A whole cord model for identification of mechanisms for the antivascular effects of DMXAA

by

Kiriana Mihi Moses

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Abstract

Endothelial cells form the inner lining of a blood vessel and their structure and functional integrity are important in maintenance of the vessel wall and circulatory function.

These cells play key roles in immune and inflammatory reactions by regulating lymphocyte and leukocyte movement into tissues; they are also main targets for antivascular agents in cancer therapy.

Endothelial cell responses to different stimuli have been previously investigated using conventional approaches, 'HUVEC in vitro culture system'.

In this study an ex vivo perfusion model was constructed and utilized using whole human umbilical cords, in attempts to replicate a more accurate in vivo microenvironment.

Assessment of the proportion and duration of endothelial cell viability in the *ex vivo* model was undertaken using a MTT 3, (4,5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide viability assay. A time baseline was successfully established for all experimental perfusions.

Endothelial cell immune response was assessed through intravenous perfusion of the endotoxin, Lipopolysaccharide (LPS). Gene expression profiles revealed a significant increase in expression levels of E-Selctin (E-Sel), Intracellular adhesion molecule (I-CAM) and Tissue Factor (TF) relative to the housekeeping gene Beta 2 Microglobulin. When LPS was administered in combination with Hypertonic Saline

Solution (HSS), expression levels declined indicating HSS interferes with the activation pathway of LPS ultimately suppressing its effectiveness on endothelial cells.

HSS impact was also recognized from perfusion experiments on resting endothelial cells. All identified genes were suppressed by HSS apart from inducible nitric oxide synthase (iNOS).

As a potential target for antivascular agents, HUVEC were then stimulated with DMXAA and gene responses of Tumour Necrosis Factor-α (TNF-α) was analysed.

DMXAA demonstrates excellent antivascular acivity in experimental tumours, so tumour conditioned media (TCM) was administered intravenously through umbilical cord segments to replicate a tumour microenvironment prior to DMXAA addition.

When cells were stimulated with Tumour conditioned media then administered DMXAA, TNF-α expression was significantly upregulated; relative to the housekeeping gene Human Proteosome subunit Y, however, when the cells were exposed to tumour conditioned media in absence of DMXAA gene expression significantly decreased. Thus, the antitumour action of DMXAA is capable of inhibiting the gene response of TNF-α replicated in a tumour environment.

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List of Abbreviations

°C	Degrees Celsius
5HIAA	5-hydroxyindole-3-acetic acid
5HT	5-hydroxytryptamine (serotonin
ACSRC	Auckland Cancer Society Research Centre
bp	basepairs
cDNA	complementry DNA
Ct	threshold value (real time PCR)
DMSO	Dimethyl Sulfoxide
DMXAA	Dimethylxanthenone-acetic acid
DNA	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
E.Coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic Acid
ESEL	E Selectin
FAA	Flavone-8-acetic acid
FCS	Fetal Calf Serum
GiTC	Guanidinium Thiocyanate
H_2O	Water
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intracellular adhesion molecule – 1
IL	Interleukin
IL-1β	Interleukin One Beta
IL-6	Interleukin Six
IL-8	Interleukin Eight
INF-α	Inducible Nuclear Factor – alpha
INF-β	Inducible Nuclear Factor – beta

iNOS	Inducible nitric oxide synthase
KCl	Potassium Chloride
KH_2PO_4	Potassium Hydrogen phosphate
LPS	Lipopolysaccharide
M	Molar
Min	Minute
mL	Mililitre
mm	Millimetre
MQ	Milli Q
mRNA	messenger Ribonucleic Acid
MTT	3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide
Na_2HPO_4	Sodium ortho-hydrogen phosphate
NaCL	Sodium Chloride
$NaHPO_4$	Sodium Hydroden phosphate
Neg	Negative
NF-κB	Nuclear factor – κB
NZM3	New Zealand Melanoma 3
OD	Optical Density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPA	Ribonuclease Protection Assay
RT-PCR	Real time – Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
TCM	Tumour conditioned Media
TE	Tris & EDTA
TF	Tissue Factor
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor Alpha
Tris	Tris(Hydroxymethyl)aminomethane
U	Unit

VCAM-1	Vascular cellular adhesion molecule – 1
VTA	Vascular Targeting Agents
XAA	Xanthenone-4-acetic acid
B2M	Beta-2-Microglobulin
β-Actin	Beta-Actin
µg	Microgram
µl	Microlitre
µmol	Micromole

Chapter One

Introduction and Literature Review

1.1 Background to Research

The potential of the tumour vasculature as a target for cancer therapy has been increasingly recognized in recent years. While much effort is being directed at developing angiogenesis inhibitors, there are two drugs currently in clinical trial which induce acute tumour blood flow reduction, and thus offer different therapeutic potential (Rewcastle et al, 1991).

One of these is 5, 6-Dimethylxanthenone-4-acetic acid (DMXAA), an experimental anticancer drug that selectively reduces tumour blood flow and induces tumouricidal cytokine responses (Baguely et al, 1997). This drug is currently in clinical development in phase I/II studies in combination with chemotherapy in New Zealand and United Kingdom.

1.2 Endothelial Cells

Blood vessel walls provide a selective barrier for the transport of molecules between blood and tissues. The endothelial lining presents a surface area for the exchange of materials between blood and tissues (Baldwin and Thurston, 2001).

All blood vessels are lined by endothelial cells, the layer being called the *endothelium.* The endothelium is a continuous monolayer formed from endothelial cells linked together by cell-to-cell junctions. These are very complex structures formed by transmembrane adhesive molecules linked to a network of cytoplasmic proteins. Three types of junctions have been described in endothelial cells: tight junctions, adheren junctions and gap junctions (Dejana et al, 1995; Schnittler, 1998).

Tight junctions are formed from closely apposed neighboring plasma membrane and have over lapping regions that seal the endothelial cell layer (Michiels C, 2003). Toxic substances are capable of opening up these junctions and allowing larger molecules to pass through the walls leading to degenerative changes in the blood vessel.

Endothelial cells are flat, with a central nucleus, and form a pavement like pattern on the inside of blood vessel cells; they play important roles in the formation of blood vessels and their degeneration (Miao et al.1996).

Endothelial cell permeability changes are associated with redistribution of surface cadherins, stabilization of adhesion bonds and activation of matrix metalloproteinases (Alexander and Elrod, 2002). Loss of this barrier function can lead to extravasation of intracellular material.

The endothelium itself plays a role in pathological states such as tumour angiogenesis and thrombotic events (Schreiber et al, 1985). The surface of a healthy endothelium is both anticoagulant and antithrombotic. Endothelial cells secrete a variety of important molecules for regulation of blood coagulation and platelet functions. Damage to the vessel or exposure to certain cytokines or proinflammatory stimuli shifts balance towards a more procoagulant/prothrombotic phenotype of the endothelial cell (Pearson, 1999). Studies of endothelial cell biology have been prompted by the establishment of in vitro culture conditions, the definition of endothelial cell phenotypes and the identification of growth factors that interact with endothelial cells (Shreiber et al. 1985).

Endothelial cells lining tumour blood vessels are the main targets for antivascular agents. Two changes that are induced by these agents are rearrangement of cytoskeletal network and the alteration of junctions that lie in between endothelial cells. Further investigation of this process includes the induction of apoptosis, leading to rupture of the vessel and extravasation of erythrocytes into the surrounding tissue (Baguley B, 2003) as shown in Figure 1.1.

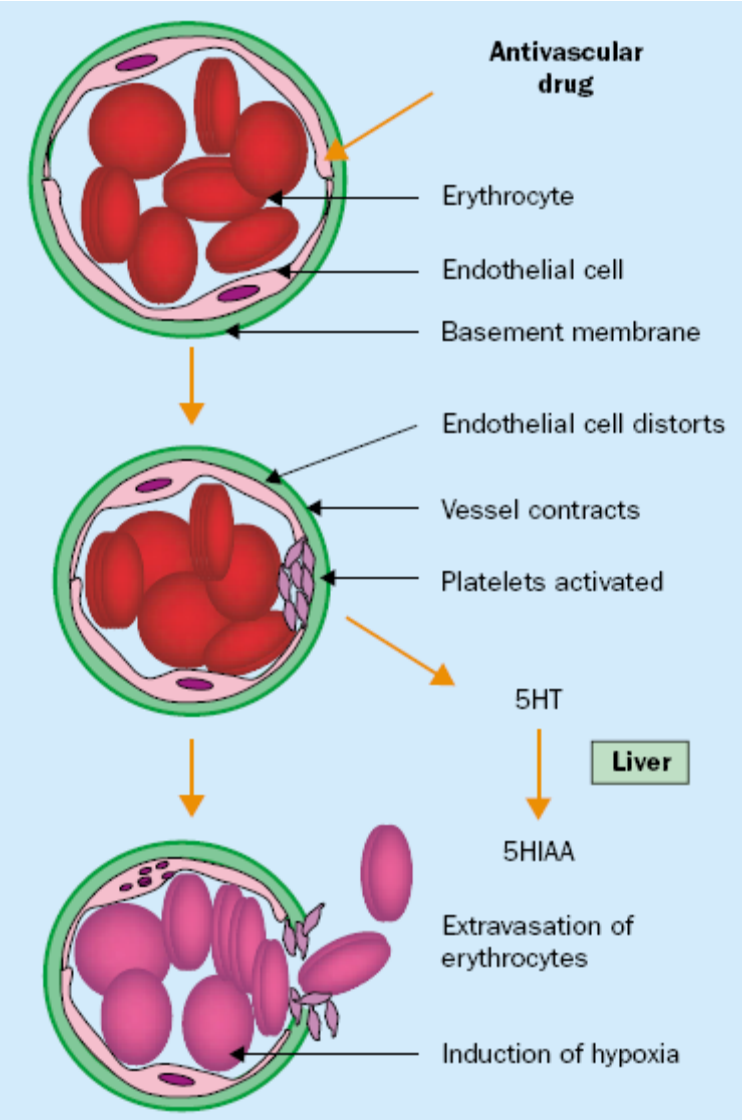


Figure 1.1. Antivascular Action
The antivascular agent causes damage or distortion to the vascular endothelial cell and the resulting exposure of basement membrane allows platelets to aggregate and release 5-hydroxytryptamine. Further disruption to the vascular endothelial cell results in rupture of the capillary, extravasations of erythrocytes, induction of hypoxia, and cessation of blood flow.

Changes in vascular endothelial cells result in the exposure of basement membrane components, which promote activation of platelets.

It is important to emphasize that there is phenotypic variation between endothelial cells in different parts of the human vasculature tree, between both the arterial and venous cells. Cells from different locations will not only express different markers but can also generate different responses to the same stimulus (Gallagher G et al,. 1997).

1.3 Human Umbilical Vein Endothelial Cells (HUVEC)

For many years research has focused on endothelial cell physiology and pathology. Endothelial cells cultured from human umbilical veins of cords are the mostly widely used cell model *in vitro* (Gerritsen M et al, 1993).

Cell Biologists rely on *in vivo* cell cultures for studies of the effects of compounds and other parameters on cells (Mulder P, 2004).

It is clear, however, that cells raised in culture flasks with medium in incubators are themselves in an environment that little resembles that of its natural situation. A result of this that cells are subjected to more stress, which will affect their growth and response to applied stimuli (Mulder P, 2004). The information obtained from *in vitro* studies has, however furthered the understanding of the molecular processes that take place.

A more accurate cell response would result if *in vitro* conditions could be made to resemble the *in vivo* environment (Mulder P, 2004).

This research project will use whole Human Umbilical Cords for *ex vivo* studies. The umbilical cord is flexible and can be easily manipulated with in the chamber setup. The ready availability of cords and also relative ease of isolation will contribute to the *in vitro* studies. Employing an *ex vivo* model will give a more accurate re-creation of the cells microenvironment, made possible with the tubing and a peristaltic pump, allowing continuous solution perfusion through the umbilical vein. Also liquid handling will be more controlled, so cells are exposed to minimal chemical and mechanical stress.

1.4 Cytokines

Cytokines are low molecular weight proteins. Secreted primarily from leukocytes, they function as messengers providing communication between leukocytes and endothelial cells. Cytokines stimulate the humoral and cellular immune responses, as well as the activation of phagocytic cells. Endothelial response to cytokines and bacterial products result in adhesion molecule expression and chemokine production, which mediate increased binding and transmigration of leukocytes across the vascular wall.

Many cytokines are initially given descriptive names but as their basic structure is identified they are renamed “interleukins” (IL). Interleukins are not only secreted by leukocytes but also able to affect the cellular responses of leukocytes. Specifically, interleukins are growth factors targeted to cells of hematopoietic origin.

In vitro, endothelial cells characteristically respond to proinflammatory stimuli like LPS and Interleukin-1 by induction of the expression of intracellular cell adhesion

molecule 1(ICAM-1)(Dustin and Springer, 1998), vascular endothelial cell adhesion molecule-1 (VCAM-1) (Bevilacque et al, 1987), and E-Selectin (E-Sel) (Osborne et al, 1998) on the cell surface, and secretion of proinflammatory cytokines such as Interleukin-6 (IL-6) (May et al, 1989) and Interleukin-8 (IL-8) (Gimbrone et al, 1989). IL-6 is produced by macrophages, fibroblasts, endothelial cells and activated T-helper cells and acts in synergy with IL-1 and TNF. Unlike IL-1, IL-2 and TNF-α, IL-6 does not induce cytokine expression; its main effect, therefore, is to augment the responses of immune cells to other cytokines.

1.4.1 Lipopolysaccharide (LPS)

Lipopolysaccharide is a complex glycoprotein constituent of the outer cell wall of Gram-negative bacteria. (Munshi et al, 2002).

LPS is recognized by Toll-like receptor 4 (TL4) leading to the induction of immune and inflammatory genes in myeloid, leukocytes and endothelial cells (Baldwin, 1996 & Siebenlist et al, 1994).

The pro-inflammatory effects seem to be mediated by the transcription factor NF-κB (Nuclear Factor- κB). NF-κB is a heterodimer consisting of p50 and p65 subunits. It is isolated in the cytoplasm bound to inhibitory protein IκB. Upon LPS stimulation IκB is phosphorylated, ubiquininated and degraded. The free NF-κB translocates to the nucleus where it is involved in activation of genes encoding inflammatory mediators, adhesion molecules and anti-apoptotic proteins in endothelial cells (Lush et al, 2000).

LPS is also responsible for endothelial cell responses including **1)** increased production of nitric acid and tissue factor; **2)** loss of monolayer integrity and barrier function; and **3)** apoptosis (Bannerman et al, 2001). The mechanism in which LPS activates apoptosis is unknown. Human endothelial cells sensitive to LPS induced apoptosis are dependent on new protein or mRNA synthesis inhibition, suggesting that either an inducible or constitutively expressed protein is responsible for protection (Hu et al, 1998).

1.4.2. Tumour Necrosis Factor–α (TNF-α)

Tumour necrosis factor (TNF-α) is a cytokine that contributes to a variety of inflammatory responses. The most important source of this cytokine is monocytes/macrophages (Rothe et al, 1993).

In vivo TNF is a mediator of systemic inflammation and immune responses. A major site of these effects is the vascular endothelium (Pober et al, 1995) where it is capable of inducing inflammatory responses by enhancing adhesion molecule expression and cytokine secretion.

These processes involve the translocation of transcription factor NF-κB from the cytoplasm to the nucleus. This translocation helps stimulate transcription of adhesion molecules E-Sel, ICAM-1 and VCAM-1 and the cytokines IL-6 and IL-8 which all posses the NF-κB elements (Collins T, 1995).

When cells are stimulated with appropriate agonists messenger RNA for TNF-α is induced and also the membrane bound precursor is produced (Imaizumi T et al, 2000).

In addition TNF-α induces the expression of other autocrine growth factors, increases cellular responsiveness to growth factors and induces signaling pathways that lead to proliferation.

The role of endothelial cells as a source of TNF-α is not yet known (Imaizumi et al, 2000).

1.5. Vascular Targeting Agents (VTAs)

The vascular endothelium of tumours differs physiologically from the endothelium of normal tissue; therefore it may be potentially exploited as a target for anticancer drugs (Matthews et al, 2006).

The initial observation that physical obstruction of the blood vessels of solid tumours led to tumour regression in mice suggested that VTAs might be created and altered to cause occulation of blood vessels (Thorpe, 2004).

Destroying the endothelium of solid tumours would result in death of tumour cells from lack of oxygen and nutrients leading to occulation of blood-transporting vessels as well as the capillary sprouts. This action leads to widespread necrosis of established tumours.

There are two types of VTAs; small molecule VTAs and ligand directed VTAs, they each have an individual approach in targeting blood vessels of solid tumours.

Small molecule VTAs are further placed into two categories; microtubule destabilizing agents and cytokine inducers.

1.5.1 Flavone Acetic Acid (FAA)

Flavone Acetic Acid is derived from the flavones group of compounds called flavonoids. Flavonoids have many roles which include influencing cell wall permeability, ion fluxes, altering enzyme activity, inducing interferons, and inducing gene expression (Havsteen, 1983; Redmond *et al*, 1986; Schneider and Zrenner, 1986; Wiltrout and Hornung, 1988).

Two hundred different flavones were chosen to be screened in the National Cancer Institute programme; however, none demonstrated antitumour activity *in vivo* against the current tumour cell lines in use (Plowman *et al*, 1986).

A new *in vivo* screening technique was developed using P388 leukaemia as a prescreen. From these results FAA was selected as the lead compound and was then taken into Phase I and Phase II clinical trials. Results from these clinical trials showed limited significant activity compared to potent antitumour activity against a wide range of murine tumours. This result suggested there may be a species difference in the biochemical target of FAA (Smith et al, 1987 & Finlay et al, 1989).

The Auckland Cancer Society Research Centre initiated a chemistry programme to synthesise analogues of FAA and screen them for induction of haemorrhagic necrosis in a transplanted colon short-term histology assay (Baguley *et al*, 1989; Gamage *et al*, 1992; Rewcastle *et al*, 1989). Significant activity was seen in analogues of xanthenone-4- acetic acid (XAA) and one of these, DMXAA, was selected as a lead compound for further clinical trials.

1.5.2. 5,6 –dimethylxanthenone-4-acetic acid (DMXAA)

Phase I clinical trials of the investigational anti-cancer drug 5,6 dimethyl xanthenone - 4- acetic acid (DMXAA) developed by the Auckland Cancer Society Research Centre (ACSRC) have been recently completed in New Zealand and United Kingdom. The structure of this compound is shown in Figure 1.2.

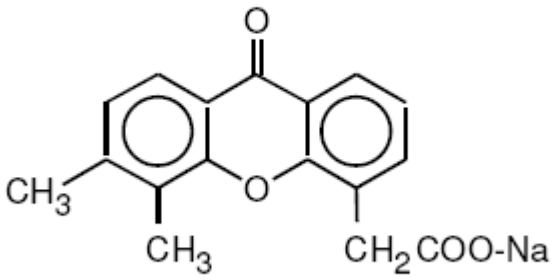


Figure 1.2 Structure of DMXAA

DMXAA acts by disrupting the tumour vasculature, directly and indirectly, causing apoptosis of tumour vasculature endothelial cells and a tumoural induction of cytokines (Baguley B, 2002).

In vitro and murine *in vivo* studies have indicated three major pharmological effects that DMXAA has; antivascular, anti-angiogenetic and immuno-modulating effects. (Zhou et al, 2002). These are dramatically represented in Figure 1.3.

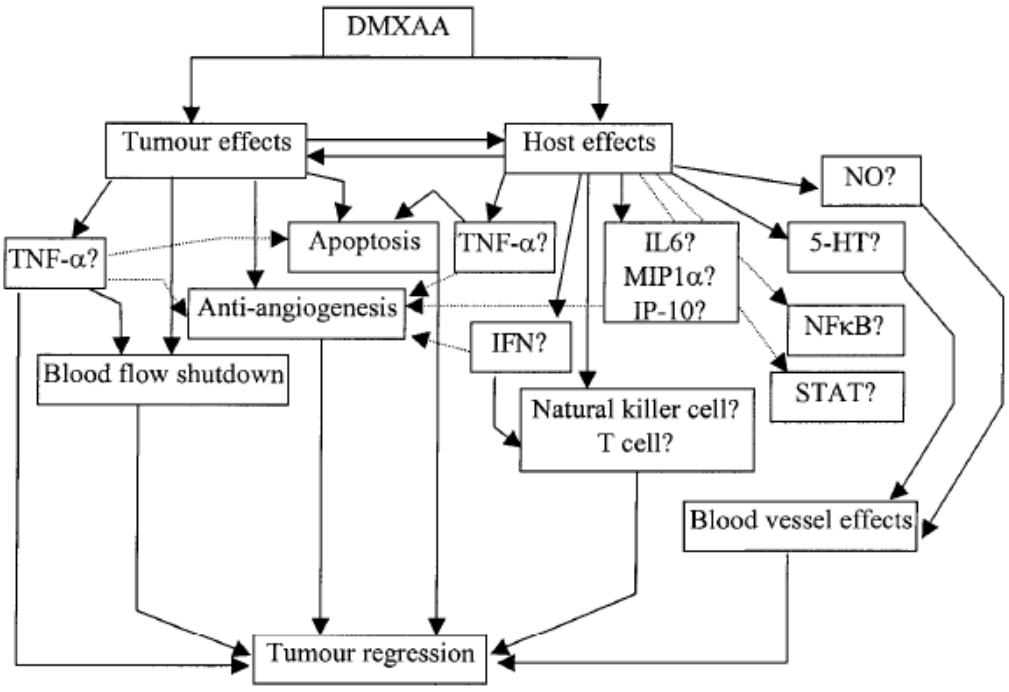


Figure 1.3 The proposed mechanism of action of DMXAA

The antivascular activity is the drugs ability to inhibit blood flow in selective tissues. In murine tumours DMXAA was shown to selectively arrest blood flow within 30 minutes of administration (Zwi L, 1994 & Lash C, 1998).

Blood modification induces tumour vascular collapse, leading to tumour necrosis (Bibby M et al, 1991 & Zwi L et al, 1989).

However, hemorrhagic necrosis induction is not capable of maintaining tumour regression long term and it is believed that stimulated immune response, in particular involvement of T cells, is essential (Hornung R, Bibby M & Pratesi G).

The immuno-modulating effects of DMXAA induce production of many cytokines and chemokines, in particular TNF-α, and Interferons (IFNs). INF-α and INF-β

activate natural killer cell activity and IFN-α and INF-β activate natural killer cell activity, IFN-γ stimulates T cell immune response (Hornung R, Sayers T & Ching L). The ability of DMXAA to induce cytokines such as Interleukin-6 and inflammatory protein-10 can also influence endothelial cell permeability and leukocyte migration.

The overall antitumour effect of DMXAA is considered to be mediated by both antivascular and immuno-modulatory effects.

Physiological Action:

A possible reason for the efficiency of DMXAA, is its ability to induce a cascade of antivascular events in tumour tissue. Necrosis of tumour tissue requires sustained inhibition of blood flow to the tumour. DMXAA may be the most effective drug in achieving this. If a cascade of events is generated within the tumour tissue then reduction in blood flow will reduce the removal rate of the agent (DMXAA) and increase the effectiveness of its action in the tissue.

Molecular Action:

The biochemical target of DMXAA is still unknown but there is evidence that its action involves the activation of the NFκB pathway in monocytes, vascular endothelial cells and various tumour cells.

This finding is taken from the observed range of cytokines and chemokines produced in response to DMXAA which is consistent with the involvement of NFκB.

Also, the induction of early changes observed in vascular endothelial cells (apoptosis) supports the idea that they could be the initial target (Ching L et al, 2002).

1.5.3. Lessons from the Clinical Trials

The two clinical trials of DMXAA have demonstrated reduction in tumour blood flow (measured with gadolinium-enhanced MRI). In addition, dose-limiting toxicity is non-overlapping with that of cytotoxic drugs. At higher dose levels in these trials, a rapid increase in the plasma concentration of the serotonin metabolite 5-hydroxyindole-acetic acid (5HIAA) was found to occur within 20 minutes of DMXAA administration, with peak levels at 4 hours. In the absence of emesis the most likely source of serotonin is platelets. This concurs with evidence from preclinical studies which suggests that acute activation of platelets may be involved in the mechanism for the tumour blood flow reduction.

Endothelial cell responses to DMXAA may therefore be an important component of the tumour blood flow changes. Evidence for a direct effect of DMXAA on endothelial cells comes from the two research groups associated with the clinical trials. Work by the Auckland Cancer Society Research Centre with a human endothelial cell line (ECV304) has shown firstly that DMXAA inhibits angiogenesis *in vivo* (Matrigel assay) and in cell culture (Cao Z et al., 1999). Furthermore DMXAA also induces endothelial cell apoptosis *in vitro*; however, the concentrations required to achieve this effect are much higher than those achieved *in vivo* and the time course is much slower than for the changes in tumour blood flow. In conjunction with the observation that platelet aggregation after DMXAA is restricted to the vasculature of tumours and spleen, this evidence suggests that the microenvironment of endothelial cells may determine their response to this class of drugs.

Ongoing studies at the ACSRC and at other centres have demonstrated synergistic antitumour activity between DMXAA and several standard cytotoxic drugs, bioreductive drugs and radiotherapy (Lash et al., 1998, Wilson et al., 1998, Siemann et al., 1999). Part of the synergy may lie in the blood flow shutdown causing trapping of the cytotoxin in the tumour, thus increasing the tumour exposure to the drug (Pruijn et al., 1997). However, for some drugs such as paclitaxel, which is known to have antiangiogenic activity in its own right, it is possible that part of the antitumour synergy with DMXAA occurs at the level of the endothelial cell.

If the mechanisms by which DMXAA reduces tumour blood flow can be determined, a rational basis would be provided to optimise its therapeutic use in further clinical trials. In addition, if current work in the ACSRC on the identification of the cellular target of DMXAA is successful, exciting opportunities will be presented for the design of new analogues. Such work, which might use the existing combinatorial chemistry facilities at the ACSRC, could generate a new range of compounds aimed specifically at exploiting this mechanism.

In general terms, the combination of agents with different and possibly complementary mechanisms of action offers the hope of more effective treatments for patients with cancer. A better understanding of the novel mechanisms of the antitumour activity of DMXAA may facilitate rational exploitation of these to maximise interactions with standard anticancer therapies.

While the murine *in vivo* models for examining mechanisms of DMXAA-induced tumour blood flow reduction are well developed, there is an urgent need to develop

robust human preclinical models, as they may bear more relevance to the antivascular activity of DMXAA in humans.

Attempts to study the effects of DMXAA using human umbilical vein endothelial cells (HUVEC), the usual human endothelial cell model, do not demonstrate the expected cellular responses, even when using co-culture with tumour-conditioned media (to replicate factors found in the tumour microenvironment. An *ex-vivo* model using human endothelial cells has been published (AD Blann *et al.*, A new whole-cord model for assessing endothelial cell physiology *ex-vivo*. XVIIth Congress of the International Society on thrombosis and haemostasis 1999). This study employed perfusion of the vein within an intact human umbilical cord, and offers the opportunity to examine responses of endothelial cells in their normal vascular anatomical relationships to other tissues, which plausibly may differ to those of cells when harvested and grown *in vitro* (HUVEC). Endothelial cell responses to damaging and activating agents was preserved in this model.

1.6. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. PCR has revolutionized the detection of RNA and DNA molecules. Traditional PCR has advanced from detection at end point of the reaction to detection while the reaction is occurring, in the technique known as Real Time PCR.

1.6.1 Reverse Transcriptase PCR (Traditional)

Traditional methods for detection of PCR products use agarose gels for detection of PCR amplification at the end point of the PCR reaction. This type of agarose gel based analysis of cDNA products of reverse transcriptase - PCR does not allow accurate quantitation, since ethidium bromide fluoresces only when irradiated in the UV part of the spectrum. However, fluorescence is not very bright making it sometimes hard to visualize, and analysis software must be used to derive comparative estimates of the amount of product present.

1.6.2. Real Time PCR

This technique is fast, precise and accurate because it monitors the amplification of products during the reaction.

Real Time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (ie. Real Time) as opposed to end point detection (Freeman et al, 1999). The signal increases in direct proportion to the amount of PCR product in a reaction. By monitoring the amount of fluorescence at each cycle, it is possible to monitor PCR reaction at exponential phase. This advantage can give a true representation of initial amount of starting material.

Other techniques in use for the quantification of mRNA include Northern Blotting or Ribonuclease Protection Assay (RPA). These two techniques require no amplification and work very well; however, they sometimes require more RNA than is made available.

1.7. Aim of Research

The main aim of this research project is to characterise the response of endothelial cells in the intact human umbilical cord to inflammatory stimuli and DMXAA.

1.8. Study Objectives

1. To confirm the viability of endothelial cells within the intact human umbilical vein when perfused over different time periods directly after delivery.

2. To characterise the response of umbilical vein endothelial cells to inflammatory stimuli and DMXAA in the presence and absence of tumour conditioned media using real time PCR

3. To compare endothelial cell responses to inflammatory stimuli and DMXAA in the intact umbilical vein with those of human umbilical vein endothelial cells cultured *in vitro*.

1.9. Maori Advisory Group Kaumatua Kaunihera Support

The investigator recognizes the importance of the collected information as Taonga and that knowledge belongs to the Tangata Whenua and must not be removed or handed on without their express approval. The Kaumatua Kunihera group have given their support and approval for this research to go ahead.

Chapter Two

Materials and Methods

2.1 General Information

2.1.1 Supplier Information

A list of all the suppliers used to source products and the reference names are listed in table 2.1. Thereafter, the suppliers are referred to only by their referred name.

Table 2.1 Supplier List

Supplier	Referred Name	Address
Invitrogen Life Technologies	Invitrogen	Carlsbad,CA,USA
Roche Applied Science	Roche	Penzberg, Germany
Promega Corporation	Promega	CA, USA
Antisoma Research Laboratories	Antisoma	London, England

2.2. General Reagents and Solutions

All chemicals used in this research, unless otherwise stated, were of analytical reagent grade.

2.2.1. General Buffers

The general buffers used in this thesis research are listed in Table 2.2.

Table 2.2. General Buffers

Buffer reference name	Components
Phosphate Buffer Solution	8.00g NaCl, 0.20g KCl, 0.25g KH_2PO_4, 1.15g Na_2HPO_4, double distilled water to make 1 litre
TE(10:1)	10mM Tris-HCL pH 7.5, 1.0mM EDTA

2.3. General Solutions

The general solutions used in this thesis research are listed in Table 2.3.

Table 2.3. General Solutions

Solution reference name	Components
Cell Lysing Solution	20% w/v sodium dodecyl sulfate (SDS;Invitrogen 15525-017) was dissolved into a solution of 50% N,N-dimethyl formamide (DMF; May & Baker D86/18/67) and 50% double distilled deionised water. The pH was adjusted to 4.7 by adding 2.5% of 80% acetic acid and 2.5% 1M HCL
Lactated Ringers Solution	0.3g KCL, 6.0g NaCl, 3.0g Sodium Lactate, 0.2g $CaCl_2$, Adjusted to pH6.5-6.6 and double distilled de-ionised water to make 1L was added
Collagenase A Solution	From Clostridium histolyticam, Working concentration = 1mg/ml(0.1% w/v)
Hypertonic Saline Solution	7.5% NaCl

2.3.1. RNA Extraction Reagents

Table 2.4 lists all the reagents used in RNA extraction method unless otherwise stated.

Table 2.4. RNA Extraction Reagents

RNA Extraction reference name	Components
5 M Guanidinium Thiocyanate (GiTC)	—
2 M Sodium Acetate	2 M Sodium Acetate, adjusted to pH 4.6 using glacial acetic acid
Phenol	Saturated with 10mM Tris HCL,pH 8.0, 1 mM EDTA Phenol phase is pH 6.7
Chloroform	Chloroform/ethyl alcohol
Isopropanol	—
Triozol Reagent	Phenol/guanidine isothiocyanate

2.3.2. Electrophoresis Reagents

The reagents used in the Gel Electrophoresis are listed in Table 2.5.

Table 2.5. Gel Electrophoresis Reagents

Reagent reference name	Components
10 x SDS Buffer	56g Boric Acid, 10g NaOH make up to 2 litres, pH 8.5
100bp Ladder(size:250µg~1.0µg/µl)	50µl ladder, 850µl TE, 100µl loading Dye
Loading Dye	10ml glycerol, 4.0 ml EDTA, 2.0 ml 10% SDS, 2.0 ml Tris, 1.5 ml H_2O

2.3.3. Dyes

The dye used for this research are listed in Table 2.6.

Table 2.6. General Dyes

Reference Name	Components
Ethidium Bromide	Stock solution was made as 25mg ethidium bromide in 500ml distilled water

2.3.4. Culture Medium

The bacterial growth mediums and the components used in this research are listed in Table 2.7.

Table 2.7. Bacterial Growth Medium

Media Reference Name	Components
M199 (100ml)	0.98g Medium 199, 20.0mls Fetal Calf Serum, 2.5mls $NaHCO_3$, 0.48g Hepes, 3.0mls GAV, 1.5mls 1N NaOH, 73ml double distilled water
HUVEC Complete (100ml)	0.98g Medium 199, 20.0mls Fetal Calf Serum, 2.5mls $NaHCO_3$, 0.48g Hepes, 3.0mls GAV, 0.2mls ECGF, 40µl Heparin, 1.5mls 1N NaOH, 76.5ml double distilled water
RPMI 1640 (100ml)	1.026g RPMI 1640, 20.0ml Fetal Calf Serum, 2.5mls $NaHCO_3$, 0.48g Hepes, 3.0mls GAV, 1.5mls 1N NaOH, 73ml double distilled water

2.4. Polymerase Chain Reaction (PCR) Reagents

Table 2.8 lists the reagents used in all PCR reactions unless otherwise stated. All reagents were supplied by Invitrogen.

Table 2.8. PCR Reagents

PCR Reference Name	Reaction Concentrations
Platinum Taq Polymerase(5U/µl)	1 Unit (U) of Platinum Taq per reaction
10 x Platinum Taq Buffer	Tris-HCl, $(NH_4)_2SO_4$,0.625µl of Taq and ~ 25µl of H2O

2.4.1. complementry DNA (cDNA) Production Reagents

The reagents used in production of cDNA from isolated RNA are listed in Table 2.9.

