

## **Population-based Screening for Severe Combined Immunodeficiency in Manitoba, Canada**

**J. Robert Thompson<sup>1</sup>, Cheryl R. Greenberg<sup>2\*</sup>, Andrew Dick<sup>1</sup>, Olga Jilkine<sup>3</sup>, Luvinia Kwan<sup>2</sup>,  
Tamar S. Rubin<sup>2</sup>, Teresa Zelinski<sup>2</sup>, Marlis L. Schroeder<sup>2</sup>, Paul Van Caesele<sup>1</sup>**

**<sup>1</sup>Cadham Provincial Laboratory, <sup>2</sup>University of Manitoba, <sup>3</sup>CancerCare Manitoba, Winnipeg,  
Manitoba, Canada**

### **\*Corresponding Author:**

Cheryl R. Greenberg, MD, CM, FRCPC, FCCMG

Distinguished Professor

Department of Pediatrics and Child Health

Max Rady College of Medicine, Rady Faculty of Health Sciences

University of Manitoba

546-715 McDermot Ave

Winnipeg, Manitoba

CANADA, R3E 3R4

Telephone (204) 977-5697

E-mail; [cgreenberg@hsc.mb.ca](mailto:cgreenberg@hsc.mb.ca)

## **Abstract**

The incidence of Severe Combined Immunodeficiency (SCID) in Manitoba, (1/15,000), is at least three to four times higher than the national average and that reported from other jurisdictions. It is overrepresented in two population groups: Mennonites (*ZAP70* founder mutation) and First Nations of Northern Cree ancestry (*IKBKB* founder mutation). We have previously demonstrated that in these two populations the most widely utilized T-cell receptor excision circle (TREC) assay is an ineffective newborn screening test to detect SCID as these patients have normal numbers of mature T-cells. We have developed a semi-automated, closed tube, high resolution DNA melting procedure to simultaneously genotype both of these mutations from the same newborn blood spot DNA extract used for the TREC assay. Parallel analysis of all newborn screening specimens utilizing both TREC analysis and the high resolution DNA procedure should provide as complete ascertainment as possible of SCID in the Manitoba population.

**Keywords:** newborn screening; SCID; TREC

## **Introduction**

Severe Combined Immunodeficiency (SCID) is the most profound form of the primary immunodeficiency diseases (PID) and is characterized by the lack of a functioning immune system. Infants born with SCID are normal at birth but invariably develop multiple severe infections which usually prove fatal in the first year of life. Treatment by hematopoietic stem cell transplantation early in life is associated with a good outcome and the highest probability of long-term survival [1]. Presymptomatic diagnosis and early intervention greatly improve the outcome of children with this condition. In March 2015, the Federal US Committee on Newborn Screening recommended the addition of Severe Combined Immunodeficiency (SCID) to the routine newborn screening panel [2]. The most widely used newborn screening test for SCID is the quantification of T-cell receptor excision circles (TREC) [3].

Canadian surveillance studies have found that SCID incidence is 3-fold higher in the province of Manitoba compared to the rest of Canada [4, 5]. In a retrospective study [5] of the 18 children diagnosed with SCID and other PID in Manitoba between 1992 and 2010, we demonstrated that more than half of these affected children would not have been identified on newborn screening by TREC analysis. The children who would have not been ascertained by TREC analysis belong to two separate population groups, each with a founder mutation that contributes to the disproportionately high frequency of SCID in Manitoba. One group is of Mennonite descent and has zeta chain-associated protein kinase (*ZAP70*) deficiency [6] and the second group is of First Nations Northern Cree ancestry with inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKBKB*) deficiency [7].

