Development of a multiplexed PCR assay to identify pathogenic variants causing severe combined immunodeficiency (SCID) in Manitoba

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Abstract

Severe combined immunodeficiency (SCID) is a primary immune deficiency characterized by T cells that are either low in number, absent, or non-functional. SCID is fatal without treatment and has a higher incidence in Manitoba compared to the rest of Canada. Two founder mutations in Manitoba cause a T-cell positive SCID phenotype that cannot be identified using the T-cell receptor excision circle (TREC) assay, which is the commonest method of newborn screening for SCID. These are mutations of the *ZAP70* and *IKBKB* genes and are present at increased frequency in the Mennonite and First Nations of Northern Cree ancestry populations, respectively. Currently, newborns in Manitoba are screened for inherited disorders by way of dried blood spot (DBS) specimens. We developed allele-specific real-time quantitative polymerase chain reaction (qPCR) assays for the detection of both variants using the DBS sample type. These assays were multiplexed together with the existing TREC assay (a multiplexed assay is an assay in which multiple targets are assessed within a single reaction mixture). This paper will give a brief introduction to the mutations of interest and describe the methodology and validation of this assay. We will also explore some of the implications of expanded newborn screening in Manitoba and Canada more broadly.

Keywords: severe combined immunodeficiency; newborn screening; First Nations and Inuit population; Manitoba

Conflict of Interest Statement: None to declare.

Introduction

Severe combined immunodeficiency (SCID) is an inherited primary immune deficiency characterized by low, absent, or non-functional T cells. Despite no clinical signs at birth, SCID is typically fatal by one year of age if untreated.[1,2] Hematopoietic stem cell transplantation in the neonatal period (first 28 days of life) has been associated with decreased morbidity and mortality for affected newborns.[3] This curative treatment highlights the importance of early identification of all SCID cases. Surveillance studies of SCID have found an incidence in Manitoba that is close to 3 times the national incidence.[1,4] Two founder mutations underlie this increased incidence of SCID in Manitoba: a frameshift mutation in the *IKBKB* gene and a splice site mutation in *ZAP70*.[5,6]

In patients of Northern Cree ancestry, a single base pair duplication (NM_001556.3: c.1292dupG p.Gln432fs) causes a frameshift mutation and results in deficiency of the gene product inhibitor of kappa B kinase 2 (IKK2) and a SCID phenotype.[5] In homozygotes, deficiency of IKK2 interferes with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway by decreasing the rate of degradation of the inhibitor of κ-B (IKB). As a result, NF-κB remains inactivated, impacting T and B cell receptor signalling and causing SCID.[5,7]

Among Canadian Mennonites, a homozygous base pair substitution (NM_001079.3: c.1624–11G>A) in the

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acceptor splice site of intron 12 of *ZAP70* produces a new acceptor splice site and results in an mRNA transcript with 9 additional nucleotides. This transcript yields an unstable gene product and an inactivated zeta chain-associated protein 70 kinase (ZAP-70).[6] The resultant deficiency of this kinase interferes with T cell maturation and receptor signalling and causes inactivity of CD4+ T cells, an absence of CD8+ T cells, and a SCID phenotype.

For a disorder to be considered eligible for inclusion on a newborn screening panel, it must meet certain criteria. The classical criteria for evaluating newborn screening candidate disorders are the Wilson and Jungner criteria, which have since been expanded to allow for the possibility of genetic testing.[8] Two of these criteria are that the facilities for diagnoses and treatment should be available and that case-finding should be a continuous process. It is important to distinguish newborn screening from diagnosis, as both are important components in case-finding. Newborn screening involves high throughput tests designed to minimize false negatives at the expense of a higher false positive rate. A positive screening test is therefore not diagnostic but raises the index of suspicion for a given disease. In the case of a positive screening test, the sample is reflexed to a diagnostic test and the patient is referred to the appropriate health care team.

