Optimisation and evaluation of Human Leucocyte Antigen-G1 expression in Placental Tissue using Real Time Polymerase Chain Reaction

(PhD in Medicine), (PhD in Medicine), (MBCHB)
Shamala Moodley¹, Ravesh Singh² and Dharshni Pillay³

¹Dept of Biomedical Sciences, Mangosuthu University of Technology, South Africa
²HPP Dept of HIV Pathogenesis
³Stanger Hospital
Short title: Quantifying HLA-G1

Corresponding Author: Shamala Moodley (PhD)
Mangosuthu University of Technology, Umlazi, KwaZulu-Natal, South Africa
Email address: shamala@mut.ac.za

Received for publication: March 20, 2014; Accepted: April 11, 2014.

Abstract
Laboratory diagnostic techniques serve as essential tools for screening diseases, and monitoring clinical outcomes. Using this as motivation, this study optimised the Real time Quantification Polymerase Chain Reaction (RT-qPCR) technique to specifically quantify Human Leucocyte Antigen-G1 expression in placental tissue. Synthesis of complementary deoxyribose nucleic acid (cDNA) was performed using ribose nucleic acid (RNA) extracted from human placental tissue. Amplification of cDNA, using specifically designed primers, complementary to the full length HLA-G1 isoform, was performed using RT-qPCR. Data analysis was concluded using the “second derivative method” (Roche Molecular Biochemicals Light Cycler Relative Quantification Software, Version 3.5). Amplification of cDNA was successfully accomplished. Samples with HLA-G1 transcripts gave a fluorescent signal for the 98-base pair (bp) PCR product, characterized by a melting peak at 86.5°C. The RT-qPCR assay is an efficient and reliable tool, readily adaptable for quantifying HLA-G1 expression in placental tissue, to ensure normal full term pregnancies.

Key words: Quantify, LightCycler, RT-qPCR, HLA-G, cDNA, placental tissue

Introduction
Human Leukocyte Antigen-G (HLA-G) is a tolerogenic molecule which is relevant in transplantation, placentation, and protection of the semiallogenic foetus from the maternal immune system [1]. The expression of HLA-G is normally restricted to the placenta during pregnancy specifically to those trophoblasts at the maternal-foetal interface [2].

The nonclassical HLA-G single primary transcript in cytotrophoblasts and other foetal tissue is alternatively spliced to generate four membrane-bound (HLA-G1, G2, G3 and G4) and three soluble (HLA-G5, G6 and G7) isoforms [3, 4, 5]. Both the membrane bound HLA-G1 and the soluble HLA-G5 isoforms, the most studied HLA-G molecules, act in diverse inflammatory conditions protecting tissue against Natural killer (NK) and T-lymphocyte cell infiltration [3,4]. These anti-inflammatory functions can become operative in conditions in which such immune cells try to attack viral infected or tumour cells [6, 7, 8].

Various immunological detection methods and preparation techniques are used to detect HLA-G specific antibodies. Gonen-Gross et al [9] used cellular models to demonstrate surface associated HLA-G proteins [9]. Cell surface expression levels of HLA-G using flow cytometry and confocal microscopy staining was explored by Pizzato et al [10]. A number of studies have successfully used RT-PCR technology and melting curve analysis for other nonclassical HLA genes such as HLA-A, HLA-B and HLA-DR [11,12]. In this study the RT-qPCR technique was adapted and optimised to quantifying the full length membrane bound HLA-G1 transcript in human placentas.
**Materials and Methods**

**Patient Population, Recruitment and Selection**
Patients attending the antenatal clinic (ANC) for general monthly antenatal examination were recruited and included, into this study, at the chosen site for sample collection. Informed consent was obtained from all participants. Approval for the study was obtained from the Ethics Committee, University of KwaZulu-Natal as well as the Clinic’s Management. A total of 55 placental samples, obtained from women post delivery, were analysed.

**Collection and Transport of Placental Samples**
Placental tissue samples were collected immediately after delivery, from all participants and placed into 10% formal saline. The samples were thereafter, transported on ice, to the laboratory, and appropriately stored at -70°C for RNA extraction and cDNA synthesis.