Table 2.9. cDNA Reagents

cDNA Reference Name	Reaction Concentrations
Oligo dt	____
5 x RT Buffer	100mM Tris-HCl(pH8.4),250mM KCl
10mM dNTPs	10mM each of dATP,dCTP,dGTP,dTTP in double distilled water at neutral pH.
0.1 M DTT	250mM Tris-HCL(pH8.3 at room temp.),375mM KCl,15mM $MgCl_2$)
Superscript III	200 units/µl
Double distilled water	___

Table 2.10. Real Time PCR Primer List

Primer Name	Forward Sequence	Reverse Sequence
Intercellular adhesion molecule-1 (I-CAM1)	CTGCAGACAGTGACCATC (892-909)	GTCCAGTTTCCCGGACAA (1211-1228)
HS E-Selectin (E-S)	CGTTGGTGAGGTGTGCTC (7-24)	TGATCTTTCCCGGAACTGC (199-216)
Interleukin -1β (IL-1β)	TACGAATCTCCGACCACCA (235-253)	GTTGCTCCATATCCTGTCCC(549-530)
Interleukin-6 (IL-6)	ATGTAGCCGCCCCACAG	CATCATCTTTTTCAGCCAT
Interleukin-8 (IL-8)	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC
Tumour Necrosis Factor α (TNFα)	CCTCCTCTCTGCCATCAAG	GAGGCGTTTGGGAAGGTT
Human proteosome subunit Y (HPSY)	CAGAACAACCACTGGGTCCT	CCCGGTATCGGTAACACATC
Tissue Factor (TF)	GGAACCCAAACCCGTCAA	TGCTTCACATCCTTCACAATCT
RNA Polymerase II (RNA Pol II)	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
Beta 2 Globulin (B2M)	AGCGTACTCCAAAGATTCAGGTT	TACATGTCTCGATCCCACTTAACTAT
Inducible nitric oxide synthase (iNOS)	AGGAAGTGGGCAGGAGGATG	GAAGGACTCTGAGGCTGTGTG

Table 2.11. Genes regulated in endothelial cells in response to injury and inflammation stimuli

Gene	Gene Function
Intercellular adhesion molecule-1 (I-CAM1)	Adhesion molecule of immunoglobulin supergene family. Binds ligands, monocytes and lymphocytes
HS E-Selectin (E-S)	Adhesion molecule of selectin family
Interleukin -1β (IL-1β)	Inflammatory Cytokoine
Interleukin-6 (IL-6)	B cell growth factor
Interleukin-8 (IL-8)	Neutrophil chemotatic Factor
Tumour Necrosis Factor α (TNFα)	Upregulated cytokine
Human proteosome subunit Y (HPSY)	Housekeeping
Tissue Factor (TF)	Co factor for activation of extrinsic pathway of coagulation
RNA Polymerase II (RNA Pol II)	Housekeeping
Beta-Actin (β-Actin)	Housekeeping
Beta 2 Globulin (B2M)	Housekeeping
Inducible nitric oxide synthase (iNOS)	Synthesis of NO

2.5. Sample Collection

Whole human umbilical cords were kindly donated, with informed consent from women undergoing elective caesarians at Waikato Hospital. Ethical approval was granted from the Northern Y Ethical committee and the University of Waikato Human Ethical Committee. Umbilical cords were collected immediately after delivery. To minimise blood clot formation the vein was flushed with iced PBS then transported in a PBS filled container compacted with ice, back to the Molecular Genetics laboratory at Waikato University. In the Molecular Genetics Lab the umbilical cord vein was cannulated using modified 18 gauge IV catheter hubs and placed into a perfusion chamber. The perfusion chamber was attached to a perfusate reservoir via luer lock tubing and a peristaltic pump (Eyela Microtube Pump MP-3) producing a flow rate through the umbilical vein of 3ml.min^{-1}. The dissolved gas content was maintained in perfusate by pumping air into reservoir through a syringe filter (Millex-HV 0.45μm Filter Unit,SLHV025LS). All perfusion equipment was maintained at 37°C by containment within an air incubator (Ratek Orbital Mixer Incubator).

2.6. General Methods

2.6.1. HUVEC Viability Assays

After the desired perfusion period, 0.1mg.mL^{-1} tetrazolium bromide (3,(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide; MTT; AppliChem 298-93-1)

was added to the perfusate reservoir and the chamber system perfused for a further two hours. To each umbilical cord segment; the vein was flushed with PBS and filled with cell lysing solution and incubated for 12 hours. Cell lysate solution was added to dissolve the insoluble purple formazan product into a coloured solution. The resultant solution was collected and absorbance analysed using a spectrophotometer (Bio-Rad SmartSpec 3000) at a wavelength of 570nm.

2.6.2. Immune Response Induction

LPS solution was prepared by Joe Cursons. Briefly, *E.Coli (DH5α)* was cultured on agar plates, then suspended in Lactated Ringers Solution and pasteurized at 65°C for 30 minutes. The pasteurized *E.Coli* was added to the perfusate reservoir and the umbilical vein was perfused for 4 hours to induce an immune response.

2.6.3. Hypertonic Saline Solution

For Hypertonic Saline Solution (HSS) treatment 2.5ml of 7.5% NaCl solution was added to perfusate reservoir.

2.6.4. DMXAA Requirements

DMXAA requirements are described in Appendix I. DMXAA working concentration for perfusion studies was 250 μM (peak plasma free drug concentration at higher doses used in trials) (Jameson et al, 2000) in 100mls PBS. Then 7.6 mg was accurately measured out and dissolved in 100mls of warm 37°C incubated PBS. This perfusate solution was used for DMXAA perfusion studies.

2.7. RNA Extraction and Isolation

To extract mRNA from umbilical vein after desired perfusion period, the vein was filled with 500µL of 5M guanidinium thiocyanate (GiTC) and left for 1 minute to lyse the HUVECs. The lysate solution was then flushed out (~400µL) and collected in an eppendorf tube. A further 100µL of 5M GiTC was added to the suspension to bring final volume to 500µL. To this, 50µL of 2M Sodium Acetate (pH 4.0) was added followed by 500µL of Phenol (pH4.0) and then mixed by vortexing and placed on ice for 5 minutes. 200µL of Chloroform was added, mixed by vortexing then left on ice for 5 minutes. The suspension was then centrifuged at 12000G for 10 minutes and the aqueous layer was pipetted off into another tube and precipitated using an equal volume of isoproponal and incubated at -20°C for 20 minutes. The suspension was then centrifuged at 12000G for 10 minutes and the isopropanol was decanted off. The RNA pellet was resuspended in 0.5ml of TRI reagent (MRC TR118) and left on ice for 5 minutes and followed by another extraction. Once isopropanol was decanted off from the second extraction, the RNA pellet was washed in 1.0ml of 70% ethanol and then centrifuged 12000G for 1 minute. The ethanol was decanted off and the pellet was resuspended in 20µL of 10mM Tris-HCl (pH 7.8),0.66mM $MnCl_2$. The RNA was then purified of DNA by adding 2µL of RQ1 RNase free DNase (1 unit/µL; Promega M610A) and the solution incubated at 37°C for 30 minutes; the DNase was then inactivated by adding 2.0µL of RQ1 stop solution (20mM EGTA; Promega M199A) followed by an incubation at 65°C for 10 minutes.

2.8. RNA Quality

The purified RNA was quantitated and purity assessed using an electrophoretic gel analysis and a Nanodrop ND-1000 spectrometer with software ND-1000 v3.1.0.

High quality RNA should exhibit sharp 18s and 28s rRNA bands in an agarose gel stained with ethidium bromide. The proportion of ribosomal bands has been viewed as the primary indicator of RNA integrity. One disadvantage of checking RNA purity using an agarose gel is sample consumption. Only small amounts of RNA were able to be extracted from each umbilical cord segment so the agarose gel technique needed to be reconsidered. More successful results were observed with the Nanodrop spectrometer.

Briefly, the Nanodrop is a full spectrum (220-750nm) spectrometer that is a capable of measuring 1μl samples. It employs surface tension alone to hold the sample in place. This advantage eliminates the need for curvettes and other sample contaminant devices. Good quality RNA will have an OD 260/280 ratio higher than 1.50 and an OD 260/230 close to 1.0. This is because nucleic acid is detected at 260nm, whereas; protein, salts and solvents are detected at 230nm and 280nm. A high OD260/280 and OD 260/230 means that the RNA was extracted successfully without any of these contaminants (Auburn R, 2004). If the OD260/280 results are considerably low the RNA was repurified and another reading taken.

2.9. cDNA Production

To produce cDNA, tube A was prepared with 5µl of RNA and added 1µl of OligodT and 4µl H2O. The sample was then heated at 65°C – 70°C (PTC -200 Peltier Thermal Cycler) for 7 minutes to remove any secondary structure from the RNA and allow the oligo dT to bind to the Poly A tail, acting as a reverse transcriptase template. The mixture was then cooled on ice for 2-5 minutes.

In a separate tube 4µl of 5xRT Buffer, 1µl 10mMdntps, 1µl 0.1M TT, 1µl SS III and 3µl H2O were combined. This solution was added to tube A and heated to

50°C for 60 minutes and then 85°C for a futher 5 minutes, to inactivate the reverse transcriptase. The cDNA quality was confirmed by performing a PCR reaction with two known housekeeping genes on endothelial cells, β-Actin and RNA Polymerase II.

2.9.1. Electrophoretic Analysis

Gel electrophoresis was a technique used to separate DNA fragments. The DNA sequences used were stained by mixing 8µl of the DNA with 2µl of loading Dye and the entire sample loaded into a single well of an agarose gel. Multiple samples were loaded next to each other for easy comparison. The agarose gels were prepared by boiling 0.50g of agarose in 35ml of 1XSDS Buffer for 10 seconds before adding 15µl of 10mg/ml Ethidium Bromide then poured into a small mould with desired well spacers and left to set. As a molecular weight standard, 0.4µl of a 100bp ladder (Invitrogen) was loaded into a single well on each gel. The electrophoresis was

conducted at a constant voltage of 120V for 35 minutes and then gels were analysed under UV light (Life Technologies TFX-35m). Images were captured using a CCD camera and intergrating image over 15 frames. The resultant image was modified using Scion imaging to improve visibility.

2.9.2. Polymerase Chain Reaction (PCR)

All Primers used for this research are listed in section 3.0, and detailed where appropriate. In general PCR reactions were carried out using a PTC-200 Peltier Thermal Cycler and the genes of interest along with the housekeeping gene were amplified out using a specified PCR programme. The volume of the PCR reactions were 20µl. In general 100µl of SYBER green master mix, 0.5µl Taq and 2µl forward/reverse primer were combined in one tube, 20µl were dispensed out into each tube including one negative control (5 tubes). Then 0.5µl of cDNA for each sample was dispensed into four of the five tubes as shown in figure 2.1. The PCR programme was as follows: 95°C for 2 mins, 55°c for 20 sec, 68°C for 35 sec, 95°C for 20 sec, 55°C for 20 sec, 68°C for 35 sec, 39 quantification cycles of; 95°C for 20 sec, 68°C for 5 mins, End.

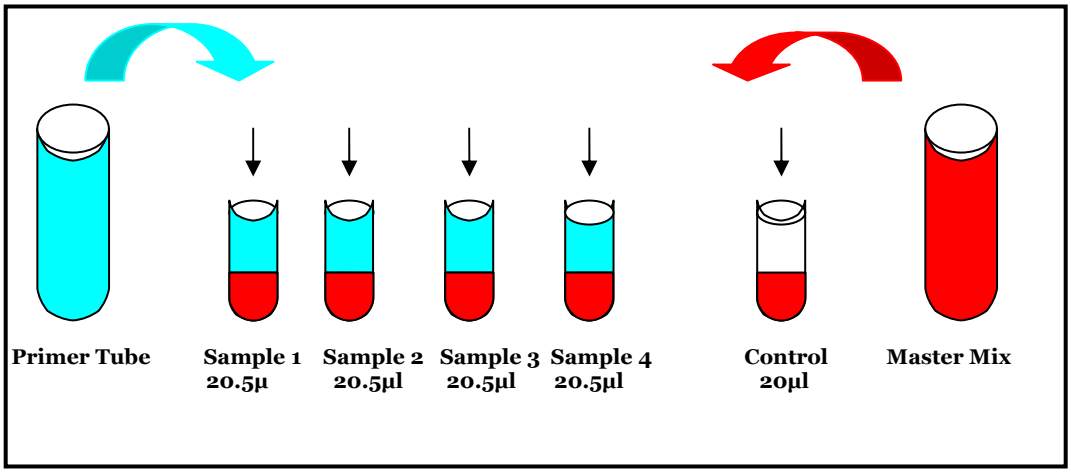


Figure 2.1. PCR reaction with 4 samples & 1 control

2.10. HUVEC Isolation

Umbilical cords were collected from Waikato hospital and brought back to the molecular genetics lab, the cord vein was then cannulated at either end. The vein was flushed with cold PBS and then further air flushed. The vein was then filled with 8ml of Collagenase A (1mg/ml) and sealed at either end with 10ml syringes. The cord was then placed in a 37°C incubator oven and left for 30 minutes. Incubator flasks were prepared by coating base with 1% gelatin to allow then endothelial cells to attach. The cord was then removed from the incubator and the vein was massaged by hand to loosen any endothelial cells still attached to the vein wall. The collagenase A solution was then flushed out with a 10ml syringe and the effluent collected in a 50ml conical centrifuge tube. 10ml of M199 medium was then flushed through the cord to collect

any further cells left behind. The resultant solution was then spun at 20,000 rpm for 10 mins (Multifuge 1 S-R, *Heraeus, Kendro*). The supernatant was tipped off leaving a pellet of cells at the bottom of centrifuge tube. This cell pellet was resuspended in fresh M199 media and transferred to a culture flask. The flask was then incubated at 37°C and the cells were checked daily. The M199 media was replaced on day 2 with 5mls of fresh complete HUVEC media. Media was changed every three to four days until cell confluence was reached.

2.10.1. HUVEC Culture Media

Isolated human umbilical vein endothelial cells were routinely maintained on HUVEC complete medium after observed cell growth on either RPMI 1640 or M199 Medium. Both M199 and HUVEC complete media were prepared by adding 0.98g M199 media, 0.48g Hepes and 76.5mls double distilled H2O into a sterile 250ml bottle. This content was filter sterilized into another sterile 250ml bottle. To this NaHCO3, GAV and Fetal Calf Serum was added, the media was then adjusted to pH 7.3 with 1 N NaOH. In HUVEC media at this stage both Heparin and endothelial cell growth factors were added as stated in table 2.7.

RPMI 1640 medium was prepared in a similar method except RPMI 1640, Hepes and double distilled H20 were autoclaved on liquid cycle at 121°C instead of filter sterilized.

2.10.2. Subculturing

HUVEC cells were subcultured once grown to confluence, old media was removed from flask, 3.0ml of trypsin was added and the flask was returned to the 37°C incubator for 5 minutes or until cells had loosened from the base of the flask. 10mls of fresh HUVEC media was added to original flask and then 5mls removed and pipetted into a new flask. Cells were checked daily and fresh HUVEC media replaced when needed.

2.10.3. Human Melanoma Cells

Human Melanoma Cancer Cells (NZM3) were kindly donated from patients at the Auckland Cancer Society Research. These were received in ITS growth medium and were passaged as previously stated in section 2.10.2 with two flasks allowed to grow to confluence. Once cell confluence was reached, cells were passaged and the media was collected in 50ml centrifuge tubes and frozen at -80°C until required for experimental purposes.

2.10.4. Freezing cells

Both HUVEC cells and NZM3 cells were allow to grow to confluence in flasks. Both cell types were frozen for bulk storage and experimental use later in the year. The cells media was removed from flasks, 3ml of trypsin/EDTA was added and it was then incubated at 37°C. Once all cells were loosened from flask base, cell suspension

was transferred to 10ml centrifuge tubes and centrifuged (Multifuge 1 S-R, *Heraeus Kendro*) at 10,000 rpm for 5 minutes. The supernatant was removed and the remaining cell pellet was resuspended in 1ml of Fetal Calf Serum/Dimethyl Sulfoxide. This solution was then transferred to a cryogenic vial and placed in a cryocontainer, then placed in -80°C freezer. Cryopreservation enabled stocks of cells to be stored, preventing the cell line to be in continuous culture during other experimental procedures.

2.10.5. Thawing Cells

Cells were thawed when required for experimental purposes. Briefly, cells were removed from the -80°C freezer and fast thawed in a heated water bath maintained at 30°C. The cells were then transferred to a 10ml centrifuge tube containing 5mls of appropriate media and then centrifuged for 5 minutes at 10,000rpm. This was to remove all DMSO present. The supernatant was tipped off and cells were resuspended in fresh media (complete HUVEC media for HUVEC cells or ITS media for human melanoma cells) and transferred to a culture flask. The culture flasks were incubated at 37°C to allow cells to once again reach confluence.

2.11. Real -Time Polymerase Chain Reaction

All primers used for this research are listed in table 3.0 and detailed where appropriate. In general, Real-time PCR was performed using a Corbett RG-3000 with Rotor-Gene (v6.0.27) software and genes the of interest were amplified out of the cDNA library using a specified PCR programme.

In general 400µl of master mix (Sybr Green), 2.5µl Taq and were combined in one tube, 35µl was dispensed in 9 tubes. Then 0.4µl of each primer was dispensed into the 9 tubes. Each tube was mixed and 10µl was dispensed from each different primer tube to two new tubes (includes replicates). The control tubes (primer tubes) were closed. To the remaining tubes 1µl of the cDNA sample was added. The PCR was run in duplicates. Please refer to Figure 2.2. for details.

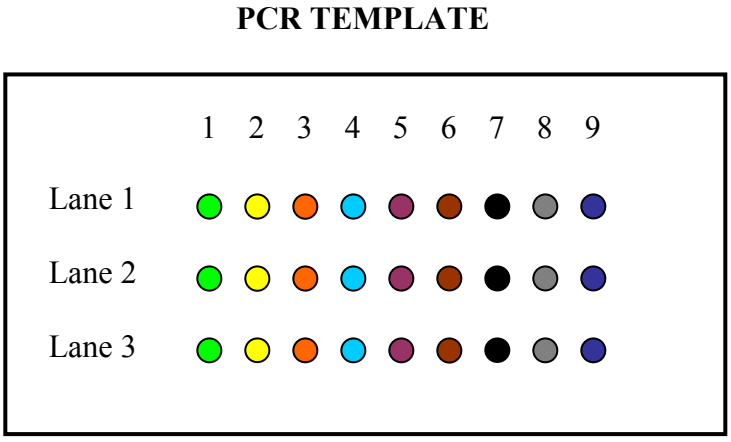


Figure. 2.2. Setup PCR Plate: Lane 1 has the nine individual primers. Lane 2 & 3; each tube contains each of the different primers and 1µl of cDNA sample

The PCR was cycled with an initial inactivation step of 7.5 minutes at 95°C followed by 40 quantification cycles of; 94°C for 10 seconds, 55°C for 10 seconds, 68°C for 30 seconds and 80°C for 20 seconds. Relative quantification was achieved by analyzing the Ct value, with data quality examined through a regression model. The approach identified the correlation between the Ct value and logrithm (Base 2) transformed concentration of template, giving a significant linear relationship for each standard curve.

Chapter Three

Results

3.1. Ex Vivo Perfusion Model

The perfusion chamber was designed previously in a summer studentship but further modified by Joe Cursons to minimise experimental variables and to also allow within cord comparison which is clearly shown in Figure 3.3.

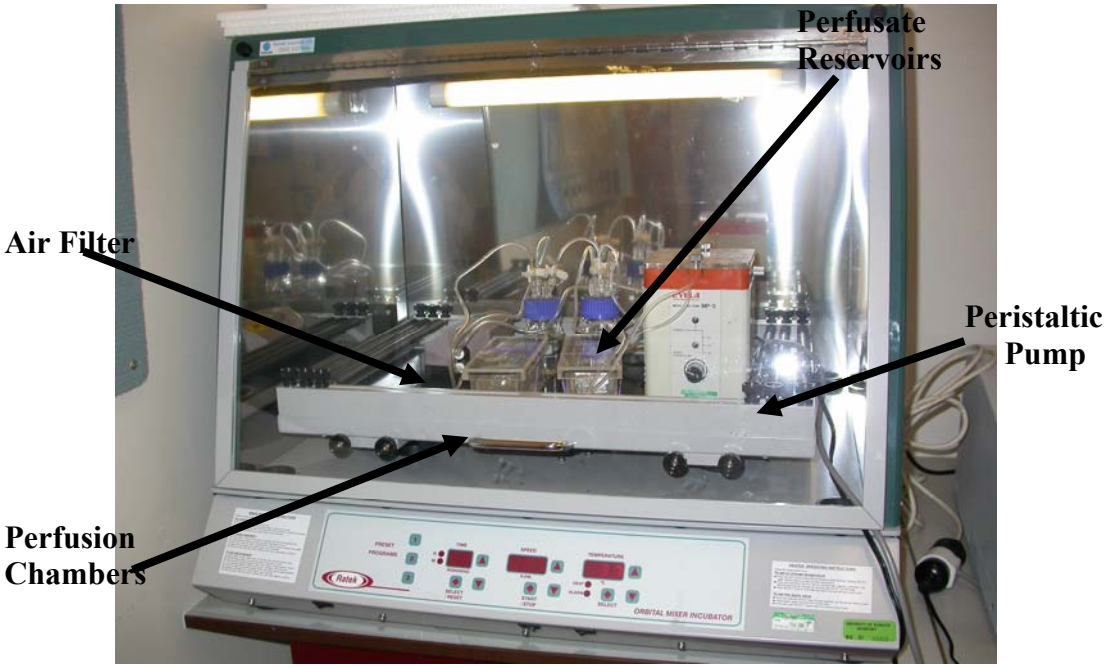


Figure 3.1 Modified Ex vivo perfusion model
Perfusion equipment maintained at 37°C by containment within an air incubator (Ratek Orbital Mixer Incubator).

Figure 3.2. Ex vivo perfusion model: Three chambers are set up running along side each other, peristaltic pump(Eyela Microtube Pump MP-3) producing a flow rate through the umbilical vein of 3ml.min^{-1}, dissolved gas content was maintained by pumping air into reservoir through a syringe filter (Millex-HV 0.45µm Filter Unit,SLHV025LS).

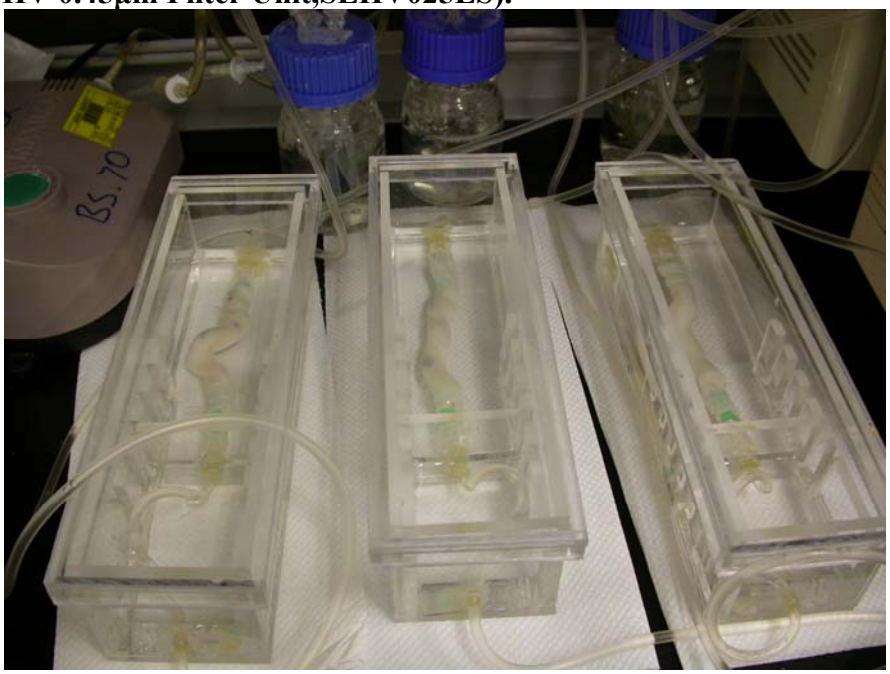


Figure 3.3. Perfusion chambers: Umbilical cord divided into 3 segments and perfused separately, to allow within cord comparison.

3.2. MTT Viability Assay.

Table 3.1. Summary of umbilical cord segment OD absorbance readings

Experiment	Cord 1		Cord 2		Cord 3		Cord 4	
Section	1	2	1	2	1	2	1	2
OD Absorbance (AU) at 570nm	1.440	2.735	0.608	0.406	0.474 (1:4)	0.784 (1:4)	0.052	0.083
Time Perfused(hrs)	2	2	2	2.5	2.25	2	2.5	5
Volume of Solution(ml)	1.9	3.0	1.5	2.5	3.0	0.9	0.15	3.0
Length(mm)	80	60	80	65	40	40	45	40
Weight(g)	10.73	9.2	11.3	11.7	10.32	5.77	5.25	9.80
OD/mm($x10^{-3}$)	18	45	7.6	6.2	11.8	19.6	1.15	2.1

Experiment	Cord 5		Cord 6		Cord 7		Cord 8	
Section	1	2	1	2	1	2	1	2
OD Absorbance (AU) at 570nm	0.123	0.049	0.223	0.149	0.078	0.090	0.120	0.080
Time Perfused(hrs)	3	4	2.5	4	4	5	5	5
Volume of Solution(ml)	1.5	1.0	.9	1.0	1.5	1.5	1.0	1.0
Length(mm)	50	40	60	50	50	50	60	55
Weight(g)	10.73	9.2	9.8	6.0	6.5	5.8	6.0	5.8
OD/mm($x10^{-3}$)	2.46	1.225	3.7	2.9	1.56	1.8	1.5	1.45

The umbilical cord segments were perfused as described in section 2.6.1.

When MTT solution was intravenously perfused through each segment the tetrazolium salts were converted to a purple formazan dye by only the active cells. The lysate solution from each segment was collected and measured using a spectrophotometer set at wavelength 570nm.

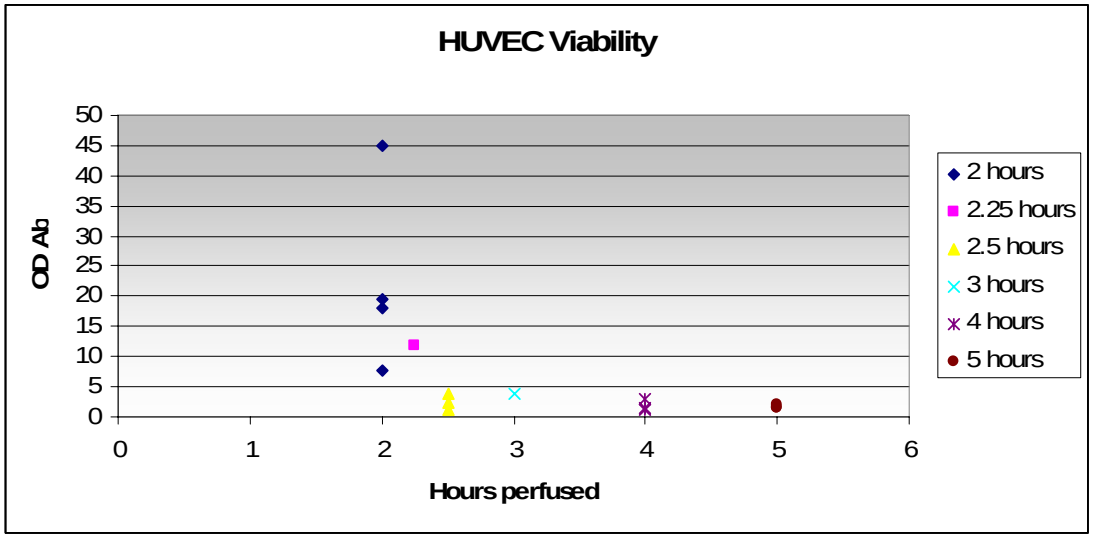


Figure 3.4. Optical Density absorbance measurements of HUVEC

Table 3.2. HUVEC Viability Over Time

% Cell Viability	Hours perfused
100	0
90	1
80	2
60	3
40	4
30	5
10	6
0	7
0	8
0	9
0	10

In Figure 3.4. the x-axis represents hours each cord segment was perfused, the y-axis represents the OD/mm ($x10^{-3}$) measurement. At 0 hours resting endothelial cells within the cord vein are presumed to be 100% viable.

The minimum perfusion time was 2 hours, after this time the cells would expect to be losing their viable state. With this assumption, 80% of cells are still viable after 2 hours. A general decreasing trend was seen in Figure 3.4., an estimation of % cell viability over time was possible. Based on the results in Table 3.1 only an estimation

of cell viability was possible. The differences in OD absorbance readings in cord 4, questioned the reliability of the results. It was shown in Table 3.1 that after 5 hours perfusion a significantly low OD absorbance reading of 0.083 is produced; however in the same cord after 2 hours perfusion a lower OD absorbance reading of 0.052 is recorded. The expected results of a longer perfusion time, decreases cell viability is clearly not displayed with cord 4. However, observation of the other perfused cords do support the general trend of, increasing time reduces cell viability, and a time frame of 2 hours perfusion was concluded.

3.3. Immune Response Induction: Cord 1

Table 3.3. Summary of umbilical cord 1 information: Segment weights, lengths, perfusate solutions used and time period.

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	Unperfused (control)	4 hours	57	10.4
2	PBS	4 hours	46	8.6
3	LPS (1μg/ml)	4 hours	50	8.9

Table 3.4. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 1)

Experiment – Nanodrop Data					
Sample ID	**concentration ng/μL**	**A260**	**260/230**	**260/280**	**Const.**
control	373.04	9.326	0.57	1.65	40
PBS	230.44	5.761	0.37	1.51	40
LPS	414.37	10.359	0.62	1.61	40

The gene expression was measured in response to PBS and LPS.

The results from the Nanodrop spectrometer in Table 3.4, indicate that the RNA isolation procedure was successful and produced RNA of an accepted quality as indicated by the (260/280) ratio values >1.50. The purified RNA from PBS and LPS samples was then used to produce cDNA as described in section 2.9. Real Time PCR was used for gene expression analysis.

Quantitation data for SYBR Green ford cord 1

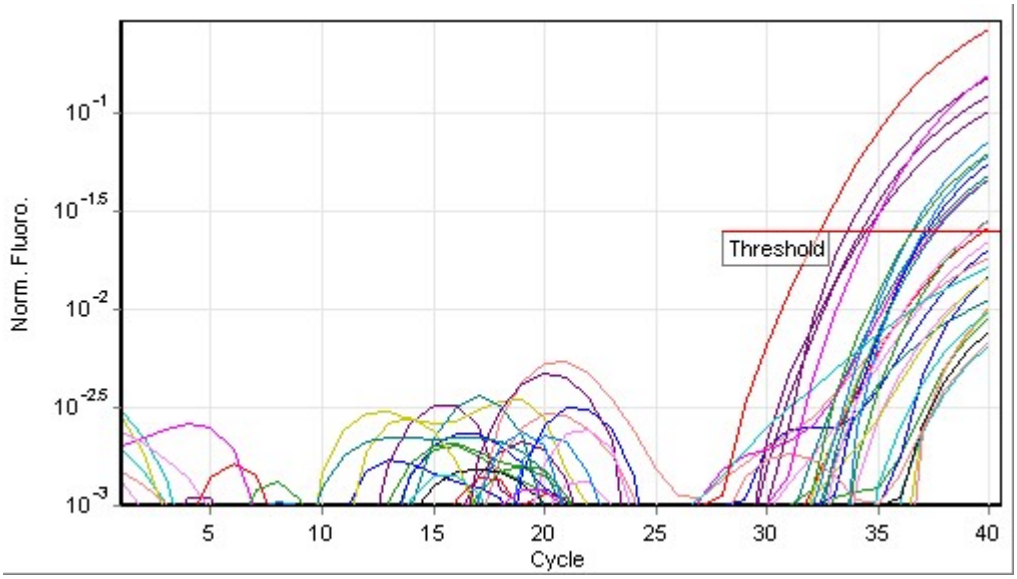


Figure 3.5. Quantitation Data for SYBR Green (cord 1)

Table 3.5. Summary of Genes and Threshold cycles Ct (cord 1)

Name	Ct	Name	Ct
B2M-neg	-	PAI1-neg	-
B2M-LPS	39.76	PAI1-LPS	37.29
B2M-PBS	32.32	PAI1-PBS	37.55
IL1B-neg	-	TLR4-neg	-
IL1B-LPS	37.55	TLR4-LPS	-
IL1B-PBS	-	TLR4-PBS	-
IL6-neg	-	TNFα-neg	-
IL6-LPS	-	TNFα-LPS	39.34
IL6-PBS	37.18	TNFα-PBS	36.55
IL8-neg	34.44	TF-neg	-
IL8-LPS	34.18	TF-LPS	37.45
IL8-PBS	33.56	TF-PBS	34.62
IL12-neg	-	ICAM-neg	-
IL12-LPS	-	ICAM-LPS	-
IL12-PBS	39.41	ICAM-PBS	-
iNOS-neg	-	ESEL-neg	-
iNOS-LPS	37.02	ESEL-LPS	-
iNOS-PBS	36.49	ESEL-PBS	-

Melt data for cord 1

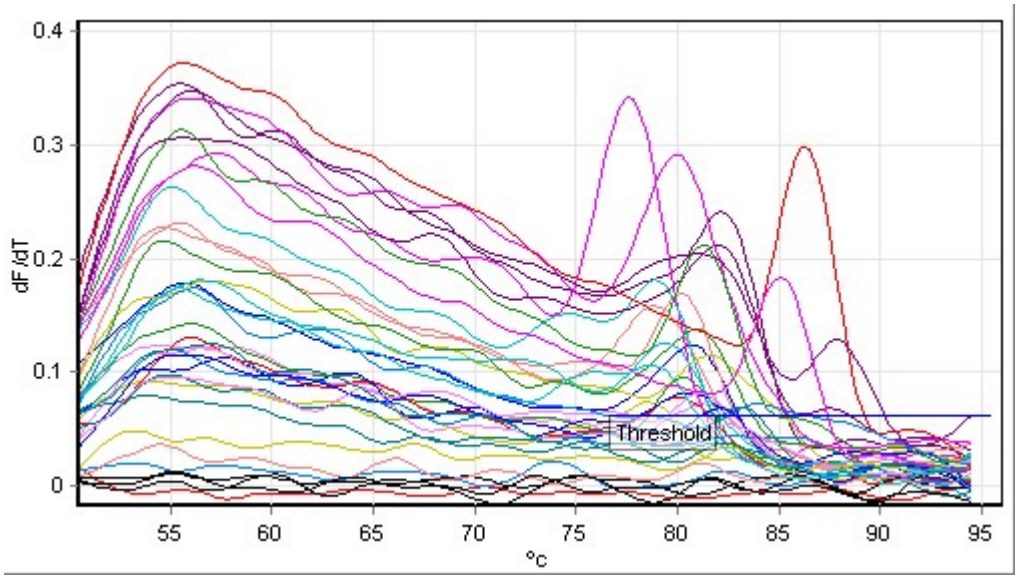


Figure 3.6. Melt Data (cord 1)

Table 3.6. Summary of Genes and Melt temperatures (cord 1)

Name	Melt temp°		Name	Melt temp°
B2M-neg	-		PAI1-neg	-
B2M-LPS	80		PAI1-LPS	-
B2M-PBS	86.3		PAI1-PBS	83.2
IL1B-neg	78.5		TLR4-neg	78.8
IL1B-LPS	81.5		TLR4-LPS	80.2
IL1B-PBS	-		TLR4-PBS	-
IL6-neg	80.7		TNFα-neg	80.3
IL6-LPS	80.5		TNFα-LPS	81
IL6-PBS	82		TNFα-PBS	81.3
IL8-neg	82.2		TF-neg	77.5
IL8-LPS	82	87.5	TF-LPS	80
IL8-PBS	81	87.8	TF-PBS	85
IL12-neg	81.5		ICAM-neg	-
IL12-LPS	81.2		ICAM-LPS	-
IL12-PBS	-		ICAM-PBS	-
iNOS-neg	-		ESEL-neg	79
iNOS-LPS	-		ESEL-LPS	79.5
iNOS-PBS	84		ESEL-PBS	78.7

The internal control standard (housekeeping gene) used in this Real Time PCR was Beta 2 Microglobulin (B2M). The background threshold was set to give a threshold cycle (Ct) for each gene. The results in Table 3.5 show very little amounts of cDNA, this made it difficult to measure gene expression.