The gold standard for newborn screening for SCID uses quantitative real-time polymerase chain reaction (qPCR) to quantify T cell receptor excision circles (TRECs) in neonatal dried blood spots. Very low TREC copy levels correspond to a positive SCID screening result and reflect the absence of mature T cells characteristic of traditional SCID.[9,10] SCID patients with the above-described *IKBKB* and *ZAP70* variants, however, are often T cell positive (T cells are present but non-functioning) with TREC levels above the upper threshold of the TREC screening test.[4,11] A high-resolution DNA melting procedure of *IKBKB* and *ZAP70* PCR amplicons has previously been described as a method to genotype these mutations.[2] We developed allele-specific quantitative real-time polymerase chain reaction (qPCR) primers and probes for the identification of these *ZAP70* and *IKBKB* variants. These components have been multiplexed with the existing TREC assay to improve the efficiency and scope of newborn screening for SCID in Manitoba. By multiplexing the allele-specific assays with the existing SCID screening test, we minimized costs associated with running multiple PCR plates (as required in a high resolution melting procedure) as well as improved ease of analysis. The aim of this paper is to describe the methodology and validation techniques for this assay.

Methods

In Manitoba, newborn screening is conducted at Cadham Provincial Laboratory (CPL) in Winnipeg using dried blood spots collected from newborns in their first days of life. A heel prick is performed with a lancet to collect and spot blood onto filter paper with known physical specifications such as absorbance. The blood spots are left undisturbed to dry before transport and then sent to CPL for processing. To perform PCR analysis of these samples, DNA must be extracted from the dried blood spot specimens. In the development of this assay, a 3.2 mm disc of saturated filter paper was used for the extraction step for each sample, standardizing extractions between newborns. For the *IKBKB* and *ZAP70* assays, multiple extraction techniques were used to assess the effect of inhibitors. These extraction techniques used Biosprint 96 DNA Blood kit reagents from QIAGEN Sciences (Hilden, Germany), DBS Extracta reagent from Quantabio (Beverley, MA, USA), and QuickExtract DNA from Lucigen (Middleton, WI, USA). After extraction, 5 µL of extracted DNA along with 15 µL of the reaction mixture containing primers, probes, and TaqMan Fast MasterMix from Life Technologies (Thermo Fischer Scientific, Waltham, MA, USA) was added to each sample well of a 96-well PCR plate for analysis.

PCR amplifies segments of DNA for which the appropriate primer is present in the reaction mixture. In qPCR, fluorescently labelled oligonucleotide probes (hydrolysis probes) bind to a specific sequence and fluoresce when this segment of DNA is amplified. Relative fluorescence units (RFU) are reported for fluorophores corresponding to each probe. A baseline is set automatically and adjusted manually to differentiate a signal from background. The threshold cycle (Ct) or quantification cycle (Cq) is defined as the PCR cycle at which an amplification curve crosses this threshold, constituting a signal.

To detect *IKBKB* and *ZAP70*, the assay was originally designed to use competitive binding of probes to differentiate wild-type and mutated variants. A hydrolysis probe was designed to bind with greater affinity to the wild-type variant and a non-fluorescing or "silent" probe was designed to outcompete the hydrolysis probe for the mutated variant.[12] Locked nucleic acids were used to increase the specificity of the probes (Table 1). This method has been published for use in other contexts including the molecular characterization of tumour cells[13] and when multiplexing newborn screening for spinal muscular atrophy with SCID.[14] Through this method, the hydrolysis probe outcompetes the silent probe in the presence of the wild-type variant and a signal is produced. The absence of a signal is expected in samples homozygous for the mutated variant, while a diminished signal should be observed in heterozygous samples.