The Mennonite *ZAP70* deficiency seen in our population results from a homozygous G>A substitution in the acceptor splice site of intron 12 of *ZAP70* (c.1264-11G>A) abolishing the usual acceptor splice site and creating a new acceptor splice sequence upstream. This results in the insertion of 9 nucleotides in the mRNA and 3 additional amino acids in the protein product, inactivating the kinase. The Northern Cree *IKBKB* mutation results from the homozygous insertion of a G at nucleotide 1292 in exon 13 of the *IKBKB* gene (c.1292dupG) causing a frameshift mutation and a premature stop codon with complete loss of kinase function of the *IKBKB* protein. The latter is integral to the nf-Kb pathway by phosphorylating inhibitors of Kb, thereby impacting T and B cell receptors [7]. A semi-automated method involving closed-tube (homogeneous) high-resolution melting analysis for the simultaneous genotyping of these two founder mutations was developed and run in parallel with the quantification of TREC on each newborn dried filter paper blood spot (DBS) collected as part of routine newborn screening. We now report details of this methodology and its validation and emphasize the importance of ensuring that ascertainment is as complete as possible when introducing universal newborn screening for SCID.

## **Materials and Methods**

### **1) Patient studies**

DNA was extracted from 46 DBS retrieved from patients known to be at risk for *ZAP70* deficiency and *IKBKB* deficiency, genotyped in previous studies as n=3 *ZAP70*, n=2 *IKBKB* homozygous affected; n=6 *ZAP70*, n=14 *IKBKB* heterozygous; and n=1 *ZAP70*, n=20 *IKBKB* homozygous normal with respect to the *ZAP70* and *IKBKB* mutations [5,6,7]. The 46 DNA eluates were then amplified and analyzed by the high-resolution melting technique as described below and assigned a genotype. The amplicons assigned homozygous affected, heterozygous or homozygous normal genotypes were then compared to the Sanger sequencing results generated from the 46 original DBS to validate the genotype assignments from the high-resolution melting technique. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Board of the University of Manitoba (H2013:180).

### **2) High Resolution Melting DNA studies**

A quantitative real-time PCR (qPCR) assay was designed to make use of the high-resolution DNA melting curves by exploiting the difference in the melt temperature ( $T_m$ ) of amplicons resulting from the single nucleotide substitutions in *ZAP70* and *IKBKB* from individuals with different genotypes. Primers were designed to ensure similar melt temperatures ( $T_m$ ) for forward and reverse primers and short amplicon product sizes of less than 100 bp, based on the rationale

that the shorter the amplicon, the greater the effect of a single base change on the melting temperature of the product. The primer sequences and their Tms were as follows:

*IKBKB* Forward Primer - 5'- **AGG AAT CTC GCC TTC TTC C-3'** Length: 19 Tm 56.52

*IKBKB* Reverse Primer - 5'- **CTG GAT GCT GTG CCA GAC-3'** Length: 18 Tm 58.10

*ZAP70* Forward Primer- 5'- **TGA GGA GGA GGA CAC TGG-3'** Length: 18 Tm 57.13

*ZAP70* Reverse Primer - 5'- **TTG CCC TGC TCG ATG AAG-3'** Length: 18 Tm 57.38

DNA was extracted from each DBS using a 3mm spot punched by an Eppendorf DBS puncher and lysed using Qiagen Biosprint 96 lysis buffers containing Proteinase K at 56°C for 60 minutes while shaking at 900 rpm. DNA was extracted from the samples over 40 minutes using the Biomerieux Easymag Nuclisens system with magnetic silica beads to produce a 25 ul eluate of purified DNA. Eluates were stored at 4°C until analysis.

Amplification of 5 µl aliquots was performed using the Biorad CFX96 Touch System using Biorad SsoFast EvaGreen Supermix (Biorad Cat. No. 1725201). Reaction mixtures (total volume 20 µL) were prepared by combining 10 µL SsoFast EvaGreen Supermix, 0.2 µL of each 20 µM forward and reverse *IKBKB* primers, 0.2 µL of each 10 µM forward and reverse *ZAP70* primers, 5 µL extracted DNA eluate from patients and controls and 4.2 µL DNase /RNase free water to bring final reaction volume to 20 µL. Product amplification was monitored by the increase in fluorescent intensity using the following cycling parameters. Initial DNA denaturation at 98°C for 2 minutes (to complete the activation of hot start polymerase) was followed by denaturation at 98°C for 5 seconds and annealing/extension at 58°C for 10 seconds. This was repeated for 39 cycles with a plate read at the end of each cycle followed by a final denaturation at 95°C for 30 seconds and final anneal/extension at 70°C for 30 seconds. Melt curves were then generated between 75°-95°C by incrementally increasing the temperature in steps of 0.2°C for 10 seconds per step followed by plate read.

