; µL=Microliter.

2.5.4.2. Standard curve construction

A real-time quantitative PCR (qPCR) assay standard curve for the current study was created by performing a dilution series of known DNA concentrations to estimate the initial starting amount of the target template in the experimental samples. Serial dilutions were prepared using phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA). This study used a DNA sample with an initial concentration of 39.86 nanograms per microliter (ng/µL) to construct the standard curves for TREC and KREC. The DNA stock solution was diluted in a series of five two-fold dilutions. To construct a standard curve for TREC, 7.5 µL TREC mixture (forward primer, reverse primer, probe, and nuclease-free water) was added to each PCR plate well, followed by 12.5 µL TaqMan™ Multiplex Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 5 µL DNA. To produce the KREC standard curve, a volume of 7.5 µL KREC mixture (forward primer, reverse primer, probe, and nuclease-free water) was added to each PCR plate well, along with a volume of 12.5 µL TaqMan™ Multiplex Master Mix and 5 µL DNA (one dilution per well). A standard curve for absolute quantification in the real-time qPCR assay was created by plotting the threshold cycles (Ct) against the log of the initial TREC or KREC amplification copies (QuantStudio 6 and 7 flex real-time PCR system software, Foster City, California).

2.5.4.3. Quantification of T-cell receptor excision circles and kappa-deleting recombination excision circles

The real-time qPCR assays used genomic DNA with concentrations that were between 6.65 ng/µL and 30.54 ng/µL. The DNA yielded from the manual DNA extraction method was utilised for quantitative real-time PCR for HEU, HUU, WLWH, and WLWOH samples. The PCR was conducted using the TaqMan™ assay multiplex PCR optimisation application guide. Each well of the PCR plate contained a total volume of 25 µL per PCR reaction. Table 4 summarizes the reagent setup. To eliminate any bubbles and ensure that all reagents were at the bottom of the wells, the PCR plate was filled with all the necessary reagents, sealed with an adhesive cover, and centrifuged at 580 × g (Hermle LaborTechnik, Wehingen, DE). Next, the PCR plate was placed in a QuantStudio™6 flex PCR instrument (Applied Biosystems, Foster City, CA, USA), and the TaqMan™ assay multiplex PCR optimization guide was used for the reaction procedure. The procedure started with an enzyme activation cycle of 20 s at 95 °C.[109] Thereafter, there were 40 cycles of 3 s denaturation steps at 95 °C.[109] Finally, 40 cycles of 30 s at 60 °C annealing and extending stages.[109]

Table 4. Reagent setup for T-cell receptor excision circles, kappa-deleting recombination excision circles, and T-cell receptor alpha constant assays.

TREC/KREC		***TCRAC***	
Sterile water (H_2O)	5.6 µL	H_2O	2.50 µL
TREC forward	0.225 µL	*TCRAC* forward	2.25 µL
TREC reverse	0.225 µL	*TCRAC* reverse	2.25 µL
TREC probe	0.500 µL	*TCRAC* probe	0.50 µL
KREC forward	0.225 µL	2x TaqMan Multiplex Master Mix	12.5 µL
KREC reverse	0.225 µL	DNA/Nuclease water	5.00 µL
KREC probe	0.500 µL	**Total**	**25.00 µL**
2x TaqMan Multiplex Master Mix	12.50 µL		
DNA/Nuclease water	5.000 µL		
Total	**25.000 µL**		

Abbreviations/symbols: H_2O=Water; TREC=T-cell receptor excision circles; KREC=kappa-deleting recombination excision circles; *TCRAC*= T-cell receptor alpha constant; µM=Micromolar; nM=Nanomolar; µL=Microliter.

Using control samples, the specificity of previously published primers was determined.[108] The *TCRAC* gene was used as the reference control gene. To assess the test validity of the TREC/KREC cutoff and assay scope, serial dilutions of samples taken from healthy controls were prepared and examined to establish the sensitivity of the results. Phosphate buffered saline was used to prepare the dilutions. As a negative control, an empty DBS was used to ensure undetectable amounts of TREC/KREC biomarkers. Intra- and inter-assay variation trials using DNA from the same sample were used to verify the reproducibility of the assay. The data were recorded on a data sheet and analysed using the QuantStudio™ delta analysis software (Applied Biosystems, Foster City, CA, USA). A summary of all the results is documented in Chapter 3, and the results are presented in full in the appendices.

2.6. Statistics

Descriptive and inferential statistical techniques were used for analyses. Data of this study were assumed to be non-parametric, and this was confirmed by Shapiro-Wilk and Shapiro-Francia tests for normality. Descriptive statistics are presented as measures of central tendency and dispersion medians with interquartile ranges, proportions, and frequencies relevant to the data. The tests for the association between contingency tables were performed using a two-tailed Chi^2 test. One-way analysis of variance (ANOVA) was performed using the Kruskal-Wallis test for non-parametric data for more than two groups, or the Mann-Whitney test when two groups were compared. Dunn's test of multiple comparisons, using rank sums, was performed to determine multiple pairwise comparisons. Bland Altman method of agreement was used to determine the DNA purity and concentration between the manual and Maxwell extraction methods.

Statistical significance was determined as a *P*-value <0.05. The analyses were conducted using StataNow 18.5 BE (Statacorp LLC, College station, TX, USA).

Chapter 3: Results

3.1. Demographics

The current investigation used a total of 107 samples (human immunodeficiency virus (HIV)-exposed uninfected (HEU): 29.9%, HIV-unexposed uninfected (HUU): 28%, women living with HIV (WLWH): 19.6%, women living without HIV (WLWOH): 22.4%) and is summarised in Table 5 and Table 6, respectively. The HEU and HUU infant groups comprised both females (HEU:44%, HEU:57%) and males (HEU:56%, HUU:43%). From a total of 107 stored samples from the Siyakhula cohort, 15 samples were randomly selected to evaluate deoxyribonucleic acid (DNA) extraction methods for validating DNA isolation from DBS samples using the Maxwell® 16 blood purification kit (automated DNA extraction method). Furthermore, the study included the paired mothers of newborn infants to further assess the reliability of the established in-house real-time polymerase chain reaction (PCR) for T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC).

The demographics for HEU and HUU infant groups include gender, gestational age, weight, length, and head circumference. Most of the infants were born between 37 and 42 weeks of gestational age. Premature birth (less than 36 weeks gestational age) accounted for 4.8 percent (%) in HEU infants and 6.50% in HUU infants for the overall infant study group. The weight of the babies at week zero after birth ranged between 1.6 and 3.9 kilograms (kg) for HEU infants and between 1.4 and 3.7 kg for the HUU infants. Body length of the babies at week zero ranged between 41 to 57 centimetres (cm) in HEU and HUU infants. Head circumference at week zero ranged between 29 and 37 cm for HEU babies and between 30 and 38 cm for the HUU babies.

The demographics of the maternal group (WLWH and WLWOH) included their age, height, and weight. The clusters of differentiation (CD)4+ count & HIV viral load were also included for the WLWH group. The age of the WLWH group ranged between 24 and 45 years, and for the WLWOH it ranged from 19 to 44 years. Height ranged between 120 and 176 cm for the WLWH group and between 144 and 170 cm for the WLWOH group. The weight at 36 weeks of pregnancy for the WLWH group ranged between 48 and 92 kg and between 53 and 88 kg for the WLWOH group. The CD4+ count and HIV viral load were also included for the WLWOH group in Table 6.

The demographic data for the infant group and maternal groups are presented in Tables 5 and 6. The comprehensive demographic characteristics of this section are provided in Appendices C and D.

Table 5. Summary of the demographic features of the research group, comparing human immunodeficiency-exposed uninfected newborn babies with human immunodeficiency-unexposed uninfected newborn babies.

Category	Statistical measure	HEU infants	HUU infants
Gender **Female**	n (%)	14 (44%)	17 (57%)
Male	n (%)	18 (56%	13 (43%)
Gestational age ≤36 weeks	n (%)	3 (9.4%)	4 (13.3%)
37 to 42 weeks	n (%)	29 (90.6%)	26 (86.7%)
Weight (kilograms (kg))	Median (week 0) IQR (week 0)	3.00 2.8 – 3.2	3.00 2.6 – 3.2
	Median (24 months) IQR (24 months)	11.0 10.1 – 11.8	11.0 10.9 – 12.8
Length (Centimetres (cm))	Median (week 0) IQR (week 0)	50.0 48.0 – 52.0	49.0 46.0 – 52.0
	Median (24 months) IQR (24 months)	84.0 83.0 – 88.0	86.0 84.3 – 89.0
Head circumference (cm)	Median (week 0) IQR (week 0)	35.0 33.0 – 36.0	34.0 33.0 – 35.0
	Median (24 months) IQR (24 months)	49.0 48.0 – 50.0	49.0 47.9 – 49.6

Abbreviation/symbols: HEU = HIV-exposed uninfected; HUU = HIV-unexposed uninfected; kg = Kilograms; cm = Centimetres; IQR = Interquartile range; % = Percent; ≤ = Less than/equal to; n = Sample size.

Table 6. Summary of the demographic features of the research group, comparing women living with human immunodeficiency virus with women living without human immunodeficiency virus.

Category	Statistical measure	WLWH	WLWOH
Age (years)	Median	38	30
	IQR	35 - 40	25 - 34
Height (cm)	Median	159	157
	IQR	156 - 161	154 - 158
Weight (kg) Before delivery (gestation age = 36 weeks)	Median	69	74
	IQR	66 - 81	64 - 80
After delivery (24 months)	Median	65	69
	IQR	58 - 77	63 - 73
CD4 count Before delivery (gestation age = 36 weeks)	Median	466	
	IQR	316 - 648	
After delivery (24 months)	Median	700	
	IQR	700 - 750	
HIV viral load (24 months after birth)	Viral load = 0	n = 21	
	Viral load > 0	n = 0	

Abbreviation/symbols: HIV = Human immunodeficiency virus; WLWH = Women living with HIV; WLWOH = Women living without HIV; IQR = Interquartile range; cm = Centimetres; kg = Kilograms; CD4 = Clusters of differentiation 4 (T helper cells), > = Greater than.

3.2. Dried blood spot deoxyribonucleic acid concentration and purity

3.2.1. Comparison of deoxyribonucleic acid extraction methods from dried blood spots

Both DNA extraction approaches (manual and automated methods) were performed on a group of 15 participants to validate the reliability of the Maxwell® 16 blood DNA purification system for dried blood spots. A comparison of automated and manual DNA extraction methods was conducted for DNA concentration and purity. Statistical measures of the mean and the standard deviation were used, as shown in Table 7. The standard deviation was used to determine the variation in the concentration/purity of the DNA values in each DNA extraction method. The less spread out the DNA concentration/purity values are, the smaller the standard deviation; the larger the standard

deviation, the more widely distributed the values are. Table 7 presents the results of the comparative study of the two DNA extraction methods. Appendix E displays all the DNA concentration and purity data for this section.

Figure 3 illustrates the graphical representation of the comparison of DNA purity between the two DNA extraction methods using violin plots. The agreement between the two DNA extraction techniques was assessed using the Passing Bablok residual plots, which are displayed in Figure 4. For the samples extracted using the automated DNA extraction method, sensitivity was assessed by detecting TREC and KREC using real-time PCR. The qualitative real-time PCR results were comparable for all examined dried blood spot (DBS) samples from both DNA extraction methods, and the sensitivity test results for the automated and manually extracted samples were compared. Figure 5 shows a representation of all the results.

Table 7. Deoxyribonucleic acid extraction results of the high-purity polymerase chain reaction template preparation kit (manual extraction) and the Maxwell® 16 blood deoxyribonucleic acid purification system (automated extraction).

Category	**Statistical measure**	**Maxwell 16 blood DNA purification system**	**High pure PCR template preparation kit**
DNA concentration (ng/µL)	Mean	11.2	14.8
	Standard deviation	3.2	3.7
DNA purity measure (260/280 ratio)	Mean	1.7	1.5
	Standard deviation	0.2	0.1

Abbreviations: DNA = Deoxyribonucleic acid; PCR = Polymerase chain reaction; ng = Nanograms; µL = Microliters.

According to the Violin plots shown in Figure 3 the automatic extraction method gave a purity ratio range between 1.6 and 1.8 whereas the purity ratio of the DNA extracted using the manual method ranged between 1.4 and 1.6.

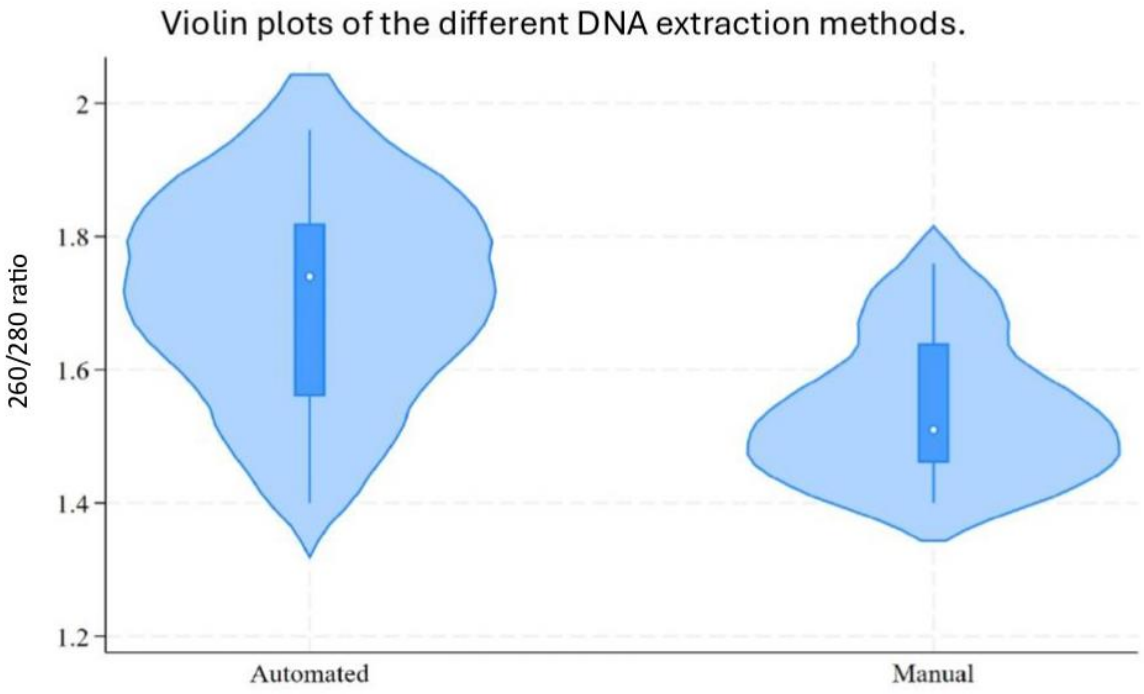

Figure 3: Comparison of the 260/280 ratio (purity) for the automated DNA extraction method (Maxwell® 16 blood DNA purification kit) and the manual DNA extraction method (High purity PCR template preparation kit).

The intra- and inter-assay precision were determined using the coefficient of variation (CV). The CV was calculated using the ratio of standard deviation to the mean/average of the data set. The criteria of acceptance for intra- and inter-assay precision were accepted as a %CV of ≤15% and ≤20%, respectively. The CV's reported by Qureshi *et al*. (2023) were used as the CV references. The DNA was measured at 260 and 280 nanometres (nm) to gauge the purity (a ratio of 1.8 is generally accepted as "pure"). Table 8 displays the Intra- and inter-assay variables for the DNA concentration.

Table 8. Statistical measures used for the assessment of intra- and inter-assay in DNA concentration.

Assay	Mean	Standard deviation	% of CV
Intra-assay	9.4 ng/µL	1.1	12%
Inter-assay			
Sample 1	6.8 ng/µL	0.99	14%
Sample 2	9.1 ng/µL	0.21	2%
Sample 3	7.9 ng/µL	0.47	6%

Abbreviations/symbols: CV = Coefficient of variation, ng = Nanograms, µL = Microliters, % = Percent.

The Passing Bablok method was used for the regression analysis to compare DBS DNA extracted using the manual and automated methods to assess if the two methods would yield similar results. This approach uses a regression line model represented as Y = A + BX. The Passing Bablok regression analysis assumes that if zero is included in the confidence interval of A (Intercept) and one is included in the confidence interval (CI) of B (Slope), the two approaches are considered similar within the measured concentration range. The slope coefficient (B) is 0.87, with a 95% CI between 0.23 and 2.34, and the intercept coefficient (A) is -1.58, with a 95% CI between -25.29 and 7.86. The regression analysis using the Passing Bablok method for the dataset (concentration and purity) measured on the DBS DNA extracted using two DNA extraction techniques (automated and manual) is illustrated in Figure 4.