Table 1. Original primers and probes for PCR multiplex assay to selectively amplify wild-type IKBKB and ZAP70 alleles in concert with TREC amplification

Target/reagent [a]	Sequence (5'–3')	Concentration (nmol/L) [b]
TREC		
Forward primer	TGACACCTCTGGTTTTTGTAA	800
Reverse primer	GTGCCAGCTGCAGGGTTTAG	800
Probe	ATG***C***A***T***AGG***C***ACCTGC	250
IKBKB		
Forward primer	AGGAATCTCGCCTTCTTCC	500
Reverse primer	CTGGATGCTGTGCCAGAC	500
Probe (wt)	TGTGGGGCC***AG***GTCTGG	100
Blocker (mt)	GTGTGGGGG***C***CA***G***GTCTG	400
ZAP70		
Forward primer	TGAGGAGGAGGACACTGG	325
Reverse primer	TTGCCCTGCTCGATGAAG	325
Probe (wt)	CTGCCC***CG***GCTTG***A***GCA	250
Blocker (mt)	CTGCCC***CA***GCTTG***A***GCA	500

[a] Bases in bold italic font denote locked nucleic acids
[b] Final concentration in PCR reaction (MasterMix)

Table 2. Adjusted primers and probes for PCR multiplex assay to selectively amplify wild-type IKBKB and ZAP70 alleles in concert with TREC amplification

Target/reagent [a]	Sequence (5'–3')	Concentration (nmol/L) [b]
TREC		
Forward primer	TGTTTCACAGCTATCCCAAG	800
Reverse primer	CTGATCTTGTCTGACATTTGC	800
Probe	AACACACTCTAOTGATGCCAGCAC	250
IKBKB		
Forward primer	TCAAGAGCCCAAGAGCAA	500
Reverse primer	CTTCCTTCAGGGTCTGGA	500
Probe (wt)	5HEX/TG***GC***CCCC***AC***A/3IABkFQ	100
ZAP70		
Forward primer	GATGAGGAGGAGGACACT	325
Reverse primer	CCGGCCCTTTCATCTTC	325
Probe (wt)	56-FAM/CCC***CGG***CTTG/3IABkFQ	250

[a] Bases in bold italic font denote locked nucleic acids
[b] Final concentration in PCR reaction (MasterMix)

TREC copy numbers follow a normal distribution and are affected by characteristics such as low birth weight or the presence of other immune deficiencies. CPL uses a TREC level of 33 TRECs/µL of whole blood as the cut-off for a positive SCID screening result. If the level of TRECs for a given specimen is below this value, and other elements of the multiplex are detected within a normal range proving successful extraction, the specimen is retested. A subsequent positive test indicates a high risk for SCID.

Primer and probe sequences and the PCR protocol were developed and optimized in-house using concentration and temperature gradients (Tables 2–3). The initial primers and probes were adjusted during validation to increase allele specificity and negate the need for a silent probe or blocker, further reducing costs and sources of error (Table 4). Candidate sequences for the custom primers and probes were selected using NCBI Primer-BLAST. Validation was carried out using two quality control standards and three blank or "no template" controls (Tables 5–6). Since these assays were multiplexed with the TREC assay, the former served as an internal control for the latter and vice versa. Successful amplification of genomic *IKBKB*, *ZAP70*, or TRECs indicated successful DNA extraction and PCR.

Table 3. qPCR protocol for the SCID Multiplex assay

Step #	Temperature	Duration
1	95°C	10 minutes
2	95°C	10 seconds
3	63.5°C	10 seconds
4	69.0°C	30 seconds
5	Plate Read; GO TO STEP 2 39×	
6	END	

Table 4. Quality controls for TREC quantification [a]

Control [b]	Contents
TREC QC 70	Dried blood spot with 70 copies of TREC per µL of whole blood
TREC QC 35	Dried blood spot with 35 copies of TREC per µL of whole blood

[a] Materials developed at Cadham Provincial Laboratory
[b] Spiked with IKBKB or ZAP70 mutated plasmid to serve as internal controls

Table 5. qPCR controls included in the SCID assay during validation

Control	Contents
No Template Control 1 (NTC1)	Filter paper blanks, DNA extraction reagents, PCR reagents
No Template Control 2 (NTC2)	DNA extraction reagents, PCR reagents
No Template Control 3 (NTC3)	PCR reagents

