UNIVERSITI PUTRA MALAYSIA

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DEPARTMENT OF BIOMEDICAL SCIENCES

FACULTY OF MEDICINE AND HEALTH SCIENCES, UNIVERSITI PUTRA MALAYSIA


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**YEOH SOO GHEE**

**A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR SCIENCES (BIOMEDICAL SCIENCE)**

**DEPARTMENT OF BIOMEDICAL SCIENCES**
**FACULTY OF MEDICINE AND HEALTH SCIENCES, UNIVERSITI PUTRA MALAYSIA**

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# DEVELOPMENT OF PARALOGUE RATIO TEST FOR KIR COPY NUMBER QUANTIFICATION AMONG MALAYSIAN HIV-1 PATIENTS

**Yeoh Soo Ghee & Suhaili Abu Bakar**

*Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,*

*Universiti Putra Malaysia*

## ABSTACT

**Background:** Killer cell immunoglobulin-like receptors (KIR) located on chromosome 19q13.4 are inhibitory and activating immune receptors, on natural killer (NK) cells. KIR plays a significant role in facilitating cytotoxic activity of NK cells against pathogenic cells. KIR activity diversifies the NK cell function in the anti-HIV immune response, so the presence of KIR may influence HIV-1 infectious. KIR exhibits Copy Number Variation (CNV) and could affect HIV susceptibility attribute to gene dosage effect. Previous studies using real-time PCR, have shown an association between higher copy number of KIR gene and reduced risk of HIV-1 in European and Caucasian populations due to the increased activation of NK cell antiviral response. Nevertheless, limited study of Paralogue Ratio Test (PRT) application on KIR CNV had been conducted particularly among Malaysian population. PRT is known as a comparative PCR based method that uses single pair of primers to amplify both the test and reference loci. **Objective:** Thus, this study generally aimed to develop the PRT assay for KIR copy number quantification and association in Malaysian HIV-1 patients. Specifically to analyse the association of KIR CNV with CD4+ level. **Methodology:** A total of 130 extracted DNA samples from HIV-1 Malaysian patients (Malay: 52, Chinese: 49 & Indian: 29) and 163 of controls (Malay : 83, Chinese : 42 & Indian : 38) have been amplified by using PRT assay. PCR products were then electrophoresed and visualized under UV illumination, followed by capillary electrophoresis for peak scanner analysis through outsource of Apical Scientific Company. The genotypes were recorded and compared with the 163 of control samples. The results obtained were analyzed using Likelihood ratio test. **Result and discussion:** PRT assay has been unambiguously assigned 0.44 % of deletion and 0.3% of duplication KIR gene copies. However, distribution of KIR variable copy number was found not significantly associated with three major ethnics in Malaysia. Consequently, case-control analysis showed no association between the KIR copy number variables gene and Malaysia HIV-1 patients. Moreover, there is no significant correlation of CD4+ level with KIR CNV in Malaysia was found. **Conclusion:** Nevertheless, this study illustrates that PRT has been successfully detected CNV of KIR gene by showing there are variable KIR copies in HIV-1 and control, while KIR CNV is independent with ethnicity and CD4+ level after HAART.

*Keywords : KIR, NK cell,CNV, HIV-1, PRT*

# PEMBANGUNAN UJIAN RATIO PARALOG UNTUK KUANTIFIKASI BILANGAN SALINAN KIR DI KALANGAN PESAKIT HIV-1 MALAYSIA

**Yeoh Soo Ghee & Suhaili Abu Bakar**

*Jabatan Sains Bioperubatan, Fakulti Perubatan dan Sains Kesihatan*

*Universiti Putra Malaysia*

## ABSTRAK

**Pengenalan:** *Killer cell immunoglobulin-like receptors* (KIR) yang terletak pada kromosom 19q13.4 adalah berfungsi sebagai penghalang dan pengaktifan reseptor imun, pada *natural killer* (NK). KIR berperanan penting dalam membantu aktiviti sitotoksik sel NK terhadap sel patogenik. Aktiviti KIR mempelbagaikan fungsi sel NK dalam tindak balas imun anti-HIV, jadi kehadiran KIR dapat mempengaruhi kejangkitan HIV-1. KIR menunjukkan Kepelbagaian Bilangan Salinan (CNV) dan boleh mempengaruhi kerentanan HIV terhadap kesan dos gen. Kajian terdahulu yang menggunakan *real-time* PCR, telah menunjukkan perhubungan antara bilangan salinan gen KIR yang lebih tinggi dengan penurunan risiko HIV-1 dalam populasi Eropah dan Kaukasia kerana peningkatan pengaktifan tindak balas antivirus sel NK. Namun begitu, kajian yang dijalankan dengan menggunakan *Paralogue Ratio Test* (PRT) adalah terhad pada KIR CNV di kalangan populasi Malaysia. PRT adalah kaedah perbandingan PCR yang menggunakan pasangan primer tunggal untuk menperbanyakkan kedua lokasi ujian dan rujukan. **Tujuan:** Oleh itu, kajian ini secara amnya bertujuan untuk mengembangkan pengujian PRT untuk pengukuran bilangan salinan KIR dan kaitannya di kalangan pesakit HIV-1 Malaysia. Seacara khususnya, untuk menganalisis hubungan KIR CNV dengan aras CD4+. **Metodologi:** Sejumlah 130 sampel DNA yang diekstrak dari pesakit HIV-1 rakyat Malaysia (Melayu: 52, Cina: 49 & India: 29) dan 163 kontrol (Melayu: 83, Cina: 42 & India: 38) telah diamplikasi dengan menggunakan kaedah PRT. Produk PCR kemudian dielektroforeskan dan divisualisasikan dengan menggunakan pencahayaan UV, diikuti dengan elektroforesis kapilari untuk analisis pengimbas puncak oleh Syarikat Apical Saintifik. Genotip dicatatkan dan dibandingkan dengan 163 sampel kontrol. Hasil yang diperoleh dianalisis dengan menggunakan ujian *likelihood ratio*. **Keputusan dan Perbincangan:** PRT secara jelas telah menunjukkan 0.44% kehilangan dan 0.3% penduaan salinan gen KIR. Walau bagaimanapun, pengagihan kepelbagaian bilangan salinan KIR didapati tidak berjaya dikaitkan dengan tiga etnik utama di Malaysia. Maka, analisis kontrol-kes tidak menunjukkan hubungan antara bilangan salinan KIR gen dalam pesakit HIV-1 Malaysia. Tambahan pula, tidak terdapat perhubungan yang signifikan di antara aras CD4+ dengan KIR CNV di Malaysia. **Kesimpulan:** Walaupun begitu, kajian ini menggambarkan bahawa PRT telah berjaya mengesan KIR CNV gen dengan menunjukkan terdapat kepelbagaian salinan KIR dalam HIV-1 dan kontrol, sementara KIR CNV tidak bergantung pada etnik dan tahap CD4+ selepas HAART.

*Kata kunci : KIR, NK cell ,CNV, HIV-1, PRT*

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| | |
|---|---|
| Bp | Base pair |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CNV | Copy Number Variation |
| DNA | Deoxyribonucleic acid |
| HAART | Highly Activated Anti-Retroviral Therapy |
| HIV | Human Immunodeficiency Virus |
| KIR | Killer cell Immunoglobin-like receptor |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative Polymerase chain reaction |
| PRT | Paralogue Ratio Test |
| UV | Ultraviolet |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of Study

Human immunodeficiency virus (HIV) is a blood-borne virus typically transmitted through sexual intercourse, shared intravenous drug paraphernalia, and mother-to-child transmission (MTCT), which can occur during the birth process or during breastfeeding cycle. HIV disease is triggered by infection with HIV-1 or HIV-2, which involves retroviruses in the Retroviridae family, Lentivirus genus (Hakim et al., 2008).HIV or AIDS remains a major threat to public health worldwide by having 37.9 million people are living with HIV in 2018 and 770 000 died with HIV in 2018 with Africa consists of the highest number of people living with HIV (67.99%) at all ages in 2018 (WHO, 2018) In Malaysia, the new HIV infections had remained static between 2010 and 2017 at average of 3,400 case per year (Suleiman et al, 2018). The importance of host genetic factors in HIV infection has been known for the last 20 years, from the role of mutations in genes that encode chemokines and their receptors to specific microRNAs that interfere with the replication cycle of viruses (Zwolińska et al., 2009). The role of genetic characteristics in HIV infection is not always clear, and research findings are sometimes arbitrary. This involves the mutation KIR encoding genes, particularly in the context of virus susceptibility (Hong et al., 2013 & Paximadis et al., 2011).Furthermore, antigen-specific CD4 + CTLs which have been detected in HIV patients (Zaunders et al., 2004) may also have antiviral activity in some scenarios, despite the high degree to which CD4+T-cells are compromised in HIV disease (Vijayan et al., 2017). HIV infection is associated with the gradual loss of CD4+T cells by their reduction in KIR production. HIV attacks the CD4+T cells and can cause acquired immune deficiency syndrome (AIDS). Viral load monitoring and CD4+T cell enumeration are the two most preferred methods to identify the onset of disease and effectiveness of treatment respectively. The CD4 count provides invaluable information about the overall health of the immune system while plasma viral load determines the level of HIV viremia (Goedert et al., 1987).

KIR comprise a family of receptors expressed on the surface of Natural Killer (NK) cells and activated or memory T cell subsets. (Thielens et al., 2012). The KIR region which are located on chromosome 19q13.4, are highly polymorphic structure in humans (Uhrberg et al., 1997) and its extensive polymorphism has been repeatedly associated with the natural history of

HIV-1 infection (Carrington et al., 2008) KIR-HLA intercourse in HIV disease. Its polymorphism has been studied in several populations globally (Norman et al., 2001, Augusto et al., 2015, 2016 & Hollenbach et al., 2013) and more than 500 KIR gene-content genotypes have been described among over 200 worldwide populations (Gonzalez et al., 2015).Previously, Pelak (2011) found that CNV of KIR3DL1S1 affects HIV control (Pelak et al., 2011) and expression differences. Besides, its interaction with HLA-C can have a profound effect on hepatitis C virus infection resolution with an odds ratio of more than 2 (Khakoo et al., 2004 & Li et al., 2008). Hence, addictive effect of gene dosage effect may affect expression frequency of KIR gene on NK cells (Li et al., 2008).

Copy number variation (CNV) is an extensive source of variation occurred between individual genomes in human and many other species. There is evidence of CNV affecting phenotype in both disease and normal population (Iafrate et al., 2004 & Redon et al, 2006). Generally, CNV includes deletion, duplication and complex multiallelic variation, but accurate, precise normalized copy number genotyping is the most problematic and technically challenging. Therefore, Armour et al (2007) has developed PRT assay for standardised measurement of copy number. PRT is a form of quantitative PCR by using single pair of primer to for amplification, one from the copy variable region of interest and one from reference locus. Furthermore, it reduces the potential error of generating different amplification efficient of test and reference amplicon (Armour et al., 2007). Careful primer design is concerned to amplify from copy of the element within the variable repeat unit, while exact one from the unlinked reference locus. By comparison, Field et al (2009) suggested that there is an effect of differential error of real time-PCR methods between cases and control (Field et al., 2009). PRT-PCR based which is simple format and inexpensive, is comparable in accuracy to those alternative methods. Particularly, it can be used as rapid copy number typing for large scale case-control studies (Hollox et al., 2017).

Although several genetic association studies have adapted PRT assay such as CCL3L1, Complement C4 , SLC2A3 and DEFB4 (Walker et al., 2009, Field et al., 2009, Aldhous et al., 2010, Fernando et al., 2010, Khan et al., 2013, Veal et al., 2014, & Carpenter et al., 2015), there is limited research of employment of PRT assay to explore the effects of all KIR genes on HIV infection and other diseases. After comparison of data from real-time PCR with PRT assay, the research concluded that effect of differential error of real-time PCR measurement

methods between cases and controls might be involved rather than to a true association (Field et al., 2009). Thus, utilization of PRT assay should be highly encouraged for CNV measurement to reduce typing error, especially KIR gene. Besides, while the presence of some variations in copy numbers may influence the expression and function of KIR, knowledge of variations in copy numbers of KIR in HIV patients remains limited. Therefore, further studies are required to determine and confirm the relationship between the copy number variation of KIR gene and HIV progression by using PRT assay.