The melt curve profile data were generated by the CFX Manager software based on measurement of the decrease in fluorescent signal at each incremental step of double stranded amplicon denaturation. The amplicon melting temperature was defined at the point at which there was a decrease of 50% in fluorescent signal intensity. The Precision Melt Analysis software interpreted the melt curve data following signal strength normalization due to the variable intensity of the starting signal between specimens.

## **Results**

Figure 1 illustrates the raw fluorescence data of the Precision Melt Analysis adjusted by the negative first derivative visualizing the melting temperatures of the *ZAP70* and *IKBKB* amplicons (Panel A) and the normalized melt curves (Panel B). In Panel A the melting temperatures of the amplicons were assigned at the point where there is a 50% loss of fluorescent signal intensity, i.e., the point where one half of the double stranded amplicon is denatured. Panel B shows the results when the Precision Melt Analysis software normalized the raw fluorescent data and set pre- and post-melt signals to relative values of 1.0 to 0. The normalized melt curves produced clear differentiation between the *ZAP70* (Top Panel B) and *IKBKB* (Bottom Panel B) amplicons.

The temperature shifted difference curves further resolved and clearly differentiated the *ZAP70* and *IKBKB* homozygous affected, heterozygous and wild type genotypes respectively (Figure 2 Panels A and B). The *ZAP70* and *IKBKB* genotype assignments from the 46 DBS were validated by Sanger sequencing of DNA extracted from the original DBS samples. The high resolution melting technique used in the DNA studies also assigned a genotype of homozygous normal to 60 unrelated, random, age-matched, unaffected control samples with 100 % accuracy (data not shown).

## **Discussion/Conclusion**

Although TREC screening should identify 100% of T-cell deficient forms of SCID and PID, we have previously shown that it will not identify the majority of T-cell positive forms of SCID and PID that are prevalent in our population. Implementation of the high-resolution DNA melting analysis described in this manuscript for the determination of *ZAP70* and *IKBKB* genotypes in all patients run in parallel with TREC/RNaseP quantification should provide as complete ascertainment as possible of SCID in the newborn population of Manitoba. The methodology is semi-automated, scalable and reproducible allowing for high throughput, rapid universal newborn screening for SCID and for those particular conditions prevalent in our population, a programme we hope to implement in the coming year.