Table 6. Results of CPL SCID assay when analyzing CDC proficiency testing specimens

Specimen Number	Description	CPL Result	Fraction Correct
119R1	Normal cord blood with medium TREC copy level	Normal	3/3
119R2	Uninterpretable. TREC and reference gene out of range. Prepared from leukocyte-depleted blood.	Failed extraction	3/3
119R3	Normal cord blood with low TREC copy-level	Normal	3/3
119R4	Normal cord blood with medium TREC copy-level	Normal	2/2
119R5	SCID-like sample with very low/undetectable TREC. Reference gene within range.	SCID screen positive	3/3

Results

The original and adjusted primer and probe sequences for detection of *IKBKB* and *ZAP70* were successfully multiplexed with the TREC primers and probe. When multiplexed, the clinical performance characteristics of the TREC assay were unaffected. The TREC quantification ability of this assay was validated using 5 specimens donated as proficiency testing samples by the Centers for Disease Control and Prevention. The samples were run in triplicate, with the experimenter blinded to the anticipated result and position of the proficiency testing samples. Accuracy was 100% as shown in Table 6. TREC quantification was further challenged with other immune deficiencies using affected DBS samples from the CPL archives. TREC-deficient SCID was successfully differentiated from CHARGE Syndrome, *IKBKB* and *ZAP70* SCID, Down syndrome, and other

lymphopenias causing low TREC levels.

Using the original primer and probe set, the multiplexed assay successfully amplified both wild-type genes ZAP70 and IKBKB as well as TREC. This was evidenced by the presence of amplification curves for each fluorophore when human genomic DNA was analyzed using this method (Figure 1). Melting curve analysis verified the amplification of *IKBKB* and *ZAP70*, as the curves are consistent with theoretical melting points determined by amplicon sequence.[2] The absence of small, extraneous peaks indicated a lack of primer dimer formation (Figure 2).

Primer efficiency was evaluated by performing the assay on a serial dilution of genomic DNA spiked with a serial dilution of TREC plasmid. Each dilution was run in triplicate on a plate and the linear regression equation was calculated for each primer set. The efficiencies of all three primer sets were found to be between 90–100%. The assay also performed equally well on samples from each of the three DNA extraction conditions described in the methods and was therefore robust to inhibitors.