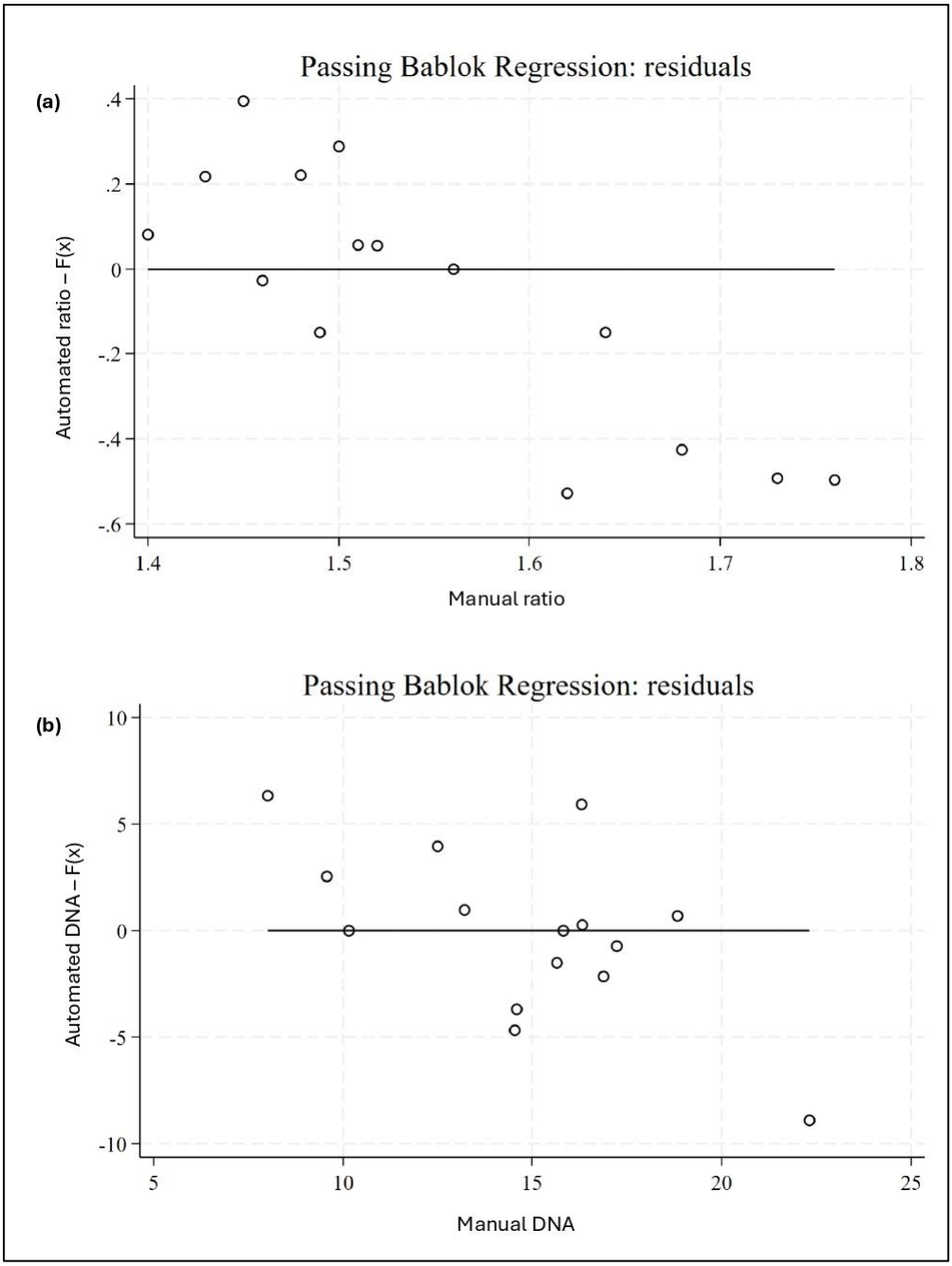

Figure 4: A Passing Bablok regression analysis to compare the automated and manual DNA extraction methods for extracted DBS DNA concentration and purity with a sample size of 15. The concentration was found to range between 6.4 and 22.3 ng/µL, and the purity was found to range between 1.4 and 2. Lin's concordance correlation coefficient of absolute agreement (r) was found to be 0.16, with a P > 0.2. **(a)** A residual plot illustrating the distribution pattern of DNA concentration

differences relative to the fitted regression line. **(b)** A residual plot depicting the distribution pattern of DNA purity differences relative to the fitted regression line.

The DNA extracted from the DBS using the automated method was tested for sensitivity by real-time PCR analysis for the ability to detect the presence of TREC and KREC in the extracted samples. Figure 5 displays the multicomponent plot of amplification cycles for TREC and KREC in one DBS DNA sample for sensitivity analysis. Figure 5 (a) shows the non-template control reaction of TREC and KREC PCR results. All other results showed similar results. In Figure 5 (b) the representative positive reaction of the TREC and KREC curves is displayed.

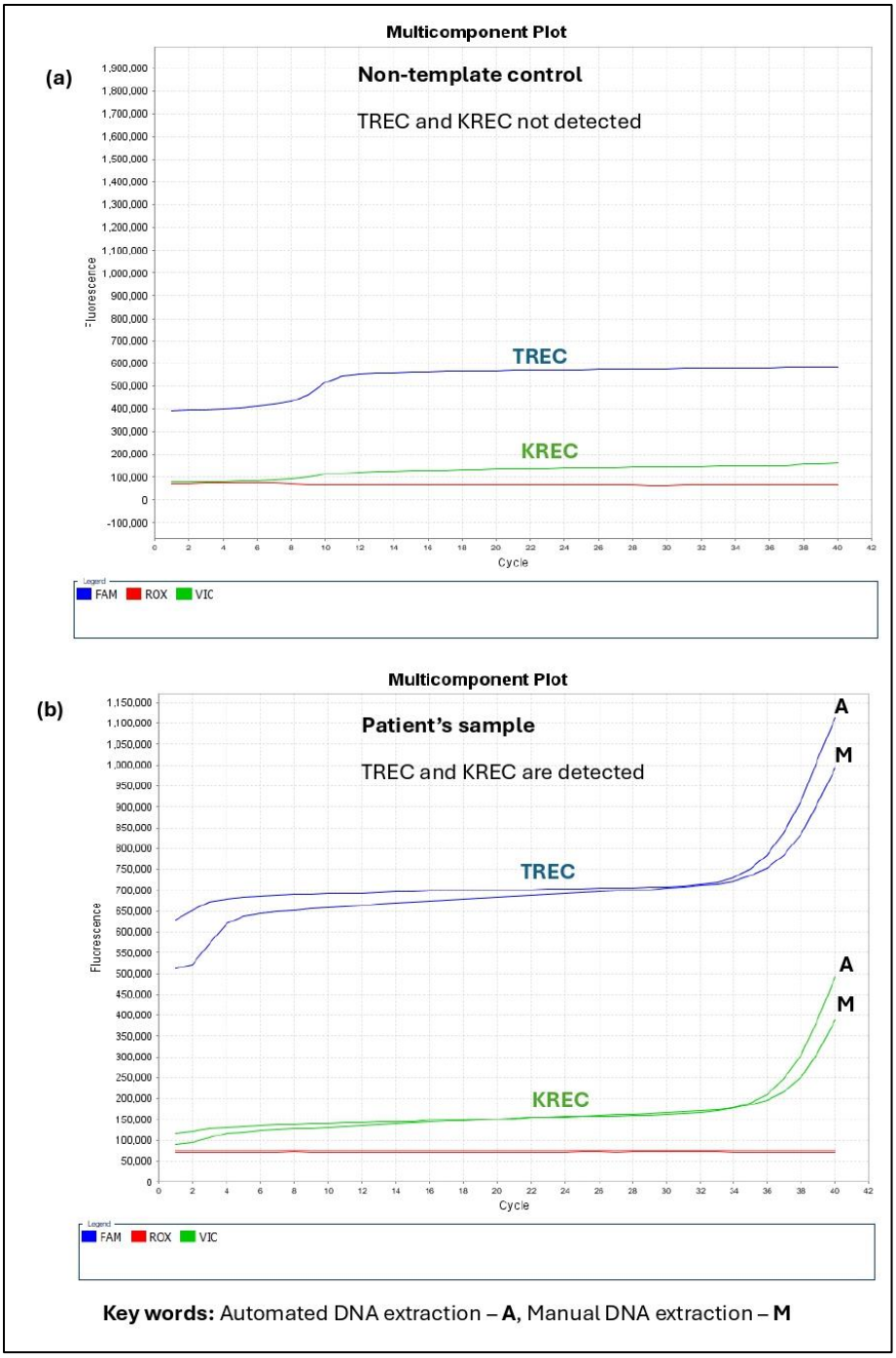

Figure 5: Multicomponent plots for TREC and KREC detection. (a) Non-template control reaction of TREC and KREC PCR results, (b) Representative TREC and KREC amplification curves.

3.2.2. Deoxyribonucleic acid extraction results for newborns and their mothers (Siyakhula cohort)

The current study used a sample size of 62 newborn babies consisting of 32 HEU and 30 HUU infants. DNA was extracted from DBS samples using a manual method (High Purity PCR Template Preparation Kit). Genomic DNA was extracted by using a validated manual procedure. Tables 9 and 10 present the DNA extraction results for the HEU and HUU infants as well as the results of the WLWH and WLWOH cohorts respectively. Appendices F and G display all the DNA concentration and purity data for this section.

Table 9. Deoxyribonucleic acid extraction results in the newborn babies' study population.

Category	Statistical measure	HEU infants	HUU infants
DNA concentration (ng/µL)	Median	18.8	17.9
	IQR	17.2 – 24.2	10.7 – 20.4
DNA purity measure (260/280 ratio)	Median	1.60	1.70
	IQR	1.5 – 1.7	1.4 -1.8

Abbreviations: DNA = Deoxyribonucleic acid, IQR = Interquartile range, ng = Nanograms, µL = Microliters, HEU = HIV-exposed uninfected, HUU = HIV-unexposed uninfected.

The present study used 45 samples from mothers of infants (WLWH: n = 21 and WLWOH: n = 24). The maternal cohort comprised WLWH (mothers of HEU infants) and WLWOH (mothers of HUU infants). Due to certain mothers' DBS samples not being available, the maternal group's sample size was smaller than that of the infant group.

Table 10. Results of deoxyribonucleic acid extraction for the study cohort of infant mothers.

Category	Statistical measure	WLMH	WLWOH
DNA concentration (ng/µL)	Median	15.2	18.5
	IQR	13.7 – 16.8	16.9 – 19.9
DNA purity measure (260/280 ratio)	Median	1.5	1.46
	IQR	1.4 – 1.5	1.40 – 1.49

Abbreviations: DNA = Deoxyribonucleic acid, IQR = Interquartile range, ng = Nanograms, µL = Microliters, WLWH = Women living with HIV, WLWOH = Women living without HIV.

3.3. Quantitative real-time polymerase chain reaction

3.3.1. Standard curve

The standard curve for the real-time quantitative PCR (qPCR) experiment was obtained by conducting a two-fold dilution series. Standard curves for TREC and KREC were constructed using a DNA sample with a starting concentration of 39.86 ng/µL. Table 11 presents the corresponding statistical measurements, and Figure 6 displays the standard curves for TREC and KREC.

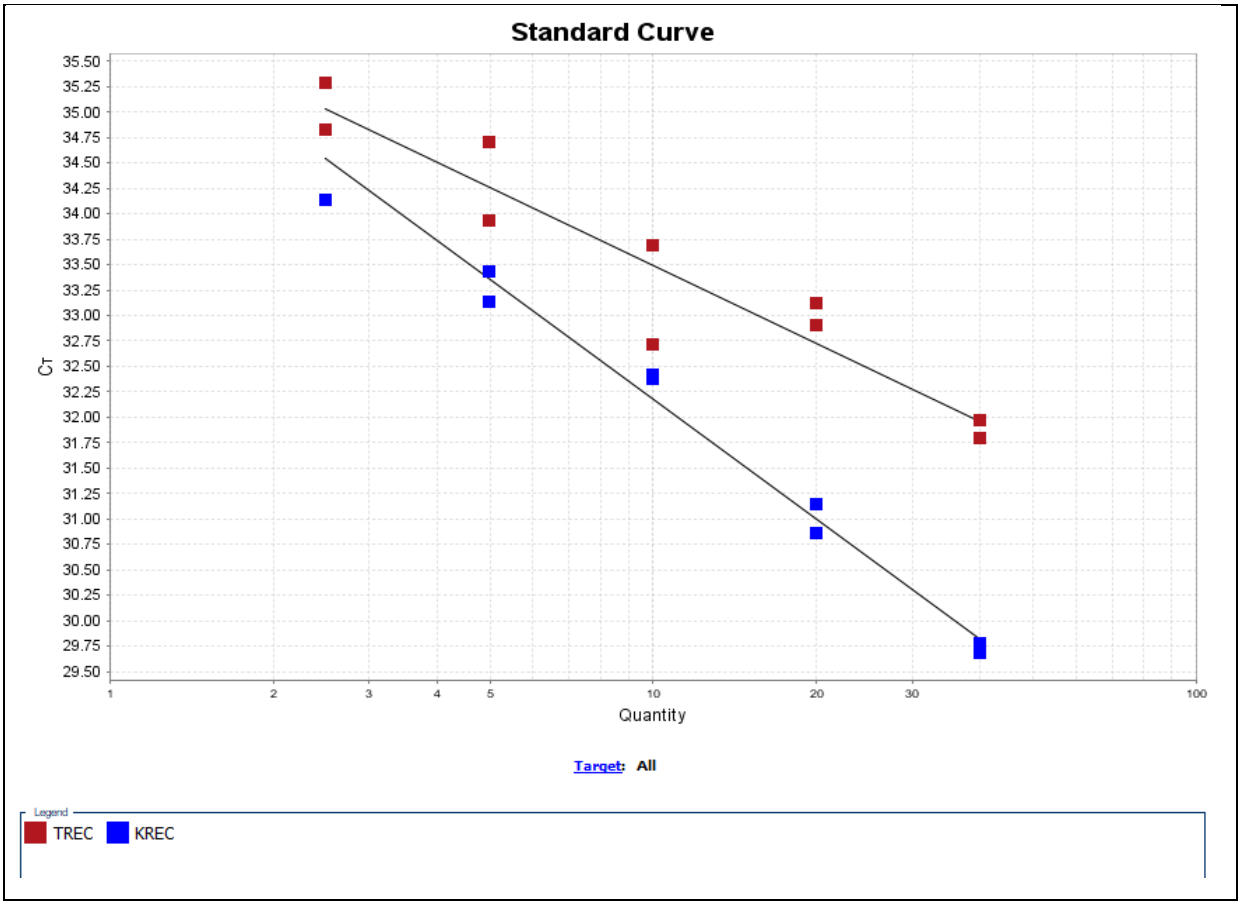

Figure 6: Standard curve for TREC and KREC circles.

Table 11. The statistical measures of T-cell receptor excision circles and kappa-deleting recombination excision circles standard curves.

	TREC standard curve	KREC standard curve
Slope	-2.55	-3.92
Y-intercept	36.04	36.09
R^2	0.90	0.98
Efficiency	146.98	79.97
Error	0.30	0.18

Abbreviations/symbols: R^2 = Coefficient of determination, TREC = T-cell receptor excision circles, KREC = kappa-deleting recombination excision circles.

3.3.2. Detection of T-cell receptor excision circles and kappa-deleting recombination excision circles in samples from newborn babies.

After constructing the standard curve, DNA extracted from DBS samples was processed for the next stage of quantitative PCR. The T-cell receptor alpha constant gene (*TCRAC*) was used as the reference control gene for quantitative PCR. Each sample was assayed twice, with one well containing a mixture of TREC and KREC (primers and probes), and the second well containing *TCRAC*. The *TCRAC* gene was identified in all samples. Clean DBS filter paper was treated with the same DNA extraction methodology, was subsequently used as a negative control. No target genes were detected in negative controls. All non-template controls (NTCs) for every PCR run yielded negative results, confirming the absence of contamination in all PCR cycles conducted in this investigation.

The mean quantity of TREC in both HEU and HUU newborn babies was found to be greater than the quantity of KREC measured in both groups. The cycle threshold (CT) values for all the samples ranged between 30 and 38. The cycle threshold is the point at which the fluorescent signal from the reaction exceeds this threshold. It is utilized to determine the initial number of DNA copies, with the CT value inversely related to the initial amount of the target. A sample with a high CT value indicates a low initial number of TREC or KREC genes. The coefficient of determination (R^2) for the TREC and KREC standard curves was 0.90 and 0.98, respectively. Coefficient of determination is used to evaluate the performance of regression models. The values of R^2 range from 0 to 1, where a value closer to one indicates a perfect fit and zero indicates no relationship. Statistical significance was determined by *P*-value was less than 0.05. The *P*-value for TREC quantification between HEU and HUU infants was

0.006, and it was 0.248 for KREC quantification. Figure 7 shows the differences between the HEU and HUU infant groups for the quantity of TREC and KREC. The statistical measurements from qPCR for both HEU and HUU infants are shown in Table 12 and Figure 7. Appendix H contains detailed quantitative PCR results for the HEU and HUU newborn study populations.

Table 12. Comparison of quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination excision circles between HIV-exposed uninfected and HIV-unexposed uninfected.

Category	**Statistical value**	**HEU infants**		**HUU infants**		***P*-value**
		TREC	**KREC**	**TREC**	**KREC**	
Target gene detection: **Detected**	n (%)	32 (100%)	32 (100%)	30 (100%)	30 (100%)	
Undetected	n (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Quantity (TREC or KREC per 2 x 10^5 copies)	Median	3	4	10	7	TREC: 0.006
	IQR	2 - 8	3 - 10	5 - 16	4 - 10	KREC: 0.248
CT	Median	35	34	34	33	
	IQR	34 - 35	32 - 34	33 - 34	32 - 34	
R^2	TREC: 0.90 KREC: 0.98					
Slope	TREC: -2.55 KREC: -3.92					

Abbreviations/symbols: n = Sample size, % = Percent, CT = Cycle threshold, R^2 = Coefficient of determination, TREC = T-cell receptor excision circles, KREC = kappa-deleting recombination excision circles, IQR = Interquartile range, HEU = HIV-exposed uninfected, HUU = HIV-unexposed uninfected.