## 1.2 Problem Statement

In Malaysia, there is limited association studies between CNV and diseases. Previously, there are large-scale of association studies between CNV and diseases. According to the studies, CNV represent an alternative source of genetic variation influencing HIV, Crohn's disease and cancers (Gonzalez et al., 2012, Fellermann et al., 2006 & Frank et al., 2017). For example, there are association of *CCL3L1* and *β-defensin* copy number variation with HIV (Jamaluddin et al., 2020, Isa et al., 2020, Yusoff et al., 2020, Fu et al., 2018, Khan et al., 2017, Liu, S et al., 2010 & Hardwick, et al., 2016). Furthermore, CNV plays significant role in the pathogenesis of autoimmune, inflammatory, and cancer (McKinney et al., 2008, Fellermann et al., 2006 & Ledet et al., 2013).

Besides, there is also lack of association study of KIR gene polymorphism with HIV particularly, in Malaysian population. Formerly, there are several studies of KIR gene and HIV-1 progression in various counties. For instance, KIR gene polymorphism involved in HIV progression in India population , Zimbabwean population, Iranian Population, Zambian Population ,Polish population ,and Caucasian population (Gaudieri et al., 2005,Rajalingam et al., 2008, Hiby et al., 2010, López-Vázquez et al.,2005,Chavan et al., 2018, Mhandire et al., 2016 & Zwolińska et al., 2016). Based on previous studies, research on KIR gene polymorphism among Malaysian population are unrelated to HIV (NurWaliyuddin et al., 2015, NurWaliyuddin et al., 2014 & Aghafar et al., 2012). Therefore, there is limited genetic association study of KIR gene polymorphism with HIV-1 in Malaysia population.

Based on preceding studies, PRT assay has been utilized for quantification of CNV in beta-defensin, Complement C4, CCL3L1/CCLAL1, SLC2A3, SIRPB1, alpha-defensin 1(DEFA1A3) ,and Amylase region (AMγ1 and AMγ2), except for KIR gene (Walker et al., 2009, Aldhous et al., 2010, Fernando et al., 2010,Khan et al., 2013, Veal et al., 2014, & Carpenter et al, 2015). Consequently, this present study plays significant role in selection of the gold method for KIR CNV quantification.

Furthermore, according to current data, there is an increase incidence of HIV in Malaysian population. This is justified by Suleiman et al (2018) by showing the incidence rate of HIV has increased to 0.43 % in 2017 compared to 0.42 % in 2016.

### 1.3 Hypothesis

1) Variable copies of KIR gene present in HIV-1 patients and control.
2) Copy Number Variation (CNV) of KIR gene is associated with Malaysian HIV-1 patients.
3) Higher variable copies of KIR gene is associated with higher CD4+ level and lower viral load.

### 1.4 Objective

**General Objective :**

To develop the PRT assay for KIR copy number quantification and association in Malaysian HIV-1 patients.

**Specific Objectives :**

1) To obtain the KIR genotype by using the optimized PRT.

2) To compare the frequency of KIR genotype in HIV-1 patients with control among three major ethnics in Malaysia.

3) To analyze the association of KIR copy number variation (CNV) with CD4+ level and viral load.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Copy Number Variation

Genetic polymorphism can be defined as the presence of two or more alleles in a single locus , with the most common allele have frequency of 99% or less in a population (Singh,2012).Copy number variation (CNV) is defined as DNA segment of one kilobase (kb) or larger that differs in copy number between individuals compared to reference genome. It is an extensive and often underappreciated source of genetic variation within species (Schrider et al., 2010). CNV has shown as a common source of genomic diversity in human and it is suspected to influence at least 12% of the human genome (Redon et al., 2006). According to the study by Schrider et al (2010), they reported that CNVs cannot be efficiently tagged by nearby SNPs because of the possibility of recurrent mutations and transposition of the duplicated genomic segments into new genomic locations. CNV plays critical role in affecting phenotype due to gene dosage effect. Elevated levels of mRNA and protein is caused by elevated number of same gene. (Hollox et al., 2014). Abundant levels of copy number variation of specific gene, and stochastic expression profiles may reflect the complexity of the KIR clusters.

Based on the study by Hardwick (2012) ,higher copy number of β-defensin is associated with increased HIV load before HAART and low immune reconstitution after HAART initiation (Hardwick et al.,2012).However, the study cited by Abujaber (2017) argued against an impact of copy number variation of the β-defensin region in the spontaneous control of HIV infection due to no significant difference between distribution of β-defensin copy number between European cases and controls (Abujaber et al., 2017).Besides, this evidence is supported by Mehlotra (2016) by showing no association between CNV of β-defensin (DEFB) genes and clinical measure of HIV in Caucasians, or African Americans.

Besides, lower copy number of CCL3L1 is associated with an increased risk of HIV-1 infection, while higher copy number is associated with reduced risk for acquiring HIV-1 (Liu et al., 2010).The role of CCL3L1 CNV is further investigated by showing association of low CCL3L1 gene copy number with improved immune reconstitution following initiation of highly active anti-retroviral therapy in HIV (Aklillu et al., 2013).

Other than CCL3L1 and β-defensin (DEFB) genes polymorphism, there were large amount of KIR polymorphism researches had been conducted. Pelak (2011) demonstrated that, in the presence of KIR3DS1 and the appropriate ligands, NK cells with multiple copies of KIR3DL1 more robustly inhibit HIV-1 replication. It was found that the CNV of KIR can influence the proportion of NK cells expressing the KIR receptor, expression level of KIR on NK cell, ligand specificity, and ability of NK cell recognition of viral infected cell (Pelak et al., 2011).

Previously, the study done by Hellman et al (2011) showed none association of KIR3DH copy numbers with neither loss of total CD4+ T cells nor loss of CD4+ T cells in rhesus macaques of Indian origin (Hellmann et al., 2011). However, for KIR2DL4, correlation of KIR2DL4 copy number variation (CNV) with CD4+ T-cell decline during primary immunodeficiency virus (SIV) infection in these monkeys was found. Therefore, higher KIR2DL4 copy numbers is associated with better survival of CD4+ T cells and increased gamma interferon (IFN-π) (Hellmann et al., 2013). In contrast, Gaudieri et al (2005) revealed higher *KIR2DS2* copy number was associated with greater $CD4^+$ T cell loss and rapid progression to AIDS (Gaudieri et al., 2005). Based on these conflicting results, further research still be needed.

Apart from that, research by Bruijnesteijn (2018) showed that alternative splicing involves an additional of complexity to the KIR gene scheme, resulting in a wider range of structural and functional KIR receptors and their isoforms that can play critical roles in health and disease. Hence, further studies on role of KIR gene for different disease by considering alternative splicing are required.

In addition, there is an association between CNV with diseases such as autoimmune disease, infectious disease, cancers ,inflammatory, neurodevelopmental disease, cardiovascular disease (Brønstad et al., 2011, Kuiper et al., 2010, Hollox et al., 2014, Swaminathan et al., 2012, Liang et al., 2016, Frenkel et al., 2020, Glessner et al., 2017 & Pollex et al., 2007). Copy number of CCL3L1 more than 2 copies which causes increased recruitment of T cells and macrophage to a site of early inflammation result in elevated response of pro-inflammatory feedback (McKinney et al., 2008). Based on Fellermann et al (2006), impaired induction of epithelial β-defensin HBD-2 in Crohn's disease may due to lower copy number of β-defensin (Fellermann et al., 2006). Additionally, there is an association of higher copy number of CDH13 with increased risk of prostate cancer by causing elevated methylation of CDH13 in cancer (Ledet et al., 2013). Moreover, low FCGR3B copy number is associated with

increased risk of systemic lupus erythematosus, microscopic and Wegener's granulomatosis (Fanciulli et al., 2007). For neurodegenerative disease, increased risk of Parkinson disease is associated with lower copy number *PARK2 (*Pankratz et al., 2011).

### 2.2 KIR (Killer cell Immunoglobin-like receptor)

KIRs are known as natural killer (NK) cell receptors that trigger lysis of any infected cell when binding to their human leukocyte antigen (HLA) ligands. KIR genes are located in 15 different loci on chromosome 19q13.4, and some of them are allelic variants, KIR3DL1/S1, 2DL2/ 2DL3, and2DS3/2DS5 (Jiang et al., 2012 & Martin et al., 2013). They are diverse family of activating and inhibitory receptors that modulate the development and activity of NK cells and some of CD8+ T cells by reacting with the class I major histocompatibility complex (MHC I) (Bjorkstrom et al., 2012). KIR which acts as activation threshold of NK cell, plays significant roles in regulating the development, tolerance and activation of NK cells. The cytolytic activity of NK cell is modulated by the interaction of inhibitory and activating KIR with MHC I. For missing-self hypothesis, the HIV infected cell evades from the elimination by T cells through downregulation of MHC I expression. Thus, NK cells with inhibitory KIR recognizes and eliminates the HIV- infected cell that fails to express self-MHC I (Uhrberg et al., 1997). The diversity of KIR in gene content, polymorphism and expression variation affects the ability of NK cells responding to virus-infected cells. Moreover, their extensive variability generate repertoire of NK cells in which KIR are expressed at the cell surface in a combinatorial fashion (Carrington et al., 2008).

Apart from that, KIR haplotypes can possess from 7 to 12 genes and may be divided into two main haplotype groups A and B on the basis of the presence of specific genes in centromeric and telomeric regions of the cluster. KIR haplotypes are highly diversified by copy number variability. Formely, there is an evidence that copy number may be important to susceptibility to certain diseases, leading to differences in expression (Norman et al., 2009). Accordingly, differential frequency of expression of KIR also may due to gene dosage effect. Higher expression of KIR repertoire causes increased activation of NK cell antiviral response. NK cell activity contributes to the immune control of HIV-1 infection by providing rapid response to viral infected cells and striking the balance between the innate and adaptive

immune systems. (Li et al., 2008, Carrington et al., 2008, Wang et al., 2018, Naranbhai et al., 2015, Middleton & Gonzelez, 2009).

There have been several studies conducted to determine the genetic association of KIR with HIV disease in various populations including Chinese population, India population , Zimbabwean population, Iranian Population, Zambian Population ,Polish population ,and Caucasian population (Jiang et al., 2013,Gaudieri et al.,2005,Rajalingam et al., 2008,Hiby et al., 2010, López-Vázquez et al., 2005, Chavan et al., 2018, Mhandire et al., 2016 & Zwolińska et al., 2016). Based on a meta-analysis study conducted by Chavan V.R (2015) in India by using PCR-SSP method, association of specific KIR genes with perinatal HIV infection was shown by high frequencies of activating gene KIR 2DS5 and inhibitory gene KIR 2DL3 in HIV exposed uninfected (EU) infants compared to HIV-1 positive infants (Chavan et al., 2015). Recently, further KIR genetic association studies with HIV-1 among certain population such as Asian, Caucasian, and East Asian have been conducted by Zhao (2019). In overall, the presence of KIR2DS4 may associate with increased risk of HIV-1 infection while KIR3DS1 may associate with reduced risk (Zhao et al., 2019). However, there is limited study reporting the association of KIR3DL1 specifically with HIV progression. There has been insufficient research to unmask the effects of all KIR genes on HIV infection. The role of genetic features in HIV infection is not always clear, and sometimes the results of research are questionable or conflicting (Zwolińska et al., 2016). It is also due to the high diversity of KIR gene and HIV variability as well as its defensive strategies (Gonzalez-Galarza et al., 2015).In addition, it is interesting that there are large amount of studies showed the combination KIR-HLA may provide protective effect to HIV than HLA or KIR alone (Qi et al., 2006, Bashirova et al, 2010, Paximadis et al., 2011, Jennes et al., 2013, Tiemessen et al., 2011, Soria et al., 2011, Jiang et al., 2013, Habegger de Sorrentino et al., 2013 & Jamil et al., 2011).This indicates that associations with HLA may be linked to interaction with KIR. However, it is still inconclusive that the significance of NK cell in killing neoplastic cells as well as increasing amount of research reporting disease connection with KIR-HLA are taken into account.