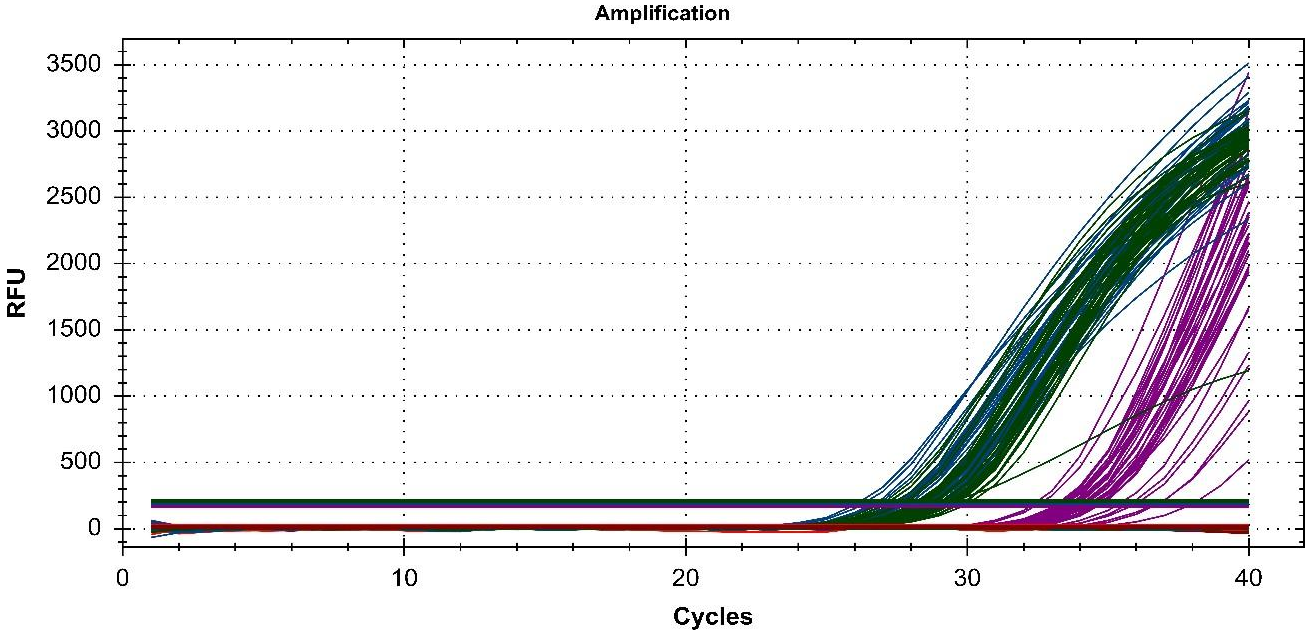

Figure 1. The amplification curves of IKBKB wild-type (Green; HEX), ZAP70 wild-type (Blue; FAM), and TREC (Purple; Cy5) for the original primer & probe (Appendix)

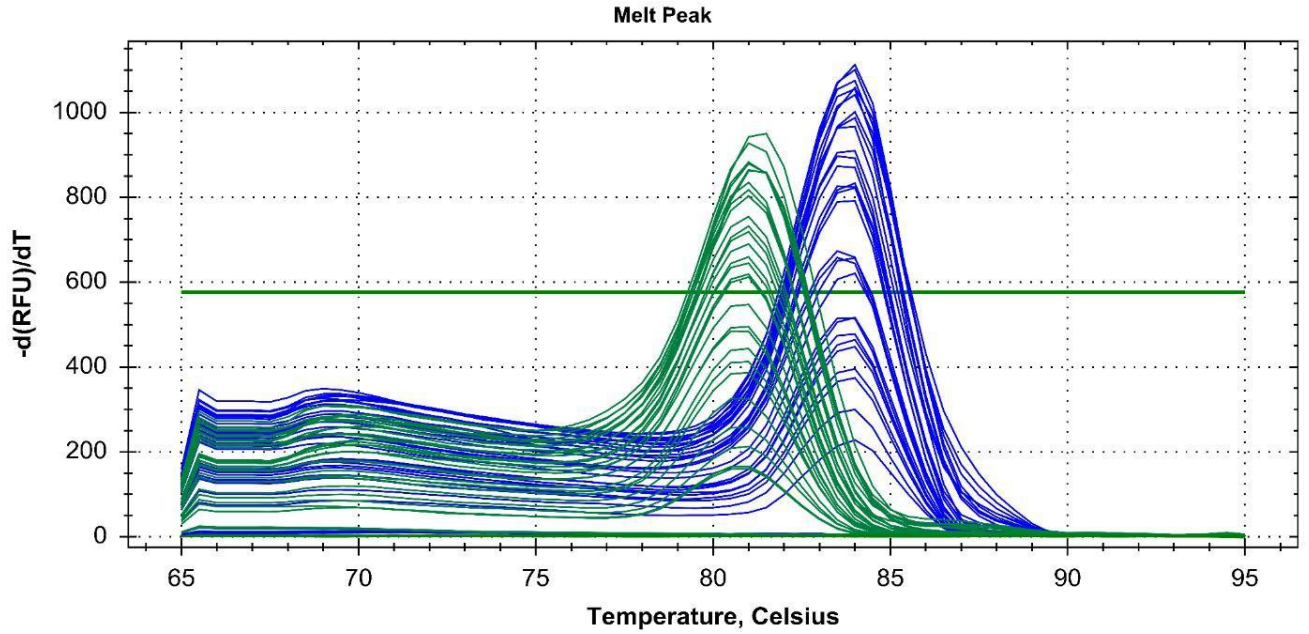

Figure 2. Post-PCR melting curves for IKBKB amplification (Green; 81°C) and ZAP70 (Blue; 84°C)

Using the second primer and probe set, the assay was able to correctly identify homozygous mutated variants of the genes in the absence of a silent probe for the mutated *IKBKB* and *ZAP70* variants (Table 7). This is evidenced by the lack of signal for the mutated allele when the assay was tested against samples from SCID patients with a known *IKBKB* or *ZAP70* mutation causing the disease (Figures 3–5).