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## **Legend to Figures**

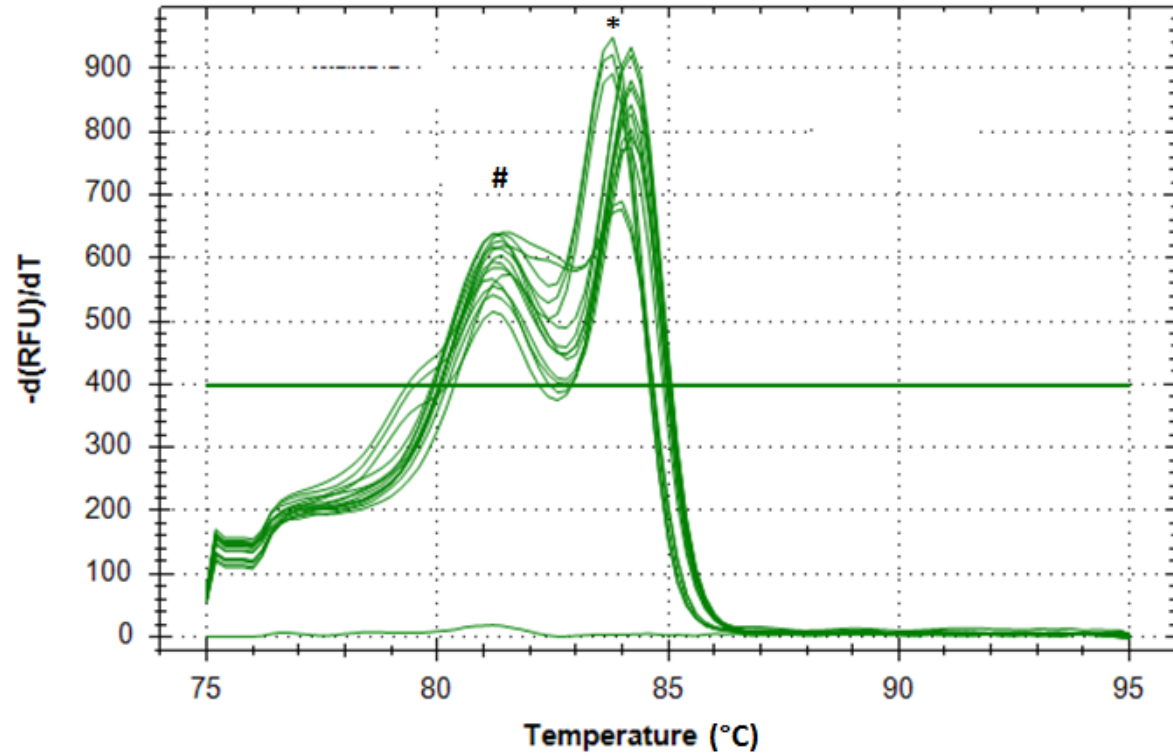
### **Figure 1 Negative First Regression Adjusted Raw Fluorescent Data of Amplified Products**

In Panel A the peaks of *ZAP70*\* and *IKBKB*# represent the melting temperatures of all the *ZAP70* and *IKBKB* amplicons where there is 50% loss of fluorescent signal intensity, i.e. the point at which one half of the double stranded amplicon DNA is denatured. The rate of change in fluorescence as the temperature rises is determined by plotting the negative first regression of relative fluorescence (RFU) vs. Temperature ( $-d(\text{RFU})/dT$ ) on the y-axis. Panel B shows the results when the Precision Melt Analysis software normalized the raw fluorescent data (Panel A) and set pre- and post-melt signals to relative values of 1.0 to 0. The normalized melt curves produced were distinct for the *ZAP70* amplicons (Top Panel B) and the *IKBKB* amplicons (Bottom Panel B).