A dissociation curve (melt curve) was produced for each sample, this was to ensure that only the desired product was detected. The melt temperatures for each sample should be within 0.5-1°C of each other. The results in Table 3.6 indicate possible DNA contamination or primer dimer taking place where two peaks appear from one sample.

Due to the inadequate amounts of cDNA detected for each gene and a non reliable Ct reading for B2M (housekeeping gene), gene expression evaluation was inconclusive.

3.4. Immune Response Induction: Cord 2

Table 3.7. Summary of umbilical cord 2 information: Segment weights, lengths, perfusate solutions used and time period.

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	105	16.057
2	LPS (1µg/ml)	4 hours	120	9.943
3	LPS + HSS	4 hours	110	10.997

Table 3.8. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 2)

Experiment – Nanodrop Data					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
PBS(control)	551.55	13.789	0.89	1.67	40
LPS	340.94	8.524	0.52	1.52	40
LPS + HSS	228.12	5.703	0.41	1.51	40

Gene expression was measured in response to LPS and combination LPS+HSS.

The isolated RNA from each sample was quality checked with the Nanodrop spectrometer and results presented in Table 3.8. All perfused segments resulted in good RNA purity with 260/280 ratio values of >1.50.readings >1.50. RNA samples were then used to produce cDNA as described in section 2.9. Real Time PCR was performed for gene expression analysis.

Quantitation data for SYBR Green for Cord 2

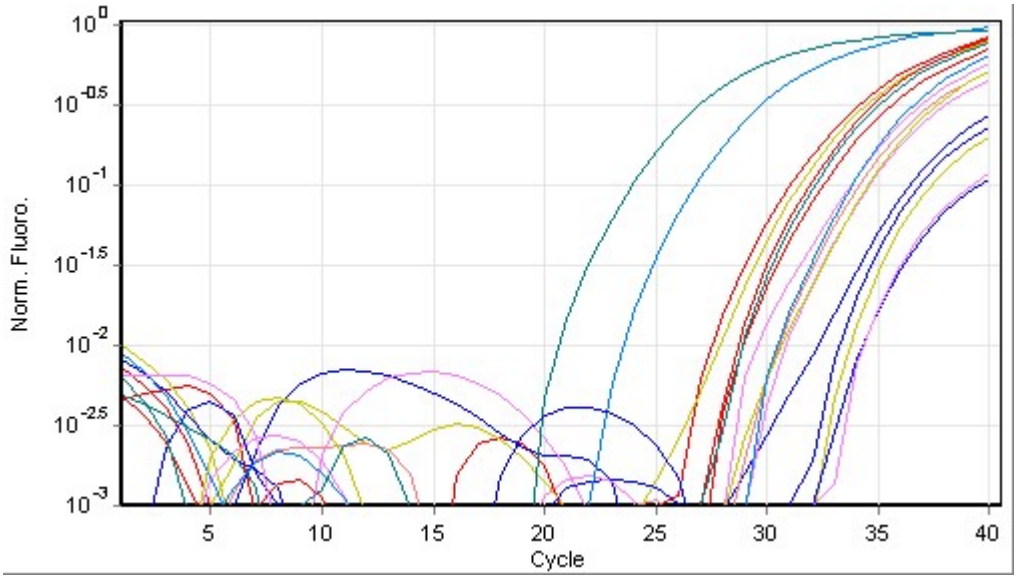


Figure 3.7. Quantitation Data for SYBR Green (cord 2)

Table 3.9. Summary of Genes and Threshold cycle Ct (cord 2)

Name	Ct
B2M-neg	-
B2M-PBS	28.21
B2M-LPS	26.80
B2M-LPS+HSS	28.08
IL1B-neg	-
IL1B-PBS	33.02
IL1B-LPS	26.91
IL1B-LPS+HSS	29.67
iNOS-neg	-
iNOS-PBS	33.43
iNOS-LPS	31.00
iNOS-LPS+HSS	32.54
TLR4-neg	-
TLR4-PBS	-
TLR4-LPS	-
TLR4-LPS+HSS	-

Name	Ct
TNF-αneg	-
TNF-α-PBS	33.61
TNF-α-LPS	30.12
TNFα-LPS+HSS	28.75
TF-neg	-
TF-PBS	-
TF-LPS	22.88
TF-LPS+HSS	29.78
ICAM-neg	-
ICAM-PBS	-
ICAM-LPS	29.78
ICAM-LPS+HSS	-
ESEL-neg	-
ESEL-PBS	-
ESEL-LPS	20.01
ESEL-LPS+HSS	28.30

Melt data for cord 2

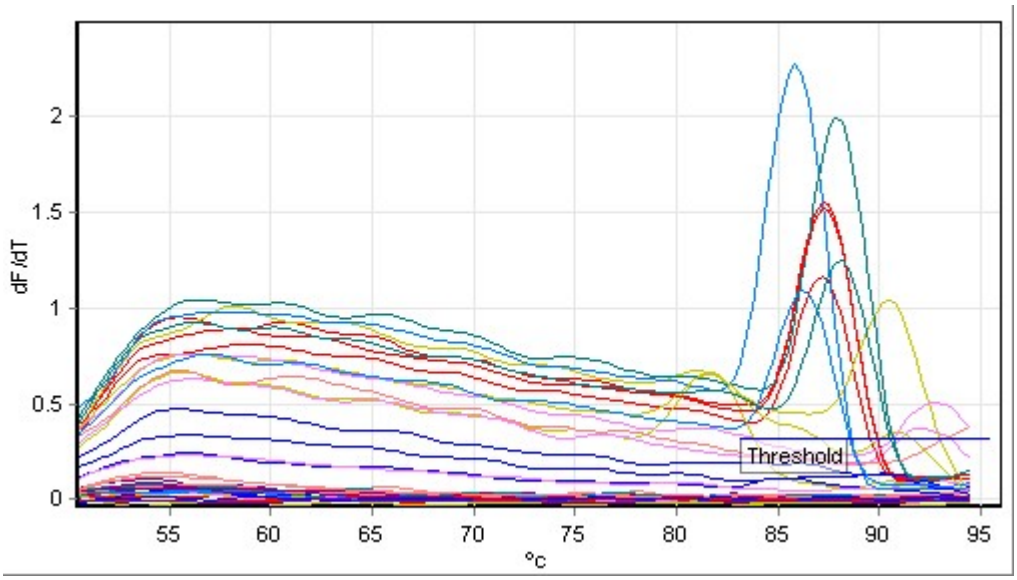


Figure 3.8. Melt Data (cord 2)

Table 3.10. Summary of Genes and Melt Temperatures (cord 2)

Name	Melt temp°
B2M-neg	-
B2M-PBS	87.2
B2M-LPS	87.3
B2M-LPS+HSS	87.3
IL1B-neg	-
IL1B-PBS	-
IL1B-LPS	90.5
IL1B-LPS+HSS	91
iNOS-neg	-
iNOS-PBS	-
iNOS-LPS	-
iNOS-LPS+HSS	-
TLR4-neg	-
TLR4-PBS	-
TLR4-LPS	-
TLR4-LPS+HSS	-

Name	Melt temp°
TNF-αneg	-
TNF-αPBS	-
TNF-αLPS	92
TNFα-LPS+HSS	92.8
TF-neg	-
TF-PBS	-
TF-LPS	85.8
TF-LPS+HSS	86.3
ICAM-neg	-
ICAM-PBS	-
ICAM-LPS	-
ICAM-LPS+HSS	-
ESEL-neg	-
ESEL-PBS	88
ESEL-LPS	88
ESEL-LPS+HSS	-

Table 3.11. Summary of Gene Expression Profile Data (cord 2)

Gene	Normalised ICN	Gene	Normalised ICN
ESel-Control	0	TF-Control	0
ESel-LPS	25.41882	TF-LPS	1.105642
ESel-LPS+HSS	0.958857	TF-LPS+HSS	0.158604
ICAM-Control	0	TLR4-Control	0
ICAM-LPS	0.762643	TLR4-LPS	0
ICAM-LPS+HSS	0	TLR4-LPS+HSS	0
IL1B-Control	0.084054	TNFa-Control	0.503114
IL1B-LPS	2.18183	TNFa-LPS	0.248113
IL1B-LPS+HSS	2.378788	TNFa-LPS+HSS	0.811941
iNOS-Control	0.121137		
iNOS-LPS	0.005642		
iNOS-LPS+HSS	0.175089		

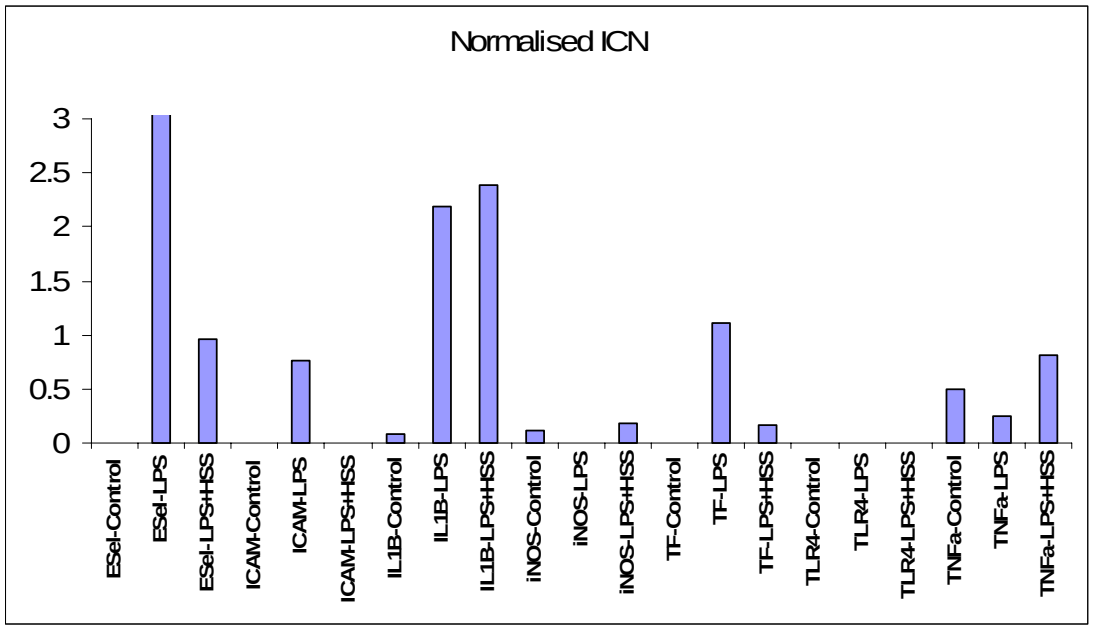


Figure 3.9. Gene Expression Profile (cord 2)

The internal control standard (housekeeping gene) used for this Real Time PCR was Beta 2 Microglobulin (B2M).

After normalizing the expression of each gene to that of the housekeeping gene, B2M, it was observed that LPS stimulated endothelial cells have unregulated ESEL expression greater than 8 - fold than that of the control (PBS). In the presence of HSS, ESEL has decreased expression to 2-fold to that of the control. LPS has also increased regulation of ICAM 2-fold, IL1B 4-fold and TF 2-fold. The impact of HSS on LPS activation is evident in gene expression of ESEL, ICAM and TF, ultimately suppressing the up regulation of these genes. ESEL, ICAM and TF cytokines are known to be induced in endothelial cells in the presence of inflammatory stimuli like LPS.

3.5. Immune Response Induction: Cord 3

Table 3.12. Summary of umbilical cord 3 information. Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	45	15.67
2	LRS	4 hours	49	15.0
3	LPS (1μg/ml)	4 hours	40	12.92
4	LPS + HSS	2 hours + 2 hours	50	12.0

Table 3.13. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 3)

Experiment					
Sample ID	**ng/μL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
Control(PBS)	330.35	8.259	0.50	1.56	40
LRS	284.62	7.116	0.44	1.48	40
LPS	217.13	5.428	0.35	1.42	40
LPS + HSS	208.66	5.217	0.33	1.39	40

Gene expression was measured in response to LRS, LPS and combination LPS+HSS. Isolated RNA from each sample was quantified using a nanodrop spectrometer. The concentrations were recorded in Table 3.13. The 260/280 ratio values are low with LPS isolated RNA sample recorded at 1.42. All RNA samples were used to produce cDNA for Real Time PCR.

Quantitation data for SYBR Green for cord 3

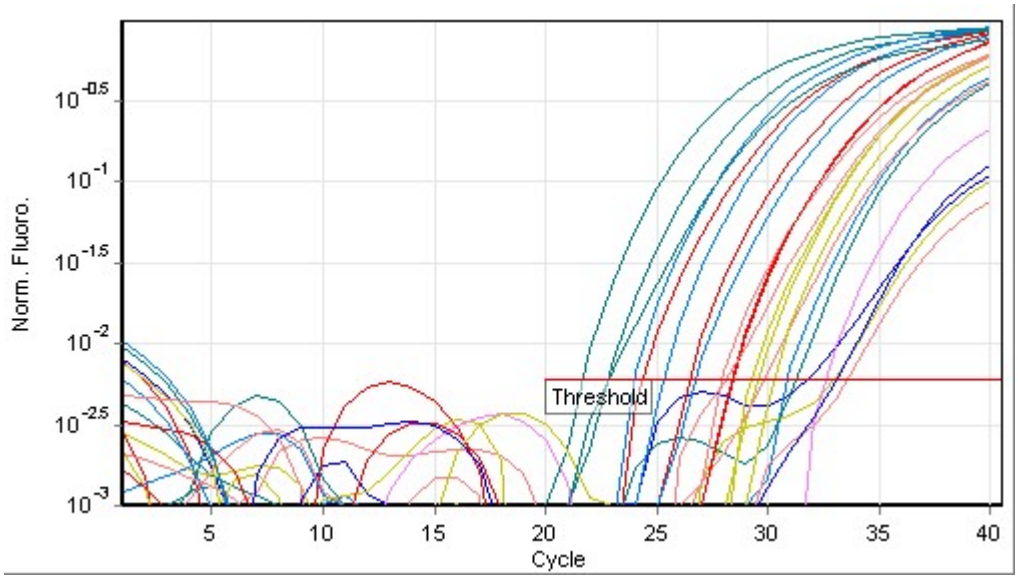


Figure 3.10. Quantitation data for SYBR Green (cord 3)

Table 3.14. Summary of genes and threshold cycle (cord 3)

Name	Ct
B2M-PBS	24.28
B2M-LPS	28.39
B2M-LPS+HSS	28.33
B2M-LRS	26.40
IL1β-PBS	32.94
IL1β-LPS	29.22
IL1β-LPS+HSS	30.26
IL1β-LRS	29.54
iNOS-PBS	31.73
iNOS-LPS	-
iNOS-LPS+HSS	33.03
iNOS-LRS	-
TLR4-PBS	-
TLR4-LPS	-
TLR4-LPS+HSS	-
TLR4-LRS	-

Name	Ct
TNFα-PBS	-
TNFα-LPS	32.50
TNFα-LPS+HSS	-
TNFα-LRS	-
TF-PBS	30.84
TF-LPS	23.89
TF-LPS+HSS	26.65
TF-LRS	25.24
ESEL-PBS	31.24
ESEL-LPS	21.63
ESEL-LPS+HSS	22.78
ESEL-LRS	22.72
ICAM-PBS	33.53
ICAM-LPS	27.87
ICAM-LPS+HSS	28.10
ICAM-LRS	29.86

Melt Data for cord 3

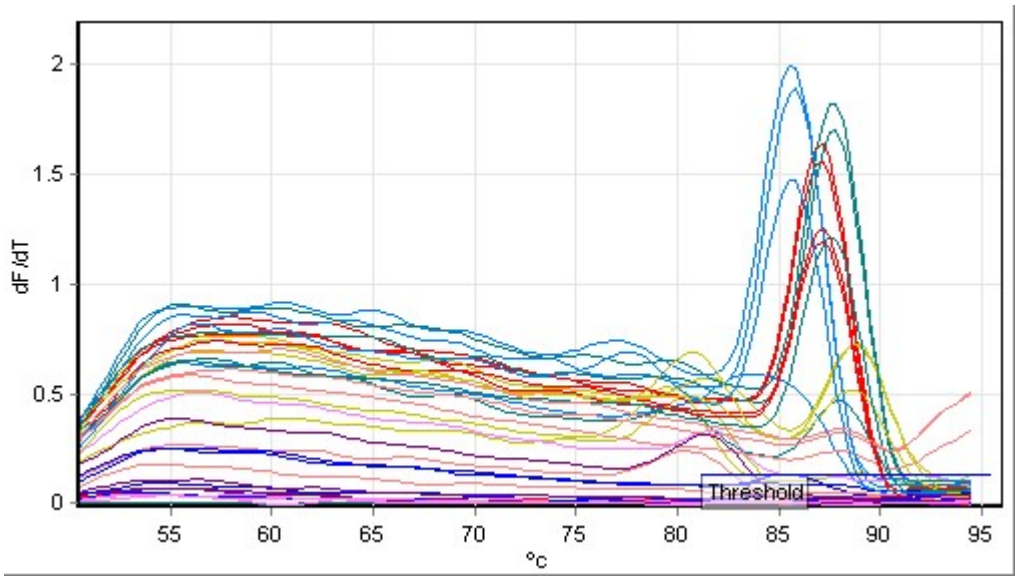


Figure 3.11. Melt data (cord 3)

Table 3.15. Summary of genes and melt temperatures (cord 3)

Name	Melt temp°	Name	Melt temp°
B2M-PBS	87	TNFα-PBS	-
B2M-LPS	87.2	TNFα-LPS	-
B2M-LPS+HSS	87.2	TNFα-LPS+HSS	-
B2M-LRS	87	TNFα-LRS	-
IL1β-PBS	-	TF-PBS	83.7
IL1β-LPS	88.8	TF-LPS	85.5
IL1β-LPS+HSS	88.5	TF-LPS+HSS	85.7
IL1β-LRS	88.7	TF-LRS	85.7
iNOS-PBS	-	ICAM-PBS	-
iNOS-LPS	-	ICAM-LPS	-
iNOS-LPS+HSS	-	ICAM-LPS+HSS	-
iNOS-LRS	-	ICAM-LRS	-
TLR4-PBS	-	ESEL-PBS	88
TLR4-LPS	-	ESEL-LPS	87.7
TLR4-LPS+HSS	-	ESEL-LPS+HSS	87.5
TLR4-LRS	-	ESEL-LRS	87.7

Table 3.16. Summary of Gene Expression Profile Data (cord 3)

Gene	Normalised ICN	Gene	Normalised ICN
ESel-Control	0.063638	iNOS-LPS	0
ESel-LPS	134.1575	iNOS-LPS+HSS	11.57995
ESel-LPS+HSS	566.1752	iNOS-LRS	0
ESel-LRS	17.62928	TF-Control	0.003004
ICAM-Control	0.030681	TF-LPS	26.156
ICAM-LPS	8.351366	TF-LPS+HSS	514766.9
ICAM-LPS+HSS	29.85016	TF-LRS	2.749562
ICAM-LRS	4.863645	TLR4-Control	0
IL1B-Control	0.003997	TLR4-LPS	0
IL1B-LPS	0.812339	TLR4-LPS+HSS	0
IL1B-LPS+HSS	7.200989	TLR4-LRS	0
IL1B-LRS	0.421942	TNFa-Control	0
iNOS-Control	0.021249	TNFa-LPS	0.041572
		TNFa-LPS+HSS	0
		TNFa-LRS	0

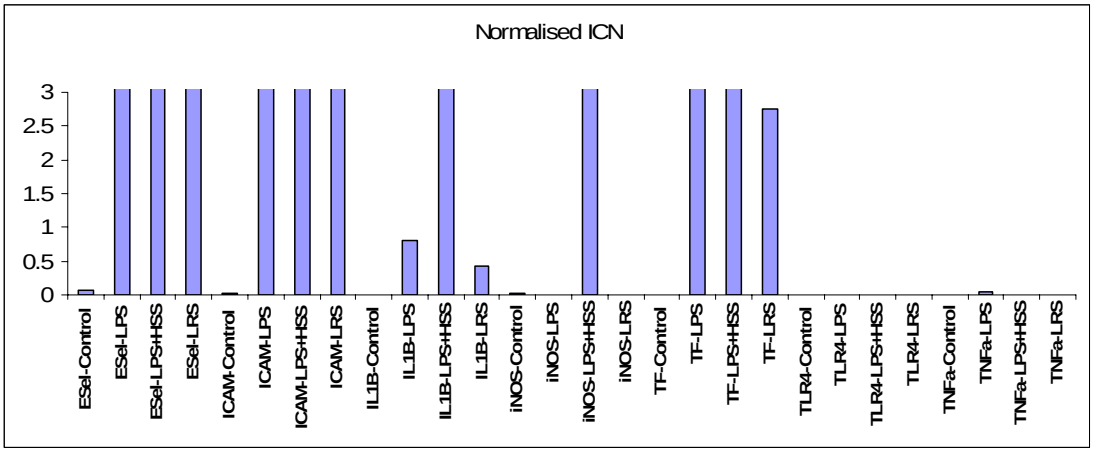


Figure 3.12. Gene Expression Profile (cord 3)

Beta-2 Microglobulin (B2M) was the internal control standard (housekeeping gene) used for Real Time PCR.

Ideally the melt temperature for each primer pair should be the same or within 0.5-1°C of each other. The recorded melt readings recorded and listed in Table 3.15 were successful results, indicating that the specific gene product is being detected with no interference or contamination.

Endothelial cell stimulation from LPS has shown an 8-fold up regulation of E-SEL, I-CAM and TF genes. The introduction of HSS also shows a positive 8-fold increase of each of these genes.

IL1B is unregulated 2-fold compared to that in the presence of HSS it is unregulated 8-fold. Inducible NOS (iNOS) had also been unregulated 8-fold with HSS but in LPS stimulation alone there is no expression of this gene.

The overall results showed that HSS contributes entirely to the gene expression of iNOS, and increases the up regulation of IL1B.

The known cytokines of endothelial cells, ESEL, ICAM and TF gene expressions have not been affected by HSS in this cord.

3.6. Immune Response Induction: Cord 4

Table 3.17. Summary of umbilical cord 4 information. Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	90	9.8
2	LPS (1μg/ml)	4 hours	110	10.5
3	LPS+HSS	2 hours + 2hours	70	7.9

Table 3.18. RNA quality check: Nanodrop spectrometer data for each cord segment (cord 4)

Experiment – Nanodrop Data					
Sample ID	**ng/μL**	**A260**	**260/230**	**260/280**	**Const.**
Control(PBS)	192.47	4.812	.32	1.48	40
LPS	447.15	11.179	.78	1.70	40
LPS+HSS	340.01	8.500	.60	1.58	40

Gene expression was measured in response to LPS and combination LPS+HSS.

RNA isolated from samples was purity assessed using the Nanodrop spectrometer. The 260/280 ratio values were recorded in table 3.18. Only PBS had the lowest 260/280 ratio value of 1.48. All RNA samples were used to produce quality cDNA for Real Time PCR.

Quantitation data for SYBR Green for cord 4

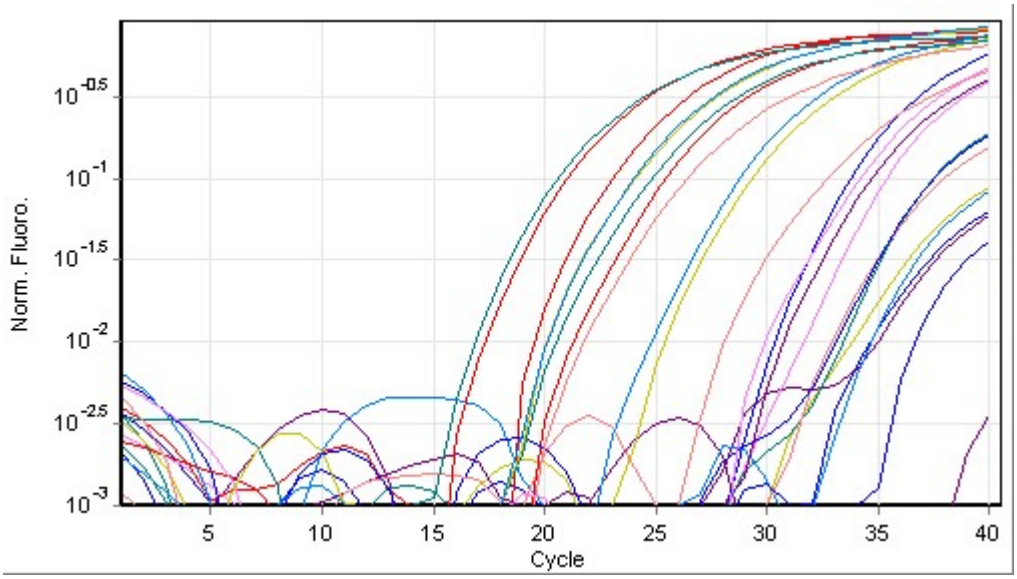


Figure 3.13. Quantitation data for SYBR Green (cord 4)

Table 3.19. Summary of genes and the threshold cycle (cord 4)

Name	Ct
B2M-PBS	18.99
B2M-LPS	20.53
B2M-LPS+HSS	16.63
IL-1β-PBS	32.48
IL-1β-LPS	24.68
IL-1β-LPS+HSS	19.53
iNOS-PBS	29.73
iNOS-LPS	35.94
iNOS-LPS+HSS	32.06
TLR4-PBS	33.09
TLR4-LPS	-
TR4-LPS+HSS	30.05

Name	Ct
TNFα-PBS	30.75
TNFα-LPS	-
TNFα-LPS+HSS	29.20
TF-PBS	33.83
TF-LPS	23.90
TF-LPS+HSS	19.50
ICAM-PBS	31.99
ICAM-LPS	27.38
ICAM-LPS+HSS	20.95
ESEL-PBS	32.51
ESEL-LPS	19.79
ESEL-LPS+HSS	16.21

Melt Data for cord 4

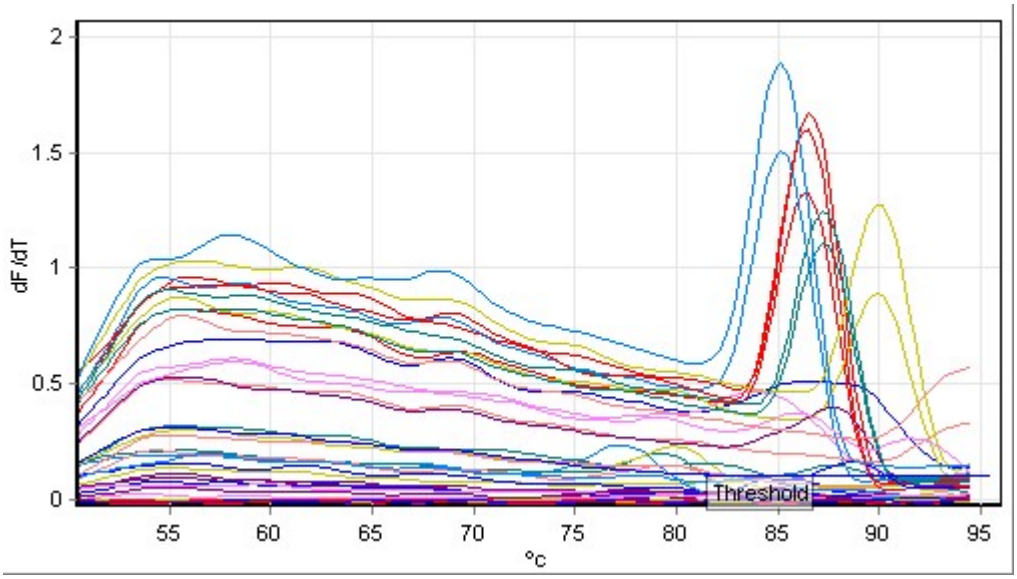


Figure 3.14. Melt data (cord 4)

Table 3.20. Summary of Genes and Melt Temperatures (cord 4)

Name	Melt temp°
B2M-PBS	86.5
B2M-LPS	86.5
B2M-LPS+HSS	86.5
IL-1β-PBS	-
IL-1β-LPS	90
IL-1β-LPS+HSS	90
iNOS-PBS	86.3
iNOS-LPS	88.5
iNOS-LPS+HSS	-
TLR4-PBS	-
TLR4-LPS	-
TLR4-LPS+HSS	87.7

Name	Melt temp°
TNFα-PBS	84
TNFα-LPS	85.2
TNFα-LPS+HSS	86
TF-PBS	-
TF-LPS	-
TF-LPS+HSS	85
ICAM-PBS	-
ICAM-LPS	-
ICAM-LPS+HSS	-
ESEL-PBS	87.7
ESEL-LPS	87.3
ESEL-LPS+HSS	87.3

Table 3.21. Summary of Gene Expression Profile Data (cord 4)

Gene	Normalised ICN	Gene	Normalised ICN
ESel-Control	736.1406	TF-Control	8158.893
ESel-LPS	2.298194	TF-LPS	0.16943
ESel-LPS+HSS	1.718896	TF-LPS+HSS	0.442395
ICAM-Control	2566.006	TLR4-Control	0.003426
ICAM-LPS	0.039796	TLR4-LPS	0
ICAM-LPS+HSS	0.265375	TLR4-LPS+HSS	0.007323
IL1B-Control	0.155308	TNFa-Control	0.078705
IL1B-LPS	0.276012	TNFa-LPS	0
IL1B-LPS+HSS	0.229002	TNFa-LPS+HSS	0.001428
iNOS-Control	0.021249		
iNOS-LPS	0.104472		
iNOS-LPS+HSS	0.000294		

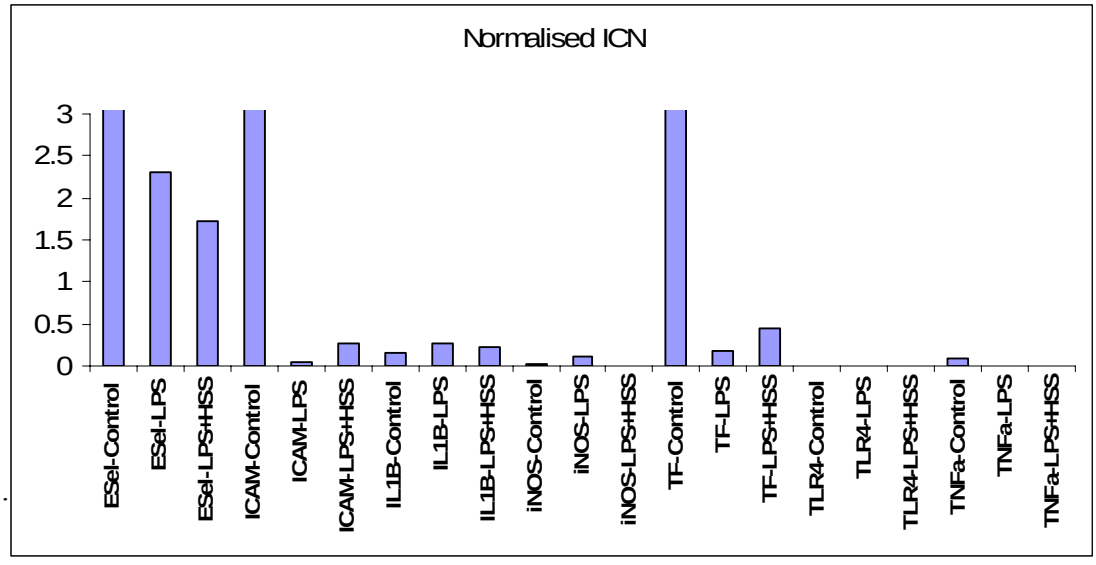


Figure 3.15. Gene expression profile (cord 4)

The internal control standard (housekeeping gene) used for this Real Time PCR was Beta 2 Microglobulin (B2M).

It was observed from the gene expression results in Figure 3.15 that ESEL, ICAM and TF have been up regulated 8-fold in the control (PBS) samples. The result of this gives evidence that ESEL, ICAM and TF expressions are induced in resting endothelial cells without any inflammatory stimuli. The large 8-fold increase may be due to individual cord heterogeneity. LPS has up regulated gene expression of ESEL 6-fold but in combination stimulation with HSS, ESEL falls to less than 4-fold.

HSS interferes with the stimulation of LPS on endothelial cells.

3.7. Immune Response Induction: Cord 5

Table 3.22. Summary of umbilical cord 5 information. Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	70	8.7
2	HSS	4 hours	95	12.8
3	LRS	2 hours	92	7.9

Table 3.23. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 5)

Experiment – Nanodrop Data					
Sample ID	**ng/µL**	**A260**	**260/230**	**260/280**	**Const.**
Control(PBS)	334.44	8.361	0.55	1.51	40
HSS	370.85	9.271	0.57	1.53	40
LRS	502.24	12.556	0.96	1.63	40

Gene expression was measured in response to HSS and LRS perfusion.

The isolated RNA samples were quantified using the Nanodrop spectrometer. Results were recorded in Table 3.23. The 260/230 ratio values were >1.5, but of low quality. The highest 260/280 ratio value was 1.63 for the Lactated Ringers (LRS) perfused segment. All RNA samples were used to produce cDNA for Real Time PCR.