Table 7. Quality controls for the detection of IKBKB and ZAP70 mutations [a]

Control	Contents
IKBKB and ZAP70 wild-type	The whole-blood TREC-spiked blood spots used as TREC standard curve in an independent PCR.
IKBKB homozygous mutated	Washed red blood cells with 55% hematocrit 3% bovine serum albumin spiked with ZAP70 wild-type plasmid and IKBKB mutated plasmid as blood spots.
ZAP70 homozygous mutated	Washed red blood cells with 55% hematocrit 3% bovine serum albumin spiked with IKBKB wild-type plasmid and ZAP70 mutated plasmid as blood spots.
IKBKB heterozygous	Whole blood dried blood spot spiked with TREC plasmid to 70 copies per µL and with IKBKB mutated plasmid.
ZAP70 heterozygous	Whole blood dried blood spot spiked with TREC plasmid to 35 copies per µL and with ZAP70 mutated plasmid.

[a] Materials developed at Cadham Provincial Laboratory

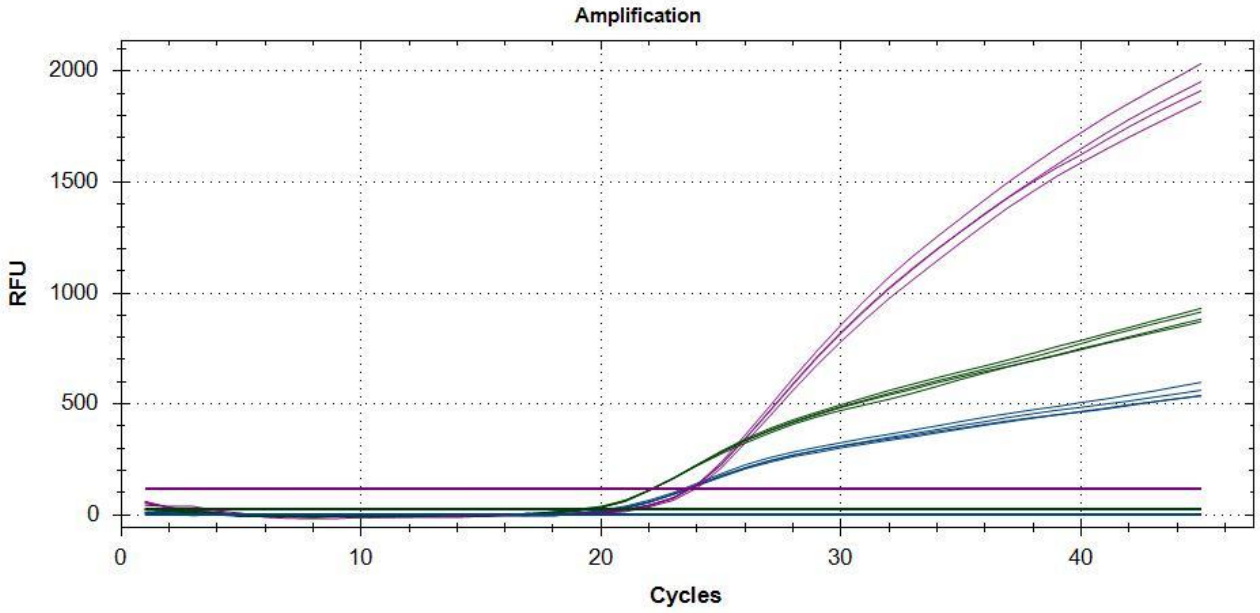

Figure 3. Commercial human genomic DNA amplification plot (purple: TREC, green: wild-type IKBKB, blue: wild-type ZAP70) using the adjusted primer and probe set