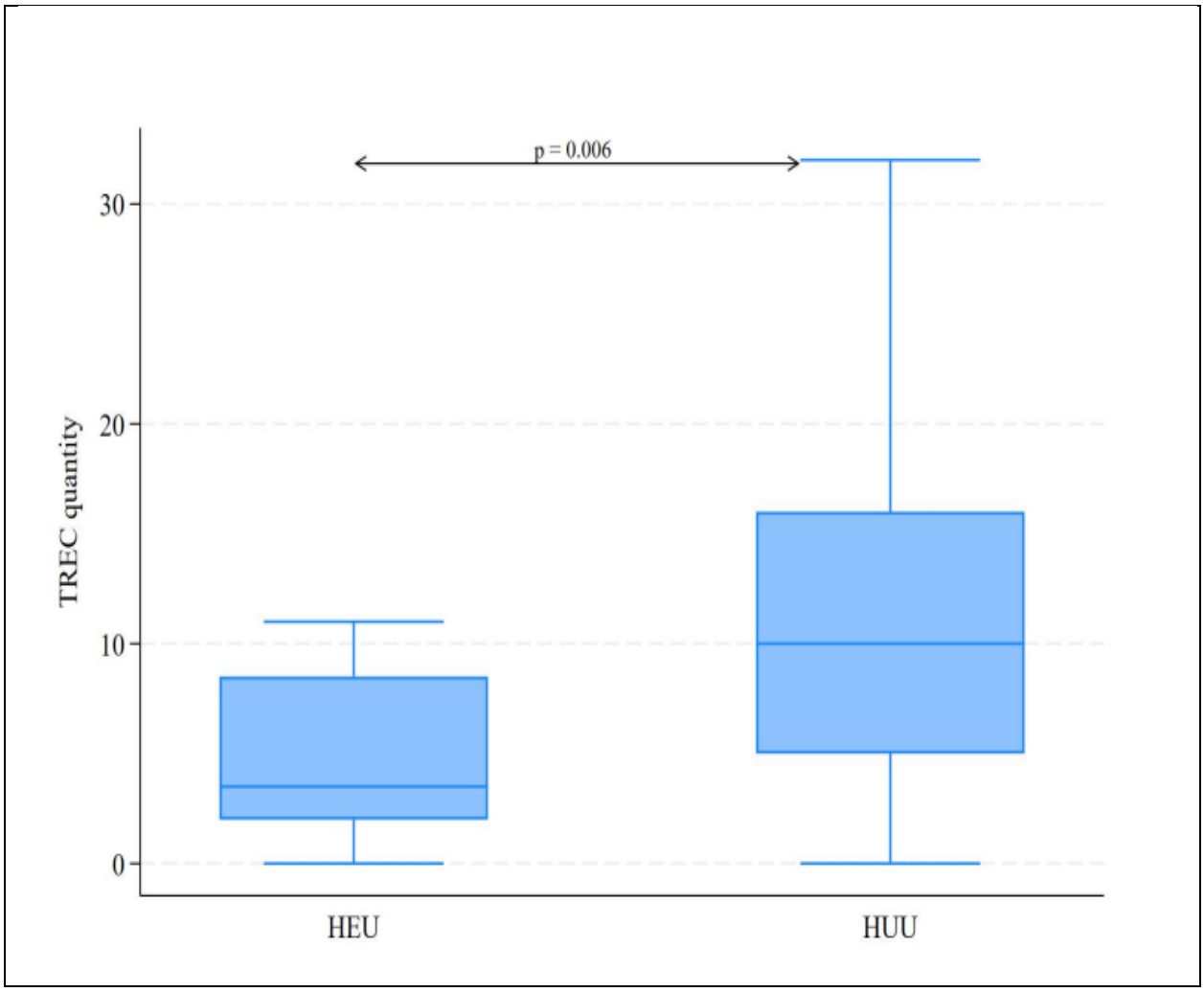

Figure 7: A box plot illustrating the statistical significance of TREC quantity between HEU and HUU newborn babies. The differences between the two infant groups were statistically significant, with a *P*-value of 0.006.

Table 13 displays the statistical measurements of CT and quantity from qPCR samples for the WLWH and WLWOH cohort. Figure 8 shows the differences in the median, IQR, minimum, and maximum TREC and KREC quantities between the WLWH and WLWOH cohorts. Appendix I contains detailed qPCR results for the WLWH and WLWOH samples. The *P*-value for TREC and KREC quantification between WLWH and WLWOH was 0.14 and 0.12, respectively.

Table 13. Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination excision circles between Women living with HIV and Women living without HIV.

Category	**Statistical value**	**WLWH**		**WLWOH**		***P*-value**
		TREC	**KREC**	**TREC**	**KREC**	
Target gene detection: **Detected**	n (%)	19 (90.5%)	20 (95.2%)	21 (87.5%))	24 (100%)	
Undetected	n (%)	2 (9.5%)	1 (4.8%)	3 (12.5%)	0 (0%)	
Quantity (TREC or KREC per 2 x 10^5 copies)	Median	0	3	1	2	TREC: 0.14
	IQR	0 - 1	1 - 5	0 - 2	1 – 4	KREC: 0.12
CT	Median	37	34	37	35	
	IQR	36 - 38	33 - 36	35 - 37	34 - 35	
R^2	TREC: 0.90 KREC: 0.98					
Slope	TREC: -2.55 KREC: -3.92					

Abbreviations: n = Sample size, % = Percent, CT = Cycle threshold, R^2 = Coefficient of determination, TREC = T-cell receptor excision circles, KREC = kappa-deleting recombination excision circles, IQR = Interquartile range, WLWH = Women living with HIV, WLWOH = Women living without HIV.

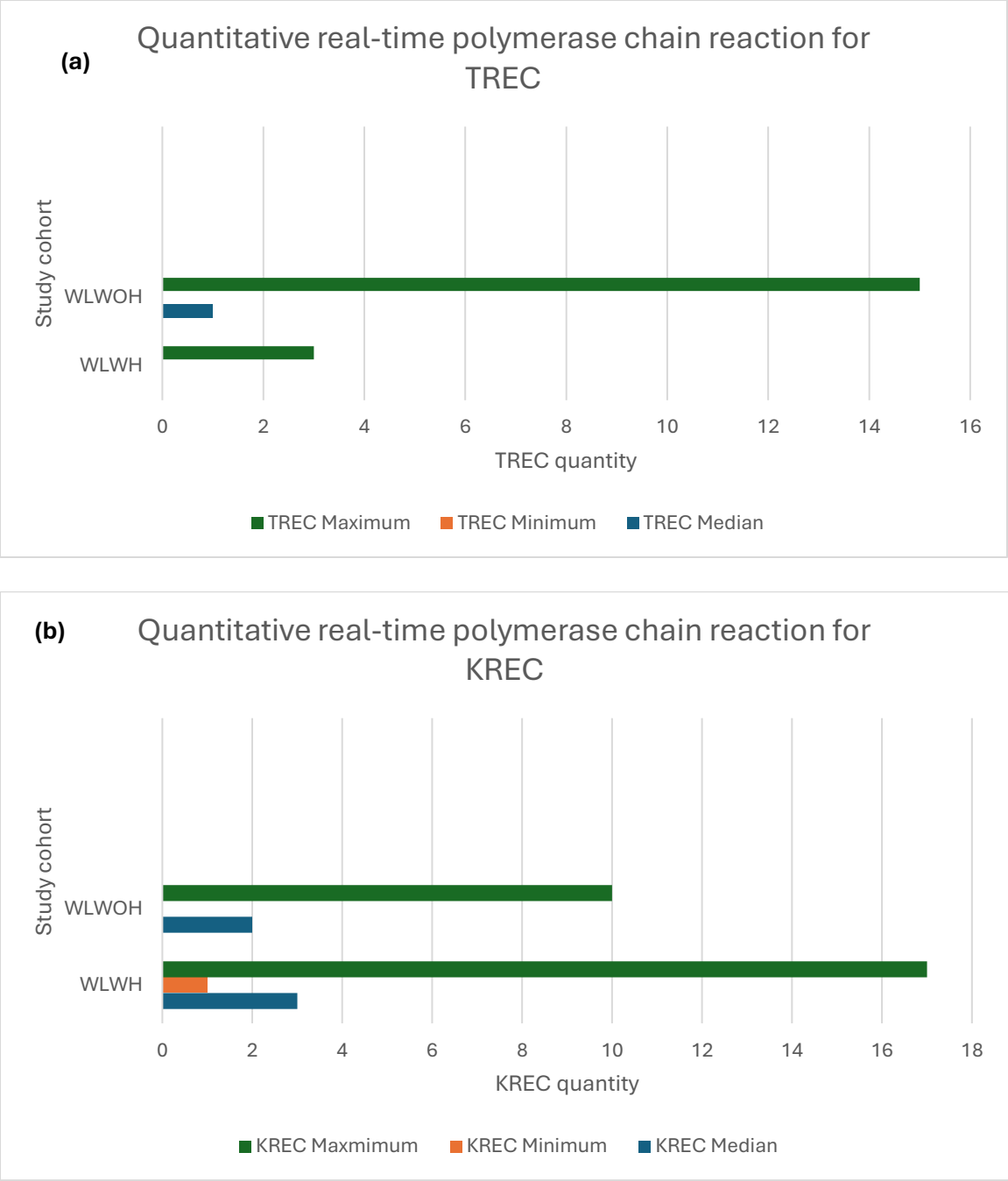

Figure 8: Comparison of TREC and KREC quantitative real-time PCR results between WLWH and WLWOH. The comparison focused on **(a)** the quantity of TREC**/** 2 x 10^5 copies and **(b)** the quantity of KREC**/** 2 x 10^5 copies between the two study groups. This is a graphic representation of the median, minimum, and maximum TREC and KREC quantities for the WLWH and WLWOH groups.

Chapter 4: Discussion and Conclusion

4.1. Discussion

Monogenic immune system abnormalities known as inborn errors of immunity (IEI) can result in immunodeficiency, autoimmunity, autoinflammation, allergies, and neoplasms.[1,10,110] Inborn errors of immunity were regarded as rare disorders.[25,110] However, the rate at which new mutations underlying IEIs are being identified is extraordinary, and the clinical phenotypes that are linked with these defects are becoming increasingly well-defined.[110-111] This is revealing the significant health risks that these diseases collectively pose.[110] Circular DNA (cDNA) fragments of T- and B-cell receptors (TREC and KREC) that arise from receptor gene rearrangement in T and B cells can be used to identify IEI.[112]

An in-house real-time polymerase PCR method for TREC and KREC was developed for screening IEI in newborn babies. Newborn screening aims to detect pre-symptomatic infants who might have life-threatening or serious health conditions that could be effectively treated, resulting in a significant reduction in morbidity and mortality.[113] The HEU and HUU cohorts were specifically chosen for this study's population because numerous research studies have shown that HEU babies are more immunologically vulnerable than HUU infants because of their exposure to HIV and antiretroviral therapy.[114-117] Assessing how TREC and KREC levels would vary in infants with a higher risk of immunological vulnerability (HEU) versus HUU neonates was one of the study's objectives to detect T- and B-cell markers in these infants.

Dried blood spot samples were used, and DNA was extracted from DBS. An automated method for DNA extraction (specifically the Maxwell® 16 blood DNA purification kit) was validated for DBS DNA extraction. An in-house real-time PCR assay for TREC and KREC quantification was developed. Using the newly developed TREC and KREC assays, TREC and KREC were detected and quantified in samples of HEU and HUU newborn babies.

4.1.1. Deoxyribonucleic acid extraction comparison

Dried blood spot refers to drops of blood on absorbent filter paper.[118-119] The DBS approach does away with the necessity for phlebotomy, which is typically uncomfortable, and for frequent, routine trips to medical facilities.[120] In contrast to plasma or serum maintained under the same conditions, RNA and DNA, antibodies or other proteins, and antiviral medications or their metabolites stay stable on

DBS cards for comparatively extended periods of time when kept with desiccant in sealed bags at room temperature.[121-122] Dried blood spot filter cards are small, lightweight, and easy to transport. They also pose little risk of infection since, as they dry out, many viruses become inactive owing to the disruption of their envelopes.[121-122]

Several manual and automated methods for DNA extraction from DBS have been validated.[123-126]. In the laboratory where the current study was conducted, the automated DNA extraction technique used was the Maxwell® 16 blood DNA purification technique (specifically, the Maxwell® 16 blood DNA purification kit on the Maxwell® 16 instrument version 4.0 (Promega Corporation, Madison, WI, USA)). The study's objectives included validating the Maxwell® 16 blood DNA purification method for DBS DNA extraction, as there is currently no literature validating this technique's application for DBS DNA extraction. Automated DNA extraction is less time-consuming and minimizes human errors and contamination. A high-purity PCR template preparation kit (Roche Life Sciences, Basel, CH) was used to compare the DBS DNA extracted by an automated method to DBS DNA extracted by a validated manual method.

The DBS samples can be maintained at room temperature (15 to 30 degree Celsius (°C)) for 7 days, provided they are not subjected to high humidity (>50%).[127-128] Alternatively, they may be preserved for up to 2 months under low-humidity conditions without compromising DNA detection via qPCR.[127-128] Li *et al*. conducted a study demonstrating the long-term stability of HIV-1 DNA in DBS preserved for over four years at ambient temperature.[129] This is consistent with the findings of the current study, where DBS samples had been stored for over four years, and DNA detection for TREC and KREC via qPCR was not compromised.

A higher DNA concentration was achieved in this study for a single DBS spot, with values ranging from 6.4 ng/µL to 18.5 ng/µL, in contrast to the study by Kumar *et al*., which reported a concentration range of 2.14 ng/µL to 4.7 ng/µL for DNA extracted from one DBS spot.[130] In a previous study, Lang *et al*. showed that the concentration of DNA extracted using an automated method was comparable to that of DNA extracted manually when following the manufacturer's instructions and adding carrier RNA treatment. However, both the chosen elution volume and the method greatly affected the integrity of the DNA.[131] This contradicts the findings of the current study, as the DNA concentration obtained from the automated method (Maxwell® 16 blood purification kit) was dissimilar to that from the manual method (High purity PCR template preparation kit).

Compared to the average DNA purity of 1.5 acquired using the manual method, the average DNA purity of samples obtained using the automated method was 1.7, which was closer to the optimal purity of 1.80. ThermoFisher Scientific reports that a 260/280 ratio of approximately 1.80 indicates pure DNA.[132] Any ratio higher or lower than 1.80 is considered abnormal and suggests contamination by the reagents used in the extraction process. If contamination is caused by the reagents, the 260/280 ratio is typically lower.[132] An insufficient amount of DNA (less than 10 ng/µL) might potentially contribute to an abnormal 260/280 ratio.[132] The DNA concentrations from the automated and manual DNA extraction methods were found to be similar, unlike the purity of the DNA extracted by the two methods. As expected, the purity of the DNA extracted using the automated method of extraction was higher than that extracted manually. This underscores the ability of automated DNA extraction in reducing human error and interaction; therefore, resulting in increased DNA purity.

The intra- and inter-assay precision adhered to the acceptance requirements of ≤15% and ≤20%, respectively. The intra- and inter-assay results align with the findings by Qureshi *et al*., which similarly reported a variation limit of ≤15% and ≤20%.[133]

To establish an agreement between the two methods of DNA extraction, the Passing Bablok method was used. The confidence interval for both the slope and intercept was found to adhere to the assumptions of the Passing Bablok method, therefore it was determined that the automated and manual DNA extraction methods are comparable for DBS DNA extraction.

Research conducted by Lang *et al*. indicated that analysing DNA extracted from DBS samples and its ability to amplify TREC sequences supports the preference for an automated extraction approach for subsequent qPCR applications.[131] This study corroborates the findings of Kumar *et al*., demonstrating that isolated DBS DNA can be effectively utilized in PCR.[130]

The choice of DNA extraction method used does influence the downstream analyses, such as real-time qPCR, to a lesser extent. The automated DNA extraction method yields DNA that has a higher TREC and KREC gene expression as compared to the manual DNA extraction method; the differences can be seen in Figure 5 in the results section. This is to be expected, as automated DNA extraction involves less human interaction than manual DNA extraction.

4.1.2. Quantification of T-cell receptor excision circles and kappa-deleting recombination excision circles

The present study established an in-house real-time PCR method aimed at newborn screening for inborn errors of immunity (IEI). This was accomplished by utilising commercially available primers and probes for TREC and KREC markers.

Several methods have been used for the quantitative assessment of TREC and KREC, including RT-PCR, which is among the most widely utilised and affordable technologies.[3,19,32,35,83,134] The implementation of KREC measurement provides an additional method for identifying B-cell lymphopenia.[19] Conversely, multiplex TREC, KREC, and internal control in a single RT-PCR reaction mitigates variability from pipetting errors and facilitates precise assessment of TREC and KREC levels in a cost-efficient manner.[135]

The quantity of TREC was found to be greater than that of KREC in both groups of infants (HEU and HUU). Due to T-cells constituting a greater proportion of total lymphocytes in the blood compared to B-cells[136], it is expected that individuals will have a higher quantity of TREC compared to that of KREC.