Furthermore, genetic association studies of KIR genes with other diseases have been widely conducted. The potential connection of KIR genes with complex malaria (CM) among northern Indians has explored. KIR receptor-HLA ligand association also assessed the seriousness of the disease as controlled by topics of uncomplicated malaria (UCM) (Prakash

et al., 2018). In previous study, KIR were associated with several types of cancer such as colorectal carcinoma, breast cancer, prostate cancer, thyroid cancer, lung cancer and ovarian cancer (Canossi et al., 2016, Jobim et al., 2013, Portela et al., 2012, Ashouri et al., 2012, Li et al., 2019 & Giebel et al., 2014). Besides, its extensive polymorphism involved in the association of Inflammatory bowel disease (IBD) by showing positive association for KIR2DL5 and KIR2DS1 (Fathollahi et al., 2018). Recently, an evidence estimated that KIR2DL3, 2DL5, 3DL3, and 2DS5 might have a potential protective role for rheumatoid arthritis disease (RA) (Aghaei et al., 2019). Similarly, Rizzo R (2019) also showed the correlation of combination KIR2DS2/KIR2DL2/C1 with severe Alzheimer's Disease status and an increased susceptibility to HHV-6A infection. (Rizzo et al., 2019). KIR2S2 and KIR2L2 may play protective roles against bladder cancer development in Iranian population by showing decreased frequency of inhibitory KIR2DL2 and activating KIR2DS2 (Jamali et al., 2018).

**2.3 Paralogue Ratio Test (PRT)**

The paralogue ratio test (PRT) is a quantitative PCR that uses single pair of primer to amplify test locus with variable of copy number and reference locus without variable of copy number. PRT which is a type of comparative PCR differs in one important aspect from the conventional method: by designing the primers that target paralogous sequences, and one primer pair is used to amplify a putative CNV target locus relative to a single copy reference locus ( Armour et al., 2007).PRT characterizes the CNV in terms of the actual integer number of copies per diploid genome to maximize the power of association studies of CNV. It also can be used for determination of multiallelic complex copy number and sequence variation by identifying 81 % of sequence variants (Forni et al.,2015). The copy number of the KIR gene was estimated according to ratio of test to reference product yields. Single-nucleotide change is used to distinguish the test amplicon from reference amplicon. Accordingly, PRT can quantify this single nucleotide difference to measure the amount of test and reference. Importantly, this strategy avoids the problems caused by the comparison between the yields of two dissimilar amplicons that may have different amplification efficiencies (Aldhous et al., 2010).

In several researches for analyzing CNV, PRT has been effectively implemented (Aldhous et al., 2010, Hollox et al., 2009, Fernando et al., 2010, Hardwick et al., 2014, Khan et al.,2013, Polley et al.,2015, Walker et al., 2009, Carpenter et al., 2011, Saldanha et al., 2011, Veal et al., 2014, Koontz et al., 2014, Carpenter et al., 2015, Hallast et al., 2013,& Royo et al., 2015). However, due to problems in assay design, its use was uncommon. Research conducted by Hollox (2017) for the analysis of copy number variation of the beta-defensin locus had been carried out by using paralogue ratio test. This test has been applied successfully at many other loci. Besides, this research also addressed approaches to use manual and bioinformatics techniques, strengths and approach weaknesses to design effective PRT assays (Hollox et al., 2017).For the study of SIRPB1 copy number variant, it was also carried out by using Paralog Ratio Test (PRT) followed by the quantification of MALDI-MS in order to determine the genotype of the CNV (Royo et al., 2015). Apart from that, to test the hypothesis of association of variation in defensin beta DEFB copy number, DEFB copy number that is variable in copy number was determined by paralogue ratio test (James et al.,2015). In the Alzheimer's disease study cited by Kucukkilic et al (2018), triplex paralogue ratio test assay for CR1 LCR copy number was performed by involving large sample size with a limited amount of DNA (Kucukkilic et al., 2018). Moreover, FCGR3A and FCGR3B copy numbers (CNs) were also determined by both a paralogue ratio test and TaqMan quantitative PCR assay (Qi et al., 2017).

Research conducted by Perne (2009) investigated the comparison of Multiplex Ligation Dependent Probe Amplification (MLPA), real-time PCR (qPCR) and Pyrosequencing-based paralog ratio test (PRT) accuracy for copy number quantification. The data for CCL3L1 and HIV susceptibility, and β-defensins and Crohn's disease showed differences in performance of the different methods by calculating the CNV of the raw data (Perne et al., 2009). For instance, for determining the association CCL3L1 copy number and type I diabetes, deviation between the data of PRT and real time-PCR was found due to effect of differential error of real-time PCR measurement methods between cases and controls (Field et al., 2009). Hence, further studies are required for quantification of gene copy number through PRT for validation.

Even though aCGH (Lemay et.al., 2019), sequence read depth analysis of next-generation sequencing (Yao et al., 2019) and analysis of hybridization signal intensity from

SNP genotyping arrays (Sudmant et al., 2010) can be used for CNV determination, but these techniques were limited in the ability of complex multiallelic CNV to correctly label an integer copy number. Micrograms of DNA are required for sequence read depth analysis approaches (Pugh et al., 2008). Therefore, it may lead to false-negative results due to overestimation of library complexity (Eijkelenboom et al.,2016). Besides, this current method is not possible to amplify entire genome (Haraksingh et al., 2011 & Shen et al., 2016) as this method is known to implement unreliable biased copy numbers. Additionally, it has relatively low detection rates (30–60%), high false detection rates (< 10–89%), and are highly dependent on the quality of the reference genome read alignment (Abyzov et al., 2011).

### 2.4 Comparison between Other Molecular Techniques and PRT

By comparison, there are several methods with different advantages and disadvantages for KIR copy number quantification such as PCR-SPP, qPCR, MLPA, and MAPH.

PCR-SPP is polymerase chain reaction that uses single specific primer for DNA amplification by permitting unidirectional genome moving from known into unknown chromosome (Shyamala et al., 1993). Previously, PCR-SPP is widely used technique for allele identification and copy number measurement (Lobashevsky et al., 1999, Gaudieri et al., 2005, Vickerman et al., 2011 Touinssi et al., 2001, & Steffensen et al., 2000). This method involves annealing non-specifically to the similar DNA sequences but dissimilar to the target DNA. However, its limitations include time consuming (8 hours), high cost and requirement of separate tubes for allele amplification (Steffensen et al., 2003).

Apart from that, real-time or quantitative PCR (qPCR) involves amplification of DNA in real time used to impute the copy number of KIR by involving TaqMan detection. The Cq value is quantitative cycle in which the fluorescent can be detected. The higher the Cq value, the lower the copy number of the target. It can be used to quantify the copy number in single loci by requiring 5 to 10ng DNA (Jiang et al, 2016). Based on previous studies, qPCR is commonly being used for CNV quantification (Hellmann et al, 2011, Jiang et al., 2012, Pontikos et al., 2014, Roberts et al., 2014 & Chaisri et al., 2018). Nevertheless, limited number of targets can be detected as well as requirement TaqMan for fluorescent detection for this method. According to previous CNV results of KIR2DL1 by qRT-PCR ,63% to 84.6% of 43 samples

showed 1 copy while none of the sample has 4 copies (Saltarrelli et al., 2015). Furthermore, one of the biggest challenges of qPCR include the difference of kinetic amplification leading to proportional changes of Cq value and copy number quantification (Fernandez-Jimenez et al., 2011).

Besides, Multiplex Ligation-Dependent Amplification (MLPA) is locus-specific multiplex technique for molecular diagnosis of several genetic diseases by detecting copy number variation of specific genes, including small intragenic rearrangements (Stuppia et al., 2012). Previously, MLPA had been used for quantification of KIR copy number (Kozlowski et al., 2007, Hellmann et al., 2011 & Vendelbosch et al., 2013). It can be used to detect 40 targets by using of single pair of primer due to the PCR amplification on the ligated probes, but not target sequences. By comparison, this method involves 100 to 200ng DNA samples, time consuming, expensive, and complicated interpretation due to unreliable probes.

By comparing MAPH with PRT, Multiplex Amplifiable Probe Hybridisation (MAPH) can be used for copy number analysis of KIR due to recovery and amplification of specific probes and after hybridization to genomic DNA immobilized on a solid matrix. Hence, time to obtain results is more than 24 hours. It requires 0.5 to 1 µg DNA sample to detect more than 40 of target loci with 100bp resolution (Armour et al., 2000 & Hollox et al., 2008).

Particularly, in comparison, the main difference of PRT with these methods is the use of single pair of designed primer for amplification of test and reference locus. Furthermore, due to the same pair of primer being used to amplify the test and reference locus, the kinetic of amplification of the two amplicons are almost same (Hollox et al., 2017).

# CHAPTER THREE

# MATERIALS AND METHODOLOGY

## 3.1 Study Design

Study population involved HIV-infected individuals from Hospital Sg. Buloh, Hospital Sultanah NurZahirah, and Hospital Kajang in Malaysia and included three main races in Malaysia: Malay, Chinese and Indian. Total of 293 samples composed of 46.1% of Malay (n=135), 31.1% of Chinese(n=91), and 34.7% of Indian(n=67).CD4+ cell count was taken from patient receiving first line of Anti-retroviral Therapy while extracted DNA from the blood samples was eluted and stored under -20˚C until further usage. This study has been approved by the Medical Research and Ethics Committee (MREC) (Reference: NMRR ID: NMRR-16-705-29043 IIR)

## 3.2 Agarose gel electrophoresis

In this study, agarose gel electrophoresis was performed for PCR optimization. It mainly was used for confirmation of the presence of DNA and the quality of the primers used to target specific sequence at the desired location based on the size of the bands shown under UV light.

Firstly, a gel with concentration of 3% was prepared by dissolving 0.8g of agarose powder in 40ml of 1X TBE buffer before boiling the solution until a clear mixture solution. After boiling, the fully dissolved solution was poured into a cassette with comb to allow it becomes harden for 20-30 minutes. Next, agarose gel was put into the electrophoresis tank which was then filled up with 1X TBE buffer until the gel fully submerged.

For visualization of the presence and absence of the targeted gene, electrophoresis was conducted by using 3% agarose for DNA fragment separation and Novel juice (GeneDireX, Inc.) for visualization of the migrated band upon UV illumination. The presence of KIR gene for test locus and reference locus are indicated by 399bp fragment and 383bp fragment respectively.

Primarily, 4µl of 100bp DNA ladder was mixed with 3µl of Novel Juice while 8µl of PCR product were mixed with 2µl of Novel Juice. After that, a constant voltage supply of 100 Volt

was run through the agarose gel inside a gel tank filling with 1X TBE buffer. DNA which is negatively charged allows the DNA band to migrate from the negative terminal to positive terminal. A mixture of 3 dye colours which consist of bromophenol blue, xylene cyanol, and orange G is used for the monitoring of the DNA migration. Lastly, the voltage supply was switched off after a complete DNA migration which is indicated by Xylene Cyanol (purple) reached the third line on the cassette.

### 3.3 Paralogue Ratio Test (PRT)

Genotyping of KIR genes was conducted by using PRT-based PCR. PRT based-PCR was performed to amplify the KIR gene by using the single pair of specific primer designed. In this study, the PRT primer was fluorescently labelled with FAM to allow detection. The primer sequences and product sizes for test and reference loci of KIR gene provided by UK Collaborator, Prof Dr Edward Hollox, Leicester University were shown in Table 1. By using UCSC Genome Browser (http://genome.ucsc.edu/), the primer sequences were checked for confirmation of the product sizes of amplification products.

Table 1 : Primer sequences and product size for PCR.

| Gene | Primer Sequence | Product size | |
|---|---|---|---|
| KIR_PRT 1 | Forward : ATTCATCCACTGATGGGCA Reverse : GAGACCAGCCTGGCTAACAT | Test locus | Reference locus |
| | | 399 bp | 383 bp |

In the 96 well plate, the total samples for each plate involves 94 DNA samples, 1 negative control, and 1 empty blank. Firstly, Master Mix with total volume of 10µl with the composition of mix as shown as Table 2 was prepared.

Table 2 : Preparation of PCR Master Mix for 1x DNA samples

| **Component** | **Volume for 1x** |
|---|---|
| 2x Taq PCR Green Premix | 5.0 µl |
| Forward primer | 0.5 µl |
| Reverse primer | 0.5 µl |
| Ultra-purewater | 1.0 µl |
| DNA sample | 3.0 µl |
| **Total Volume** | **7.0 µl** |

After filling the prepared Master Mix into the 96 well plate, the plate was placed into the thermocycler for gene amplification with PCR reaction as shown as Table 3.

Table 3 : PCR Cycling Condition

| Process | Temperature ( ˚C ) | Time | |
|---|---|---|---|
| Initial denaturation | 95 | 1 min | |
| Denaturation | 95 | 30 sec | 30 cycles |
| Annealing | 55 | 30 sec | |
| Extension | 70 | 1 min | |
| Final annealing | 70 | 40 min | |
| Final extension | 15 | - | |

After pre-denaturation at 95˚C for 1 minute, the amplification is followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds and extension at 70˚C for 1 minute. The amplification is completed at 70˚C for 40 minutes for final annealing while at 15 ˚C for final extension until further usage.