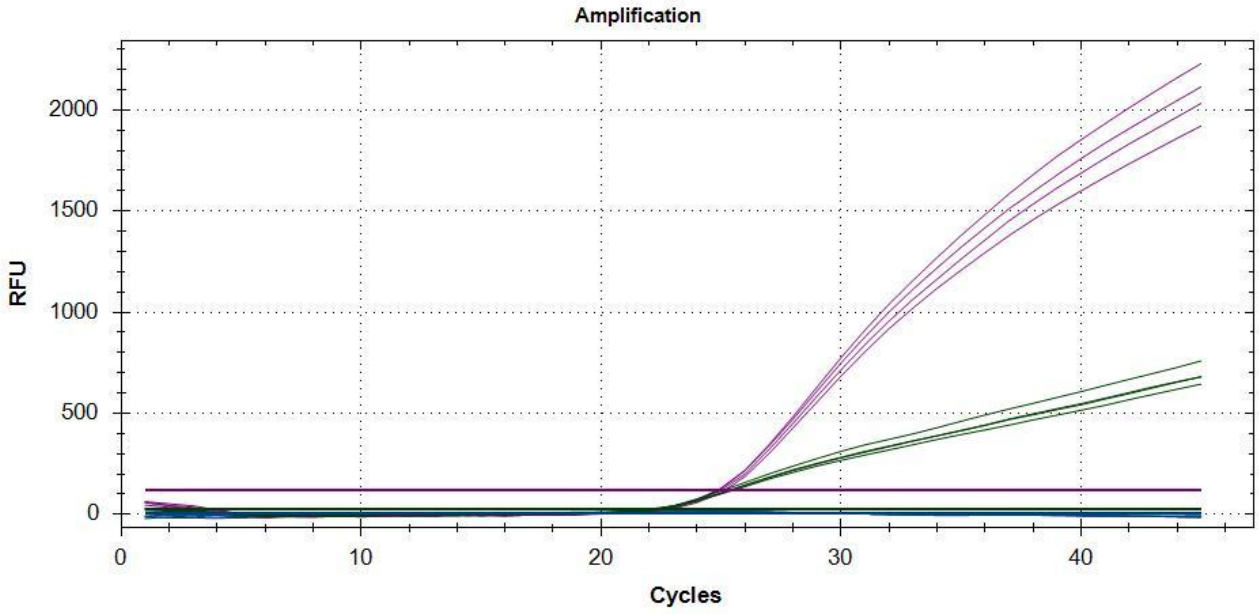

Figure 4. *ZAP70* mutated SCID DNA amplification plot (purple: TREC, green: wild-type IKBKB, blue: wild-type ZAP70) using the adjusted primer and probe set