Quantitation data for cord 5

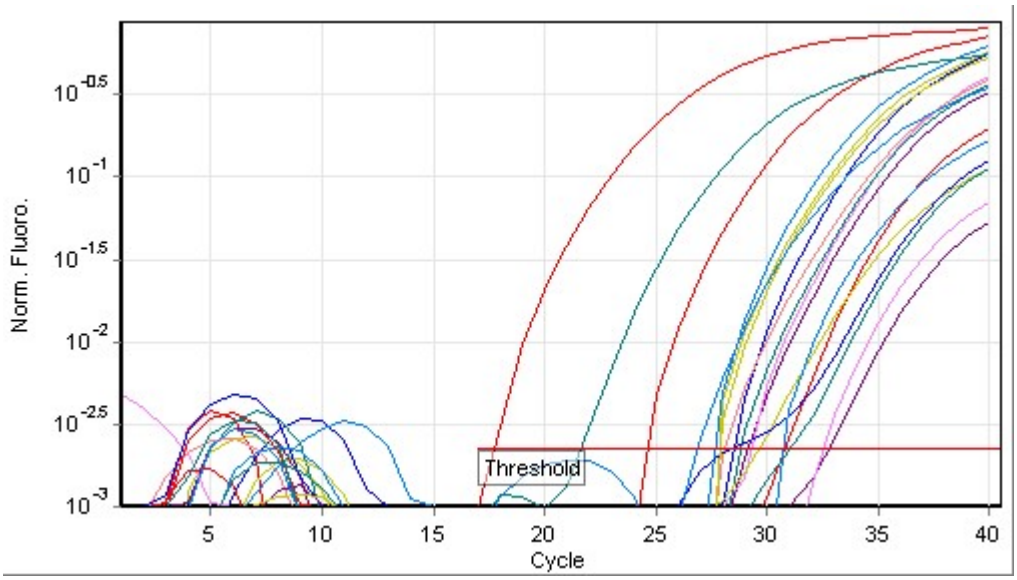


Figure 3.16. Quantitation data for SYBR Green (cord 5)

Table 3.24. Summary of genes and threshold cycle Ct (cord 5)

Name	Ct
B2M-neg	-
B2M-PBS	17.81
B2M-LRS	30.82
B2M-HSS	24.46
IL1B-neg	-
IL1B-PBS	27.57
IL1B-LRS	29.67
IL1B-HSS	27.73
iNOS-neg	-
iNOS-PBS	28.44
iNOS-LRS	28.32
iNOS-HSS	-
TLR4-neg	-
TLR4-PBS	29.15
TLR4-LRS	32.75
TLR4-HSS	-

Name	Ct
TNFα-neg	-
TNFα-PBS	29.23
TNFα-LRS	32.45
TNFα-HSS	-
TF-neg	-
TF-PBS	27.49
TF-LRS	30.61
TF-HSS	26.85
ESEL-neg	-
ESEL-PBS	28.75
ESEL-LRS	31.07
ESEL-HSS	21.68
ICAM-neg	-
ICAMP-PBS	28.06
ICAM-LRS	-
ICAM-HSS	-

Melt data for cord 5

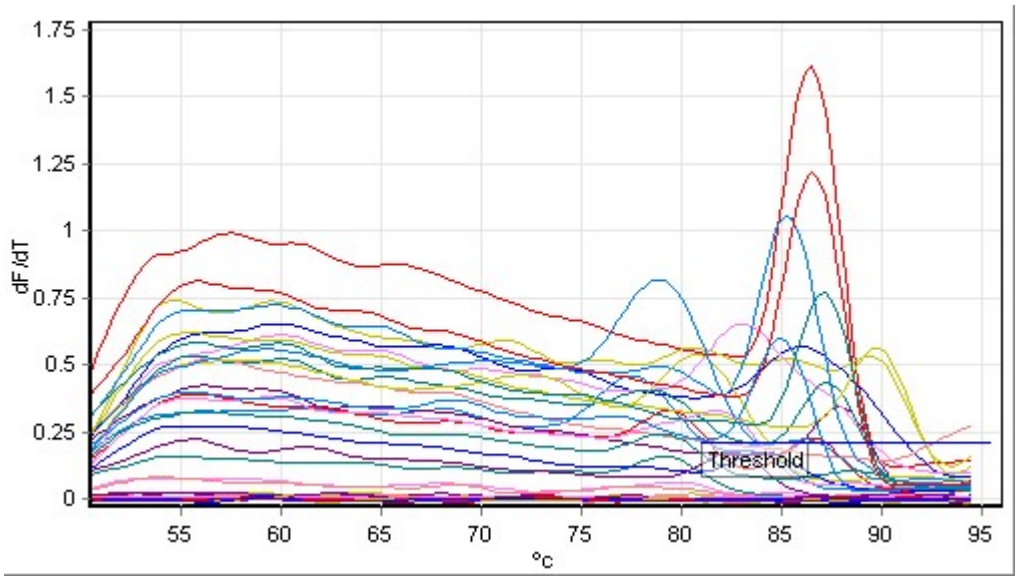


Figure 3.17. Melt data (cord 5)

Table 3.25. Summary of genes and melt temperatures (cord 5)

Name	Melt temp°	
B2M-neg	-	
B2M-PBS	84.8	
B2M-HSS	85	89.7
B2M-LRS	85.2	
IL1B-neg	-	
IL1B-PBS	-	
IL1B-HSS	-	
IL1B-LRS	-	
iNOS-neg	-	
iNOS-PBS	82.5	87.3
iNOS-HSS	83.7	
iNOS-LRS	83.3	
TLR4-neg	-	
TLR4-PBS	-	
TLR4-HSS	-	
TLR4-LRS	-	

Name	Melt temp°	
TNFα-neg	89.3	
TNFα-PBS	83	89.7
TNFα-HSS	-	
TNFα-LRS	83.3	90
TF-neg	-	
TF-PBS	-	
TF-LPS	-	
TF-LRS	-	
ESEL-neg	87.5	
ESEL-PBS	-	
ESEL-HSS	-	
ESEL-LRS	88.2	
ICAM-neg	82	91
ICAMP-PBS	83	91.7
ICAM-HSS	-	
ICAM-LRS	82.8	91.5

Table 3.26. Summary of Gene Expression Profile Data (cord 5)

Gene	Normalised ICN	Gene	Normalised ICN
ESel-Control	0.0034	TF-Control	0.001789
ESel-HSS	9.446954	TF-HSS	0.958624
ESel-Ringers	2634380	TF-Ringers	977525
ICAM-Control	0.010927	TLR4-Control	0.010607
ICAM-HSS	0	TLR4-HSS	0
ICAM-Ringers	0	TLR4-Ringers	3854187
IL1B-Control	0.002452	TNFa-Control	0.004521
IL1B-HSS	0.083368	TNFa-HSS	0
IL1B-Ringers	1367010	TNFa-Ringers	0.981007
iNOS-Control	0.006335		
iNOS-HSS	0		
iNOS-Ringers	12.15964		

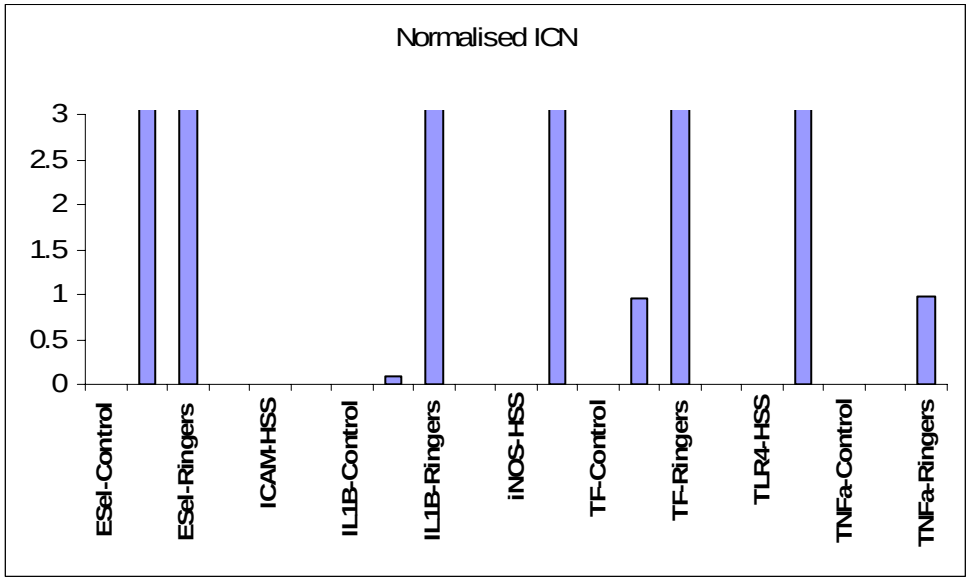


Figure 3.18. Gene Expression Profile (cord 5)

The internal control standard (housekeeping gene) used in this Real Time PCR was Beta 2 Microglobulin.

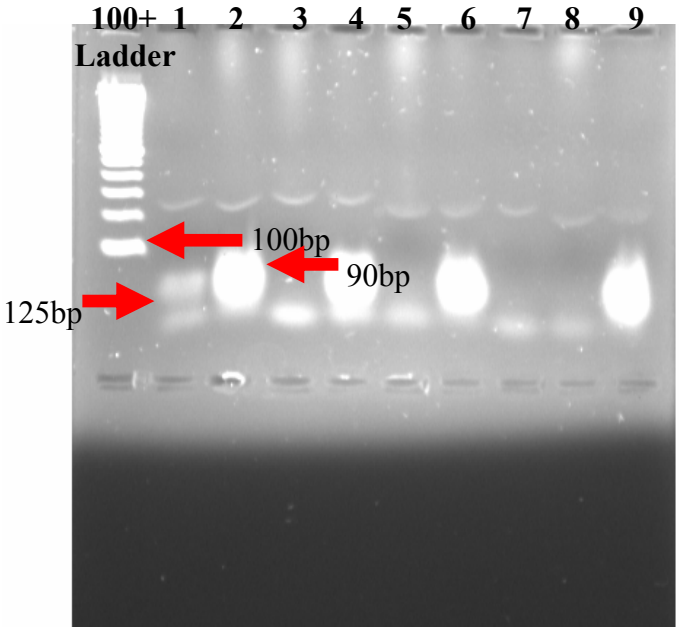
Evidence from the gene expression profile in Figure 3.18 shows an 8-fold up regulation of ESEL when endothelial cells are exposed to HSS and LRS. There is also an 8-fold up regulation of IL1B, iNOS and TF for LRS stimulation. TNF-α gene is also expressed 2-fold in the presence of LRS but is not expressed in resting endothelial cells or stimulation with HSS. The gene expression of ICAM is suppressed in all samples.

The gene expression for the PBS (control) samples were suppressed, this result may of been due to gene heterogeneity of each individual umbilical cord.

3.8. DMXAA & TCM Responses for cord 6

Table 3.27. Summary of umbilical cord 6 information. Segment weights, lengths, perfusate solutions used and time period

Perfusion Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	70	7.178
2	TCM	4 hours	75	6.281
3	TCM + DMXAA (250µg/ml)	2 hours + 4 hours	48	6.545
4	DMXAA (250µg/ml)	4 hours	56	7.808



Lane Number	Description	Size (bp)
100bp+	Ladder	-
1	RNA Pol	125
2	β Actin	90
3	PBS (RNA Pol)	125
4	PBS (β actin)	90
5	TCM (RNA Pol)	125
6	TCM (β actin)	90
7	DMXAA(RNA Pol)	125
8	DMXAA + TCM(RNA Pol)	125
9	DMXAA + TCM(β actin)	90

Figure 3.19. cDNA gel elctrophoresis (cord 6)
The agarose gel was loaded with 5µl 100bp+ molecular weight standard ladder, 8µl of both housekeeping genes (RNA Pol & β Actin) were loaded into lanes 1 & 2. Samples (8µl) were loaded into lanes 3-9.

The cord segments were perfused successfully with appropriate solutions stated in Table 3.27. Confirmation of. cDNA with 2 housekeeping genes (β Actin & RNA Pol II) was performed and quantitated by agarose gel electrophoresis method described in section 2.9.1. The cDNA produced as described in section 2.9 was run on a 1% acrylimide gel and bands visualized with ethidium bromide. In lanes 2 & 3 the bands are clearly visible in the gel. The β Actin detected at approximately 90bp and RNA Pol II detected at 125bp. The cDNA samples loaded into gel lanes 3-9 detected each housekeeping gene of interest with strong visual bands. The samples were then prepared for Real Time PCR as described in section 2.11.

3.8.1. Tumour conditioned Media Perfusion for cord 6

cDNA sample from tumour conditioned media perfused segment was analysed for expression of specific genes. Primers used are previously described in Table 2.10.

Quantitation Data for SYBR Green for cord 6

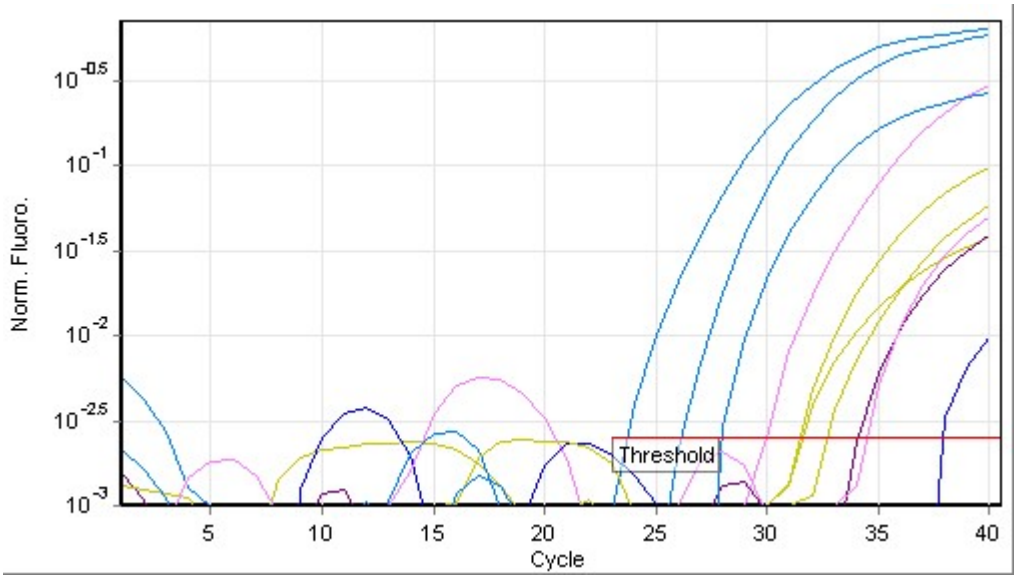


Figure 3.20. Quantitation data for SYBR Green (TCM cord 6)

Table 3.28. Summary of Genes and Threshold cycle Ct (TCM cord 6)

Name	Ct
HPSY - neg	NTC
HPSY –TCM	28..90
HPSY- TCM	NTC
E-Sel - neg	31.40
E-Sel – TCM	31.49
E-Sel – TCM	32.56
ICAM - neg	37.76
ICAM – TCM	-
ICAM – TCM	-
IL-1β – neg	-
IL-1β – TCM	34.04
IL-1β - TCM	-
IL-6 – neg	29.99
IL-6 – TCM	-
IL-6 – TCM	34.38

Name	Ct
IL-8 neg	23.58
IL-8 – TCM	27.89
IL-8 - TCM	26.09
TNF – neg	-
TNF – TCM	-
TNF – TCM	-
TF – neg	-
TF – TCM	-
TF – TCM	-
RNA Pol-neg	-
RNA Pol-TCM	-
RNA Pol-TCM	-

*NTC=No template Control

Melt data for cord 6

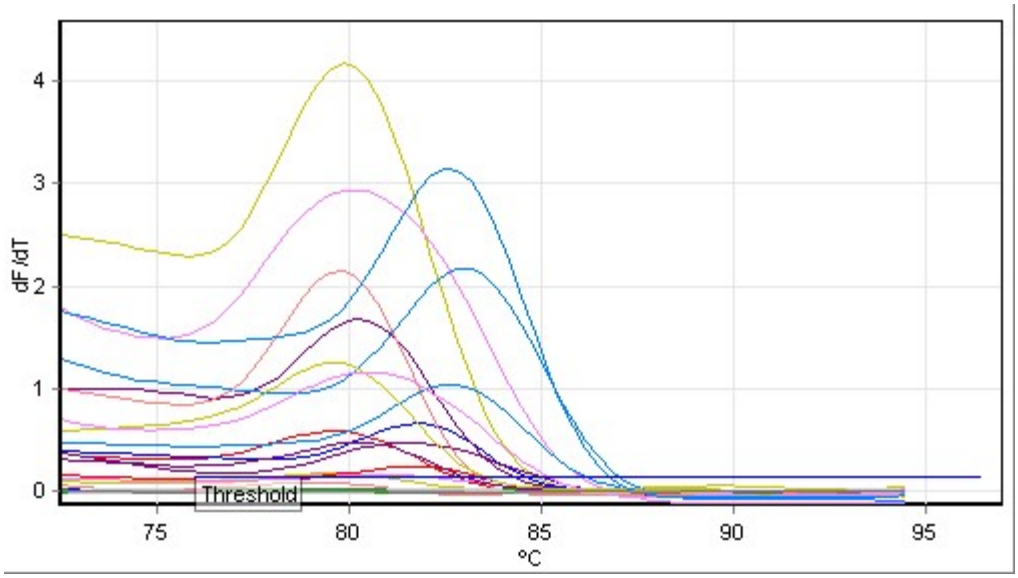


Figure 3.21. Melt data (TCM cord 6)

Table 3.29. Summary o f Genes and Melt Temperatures (TCM cord 6)

Name	Melt temp °
HPSY - neg	79.5
HPSY- TCM	-
HPSY- TCM	81.7
E-Sel - neg	80.0
E-Sel – TCM	79.6
E-Sel – TCM	79.5
ICAM - neg	81.7
ICAM – TCM	-
ICAM – TCM	-
IL-1β – neg	80.3
IL-1β – TCM	81.5
IL-1β - TCM	80.2
IL-6 – neg	80.2
IL-6 – TCM	80.3
IL-6 – TCM	80.5

Name	Melt temp °
IL-8 neg	83.0
IL-8 – TCM	82.7
IL-8 - TCM	82.5
TNF – neg	-
TNF – TCM	-
TNF – TCM	-
TF – neg	-
TF – TCM	79.7
TF – TCM	-
RNA Pol-neg	-
RNA Pol-TCM	-
RNA Pol-TCM	-

Table 3.30. Summary of Gene Expression Profile Data (TCM cord 6)

Gene	Normalised ICN	log(2) ICN
ESEL-TCM	2.83562E-09	-28.3937
ICAM-TCM	1.53782E-07	-22.6326
IL1B-TCM	4.02199E-05	-14.6017
IL6-TCM	3.95697E-06	-17.9472
IL8-TCM	6.82987E-15	-47.0571
RNAPol-TCM	0.000116602	-13.0661
TF-TCM	1.77965E-17	-55.6412
TNF-TCM	8.95688E-10	-30.0563

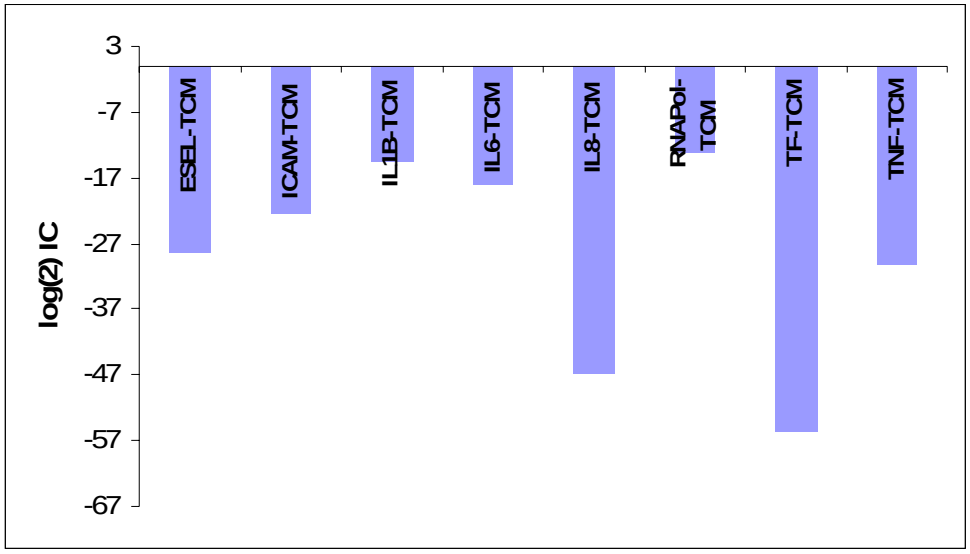


Figure 3.22. Gene Expression Profile (TCM cord 6)

The internal control standard (housekeeping gene) used in this Real Time PCR was Human Proteosome subunit Y (HPSY). The Ct values for each gene were recorded in Table 3.28. It was observed that Ct results were recorded for negative controls of ESEL, IL8 and IL6, this indicates possible contamination, from observing the melt data in Table 3.29, specific products were detected cofirming reaction contamination for all 3 of these genes.

The gene expression profile was produced by normalizing the genes to that of the primary housekeeping gene HPSY. It was clearly visible in Figure 3.22 that the response of endothelial cells when stimulated with tumour conditioned media (TCM) significantly effected the expression of all genes. TF gene expression was down regulated the most. The results from this perfusion experiment provided evidence of how effective TCM is on gene regulation in HUVEC. The gene expression of the contaminated genes ESEL, IL6 and IL8 are inconclusive due to contamination.

3.8.2. Tumour conditioned Media Perfusion + DMXAA for cord 6

cDNA sample from Tumour conditioned media + DMXAA perfused segment was analysed for expression of specific genes.

Quantitaion data for SYBR Green for cord 6

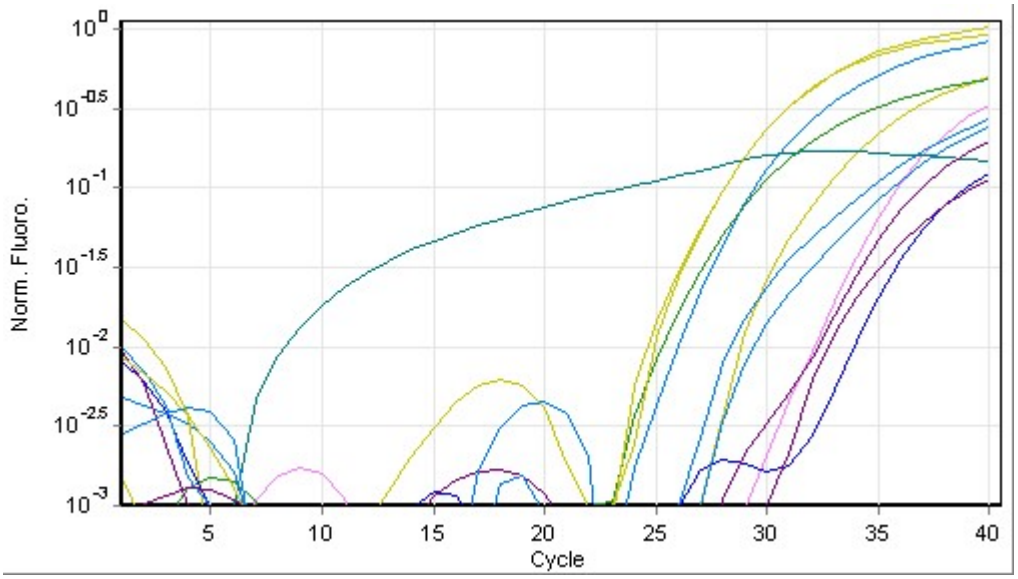


Figure 3.23. Quantitation data for SYBR Green (TCM+DMXAA cord 6)

Table 3.31. Summary of Genes and Threshold cycles (TCM+DMXAA cord 6)

Name	Ct
HPSY - neg	-
HPSY – TCM+DMXAA	-
HPSY- TCM+DMXAA	-
E-Sel - neg	27.93
E-Sel – TCM+DMXAA	23.55
E-Sel – TCM+DMXAA	24.10
ICAM - neg	-
ICAM – TCM+DMXAA	32.22
ICAM – TCM+DMXAA	-
IL-1β – neg	-
IL-1β – TCM+DMXAA	31.28
IL-1β - TCM+DMXAA	29.87
IL-6 – neg	30.53
IL-6 – TCM+DMXAA	-
IL-6 – TCM+DMXAA	-

Name	Ct
IL-8 neg	24.64
IL-8 – TCM+DMXAA	27.1
IL-8 - TCM+DMXAA	27.95
TNF – neg	-
TNF – TCM+DMXAA	6.61
TNF – TCM+DMXAA	-
TF – neg	-
TF – TCM+DMXAA	-
TF – TCM+DMXAA	-
RNA Pol-neg	23.84
RNA Pol- TCM+DMXAA	-
RNA Pol- TCM+DMXAA	-

Melt data for cord 6

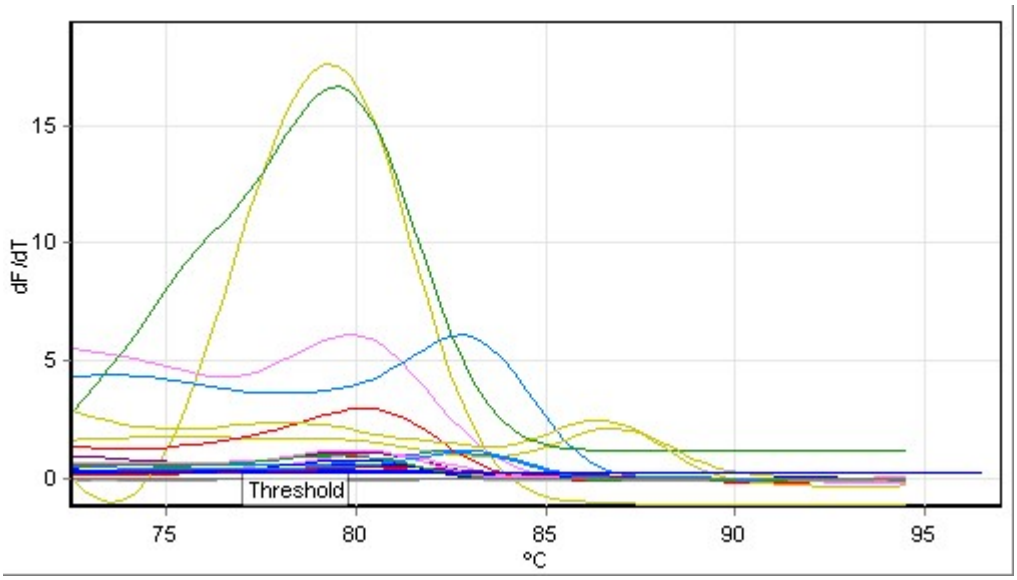


Figure 3.24. Melt data (TCM+DMXAA cord 6)

Table 3.32. Summary of Genes and Melt temperatures (TCM+DMXAA cord 6)

Name	Melt Temp °	
HPSY - neg	80.0	
HPSY – TCM+DMXAA	79.3	
HPSY- TCM+DMXAA	80.2	
E-Sel - neg	79.3	
E-Sel – TCM+DMXAA	78.7	86.8
E-Sel – TCM+DMXAA	78.0	86.5
ICAM - neg	80.5	
ICAM – TCM+DMXAA	80.5	
ICAM – TCM+DMXAA	80.0	
IL-1β – neg	-	
IL-1β – TCM+DMXAA	80.8	
IL-1β - TCM+DMXAA	80.0	85.0
IL-6 – neg	79.8	
IL-6 – TCM+DMXAA	80.5	
IL-6 – TCM+DMXAA	80.0	

Name	Melt temp °	
IL-8 neg	82.7	
IL-8 – TCM+DMXAA	82.5	
IL-8 - TCM+DMXAA	82.7	
TNF – neg	-	
TNF – TCM+DMXAA	-	
TNF – TCM+DMXAA	-	
TF – neg	-	
TF – TCM+DMXAA	-	
TF – TCM+DMXAA	-	
RNA Pol-neg	79.5	88.5
RNA Pol- TCM+DMXAA	79.5	
RNA Pol- TCM+DMXAA	79.5	

Table 3.33. Summary of Gene Expression Profile Data (TCM+DMXAA cord 6)

Gene	Normalised ICN	log(2) ICN
ESEL-TCM/DMXAA	128.3869157	7.004354
ICAM-TCM/DMXAA	4.66197E-05	-14.3887
IL1B-TCM/DMXAA	4.065873325	2.023565
IL6-TCM/DMXAA	1436.55199	10.48839
IL8-TCM/DMXAA	8.124346329	3.022252
RNAPol-TCM/DMXAA	84881840.91	26.33895
TF-TCM/DMXAA	2.231986514	1.158328
TNF-TCM/DMXAA	4.38523E+11	38.67386

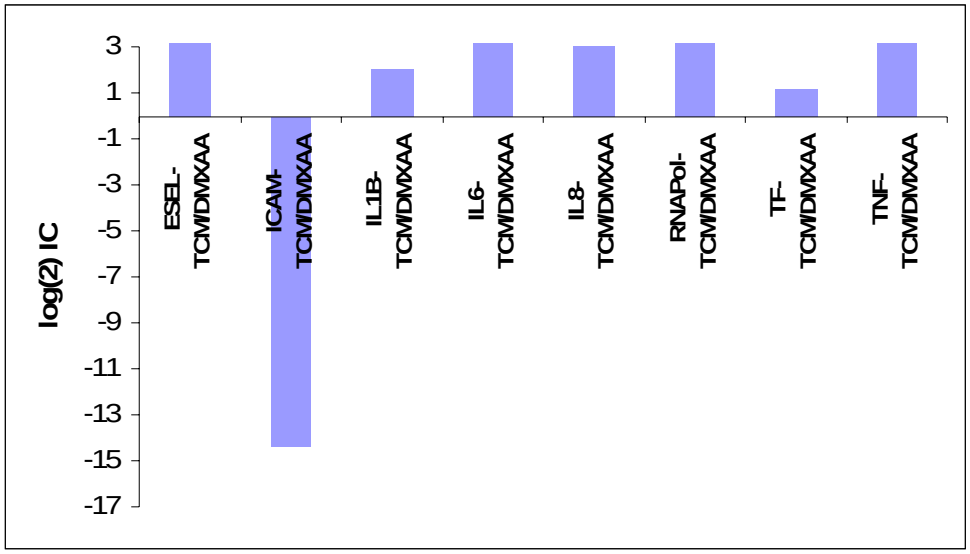


Figure 3.25. Gene Expression Profile (TCM+DMXAA cord 6)

The internal control standard (housekeeping gene) used in this Real Time PCR was RNA Polymerase II, this was the secondary housekeeping gene. The primary housekeeping gene Human Proteosome subunit Y was an unreliable internal control because there was no recorded Ct value for any of the samples.

The impact of DMXAA is identified in the gene expression profile displayed in Figure 3.25. The TNF-α gene observed up regulated 8-fold. ICAM is the only gene that displayed down regulation. The results indicate, DMXAA is suppressing the effective action of TCM, and initiating genes to be expressed and up regulated. This experiment with samples from TCM perfusion and DMXAA perfusion provided an ideal within cord comparison limiting the factors influencing results (ie. heterogeneity between cords).

TNF-α in particular is known to be induced by DMXAA and this is replicated in the results from Figure 3.25. However, the recorded Ct values for the controls of IL6 and IL8 and ESEL seen in Table 3.31 indicated contamination which had ultimately produced misleading results of gene expression for each of these genes shown in the gene expression profile (Figure 3.25.)

3.9. DMXAA & TCM Responses for cord 7

Table 3.34. Summary of umbilical cord 7 information. Segment weights, lengths, perfusate solutions used and time period

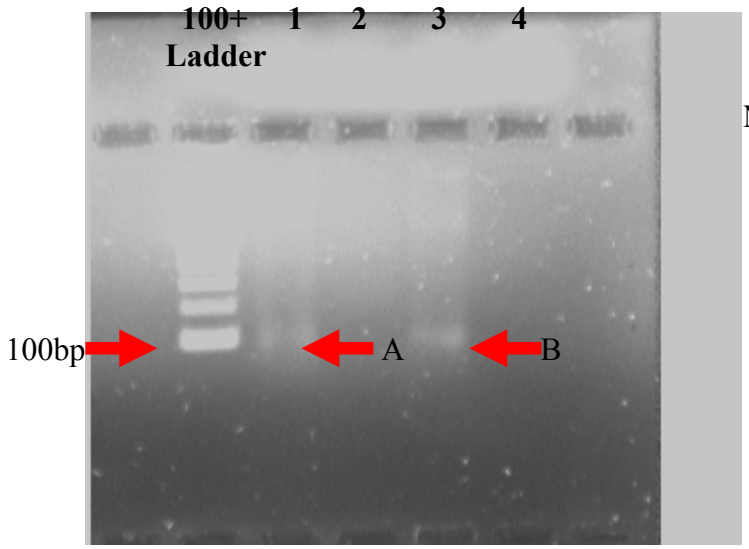
Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS	4 hours	50	9.90
2	DMXAA (125µm)	4 hours	45	16.6
3	DMXAA (250µM)	4 hours	50	10.28
4	TCM / DMXAA (250µM)	2 hours / 4 hours	65	16.20

Table 3.35. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 7)

Experiment					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
control	741.65	18.541	0.27	1.21	40
DMXAA (125µM)	2104.6	52.616	0.53	1.34	40
DMXAA (250µM)	1126.6	28.167	0.33	1.28	40
TCM + DMXAA (250µM)	484.72	12.188	0.87	1.52	40

Table 3.36. Nanodrop Spectrometer Data 2: RNA quality check for each cord segment (cord 7)

Experiment - Nanodrop					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
control	559.36	13.984	1.41	1.74	40
DMXAA (125µM)	419.79	12.245	0.92	1.66	40
DMXAA (250µM)	219.84	5.496	0.37	1.53	40
TCM + DMXAA (250µM)	548.23	13.706	1.27	1.77	40



Lane Number	Description	Size (bp)
100bp+	Ladder	-
1	PBS	-
2	DMXAA 125µM	-
3	DMXAA 250µM	-
4	TCM + DMXAA (250µM)	-

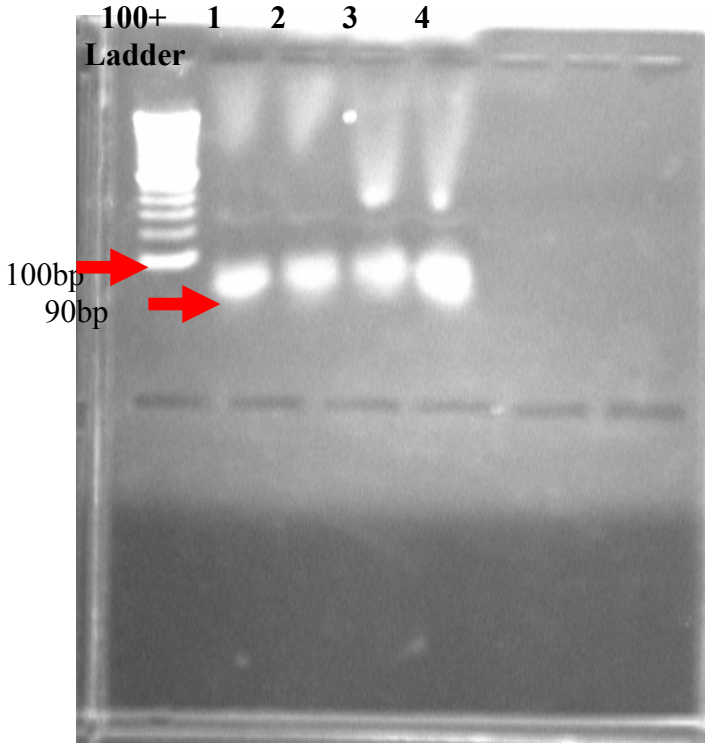
Figure 3.26. RNA Gel Electrophoresis (cord 7)
The agarose gel was loaded with 4µl 100bp+ molecular weight standard Ladder, Samples (2µl) were loaded into lanes 1-4.

The RNA isolated from each cord segment was assessed on an agarose gel to check the quality of extracted RNA. In Figure 3.26. only 1 band can be visualized in lane 1 and lane 3. There are no sharp 18s or 28s bands so the RNA was then checked with the Nanodrop spectrometer. The results in Table 3.35 indicate a low 260/280 ratio, with results for PBS, and both DMXAA perfusions < 1.50, so the RNA isolation procedure was repeated. The final RNA quality results are shown in Table 3.36, the 260/280 ratio value of all 4 segments were > 1.5, this result was suitable to produce cDNA. The cDNA samples were run on an agarose gel for 30 mins (Figure 3.31) with housekeeping gene RNA Pol II. There were 2 bands seen for TCM+DMXAA only; however, there was no band identified for the housekeeping gene. This experimental procedure was inconclusive.

3.10. DMXAA & TCM Responses for cord 8

Table 3.37. Summary of umbilical cord 8 information. Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	105	15.67
2	DMXAA (250µM)	2 hours	115	12.92
3	TCM	2 hours	80	9.26



Lane Number	Description	Size (bp)
100bp+	Ladder	-
1	β Actin	90
2	PBS(β-actin)	90
3	TCM(β-actin)	90
4	DMXAA(β-actin)	90

Figure 3.27. cDNA Gel Electrophoresis (cord 8)
The agarose gel was loaded with 8µl 100bp+ molecular weight standard ladder, then 8µl of housekeeping gene β -actin in lane 1. Samples (8µl) were loaded into lanes 2-4.

The gene expression was measured in response to DMXAA and TCM.

The RNA was isolated from each sample successfully and used to produce cDNA. As described in section 2.9. The quality of cDNA was confirmed on an agarose gel performed with housekeeping gene β-actin as seen in Figure 3.27.

The housekeeping gene β-actin was positively detected at 90bp. A single band was detected at approximately 90bp for each sample, indicating the cDNA quality was suitable to measure gene expression using Real Time PCR.