### 3.4 Capillary Electrophoresis

Capillary electrophoresis involves application of high voltage up to 30 kV for electrokinetic separation of proteins, peptides, amino acids, oligonucleotides, nucleosides and various small organic molecules at extremely high resolution (Kasper et al, 1988). In this study, the PCR product will be sent for capillary electrophoresis service at the Apical Scientific Company. CE is used for efficient separation of PCR products based on their product sizes to obtain an electropherogram. It involves 96 capillaries for high-throughput capillary DNA sequencing.

Firstly, 1.5µl of PCR products will be mixed with 10ul HiDi formamide and ROX-marker before the PCR products co-injected into the capillary electrophoresis system for analysis. The migration of the negatively-charged DNA to the positively-charged electrode was initiated by the application of high voltage power supply. According to these, the fluorescently-labelled DNA will be separated according to their product sizes. The smaller DNA fragments move more quickly than the larger. Then, the fluorescently labelled PCR product will be passed through the laser beam along the pathway to positively-charged electrode. The end-label dyes which were activated by the laser beam in the detector window will result in dye signal with different fluorescence wavelength .CCD camera was used for detection of the dye signals separated by diffraction system. Hence, the output of the detector was sent to the computer by converting the dye signals into digital data. The data was then displayed as an electropherogram by reporting the peak height. Based on the peak height shown at target product sizes, the copy number of the DNA sample was estimated.

### 3.5 Data Analysis

By using Peak Scanner Software Version 1, peak areas and height corresponding to the 380bp, 383bp, 396bp and 399bp PCR product were recorded. For the quantification of copy number of DNA samples, the copy number of the DNA samples were estimated and assumed according to the ratio of height of test to reference at targeted product sizes.

### 3.6 Statistical Analysis

For the collected data analysis, the Statistical Package for Social Sciences (SPSS) software version 25.00 was run. By using Likelihood ratio test, association of CNV of KIR with HIV-1 was studied as well as the association between CNV of KIR gene and CD4+ level after HAART. P-value less than 0.005 ($p < 0.005$) was defined as statistically significant.

# CHAPTER FOUR
# RESULTS

## 4.1 Distribution of human subjects

A total of 130 extracted DNA samples from HIV-1 Malaysian patients and 163 of control samples were used in this study. From 130 of HIV-1 samples, the study involved 40.0 % of Malay, 37.7 % of Chinese and 22.3 % of Indian.

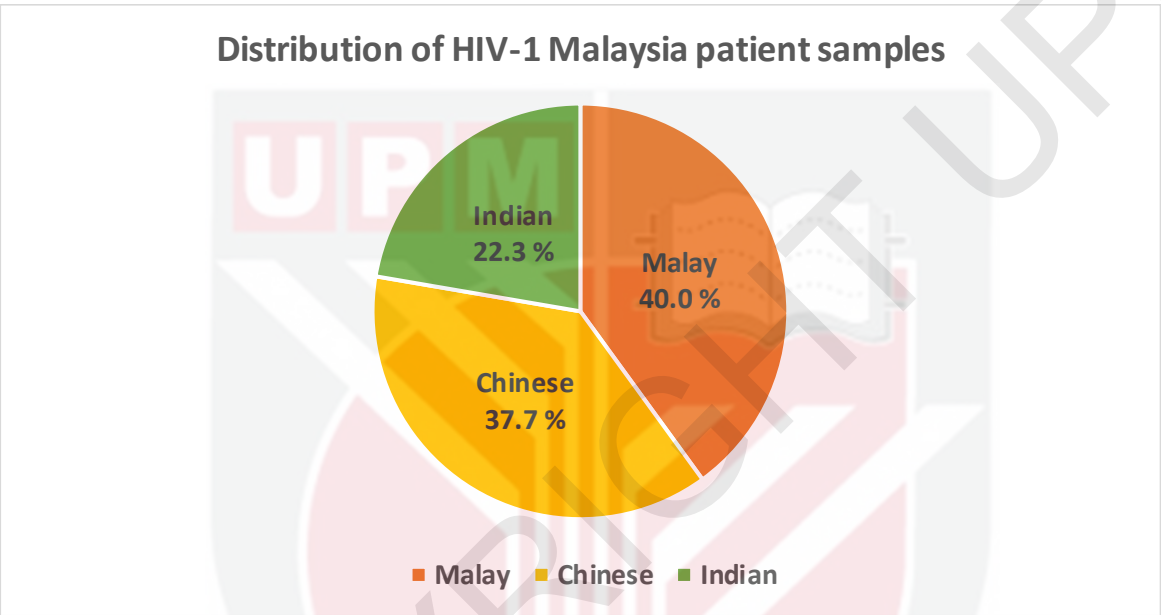


Figure 1 : Pie chart for distribution of HIV-1 patient samples among the three major ethnics in Malaysia.

## 4.2 Agarose Gel Electrophoresis

For the confirmation of amplification product based on the desired product size, PCR optimization was performed by proceeding the PCR product to agarose gel electrophoresis before performing the capillary electrophoresis. The result of the gel electrophoresis was showed as Figure 2 below. The figure showed the two distinct bands at 383bp and 399bp. Due to small difference between the two bands, the result showed single thicker band with unclear separation between the two bands.

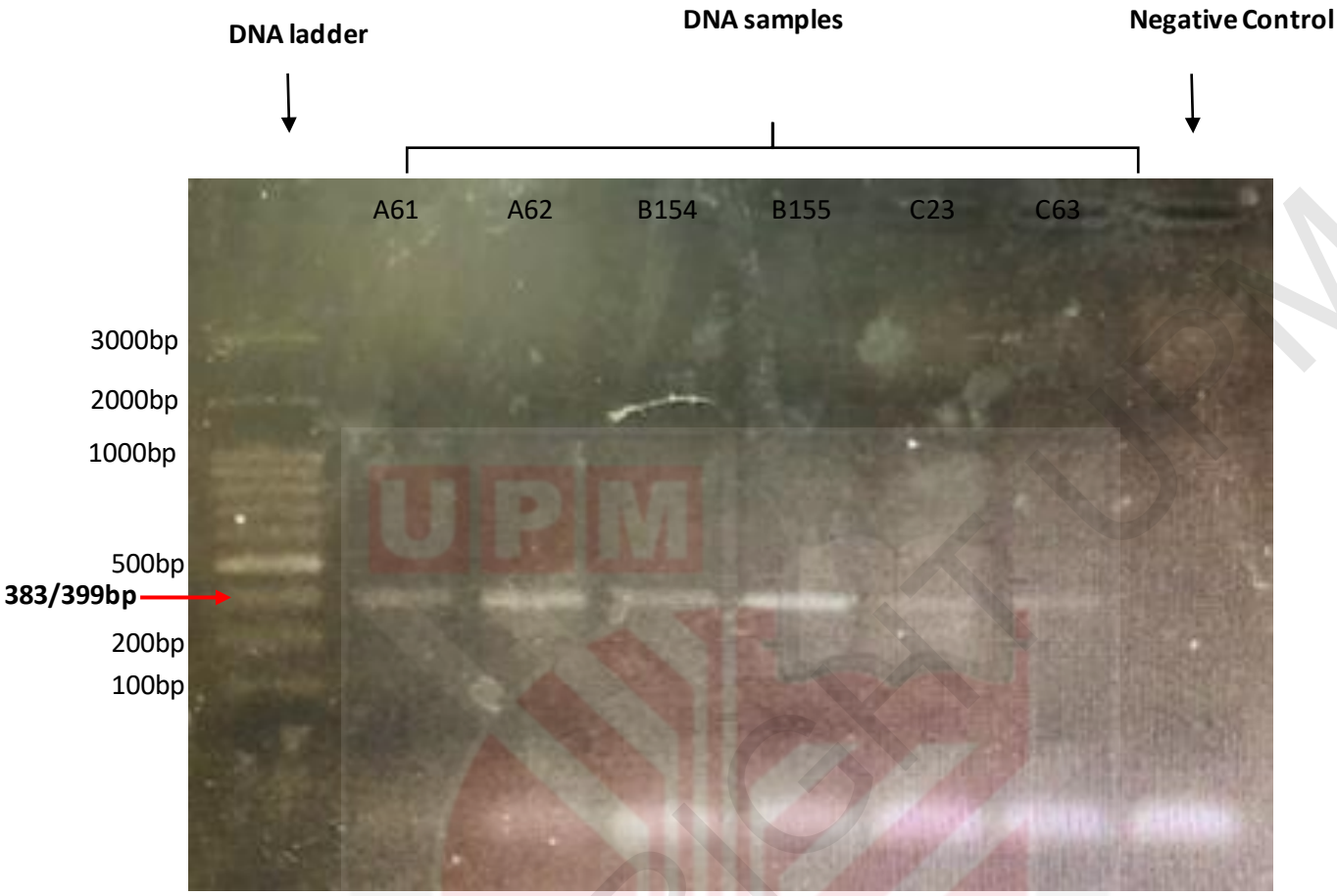


Figure 2 : PCR product visualized under 3 % gel electrophoresis for samples ( A61, A62, B154, B155 C23, C63, and negative control.

### 4.3 Capillary Electrophoresis

According to the results analysed through Peak Scanner Software Version 1, the peaks for height and area of the DNA samples showed respective sizes ; 383/396bp, 383/399bp, 380/396bp,and 380/399bp. The analysed results for samples with 1 , 3 and 4 copy numbers via Peak Scanner Software version 1.0 separated based on the sizes were shown on Figure 3.

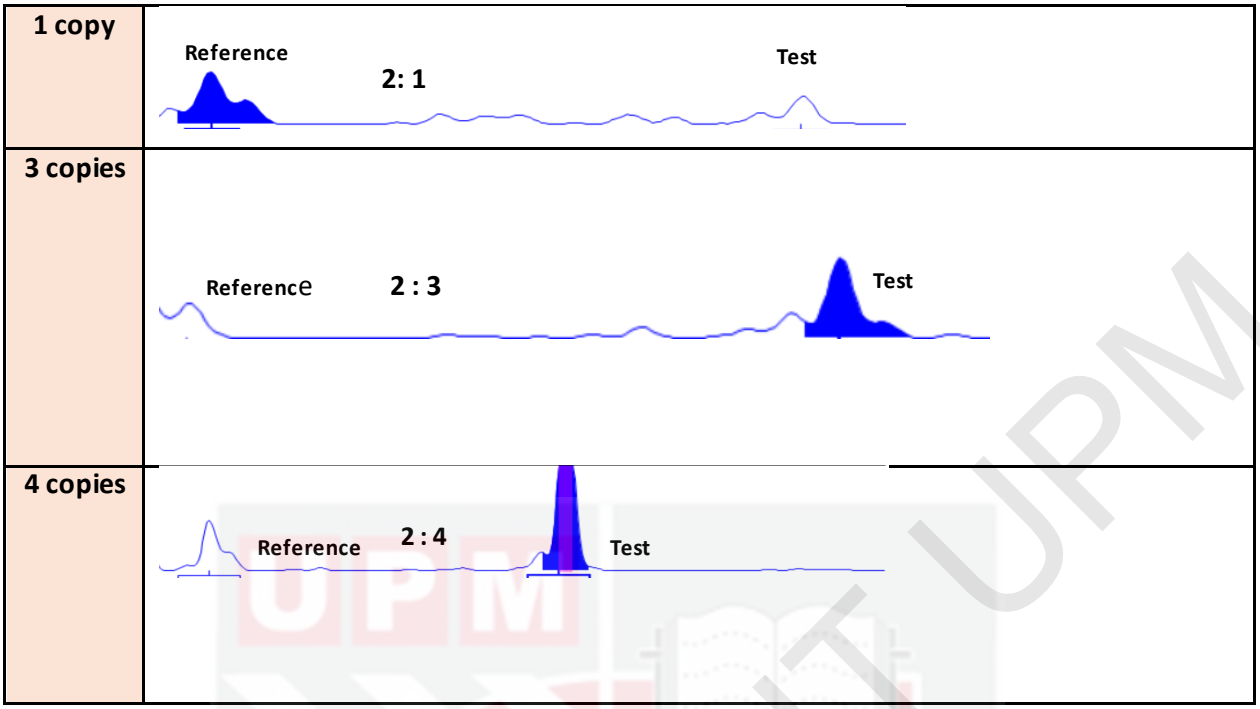

Figure 3 : Peak analysis of amplification products via Peak Scanner Software version 1.0.