The TREC and KREC markers were detected in all the newborn babies included in this study. This was expected as no adverse health conditions were noted for any of the infants included in the current study over the duration of the study. The HUU infant group were found to have significantly higher levels of TREC than the HEU infant group. Given that HEU infants have altered cell-mediated immunity, including impaired T-cell maturation and documented hypo- and hyper-responsiveness to T-cell activation results of the current study are similar to those reported by Evans *et al*.[71] Studies have shown that HEU children are more likely to be born prematurely and small for their gestational age, and they generally have poor growth and developmental outcomes compared to HUU infants.[71,115,117] This may be due to *in utero* exposure to HIV as well as antiretroviral therapy. Although slightly higher in the HEU infants, the levels of KREC were not statistically different between the HEU and HUU infant groups.

The TREC gene was detected in 90.5% of the women living with HIV (WLWH) included in the maternal study group; two samples had undetectable TREC. All of the WLWH selected in the current study had low detectable levels (LDL) of HIV. The undetectable TREC in WLWH was unexpected. The HIV targets and eradicates CD4+ T-cells, hence diminishing the body's immune response to infection.[137] Nonetheless, a low or undetectable viral load should yield detectable TREC levels.[137] While a low

detectable viral load or zero HIV viral load may not always indicate complete immunological reconstitution,[138] TREC should be detectable and quantifiable, even in small quantities, to demonstrate thymic function.

The KREC gene was not detected in 4.8% of WLWH. The HIV infection is reported to cause disruptions in all primary cell types of the immune system, including B-lymphocytes.[139] Since the WLWH in this study had a LDL viral load, undetectable TREC and KREC levels were not expected.

The study group of women living without HIV (WLWOH) were included as healthy control participants. A percentage of 12.5% of the WLWOH showed undetectable expression of the TREC gene, while 100% of the samples expressed the KREC gene. Given that the WLWOH samples came from healthy individuals, one would expect to detect both TREC and KREC in all samples, however, this was not the case. Both healthy individuals and HIV-infected participants between the ages of 20 and 65 were found to have a 95% reduction in TREC levels, according to research by Goronzy *et al*. and Naylor *et al*.[140-141]

No statistically significant differences were observed in the levels of both TREC and KREC between the WLWH and the WLWOH groups. The DNA concentration and purity of samples with detected TREC and KREC were comparable to those of samples with detectable and quantifiable TREC and KREC. The assessment of TREC and KREC quantification and standard curves requires further analysis to determine why TREC and KREC cannot be detected in certain samples while simultaneously being unquantifiable for both markers.

4.2. Conclusion

Automated DNA extraction using the Maxwell system can be used to extract sufficient DNA with adequate purity from DBS samples, which is suitable for qPCR.

The newly developed in-house real-time PCR method effectively quantified TREC and KREC in newborn babies. The assays of TREC and KREC have demonstrated significant potential for IEI screening, and the simultaneous detection and quantification of TREC/KREC is a cost-effective method that utilizes fewer resources. Incorporating TREC and KREC tests into newborn screening can significantly enhance the chance of detecting IEI at birth. Quantifying these biomarkers may allow for the early identification of IEI, which, in turn, would allow for timely therapeutic interventions.

There were differences observed between HUU and HEU infants. These results indicate that immunologically vulnerable babies (HEU) have reduced expression of TREC. This suggests that babies born to mothers living with HIV are at a higher risk of T-cell dysfunction due to exposure to HIV and antiretroviral therapy.

4.3. Strengths of the study

A quantitative real-time PCR assay was used in the current study; it requires no post-PCR analysis and uses fewer resources and less time. The quantification of TREC and KREC occurred in real-time instead of at the end of the procedure, as is the case with conventional PCR methods. TREC and KREC qPCR techniques have been successfully used in other studies to screen newborn babies for IEI.

4.4. Limitations of the study

The establishment of cut-off values for TREC and KREC quantities to differentiate between abnormal and normal samples was not feasible, nor was it possible to determine the expected ranges of TREC and KREC quantities in paediatric and adult populations due to the limited sample size. This objective could have been more readily achieved had the study groups encompassed children and adults of various age groups, as well as a subset of participants with confirmed diagnoses of IEI.

Furthermore, it was not feasible to monitor the longitudinal changes in TREC and KREC quantities during infant development, as no single infant participant provided a DBS sample for all nine neonatal visit time points.

A positive control, specifically a sample from an individual with IEI, was not available. Consequently, it was not possible to compare a sample from a healthy individual with that of a confirmed case of IEI.

4.5. Recommendations

The quantification process of the TRECs and KRECs requires further refinement, as the limited instances where detection failed could potentially be attributed to design or procedural issues in the standard curve.

The findings of the current study emphasize the need for a more extensive investigation into the South African population to establish reference ranges for TREC and KREC expression in both paediatric and adult groups. Given the broad spectrum of conditions classified as IEI, it is crucial to conduct further research on TREC and KREC assays across various manifestations of these genetic disorders.

This expanded research could provide valuable insights into the prevalence and characteristics of IEI in South Africa, potentially leading to improved diagnostic strategies and earlier interventions. Additionally, longitudinal studies tracking TREC and KREC levels over time in individuals with suspected or confirmed IEI could enhance our understanding of disease progression and treatment efficacy. Such comprehensive data would not only benefit the South African healthcare system but also contribute to the global knowledge base on IEI, facilitating more accurate comparisons and collaborations across different populations and regions.

References

1. Bousfiha A, Moundir A, Tangye SG, Picard C, Jeddane L, Al-Herz W, Rundles CC, Franco JL, Holland SM, Klein C, Morio T, Oksenhendler E, Puel A, Puck J, Seppänen MRJ, Somech R, Su HC, Sullivan KE, Torgerson TR, Meyts I. The 2022 update of iuis phenotypical classification for human inborn errors of immunity. J Clin Immunol. 2022; 42(7):1508-20. doi:10.1007/s10875-022-01352-z

2. Dasouki M, Jabr A, AlDakheel G, Elbadaoui F, Alazami AM, Al-Saud B, Arnaout R, Aldhekri H, Alotaibi I, Al-Mousa H, Hawwari A. Trec and krec profiling as a representative of thymus and bone marrow output in patients with various inborn errors of immunity. Clin Exp Immunol. 2020; 202(1):60-71. doi:10.1111/cei.13484

3. Korsunskiy I, Blyuss O, Gordukova M, Davydova N, Zaikin A, Zinovieva N, Zimin S, Molchanov R, Salpagarova A, Eremeeva A, Filipenko M, Prodeus A, Korsunskiy A, Hsu P, Munblit D. Expanding trec and krec utility in primary immunodeficiency diseases diagnosis. Front Immunol. 2020; 11:320. doi:10.3389/fimmu.2020.00320

4. Yu JE. New primary immunodeficiencies 2023 update. Curr Opin Pediatr. 2024; 36(1):112-23. doi:10.1097/mop.0000000000001315

5. Bruton OC. Agammaglobulinemia. Pediatrics. 1952; 9(6):722-8.

6. Glanzmann E, Riniker P. [essential lymphocytophthisis; new clinical aspect of infant pathology]. Ann Paediatr. 1950; 175(1-2):1-32.

7. Hitzig WH, Biro Z, Bosch H, Huser HJ. [agammaglobulinemia & alymphocytosis with atrophy of lymphatic tissue]. Helv Paediatr Acta. 1958; 13(6):551-85.

8. Hitzig WH, Barandun S, Cottier H. [the swiss type of agammaglobulinemia]. Ergeb Inn Med Kinderheilkd. 1968; 27:79-154.

9. Cooper MD. A life of adventure in immunobiology. Annu Rev Immunol. 2010; 28:1-19. doi:10.1146/annurev-immunol-030409-101248

10. Fischer A, Provot J, Jais JP, Alcais A, Mahlaoui N. Autoimmune and inflammatory manifestations occur frequently in patients with primary immunodeficiencies. J Allergy Clin Immunol. 2017; 140(5):1388-93.e8. doi:10.1016/j.jaci.2016.12.978

11. Notarangelo LD, Bacchetta R, Casanova JL, Su HC. Human inborn errors of immunity: An expanding universe. Sci Immunol. 2020; 5(49) doi:10.1126/sciimmunol.abb1662

12. Bonilla FA, Khan DA, Ballas ZK, Chinen J, Frank MM, Hsu JT, Keller M, Kobrynski LJ, Komarow HD, Mazer B, Nelson RP, Jr., Orange JS, Routes JM, Shearer WT, Sorensen RU, Verbsky JW, Bernstein DI,

Blessing-Moore J, Lang D, Nicklas RA, Oppenheimer J, Portnoy JM, Randolph CR, Schuller D, Spector SL, Tilles S, Wallace D. Practice parameter for the diagnosis and management of primary immunodeficiency. J Allergy Clin Immunol. 2015; 136(5):1186-205.e1-78. doi:10.1016/j.jaci.2015.04.049

13. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, Franco JL, Holland SM, Klein C, Morio T, Ochs HD, Oksenhendler E, Picard C, Puck J, Torgerson TR, Casanova JL, Sullivan KE. Human inborn errors of immunity: 2019 update on the classification from the international union of immunological societies expert committee. J Clin Immunol. 2020; 40(1):24-64. doi:10.1007/s10875-019-00737-x

14. Baloh CH, Chong H. Inborn errors of immunity. Prim Care. 2023; 50(2):253-68. doi:10.1016/j.pop.2022.12.001

15. El-Sayed ZA, Radwan N. Newborn screening for primary immunodeficiencies: The gaps, challenges, and outlook for developing countries. Front Immunol. 2019; 10:2987. doi:10.3389/fimmu.2019.02987

16. Kutlug S, Karadag Alpaslan M, Hancioglu G, Elif Ozyazici Ozkan S, Cemile Yesilirmak D, Bulut H, Aygun C, Ogur G, Yildiran A. Multiplex pcr-based newborn screening for severe t and b-cell lymphopenia: The first pilot study in turkey. Sisli Etfal Hastan Tip Bul. 2021; 55(4):551-9. doi:10.14744/semb.2020.09623

17. Chong-Neto HJ, Radwan N, Condino-Neto A, Rosário Filho NA, Ortega-Martell JA, El-Sayed ZA. Newborn screening for inborn errors of immunity: The status worldwide. World Allergy Organ J. 2024; 17(6):100920. doi:10.1016/j.waojou.2024.100920

18. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, Kapoor N, Hanson IC, Filipovich AH, Jyonouchi S, Sullivan KE, Small TN, Burroughs L, Skoda-Smith S, Haight AE, Grizzle A, Pulsipher MA, Chan KW, Fuleihan RL, Haddad E, Loechelt B, Aquino VM, Gillio A, Davis J, Knutsen A, Smith AR, Moore TB, Schroeder ML, Goldman FD, Connelly JA, Porteus MH, Xiang Q, Shearer WT, Fleisher TA, Kohn DB, Puck JM, Notarangelo LD, Cowan MJ, O'Reilly RJ. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med. 2014; 371(5):434-46. doi:10.1056/NEJMoa1401177

19. Kwok JSY, Cheung SKF, Ho JCY, Tang IWH, Chu PWK, Leung EYS, Lee PPW, Cheuk DKL, Lee V, Ip P, Lau YL. Establishing simultaneous t cell receptor excision circles (trec) and k-deleting recombination excision circles (krec) quantification assays and laboratory reference intervals in healthy individuals of different age groups in hong kong. Front Immunol. 2020; 11:1411. doi:10.3389/fimmu.2020.01411

20. Blom M, Bredius RGM, van der Burg M. Future perspectives of newborn screening for inborn errors of immunity. Int J Neonatal Screen. 2021; 7(4) doi:10.3390/ijns7040074

21. Pavey AR, Bodian DL, Vilboux T, Khromykh A, Hauser NS, Huddleston K, Klein E, Black A, Kane MS, Iyer RK, Niederhuber JE, Solomon BD. Utilization of genomic sequencing for population screening of immunodeficiencies in the newborn. Genet Med. 2017; 19(12):1367-75. doi:10.1038/gim.2017.57

22. Stray-Pedersen A, Sorte HS, Samarakoon P, Gambin T, Chinn IK, Coban Akdemir ZH, Erichsen HC, Forbes LR, Gu S, Yuan B, Jhangiani SN, Muzny DM, Rødningen OK, Sheng Y, Nicholas SK, Noroski LM, Seeborg FO, Davis CM, Canter DL, Mace EM, Vece TJ, Allen CE, Abhyankar HA, Boone PM, Beck CR, Wiszniewski W, Fevang B, Aukrust P, Tjønnfjord GE, Gedde-Dahl T, Hjorth-Hansen H, Dybedal I, Nordøy I, Jørgensen SF, Abrahamsen TG, Øverland T, Bechensteen AG, Skogen V, Osnes LTN, Kulseth MA, Prescott TE, Rustad CF, Heimdal KR, Belmont JW, Rider NL, Chinen J, Cao TN, Smith EA, Caldirola MS, Bezrodnik L, Lugo Reyes SO, Espinosa Rosales FJ, Guerrero-Cursaru ND, Pedroza LA, Poli CM, Franco JL, Trujillo Vargas CM, Aldave Becerra JC, Wright N, Issekutz TB, Issekutz AC, Abbott J, Caldwell JW, Bayer DK, Chan AY, Aiuti A, Cancrini C, Holmberg E, West C, Burstedt M, Karaca E, Yesil G, Artac H, Bayram Y, Atik MM, Eldomery MK, Ehlayel MS, Jolles S, Flatø B, Bertuch AA, Hanson IC, Zhang VW, Wong LJ, Hu J, Walkiewicz M, Yang Y, Eng CM, Boerwinkle E, Gibbs RA, Shearer WT, Lyle R, Orange JS, Lupski JR. Primary immunodeficiency diseases: Genomic approaches delineate heterogeneous mendelian disorders. J Allergy Clin Immunol. 2017; 139(1):232-45. doi:10.1016/j.jaci.2016.05.042

23. King JR, Notarangelo LD, Hammarström L. An appraisal of the wilson & jungner criteria in the context of genomic-based newborn screening for inborn errors of immunity. J Allergy Clin Immunol. 2021; 147(2):428-38. doi:10.1016/j.jaci.2020.12.633

24. Padilla CD, Krotoski D, Therrell BL, Jr. Newborn screening progress in developing countries--overcoming internal barriers. Semin Perinatol. 2010; 34(2):145-55. doi:10.1053/j.semperi.2009.12.007

25. Bousfiha A, Jeddane L, Picard C, Al-Herz W, Ailal F, Chatila T, Cunningham-Rundles C, Etzioni A, Franco JL, Holland SM, Klein C, Morio T, Ochs HD, Oksenhendler E, Puck J, Torgerson TR, Casanova JL, Sullivan KE, Tangye SG. Human inborn errors of immunity: 2019 update of the iuis phenotypical classification. J Clin Immunol. 2020; 40(1):66-81. doi:10.1007/s10875-020-00758-x

26. Liao HC, Liao CH, Kao SM, Chiang CC, Chen YJ. Detecting 22q11.2 deletion syndrome in newborns with low t cell receptor excision circles from severe combined immunodeficiency screening. J Pediatr. 2019; 204:219-24.e1. doi:10.1016/j.jpeds.2018.08.072

27. Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. J Nutr. 2001; 131(5):1631s-6s. doi:10.1093/jn/131.5.1631S

28. Aranda CS, Guimarães RR, de Gouveia-Pereira Pimentel M. Combined immunodeficiencies. J Pediatr (Rio J). 2021; 97 Suppl 1(Suppl 1):S39-s48. doi:10.1016/j.jped.2020.10.014

29. Al-Herz W, Al-Mousa H. Combined immunodeficiency: The middle east experience. J Allergy Clin Immunol. 2013; 131(3):658-60. doi:10.1016/j.jaci.2012.11.033

30. Cossu F. Genetics of scid. Ital J Pediatr. 2010; 36:76. doi:10.1186/1824-7288-36-76

31. Gennery AR. Predicting the future with trecs. Blood. 2018; 132(17):1731-3. doi:10.1182/blood-2018-09-873638

32. Kanegae MP, Barreiros LA, Mazzucchelli JT, Hadachi SM, de Figueiredo Ferreira Guilhoto LM, Acquesta AL, Genov IR, Holanda SM, Di Gesu RS, Goulart AL, Dos Santos AM, Bellesi N, Costa-Carvalho BT, Condino-Neto A. Neonatal screening for severe combined immunodeficiency in brazil. J Pediatr (Rio J). 2016; 92(4):374-80. doi:10.1016/j.jped.2015.10.006

33. Korsunskiy I, Blyuss O, Gordukova M, Davydova N, Gordleeva S, Molchanov R, Asmanov A, Peshko D, Zinovieva N, Zimin S, Lazarev V, Salpagarova A, Filipenko M, Kozlov I, Prodeus A, Korsunskiy A, Hsu P, Munblit D. Trec and krec levels as a predictors of lymphocyte subpopulations measured by flow cytometry. Front Physiol. 2018; 9:1877. doi:10.3389/fphys.2018.01877