**Ribose Nucleic Acid Extraction from Placental Tissue**
Placental tissue samples were homogenized using a modified Trizol method by Perou followed by a total RNA extraction using the Arum protocol (BIORAD) according to manufacturer’s recommendations [13]. To increase the yield of RNA, placental tissue was cut in 100 mg portions and homogenized in 1 ml Trizol reagent. Homogenates were placed into cryoshredder tubes and spun at 12 000 rpm for 2 minutes at 4°C. Tubes were removed from the centrifuge and left to stand for 10 minutes at room temperature. This was followed by centrifugation for 10 minutes at 12 000 rpm at 4°C. After centrifugation 200μl of chloroform/1ml Trizol were added to the tubes and shaken vigorously for 30 seconds, followed by a standing time of 10 minutes at room temperature. Thereafter, samples were spun for 15 minutes at 12 000 (4°C). The chloroform aqueous layer was removed and added to equal volumes of 60% ethanol in diethyl pyrocarbonate (DEPC) water. Binding columns (RNA) were inserted into 2ml capless tubes. Lysates, from the tubes, were transferred into columns and centrifuged for 60 seconds. The filtrates were discarded post centrifugation. Low stringency wash solution (700μl) was added to each tube and spun for 60 seconds. Again the filtrates were discarded; 80μl of DNase 1 (1:15 dilution) was added to each tube and incubated at room temperature for 25 minutes. The columns were centrifuged for 30 seconds and the filtrate discarded. High stringency wash (700μl) was added to the tubes and centrifuged for a further 30 seconds. Thereafter, the filtrate was discarded and 700μl of low stringency wash was added to the binding columns. A further 30 seconds centrifugation followed after which, the filtrate was discarded and the tubes were subjected to an additional 1 minute spin. The RNA binding columns were placed into 1.5 ml capped tubes and 80μl (70°C) elution solution was placed onto each membrane stack. Tubes were incubated for a further 3 minutes and centrifuged for 2 minutes to elute. The concentration of the total RNA was measured at absorbance 260 nm.

**Determination of Ribose Nucleic Acid Concentration**
The concentration of RNA was determined by Nanodrop (ND1000 spectrophotometer). Absorbance was taken at 260 nm and 280 nm and a factor was used (40RNA). Purity of all RNA samples was determined by looking at the 260/280 ratio and the value of 2 was considered acceptable. Sample value < 2 was not considered.

**Confirmation of Ribose Nucleic Acid (RNA) Using Gel Electrophoresis**
Confirmation for the presence of RNA was performed on 2% agarose gel using electrophoresis. Briefly, a 2% w/v agarose gel was prepared and the mixture was poured into casting trays with the comb inserted into the gel at 10mm from one end of the tray. Polymerisation of gel was achieved between 30-40 minutes at room temperature, prior to use. The polymerized gel was placed in a gel tank containing 1X tris-borat/EDTA (TBE) buffer (TBE must cover the gel). PCR product (10μl) with 2μl of bromophenol blue loading dye (0.25% and 40% (w/v sucrose) was loaded into an appropriate well in the gel. DNA molecular marker (Fermentas # SMO373, GeneRuler 50 base pairs (-bp) DNA Ladder was used as control. The gel was subjected to electrophoresis for 60 minutes at 80 volts (V) and thereafter, viewed using the Chemi-Doc XRS UV-transilluminator (BIORAD) and photographed. RNA was stored at -70 °C for preparation of cDNA.

**Complementary Deoxyribose Nucleic Acid (cDNA) Synthesis using Reverse Transcriptase Polymerase Chain Reaction**
The BIORAD iScript cDNA (Cat.No.1708891) synthesis kit was used to produce cDNA from the RNA sample. The procedure was performed according to manufacturer’s protocol. Complementary Deoxyribose Nucleic Acid was synthesized using 1μg of extracted tissue RNA. The reaction mixture was constituted using 4μl of 5x iScript reaction mix, 1μl of iScript reverse transcriptase, and 8μl of nuclease free water and 7μl of RNA template. All the reagents were added to a sterile PCR tube placed on ice. The reaction mixture was incubated for 25 °C for 5 minutes; 42 °C for 30 minutes and 85 °C for 5 minutes with a hold cycle at 4°C for 45 minutes (GeneAmp 9700 PCR system, Applied Biosystems).
Quantifying Human Leucocyte Antigen-G1 Using Real Time Polymerase Chain Reaction