3.10.1 PBS (control) Perfusion for cord 8

cDNA sample from PBS perfused segment was analysed for expression of specific genes. Primers used are previously described in Table 2.10.

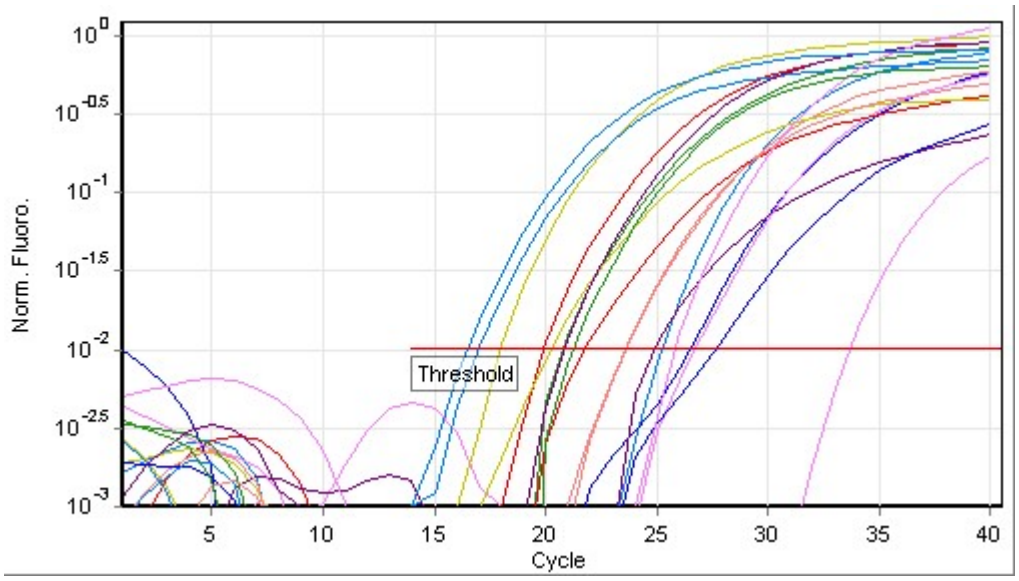


Figure 3.28. Quantitation data for SYBR Green (PBS cord 8)

Table 3.38. Summary of Genes and Threshold cycle Ct (PBS cord 8)

Name	Ct
HPSY - neg	NTC
HPSY –PBS	19.91
HPSY- PBS	21.77
E-Sel - neg	NTC
E-Sel – PBS	17.93
E-Sel – PBS	20.26
ICAM – neg	NTC
ICAM – PBS	26.50
ICAM – PBS	27.68
IL-1β – neg	NTC
IL-1β – PBS	24.88
IL-1β - PBS	20.85
IL-6 - neg	33.64
IL-6 -PBS	26.73
IL-6 -PBS	25.76

Name	Ct
IL-8 - neg	25.22
IL-8 – PBS	16.95
IL-8 – PBS	16.44
TNF – neg	-
TNF – PBS	-
TNF – PBS	-
TF – neg	-
TF – PBS	23.85
TF - PBS	23.54
RNA Pol- neg	-
RNA Pol-PBS	20.77
RNA Pol-PBS	21.26

*NTC=No template Control

Melt data for cord 8

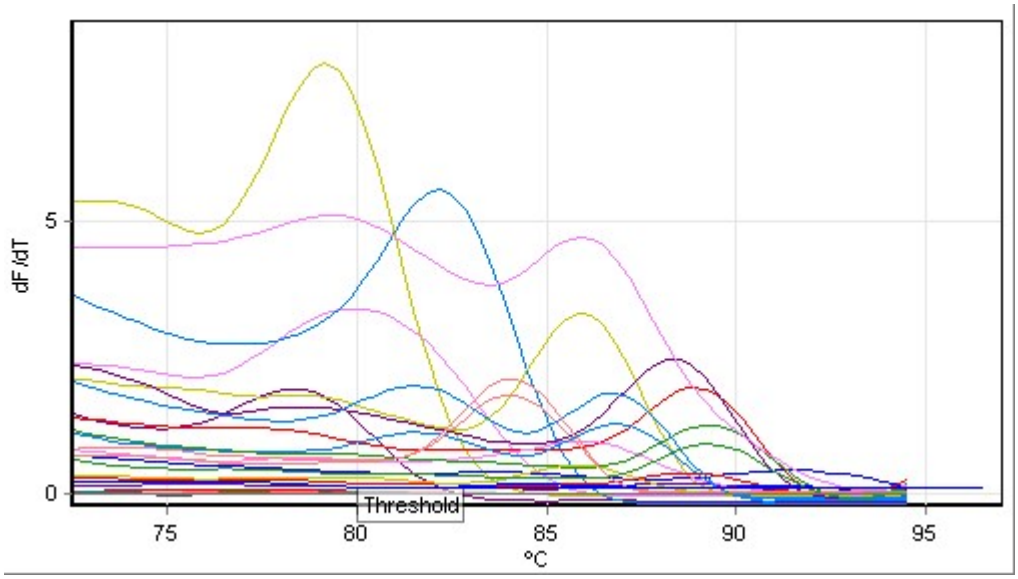


Figure 3.29. Melt data (PBS cord 8)

Table 3.39. Summary of Genes and Melt Temperatures (PBS cord 8)

Name	Melt temp°	
HPSY - neg	-	
HPSY –PBS	88.8	
HPSY- PBS	88.7	
E-Sel - neg	-	
E-Sel – PBS	86.0	
E-Sel – PBS	85.8	
ICAM – neg	-	
ICAM – PBS	83.5	91.5
ICAM – PBS	84.5	91.2
IL-1β – neg	-	
IL-1β – PBS	88.5	
IL-1β – PBS	88.3	
IL-6 - neg	-	
IL-6 –PBS	86.0	
IL-6- PBS	86.0	

Name	Melt temp°	
IL-8 - neg	82.2	
IL-8 – PBS	81.5	81.5
IL-8 – PBS	81.5	81.5
TNF – neg	-	
TNF – PBS	-	
TNF – PBS	-	
TF– neg	-	
TF – PBS	84.0-	
TF - PBS	84.0-	
RNA Pol- neg	-	
RNA Pol-PBS	89.3	
RNA Pol-PBS	81.0	89.3

Table 3.40. Summary of Gene Expression Profile Data (PBS cord 8)

Gene	Normalised ICN	log(2) ICN
ESEL-PBS	1.57524985	0.655581
ICAM-PBS	0.209713042	-2.25351
IL1B-PBS	2.962296254	1.566716
IL6-PBS	0.018156277	-5.78339
IL8-PBS	0.663015378	-0.59289
RNAPol-PBS	0.186369385	-2.42376
TF-PBS	0.183677127	-2.44476
TNF-PBS	0	0

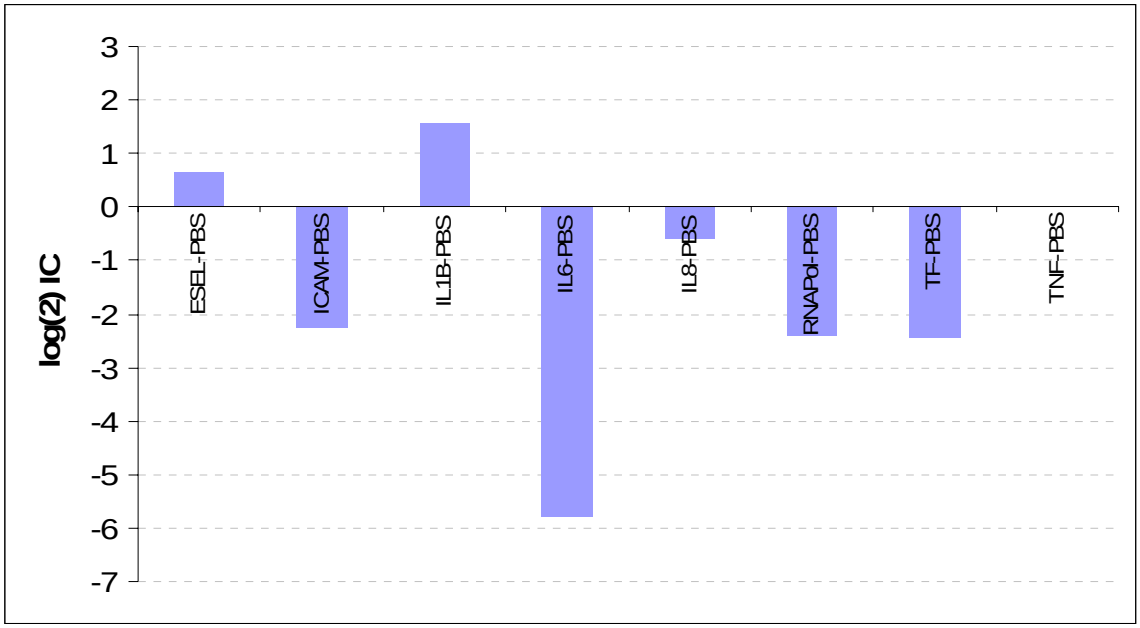


Figure 3.30 Gene Expression Profile (PBS cord 8)

The internal control standard (housekeeping gene) used for this Real Time PCR was Human Proteosome subunit Y. The results for gene expression measured in response to PBS perfusion are represented in Figure 3.30.

The Ct values for each gene was recorded in Table 3.38 and revealed Ct values for negative controls for both IL6 and IL8, this is possible DNA contamination. From observing the melt temperatures values recorded in Table 3.39, IL6 had no recorded melt temperature for the negative but IL8 recorded an ideal melt temperature. This indicated the specific product was being detected and contamination was confirmed. The gene expression analysis for IL8 was unreliable.

IL1β was expressed the most with a 3-fold up regulation. ESEL expression was up regulated 1-fold. TNF-α gene expression is not detected, providing evidence it is not induced in the resting endothelial cells of this cord.

3.10.2 Tumour Conditioned Media Perfusion for cord 8

cDNA sample from Tumour conditioned media perfused segment was analysed for specific gene expression.

Quantitation data for SYBR Green for cord 8

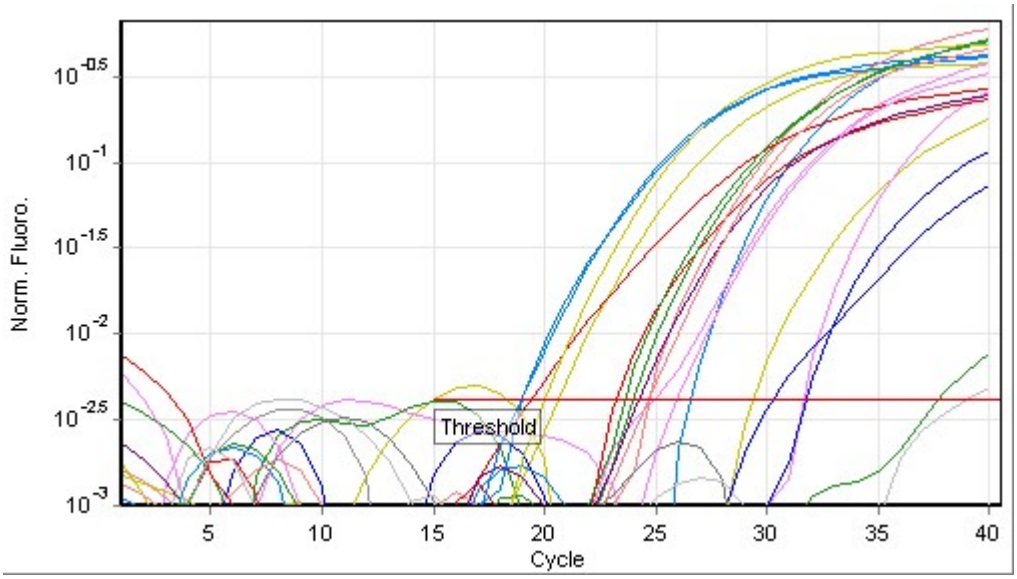


Figure 3.31. Quantitation data for SYBR Green (TCM cord 8)

Table 3.41. Summary of Genes and Threshold cycle Ct (TCM cord 8)

Name	Ct
HPSY - neg	-
HPSY –TCM	23.16
HPSY- TCM	19.41
E-Sel - neg	-
E-Sel – TCM	20.63
E-Sel – TCM	20.01
ICAM – neg	-
ICAM – TCM	31.70
ICAM – TCM	30.29
IL-1β – neg	-
IL-1β – TCM	-
IL-1β – TCM	24.20

Name	Ct
IL-6 - neg	31.52
IL-6 –TCM	24.96
IL-6- TCM	25.92
IL-8 - neg	26.28
IL-8 – TCM	18.98
IL-8 – TCM	18.95
TNF – neg	-
TNF – TCM	-
TNF – TCM	-
TF– neg	-
TF – TCM	24.60
TF – TCM	24.66
RNA Pol- neg	37.72
RNA Pol-TCM	23.93
RNA Pol-TCM	23.53

Melt data for cord 8

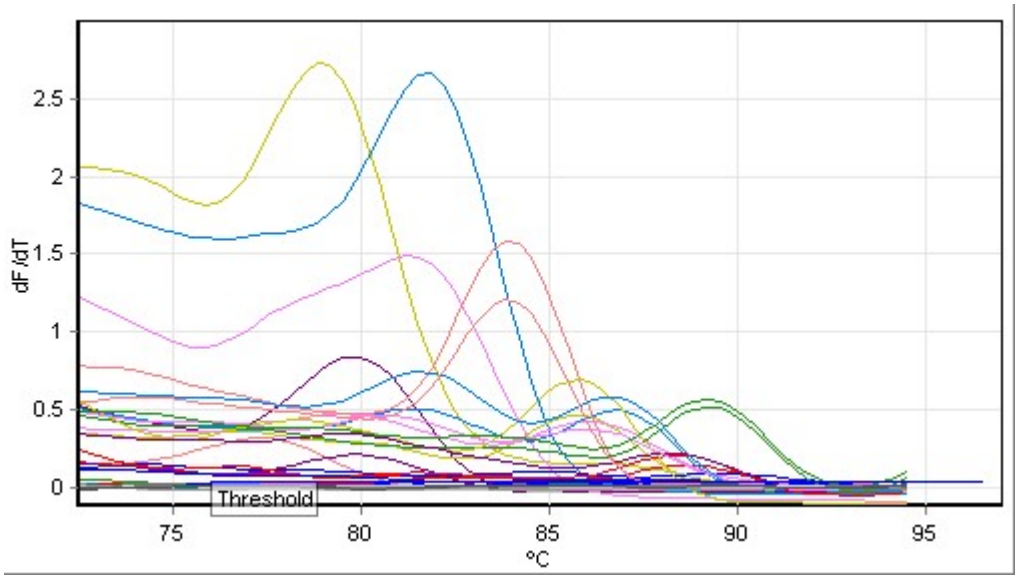


Figure 3.32. Melt data (TCM cord 8)

Table 3.42. Summary of Genes and Melt Temperatures (TCM cord 8)

Name	Melt temp °	
HPSY - neg	NTC	
HPSY –TCM	88.7	
HPSY- TCM	76.5	
E-Sel - neg	79.0	86.3
E-Sel – TCM	76.7	85.7
E-Sel – TCM	78.3	85.7
ICAM – neg	79.2	
ICAM – TCM	84.7	89.5
ICAM – TCM	77.8	85.8
IL-1β – neg	79.8	
IL-1β – TCM	79.8	
IL-1β – TCM	79.5	88.2

*NTC=No template Control

Name	Melt temp°		
IL-6 - neg	81.3		
IL-6 –TCM	79.5	86.0	
IL-6- TCM	80.5	86.0	
IL-8 - neg	81.7		
IL-8 – TCM	81.3	86.7	
IL-8 – TCM	81.5	86.5	
TNF – neg	-		
TNF – TCM	-		
TNF – TCM	-		
TF– neg	77.3		
TF – TCM	84.0		
TF – TCM	83.8	91.5	
RNA Pol- neg	-		
RNA Pol-TCM	76.2	83.5	89.2
RNA Pol-TCM	83.0	89.2	

Table 3.43. Summary of Gene Expression Profile data (TCM cord 8)

Gene	Normalised ICN	log(2) ICN
ESEL-TCM	0.000914758	-10.0943
ICAM-TCM	2.65858E-06	-18.5209
IL1B-TCM	7.162358061	2.840435
IL6-TCM	0.000495031	-10.9802
IL8-TCM	0.008274252	-6.91716
RNAPol-TCM	0.010767565	-6.53716
TF-TCM	0.009778199	-6.67622
TNF-TCM	0	0

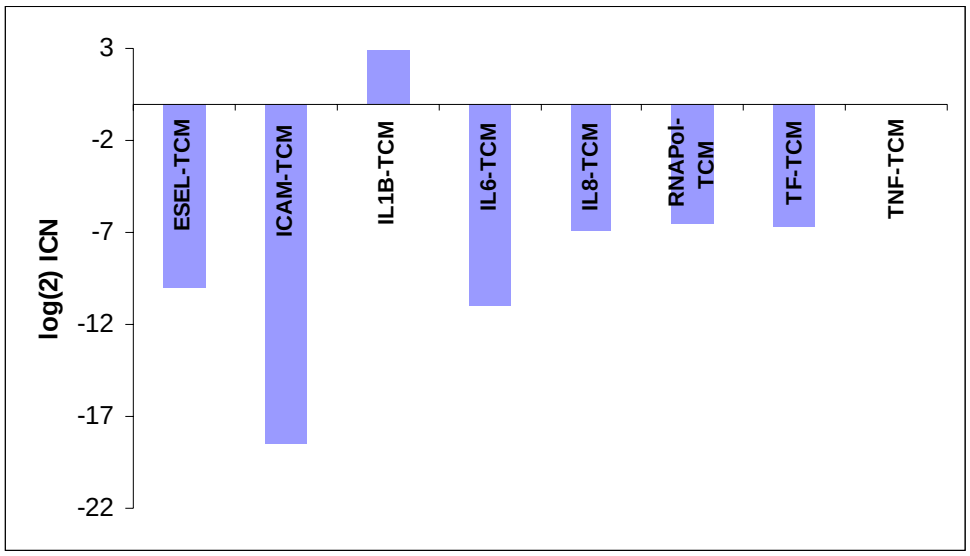


Figure 3.33. Gene Expression Profile (TCM cord 8)

The internal control standard (housekeeping gene) used for this Real Time PCR.

The gene expression profile results of TCM perfusion are shown in Figure 3.33. There is evidence of an 8-fold up regulation of IL1B but the overall trend identifies the decreasing effect tumour conditioned media has on gene regulation. ESEL, ICAM and TF, cytokines expressed in resting endothelial cells have all been significantly down regulated.

The gene expression of TNF-α has been suppressed when HUVEC was stimulated with tumour conditioned media.

Interleukin-6 (IL6), IL8 and RNA Pol showed reaction contamination and were excluded from gene expression analysis.

3.10.3. DMXAA Perfusion for cord 8

cDNA sample from DMXAA perfused segment was analysed for expression of specific genes.

Quantitation data for SYBR Green for cord 8

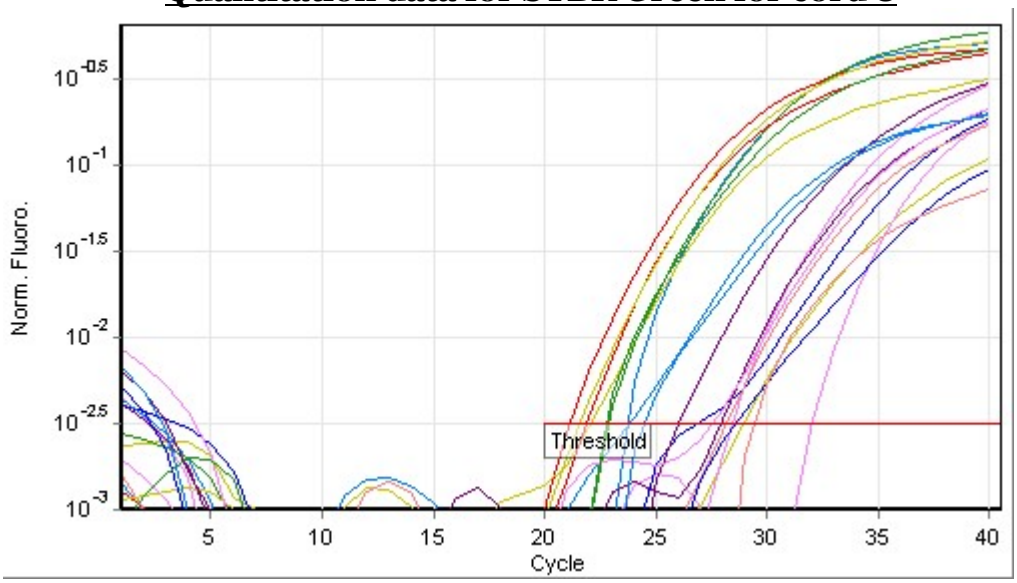


Figure 3.34. Quantitation data for SYBR Green (DMXAA cord 8)

Table 3.44. Summary of Genes and Threshold cycles Ct (DMXAA cord 8)

Name	Ct
HPSY - neg	-
HPSY- DMXAA	21.13
HPSY- DMXAA	21.90
E-Sel - neg	28.93
E-Sel – DMXAA	22.08
E-Sel – DMXAA	21.59
ICAM – neg	-
ICAM -DMXAA	26.76
ICAM–DMXAA	28.60
IL-1β – neg	
IL-1β –DMXAA	25.99
IL-1β –DMXAA	27.92

Name	Ct
IL-6 - neg	31.94
IL-6 - DMXAA	28.41
IL-6 - DMXAA	27.57
IL-8 - neg	23.58
IL-8 – DMXAA	23.78
IL-8 – DMXAA	
TNF – neg	-
TNF – DMXAA	-
TNF – DMXAA	-
TF– neg	-
TF – DMXAA	28.16
TF – DMXAA	29.38
RNA Pol- neg	-
RNA Pol-DMXAA	22.78
RNA Pol-DMXAA	22.62

Melt Data for cord 8

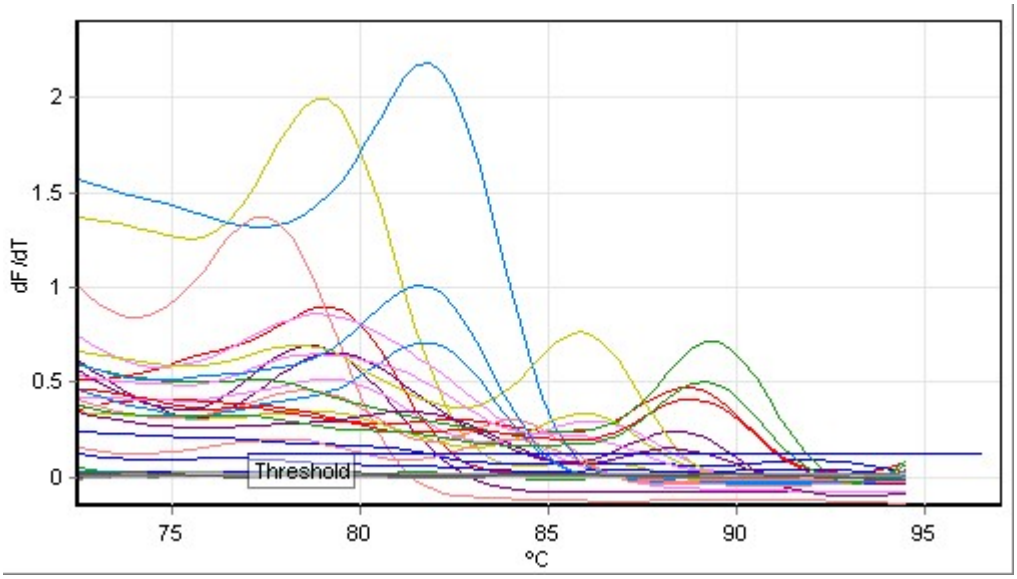


Figure 3.35. Melt data (DMXAA cord 8)

Table 3.45. Summary of Genes and Melt Temperatures (DMXAA cord 8)

Name	Melt temp°	
HPSY - neg	79.0	92.5
HPSY- DMXAA	82.5	88.7
HPSY- DMXAA	82.7	88.8
E-Sel - neg	79.0	
E-Sel – DMXAA	78.7	86.0
E-Sel – DMXAA	78.5	85.8
ICAM – neg	-	
ICAM -DMXAA	-	
ICAM–DMXAA	-	
IL-1β – neg	78.7	
IL-1β –DMXAA	79.3	88.5
IL-1β –DMXAA	81.3	88.3

Name	Melt temp°	
IL-6 - neg	79.0	
IL-6 - DMXAA	79.3	86.0
IL-6 - DMXAA	78.8	
IL-8 - neg	81.7	
IL-8 – DMXAA	81.7	
IL-8 – DMXAA	81.5	
TNF – neg	-	
TNF – DMXAA	-	
TNF – DMXAA	-	
TF– neg	77.5	
TF – DMXAA	78.5	84.0
TF – DMXAA	78.0	84.0
RNA Pol- neg	-	
RNA Pol-DMXAA	77.2	89.3
RNA Pol-DMXAA	89.0	

The internal control standard (housekeeping gene) used in this Real Time PCR was Human Proteosome subunit Y.

The Ct values for each gene are represented in Table 3.44 and showed adequate amounts of cDNA; however, for IL8 and ESEL control genes, a Ct value was recorded, this indicates contamination. This was also confirmed from observing the melt temperature results recorded in Table 3.35. IL8 produced a melt temperature matching the melt temperature of the 2 replicate samples. This indicated the correct product had been detected, confirming contamination. Gene expression analysis was inconclusive from these results.

3.11. DMXAA & TCM Responses for cord 9

Table 3.46. Summary of umbilical cord 9 information: Segment weights, lengths, perfusate solutions used and time period

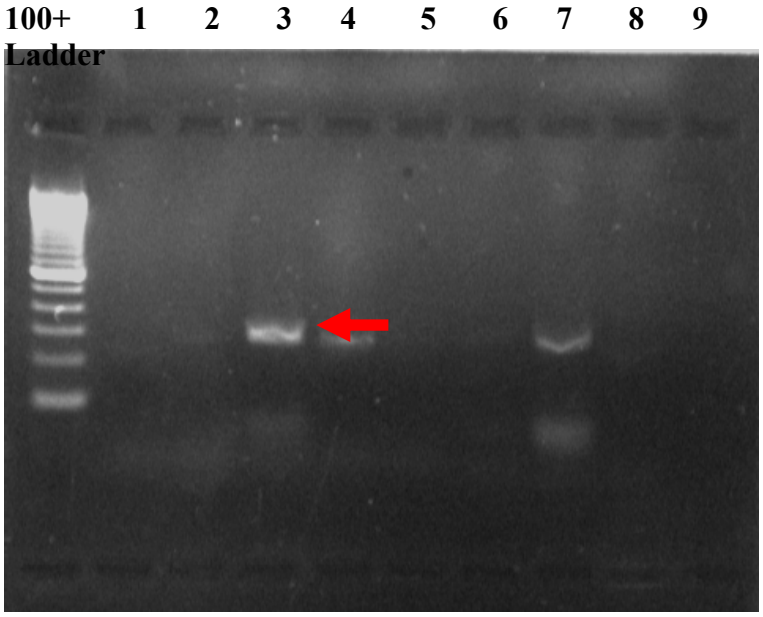
Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	50	16.057
2	DMXAA (250µM)	4 hours	45	9.943
3	TCM / DMXAA (250µM)	2 hours / 4 hours	30	10.997

Table 3.47. Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 9)

Experiment					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
control	385.87	9.647	0.66	1.43	40
DMXAA (250µM)	273.05	6.826	0.44	1.37	40
TCM + DMXAA (250µM)	264.66	6.617	0.44	1.18	40

Gene expression was measured in response to DMXAA and TCM+DMXAA.

RNA was successfully isolated from all samples and purity assessed on the nanodrop spectrometer. The cord segment perfused with PBS (control) was shown to give the highest 260/280 ratio value of 1.43 seen in Table 3.47. indicating the purity of RNA extracted. However, the results from all 3 perfused segments were very low



Lane Number	Description	Size (bp)
100bp+	Ladder	-
1	RNA Pol II	-
2	PBS (RNA Pol)	-
3	TCM + DMXAA(RNA Pol)	90
4	DMXAA(RNA Pol)	90
5	-	-
6	PBS(RNA Pol)	-
7	TCM + DMXAA(RNA Pol)	90
8	DMXAA 250µM(RNA Pol)	-
9	DMXAA 125µl(RNA Pol)	-

Figure 3.36. cDNA Gel Electrophoresis (cord 9)
The agarose was loaded with 5µl 100bp+ molecular weight standard ladder and 8µl of housekeeping gene (RNA Pol II) in lane 1. Samples (8µl) were loaded into lanes 2-4. cDNA samples from table 3.34 (cord 7) perfusion experiment were loaded into lanes 6-9.

The RNA samples were used to produce cDNA as outlined in section 2.9. The cDNA samples were then quality assessed on an agarose gel shown in Figure 3.36 with housekeeping gene RNA Pol II. The brightest band in lane 3 was detected representing the cDNA sample for TCM+DMXAA (shown by the arrow) There was no visual band seen for the housekeeping gene which made it difficult to compare samples. The poor result produced from the agarose gel seen in Figure 3.36 prevented Real Time PCR gene expression analysis.

3.12. DMXAA & TCM Responses for cord 10

Table 3.48. Summary of umbilical cord 10 information. Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	100	15.67
2	DMXAA (250µM)	2 hours	98	12.92
3	TCM	2 hours	80	9.26

Table 3.49. Nanodrop spectrometer data: RNA quality check for each cord segment (cord 10)

Experiment					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
control	383.07	9.577	0.59	1.54	40
DMXAA (250µM)	499.2	12.481	0.73	1.70	40
TCM + DMXAA (250µM)	193.39	4.835	0.37	1.36	40

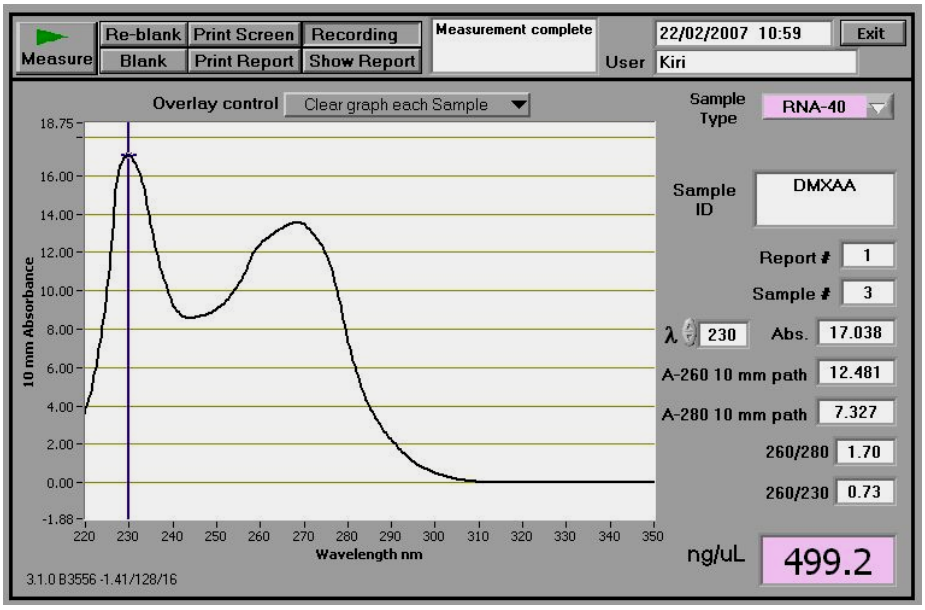
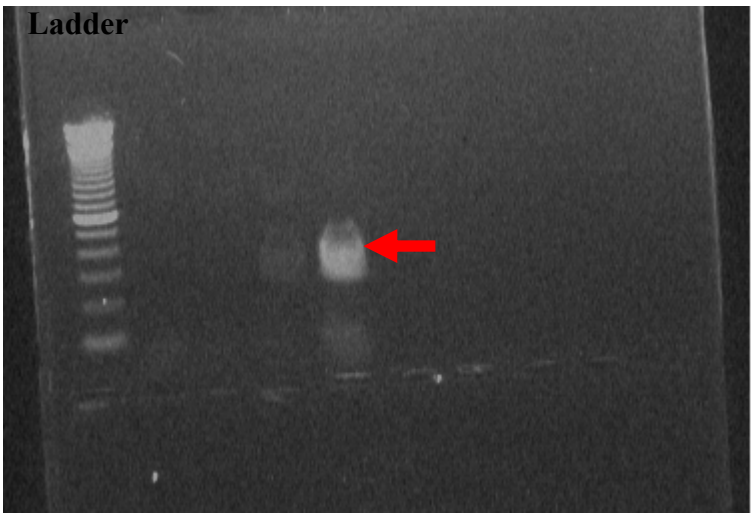


Figure 3.37. Optical Density (OD) Measurements



Lane Number	Description	Size
100bp+	Ladder	-
1	RNA Pol	125
2	PBS(RNA Pol)	-
3	TCM + DMXAA(RNA Pol)	-
4	DMXAA(RNA Pol)	-

Figure 3.38. cDNA Gel Electrophoresis (cord 10)
The agarose gel was loaded with 5µl 100bp+ molecular weight standard ladder.Lane 1 loaded with 8µl of both housekeeping genes (RNA Pol & β Actin). Samples (8µl) were loaded into lanes 2-4.

The isolated RNA from perfused samples in table 3.48 was purity assessed with the Nanodrop spectrometer. RNA from TCM+DMXAA samples produced a low 260/280 ratio value of <1.50. DMXAA gave an ideal reading for RNA purity with 260/280 ratio value of >1.7, supported with 2 clear peaks (18s and 28s) in Figure 3.37. All 3 RNA samples were used to produce cDNA and an agarose gel performed with RNA Pol II as an internal standard control (housekeeping gene). Only a single band was detected for DMXAA sample as indicated by the arrow; however, RNA Pol II was undetected. Only PBS and DMXAA cDNA samples were used for gene analysis in Real Time PCR.

3.12.1. PBS (control) Perfusion for cord 10

cDNA sample from PBS perfused segment was analysed for expression of specific genes. Primers used are previously described in Table 2.10.