34. Trück J, Prader S, Natalucci G, Hagmann C, Brotschi B, Kelly J, Bassler D, Steindl K, Rauch A, Baumgartner M, Fingerhut R, Hauri-Hohl M, Güngör T, Pachlopnik Schmid J, Berger C, Reichenbach J. Swiss newborn screening for severe t and b cell deficiency with a combined trec/krec assay - management recommendations. Swiss Med Wkly. 2020; 150:w20254. doi:10.4414/smw.2020.20254

35. Baker MW, Grossman WJ, Laessig RH, Hoffman GL, Brokopp CD, Kurtycz DF, Cogley MF, Litsheim TJ, Katcher ML, Routes JM. Development of a routine newborn screening protocol for severe combined immunodeficiency. J Allergy Clin Immunol. 2009; 124(3):522-7. doi:10.1016/j.jaci.2009.04.007

36. Cirillo E, Giardino G, Gallo V, D'Assante R, Grasso F, Romano R, Di Lillo C, Galasso G, Pignata C. Severe combined immunodeficiency--an update. Ann N Y Acad Sci. 2015; 1356:90-106. doi:10.1111/nyas.12849

37. Froňková E, Klocperk A, Svatoň M, Nováková M, Kotrová M, Kayserová J, Kalina T, Keslová P, Votava F, Vinohradská H, Freiberger T, Mejstříková E, Trka J, Sedivá A. The trec/krec assay for the diagnosis and monitoring of patients with digeorge syndrome. PLoS One. 2014; 9(12):e114514. doi:10.1371/journal.pone.0114514

38. Kuo CY, Signer R, Saitta SC. Immune and genetic features of the chromosome 22q11.2 deletion (digeorge syndrome). Curr Allergy Asthma Rep. 2018; 18(12):75. doi:10.1007/s11882-018-0823-5

39. Altshuler E, Saidi A, Budd J. Digeorge syndrome: Consider the diagnosis. BMJ Case Rep. 2022; 15(2) doi:10.1136/bcr-2021-245164

40. Bayat M, Bayat A. Neurological manifestation of 22q11.2 deletion syndrome. Neurol Sci. 2022; 43(3):1695-700. doi:10.1007/s10072-021-05825-8

41. Gathmann B, Grimbacher B, Beauté J, Dudoit Y, Mahlaoui N, Fischer A, Knerr V, Kindle G. The european internet-based patient and research database for primary immunodeficiencies: Results 2006-2008. Clin Exp Immunol. 2009; 157 Suppl 1(Suppl 1):3-11. doi:10.1111/j.1365-2249.2009.03954.x

42. Javier FC, 3rd, Moore CM, Sorensen RU. Distribution of primary immunodeficiency diseases diagnosed in a pediatric tertiary hospital. Ann Allergy Asthma Immunol. 2000; 84(1):25-30. doi:10.1016/s1081-1206(10)62736-6

43. Kobrynski L, Powell RW, Bowen S. Prevalence and morbidity of primary immunodeficiency diseases, united states 2001-2007. J Clin Immunol. 2014; 34(8):954-61. doi:10.1007/s10875-014-0102-8

44. Coulter TI, Chandra A, Bacon CM, Babar J, Curtis J, Screaton N, Goodlad JR, Farmer G, Steele CL, Leahy TR, Doffinger R, Baxendale H, Bernatoniene J, Edgar JD, Longhurst HJ, Ehl S, Speckmann C, Grimbacher B, Sediva A, Milota T, Faust SN, Williams AP, Hayman G, Kucuk ZY, Hague R, French P, Brooker R, Forsyth P, Herriot R, Cancrini C, Palma P, Ariganello P, Conlon N, Feighery C, Gavin PJ, Jones A, Imai K, Ibrahim MA, Markelj G, Abinun M, Rieux-Laucat F, Latour S, Pellier I, Fischer A, Touzot F, Casanova JL, Durandy A, Burns SO, Savic S, Kumararatne DS, Moshous D, Kracker S, Vanhaesebroeck B, Okkenhaug K, Picard C, Nejentsev S, Condliffe AM, Cant AJ. Clinical spectrum and features of activated phosphoinositide 3-kinase δ syndrome: A large patient cohort study. J Allergy Clin Immunol. 2017; 139(2):597-606.e4. doi:10.1016/j.jaci.2016.06.021

45. Lougaris V, Soresina A, Baronio M, Montin D, Martino S, Signa S, Volpi S, Zecca M, Marinoni M, Baselli LA, Dellepiane RM, Carrabba M, Fabio G, Putti MC, Cinetto F, Lunardi C, Gazzurelli L, Benvenuto A, Bertolini P, Conti F, Consolini R, Ricci S, Azzari C, Leonardi L, Duse M, Pulvirenti F, Milito

C, Quinti I, Cancrini C, Finocchi A, Moschese V, Cirillo E, Crescenzi L, Spadaro G, Marasco C, Vacca A, Cardinale F, Martire B, Trizzino A, Licciardello M, Cossu F, Di Matteo G, Badolato R, Ferrari S, Giliani S, Pession A, Ugazio A, Pignata C, Plebani A. Long-term follow-up of 168 patients with x-linked agammaglobulinemia reveals increased morbidity and mortality. J Allergy Clin Immunol. 2020; 146(2):429-37. doi:10.1016/j.jaci.2020.03.001

46. Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood. 2012; 119(7):1650-7. doi:10.1182/blood-2011-09-377945

47. Tang P, Upton JEM, Barton-Forbes MA, Salvadori MI, Clynick MP, Price AK, Goobie SL. Autosomal recessive agammaglobulinemia due to a homozygous mutation in pik3r1. J Clin Immunol. 2018; 38(1):88-95. doi:10.1007/s10875-017-0462-y

48. Winkelstein JA, Marino MC, Lederman HM, Jones SM, Sullivan K, Burks AW, Conley ME, Cunningham-Rundles C, Ochs HD. X-linked agammaglobulinemia: Report on a united states registry of 201 patients. Medicine (Baltimore). 2006; 85(4):193-202. doi:10.1097/01.md.0000229482.27398.ad

49. Firtina S, Ng YY, Ng OH, Kiykim A, Ozek EY, Kara M, Aydiner E, Nepesov S, Camcioglu Y, Sayar EH, Gungoren EY, Reisli I, Torun SH, Haskologlu S, Cogurlu T, Kaya A, Cekic S, Baris S, Ozbek U, Ozen A, Sayitoglu M. Primary antibody deficiencies in turkey: Molecular and clinical aspects. Immunol Res. 2022; 70(1):44-55. doi:10.1007/s12026-021-09242-z

50. Demirdag YY, Gupta S. Update on infections in primary antibody deficiencies. Front Immunol. 2021; 12:634181. doi:10.3389/fimmu.2021.634181

51. de Felipe B, Olbrich P, Lucenas JM, Delgado-Pecellin C, Pavon-Delgado A, Marquez J, Salamanca C, Soler-Palacin P, Gonzalez-Granado LI, Antolin LF, Borte S, Neth O. Prospective neonatal screening for severe t- and b-lymphocyte deficiencies in seville. Pediatr Allergy Immunol. 2016; 27(1):70-7. doi:10.1111/pai.12501

52. Kanegae MPP, Barreiros LA, Sousa JL, Brito MAS, Oliveira EBJ, Soares LP, Mazzucchelli JTL, Fernandes DQ, Hadachi SM, Holanda SM, Guimarães F, Boacnin M, Pereira MAL, Bueno JMC, Grumach AS, Gesu R, Santos A, Bellesi N, Costa-Carvalho BT, Condino-Neto A. Newborn screening for severe combined immunodeficiencies using trecs and krecs: Second pilot study in brazil. Rev Paul Pediatr. 2017; 35(1):25-32. doi:10.1590/1984-0462/;2017;35;1;00013

53. El-Sayed ZA, Abramova I, Aldave JC, Al-Herz W, Bezrodnik L, Boukari R, Bousfiha AA, Cancrini C, Condino-Neto A, Dbaibo G, Derfalvi B, Dogu F, Edgar JDM, Eley B, El-Owaidy RH, Espinosa-Padilla

SE, Galal N, Haerynck F, Hanna-Wakim R, Hossny E, Ikinciogullari A, Kamal E, Kanegane H, Kechout N, Lau YL, Morio T, Moschese V, Neves JF, Ouederni M, Paganelli R, Paris K, Pignata C, Plebani A, Qamar FN, Qureshi S, Radhakrishnan N, Rezaei N, Rosario N, Routes J, Sanchez B, Sediva A, Seppanen MR, Serrano EG, Shcherbina A, Singh S, Siniah S, Spadaro G, Tang M, Vinet AM, Volokha A, Sullivan KE. X-linked agammaglobulinemia (xla):Phenotype, diagnosis, and therapeutic challenges around the world. World Allergy Organ J. 2019; 12(3):100018. doi:10.1016/j.waojou.2019.100018

54. Broderick L, Yost S, Li D, McGeough MD, Booshehri LM, Guaderrama M, Brydges SD, Kucharova K, Patel NC, Harr M, Hakonarson H, Zackai E, Cowell IG, Austin CA, Hügle B, Gebauer C, Zhang J, Xu X, Wang J, Croker BA, Frazer KA, Putnam CD, Hoffman HM. Mutations in topoisomerase iiβ result in a b cell immunodeficiency. Nat Commun. 2019; 10(1):3644. doi:10.1038/s41467-019-11570-6

55. Yazdani R, Habibi S, Sharifi L, Azizi G, Abolhassani H, Olbrich P, Aghamohammadi A. Common variable immunodeficiency: Epidemiology, pathogenesis, clinical manifestations, diagnosis, classification, and management. J Investig Allergol Clin Immunol. 2020; 30(1):14-34. doi:10.18176/jiaci.0388

56. Bagheri Y, Vosughi A, Azizi G, Yazdani R, Kiaee F, Hafezi N, Alimorad S, Khoshmirsafa M, Seif F, Hassanpour G, Abolhassani H, Aghamohammadi A. Comparison of clinical and immunological features and mortality in common variable immunodeficiency and agammaglobulinemia patients. Immunol Lett. 2019; 210:55-62. doi:10.1016/j.imlet.2019.05.001

57. Yazdani R, Abolhassani H, Kiaee F, Habibi S, Azizi G, Tavakol M, Chavoshzadeh Z, Mahdaviani SA, Momen T, Gharagozlou M, Movahedi M, Hamidieh AA, Behniafard N, Nabavi M, Bemanian MH, Arshi S, Molatefi R, Sherkat R, Shirkani A, Amin R, Aleyasin S, Faridhosseini R, Jabbari-Azad F, Mohammadzadeh I, Ghaffari J, Shafiei A, Kalantari A, Mansouri M, Mesdaghi M, Babaie D, Ahanchian H, Khoshkhui M, Soheili H, Eslamian MH, Cheraghi T, Dabbaghzadeh A, Tavassoli M, Kalmarzi RN, Mortazavi SH, Kashef S, Esmaeilzadeh H, Tafaroji J, Khalili A, Zandieh F, Sadeghi-Shabestari M, Darougar S, Behmanesh F, Akbari H, Zandkarimi M, Abolnezhadian F, Fayezi A, Moghtaderi M, Ahmadiafshar A, Shakerian B, Sajedi V, Taghvaei B, Safari M, Heidarzadeh M, Ghalebaghi B, Fathi SM, Darabi B, Bazregari S, Bazargan N, Fallahpour M, Khayatzadeh A, Javahertrash N, Bashardoust B, Zamani M, Mohsenzadeh A, Ebrahimi S, Sharafian S, Vosughimotlagh A, Tafakoridelbari M, Rahim M, Ashournia P, Razaghian A, Rezaei A, Samavat A, Mamishi S, Khazaei HA, Mohammadi J, Negahdari B, Parvaneh N, Rezaei N, Lougaris V, Giliani S, Plebani A, Ochs HD, Hammarström L, Aghamohammadi A. Comparison of common monogenic defects in a large predominantly antibody deficiency cohort. J Allergy Clin Immunol Pract. 2019; 7(3):864-78.e9. doi:10.1016/j.jaip.2018.09.004

58. Grumach AS, Kirschfink M. Are complement deficiencies really rare? Overview on prevalence, clinical importance and modern diagnostic approach. Mol Immunol. 2014; 61(2):110-7. doi:10.1016/j.molimm.2014.06.030

59. Botto M, Kirschfink M, Macor P, Pickering MC, Würzner R, Tedesco F. Complement in human diseases: Lessons from complement deficiencies. Mol Immunol. 2009; 46(14):2774-83. doi:10.1016/j.molimm.2009.04.029

60. Modell V, Gee B, Lewis DB, Orange JS, Roifman CM, Routes JM, Sorensen RU, Notarangelo LD, Modell F. Global study of primary immunodeficiency diseases (pi)--diagnosis, treatment, and economic impact: An updated report from the jeffrey modell foundation. Immunol Res. 2011; 51(1):61-70. doi:10.1007/s12026-011-8241-y

61. Naidoo R, Ungerer L, Cooper M, Pienaar S, Eley BS. Primary immunodeficiencies: A 27-year review at a tertiary paediatric hospital in cape town, south africa. J Clin Immunol. 2011; 31(1):99-105. doi:10.1007/s10875-010-9465-7

62. Kolukısa B, Barış S. Primary immune regulatory disorders and targeted therapies. Turk J Haematol. 2021; 38(1):1-14. doi:10.4274/tjh.galenos.2021.2020.0724

63. Baris S, Schulze I, Ozen A, Karakoç Aydıner E, Altuncu E, Karasu GT, Ozturk N, Lorenz M, Schwarz K, Vraetz T, Ehl S, Barlan IB. Clinical heterogeneity of immunodysregulation, polyendocrinopathy, enteropathy, x-linked: Pulmonary involvement as a non-classical disease manifestation. J Clin Immunol. 2014; 34(6):601-6. doi:10.1007/s10875-014-0059-7

64. Cepika AM, Sato Y, Liu JM, Uyeda MJ, Bacchetta R, Roncarolo MG. Tregopathies: Monogenic diseases resulting in regulatory t-cell deficiency. J Allergy Clin Immunol. 2018; 142(6):1679-95. doi:10.1016/j.jaci.2018.10.026

65. Verbsky JW, Chatila TA. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked (ipex) and ipex-related disorders: An evolving web of heritable autoimmune diseases. Curr Opin Pediatr. 2013; 25(6):708-14. doi:10.1097/mop.0000000000000029

66. Tsilifis C, Slatter MA, Gennery AR. Too much of a good thing: A review of primary immune regulatory disorders. Front Immunol. 2023; 14:1279201. doi:10.3389/fimmu.2023.1279201

67. DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. Lancet. 2018; 391(10138):2449-62. doi:10.1016/s0140-6736(18)31320-5

68. Terrell DR, Beebe LA, Vesely SK, Neas BR, Segal JB, George JN. The incidence of immune thrombocytopenic purpura in children and adults: A critical review of published reports. Am J Hematol. 2010; 85(3):174-80. doi:10.1002/ajh.21616

69. Kuruvilla M, de la Morena MT. Antibiotic prophylaxis in primary immune deficiency disorders. J Allergy Clin Immunol Pract. 2013; 1(6):573-82. doi:10.1016/j.jaip.2013.09.013

70. Kuo CY, Kohn DB. Overview of the current status of gene therapy for primary immune deficiencies (pids). J Allergy Clin Immunol. 2020; 146(2):229-33. doi:10.1016/j.jaci.2020.05.024

71. Evans C, Jones CE, Prendergast AJ. Hiv-exposed, uninfected infants: New global challenges in the era of paediatric hiv elimination. Lancet Infect Dis. 2016; 16(6):e92-e107. doi:10.1016/s1473-3099(16)00055-4

72. (UNAIDS) TJUNPoHA [Internet]. 2015 progress report on the global plan towards the elimination of new hiv infections among children and keeping their mothers alive. 2015 [cited 2024 10 July 2024]. Available from: https://www.unaids.org/en/resources/documents/2015/JC2774_2015ProgressReport_GlobalPlan.

73. Kourtis AP, Wiener J, Kayira D, Chasela C, Ellington SR, Hyde L, Hosseinipour M, van der Horst C, Jamieson DJ. Health outcomes of hiv-exposed uninfected african infants. Aids. 2013; 27(5):749-59. doi:10.1097/QAD.0b013e32835ca29f

74. Kuhn L, Sinkala M, Semrau K, Kankasa C, Kasonde P, Mwiya M, Hu CC, Tsai WY, Thea DM, Aldrovandi GM. Elevations in mortality associated with weaning persist into the second year of life among uninfected children born to hiv-infected mothers. Clin Infect Dis. 2010; 50(3):437-44. doi:10.1086/649886

75. Wei R, Msamanga GI, Spiegelman D, Hertzmark E, Baylin A, Manji K, Fawzi WW. Association between low birth weight and infant mortality in children born to human immunodeficiency virus 1-infected mothers in tanzania. Pediatr Infect Dis J. 2004; 23(6):530-5. doi:10.1097/01.inf.0000129691.42964.eb

76. Slyker JA, Patterson J, Ambler G, Richardson BA, Maleche-Obimbo E, Bosire R, Mbori-Ngacha D, Farquhar C, John-Stewart G. Correlates and outcomes of preterm birth, low birth weight, and small for gestational age in hiv-exposed uninfected infants. BMC Pregnancy Childbirth. 2014; 14:7. doi:10.1186/1471-2393-14-7

77. Miyamoto M, Gouvêa A, Ono E, Succi RCM, Pahwa S, Moraes-Pinto MI. Immune development in hiv-exposed uninfected children born to hiv-infected women. Rev Inst Med Trop Sao Paulo. 2017; 59:e30. doi:10.1590/s1678-9946201759030

78. Warszawski J, Thomas C, Dialla O, Garrait V, Dollfus C, Reliquet V, Clech L, Dert C, Mandelbrot L, Audrain M, Blanche S. T-cell receptor excision circles in hiv-exposed, uninfected newborns

measured during a national newborn screening program for severe combined immunodeficiency. J Pediatr. 2018; 202:311-4.e2. doi:10.1016/j.jpeds.2018.06.008

79. Clerici M, Saresella M, Colombo F, Fossati S, Sala N, Bricalli D, Villa ML, Ferrante P, Dally L, Vigano A. T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to hiv. Blood. 2000; 96(12):3866-71.