According to Figure 4.3, the first individual with one peak showed less than two copy number of KIR gene while the second and third individuals showed more than two KIR copy number.

### 4.4 Distribution of KIR variables copy number among three major ethnics in Malaysia

Copy number of KIR gene was analysed according to the three ethnics in Malaysia: Malay, Chinese, and Indian. Distribution of KIR copy number variation among three major ethnics in Malaysia for HIV-1 patient and control was shown as Table 5.

Among the three ethnics in HIV-1 patient, KIR with more than 2 copies is the most common copy number of KIR gene. Malay is the most frequent ethnic with KIR gene more than 2 copies number compared to Chinese and Indian. Nevertheless, comparison of CNV of KR gene in Malaysia ethnicity demonstrated that there was no significant association of KIR CNV with three ethnics in Malaysia, neither for HIV nor control groups ($p>0.005$ ), as shown in Table 5.

| Status | Number of KIR copy number | Ethnics N % | | | Total | Likelihood ratio ($X^2$) | p-value |
|---|---|---|---|---|---|---|---|
| | | Malay N=135 | Chinese N=91 | Indian N=67 | | | |
| HIV M (n= 52) C (n = 49) I (n=29) | <2 | 1 (0.8) | 0(0.0) | 0(0.0) | 1(0.8) | 1.844 | 0.398 |
| | >2 | 51(39.5) | 49(38.0) | 29(22.5) | 129(99.2) | | |
| | Total | 52(40.0) | 49(37.7) | 29(22.3) | 130(100.0) | - | - |
| Control M (n= 83) C (n = 42) I (n=38) | <2 | 2(1.2) | 0(0.0) | 0(0.0) | 2(1.2) | 2.724 | 0.256 |
| | >2 | 81(50.3) | 42(26.0) | 38(23.6) | 161(98.8) | | |
| | Total | 83(50.9) | 42(25.8) | 38(23.3) | 163(100.0) | - | - |
| Total of HIV and Control | | 135(46.6) | 91(31.1) | 67(34.7) | 293(100.0) | - | - |

Table 4 : Distribution of KIR variables copy number among three major ethnics in Malaysia for HIV-1 patient and control.

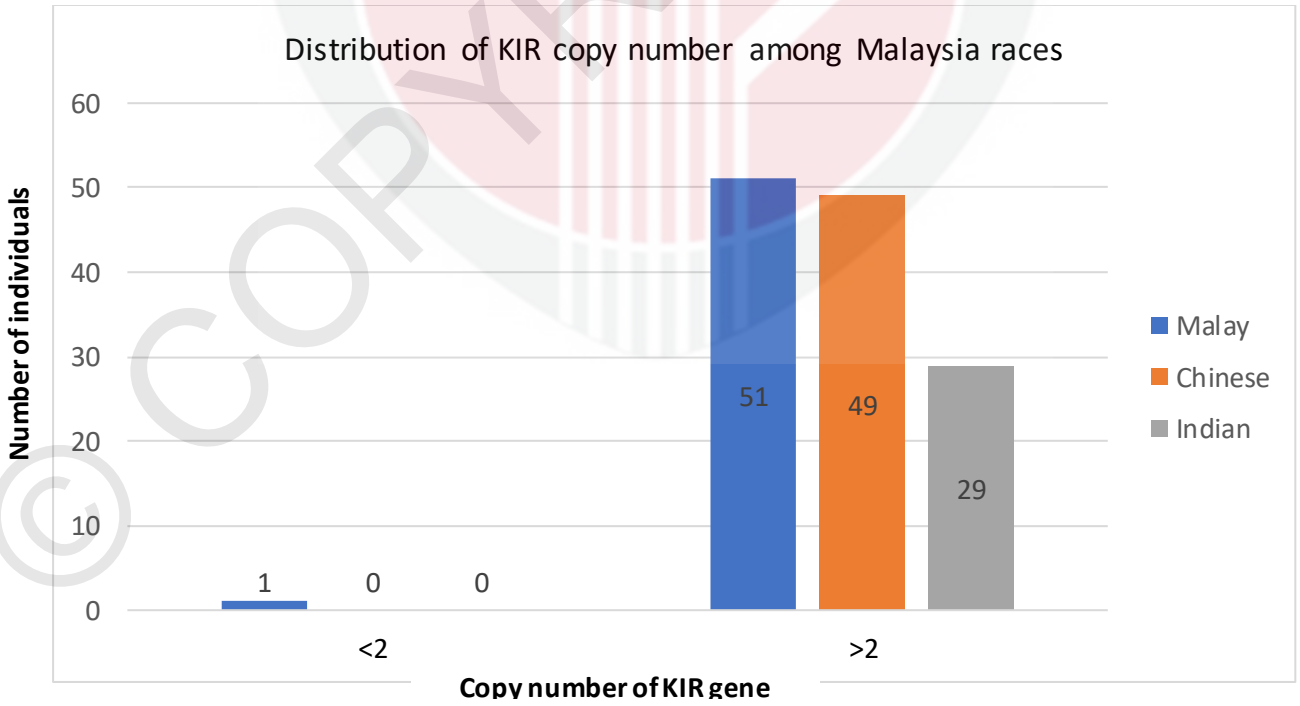


Figure 4 : Histogram of KIR copy number variation among three major ethnics in Malaysia for HIV.

### 4.5 Distribution of KIR variables copy number in Malaysia HIV-1 patient and control

As shown in Table 4, we obtained 129 of duplication of KIR gene copy with frequency of 44.0% and single one deletion of KIR gene copy copies with low frequency of 0.3% in HIV patients. In control samples, the frequency of the duplication and deletion of KIR gene are 54.9 % and 0.7 % respectively. By comparing the CNV of KIR gene for HIV patients with non-HIV, there was no significant association in KIR CNV between HIV-1 patients and control (p>o.oo5).

| Copy number of KIR gene | Case N % | Control N % | Total | Likelihood ratio ($X^2$) | p-value |
|---|---|---|---|---|---|
| <2 | 1 (0.3) | 2 (0.7) | 3 (1.0) | 0.153 | 0.695 |
| >2 | 129 (44.0) | 161 (54.9 ) | 290 (99.0) | | |
| Total | 130 (100.0) | 163 (100.0 ) | 293(100.0) | - | - |

Table 5 : Frequency of KIR genotype in HIV and control .

### 4.6 CNV of KIR gene with CD4+ level after HAART

CNV of KIR gene was classified based on the three stages of CD4+ level shown by Ministry of Health Malaysia (2011). The CD4+ levels are classified into three stages : <200 cells /mm $^3$, 200-350 cells/mm$^3$, and >350 cells/mm$^3$. Figure 5 showed the distribution of KIR copy number with CD4+ level after HAART.

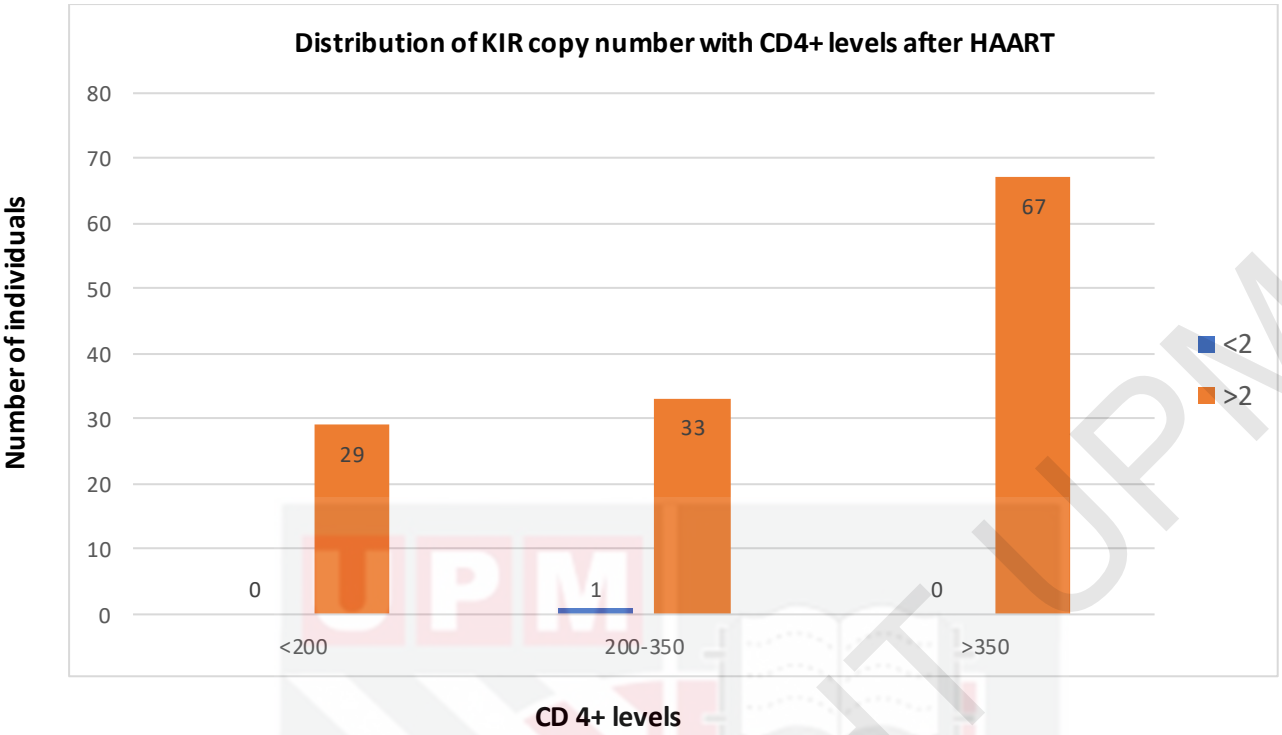


Figure 5 : Histogram of distribution of KIR copy number variation with CD4+ levels after HAART.

Based on Table 6, by likelihood ratio analysis, no significant correlation between the KIR copy number variation and CD4+ level after HAART was found ( p-value > 0.005).

| Number of KIR copy number | CD4+ cell court after HAART (cells /mm ³) | | | Total | Likelihood ratio ($X^2$) | p-value |
|---|---|---|---|---|---|---|
| | < 200 | 200-350 | >350 | | | |
| **<2** | 0 | 1 | 0 | 1 | 2.704 | 0.259 |
| **>2** | 29 | 33 | 67 | 129 | | |
| **Total** | 29 | 34 | 67 | 130 | | |

Table 6 : Distribution of KIR coy number with CD4+ level after HAART.

# CHAPTER FIVE
# DISCUSSIONS

## 5.1 Utilization of PRT for KIR Genotyping

Very few studies have evaluated KIR CNV, and to date, no study has quantified KIR CNV through PRT system. In this study, CNV of KIR gene was successfully quantified by using the paralogue ratio test (PRT), with representing 1, 3, and 4 copies of KIR gene. Based on the results, PRT can be used to detect deletion and duplication of variables copy number for KIR gene in acceptable level of accuracy. For both HIV and control samples, most of the samples showed 3-4 copies while there is only single sample being quantified as 1 copy. Particularly, none of the DNA samples showed KIR of 2 copies even for the control sample. Higher copy numbers might be more error-prone, it could lead to an artefactual in the KIR copy number quantification between cases and controls. From previous researches, constant copy number of reference loci and absence of mismatched at primer sites are the main criteria to obtain accurate PRT data (Walker et al., 2009). PRT system is a complex assay due to the primer design that is being used to amplify only both the specific reference and test loci (Walker et al., 2009). Thus, primer mismatches and increase of copy number of the test loci could result in failure to amplify 2 copies of reference loci (Walker et al., 2009). There is high possibility for this failure to be occurred in this study by observing the peaks through Peak Scanner (Figure 3). Nonetheless, additional measurement error may appear to be consequence of measurement of higher copy numbers due to complexity of KIR gene. Thus, it cannot be only explained simply by either mismatch at primer sites or copy number variants at test loci.

In addition, failure to quantify 2 copies from the samples especially control groups, might due to the DNA degradation because PRT is highly sensitive to DNA degradation compared to conventional methods such as qPCR. Apart from that, PRT which is very sensitive to DNA degradation, is due to the longer amplicon for optimization of sensitivity and specificity of paralogue sequence (Fernandez-Jimenez et al., 2011). Although PRT has been recognised as robust assay for CNV quantification, high quality DNA samples still be required to obtain accurate data as difference in DNA source and quality can cause minor changes in copy number measurement (Craddock et al., 2010).