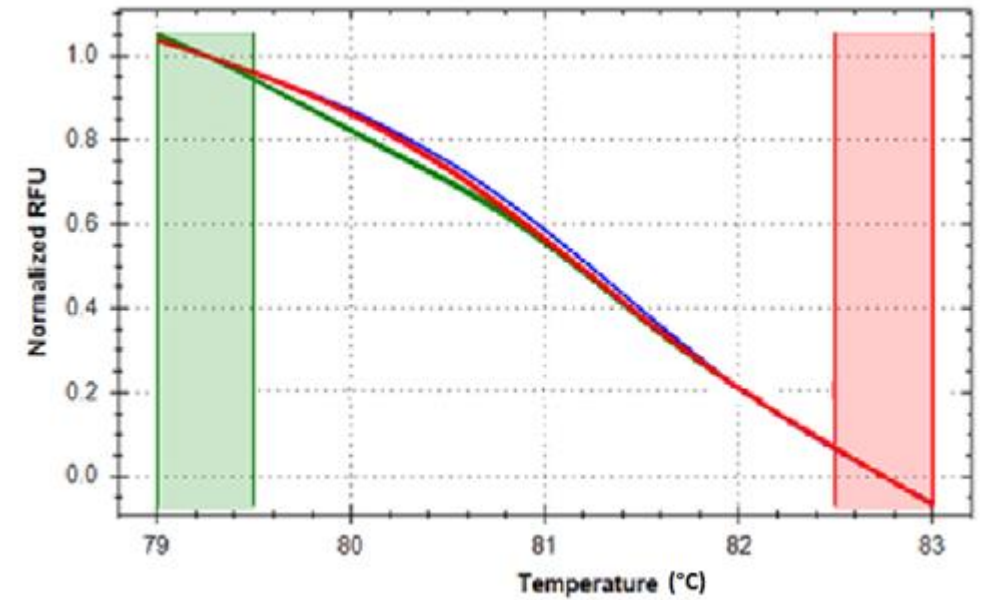
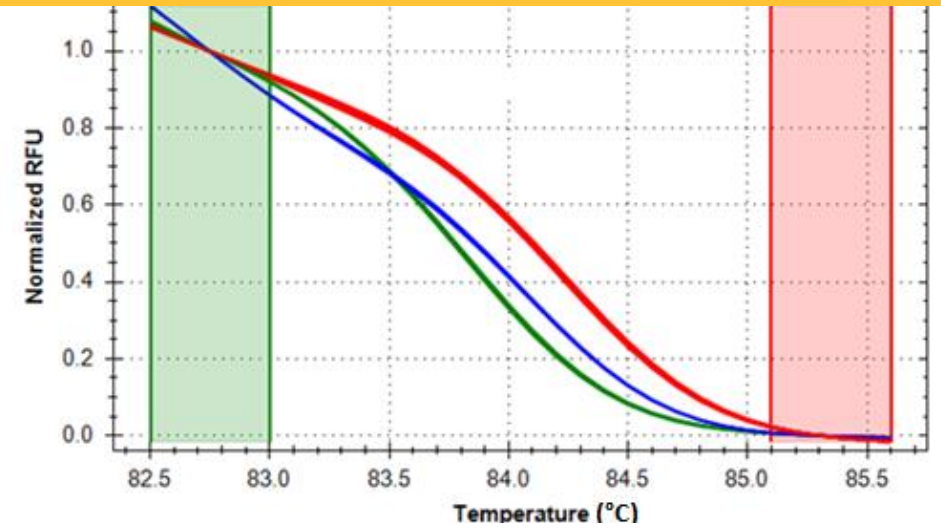
### Figure 2 Representative Temperature Shifted Difference Curves

The temperature shifted difference curves further resolve clusters of the same genotype and clearly differentiate the *ZAP70* homozygous affected (green line), heterozygous (blue line) and wild type (red line) genotypes (Panel A) and the *IKBKB* homozygous affected (blue line), heterozygous (green line) and wild type (red line) genotypes (Panel B).

Figure 1.