80. Locke BA, Dasu T, Verbsky JW. Laboratory diagnosis of primary immunodeficiencies. Clin Rev Allergy Immunol. 2014; 46(2):154-68. doi:10.1007/s12016-014-8412-4

81. Quiros-Roldan E, Serana F, Chiarini M, Zanotti C, Sottini A, Gotti D, Torti C, Caimi L, Imberti L. Effects of combined antiretroviral therapy on b- and t-cell release from production sites in long-term treated hiv-1+ patients. J Transl Med. 2012; 10:94. doi:10.1186/1479-5876-10-94

82. Al-Harthi L, Marchetti G, Steffens CM, Poulin J, Sékaly R, Landay A. Detection of t cell receptor circles (trecs) as biomarkers for de novo t cell synthesis using a quantitative polymerase chain reaction-enzyme linked immunosorbent assay (pcr-elisa). J Immunol Methods. 2000; 237(1-2):187-97. doi:10.1016/s0022-1759(00)00136-8

83. Tagliaferri L, Kunz JB, Happich M, Esposito S, Bruckner T, Hübschmann D, Okun JG, Hoffmann GF, Schulz A, Kappe J, Speckmann C, Muckenthaler MU, Kulozik AE. Newborn screening for severe combined immunodeficiency using a novel and simplified method to measure t-cell excision circles (trec). Clin Immunol. 2017; 175:51-5. doi:10.1016/j.clim.2016.11.016

84. Ravkov E, Slev P, Heikal N. Thymic output: Assessment of cd4(+) recent thymic emigrants and t-cell receptor excision circles in infants. Cytometry B Clin Cytom. 2017; 92(4):249-57. doi:10.1002/cyto.b.21341

85. Ramos BC, Aranda CS, Sobrinho AO, Sole D, Condino-Neto A. Trecs/krecs: Beyond the diagnosis of severe combined immunodeficiency. J Clin Immunol. 2023; 43(1):80-1. doi:10.1007/s10875-022-01363-w

86. Steffens CM, Al-Harthi L, Shott S, Yogev R, Landay A. Evaluation of thymopoiesis using t cell receptor excision circles (trecs): Differential correlation between adult and pediatric trecs and naïve phenotypes. Clin Immunol. 2000; 97(2):95-101. doi:10.1006/clim.2000.4938

87. Centerwall SA, Centerwall WR. The discovery of phenylketonuria: The story of a young couple, two retarded children, and a scientist. Pediatrics. 2000; 105(1 Pt 1):89-103. doi:10.1542/peds.105.1.89

88. King JR, Grill K, Hammarström L. Genomic-based newborn screening for inborn errors of immunity: Practical and ethical considerations. Int J Neonatal Screen. 2023; 9(2) doi:10.3390/ijns9020022

89. Følling I. The discovery of phenylketonuria. Acta Paediatr Suppl. 1994; 407:4-10. doi:10.1111/j.1651-2227.1994.tb13440.x

90. Schmid-Rüter E. [early diagnosis of hereditary metabolic diseases in the newborn by means of microbiological guthrie tests]. Fortschr Med. 1978; 96(24):1289-93.

91. Cornel MC, Rigter T, Jansen ME, Henneman L. Neonatal and carrier screening for rare diseases: How innovation challenges screening criteria worldwide. J Community Genet. 2021; 12(2):257-65. doi:10.1007/s12687-020-00488-y

92. Jiang T, Li Z, Zhang Q. Advances in neonatal screening for primary immune deficiencies. Exp Ther Med. 2016; 11(5):1542-4. doi:10.3892/etm.2016.3119

93. Puck JM. Newborn screening for severe combined immunodeficiency and t-cell lymphopenia. Immunol Rev. 2019; 287(1):241-52. doi:10.1111/imr.12729

94. Haddad E, Logan BR, Griffith LM, Buckley RH, Parrott RE, Prockop SE, Small TN, Chaisson J, Dvorak CC, Murnane M, Kapoor N, Abdel-Azim H, Hanson IC, Martinez C, Bleesing JJH, Chandra S, Smith AR, Cavanaugh ME, Jyonouchi S, Sullivan KE, Burroughs L, Skoda-Smith S, Haight AE, Tumlin AG, Quigg TC, Taylor C, Dávila Saldaña BJ, Keller MD, Seroogy CM, Desantes KB, Petrovic A, Leiding JW, Shyr DC, Decaluwe H, Teira P, Gillio AP, Knutsen AP, Moore TB, Kletzel M, Craddock JA, Aquino V, Davis JH, Yu LC, Cuvelier GDE, Bednarski JJ, Goldman FD, Kang EM, Shereck E, Porteus MH, Connelly JA, Fleisher TA, Malech HL, Shearer WT, Szabolcs P, Thakar MS, Vander Lugt MT, Heimall J, Yin Z, Pulsipher MA, Pai SY, Kohn DB, Puck JM, Cowan MJ, O'Reilly RJ, Notarangelo LD. Scid genotype and 6-month posttransplant cd4 count predict survival and immune recovery. Blood. 2018; 132(17):1737-49. doi:10.1182/blood-2018-03-840702

95. Baris S, Abolhassani H, Massaad MJ, Al-Nesf M, Chavoshzadeh Z, Keles S, Reisli I, Tahiat A, Shendi HM, Elaziz DA, Belaid B, Al Dhaheri F, Haskologlu S, Dogu F, Ben-Mustapha I, Sobh A, Galal N, Meshaal S, Elhawary R, El-Marsafy A, Alroqi FJ, Al-Saud B, Al-Ahmad M, Al Farsi T, Al Sukaiti N, Al-Tamemi S, Mehawej C, Dbaibo G, ElGhazali G, Kilic SS, Genel F, Kiykim A, Musabak U, Artac H, Guner SN, Boukari R, Djidjik R, Kechout N, Cagdas D, El-Sayed ZA, Karakoc-Aydiner E, Alzyoud R, Barbouche MR, Adeli M, Wakim RH, Reda SM, Ikinciogullari A, Ozen A, Bousfiha A, Al-Mousa H, Rezaei N, Al-Herz W, Geha RS. The middle east and north africa diagnosis and management guidelines for inborn errors of immunity. J Allergy Clin Immunol Pract. 2023; 11(1):158-80.e11. doi:10.1016/j.jaip.2022.10.003

96. Aghamohammadi A, Rezaei N, Yazdani R, Delavari S, Kutukculer N, Topyildiz E, Ozen A, Baris S, Karakoc-Aydiner E, Kilic SS, Kose H, Gulez N, Genel F, Reisli I, Djenouhat K, Tahiat A, Boukari R, Ladj S, Belbouab R, Ferhani Y, Belaid B, Djidjik R, Kechout N, Attal N, Saidani K, Barbouche R, Bousfiha A, Sobh A, Rizk R, Elnagdy MH, Al-Ahmed M, Al-Tamemi S, Nasrullayeva G, Adeli M, Al-Nesf M, Hassen A, Mehawej C, Irani C, Megarbane A, Quinn J, Maródi L, Modell V, Modell F, Al-Herz W, Geha RS, Abolhassani H. Consensus middle east and north africa registry on inborn errors of immunity. J Clin Immunol. 2021; 41(6):1339-51. doi:10.1007/s10875-021-01053-z

97. Modell V, Knaus M, Modell F. An analysis and decision tool to measure cost benefit of newborn screening for severe combined immunodeficiency (scid) and related t-cell lymphopenia. Immunol Res. 2014; 60(1):145-52. doi:10.1007/s12026-014-8485-4

98. Chan K, Davis J, Pai SY, Bonilla FA, Puck JM, Apkon M. A markov model to analyze cost-effectiveness of screening for severe combined immunodeficiency (scid). Mol Genet Metab. 2011; 104(3):383-9. doi:10.1016/j.ymgme.2011.07.007

99. Kobrynski LJ. Newborn screening in the diagnosis of primary immunodeficiency. Clin Rev Allergy Immunol. 2022; 63(1):9-21. doi:10.1007/s12016-021-08876-z

100. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, Baker M, Ballow M, Bartoshesky LE, Bonilla FA, Brokopp C, Brooks E, Caggana M, Celestin J, Church JA, Comeau AM, Connelly JA, Cowan MJ, Cunningham-Rundles C, Dasu T, Dave N, De La Morena MT, Duffner U, Fong CT, Forbes L, Freedenberg D, Gelfand EW, Hale JE, Hanson IC, Hay BN, Hu D, Infante A, Johnson D, Kapoor N, Kay DM, Kohn DB, Lee R, Lehman H, Lin Z, Lorey F, Abdel-Mageed A, Manning A, McGhee S, Moore TB, Naides SJ, Notarangelo LD, Orange JS, Pai SY, Porteus M, Rodriguez R, Romberg N, Routes J, Ruehle M, Rubenstein A, Saavedra-Matiz CA, Scott G, Scott PM, Secord E, Seroogy C, Shearer WT, Siegel S, Silvers SK, Stiehm ER, Sugerman RW, Sullivan JL, Tanksley S, Tierce MLt, Verbsky J, Vogel B, Walker R, Walkovich K, Walter JE, Wasserman RL, Watson MS, Weinberg GA, Weiner LB, Wood H, Yates AB, Puck JM, Bonagura VR. Newborn screening for severe combined immunodeficiency in 11 screening programs in the united states. Jama. 2014; 312(7):729-38. doi:10.1001/jama.2014.9132

101. Justiz Vaillant AA, Mohseni M. Severe combined immunodeficiency. Statpearls. Treasure Island (FL) with ineligible companies. Disclosure: Michael Mohseni declares no relevant financial relationships with ineligible companies.: StatPearls Publishing

102. Marinova M, Georgyeva A, Yordanova V, Ivanov N, Atanasova V, Naumova E, Kandilarova SM. Implementation of trec/krec detection protocol for newborn scid screening in bulgaria: A pilot study. Cent Eur J Immunol. 2022; 47(4):339-49. doi:10.5114/ceji.2022.124396

103. Shinwari K, Bolkov M, Tuzankina IA, Chereshnev VA. Newborn screening through trec, trec/krec system for primary immunodeficiency with limitation of trec/krec. Comprehensive review. Antiinflamm Antiallergy Agents Med Chem. 2021; 20(2):132-49. doi:10.2174/1871523019999200730171600

104. Currier R, Puck JM. Scid newborn screening: What we've learned. J Allergy Clin Immunol. 2021; 147(2):417-26. doi:10.1016/j.jaci.2020.10.020

105. Buckley RH. Scid: A pediatric emergency. N C Med J. 2019; 80(1):55-6. doi:10.18043/ncm.80.1.55

106. Taki M, Miah T, Secord E. Newborn screening for severe combined immunodeficiency. Immunol Allergy Clin North Am. 2021; 41(4):543-53. doi:10.1016/j.iac.2021.07.007

107. Waltenburg R, Kobrynski L, Reyes M, Bowen S, Khoury MJ. Primary immunodeficiency diseases: Practice among primary care providers and awareness among the general public, united states, 2008. Genet Med. 2010; 12(12):792-800. doi:10.1097/GIM.0b013e3181f3e2c9

108. Sottini A, Serana F, Bertoli D, Chiarini M, Valotti M, Vaglio Tessitore M, Imberti L. Simultaneous quantification of t-cell receptor excision circles (trecs) and k-deleting recombination excision circles (krecs) by real-time pcr. J Vis Exp. 2014; (94) doi:10.3791/52184

109. Scientific T. Taqman™ assay multiplex pcr optimization

application guide. 2022;

110. Akalu YT, Bogunovic D. Inborn errors of immunity: An expanding universe of disease and genetic architecture. Nat Rev Genet. 2024; 25(3):184-95. doi:10.1038/s41576-023-00656-z

111. Tangye SG, Al-Herz W, Bousfiha A, Cunningham-Rundles C, Franco JL, Holland SM, Klein C, Morio T, Oksenhendler E, Picard C, Puel A, Puck J, Seppänen MRJ, Somech R, Su HC, Sullivan KE, Torgerson TR, Meyts I. Human inborn errors of immunity: 2022 update on the classification from the international union of immunological societies expert committee. J Clin Immunol. 2022; 42(7):1473-507. doi:10.1007/s10875-022-01289-3

112. Cheremokhin DA, Shinwari K, Deryabina SS, Bolkov MA, Tuzankina IA, Kudlay DA. Analysis of the trec and krec levels in the dried blood spots of healthy newborns with different gestational ages and weights. Acta Naturae. 2022; 14(1):101-8. doi:10.32607/actanaturae.11501

113. Chiarini M, Zanotti C, Serana F, Sottini A, Bertoli D, Caimi L, Imberti L. T-cell receptor and k-deleting recombination excision circles in newborn screening of t- and b-cell defects: Review of the literature and future challenges. J Public Health Res. 2013; 2(1):9-16. doi:10.4081/jphr.2013.e3

114. Abu-Raya B, Kollmann TR, Marchant A, MacGillivray DM. The immune system of hiv-exposed uninfected infants. Front Immunol. 2016; 7:383. doi:10.3389/fimmu.2016.00383

115. Afran L, Garcia Knight M, Nduati E, Urban BC, Heyderman RS, Rowland-Jones SL. Hiv-exposed uninfected children: A growing population with a vulnerable immune system? Clin Exp Immunol. 2014; 176(1):11-22. doi:10.1111/cei.12251

116. Dirajlal-Fargo S, Mussi-Pinhata MM, Weinberg A, Yu Q, Cohen R, Harris DR, Bowman E, Gabriel J, Kulkarni M, Funderburg N, Chakhtoura N, McComsey GA. Hiv-exposed-uninfected infants have increased inflammation and monocyte activation. Aids. 2019; 33(5):845-53. doi:10.1097/qad.0000000000002128

117. Filteau S. The hiv-exposed, uninfected african child. Trop Med Int Health. 2009; 14(3):276-87. doi:10.1111/j.1365-3156.2009.02220.x

118. Klak A, Pauwels S, Vermeersch P. Preanalytical considerations in therapeutic drug monitoring of immunosuppressants with dried blood spots. Diagnosis (Berl). 2019; 6(1):57-68. doi:10.1515/dx-2018-0034

119. Lee YR, Lee J, Kang HG. Presoaking dried blood spot with water improves efficiency for small-molecule analysis. Biotechniques. 2019; 67(5):219-28. doi:10.2144/btn-2019-0043

120. Ryšavá L, Dvořák M, Kubáň P. Dried blood spot self-sampling with automated capillary electrophoresis processing for clinical analysis. Angew Chem Int Ed Engl. 2021; 60(11):6068-75. doi:10.1002/anie.202012997

121. Ait Belkacem I, Mossadegh-Keller N, Bourgoin P, Arnoux I, Loosveld M, Morange PE, Markarian T, Michelet P, Busnel JM, Roulland S, Galland F, Malergue F. Cell analysis from dried blood spots: New opportunities in immunology, hematology, and infectious diseases. Adv Sci (Weinh). 2021; 8(18):e2100323. doi:10.1002/advs.202100323

122. Snijdewind IJ, van Kampen JJ, Fraaij PL, van der Ende ME, Osterhaus AD, Gruters RA. Current and future applications of dried blood spots in viral disease management. Antiviral Res. 2012; 93(3):309-21. doi:10.1016/j.antiviral.2011.12.011

123. Göhring K, Dietz K, Hartleif S, Jahn G, Hamprecht K. Influence of different extraction methods and pcr techniques on the sensitivity of hcmv-DNA detection in dried blood spot (dbs) filter cards. J Clin Virol. 2010; 48(4):278-81. doi:10.1016/j.jcv.2010.04.011

124. Lang PO, Govind S, Dramé M, Aspinall R. Measuring the trec ratio in dried blood spot samples: Intra- and inter-filter paper cards reproducibility. J Immunol Methods. 2013; 389(1-2):1-8. doi:10.1016/j.jim.2012.12.003

125. Nsojo A, Aboud S, Lyamuya E. Comparative evaluation of amplicor hiv-1 DNA test, version 1.5, by manual and automated DNA extraction methods using venous blood and dried blood spots for hiv-1 DNA pcr testing. Tanzan J Health Res. 2010; 12(4):229-35. doi:10.4314/thrb.v12i4.58621

126. Wen W, Guo M, Peng HB, Ma L. Optimization and application of a dried blood spot-based genetic screening method for thalassemia in shenzhen newborns. World J Pediatr. 2019; 15(6):610-4. doi:10.1007/s12519-018-00222-2

127. Lang PO, Mitchell WA, Govind S, Aspinall R. Real time-pcr assay estimating the naive t-cell pool in whole blood and dried blood spot samples: Pilot study in young adults. J Immunol Methods. 2011; 369(1-2):133-40. doi:10.1016/j.jim.2011.05.002

128. McDade TW, Williams S, Snodgrass JJ. What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography. 2007; 44(4):899-925. doi:10.1353/dem.2007.0038

129. Li CC, Beck IA, Seidel KD, Frenkel LM. Persistence of human immunodeficiency virus type 1 subtype b DNA in dried-blood samples on fta filter paper. J Clin Microbiol. 2004; 42(8):3847-9. doi:10.1128/jcm.42.8.3847-3849.2004

130. Kumar A, Mhatre S, Godbole S, Jha P, Dikshit R. Optimization of extraction of genomic DNA from archived dried blood spot (dbs): Potential application in epidemiological research & bio banking. Gates Open Res. 2018; 2:57. doi:10.12688/gatesopenres.12855.3

131. Lang PO, Govind S, Dramé M, Aspinall R. Comparison of manual and automated DNA purification for measuring trec in dried blood spot (dbs) samples with qpcr. J Immunol Methods. 2012; 384(1-2):118-27. doi:10.1016/j.jim.2012.07.016

132. Scientific T [Internet]. Interpretation of nucleic acid 260/280 ratios. Wilmington, Delaware USA Thermo Scientific; 2012 [cited 2024 05 February 2024]. Available from: www.thermoscientific.com/nanodrop.