Importantly, PRT optimization should be followed systematically to obtain clear specific amplification of test and reference amplicon, whilst to achieve the test to reference ratio covaries with gene copy number (Hollox et al., 2017). There might exist the possibility that misestimation of the gel band size as shown in Figure leading to inaccurate PRT optimization. Thus, misinterpretation of the peaks product size in Peak Scanner may be expected, leading to either over- or under-estimation of integer copy number. Nevertheless, it is inconclusive for the present error in this study due to limited previous study (Carpenter et al, 2011). Furthermore, one of the major limitations in this study involves low sample size that might reduce the power for detection of disease association and increase the margin of error. Although the sample size was too small to observe the group difference, we did not even observe the apparent trend toward an effect of KIR CNV when comparing among control and HIV group.

According to our result, there is lower possibility for the differential bias to be occurred. Based on the previous studies, causes of differential bias include different DNA extraction method used (Craddock et al., 2010), storage and handling (Clayton et al., 2005). However, this might not be our possible cause as the extraction method used from peripheral blood was the same for both patients and control groups. Apart from that, there is low batch effect in our study due to random sampling of DNA sample for each set PCR plates for capillary electrophoresis by interspersing case and control samples in the same plates.

Utilization of PRT system in this study for KIR copy number assignment has proven the robustness and usefulness of PRT mentioned in previous studies (Aldhous et al., 2010 & Cantsilieris et al., 2013). Advantages of PRT involve time-saving (4 hours), low labour requirement, less amount of DNA sample required (3µL), and cost effective due to utilization of typically equipment in molecular genetic laboratory. Importantly, it is useful for annealing of the single pair of PRT primer to only the reference locus and test locus by showing the peaks at product size 383bp and 396bpp in the Peak Scanner. Hence, it resonates the statements shown by Aldhous et al (2010) and Hollox et al (2017) as it prevents the amplification of additional of pseudogene and other reference locus as well as test locus. Therefore, the kinetic amplification of the two amplicons are almost similar due to the similar sequence of the two amplicons. PRT-PCR which is inexpensive test can be carried out in microtitre plate format to produce accurate data, even though further clarification is needed.

In addition, multiple probe sets should be recommended for the future PRT system in order to uncover the knowledge of KIR CNV.

### 5.2 CNV of KIR among three major ethnics in Malaysia

The distribution of KIR variable copy number was analysed among the three major ethnics in Malaysia (Malay, Chinese, and Indian) for HIV-1 patient and control. Careful analysis shown in Table 4 demonstrated that there is no significant difference among the three major ethnics in Malaysia for HIV-1 patients (p=0.398). However, the data for this study is inconclusive due to low sample size for our study particularly, Chinese(n=49) and Indian (n=29) HIV-1 patient samples.

According to table 5, both duplication and deletion of KIR variables copy number in HIV-1 patient varies among three ethnics. For duplication of KIR gene, it showed that Malay (39.5%) has the highest frequency of KIR gene compared to Chinese (38.0%), and Indian(22.5%).This variation might reflect the polymorphism of KIR gene can be affected by ethnicity due to gene flow and founder effect (NurWaliyuddin et al., 2014).

By comparison with the previous study by Lee et al (2008), our result is inconsistent with the frequency shown among Malay (31.3%), Chinese (39.6%), and Indian (8.8%) in Singapore. Nevertheless, by using PCR-SSP typing method, the study by Lee et al (2008) showed the distribution of KIR genotypes with several haplotypes (AA, AB, and BB) for larger sample size of control population only (Malay=80, Chinese=210, and Indian=80) is unrelated to CNV of KIR. Thus, it is indecisive, particularly due to limited study of CNV of KIR among Malaysia ethnics.

### 5.3 Role of KIR in HIV progression

This study performed the case-control association analysis by likelihood ratio test as shown in Table 5, none association between CNV of KIR and Malaysia HIV-1 patient was observed. Based on data, observed difference in KIR CNV was very small and practically not relevant, so even larger numbers with similar observed frequency values would probably not obtain significant P-values. Accordingly, our findings showed KIR CNV does not influence HIV

progression. Multiple copies of KIR gene does not increase the NK cell expression in innate and adaptive systems for reducing the risk of HIV infection. However, it is irreconcilable with the previous researches that had applied PCR-SPP and real time-PCR (Pelak et al., 2011, Hellmann et al., 2011, Jiang et al., 2013 & Hellmann et al., 2013). Pelak et al (2011) found association of increased KIR copy number with lower viral set point ($p = 0.00028$), in contrast, our findings showed no clear association ($p=1.844$).

The most striking difference between our results and the Pelak et al (2011) might due to the difference of copy number genotyping method. Real time-PCR may be affected by additional factors such as physiochemical properties of DNA, it then results in false impression of accurate measurement. Besides, rather than to a true association, it might involve effect of differential error of real-time PCR measurement methods between cases and controls. Additionally, copy number estimated by the real-time PCR–based showed association with HIV infection, whereas the PRT method revealed no association; this discrepancy seems to be explained by systematic differences in DNA degradation between case and control samples, in which degradation or shearing of DNA leads to systematic overestimation of copy number by the PRT method specifically.

Although the studies utilized PCR-SPP and qPCR that are almost similar for copy number quantification, different range of KIR copy number was observed. Pelak et al (2011) showed up to 3 KIR copies through qPCR while Hellmann et al (2011) revealed wider range of KIR copy number by having up to 12 copies. Unfortunately, higher range of copy numbers which is inherently more error-prone could lead to invalid KIR copy number measurement, mimicking true significant association. Furthermore, the discrepancy between these findings and ours could also be explained by low sample size that precluded the detection of real association of KIR CNV with HIV-1. Thus, further researches should be conducted for further KIR CNV evaluation.

### 5.4 CNV of KIR with CD4+ level

CD4+ T cell which is a cellular marker acts as HIV progression predictor through CD4 cell count measurement to predict the risk of HIV-1 infection. (Goedert et al., 1987, Fahey et

al., 1990 & Ford et al., 2015). It is the main target of HIV infection, consequently reduction of CD4+ count is required for the development of AIDS (Fahey et al., 1984).

Based on our results, none significant dependency of KIR CNV with CD4+ level in this study is inconsistent with the previous study (Hellmann et al., 2011, Gaudieri et al., 2005, Middleton et al., 2007, Hellmann et al., 2013 & Jiang et al., 2013). Studies showed an association of higher KIR copies with decrease of CD4+ level with low viral load (Gaudieri et al., 2005 & Middleton et al., 2007). In contrast with this observation, other investigators revealed increased KIR CNV was associated with high CD4+ level, indicating a protective effect (Hellmann et al., 2011, Hellmann et al., 2013 & Jiang et al., 2013). However, initial studies that find significant associations of change in CD4+ level still require further confirmation in independent populations. Accordingly, there is conflicting results for these studies, therefore, it is still inconclusive. By using different KIR genotyping method, there is an inability to replicate such studies with conflicting results reported and uncertainty over what might be real associations. For instance, real-time PCR may not be insufficiently accurate to differentiate between KIR copy number, especially in large scale studies. Furthermore, differences in DNA storage or degradation will be expected to obtain false positive association unless DNA amounts are carefully concerned. Therefore, differences in DNA quality or concentration may systematically increase or decrease the inferred number of KIR copies. As a result, we have not been able to confirm the previous finding of association of KIR CNV with CD4+ level.

# CHAPTER SIX
# CONCLUSION

In conclusion, PRT system can be successfully utilised for efficiently quantification copy number of single loci of KIR gene with accurate assay design by reporting the copy umber of 1, 3 and 4 of KIR gene. Therefore, it can act as an alternative method for gene copy number quantification as it is useful for annealing of the single pair of PRT primer to only the reference locus and test locus. Furthermore, this present study shows that variable copies of KIR gene is present in HIV-1 patients and control. Besides, ethnic groups in Malaysia have distinct KIR gene frequencies from each other. Nevertheless, CNV of KIR gene is not significantly associated with Malaysian HIV-1 patients. Furthermore, CNV of KIR gene found to be independent with ethnicity in Malaysia as well as CD4+ level after HAART.

This study represents the first step in quantifying the CNV of KIR gene through PRT system. Thus, limitations discussed in chapter five should be resolved by including higher sample size, peculiarly Chinese and Indian samples. For recommendation of future studies, improved handling, storage, and extraction of DNA samples should be practiced to prevent DNA degradation that can interfere the generation of accurate quantification of CNV. In addition, lack of verification of reference result such as reference samples of known copy number might be supportive for future study to access the true accuracy of the PRT assay. For future studies of CNV KIR gene through PRT assay, it is recommended to allow full reporting of the unrounded copy number estimates to increase accuracy and robustness of PRT system. For effective HIV infection monitoring, further evaluation of additional parameter for HIV disease progression such as viral load and CD8 level should be concerned (Li et al., 2009, Betts et al.,2001 & Mocroft et al., 1997). Other than that, multiple primer-probe sets and increasing the replicates pairs for PRT system should be commended. Furthermore, to lower the risk of false positive association for large scale study, longer amplicon should be involved to optimize the sensitivity and specificity.

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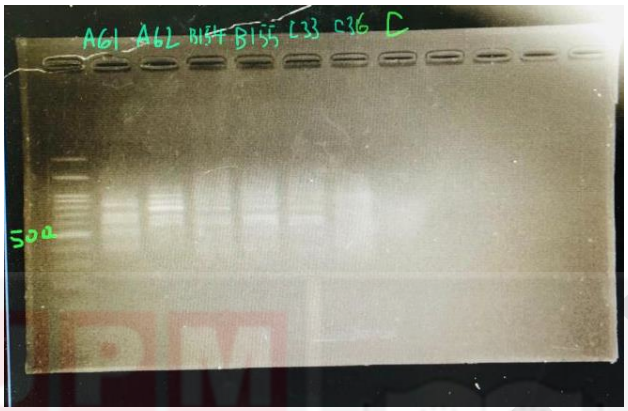
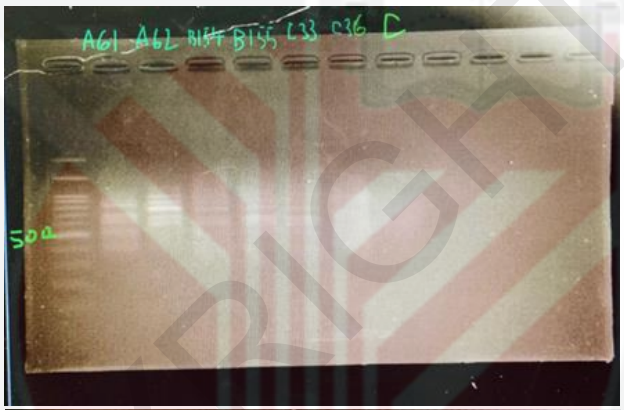
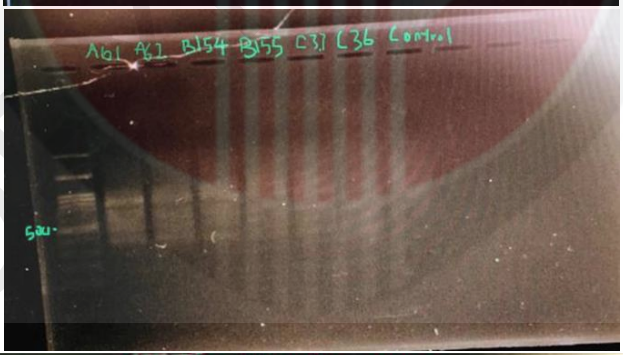
# APPENDICES

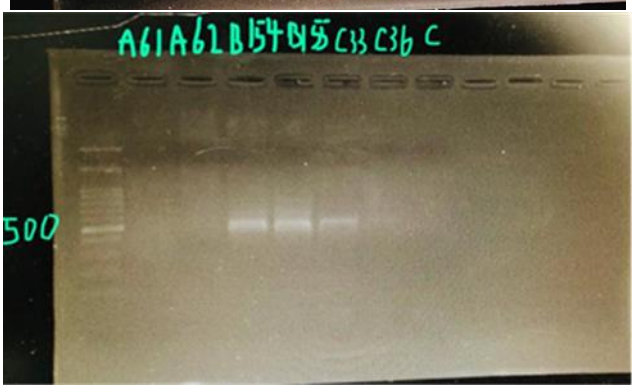
## Images of Gel Electrophoresis of PCR Products