Quantitation data for SYBR Green for cord 10

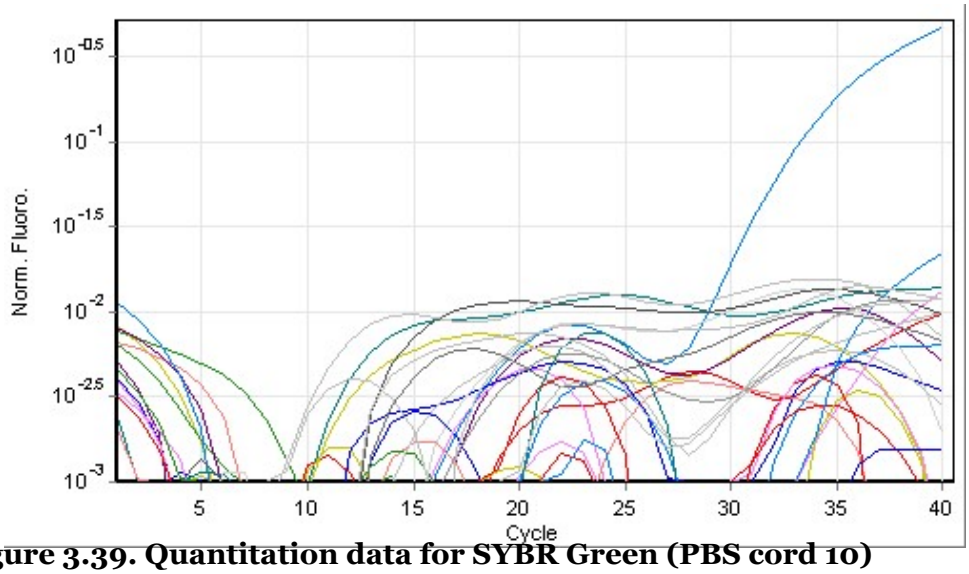


Figure 3.39. Quantitation data for SYBR Green (PBS cord 10)

Table 3.50. Summary o f Genes and Threshold cycle Ct (PBS cord 10)

Name	Ct
HPSY - neg	19.49
HPSY –PBS	-
HPSY- PBS	-
E-Sel - neg	-
E-Sel – PBS	5.02
E-Sel – PBS	-
ICAM – neg	-
ICAM – PBS	3.08
ICAM – PBS	-
IL-1β – neg	17.24
IL-1β – PBS	3.14
IL-1β – PBS	5.56

Name	Ct
IL-6 - neg	-
IL-6 –PBS	-
IL-6- PBS	3.51
IL-8 - neg	16.74
IL-8 – PBS	-
IL-8 – PBS	-
TNF – neg	10.57
TNF – PBS	-
TNF – PBS	-
TF– neg	-
TF – PBS	6.45
TF - PBS	-
RNA Pol- neg	8.65
RNA Pol-PBS	5.13
RNA Pol-PBS	3.46

Melt data for cord 10

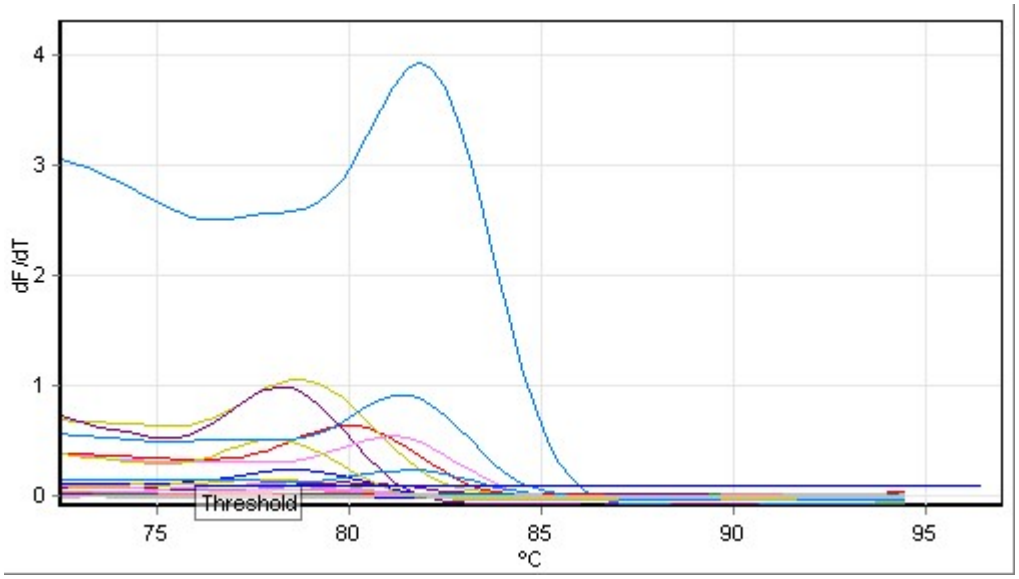


Figure 3.40. Melt data (PBS cord 10)

Table 3.51. Summary of Genes and Temperatures (PBS cord 10)

Name	Melt temp °
HPSY - neg	80.0
HPSY –PBS	-
HPSY- PBS	-
E-Sel - neg	78.7
E-Sel – PBS	78.0
E-Sel – PBS	78.0
ICAM – neg	78.5
ICAM – PBS	-
ICAM – PBS	-
IL-1β – neg	78.2
IL-1β – PBS	80.0
IL-1β - PBS	-

Name	Melt temp°	
IL-6 - neg	76.5	81.0
IL-6 -PBS	-	
IL-6 -PBS	77.8	
IL-8 - neg	81.8	
IL-8 – PBS	76.5	81.5
IL-8 – PBS	77.2	81.5
TNF – neg	-	
TNF – PBS	-	
TNF – PBS	-	
TF – neg	-	
TF – PBS	-	
TF - PBS	-	
RNA Pol- neg	-	
RNA Pol-PBS	-	
RNA Pol-PBS	-	

The cDNA from PBS perfused segments were prepared as described in section 2.92. Low Ct values of < 10 were recorded in Table 3.50. The poor recorded Ct values indicate clear contamination of the reaction.

The melt curve produced displayed signs of DNA contamination and primer dimer. The results of this Real Time PCR are inconclusive preventing any gene expression analysis the PBS (control) sample.

3.12.2. DMXAA Perfusion for cord 10

cDNA sample from DMXAA perfused segment was analysed for expression of specific genes. Primers used are previously described in Table 2.10.

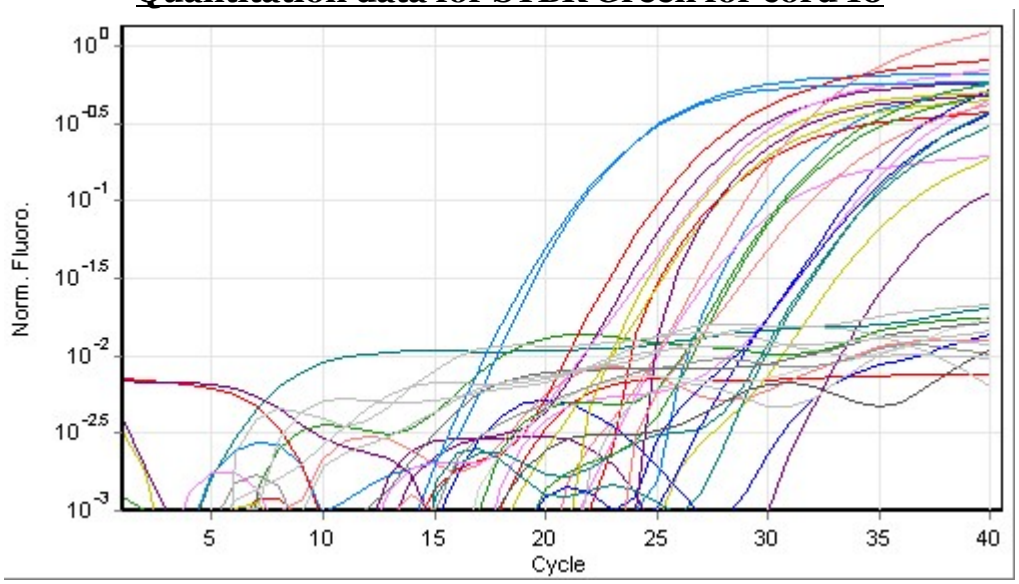


Figure 3.41. Quantitation data for SYBR Green (DMXAA cord 10)

Table 3.52. Summary of Genes and Threshold cycle (DMXAA cord 10)

Name	Ct
HPSY - neg	-
HPSY –DMXAA	-
HPSY- DMXAA	20.44
E-Sel - neg	28.86
E-Sel – DMXAA	22.19
E-Sel – DMXAA	22.41
ICAM – neg	33.06
ICAM -DMXAA	27.29
ICAM- DMXAA	28.13
IL-1β – neg	32.59
IL-1β –DMXAA	21.06
IL-1β –DMXAA	24.62

Name	Ct
IL-6 - neg	26.57
IL-6 –DMXAA	24.37
IL-6- DMXAA	20.86
IL-8 - neg	25.96
IL-8 – DMXAA	17.29
IL-8 – DMXAA	16.71
TNF – neg	8.40
TNF – DMXAA	29.04
TNF – DMXAA	29.04
TF– neg	-
TF – DMXAA	24.19
TF – DMXAA	25.31
RNA Pol-neg	-
RNA Pol-DMXAA	-
RNA Pol-DMXAA	-

Melt data for cord 10

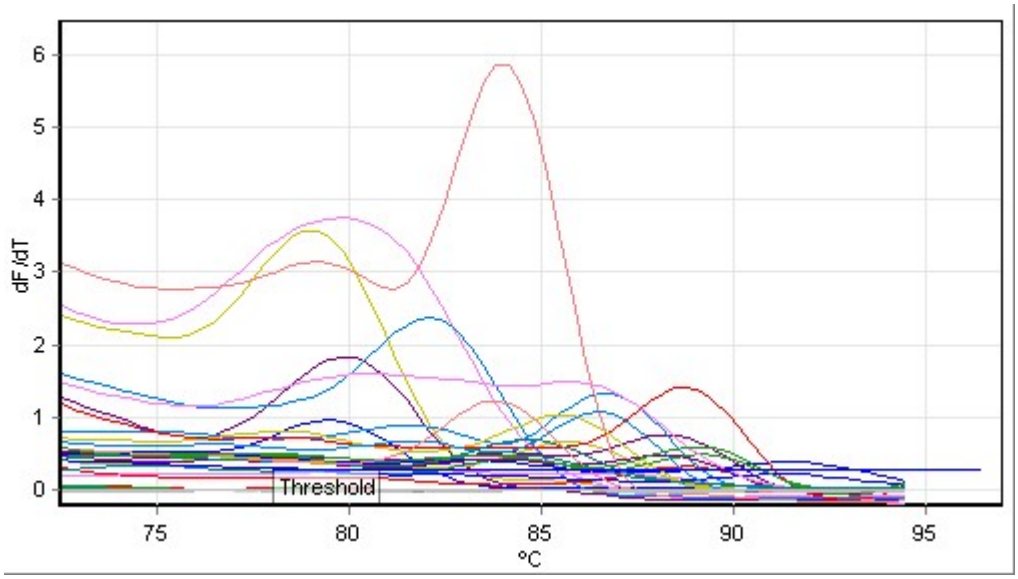


Figure 3.42. Melt data (DMXAA cord 10)

Table 3.53. Summary of Genes and Temperatures (DMXAA cord 10)

Name	Melt temp °		
HPSY - neg	-		
HPSY –DMXAA	88.8		
HPSY- DMXAA	88.7		
E-Sel - neg	79.0		
E-Sel – DMXAA	85.7		
E-Sel – DMXAA	78.2	85.5	
ICAM – neg	79.5		
ICAM -DMXAA	83.0	91.5	
ICAM -DMXAA	-		
IL-1β – neg	80.0		
IL-1β – DMXAA	79.0	84.2	88.3
IL-1β - DMXAA	83.8	88.2	

Name	Melt temp°	
IL-6 - neg	79.8	
IL-6 –DMXAA	-	
IL-6 - DMXAA	80.5	85.7
IL-8 - neg	82.0	
IL-8 – DMXAA	81.5	86.7
IL-8 – DMXAA	83.8	88.2
TNF – neg	-	
TNF – DMXAA	84.5	
TNF – DMXAA	84.5	
TF – neg	-	
TF – DMXAA	79.2	84.0
TF – DMXAA	83.8	
RNA Pol- neg	-	
RNA Pol-DMXAA	83.8	89.2
RNA Pol-DMXAA	83.5	89.0

The internal control gene (housekeeping gene) used in this Real Time PCR was Human Proteome subunit Y (HPsY). The Ct values for each gene were recorded in Table 3.52. and produced suitable Ct values of < 33; however the negative control for each gene was detected indicating contamination of the reaction. There should be no Ct values recorded for any of the negative controls. A gene expression profile could not be produced from this Real Time PCR.

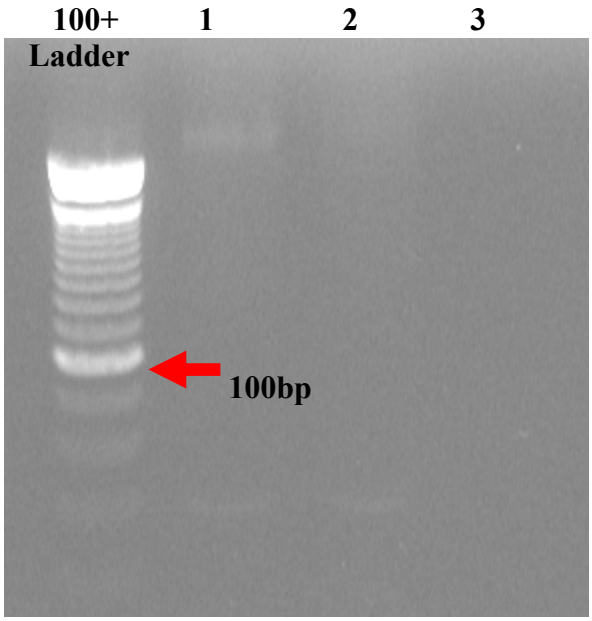
3.13. DMXAA & TCM Responses for cord 11

Table 3.54. Summary of umbilical cord 11 information: Segment weights, lengths, perfusate solutions used and time period

Experiment				
Section	**Perfused**	**Time**	**Length(mm)**	**Weight(g)**
1	PBS (control)	4 hours	100	16.06
2	TCM	2 hours	100	9.26
3	DMXAA (250µg/ml)	4 hours	80	11.78

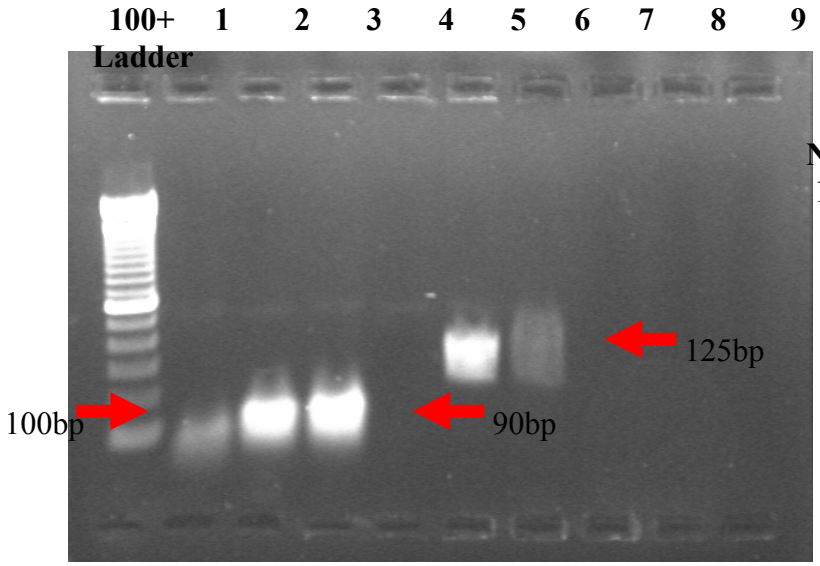
Table 3.55.Nanodrop Spectrometer Data: RNA quality check for each cord segment (cord 11)

Experiment - Nanodrop					
Sample ID	**ng/µL Concentration**	**A260**	**260/230**	**260/280**	**Const.**
PBS (control)	334.44	8.361	0.55	1.51	40
TCM	643.34	16.083	0.28	1.07	40
DMXAA (250µg/ml	370.85	9.271	0.57	1.53	40



Lane Number	Description	Size
100bp+	Ladder	-
1	PBS	-
2	DMXAA	-
3	TCM	-

Figure 3.43. RNA Gel Electrophoresis (cord 11)
The agarose gel was loaded with 4µl 100bp+ molecular weight standard ladder, RNA samples (2µl) were loaded into lanes 1-3.



Lane Number	Description	Size
100bp+	Ladder	-
1	β Actin	90
2	PBS(β actin)	90
3	DMXAA(β actin	90
4	RNA Pol II	125
5	PBS(RNA Pol)	125
6	DMXAA(RNAPol)	125

Figure 3.44. cDNA Gel Electrophoresis (cord 11)
The agarose was loaded with 5µl 100bp+ molecular weight standard ladder The housekeeping gene (β Actin) loaded in lane 1 & 8µl of housekeeping gene (RNA Pol II) in lane 4. The cDNA samples (8µl) were loaded into lanes 2,3,5,6.

Gene expression responses to TCM and DMXAA were measured using Real Time PCR. The RNA isolated from each sample were purity assessed on an agarose gel displayed in Figure 3.43. Each sample was loaded in lanes 1-3. No bands were detected in the gel, this indicated no RNA present or small amounts isolated undetectable on the gel. The RNA was reisolated as described in section 2.7. Re isolated RNA was the assessed using the Nanodrop spectrometer. The 260/280 ratio values for this isolation shown in Table 3.55 revealed low quality RNA. TCM RNA sample recorded the lowest 260/280 ratio value of 1.07. Only PBS and DMXAA samples were used to produce cDNA and quality confirmed on an agarose gel seen in Figure 3.44. Bands were positively identified for each housekeeping gene.

3.13.1. PBS (control) Perfusion for cord 11

cDNA sample from PBS perfused segment was analysed for expression of specific genes. Primers used are previously described in Table 2.10.

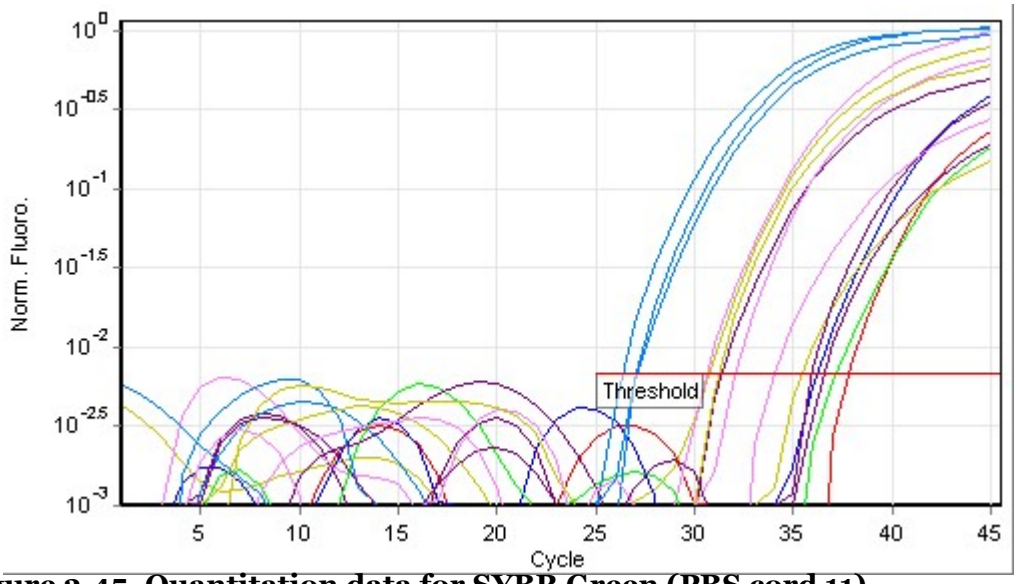


Figure 3.45. Quantitation data for SYBR Green (PBS cord 11)

Table 3.56. Table of Genes and Threshold cycle Ct (PBS cord 11)

Name	Ct
HPSY - neg	37.80
HPSY –PBS	-
HPSY- PBS	-
E-Sel - neg	30.75
E-Sel – PBS	35.46
E-Sel – PBS	31.20
ICAM – neg	36.22
ICAM – PBS	-
ICAM – PBS	-
IL-1β – neg	31.34
IL-1β – PBS	35.93
IL-1β – PBS	36.54

Name	Ct
IL-6 - neg	30.55
IL-6 –PBS	32.02
IL-6- PBS	34.00
IL-8 - neg	26.32
IL-8 – PBS	27.07
IL-8 – PBS	27.13
TNF – neg	-
TNF – PBS	-
TNF – PBS	-
TF– neg	37.18
TF – PBS	-
TF - PBS	-

Melt Data for Melt A.Green

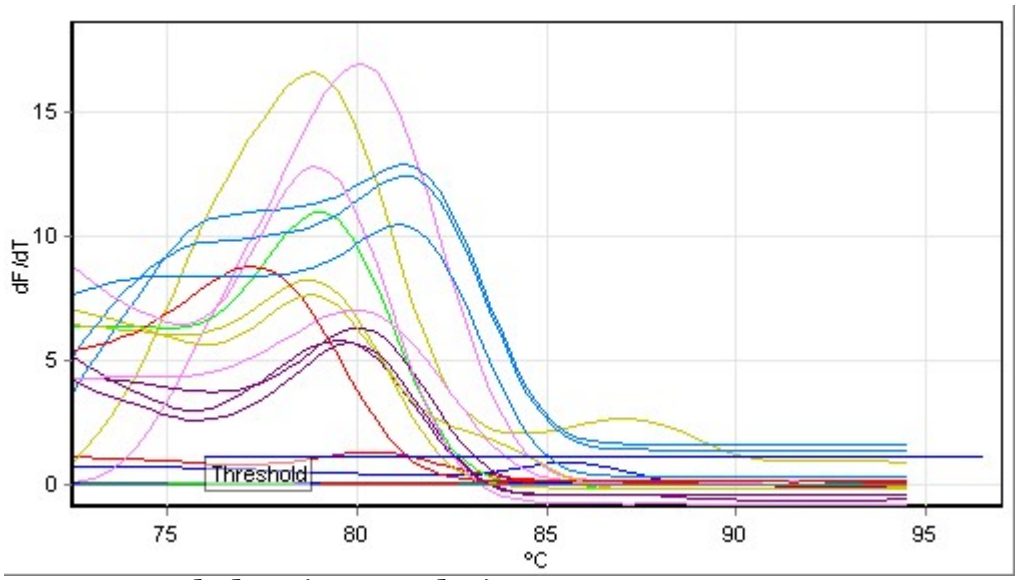


Figure 3.46. Melt data (PBS cord 11)

Table 3.57. Summary of Genes and Temperatures (PBS cord 11)

Name	Melt temp °
HPSY - neg	80.3
HPSY –PBS	77.2
HPSY- PBS	-
E-Sel - neg	78.8
E-Sel – PBS	78.7
E-Sel – PBS	78.8
ICAM – neg	-
ICAM – PBS	-
ICAM – PBS	-
IL-1β – neg	79.5
IL-1β – PBS	80.0
IL-1β – PBS	79.8

Name	Melt temp °
IL-6 - neg	80.0
IL-6 –PBS	80.0
IL-6- PBS	79.0
IL-8 - neg	81.2
IL-8 – PBS	81.2
IL-8 – PBS	81.0
TNF – neg	-
TNF – PBS	-
TNF – PBS	-
TF– neg	79.0
TF – PBS	-
TF - PBS	-

The internal control standard (housekeeping gene) used in this Real Time PCR was Human Proteosome subunit Y (HPSY) there was no secondary housekeeping gene used.. The recorded Ct values were represented in Table 3.56. The negative control for each gene except TNF-α, which was undetected, produced a Ct value which indicates DNA contamination or contamination with the reaction. Observation of the melt data presented in Table 3.57 confirms contamination, ultimately preventing any reliable gene analysis profile to be produced. The results from this Real Time PCR were unsuccessful and inconclusive.

3.13.2. Tumour Conditioned Media Perfusion for cord 11

cDNA sample from Tumour conditioned media perfused segment was analysed for expression of specific genes. Primers used are previously described in table 2.10.

Quantitation data for Cycling B.Green

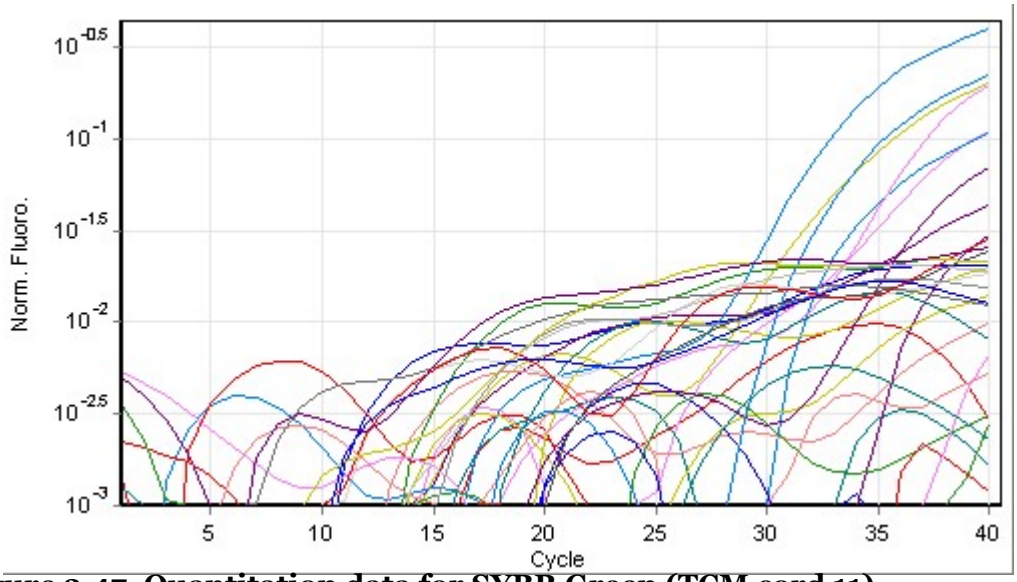


Figure 3.47. Quantitation data for SYBR Green (TCM cord 11)

Table .3.58. Summary of Genes and Threshold cycle Ct (TCM cord 11)

Name	Ct
HPSY - neg	-
HPSY –TCM	-
HPSY- TCM	-
E-Sel - neg	-
E-Sel – TCM	21.19
E-Sel – TCM	33.42
ICAM – neg	13.15
ICAM – TCM	-
ICAM – TCM	-
IL-1β – neg	18.49
IL-1β – TCM	35.52
IL-1β – TCM	32.47

Name	Ct
IL-6 - neg	27.15
IL-6 –TCM	23.83
IL-6- TCM	39.26
IL-8 - neg	30.93
IL-8 – TCM	19.97
IL-8 – TCM	29.48
TNF – neg	-
TNF – TCM	20.41
TNF – TCM	-
TF– neg	-
TF – TCM	39.33
TF - TCM	-
RNA Pol-neg	15.71
RNA Pol-TCM	-
RNA-Pol-TCM	-

Melt data for Cycling A.Green

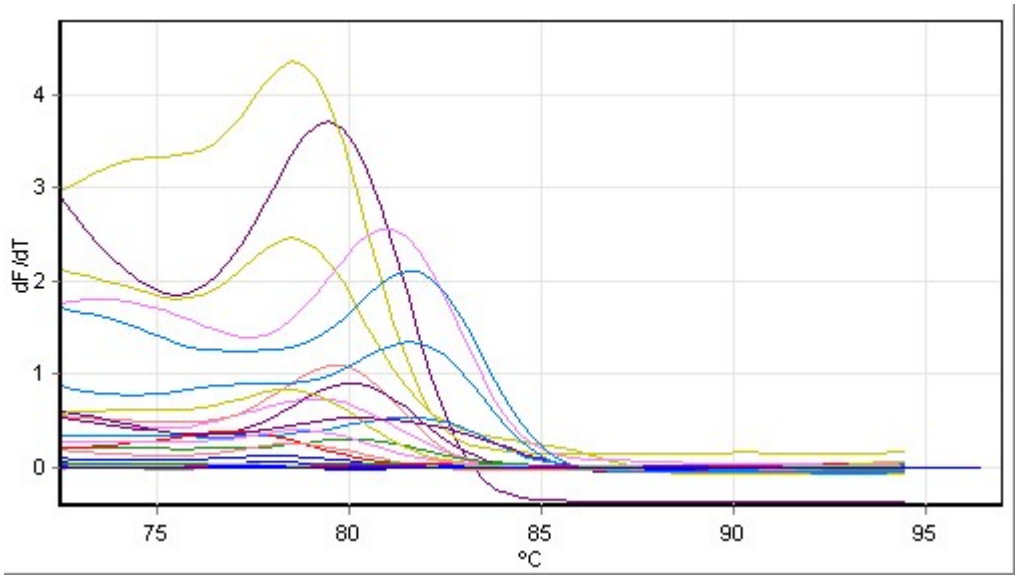


Figure 3.48. Melt data (TCM cord 11)

Table 3.59. Summary of Genes and Melt temperatures (TCM cord 11)

Name	Melt temp °			
HPSY - neg	77.5	82.5	88.0	93.5
HPSY –TCM	78.2	82.0	87.0	
HPSY- TCM	77.0	87.3	91.7	
E-Sel - neg	78.5			
E-Sel – TCM	78.5	90.3		
E-Sel – TCM	78.5	86.5		
ICAM – neg	78.0	84.3	89.0	93.5
ICAM – TCM	78.0	85.0		
ICAM – TCM	76.5	83.0	90.2	
IL-1β – neg	79.5			
IL-1β – TCM	80.0			
IL-1β – TCM	80.2			

Name	Melt temp °			
IL-6 - neg	79.2	87.0	90.0	
IL-6 –TCM	73.5	81.0		
IL-6- TCM	73.8	78.7	88.3	
IL-8 - neg	81.5	90.7		
IL-8 – TCM	81.5			
IL-8 – TCM	81.5			
TNF – neg	76.8	82.5	89.5	
TNF – TCM	75.7	78.0	85.3	91.0
TNF – TCM	73.8	80.0	82.8	87.7
TF– neg	79.7			
TF – TCM	78.0	84.0	91.5	
TF – TCM	78.5			
RNA Pol-TCM	74.0	80.0	87.5	92.2
RNA Pol-TCM	75.0	83.5	89.0	
RNA Pol-TCM	75.5	80.7	88.2	

The internal control standard used in this Real Time PCR was Human Proteosome subunit Y. The Ct value results recorded in Table 3.58 revealed no detection for the primary housekeeping gene HPSY and the secondary housekeeping gene RNA Pol II produced a result for the negative control. The melt data displayed in Table 3.59 showed significant DNA contamination and primer dimer, all gene samples detected more than 1 peak suggesting more than the specific product is being detected. The result of an undetected primary housekeeping gene and contaminated secondary housekeeping gene prevented a reliable gene expression analysis.

Chapter Four

Discussion

4.1. Method Development

The aim of this study was to develop an ex vivo method in order to characterise endothelial cell responses to inflammatory stimuli and DMXAA (presence and absence of tumour conditioned media) using whole human umbilical cords.

4.2. Ex Vivo Perfusion Model

The ex vivo perfusion model was compatible with each cord sample collected. The adjustable features were able to accommodate for different cord lengths and size. The advantage of using this model when exposing the cells to various stimuli was that the endothelial cells remained in their normal vascular anatomical relationship to other tissues while maintaining cell viability. Another advantage was the ability to perform within cord comparisons running three separate chambers together along with the continuous intravenous infusion of solutions which minimised handling contamination. This was an important factor because heterogeneity between

endothelial cells from different cords is evident due to differences in the history of the pregnancy which can make the critical interpretation of results difficult (Hughes S.E, 1996).

The antivascular effects of DMXAA seem to be mediated through direct and indirect effects on tumour vascular endothelial cells in vivo (Ching yet al, 2002). Replicating an *in vivo* environment with the *ex vivo* perfusion model allowed results to bear significant relevance to the antivascular activity of DMXAA in humans.

4.3. MTT Viability Assay

There are a number of methods that have been developed to study endothelial cell viability in cell populations. The modern assays have been developed in a microplate format (96-well plate) which allows many samples to be analysed rapidly and simultaneously.

Trypan Blue also called 'Dye Exclusion Method' is another cell viability assay commonly used; this assay measures the number of dead cells in a sample. Trypan blue is a vital stain that selectively colours dead tissue and cells blue which is then quantitated under a microscope. Because cells are selective in compounds that pass through the membrane, viable cells do not absorb trypan blue, so the dead cells are measured exclusively. The limitation of this technique is high estimates of viable cells present in a sample (Cell isolation techniques, 2004).

The viability assay chosen for this research had to be rapid and convenient for it to work well with the perfusion chamber set up.

Tetrazolium Salt MTT (3, (4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) colourmetric viability assay was the preferred method. The succinate - tetrazolium reductase system which belongs to a cells mitochondria and only active in viable cells, cleaves MTT to form non-water soluble violet formazan crystals (Altman, 1979).

The purple formazan formed gives an estimate of the number of mitochondria present hence the number of living cells. This dye solution was measured using a spectrophotometer with a wavelength of 570nm. The OD Absorbance reading obtained was proportional to the amount of converted dye.

This technique was suitable as it required no washing or harvesting of cells.

Increased OD = Increased Active Cells

In this research eight cords were collected and measured for cell viability. Only seven of these umbilical cords were perfused successfully. Each individual cord was divided into two segments to allow within cord comparison and perfused for various time periods to give an indication of a time frame in which the HUVEC would still be at a viable state (stationary phase).

Each cord was perfused for the indicated time period in Table 3.1. The MTT solution was then added and perfused for an additional 2 hours. The cords length was recorded and the OD absorbance of the resultant solution was divided by this figure to help give a true and comparable result.

The graph seen in Figure 3.4. represents the OD absorbance measurements for each cord segment. The highest measurement (OD/mm($x10^{-3}$) was recorded at 45, after only 2 hours perfusion in contrast to the low values recorded after 5 hours perfusion of 1.45 & 1.5 detailed in Table 3.1.

The perfusion period needed to be long enough to allow combination perfusions to also take place (ie.TCM+DMXAA). Evidence from Figure 3.4 show that cells after 2 hours are still in a metabolically active state, however, perfusion periods exceeding 5 hours show a decline in OD Absorbance readings indicating that fewer cells are capable of reducing MTT to a coloured formazan losing their viable state. However, the OD absorbance measurements of cord 4 question the consistency of using MTT assay as a method for testing cell viability. Cord 4, segment 1 in the MTT viability experiment recorded an OD of 0.052 after 2.5 hours perfusion, but within the same cord, segment 2 recorded an OD of 0.083 after 5 hours perfusion. This result indicates cell viability increased from 2 hours to 5 hours. A possible cause for this result could be experimental error or unusual perfusion history as the results from other perfused cord segments reflect the general trend that cell viability decreases over time. With these results it was estimated that endothelial cells within the umbilical cord are still alive and active after 2 hours perfusion but cell viability slowly declines with increasing time.

The overall conclusion was that after 6 hours perfusion there are few viable cells remaining, capable of metabolizing MTT, so perfusion experiments did not exceed 6 hours.

This assay method has been previously used in other studies to determine endothelial cell proliferation (Busby et al, 2003).

One advantage to using the MTT assay was the cells ability to reduce the salt to give a coloured appearance which was easily observed through the chambers and any problems (ie.leakage) during the perfusion period of MTT could also be identified.

It was important to establish a baseline to work from, and the generally consistent results for each cord were able to give an estimation of this time frame.

4.4. Isolating HUVEC

In this study a key objective was to characterise responses of endothelial cells to certain stimuli, and compare these results from an *ex vivo* perfusion model with the traditional *in vitro* HUVEC model. This was unsuccessful; a complete monolayer was difficult to maintain and cells were sensitive to subculturing treatment.

HUVECs were isolated from seven separate umbilical cords by collagenase digestion method described in section 2.10 based on the HUVEC isolation method in Jaffe et al, 1973.

Due to the poor results seen with the first two isolations the method was altered to try and maximise cell yield. These changes included extended incubation periods with collagenase A solution still infused in the umbilical vein.

Collagenase A is prepared from extracellular culture filtrate of Clostridium histolyticum containing other proteases including clostripain, a trypsin-like activity and a neutral protease. This mixture of enzymes allows gentle dissociation of tissue to generate single cells (In Vitro, 2004).