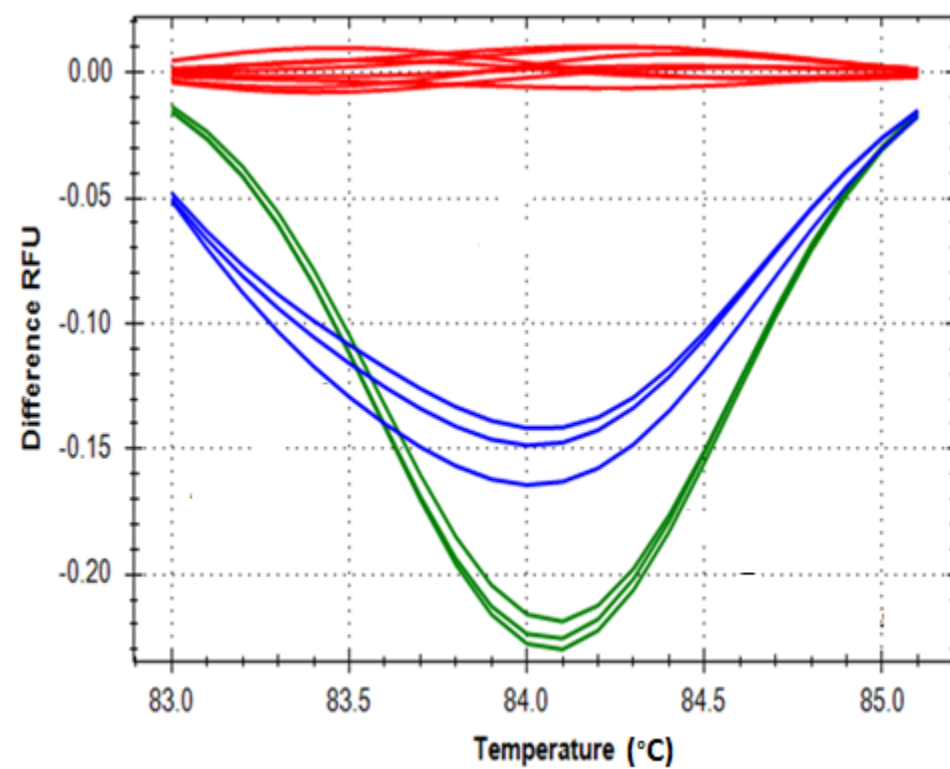
Panel A



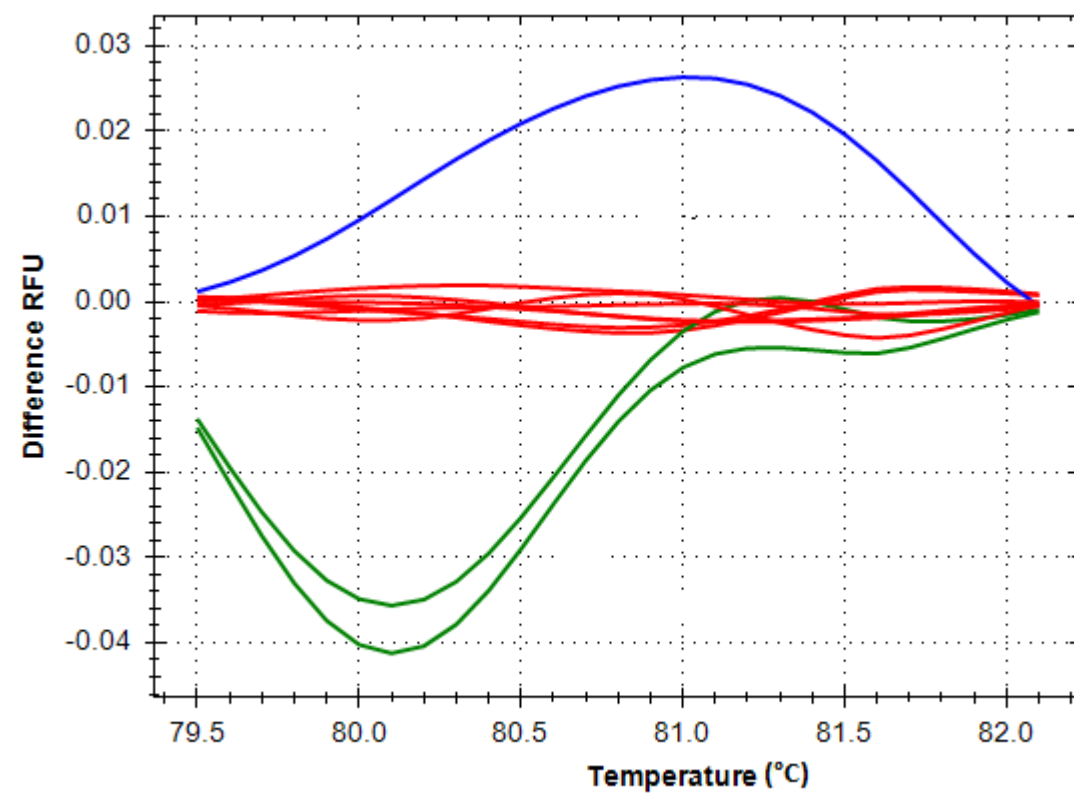
Panel B



Figure 2.



Panel A



Panel B