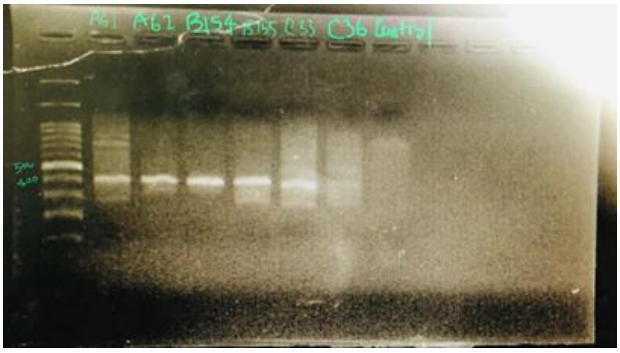
### Unlabelled KIR 1



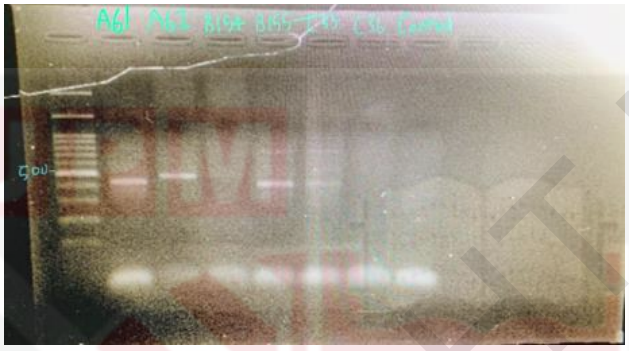



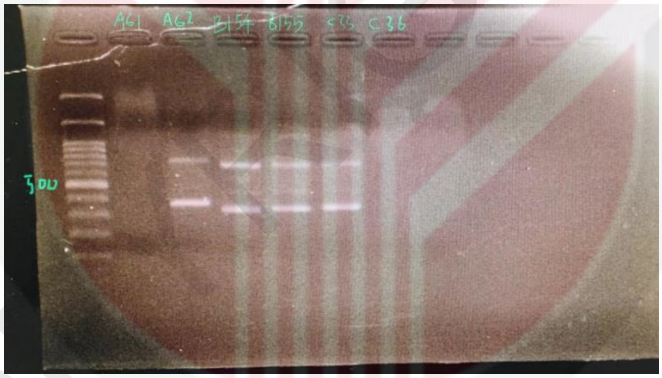





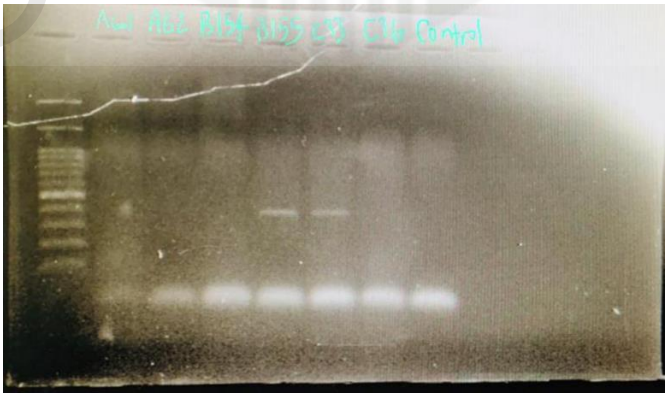
**Labelled KIR 1**



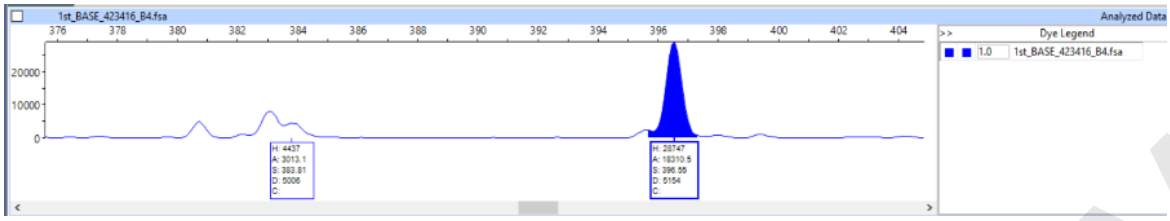
**Unlabelled KIR 2**

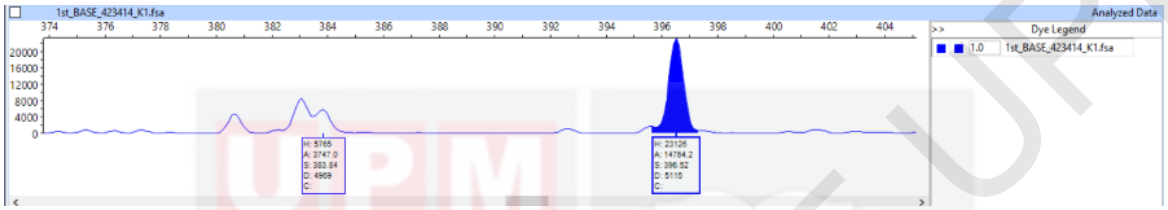


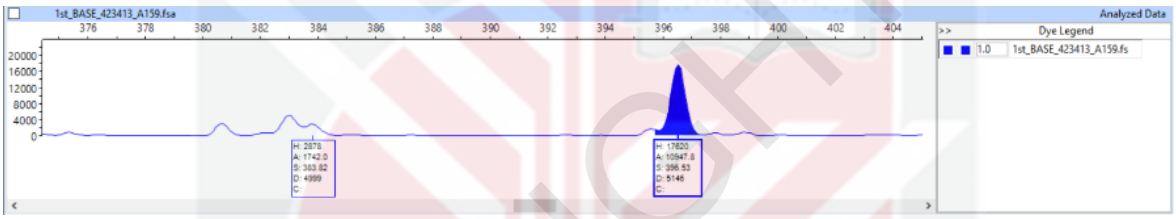
**Labelled KIR 2**

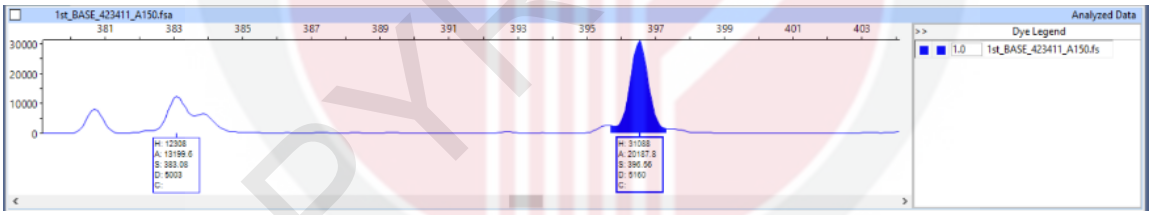


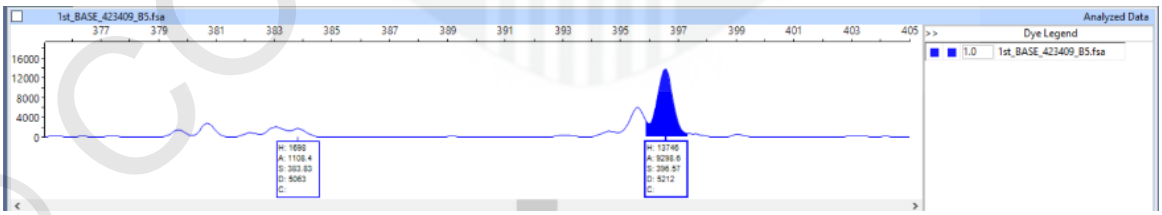
**Images of Peak analysis of amplification products via Peak Scanner Software version 1.0**