RT-PCR amplifications were carried out using HLA-G1 specific primers, designed to amplify and detect a 98-bp segment of the non classical class I HLA-G gene (Inqaba, SA). Primer sequences were as follows: HLA-G: 5' - CTGGTTTGCCTTTGCAAGCTTAG -3' (forward) and 5' - CCTTTTCAATCTGAGCTCTTTTCT -3' (reverse). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as reference gene to normalise data with the primer sequence, 5' - AAGGTCCGAGTCAACCGATT -3' (forward) and 5' - CTCTGGAAAGATGGTGATGG -3' (reverse). For practicality purposes, only GAPDH was used because of its stability. Furthermore, it was cost effective because only one target gene (HLA-G1) was assessed in a single tissue type (placental tissue).

Optimised Experimental Protocol

To quantify HLA-G1, a series of experiments were carried out to construct a LightCycler protocol for Real-Time qPCR, based on a standard curve of known concentration. To ensure that there was no amplification of any contaminating DNA, the reference gene (GAPDH) were run without reverse transcriptase, using PCR grade water. Normalisation was performed using the equation HLA-G1 value/GAPDH value. Starting material was 1μg of cDNA for both HLA-G1 and GAPDH genes.

Following optimisation, the PCR procedure was performed as described for a 20μl of standard reaction. The required number of LightCycler capillaries were sealed with stoppers and placed in pre-cooled centrifuge adapters. Adapters containing the capillaries were placed in a standard microcentrifuge and samples were spun at 3000 rpm for 5 seconds. Thereafter, the capillaries were transferred into the sample carousel of the LightCycler Instrument and cycled.

PCR mix for one 20μl reaction was prepared in a 1.5ml reaction tube held on ice. The following components were added to the capillaries in the order as mentioned. Water (PCR grade) 5.8μl, MgCl2 1.2μl, PCR primers forward and reverse 0.5μl (0.5 μM) each, 1.0μl of Fast Start SYBR GREEN I (Roche Diagnostics) and the LightCycler Master mix of 9.0μl. Finally, 2.5μl of cDNA (reverse transcribed from 4ng of total RNA) was added. The PCR mix was prepared for the required number of reactions by using the volume for one reaction and multiplying it by the number of reactions. For each reaction water and reverse transcriptase was used as negative controls.

Real time reaction was performed at one cycle (95 °C for 10 minutes) followed by 40 cycles of PCR, which included denaturation at 95 °C for 10 seconds, annealing at 60 °C for 50 seconds and extension at 72°C for 16 seconds and 85°C for 5 seconds with a single fluorescence measurement. All temperature transition rates were programmed at 20°C/s. After amplification was complete a final melting curve was recorded at 95°C for 30 seconds and 80°C for 15 seconds (20°C/s). Slow heating of the sample was accomplished at 95°C with a ramp rate of 0.1C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the SYBR Green I. This was followed by a final cooling step to 40°C. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescent channel with respect to temperature against temperature [-d (F1)/dT vs T]. Excluding preparation time the entire Light Cycler process took 2 hours. Melting point (Tm) calculations were performed using the Roche Molecular Biochemical Light Cycler Relative Quantification Software, Version 3.5. To improve the SYBR Green quantification, high temperature fluorescence measurement at 85°C for HLA-G1 was performed. This step eliminates non-specific fluorescence signals and non-specific PCR products such as primer dimers below the chosen temperature are eliminated, thus ensuring accurate quantifying of the HLA-G1 and GAPDH real-time products.

Verification of Polymerase Chain Reaction Products

The fragment size of the PCR product obtained from real time-qPCR was verified by electrophoresis. Product separation was achieved using the technique described by Maniatis et.al. (1989) [14]. Prepared PCR product and a known molecular weight marker (Fermentas# SMO373, GeneRuler 50-bp to 2000-bp DNA Ladder) were loaded into wells and electrophoresis was performed at 100V for 60 minutes at room temperature. Visualisation of the separation bands on the agarose gel was performed using the Chemi-Doc XRS UV-transilluminator (BIORAD). Capture of image and product size was completed with the aid of Quantity One software (BIORAD). The product size was compared to the molecular weight marker and the fragment size calculated (Fig.1).