133. Qureshi F, Hu W, Loh L, Patel H, DeGuzman M, Becich M, Rubio da Costa F, Gehman V, Zhang F, Foley J, Chitnis T. Analytical validation of a multi-protein, serum-based assay for disease activity assessments in multiple sclerosis. Proteomics Clin Appl. 2023; 17(3):e2200018. doi:10.1002/prca.202200018

134. Borte S, von Döbeln U, Fasth A, Wang N, Janzi M, Winiarski J, Sack U, Pan-Hammarström Q, Borte M, Hammarström L. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time pcr. Blood. 2012; 119(11):2552-5. doi:10.1182/blood-2011-08-371021

135. Sottini A, Ghidini C, Zanotti C, Chiarini M, Caimi L, Lanfranchi A, Moratto D, Porta F, Imberti L. Simultaneous quantification of recent thymic t-cell and bone marrow b-cell emigrants in patients with primary immunodeficiency undergone to stem cell transplantation. Clin Immunol. 2010; 136(2):217-27. doi:10.1016/j.clim.2010.04.005

136. Sender R, Weiss Y, Navon Y, Milo I, Azulay N, Keren L, Fuchs S, Ben-Zvi D, Noor E, Milo R. The total mass, number, and distribution of immune cells in the human body. Proc Natl Acad Sci U S A. 2023; 120(44):e2308511120. doi:10.1073/pnas.2308511120

137. Pietrangelo A [Internet]. What's the connection between viral load and risk of hiv transmission? 2020 [cited 2024 22 November 2024]. Available from: https://www.healthline.com/health/hiv-aids/undetectable-viral-load-transmission-risk.

138. Zhang W, Ruan L. Recent advances in poor hiv immune reconstitution: What will the future look like? Front Microbiol. 2023; 14:1236460. doi:10.3389/fmicb.2023.1236460

139. Moir S, Fauci AS. B cells in hiv infection and disease. Nat Rev Immunol. 2009; 9(4):235-45. doi:10.1038/nri2524

140. Goronzy JJ, Shao L, Weyand CM. Immune aging and rheumatoid arthritis. Rheum Dis Clin North Am. 2010; 36(2):297-310. doi:10.1016/j.rdc.2010.03.001

141. Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ. The influence of age on t cell generation and tcr diversity. J Immunol. 2005; 174(11):7446-52. doi:10.4049/jimmunol.174.11.7446

Appendices

Appendix A: Ethics approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

20 November 2024

Approval Certificate
Annual Renewal

Dear Miss SS Singo,

Ethics Reference No.: 573/2023 – Line 2
Title: Establishing an in-house real-time polymerase chain reaction assay to quantify T-cell receptor excision circles and kappa-deleting recombination excision circles for use in screening newborn babies for inborn errors of immunity

The **Annual Renewal** as supported by documents received between 2024-10-08 and 2024-11-18 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-11-18 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-11-20.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (573/2023) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor Theresa (TM) Rossouw
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag X323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tsa Maphelo

Appendix B: Reproduction of the formation of TREC diagram from another article permission slip

Clinical Immunology
Vol. 97, No. 2, November, pp. 95–101, 2000
doi:10.1006/clim.2000.4938, available online at http://www.idealibrary.com on IDEAL®

Evaluation of Thymopoiesis Using T Cell Receptor Excision Circles (TRECs): Differential Correlation between Adult and Pediatric TRECs and Naïve Phenotypes

Carolyn M. Steffens,* Lena Al-Harthi,*,[1] Susan Shott,† Ram Yogev,‡ and Alan Landay*

*Department of Immunology/Microbiology and †Section of Biostatistics, Department of Obstetrics and Gynecology at Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612; and ‡Department of Pediatrics, Division of Infectious Diseases, Children's Memorial Hospital/Northwestern University Medical School, Chicago, Illinois 60614

[1] To whom correspondence and reprint requests should be addressed at Rush-Presbyterian-St. Luke's Medical Center, Department of Immunology/Microbiology, 1653 West Congress Parkway, Rm. 1577 JSC, Chicago, IL 60612. Fax: 312-942-2808. E-mail: lalharth@rush.edu.

Permision to use a figure from your article External Inbox ×

Shudufhadzo Singo <u18017411@tuks.co.za> Wed, 26 Apr 2023, 09:48
to lalharth

Dear sir/madam

My name is Shudufhadzo Sharon Singo and I am an MSc student at the University of Pretoria medical immunology department doing a study on T cell receptor excision circles and Kappa-deleting recombination excision circles. I would like to use a figure from your article, "Evaluation of Thymopoiesis Using T Cell Receptor Excision Circles (TRECs): Differential Correlation between Adult and Paediatric TRECs and Naive Phenotypes," that was published in 2000 issue number 2 under the clinical immunology journal. I am asking for permission to use "Fig. 1: Generation of Coding Joint TRECs" for my study. The figure will be referenced accordingly. Your response will be highly appreciated.

Kind regards
Shudufhadzo Singo

Lena Al-Harthi <Lena_Al-Harthi@rush.edu> Wed, 26 Apr 2023, 16:21
to me

Sure you can use the figure, although I am not sure if the journal owns that copy right now? did not have this issue come up and I am ok with it

Good luck

Appendix C: Demographic characteristics of the human immunodeficiency virus-exposed uninfected and human immunodeficiency virus-unexposed uninfected newborn cohorts: this represents the average value of all the neonatal hospital visits for week 0 and 24 months.

HEU infants	**Gender**	**Appearance, Pulse, Grimace, Activity, and Respiration (APGAR) score**	**Weight (kg)**		**Length (cm)**		**Head circumference (HC) (cm)**		**Mid-upper arm circumference (MUAC) (cm)**	
			Week 0	**24 months**	**Week 0**	**24 months**	**Week 0**	**24 months**	**Week 0**	**24 months**
HEU 20	Female	6	2.76		48	N/A	35	N/A	12	N/A
HEU 34	Male	9	2.66	9.7	50	85.5	34	47.8	N/A	15
HEU 50	Male	9	2.285	12.07	45	69.9	35	50	N/A	17
HEU 63	Female	8.5	1.575	9.4	41	77	30	46.3	N/A	16.4
HEU 75	Female	9	3.03	11.3	51	79.5	33	48.1	N/A	15.9
HEU 79	Male	9	3.855	8.83	50	74.9	37	47.1	N/A	17.1
HEU 84	Female	8.5	3.61	10.2	55	93	36	48.9	N/A	15.5
HEU 93	Female	Not available (N/A)	3.39	11.2	52	85.6	33	46	N/A	15.4
HEU 111	Male	9.5	3.095	13.48	54	93	34	49.4	N/A	16.2
HEU 133	Female	9	2.14	10	48	82.5	29	45	N/A	16
HEU 138	Male	8.5	3.85	N/A	57	N/A	37	N/A	N/A	N/A
HEU 143	Female	9.5	3.32	11.25	49	85.8	33	49.2	N/A	17

HEU 149	Male	Not recorded	3.14	9.5	N/A	81.2	N/A	50.1	N/A	14
HEU 150	Female	9.5	2.32	11.8	48	91	32	48.2	N/A	15.4
HEU 172	Male	8.5	3.235	10.88	50	86.4	36	48.2	30	16.1
HEU 182	Male	8.5	3.29	14.15	48	90.6	35	50	N/A	18
HEU 198	Male	9	2.94	10.1	N/A	84.5	36	48.6	N/A	14.5
HEU 199	Female	9.5	2.8	12.04	48	91.6	33	50	N/A	17
HEU 203	Male	9	3.06	12.59	49	88.7	35	49.3	N/A	16.8
HEU 206	Male	8.5	2.6	N/A	51	N/A	32	N/A	N/A	N/A
HEU 207	Male	N/A	3.2	9.4	52	83	35	50	N/A	15
HEU 219	Female	9	3.1	11.83	N/A	89	32	50	N/A	14.5
HEU 241	Female	8.5	2.25	N/A	47	N/A	34	N/A	N/A	N/A
HEU 243	Male	9	3.02	11.6	54	81	36	50	N/A	16
HEU 249	Male	8.5	2.98	10.54	51	84.5	35	48.2	N/A	15
HEU 258	Male	9	2.91	N/A	50	N/A	32	N/A	N/A	N/A
HEU 260	Female	10	3.4	11.33	49	84.3	36	49.2	N/A	17
HEU 262	Female	9	3.23	11.77	50	84.4	N/A	48	N/A	17
HEU 268	Female	9.5	3.036	N/A	54	N/A	N/A	N/A	N/A	N/A
HEU 273	Male	9.5	2.975	11.64	49	83.2	35	48.6	N/A	16.8
HEU 278	Male	9	3.45	10.41	54	83.6	34	48	N/A	14.5
HEU 291	Male	8.5	3.2	12.42	51	83.8	36	48.2	N/A	16

HUU infants	Gender	Appearance, Pulse, Grimace, Activity, and Respiration (APGAR) score	Weight (kg)		Length (cm)		Head circumference (cm)		Mid-upper arm circumference (cm)	
			Week 0	24 months	Week 0	24 months	Week 0	24 months	Week 0	24 months
HUU 07	Female	9	2.66	11.4	48	86	33	47.6	N/A	16
HUU 08	Male	8.5	2.58	14	50	92	34	52	N/A	18
HUU 25	Male	8.5	3.4	13.2	56	88.5	36	47.9	N/A	18
HUU 119	Female	N/A	3.1	11.1	N/A	86.2	N/A	49.8	N/A	16.4
HUU 125	Female	9.5	3.2	11.1	N/A	85.7	38	51	N/A	16
HUU 128	Female	N/A	3.54	12.5	N/A	89.1	N/A	47.2	N/A	17.2
HUU 135	Male	9.5	1.44	13.2	41	89	N/A	49.5	N/A	15.8
HUU 137	Female	N/A	3.7	12.8	52	78.8	36	49	N/A	17.4
HUU 142	Female	9.5	2.68	10.6	46	85	33	47.2	N/A	14.7
HUU 144	Female	9.5	3.1	12.1	50	89	34	48.6	N/A	16.4
HUU 192	Female	N/A	3.115	12.8	N/A	90	N/A	49.5	N/A	17
HUU 197	Male	9.5	3.02	12.45	49	86.9	35	48	N/A	16
HUU 205	Male	8.5	3.47	11.31	52	92.3	33	49.1	N/A	15.3
HUU 214	Female	8.5	2.22	N/A	N/A	N/A	33	N/A	N/A	N/A

HUU 216	Female	N/A	2.6	10.69	46	84.3	34	47	N/A	15
HUU 218	Female	9	2.7	12.4	46	84.5	34	50	N/A	19
HUU 220	Female	9	2.8	10.92	52	84	33	49	N/A	17
HUU 222	Female	9.5	2.98	10.65	49	82.5	34	42	N/A	15
HUU 223	Male	8.5	2.99	11.11	48	84.1	35	48.5	N/A	17
HUU 236	Male	6	3.34	N/A	56	N/A	32	N/A	N/A	N/A
HUU 245	Female	9	2.52	9.45	44	86	30	47	N/A	14.9
HUU 246	Female	9.5	2.36	10.06	46	85	36	48.6	N/A	15
HUU 251	Male	9.5	3.28	10.92	57	84.1	36	49	N/A	14.6
HUU 253	Male	8.5	3.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A
HUU 254	Female	9	2.22	N/A	48	N/A	34	N/A	N/A	N/A
HUU 255	Male	9	3.76	N/A	N/A	N/A	N/A	N/A	N/A	N/A
HUU 261	Male	9	2.72	11.62	49	84.9	34	49.6	N/A	17.2
HUU 271	Male	6	3.2	13.14	57	86	35	52.5	N/A	17
HUU 275	Female	5	2.78	14.92	49	89.2	30	50	N/A	18.5
HUU 293	Male	9.5	2.475	9.73	46	76.4	35	47.8	N/A	15.8

Appendix D: The demographic characteristics of the human immunodeficiency virus-exposed uninfected and human immunodeficiency virus-unexposed uninfected infants' mothers study population, which includes women living with human immunodeficiency and women living without human immunodeficiency virus, represent the maternity visits week 36 of pregnancy and 24 months after birth.

WLWH	Age	HIV status	Height	Weight		MUAC		Viral load	CD4 count	
				Before delivery	After delivery	Before delivery	After delivery		Before delivery	After delivery
WLWH 50	40	Positive	155	69.5	50.3	30	25	0	271	N/A
WLWH 63	38	Positive	161	56	53.9	29	26	0	455	301
WLWH 79	35	Positive	135	72	N/A	31	N/A	0	510	750
WLWH 93	31	Positive	164	79	90	N/A	36.5	0	848	1122
WLWH 111	37	Positive	161	88.8	68.2	34	30	0	N/A	671
WLWH 143	39	Positive	159	70	70.4	31	30.3	0	332	332
WLWH 150	35	Positive	155	68	59.4	30	28	0	351	701
WLWH 182	27	Positive	162	81.9	76.4	30	31	0	505	505
WLWH 198	41	Positive	161	92.3	97.8	36	37.6	0	280	300
WLWH 199	31	Positive	172	70.9	67.8	36	26	0	819	819
WLWH 203	37	Positive	120	71	61.1	31	26.4	0	569	700
WLWH 206	45	Positive	158	61	N/A	28	N/A	0	948	948
WLWH 207	43	Positive	156	64.9	57.4	29	28.2	0	299	284
WLWH 241	27	Positive	157	68	N/A	31	N/A	0	575	595
WLWH 249	41	Positive	154	48.3	N/A	24	N/A	0	721	721
WLWH 258	39	Positive	167	65.7	N/A	N/A	N/A	0	N/A	N/A
WLWH 260	38	Positive	159	66.4	53.1	33	26	0	179	121

WLWH 262	38	Positive	160	72.1	58.8	28	28	0	N/A	900
WLWH 273	43	Positive	161	81.3	85.7	30	32.2	0	N/A	860
WLWH 278	39	Positive	157	70.1	76	38	33.6	0	N/A	500
WLWH 291	33	Positive	162	N/A	77.8	32	31	0	N/A	444

WLWOH	**Age**	**HIV status**	**Height**	**Weight**		**MUAC**	
				Before delivery	**After delivery**	**Before delivery**	**After delivery**
WLWOH 07	41	Negative	149	65.5	65.1	26	31
WLWOH 25	21	Negative	157	80	74.9	33	33.4
WLWOH 125	26	Negative	144	85	82.7	37	36
WLWOH 128	40	Negative	158	88	88.6	35	34
WLWOH 135	34	Negative	168	79	72.3	29	28.2
WLWOH 142	21	Negative	148	63	56.1	26	27.6
WLWOH 144	34	Negative	158	80	64.9	31	31.5
WLWOH 192	33	Negative	157	73.9	67	32	29.8
WLWOH 197	26	Negative	156	67.8	70.2	30	28
WLWOH 205	24	Negative	170	75.5	63.9	28	28.2
WLWOH 214	35	Negative	159	N/A	N/A	N/A	N/A
WLWOH 216	26	Negative	156	65.3	59.5	31	29
WLWOH 218	41	Negative	153	86.5	95.4	22	36
WLWOH 220	31	Negative	157	77.3	72.3	35	32
WLWOH 223	23	Negative	152	57.4	51.7	28	27
WLWOH 236	24	Negative	168	70.8	N/A	30	N/A
WLWOH 245	33	Negative	158	69.1	62.9	29	N/A

WLWOH 246	29	Negative	162	78.7	76.1	32	32
WLWOH 251	24	Negative	144	58.7	61.7	29	31
WLWOH 253	27	Negative	158	58.1	N/A	29	N/A
WLWOH 255	41	Negative	155	62.9	N/A	26	N/A
WLWOH 271	25	Negative	165	73.3	66.2	30	30
WLWOH 275	39	Negative	156	N/A	71.8	33	31
WLWOH 293	30	Negative	154	53	45.6	24	26.1

Appendix E: Deoxyribonucleic acid concentration and purity, comparison between the high pure polymerase chain reaction template preparation kit (manual extraction) and the Maxwell 16 blood deoxyribonucleic acid purification system (automated extraction).