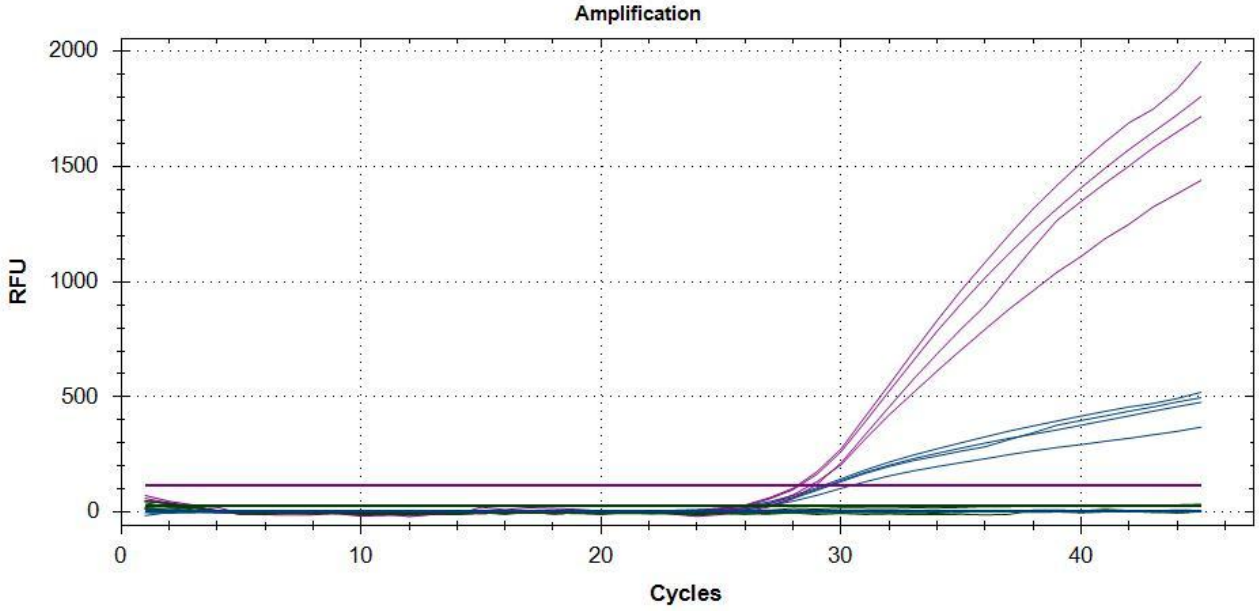

Figure 5. *IKBKB* mutated SCID DNA amplification plot (purple: TREC, green: wild-type IKBKB, blue: wild-type ZAP70) using the adjusted primer and probe set

Discussion

This assay serves as proof of concept for the use of allele-specific qPCR without a silent probe. Due to the low prevalence of SCID overall, and a resultant lack of positive control material, ongoing quality testing with in-house developed control material is required to assess the statistical power of this assay (Table 7). This will be performed in tandem with the use of the assay for newborn screening, which was implemented at CPL in September 2020. Since this time, all newborns born in Manitoba whose parents have not opted-out of newborn screening are screened for SCID using this multiplex assay.

The rollout of this assay addresses ongoing calls for a tailored SCID screening modality and provides a framework for expanded newborn screening in other provinces, particularly in the adjacent provinces of Ontario and Saskatchewan where the *IKBKB* mutation is also found.[15,16] Newborn Screening Programs for SCID currently exist in only five Canadian provinces and one territory: Alberta, Manitoba, Ontario, New Brunswick, Nova Scotia, and the Northwest Territories.[17] Although CPL receives some specimens from

births in adjacent provinces, expanded screening in Ontario and the implementation of a SCID screening protocol in Saskatchewan is needed to ensure all newborns born with SCID are identified before symptoms present. Currently, CPL only receives out-of-province samples if the newborn is either born in Manitoba, despite being from out-of-province, or if the sample is sent to CPL due to proximity. This means that robust provincial screening must be implemented to identify the greatest number of affected newborns.

Beyond the relevance of screening to decrease the direct morbidity and mortality associated with SCID, the inclusion of screening for the *IKBKB* variant has special clinical relevance because of the use of the live-attenuated *M. bovis* bacille Calmette-Guérin (BCG) vaccine in First Nations communities.[18] The BCG vaccine is contraindicated in patients with SCID and a Manitoba case series found that all IKK2 deficient infants who received BCG vaccination developed fatal disseminated mycobacterial disease.[19,20] Vaccination with BCG in the affected communities has subsequently been avoided until a negative newborn screening result is confirmed, which has been made possible through the implementation of expanded newborn screening for SCID.

To summarize, a high incidence of SCID in Manitoba is related to two founder mutations that exist at increased frequency in specific populations. We developed and validated a multiplexed qPCR assay to combine targeted, allele-specific identification of these mutations with the gold standard for screening of T cell negative SCID, TREC quantification. The implementation of this assay within the newborn screening program at CPL will decrease the morbidity and mortality of SCID in Manitoban neonates and establish a precedent for future population-specific screening programs in Canada.

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