Concentration of double stranded DNA (GM) = [absorbance at 260 nm] x [nanodrop concentration μg/ml] x [dilution factor] . Average molecular weight (MW) of a DNA base pair: 660 (Da), mm or MW of dsDNA= [number of base pairs] x [660Da]. Number of moles (n) = Concentration of double stranded DNA (GM)/Average molecular weight (MW) of dsDNA (mm).

Number of moles = number of moles (n) x [6.022 x 10 23] (Avogadro’s number) The calculations were performed using the following values obtained from the quotation procedure: Absorbance 260 = 0.787,
Figure 1: A representative gel electrophoresis (2% agar gel, stained with ethidium bromide) showing successful amplification of the 98-bp HLA-G1 PCR product obtained using the LightCycler (lanes 4, 5, 6). Lane (M) identifies the molecular weight marker (Fermentas #SMO373, GeneRuler, 50-bp to 2000-bp Ladder). Lane (1) is the negative control (water); Lanes (2, 3) is the standard (GAPDH), successfully amplified as indicated by the presence of a 257-bp fragment.

Concentration = 983.8 µg/ml, Dilution factor is 20, Volume used in Gene Quant = 0.1 ml, Ratio (purity) = 1.948 (1.8 to 2.0), Ds = [1.123], Concentration of double stranded (ds) DNA: Ds [ ] = (0.787) x 983.8µg/ml x (20) x (0.1ml) = 1548.5012 µg, Molecular weight = 98 bp x 660mm = 64680, Number of moles (n) = Gm/mm1548.5012 µg /64680 mm = 0.0239 µg/mm, Number of moles (Avogadro’s number) = 0.0239 x 6.022 x10\(^{23}\) =1.44 x 10\(^{23}\)

HLA-G1 levels were calculated as per above equation. The calculated number of moles was 1.44 x10\(^{23}\). A standard curve was generated for HLA-G1 and GAPDH with 10 fold serial dilution of the DNA standards (RT-qPCR product) ranging from 10\(^{10}\) to 10\(^{11}\) copies per PCR reaction. Quantitative analysis of data was done using the Light Cycler analysis software (Version 3.5, Roche Diagnostics). Melting curve analysis was used to determine the presence of non-specific amplification products. After construction of the standard curve, unknown samples were run with a calibrator, to ensure that the PCR conditions were controlled. At the end of the experiment the standard curve was imported, to calculate the sample concentration. Standard concentrations used ranged from 1.44 x 10\(^{15}\) to 1.44 x 10\(^{20}\) molecules (Fig.2).

Figure 2: LightCycler amplification curve of HLA-G standards using SYBR Green dye I. Dilution concentrations range from 1.44 x10\(^{15}\) to 1.44 x 10\(^{20}\). Reactions show a series of equidistance curves in order of dilution. The flat line represents the water control. The exponential phase of the PCR corresponds to the log-linear segment of the curves.
Linear regression analysis indicated the slope of the reaction curve in the exponential phase. The slope was -3.452 and the amplification efficiency (E) calculated using the formula $E = 10^{-\frac{1}{\text{slope}}}$ was 1.94. A theoretical efficiency of 2, suggests that 100% of the template is converted to products (Fig.3). HLA-G1 levels were calculated using HLA-G/GAPDH values. Calculations were performed using the “second derivative method”. Reproducibility of the assays was assessed by performing triplicate PCR amplified reactions of a representative sample in the LightCycler and calculating the inter-sample variation.

**Figure 3:** Linear regression analysis, of data, generated in LightCycler amplification of HLA-G standards. The calibration curve shows the quantifying cycle (Cq) of each standard plotted against the logarithmic concentration to produce a standard curve. The error value was 0.0451 with an R value of 1.00 and the efficiency of the standard curve was 1.94. The slope for this analysis was -3.452.