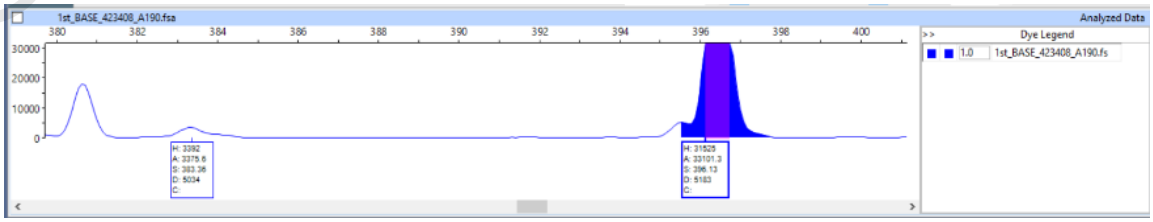


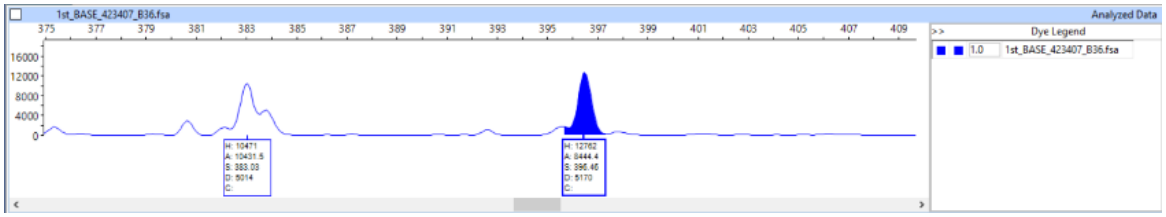



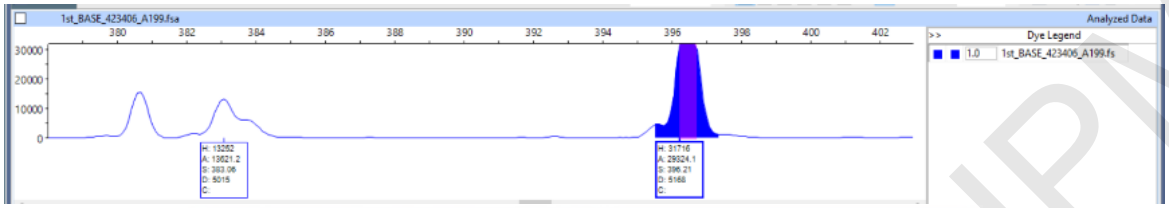



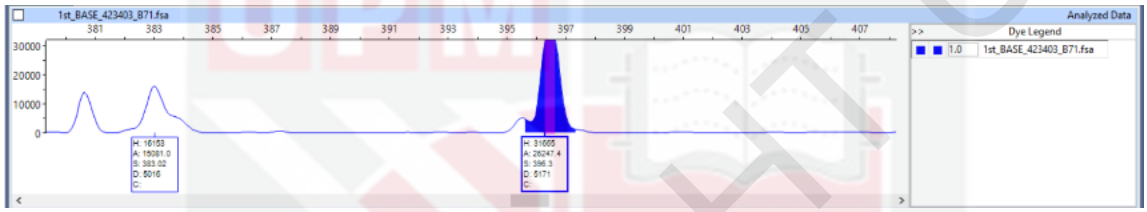



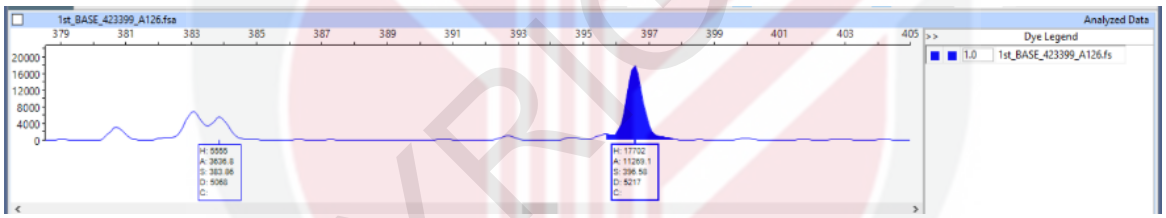



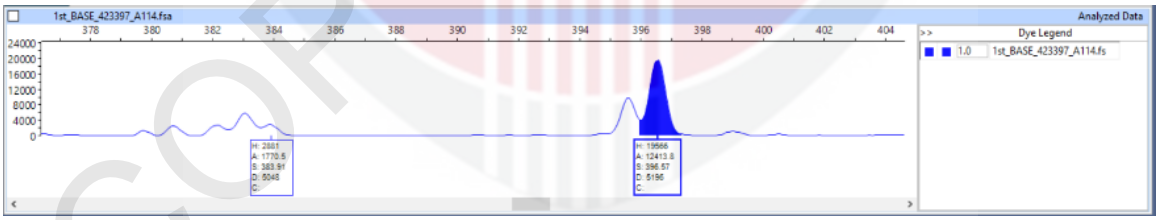



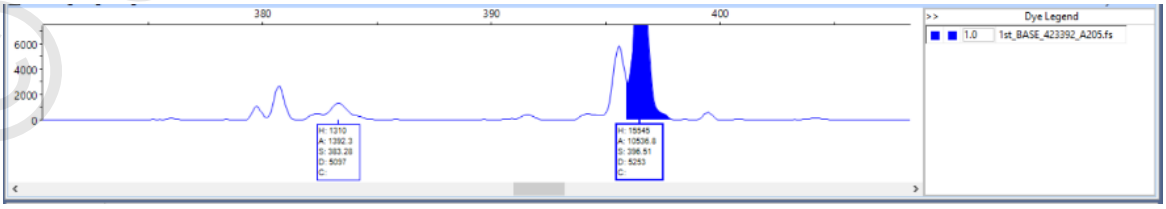


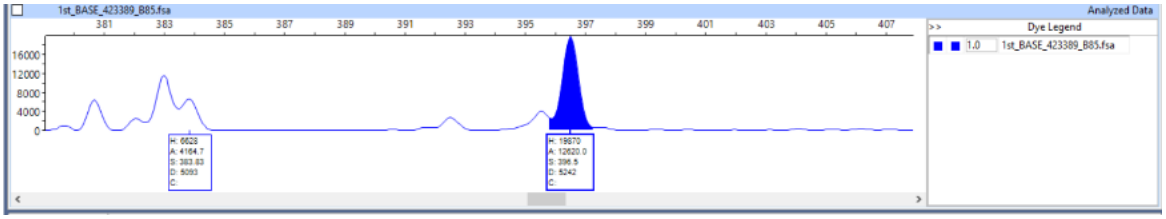



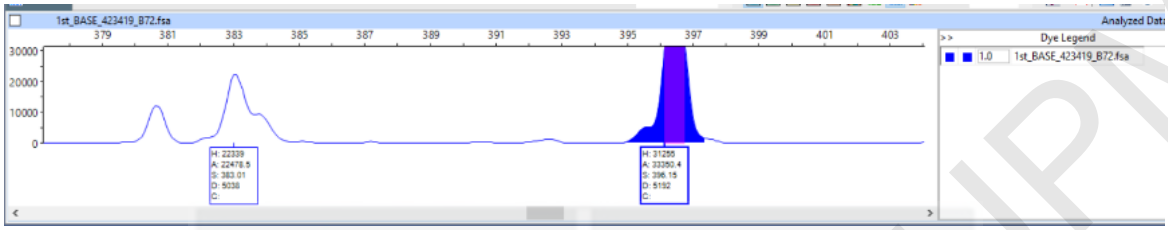



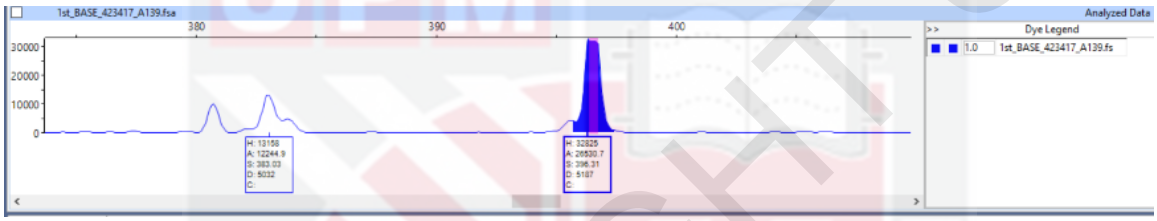









## Raw Data of PCR products

| No. | Sample Code | Ethnic | Copy Number of KIR gene | Deletion / Duplication | CD4 + level after HAART |
|---|---|---|---|---|---|
| 1. | B101 | Malay | >2 | Duplication | <200 |
| 2. | B103 | Malay | >2 | Duplication | 200-350 |
| 3. | B104 | Malay | >2 | Duplication | >350 |
| 4. | B110 | Malay | >2 | Duplication | <200 |
| 5. | B111 | Malay | >2 | Duplication | >350 |
| 6. | B112 | Malay | >2 | Duplication | >350 |
| 7. | B113 | Malay | >2 | Duplication | >350 |
| 8. | B114 | Malay | >2 | Duplication | >350 |
| 9. | B115 | Malay | >2 | Duplication | >350 |
| 10. | B116 | Malay | >2 | Duplication | >350 |
| 11. | B119 | Malay | >2 | Duplication | >350 |
| 12. | B12 | Malay | >2 | Duplication | >350 |
| 13. | B120 | Malay | >2 | Duplication | >350 |
| 14. | B122 | Malay | >2 | Duplication | >350 |
| 15. | B13 | Malay | >2 | Duplication | <200 |
| 16. | B137 | Malay | >2 | Duplication | 200-350 |
| 17. | B141 | Malay | >2 | Duplication | >350 |
| 18. | B143 | Malay | >2 | Duplication | <200 |
| 19. | B146 | Malay | >2 | Duplication | >350 |
| 20. | B147 | Malay | >2 | Duplication | >350 |

| | | | | | |
|---|---|---|---|---|---|
| 21. | B156 | Malay | >2 | Duplication | >350 |
| 22. | B16 | Malay | >2 | Duplication | 200-350 |
| 23. | B19 | Malay | >2 | Duplication | <200 |
| 24. | B2 | Malay | >2 | Duplication | <200 |
| 25. | B21 | Malay | >2 | Duplication | <200 |
| 26. | B23 | Malay | >2 | Duplication | 200-350 |
| 27. | B31 | Malay | >2 | Duplication | >350 |
| 28. | B32 | Malay | >2 | Duplication | <200 |
| 29. | B40 | Malay | >2 | Duplication | >350 |
| 30. | B45 | Malay | >2 | Duplication | 200-350 |
| 31. | B52 | Malay | >2 | Duplication | 200-350 |
| 32. | B62 | Malay | >2 | Duplication | <200 |
| 33. | B66 | Malay | >2 | Duplication | >350 |
| 34. | B68 | Malay | >2 | Duplication | <200 |
| 35. | B7 | Malay | <2 | Deletion | 200-350 |
| 36. | B71 | Malay | >2 | Duplication | 200-350 |
| 37. | B72 | Malay | >2 | Duplication | >350 |
| 38. | B73 | Malay | >2 | Duplication | >350 |
| 39. | B74 | Malay | >2 | Duplication | <200 |
| 40. | B75 | Malay | >2 | Duplication | >350 |
| 41. | B76 | Malay | >2 | Duplication | >350 |
| 42. | B80 | Malay | >2 | Duplication | 200-350 |
| 43. | B82 | Malay | >2 | Duplication | 200-350 |
| 44. | B85 | Malay | >2 | Duplication | <200 |
| 45. | B87 | Malay | >2 | Duplication | <200 |
| 46. | B88 | Malay | >2 | Duplication | >350 |
| 47. | B89 | Malay | >2 | Duplication | >350 |
| 48. | B90 | Malay | >2 | Duplication | >350 |
| 49. | B91 | Malay | >2 | Duplication | >350 |
| 50. | B98 | Malay | >2 | Duplication | >350 |
| 51. | K1 | Malay | >2 | Duplication | <200 |
| 52. | K5 | Malay | >2 | Duplication | <200 |
| 53. | B79 | Indian | >2 | Duplication | >350 |
| 54. | B10 | Indian | >2 | Duplication | 200-350 |
| 55. | B124 | Indian | >2 | Duplication | >350 |
| 56. | B126 | Indian | >2 | Duplication | 200-350 |
| 57. | B127 | Indian | >2 | Duplication | >350 |
| 58. | B128 | Indian | >2 | Duplication | 200-350 |
| 59. | B130 | Indian | >2 | Duplication | <200 |
| 60. | B132 | Indian | >2 | Duplication | 200-350 |
| 61. | B136 | Indian | >2 | Duplication | <200 |
| 62. | B145 | Indian | >2 | Duplication | >350 |
| 63. | B149 | Indian | >2 | Duplication | >350 |
| 64. | B151 | Indian | >2 | Duplication | >350 |
| 65. | B152 | Indian | >2 | Duplication | <200 |

| | | | | | |
|---|---|---|---|---|---|
| 66. | B153 | Indian | >2 | Duplication | 200-350 |
| 67. | B158 | Indian | >2 | Duplication | <200 |
| 68. | B160 | Indian | >2 | Duplication | 200-350 |
| 69. | B25 | Indian | >2 | Duplication | 200-350 |
| 70. | B27 | Indian | >2 | Duplication | 200-350 |
| 71. | B32 | Indian | >2 | Duplication | <200 |
| 72. | B35 | Indian | >2 | Duplication | <200 |
| 73. | B46 | Indian | >2 | Duplication | >350 |
| 74. | B48 | Indian | >2 | Duplication | >350 |
| 75. | B49 | Indian | >2 | Duplication | 200-350 |
| 76. | B50 | Indian | >2 | Duplication | >350 |
| 77. | B64 | Indian | >2 | Duplication | >350 |
| 78. | B65 | Indian | >2 | Duplication | >350 |
| 79. | B86 | Indian | >2 | Duplication | 200-350 |
| 80. | B92 | Indian | >2 | Duplication | >350 |
| 81. | B99 | Indian | >2 | Duplication | >351 |
| 82. | B1 | Chinese | >2 | Duplication | >350 |
| 83. | B102 | Chinese | >2 | Duplication | >352 |
| 84. | B105 | Chinese | >2 | Duplication | 200-350 |
| 85. | B106 | Chinese | >2 | Duplication | <200 |
| 86. | B107 | Chinese | >2 | Duplication | >350 |
| 87. | B108 | Chinese | >2 | Duplication | <200 |
| 88. | B109 | Chinese | >2 | Duplication | >350 |
| 89. | B117 | Chinese | >2 | Duplication | >350 |
| 90. | B118 | Chinese | >2 | Duplication | 200-350 |
| 91. | B121 | Chinese | >2 | Duplication | 200-350 |
| 92. | B123 | Chinese | >2 | Duplication | 200-350 |
| 93. | B129 | Chinese | >2 | Duplication | 200-350 |
| 94. | B131 | Chinese | >2 | Duplication | 200-350 |
| 95. | B138 | Chinese | >2 | Duplication | 200-350 |
| 96. | B139 | Chinese | >2 | Duplication | <200 |
| 97. | B140 | Chinese | >2 | Duplication | >350 |
| 98. | B142 | Chinese | >2 | Duplication | 200-350 |
| 99. | B144 | Chinese | >2 | Duplication | <200 |
| 100. | B154 | Chinese | >2 | Duplication | >350 |
| 101. | B155 | Chinese | >2 | Duplication | 200-350 |
| 102. | B157 | Chinese | >2 | Duplication | 200-350 |
| 103. | B159 | Chinese | >2 | Duplication | <200 |
| 104. | B161 | Chinese | >2 | Duplication | >350 |
| 105. | B24 | Chinese | >2 | Duplication | >350 |
| 106. | B26 | Chinese | >2 | Duplication | >350 |
| 107. | B27 | Chinese | >2 | Duplication | 200-350 |
| 108. | B34 | Chinese | >2 | Duplication | 200-350 |
| 109. | B37 | Chinese | >2 | Duplication | >350 |
| 110. | B38 | Chinese | >2 | Duplication | <200 |

| | | | | | |
|---|---|---|---|---|---|
| 111. | B4 | Chinese | >2 | Duplication | >350 |
| 112. | B43 | Chinese | >2 | Duplication | >350 |
| 113. | B47 | Chinese | >2 | Duplication | >350 |
| 114. | B53 | Chinese | >2 | Duplication | >350 |
| 115. | B54 | Chinese | >2 | Duplication | >350 |
| 116. | B55 | Chinese | >2 | Duplication | >350 |
| 117. | B57 | Chinese | >2 | Duplication | 200-350 |
| 118. | B58 | Chinese | >2 | Duplication | >350 |
| 119. | B59 | Chinese | >2 | Duplication | >350 |
| 120. | B60 | Chinese | >2 | Duplication | >350 |
| 121. | B61 | Chinese | >2 | Duplication | >350 |
| 122. | B69 | Chinese | >2 | Duplication | >350 |
| 123. | B83 | Chinese | >2 | Duplication | 200-350 |
| 124. | B93 | Chinese | >2 | Duplication | <200 |
| 125. | B94 | Chinese | >2 | Duplication | >350 |
| 126. | B95 | Chinese | >2 | Duplication | >351 |
| 127. | B96 | Chinese | >2 | Duplication | >352 |
| 128. | B97 | Chinese | >2 | Duplication | >353 |
| 129. | K9 | Chinese | >2 | Duplication | >354 |
| 130. | T8 | Chinese | >2 | Duplication | <200 |