Another additional change was massaging the cord by hand for an extended period of time. This technique used by Galley et al, 1999, assisted in the release of the attached vein endothelial cells. Both of these techniques were used and contributed to an increase in endothelial cell yield.

The endothelial cells extracted were identified as large polygonal cells, visualized through a phase contrast microscope (x40 objectives) and later confirmed from paper *Culture of Human Endothelial Cells Derived from Umbillical Veins: Identification by and immunologic criteria, Jaffe et al, 1973*.

The isolated HUVEC were maintained for the first day on RPMI 1640 culture medium to establish cell growth thereafter passaged with HUVEC (Complete) medium which contained endothelial cell growth factors. The media was changed every two days or when required. After the second passage it was difficult to maintain cell confluence and cell numbers decreased eventually dying.

This trend was observed with two additional HUVEC isolations. The medium previously used was changed to M199 (Wasserman S et al, 2002). There was a positive outcome from this media change resulting in an increase in cell numbers and a more confluent monolayer observed. These cells were maintained with HUVEC (complete) medium after the first passage; however, after the second passage cell confluence and cell numbers decreased again resulting in cell death.

The isolation of human endothelial cells is known to be time consuming and yields can often be low, in addition, the use of primary cells have a number of disadvantages such as the presence of contaminating cells, progressive loss of viability and differences in the genetic background of the isolates. (Unger et al, 2002).

In this research the ability to maintain a working endothelial cell culture isolated from individual human umbilical cords was unsuccessful.

4.5. Gene Expression Results

4.5.1. RNA Extraction

An important first step in RNA extraction was finding the appropriate method of cellular disruption for starting material to obtain maximum yield of RNA. In this study GiTC lysis solution was used for extraction of endothelial cells from the whole umbilical cords. This is a slow cellular disruption solution, ensuring that all RNA in the sample is collected for purification. However, in cord 7 RNA quality was poor or undetectable so re-isolation procedure was necessary (Table 3.36).

There are a number of possibilities for poor quality RNA starting with choice of extracting solution used.

GiTC solution, if left too long, is capable of degrading RNA by releasing endogeneous RNases internally before purification (The Basics: RNA Isolation, Ambion, 2007) however, if left for a short period it may result in incomplete disruption and decreased yield because some of the RNA in the sample will still remain in intact cells and therefore is unavailable for subsequent purification (Cell Disruption: Getting the RNA out, Ambion 2007).

The RNA isolation procedure used in this research was very tedious but all steps were necessary, it was found that if RNA quality was poor, re-isolation was capable of purifying the RNA even more to give acceptable results.

4.5.2. DMXAA & Tumour Conditioned Media

DMXAA was prepared as described in section 2.6.4.

Tumour conditioned media was collected from NZM3 culture flasks once cells had reached confluence. This media was perfused through the umbilical cord veins prior to DMXAA perfusion. The addition of tumour conditioned media assisted in replicating a tumour microenvironment for the HUVECs and offered response differences to DMXAA.

4.5.3. Real-Time PCR

Real time PCR is one of the most sensitive and reproducible quantification methods for gene expression analysis. It provides simultaneous movement of gene expression in many different samples for a number of genes (Silver et al, 2006).

Housekeeping genes can effect Real Time PCR results. The ideal housekeeping gene should be constantly transcribed in all cell types and tissues and remain stable between samples that are taken from different time points and under different experimental conditions. This is almost impossible so it is critical that an appropriate housekeeping gene is selected.

Relative quantification was achieved by analyzing the Ct value, with data quality examined through a regression model. The approach identified the correlation between the Ct value and logrithm (Base 2) transformed concentration of template, giving a significant linear relationship for each standard curve (Yuan J, 2005) shown in Figure 4.1 & 4.2. This data was then was then interpreted in to a bar graph.

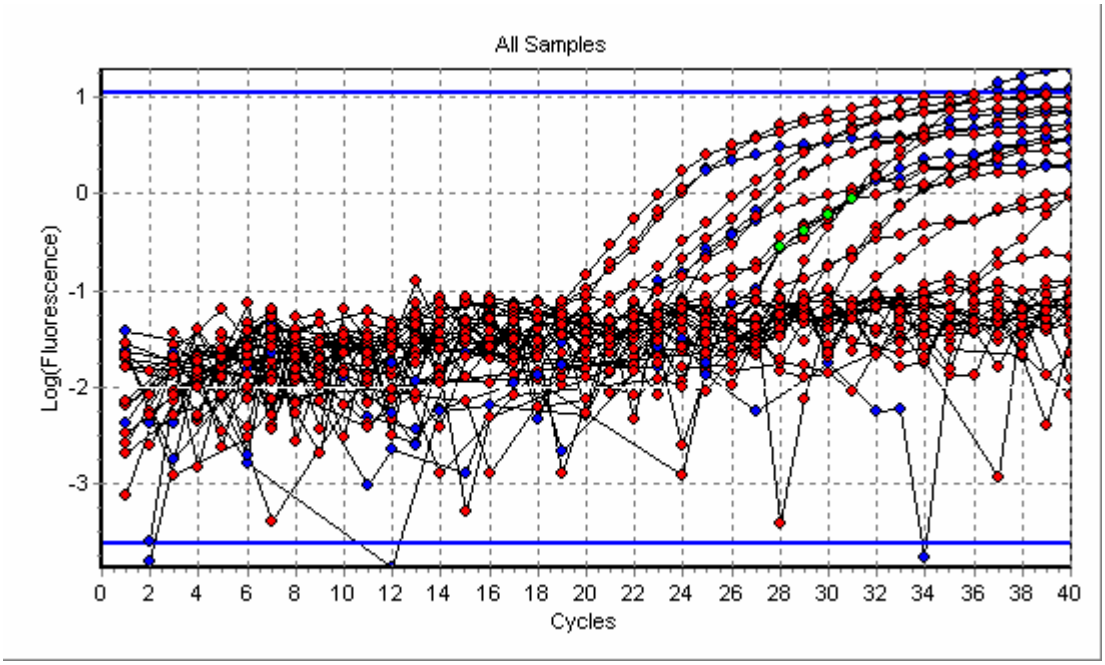


Figure 4.1. Linear Regression PCR data: All genes displayed for PBS perfusion

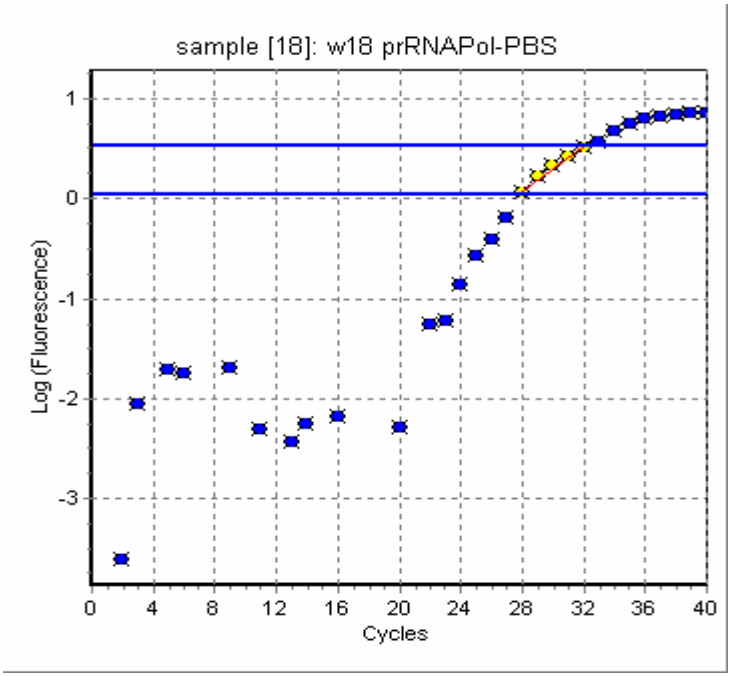


Figure 4.2. Linear Regression PCR data: Individual sample (RNAPol II – PBS)

Beta-2 Microglobulin (B2M) was the initial housekeeping gene of choice for immune response experiments but was subsequently shown to be unreliable for endothelial cells.

Human Proteosome Subunit Y (HPSY) was the housekeeping gene of choice for TCM and DMXAA perfusion experiments with RNA Pol II as the secondary housekeeping gene.

Dissociation curves (melt temperatures) were also run to ensure that the desired amplicon was being detected. This curve data was also required because the methodology process included the use of SYBR green which is capable of detecting any double stranded DNA including primer dimers, contaminating DNA and PCR products from misannealed primers (Real time PCR handbook, 2003). This could give false positive results in gene analysis.

Ideally samples should yield a sharp peak at the melting temperature indicating the products are specific, if a melt curve reveals a series of peaks this indicates there is not enough discrimination between specific and non-specific reaction products (Ambion, 2004)

4.5.4. Immune Responses

LPS induces a severe inflammatory response by initiating multiple intracellular signaling events including NF-κB which ultimately leads to synthesis and release of pro-inflammatory mediators and adhesion molecules, TNF-α and ICAM (Ulevitch R & Schletter J, 1995).

Beta-2 Microglobulin (B2M) was used as the internal control standard (housekeeping gene) for all immune response gene analysis.

4.5.4.1. Immune Response: Cord 1

In this experiment gene response was measured for LPS induced inflammatory response. Isolated RNA from perfusion samples were quantitated using the Nanodrop spectrometer. Each section produced good quality RNA with suitable 260/280 ratio values. PBS gave the lowest reading of 1.51 but was still used to produce cDNA.

Real Time PCR was performed with housekeeping gene B2M, quantitation data presented in table 3.5 indicate very little cDNA present in each sample with Ct values >34. The B2M (housekeeping gene) for LPS cDNA sample had the highest Ct (threshold cycle) value of 39.76. This indicated the need to use a higher concentration of cDNA in the Real Time PCR.

A melt curve (dissociation curve) was also produced and data displayed in Table 3.6. The results reveal possible contamination as temperatures do not lie within 0.5-1°C of each other and also possible primer dimer for both IL8-PBS & IL8-LPS. Both of these gene samples reveal 2 peaks which can be a result of self-annealed primers.

The overall results from this Real Time PCR could not be used to evaluate gene expression efficiently and based on the cDNA control, PCR reaction, the concentration of cDNA was adjusted for cord 2.

4.5.4.2. Immune Response: Cord 2

In this experiment gene response to LPS stimuli and LPS +HSS combination was measured. The RNA was successfully isolated from each cord segment with all 260/280 ratio values > 1.5. The cDNA was then produced from each RNA sample for Real Time PCR. All genes were successfully detected and Ct values recorded in Table 3.9. TLR4 gene samples recorded no conclusive Ct values. The genes expressed were normalized to housekeeping gene Beta-2 Microglobulin (B2M) and results

represented in Figure 3.9. This revealed an 8-fold up regulation of E-SEL when stimulated with LPS but only a 2-fold increase in the presence of HSS.

Tissue Factor (TF), ICAM & IL1B were also upregulated with LPS stimulation.

A 6-fold decrease in expression of E-SEL when perfused with combination LPS+HSS indicates HSS has an effective impact on LPS stimulation, suppressing its action on endothelial cells.

4.5.4.3. Immune Response: Cord 3

Gene expression was measured in response to Lactated Ringers (LRS), LPS & combination LPS+HSS.The isolated RNA from each sample revealed suitable 260/280 ratio values recorded in Table 3.13. The cord was divided into small

segments for 4 separate perfusion setups, this made the RNA isolation procedure difficult as there was minimal RNA to start with. The cDNA was produced from isolated RNA samples for Real Time PCR.

The detected Ct values represented in Table 3.14. showed suitable amounts of cDNA, with only the TLR4 gene undetected. A melt curve was produced and data displayed in Table 3.15. The melt readings for IL1β, B2M and ESEL indicated the specific amplicon was being detected.

An 8-fold up regulation of E-SEL & ICAM is shown in Figure 3.12. for both LPS and LPS+HSS. HSS appears to have no effect on LPS for E-SEL in this cord.

4.5.4.4. Immune Response: Cord 4

Gene expression was measured in response to LPS and combination LPS+HSS.

RNA was isolated from each sample and purity assessed with nanodrop spectrometer. The PBS (control) segment gave the lowest RNA 260/280 ratio value of 1.48. All samples were used to produce cDNA for Real Time PCR.

The poor RNA quality from PBS (control) sample was evident in Table 3.19, Ct values for each gene were high indicating inadequate amounts of cDNA. The melt data represented in Table 3.20 show possible DNA contamination for iNOS and TNF-α.

Ideally melt results should be identical or 0.5-1°C of each other. iNOS and TNF-α have a 2°C difference for each sample. This may be a result of DNA contamination, total RNA preparation or non specific amplification.

LPS stimulation reveals a 4-fold up regulation of E-SEL. In combination perfusion with HSS a 1-fold decrease in expression of E-SEL is observed. Control (PBS) samples show an 8-fold expression increase in E-SEL, ICAM & TF. These gene expression results indicate that ESEL, ICAM and TF genes are significantly being expressed in these HUVEC without any inflammatory stimulation, and which may represent a prior inflammatory history of the pregnant women or "problems" during delivery.

4.5.4.5. Immune Response: Cord 5

In this experiment gene expression responses to HSS and LRS were measured. All 3 RNA samples were used to produce cDNA. The melt temperatures of each gene were recorded in Table 3.25., this showed possible DNA contamination or primer dimer for gene samples TNFα-PBS, TNF-α LRS, ICAM PBS and ICAM-LRS.

Gene expression profile (Figure 3.18) shows an 8-fold up regulation of TLR4, TF, iNOS, IL1B and ESEL when stimulated by LRS. No genes were expressed for PBS (control) samples and stimulation with HSS revealed an 8-fold up regulation of E-SEL. Intracellular Adehesion Molecule (ICAM) was the only gene response fully suppressed.

Final Discussion

There is evidence from cords 2, 3 and 4 that E-SEL is over expressed when stimulated with LPS, but down regulated with the combination of LPS+HSS. The

impact of HSS appears to suppress the full effectiveness of LPS stimulation on endothelial cells.The up regulation of genes in resting endothelial cells (control) was seen in cord 4. This presents evidence that prior inflammatory stimulation of the endothelial cells may have taken place before the cord was collected. This may be a reflection of the mother's pregnancy or "problems" during the delivery of the baby.

4.5.5. DMXAA & TCM Perfusion Responses

The experimental results for DMXAA & Tumour Conditioned Media (TCM) perfusion samples were adjusted by normalizing them to housekeeping gene Human Proteasome subunit Y. A secondary housekeeping gene RNA POl II was also used in Real Time PCR analysis.

4.5.5.1. DMXAA & TCM Responses: Cord 6

Gene expression responses to TCM, DMXAA and combination TCM+DMXAA were measured. Isolated RNA from each sample was used to produce cDNA. The cDNA confirmation was performed on an agarose gel with 2 housekeeping genes, β-Actin & RNA Pol II. The β-actin gene was detected at 90 bp and RNA Pol II detected at 125bp. All samples loaded into gel wells gave positive readings and gene can be clearly identified by bands in Figure 3.9.

Tumour Conditioned Media (TCM) Perfusion

Tumour Conditioned Media (TCM) cDNA samples was used in Real Time PCR. The Ct values for each gene were recorded in Table 3.28, and each gene was normalized

to the primary housekeeping gene there was no Ct value detected for the secondary housekeeping gene RNA Pol II. The negative controls for IL6 and IL8 recorded Ct values indicating possible DNA contamination. The melt data in Table 3.29 support the evidence of reaction contamination for both of these 2 genes.

The gene expression profile represented in Figure 3.22 shows down regulation of all genes, excluding ESEL, IL6 and IL8 because of contamination in the control.. The impact of Tumour Conditioned Media (TCM) suggests that TCM causes down regulation of the genes investigated

TCM & DMXAA Perfusion

The combination perfusion samples with TCM+DMXAA were also used in Real Time PCR. The recorded Ct values in Table 3.31 revealed suitable Ct results < 32, but the housekeeping gene HPSY has no recorded Ct; however, the secondary housekeeping gene (RNA POl II) detected a Ct result. Using RNA Pol II as the internal control standard, a gene expression profile was produced shown in Figure 3.22 An up regulation of genes was evident in the presence of DMXAA. ICAM is still down regulated, but TNF-α, showed an 8-fold up regulation.

ESEL, IL8 and IL6 are contaminated so gene expression results are not conclusive for these genes.

The experiment with cord 8 is an ideal within cord comparison on the effectiveness of both DMXAA and TCM. The initial down regulation of gene expression from TNF is effectively interrupted with addition of DMXAA.

4.5.5.2. DMXAA & TCM Responses: Cord 7

In this experiment gene expression responses to DMXAA, TCM+DMXAA and DMXAA (125μM) were measured. The first round of isolated RNA was measured on an agarose gel shown in Figure 3.26. Only 2 bands were detected in lanes 1 & 3, but there are no signs of sharp 18S or 28S bands present. The RNA samples were then checked with the Nanodrop spectrometer. These results were recorded in Table 3.35. The 260/280 ratio values were < 1.5, RNA isolation was then repeated. The final results were recorded in Table 3.36. A significant increase in 260/280 ratio values >1.5 were observed.

The cDNA was produced from each RNA sample and quality confirmed on an agarose gel shown in Figure 3.31 with housekeeping gene RNA Pol II. There were 2 bands detected for TCM+DMXAA segment only, but no band was observed from the RNA Pol II control. This experimental procedure was unsuccessful.

4.5.5.3. DMXAA & TCM Responses: Cord 8

Gene responses to DMXAA and TCM were measured in this experiment. RNA was successfully isolated from each sample and was used to produce cDNA. The cDNA was then confirmed on an agarose gel shown in Figure 3.27with housekeeping gene β-actin. Three bright bands are positively identified with PBS, DMXAA and TCM samples for β-actin at 90bp. All cDNA samples were then used for Real Time PCR.

PBS Perfusion

PBS perfused cDNA samples were performed in Real Time PCR and Ct values displayed in Table 3.38. Overall there appeared to be significant amounts of detected

cDNA with Ct values of <33, to give conclusive gene expression results; however Ct values of negative control were recorded for IL6 and IL8, contamination of these genes was confirmed from the melt data presented in Table 3.39. The close temperature melts for IL8 indicate this specific amplicon is being detected. The melt temperatures observed also indicate possible contamination or primer dimer for gene samples I-CAM, IL1B & RNA Pol II.

A gene expression profile shown in Figure 3.30.was produced by normalizing gene Ct values to the housekeeping gene HSPY. Results indicate IL1B gene expression was up regulated. TNF-α expression was suppressed suggesting it is not expressed in the resting endothelial cells of this cord.

TCM Perfusion

TCM perfused segments were used to produce cDNA for Real Time PCR.

The gene expression analysis represented in Figure 3.33 clearly shows an overall down regulation of gene expression. IL1B is the only gene which has been upregulated 8-fold. The melt temperatures for each gene recorded in Table 3.42. show more than one value being recorded this could be possible primer dimer or contamination of the reaction. Another possibility is the temperature threshold was set too high, ultimately detecting more than one peak in the reaction. The genes IL6, IL8 and RNA Pol showed contamination revealed from Ct values recorded for each negative control. These genes were excluded from gene expression analysis.

DMXAA Perfusion

The DMXAA perfused segment was used to produce cDNA for Real Time pCR. The Ct values were recorded in Table 3.44 and showed adequate cDNA for gene expression analysis; however, reaction contamination is evident from the negative control Ct values detected for genes ESEL, IL6 and IL8. Gene expression analysis was inconclusive from these results.

4.5.5.4. DMXAA & TCM Responses: Cord 9

Gene responses to DMXAA and TCM+DMXAA were measured in this experiment. RNA was successfully isolated and purity assessed on the Nanodrop spectrometer. The 260/280 ratio values were <1.50 which can indicate possible contamination or not enough RNA has been extracted from the sample. The cord segment lengths were very short in this experimental procedure and would account for the lack of extracted RNA. RNA samples were then used to produce cDNA and the quality was checked on an agarose gel shown in Figure 3.36. One band was detected for TCM+DMXAA the cDNA sample; however, there was no bands detected for the housekeeping gene RNA Pol II. This made it difficult to assess the quality of the cDNA. From the poor RNA purity results and cDNA results, Real Time PCR was not performed for gene expression analysis.

4.5.5.5. DMXAA & TCM Responses: Cord 10

Gene expression analysis was measured in response to TCM and TCM+DMXAA. RNA was successfully isolated and quantitated using the Nanodrop spectrometer as

described in section 2.8. The RNA for DMXAA sample recorded a good 260/280 ratio value of 1.70, which is also displayed in Figure 3.37. The two sharp peaks in Figure 3.37 represent the 18S & 28S rRNA. The cDNA was produced from all 3 samples and confirmed on an agarose gel run with housekeeping gene RNA Pol II. The control gene RNA Pol II was undetected, and only the DMXAA cDNA sample produced a small visual band in the gel.

Due to the poor cDNA gel results, TCM+DMXAA samples were not used to perform Real Time PCR.

PBS Perfusion

The cDNA from PBS perfused segments were prepared as described in section 2.92. Low Ct values of < 10 were recorded in Table 3.50. and clearly indicated contamination. A gene expression profile was inconclusive for the PBS (control) perfused segment.

DMXAA Perfusion

The cDNA from DMXAA perfused samples were also used in Real Time PCR.

The Ct values for each gene are recorded in Table 3.52. and indicate suitable amounts of cDNA with Ct values < 33 recorded; however, the Ct values for the negative control of each gene except RNA Pol which was undetected and TF were observed. This indicated reaction contamination so no gene analysis profile was produced as results would be inconclusive and misleading.

4.5.5.6. DMXAA & TCM Responses: Cord 11

Gene expression was measured in response to DMXAA and TCM stimulation. RNA was isolated from each sample and assessed on an agarose gel to confirm RNA quality. No bands were detected from any of the samples seen in Figure 3.43. RNA was then re-isolated following the method described in section 2.7. All re isolated RNA samples were confirmed using the Nanodrop spectrometer. The isolated RNA from TCM sample gave a poor 260/280 ratio value of 1.07 seen in Table 3.55. PBS and DMXAA recorded low 260/280 ratio values of 1.51 and 1.53 respectively.

Only PBS and DMXAA RNA samples were used to produce cDNA. The cDNA quality was confirmed in an agarose gel, performed with 2 housekeeping genes β-actin and RNA Poll II. The gene β-actin was successfully detected at 90 bp, and 2 positive bands were identified from both PBS and DMXAA samples, seen in Figure 3.44. The secondary housekeeping gene RNA Pol II was undetected in the gel, but single bands were detected for both PBS and DMXAA samples at approximately 125bp.

PBS Perfusion

The cDNA from PBS (control) sample was used in Real Time PCR. The Ct values for the negative controls of each gene except TNF-α, which was undetected, was produced. This ultimately indicates reaction contamination. The results from this Real Time PCR were inconclusive and a gene expression profile could not be produced.

TCM Perfusion

The TCM cDNA samples were also used in Real Time PCR. The Ct value results recorded in Table 3.58 revealed no detection of primary housekeeping gene HPSY, and secondary housekeeping gene RNA Pol II was contaminated, evidence given from a recorded Ct value for the negative control. Interleukin (IL6), IL8, ICAM and IL1B also produced Ct values for negative control samples indicating contamination. The melt data displayed in Table 3.59 also showed significant DNA contamination and primer dimer, all gene samples detected more than one peak suggesting more than the specific product is being detected. The results from this Real Time PCR prevented a reliable gene expression analysis.

Final Discussion

Gene expression analysis quantified with Real Time PCR was capable of producing reliable results only when the reaction conditions were correct, minimizing all possible contamination that could effect the results. Problems observed were reaction contamination possibly due to inadequate amounts of cDNA, primer contamination or DNA contamination in the cDNA preparation method.

However, it was observed from TCM perfused experiments, the impact of tumour conditioned media on gene regulation, gene expression appear to be down regulated in the presence of TCM but in combination with DMXAA they are elevated. Also observed was the upregulation of TNF-α in the presence of DMXAA, which was not observed in TCM samples. This finding was also reported in paper Joseph et al, 1999 & Ching et al, 1999, "feature of DMXAA activity is ability to stimulate production of TNF-α within tumour microenvironment".

Possibilities for poor gene expression results.

Changes in gene expression can occur as early as sample harvest/handling and during RNA isolation (McGarvey et al,).

Stabilizing samples after isolation is critical in preventing artifacts and in preserving the gene expression patterns. Cell death and enzymatic degradation of RNA can limit the efficiency of Real Time and reduce yield. This results in specific genes being induced or down regulated, and non-specific and specific reduction in mRNA species (McGarvey et al,).

4.6. Conclusion

The aim of this study was to characterize responses of endothelial cells to inflammatory stimuli and DMXAA, and compare these results to two separate models: a traditional in vitro model using HUVEC and an *ex vivo* model using the intact umbilical vein.

Isolating primary endothelial cells from whole umbilical cords was found to be a very tedious and very sensitive process. Failure to culture HUVEC successfully prevented experimental comparison between the two models. Focus was then directed towards the ex vivo model, employing the technique of a whole intact umbilical vein perfusion.

Dividing the cord into a number of segments was successful in permitting control and experimental conditions in the same tissue. Each individual perfusion had limitations but it was overall successful.

Inflammatory agents and drug administration by intravenous perfusion provided an easy and reproducible method to cellular exposure minimizing cell contamination and sample handling.

The gene expression profiles resulting from the immune response study successfully shows the impact of the inflammatory stimuli LPS on endothelial cells of the umbilical cord. The results show an upregulation of ESEL, ICAM and TF; however in the presence of Hypertonic Saline Solution, gene expressions are down regulated.

This evidence suggests that HSS intercepts in the activation pathway of LPS ultimately suppressing its effect on endothelial cells.

There is also evidence from gene regulation of unstimulated endothelial cells that supports the idea of heterogeneity between cords. This may be caused from inflammatory stimuli of the umbilical cord endothelial cells during pregnancy prior to receiving the cord for experimental manipulation.

Enhanced induction of TNF-α was observed with combination of TCM + DMXAA perfusion; however not observed when TCM was perfused alone, suggesting that the enhancement of gene expression lies within the action of DMXAA. The limitations of the DMXAA and TCM perfusion experiments was avoiding contamination of each Real Time PCR reaction. This produced in some experiments, unreliable and misleading gene expression profiles, which could not give conclusive results.

4.7. Future Directions

The biggest limitation for extended work in this research was the availability of whole umbilical cords. A fresh umbilical cord sample was required for each experimental procedure.

Establishing a successful new system for ex vivo cell response analysis will open the door to new opportunities in cell biology in particular cancer gene therapy where there is an urgent need to develop robust human preclinical models.

Appendix 1

DMXAA Requirements

Assumptions:

- To allow for assay development, replicates and additional endothelial cell analysis experiments, 25 cords will be required.
- Each cord is divided into three segments (control + 2 different concentrations of DMXAA), plus additional extraction and culture of HUVEC.
- Only one cord can be processed at a time due to limitations of the perfusion chamber.
- Perfusate volume for each segment of cord is 100ml; volume of media required for HUVEC is minimal (a few ml)
- DMXAA for each cord experiment will be taken from the same vial, which will only be used immediately upon opening in order to ensure sterility and stability.
- A contingency allowance of 50% extra is included in requirements

DMXAA Concentration Proposed

- Zero (Control)
- 25μM (peak plasma free drug concentration at doses used in current clinical trial)
- 250 μM (peak plasma free drug concentration at higher doses used in trials)

Formulation

Clinical formulation = 200mg/ml, 10ml vial, 10 vials per box
Molecular Weight: 304

DMXAA Vial Requirement Calculations

25μM x 100ml = 2.5μmol = 0.76mg
250μM x 100ml = 25μmol = 7.6mg

One vial will amply cover there requirements for each cord experimental procedure

25 cords + 50% contingency allowance = 38 vials

Conclusion

4 Boxes x 10 vials each required

Appendix 2

Real Time PCR Data

Data for Cord 1

14 Hilly Street Mortlake NSW 2137 Australia
T +61 2 9736 1320
F +61 2 9736 1364
W www.corbettlifescience.com

Quantitation Report

Experiment Information

Run Name	Joe LPS Run 2 2006-01-24 (1)
Run Start	24/01/2006 4:09:50 p.m.
Run Finish	24/01/2006 6:20:57 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

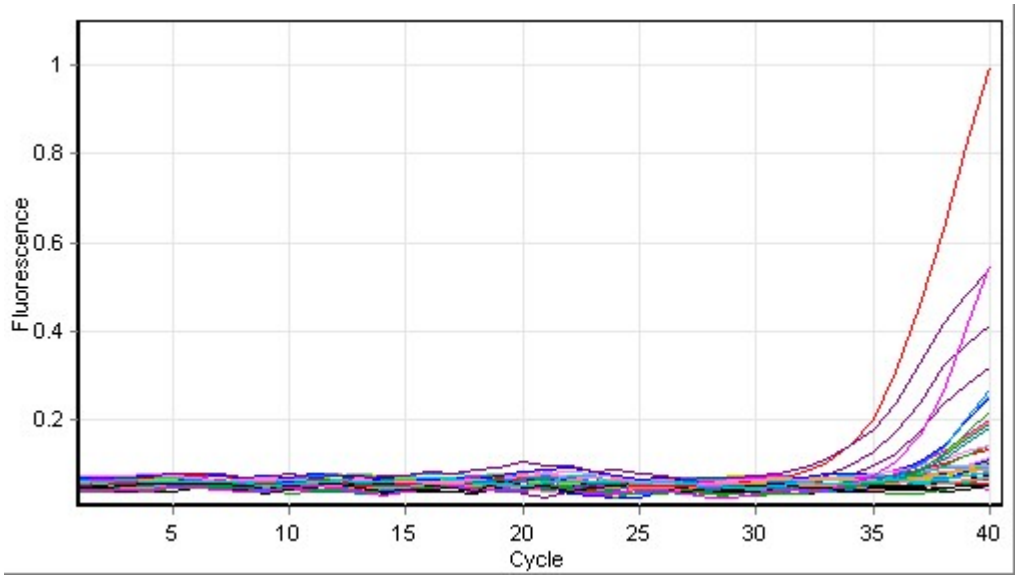
Threshold	0.0251
Left Threshold	28.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

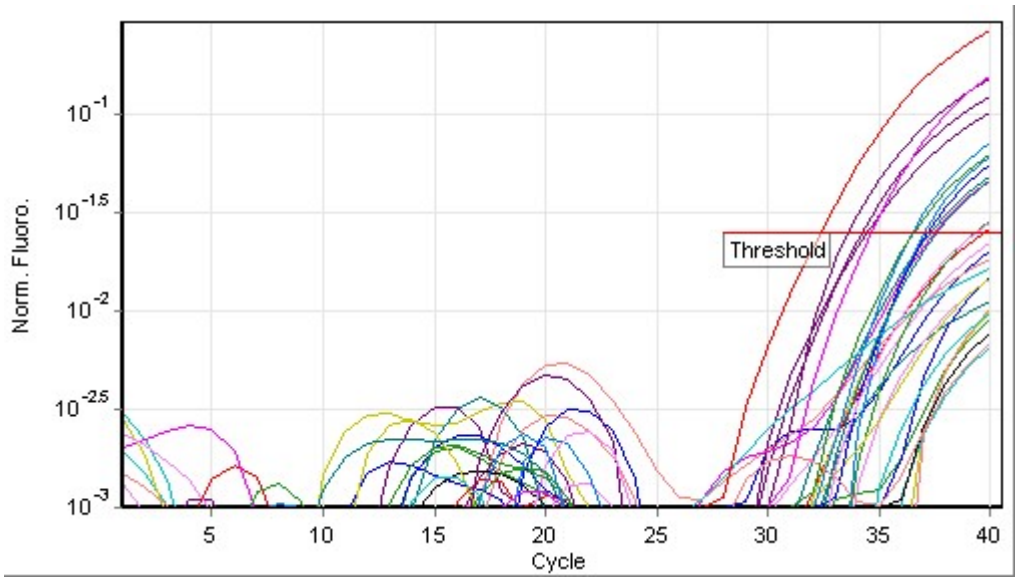
Messages

Profile

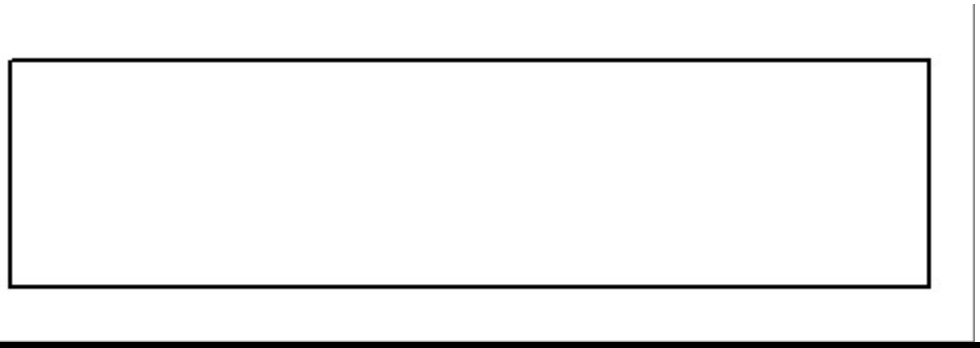
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		B2M-Blank	Unknown	NEG (NTC)			
2		IL1B-Blank	Unknown				
3		IL6-Blank	Unknown				
4		IL8-Blank	Unknown	34.44			
5		IL12-Blank	Unknown				
6		iNOS-Blank	Unknown	NEG (NTC)			
7		PAI1-Blank	Unknown				
8		TLR4-Blank	Unknown				

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		TNFa-Blank	Unknown				
10		TF-Blank	Unknown	NEG (NTC)			
11		ICAM1-Blank	Unknown	NEG (NTC)			

12		ESel-Blank	Unknown				
13		B2M-LPS	Unknown	39.76			
14		IL1B-LPS	Unknown	37.55			
15		IL6-LPS	Unknown				
16		IL8-LPS	Unknown	34.18			
17		IL12-LPS	Unknown				
18		iNOS-LPS	Unknown	37.02			
19		PAI1-LPS	Unknown	37.29			
20		TLR4-LPS	Unknown				
21		TNFa-LPS	Unknown	39.34			
22		TF-LPS	Unknown	37.45			
23		ICAM1-LPS	Unknown				
24		ESel-LPS	Unknown				
25		B2M-Control	Unknown	32.32			
26		IL1B-Control	Unknown				
27		IL6-Control	Unknown	37.18			
28		IL8-Control	Unknown	33.56			
29		IL12-Control	Unknown	39.41			
30		iNOS-Control	Unknown	36.49			
31		PAI1-Control	Unknown	37.55			
32		TLR4-Control	Unknown				
33		TNFa-Control	Unknown	36.55			
34		TF-Control	Unknown	34.62			
35		ICAM1-Control	Unknown	NEG (NTC)			
36		ESel-Control	Unknown				

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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14 Hilly Street Mortlake NSW 2137 Australia
T +61 2 9736 1320
F +61 2 9736 1364
W www.corbettlifescience.com

Melt Report

Experiment Information

Run Name	Joe LPS Run 2 2006-01-24 (1)
Run Start	24/01/2006 4:09:50 p.m.
Run Finish	24/01/2006 6:20:57 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	76.7°c
Threshold	0.062