**Results:**

**Light Cycler Melt Curve Analysis of HLA-G1 and GAPDH Genes**

The concentration of samples was calculated, from the quantifying cycle (Cq) of the standards for HLA-G1 and GAPDH (Fig.4). Positive HLA-G1 transcription gave a fluorescent signal for a 98-bp PCR product, characterized by a single product-specific melting peak at 86.5°C. The melt peak for the GAPDH, 257-bp PCR product was recorded at 84.5°C. Despite the lower fragment length, HLA-G1 specific PCR product (98-bp) yielded a higher $T_m$ value when compared with GAPDH 257-bp product (Fig.5). There was no generation of primer dimer formations during the PCR amplification cycles.

**Figure 4:** Representative graph showing the logarithmic plot of fluorescence versus cycle numbers during quantification of HLA-G1, together with the representative profile of sample data and their calculated concentration.
Discussion

Both, membrane bound and soluable HLA-G (sHLA-G) molecules exert strong inhibiting signals when interacting with receptors expressed by Natural Killer cells, T cells, and antigen-presenting cells [15]. Clinical studies have demonstrated that HLA-G expression is also relevant in allograft acceptance, after transplantation procedures, and implantation and development of embryos. In view of the diagnostic potential of HLA-G, there is increased interest in finding reliable methods for the measurement of HLA-G molecules in various human tissue and body fluids [16, 17].

Some studies have used antibodies detection and immunocytochemical staining techniques to identify the presence of HLA-G [18, 19]. The focus of this study was to optimise an RT-qPCR assay for quantifying HLA-G1. A RT-qPCR based assay was appropriate because the assay combines accuracy and precision and has the ability to amplify low copy gene sequences [20]. A further advantage of RT-qPCR is its reproducibility of sample results. Its performance in a closed system with direct analysis of results reduces the number of manipulations and the risk of contamination within the working environment [20]. Optimisation was successfully achieved using the LightCycler HLA-G1 was quantified for analysis. The current assay targets a 98-bp HLA-G1 transcript and can be modified to assess other HLA-G isoforms. However, according to literature on the topic, this is the first successful attempt of the RT-qPCR technique on placental tissue, thus offering an alternative laboratory diagnostic tool to assist clinical management of patients.

Since the results are based on data from a small sample size, strong conclusions cannot be drawn. However, it contributes to the existing knowledge of HLA-G diagnostic techniques, specifically in placental tissue. Another important aspect of the study is that the experimental protocol could be utilised to establish normal HLA-G ranges in pregnant women. It has been demonstrated that successful foetal implantation and survival, to some extent, is associated with HLA-G expression at the placental interface [22]. Established normal HLA-G ranges can be a valuable tool in clinical management of ‘at risk’ pregnancies, particularly in spontaneous abortions and miscarriages [21]. Such an improvement would be expected to allow clinicians to see a quantitative difference between the effects of low and high HLA-G expression more readily.

According to some studies, the presence of HLA-G is evident in certain pathologic conditions, such as cell malignancies, transplantation and autoimmunity [23, 24, 25]. Therefore, the diagnostic utility of RT-qPCR in both identifying and quantifying the presence of HLA-G as a potential tumor-associated marker is strengthened. Carosella et al (2008) has reported that one of the main goals of current research is to use HLA-G in the clinic, either for diagnosis or as a therapeutic tool. Accordingly, knowledge on the nature of HLA-G is essential [23]. This strengthens the case for developing techniques which would provide knowledge on levels and quantities of HLA-G and its clinical relevance.

In conclusion the data presented in this study suggest that RT-qPCR is a useful molecular technique to quantify HLA-G1. The importance of the current work is that this methodology can also be modified and applied to quantifying other placental HLA–G isoforms and mRNA of all pregnant women. As the methodology underpinning this study is extensively utilised, the expectations are that more research will be undertaken allowing for more rigorous evaluation.

Figure 5: Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence channel with respect to temperature against temperature [-d (F1)/dT vs T]. Melting peaks for GAPDH (reference gene) was recorded at a $T_m$ of 84.6°C (A). The melting temperature ($T_m$) of the single product specific HLA-G1 gene was measured at 86.5°C (B).
Conflict of Interest: None Declared
Acknowledgments
This study was supported by the National Research Foundation (SA) and Mangosuthu University of Technology Research Grant (KwaZulu- Natal, SA). Thank you to Dr ME Essa and her staff for her assistance in specimen collection and Mr R Singh for his technical support.

References