Sample identification	**Number of blood spots used**	**Automated DNA extraction (Maxwell®16 blood DNA purification kit)**		**Manually extracted DNA (High pure PCR template preparation kit)**	
		DNA yield (ng/µL)	**260/280 ratio**	**DNA yield (ng/µL)**	**260/280 ratio**
Sample 1	6 mm x 1 spot	18.53	1.82	16.31	1.64
Sample 2	6 mm x 1 spot	12.89	1.67	16.33	1.73
Sample 3	6 mm x 1 spot	12.69	1.8	17.24	1.56
Sample 4	6 mm x 1 spot	11.72	1.73	8.01	1.76
Sample 5	6 mm x 1 spot	10.87	1.63	13.2	1.68
Sample 6	6 mm x 1 spot	10.96	1.74	16.89	1.43
Sample 7	6 mm x 1 spot	15.5	1.75	18.84	1.51
Sample 8	6 mm x 1 spot	13.24	1.4	12.49	1.62
Sample 9	6 mm x 1 spot	10.53	1.84	15.66	1.48
Sample 10	6 mm x 1 spot	8.93	1.77	22.32	1.52
Sample 11	6 mm x 1 spot	12.19	1.5	15.83	1.49
Sample 12	6 mm x 1 spot	7.43	1.96	14.6	1.45
Sample 13	6 mm x 1 spot	6.4	1.6	14.55	1.5
Sample 14	6 mm x 1 spot	7.25	1.54	10.15	1.4
Sample 15	6 mm x 1 spot	9.29	1.56	9.57	1.46

Appendix F: Deoxyribonucleic acid concentration and purity for the human immunodeficiency exposed uninfected and unexposed uninfected newborns.

HIV exposed uninfected newborn babies DBS DNA concentration and purity					
Sample ID	**ng/µL**	**A260**	**A280**	**260/280**	**260/230**
HEU 20	29.05	0.581	0.352	1.65	0.93
HEU 34	24.48	0.490	0.327	1.50	0.71
HEU 50	16.15	0.323	0.200	1.61	0.60
HEU 63	18.84	0.377	0.248	1.52	0.67
HEU 75	14.81	0.296	0.296	1.63	0.68
HEU 79	21.84	0.437	0.286	1.53	0.64
HEU 84	11.62	0.232	0.138	1.68	0.66
HEU 93	18.05	0.361	0.361	1.60	0.66
HEU 111	19.54	0.391	0.391	1.68	0.66
HEU 133	24.18	0.484	0.319	1.52	0.67
HEU 138	29.92	0.598	0.386	1.55	0.70
HEU 143	18.78	0.376	0.240	1.57	0.61
HEU 149	17.31	0.346	0.346	1.53	0.68
HEU 150	20.90	0.418	0.267	1.56	0.64
HEU 172	17.49	0.350	0.230	1.52	0.63
HEU 172	14.59	0.292	0.178	1.64	0.67
HEU 182	16.90	0.338	0.175	1.93	0.64
HEU 198	26.63	0.533	0.322	1.65	0.71
HEU 199	18.09	0.362	0.209	1.73	0.74
HEU 203	19.10	0.382	0.258	1.48	0.71
HEU 206	13.59	0.272	0.163	1.67	0.69
HEU 207	19.58	0.392	0.315	1.24	0.78
HEU 219	18.47	0.369	0.216	1.71	0.67
HEU 241	28.52	0.570	0.363	1.57	0.81
HEU 243	17.70	0.354	0.207	1.71	0.69
HEU 249	16.85	0.337	0.337	1.54	0.66
HEU 258	24.75	0.496	0.321	1.54	0.70
HEU 260	24.14	0.482	0.336	1.44	0.66
HEU 262	13.56	0.271	0.149	1.82	0.64
HEU 268	6.65	0.133	0.062	2.15	0.57

HEU 273	24.34	0.487	0.314	1.55	0.71
HEU 278	18.59	0.372	0.252	1.48	0.66
HEU 291	19.08	0.382	0.227	1.68	0.64

HIV unexposed uninfected newborn babies DBS DNA concentration and purity					
Sample ID	**ng/µL**	**A260**	**A280**	**260/280**	**260/230**
HUU 07	14.52	0.290	0.191	1.52	0.72
HUU 08	17.46	0.349	0.228	1.53	0.66
HUU 25	15.15	0.303	0.180	1.68	0.67
HUU 119	20.27	0.405	0.244	1.66	0.80
HUU 125	19.13	0.383	0.212	1.81	0.75
HUU 128	16.68	0.334	0.202	1.65	0.66
HUU 135	26.31	0.526	0.298	1.76	0.99
HUU 137	16.61	0.332	0.186	1.79	0.74
HUU 142	16.64	0.333	0.212	1.57	0.69
HUU 144	10.74	0.215	0.135	1.60	0.68
HUU 192	16.00	0.320	0.189	1.69	0.69
HUU 197	17.57	0.351	0.220	1.60	0.73
HUU 205	17.86	0.357	0.204	1.75	0.76
HUU 214	18.55	0.371	0.222	1.67	0.84
HUU 216	18.40	0.368	0.209	1.76	0.70
HUU 218	19.91	0.398	0.262	1.52	0.69
HUU 220	16.13	0.323	0.163	1.97	0.77
HUU 222	17.24	0.345	0.240	1.44	0.68
HUU 223	20.76	0.415	0.249	1.67	0.76
HUU 236	16.80	0.336	0.199	1.69	0.75
HUU 245	14.18	0.284	0.192	1.48	0.78
HUU 246	17.43	0.349	0.197	1.77	0.94
HUU 251	21.59	0.432	0.266	1.62	0.78
HUU 253	19.29	0.386	0.220	1.75	0.67
HUU 254	21.78	0.436	0.281	1.55	0.73

HUU 255	23.69	0.474	0.313	1.51	0.82
HUU 261	20.37	0.407	0.254	1.61	0.66
HUU 271	12.53	0.251	0.133	1.88	0.72
HUU 275	27.26	0.545	0.347	1.57	0.84
HUU 293	27.42	0.548	0.339	1.62	0.93

Appendix G: Deoxyribonucleic acid concentration and purity for the women living with human immunodeficiency virus and those living without human immunodeficiency cohorts.

Women living with HIV DBS DNA concentration and purity					
Sample ID	**ng/µL**	**A260**	**A280**	**260/280**	**260/230**
WLWH 50	14.8	0.296	0.129	2.29	0.65
WLWH 63	25.37	0.507	0.349	1.45	0.73
WLWH 79	16.78	0.336	0.228	1.47	0.67
WLWH 93	15.86	0.317	0.228	1.39	0.63
WLWH 111	10.9	0.218	0.149	1.46	0.68
WLWH 143	13.73	0.275	0.193	1.42	0.61
WLWH 150	15.24	0.305	0.197	1.54	0.65
WLWH 182	14.94	0.299	0.2	1.5	0.77
WLWH 198	15.42	0.308	0.22	1.4	0.67
WLWH 199	15.11	0.302	0.208	1.45	0.66
WLWH 203	13.73	0.275	0.186	1.47	0.67
WLWH 206	16.78	0.336	0.217	1.55	0.67
WLWH 207	30.54	0.611	0.439	1.39	0.67
WLWH 241	15.57	0.311	0.213	1.46	0.76
WLWH 249	10.11	0.202	0.146	1.38	0.7
WLWH 258	17.74	0.355	0.259	1.37	0.64
WLWH 260	12.13	0.243	0.186	1.3	0.62
WLWH 262	14.67	0.293	0.203	1.45	0.72
WLWH 273	10.55	0.211	0.136	1.55	0.66
WLWH 278	25.81	0.516	0.371	1.39	0.69
WLWH 291	22.75	0.455	0.282	1.62	0.74

Women living without HIV DBS DNA concentration and purity					
Sample ID	**ng/µL**	**A260**	**A280**	**260/280**	**260/230**
WLWOH 07	8.31	0.166	0.127	1.31	0.65
WLWOH 25	16.42	0.328	0.218	1.51	0.71
WLWOH 125	17.68	0.354	0.258	1.37	0.68
WLWOH 128	17.16	0.343	0.251	1.37	0.73
WLWOH 135	18.43	0.369	0.254	1.45	0.72
WLWOH 142	16.42	0.328	0.235	1.4	0.68
WLWOH 144	18.13	0.363	0.252	1.44	0.66
WLWOH 192	18.52	0.37	0.264	1.4	0.73
WLWOH 197	17.37	0.347	0.237	1.46	0.69
WLWOH 205	18.91	0.378	0.272	1.39	0.72
WLWOH 214	20.39	0.408	0.275	1.48	0.71
WLWOH 216	22.22	0.444	0.302	1.47	0.73
WLWOH 218	17.04	0.341	0.247	1.38	0.63
WLWOH 220	14.13	0.283	0.196	1.44	0.71
WLWOH 223	19.21	0.384	0.251	1.53	0.73
WLWOH 236	16.64	0.333	0.227	1.47	0.73
WLWOH 245	19.14	0.383	0.257	1.49	0.75
WLWOH 246	19.64	0.383	0.272	1.44	0.82
WLWOH 251	20.48	0.41	0.264	1.55	0.8
WLWOH 253	26.9	0.538	0.36	1.49	0.96
WLWOH 255	15.28	0.306	0.214	1.43	0.67
WLWOH 271	19.75	0.395	0.265	1.49	0.73
WLWOH 275	22.01	0.44	0.292	1.51	0.86
WLWOH 293	21.97	0.439	0.277	1.59	0.86

Appendix H: Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination circles detection in newborn samples.

HEU infants	CT		Quantity		Threshold		R^2		Slope	
	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC
HEU 20	31.30	31.00	72.11	20.04	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 34	34.01	35.22	6.22	1.67	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 50	37.93	35.42	0.18	1.48	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 63	35.57	34.40	1.53	2.72	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 75	34.77	33.84	3.14	3.76	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 79	36.07	34.57	0.97	2.45	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 84	33.34	35.49	11.42	1.42	0.184	0.040	0.903	0.984	-2.547	-3.918
HEU 93	33.50	31.99	9.94	11.17	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 111	33.57	31.41	9.33	15.72	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 133	34.83	32.34	2.99	9.07	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 138	33.94	33.67	6.68	4.15	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 143	34.48	33.80	4.08	3.87	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 149	36.09	33.65	0.96	4.22	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 150	34.66	33.74	3.48	3.99	0.362	1.375	0.903	0.984	-2.547	-3.918

HEU 172	35.50	34.30	1.63	2.88	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 182	32.72	32.20	20.14	9.87	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 198	33.57	31.61	9.33	13.92	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 199	34.26	31.95	4.97	11.41	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 203	34.89	33.03	2.81	6.05	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 206	33.73	33.15	8.04	5.63	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 207	35.53	33.47	1.57	4.68	0.362	1.375	0.903	0.984	-2.547	-3.918
HEU 219	33.72	32.27	8.14	9.44	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 241	32.71	32.12	20.22	10.32	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 243	35.64	31.76	1.43	12.73	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 249	35.46	35.24	1.68	1.65	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 258	35.30	34.16	1.95	3.11	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 260	32.45	31.98	25.56	11.24	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 262	34.93	36.50	2.73	0.79	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 268	35.13	35.57	2.26	1.36	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 273	34.21	33.61	5.20	4.30	0.570	1.297	0.903	0.984	-2.547	-3.918
HEU 278	34.68	32.87	3.41	6.63	0.040	1.879	0.903	0.984	-2.547	-3.918

HEU 291	37.25	37.25	0.31	0.51	0.040	1.879	0.903	0.984	-2.547	-3.918

HUU	**CT**		**Quantity**		**Threshold**		**R^2**		**Slope**	
infants	**TREC**	**KREC**	**TREC**	**KREC**	**TREC**	**KREC**	**TREC**	**KREC**	**TREC**	**KREC**
HUU 07	33.98	30.93	6.42	20.84	0.184	0.040	0.903	0.984	-2.547	-3.918
HUU 08	36.16	30.46	0.90	27.35	0.184	0.040	0.903	0.984	-2.547	-3.918
HUU 25	34.26	30.88	4.99	21.43	0.184	0.040	0.903	0.984	-2.547	-3.918
HUU 119	33.99	32.93	6.38	6.40	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 125	33.54	32.86	9.53	6.70	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 128	33.87	33.24	7.09	5.34	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 135	33.00	31.31	15.68	16.60	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 137	33.33	33.32	11.53	5.09	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 142	32.70	33.40	20.49	4.88	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 144	34.28	30.91	4.88	21.01	0.184	0.040	0.903	0.984	-2.547	-3.918
HUU 192	33.23	32.62	12.61	7.72	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 197	33.00	32.86	15.78	6.68	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 205	32.89	32.49	17.23	8.29	0.040	1.879	0.903	0.984	-2.547	-3.918

HUU 214	34.52	35.46	3.94	1.45	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 216	33.91	32.66	6.86	7.52	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 218	36.18	37.70	0.88	0.39	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 220	33.34	32.45	11.49	8.54	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 222	35.28	31.94	1.98	11.46	0.184	0.040	0.903	0.984	-2.547	-3.918
HUU 223	32.22	33.98	31.56	3.47	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 236	33.44	32.94	10.43	6.39	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 245	32.84	32.97	17.93	6.28	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 246	33.69	34.00	8.32	3.42	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 251	33.26	32.12	12.27	10.35	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 253	34.23	36.53	5.13	0.77	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 254	32.59	34.02	22.60	3.39	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 255	32.62	33.72	21.86	4.03	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 261	36.96	38.28	0.44	0.28	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 271	33.22	33.41	12.75	4.85	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 275	34.05	32.85	6.00	6.75	0.040	1.879	0.903	0.984	-2.547	-3.918
HUU 293	30.96	32.12	98.78	10.30	0.040	1.879	0.903	0.984	-2.547	-3.918

Appendix I: Quantitative real-time polymerase chain reaction results for T-cell receptor excision circles and kappa-deleting recombination circles detection in samples of women living with human immunodeficiency virus and those living without human immunodeficiency virus.

WLWH	CT		Quantity		Threshold		R^2		Slope	
	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC
WLWH 50	36.99	33.30	0.42	5.17	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 63	37.12	32.49	0.37	8.29	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 79	35.59	31.32	1.50	16.52	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 93	38.13	35.53	0.15	1.39	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 111	37.71	36.99	0.22	0.59	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 143	35.80	35.50	1.23	1.41	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 150	35.97	32.65	1.06	7.58	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 182	36.05	33.41	0.99	4.85	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 198	37.76	36.95	0.21	0.60	0.121	0.040	0.903	0.984	-2.547	-3.918

WLWH 199	38.11	36.67	0.15	0.71	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 203	35.90	34.31	1.13	2.85	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 206	37.36	35.40	0.30	1.50	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 207	37.45	34.45	0.28	2.62	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 241	34.77	32.06	3.14	10.71	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 249	36.67	36.26	0.57	0.91	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 258	37.27	35.24	0.33	1.65	0.121	0.040	0.903	0.984	-2.547	-3.918
WLWH 260	Undetermined	33.52		4.53	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 262	Undetermined	Undetermined			0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 273	37.68	33.67	0.23	4.16	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 278	36.97	33.83	0.43	3.79	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWH 291	36.17	33.87	0.88	3.69	0.044	0.040	0.903	0.984	-2.547	-3.918

WLWOH	CT		Quantity		Threshold		R^2		Slope	
	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC	TREC	KREC
WLWOH 07	37.54	34.12	0.26	3.20	0.184	0.040	0.903	0.984	-2.547	-3.918
WLWOH 25	37.41	35.23	0.29	1.66	0.184	0.040	0.903	0.984	-2.547	-3.918
WLWOH 125	Undetermined	35.71		1.25	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 128	35.81	34.17	1.23	3.10	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 135	37.13	33.36	0.37	4.98	0.184	0.040	0.903	0.984	-2.547	-3.918
WLWOH 142	37.16	34.44	0.36	2.65	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 144	33.07	33.30	14.63	5.16	0.184	0.040	0.903	0.984	-2.547	-3.9
WLWOH 192	37.30	35.60	0.32	1.34	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 197	Undetermined	35.81		1.18	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 205	35.64	32.91	1.43	6.50	0.044	0.040	0.903	0.984	-2.547	-3.918

WLWOH 214	36.83	35.41	0.49	1.49	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 216	35.28	33.86	1.98	3.71	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 218	36.90	36.72	0.46	0.69	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 220	36.66	33.74	0.57	3.99	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 223	35.49	34.99	1.63	1.91	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 236	37.10	36.65	0.38	0.72	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 245	36.51	35.33	0.65	1.56	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 246	35.38	34.68	1.81	2.29	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 251	33.56	32.26	9.41	9.52	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 253	33.94	33.90	6.63	3.64	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 255	Undetermined	37.33		0.48	0.044	0.040	0.903	0.984	-2.547	-3.918
WLWOH 271	36.85	34.91	0.48	2.01	0.184	0.040	0.903	0.984	-2.547	-3.918

WLWO H 275	36.64	34.94	0.58	1.97	0.184	0.040	0.903	0.984	-2.547	-3.918
WLWO H 293	36.21	34.83	0.86	2.11	0.184	0.040	0.903	0.984	-2.547	-3.918