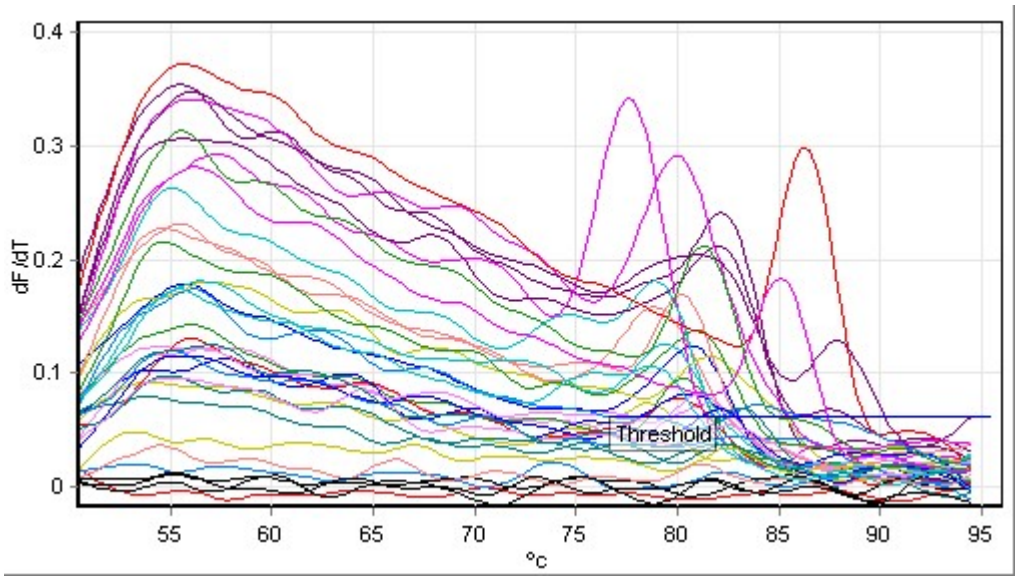
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	B2M-Blank			
2	IL1B-Blank		78.5	
3	IL6-Blank		80.7	
4	IL8-Blank		82.2	
5	IL12-Blank		81.5	
6	iNOS-Blank			
7	PAI1-Blank			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	TLR4-Blank		78.8	
9	TNFa-Blank		80.3	

10	TF-Blank		77.5	
11	ICAM1-Blank			
12	ESel-Blank		79.0	
13	B2M-LPS		80.0	
14	IL1B-LPS		81.5	
15	IL6-LPS		80.5	
16	IL8-LPS		82.0	87.5
17	IL12-LPS		81.2	
18	iNOS-LPS			
19	PAI1-LPS			
20	TLR4-LPS		80.2	
21	TNFa-LPS		81.0	
22	TF-LPS		80.0	
23	ICAM1-LPS			
24	ESel-LPS		79.5	
25	B2M-Control		86.3	
26	IL1B-Control			
27	IL6-Control		82.0	
28	IL8-Control		81.0	87.8
29	IL12-Control			
30	iNOS-Control		84.0	
31	PAI1-Control		83.2	
32	TLR4-Control			
33	TNFa-Control		81.3	
34	TF-Control		85.0	
35	ICAM1-Control			
36	ESel-Control		78.7	

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for cord 2

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T + 61 2 9736 1320
F + 61 2 9736 1364
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Quantitation Report

Experiment Information

Run Name	Joe 13_02 LPS + HSS 2006-02-15 (2)
Run Start	15/02/2006 11:33:37 a.m.
Run Finish	15/02/2006 1:43:55 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

Threshold	0.00483
Left Threshold	19.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

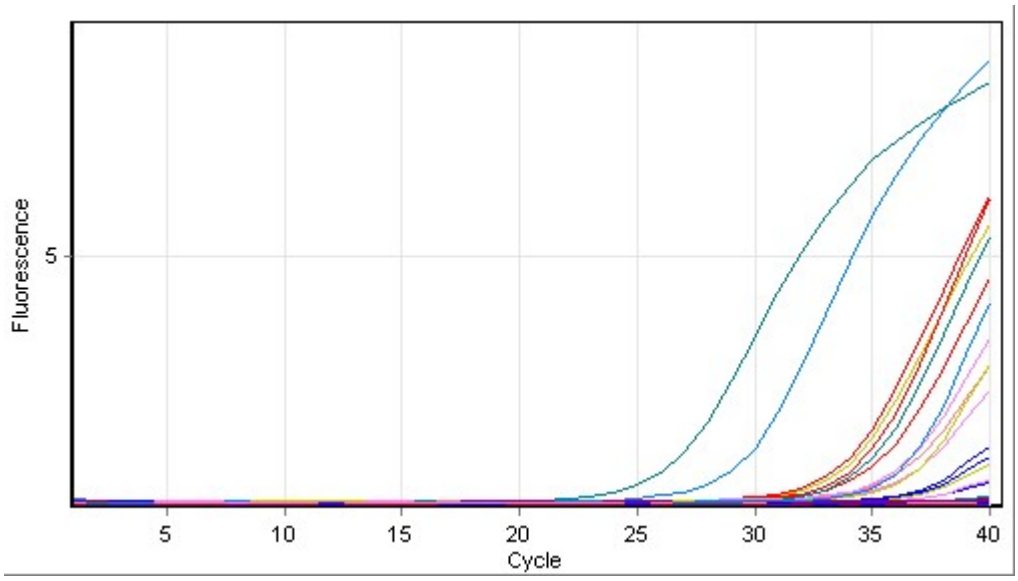
Messages

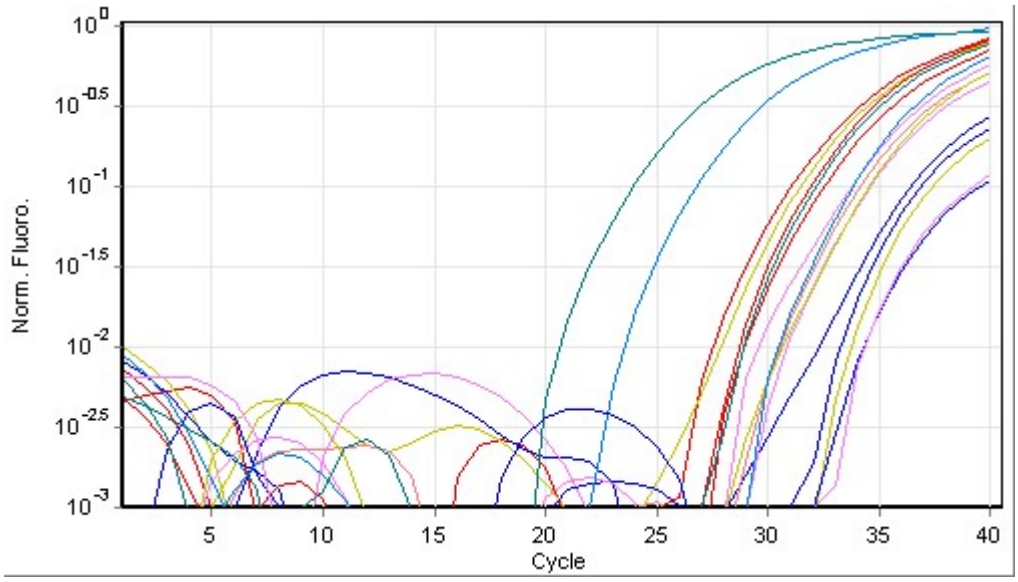
Message

Profile

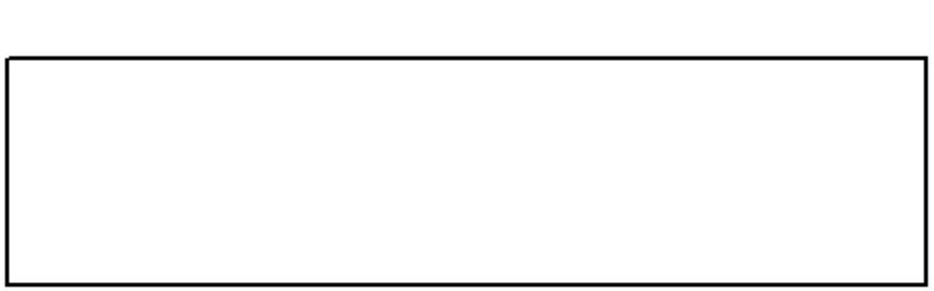
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
1		B2M-Blank	Unknown	NEG (NTC)			
2		IL1B-Blank	Unknown	NEG (NTC)			
3		iNOS-Blank	Unknown	NEG (NTC)			
4		TLR4-Blank	Unknown	NEG (NTC)			
5		TNFa-Blank	Unknown	NEG (NTC)			
6		TF-Blank	Unknown	NEG (NTC)			
7		ESel-Blank	Unknown	NEG (NTC)			
8		ICAM-Blank	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
9		B2M-Control	Unknown	28.21			
10		IL1B-Control	Unknown	33.02			
11		iNOS-Control	Unknown	33.43			

12		TLR4-Control	Unknown	NEG (NTC)			
13		TNFa-Control	Unknown	33.61			
14		TF-Control	Unknown	NEG (NTC)			
15		ESel-Control	Unknown	NEG (NTC)			
16		ICAM-Control	Unknown	NEG (NTC)			
17		B2M-LPS	Unknown	26.80			
18		IL1B-LPS	Unknown	26.91			
19		iNOS-LPS	Unknown	31.00			
20		TLR4-LPS	Unknown	NEG (NTC)			
21		TNFa-LPS	Unknown	30.12			
22		TF-LPS	Unknown	22.88			
23		ESel-LPS	Unknown	20.01			
24		ICAM-LPS	Unknown	29.78			
25		B2M-LPS+HSS	Unknown	28.08			
26		IL1B-LPS+HSS	Unknown	29.67			
27		iNOS-LPS+HSS	Unknown	32.54			
28		TLR4-LPS+HSS	Unknown	NEG (NTC)			
29		TNFa-LPS+HSS	Unknown	28.75			
30		TF-LPS+HSS	Unknown	29.78			
31		ESel-LPS+HSS	Unknown	28.30			
32		ICAM-LPS+HSS	Unknown	NEG (NTC)			
33		Empty	Unknown	NEG (NTC)			
34		Empty	Unknown	NEG (NTC)			
35		Empty	Unknown	NEG (NTC)			
36		Empty	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	Joe 13_02 LPS + HSS 2006-02-15 (2)
Run Start	15/02/2006 11:33:37 a.m.
Run Finish	15/02/2006 1:43:55 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	83.2°c
Threshold	0.31773

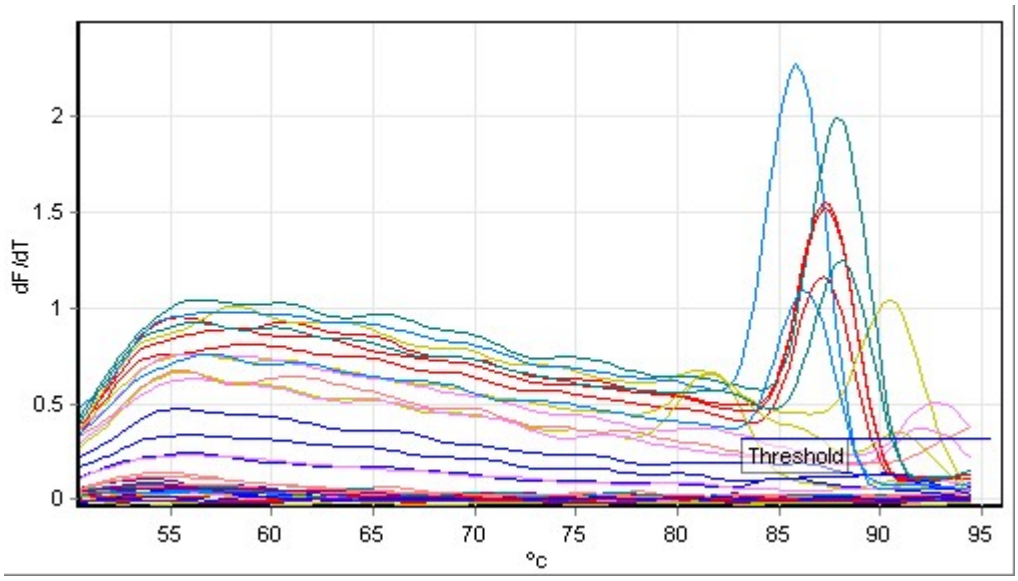
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1
1	B2M-Blank		
2	IL1B-Blank		
3	iNOS-Blank		
4	TLR4-Blank		
5	TNFa-Blank		
6	TF-Blank		
7	ESel-Blank		

(Continued on next page)...

No.	Name	Genotype	Peak 1
8	ICAM-Blank		
9	B2M-Control		87.2

10	IL1B-Control		
11	iNOS-Control		
12	TLR4-Control		
13	TNFa-Control		
14	TF-Control		
15	ESel-Control		
16	ICAM-Control		
17	B2M-LPS		87.3
18	IL1B-LPS		90.5
19	iNOS-LPS		
20	TLR4-LPS		
21	TNFa-LPS		92.0
22	TF-LPS		85.8
23	ESel-LPS		88.0
24	ICAM-LPS		
25	B2M-LPS+HSS		87.3
26	IL1B-LPS+HSS		91.0
27	iNOS-LPS+HSS		
28	TLR4-LPS+HSS		
29	TNFa-LPS+HSS		92.8
30	TF-LPS+HSS		86.3
31	ESel-LPS+HSS		88.0
32	ICAM-LPS+HSS		
33	Empty		
34	Empty		
35	Empty		
36	Empty		

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (Cord 2)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
9	B2M	Control	28.21	-	1.26E-09	1.78176	-	0.995626	939.2092	HK
17	B2M	LPS	26.8	-	1.12E-08	1.700212	-	0.99637	7442.775	HK
25	B2M	LPS+HSS	28.08	-	1.13E-09	1.798595	-	0.997176	777.4815	HK
15	ESel	Control		-	0.00338	1.064516	-	0.356879	0	0
23	ESel	LPS	20.01	-	3.32E-07	1.731798	-	0.992513	189186.6	25.41882
31	ESel	LPS+HSS	28.3	-	1.08E-09	1.793066	-	0.996362	745.4934	0.958857
16	ICAM	Control		-	0.005607	1.014962	-	0.026406	0	0
24	ICAM	LPS	29.78	-	1.27E-08	1.627005	-	0.998088	5676.183	0.762643
32	ICAM	LPS+HSS		-	0.009618	0.996541	-	0.016295	0	0
10	IL1B	Control	33.02	-	1.15E-10	1.76554	-	0.987599	78.94462	0.084054
18	IL1B	LPS	26.91	-	2.49E-08	1.648048	-	0.907754	16238.87	2.18183
26	IL1B	LPS+HSS	29.67	-	1.83E-09	1.692727	-	0.945766	1849.464	2.378788
11	iNOS	Control	33.43	-	1.37E-10	1.734211	-	0.992872	113.773	0.121137
19	iNOS	LPS	31	-	1.68E-11	1.869857	-	0.998463	41.99131	0.005642
27	iNOS	LPS+HSS	32.54	-	1.91E-10	1.750844	-	0.993907	136.1287	0.175089
14	TF	Control		-	0.00124	1.084359	-	0.263305	0	0
22	TF	LPS	22.88	-	8.42E-09	1.85391	-	0.996439	8229.041	1.105642
30	TF	LPS+HSS	29.78	-	8.94E-11	1.850263	-	0.999345	123.3113	0.158604
12	TLR4	Control		-	0.056649	0.981779	-	0.0382	0	0
20	TLR4	LPS		-	0.013683	1.012378	-	0.023781	0	0
28	TLR4	LPS+HSS		-	0.020666	0.988244	-	0.027114	0	0
13	TNFa	Control	33.61	-	8.89E-10	1.657381	-	0.994438	472.529	0.503114
21	TNFa	LPS	30.12	-	2.91E-09	1.679553	-	0.9972	1846.65	0.248113
29	TNFa	LPS+HSS	28.75	-	4.54E-10	1.78706	-	0.995395	631.2691	0.811941

Data for cord 3

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F +61 2 9736 1364
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Quantitation Report

Experiment Information

Run Name	20_02 Full expt 2006-02-21 (1)
Run Start	21/02/2006 4:35:33 p.m.
Run Finish	21/02/2006 6:47:36 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

Threshold	0.00597
Left Threshold	20.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

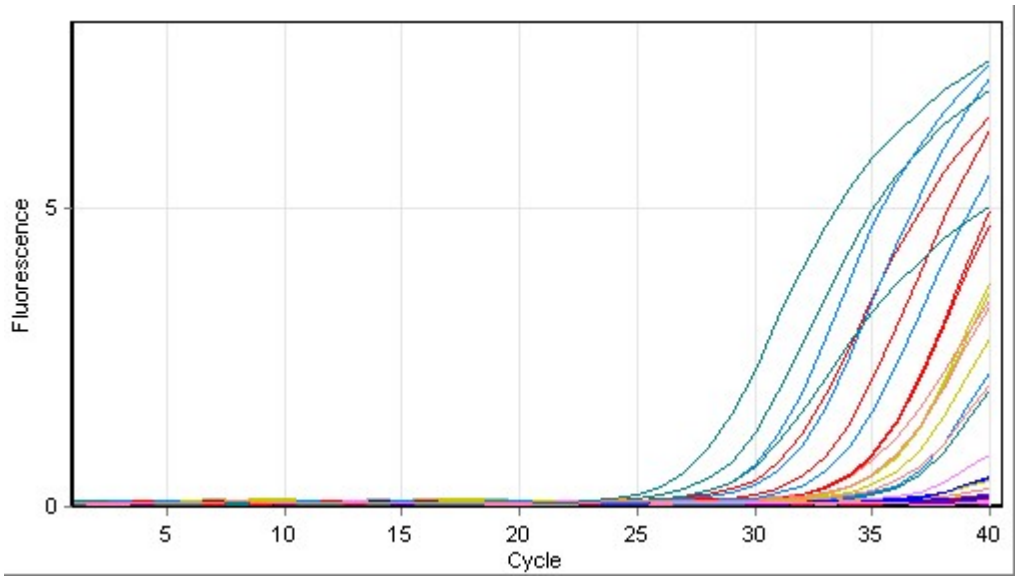
Messages

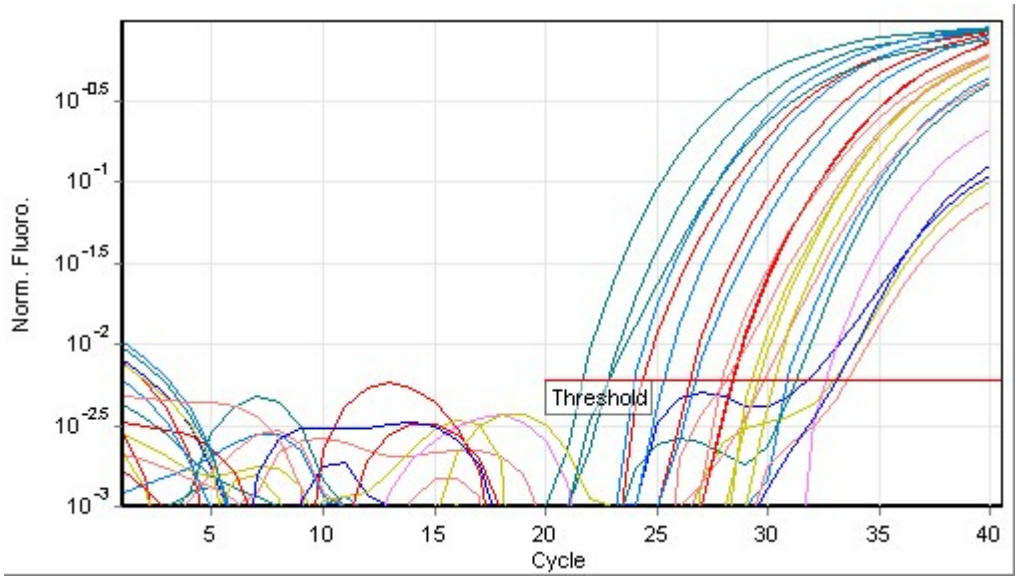
Message

Profile

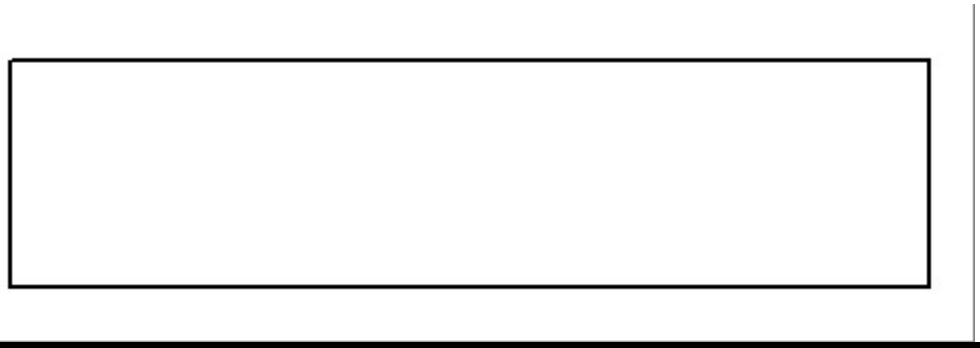
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
1		B2M-Blank	Unknown	NEG (NTC)			
2		IL1B-Blank	Unknown	NEG (NTC)			
3		ESel-Blank	Unknown	NEG (NTC)			
4		ICAM-Blank	Unknown	NEG (NTC)			
5		B2M-Control	Unknown	24.28			
6		IL1B-Control	Unknown	32.94			
7		iNOS-Control	Unknown	31.73			
8		TLR4-Control	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
9		TNFa-Control	Unknown	NEG (NTC)			
10		TF-Control	Unknown	30.84			
11		ESel-Control	Unknown	31.24			

12		ICAM-Control	Unknown	33.53			
13		B2M-LRS	Unknown	26.40			
14		IL1B-LRS	Unknown	29.54			
15		iNOS-LRS	Unknown	NEG (NTC)			
16		TLR4-LRS	Unknown	NEG (NTC)			
17		TNFa-LRS	Unknown	NEG (NTC)			
18		TF-LRS	Unknown	25.24			
19		ESel-LRS	Unknown	22.72			
20		ICAM-LRS	Unknown	29.86			
21		B2M-LPS	Unknown	28.39			
22		IL1B-LPS	Unknown	29.22			
23		iNOS-LPS	Unknown	NEG (NTC)			
24		TLR4-LPS	Unknown	NEG (NTC)			
25		TNFa-LPS	Unknown	32.50			
26		TF-LPS	Unknown	23.89			
27		ESel-LPS	Unknown	21.63			
28		ICAM-LPS	Unknown	27.87			
29		B2M-LPS+HSS	Unknown	28.33			
30		IL1B-LPS+HSS	Unknown	30.26			
31		iNOS-LPS+HSS	Unknown	33.03			
32		TLR4-LPS+HSS	Unknown	NEG (NTC)			
33		TNFa-LPS+HSS	Unknown	NEG (NTC)			
34		TF-LPS+HSS	Unknown	26.65			
35		ESel-LPS+HSS	Unknown	22.78			
36		ICAM-LPS+HSS	Unknown	28.10			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	20_02 Full expt 2006-02-21 (1)
Run Start	21/02/2006 4:35:33 p.m.
Run Finish	21/02/2006 6:47:36 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	81.2°c
Threshold	0.13391

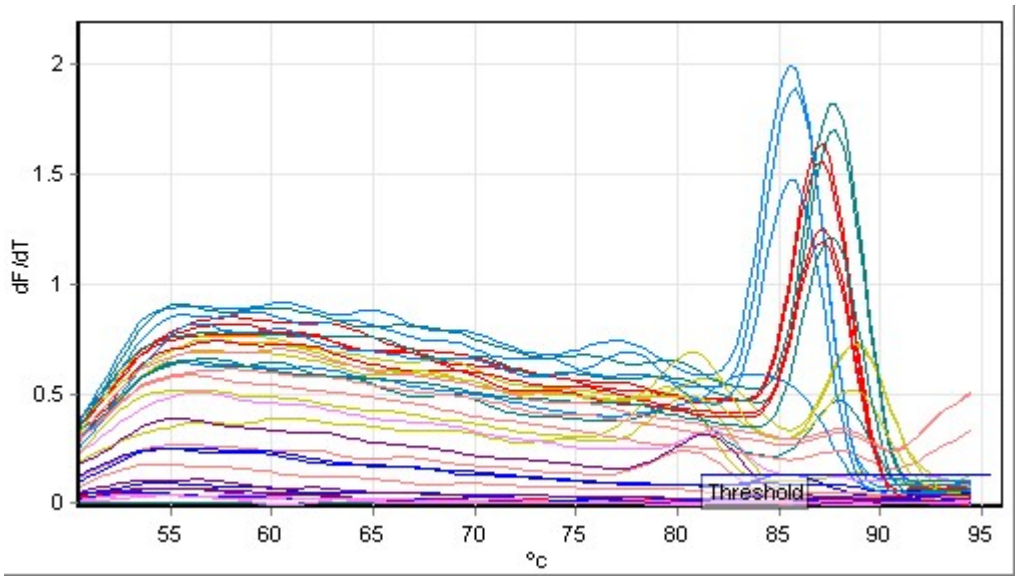
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	B2M-Blank			
2	IL1B-Blank			
3	ESel-Blank			
4	ICAM-Blank			
5	B2M-Control		87.0	
6	IL1B-Control		89.0	
7	iNOS-Control			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	TLR4-Control			
9	TNFa-Control			

10	TF-Control		83.7	
11	ESel-Control		88.0	
12	ICAM-Control			
13	B2M-LRS		87.0	
14	IL1B-LRS		81.5	88.7
15	iNOS-LRS			
16	TLR4-LRS			
17	TNFa-LRS			
18	TF-LRS		85.7	
19	ESel-LRS		87.7	
20	ICAM-LRS		87.8	
21	B2M-LPS		87.2	
22	IL1B-LPS		88.8	
23	iNOS-LPS			
24	TLR4-LPS			
25	TNFa-LPS		81.5	
26	TF-LPS		85.5	
27	ESel-LPS		87.7	
28	ICAM-LPS		88.0	
29	B2M-LPS+HSS		87.2	
30	IL1B-LPS+HSS		88.5	
31	iNOS-LPS+HSS			
32	TLR4-LPS+HSS		81.3	
33	TNFa-LPS+HSS			
34	TF-LPS+HSS		85.7	
35	ESel-LPS+HSS		87.5	
36	ICAM-LPS+HSS		88.0	

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (Cord 3)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
5	B2M	Control	24.28	-	1.77E-09	1.876198	-	0.99624	2594.264	HK
21	B2M	LPS	28.39	-	7.56E-11	1.939046	-	1	76.66357	HK
29	B2M	LPS+HSS	28.33	-	8.84E-11	1.918146	-	0.996617	108.437	HK
13	B2M	LRS	26.4	-	4.19E-10	1.902005	-	0.991418	476.4622	HK
11	ESel	Control	31.24	-	2.28E-10	1.781098	-	0.998544	165.0947	0.063638
27	ESel	LPS	21.63	-	1.34E-08	1.901532	-	0.99579	10284.99	134.1575
35	ESel	LPS+HSS	22.78	-	6.29E-08	1.701971	-	0.994204	61394.36	566.1752
19	ESel	LRS	22.72	-	8.21E-09	1.860306	-	0.994198	8399.685	17.62928
12	ICAM	Control	33.53	-	5.49E-11	1.749913	-	0.999597	79.59341	0.030681
28	ICAM	LPS	27.87	-	5.36E-10	1.819201	-	0.995664	640.2456	8.351366
36	ICAM	LPS+HSS	28.1	-	3.11E-09	1.708866	-	0.993007	3236.864	29.85016
20	ICAM	LRS	29.86	-	2.77E-09	1.674373	-	0.997155	2317.343	4.863645
6	IL1B	Control	32.94	-	3.74E-12	1.880357	-	0.929668	10.36916	0.003997
22	IL1B	LPS	29.22	-	4.42E-11	1.916498	-	0.990651	62.27679	0.812339
30	IL1B	LPS+HSS	30.26	-	1.38E-09	1.723873	-	0.996375	780.854	7.200989
14	IL1B	LRS	29.54	-	2.25E-10	1.82902	-	0.999527	201.0396	0.421942
7	iNOS	Control	31.73	-	1.4E-11	1.827385	-	0.998652	55.12499	0.021249
23	iNOS	LPS		-	0.026669	1.017908	-	0.096116	0	0
31	iNOS	LPS+HSS	33.03	-	1.86E-09	1.623397	-	1	1255.696	11.57995
15	iNOS	LRS		-	0.003848	1.035147	-	0.05218	0	0
10	TF	Control	30.84	-	4.28E-12	1.981234	-	0.993348	7.793277	0.003004
26	TF	LPS	23.89	-	1.77E-09	1.916116	-	0.99248	2005.212	26.156
34	TF	LPS+HSS	26.65	-	0.000392	1.220099	-	0.651009	55819799	514766.9
18	TF	LRS	25.24	-	1.21E-09	1.88209	-	0.993044	1310.063	2.749562
8	TLR4	Control		-	0.015083	1.020842	-	0.038403	0	0
24	TLR4	LPS		-	4.54E-14	2.000911	-	0.732345	0	0
32	TLR4	LPS+HSS		-	3.28E-11	1.761961	-	0.888133	0	0
16	TLR4	LRS		-	0.026571	1.012551	-	0.02076	0	0
9	TNFa	Control		-	0.021785	0.977704	-	0.059534	0	0
25	TNFa	LPS	32.5	-	1.91E-12	1.966608	-	0.999628	3.187054	0.041572
33	TNFa	LPS+HSS		-	0.007067	1.007046	-	0.00251	0	0
17	TNFa	LRS		-	0.01229	0.98441	-	0.097089	0	0

Data for cord 4

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Quantitation Report

Experiment Information

Run Name	Joe HSS+LPS 02_02 2005 2006-02-08 (2)
Run Start	8/02/2006 11:35:05 a.m.
Run Finish	8/02/2006 1:45:47 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

Threshold	0.0055
Left Threshold	14.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

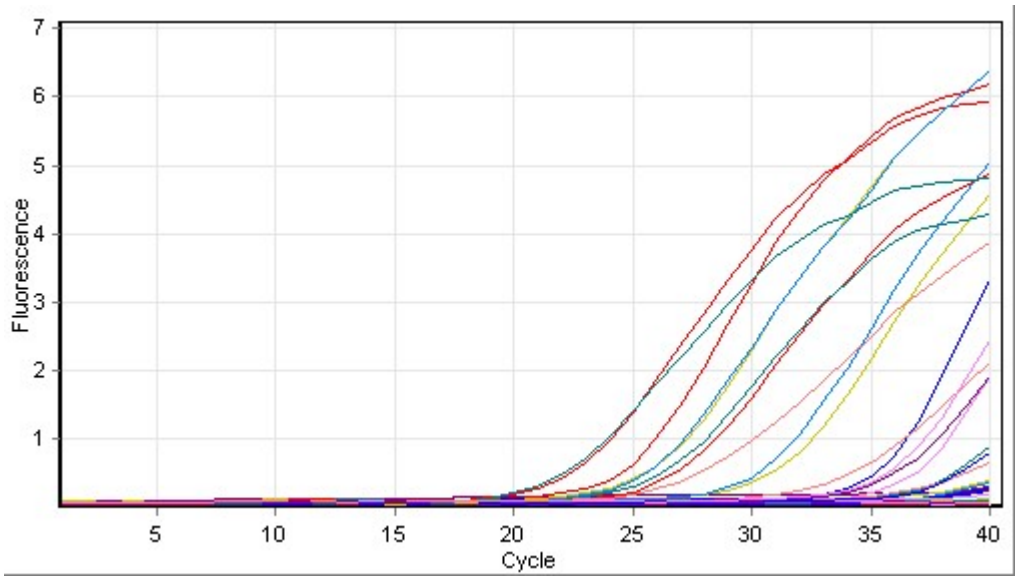
Messages

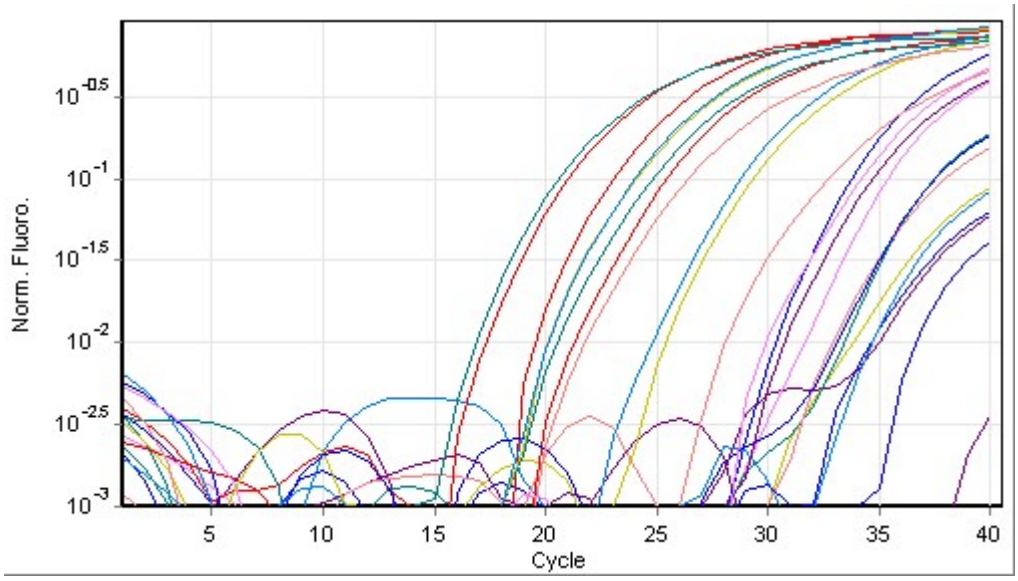
Message

Profile

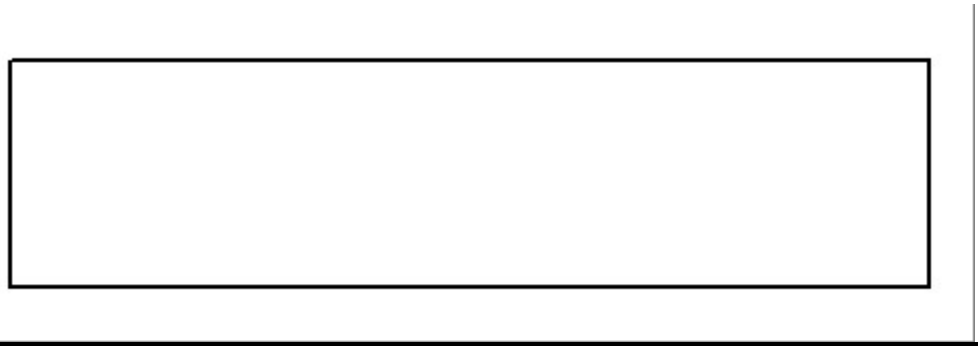
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		B2M-Blank	Unknown	NEG (NTC)			
2		IL1B-Blank	Unknown	NEG (NTC)			
3		iNOS-Blank	Unknown	33.54			
4		TLR4-Blank	Unknown				
5		TNFa-Blank	Unknown	NEG (NTC)			
6		TF-Blank	Unknown	NEG (NTC)			
7		ESel-Blank	Unknown	NEG (NTC)			
8		ICAM-Blank	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		B2M-Control	Unknown	18.99			
10		IL1B-Control	Unknown	32.48			
11		iNOS-Control	Unknown	29.73			

12		TLR4-Control	Unknown	33.09			
13		TNFa-Control	Unknown	30.75			
14		TF-Control	Unknown	33.83			
15		ESel-Control	Unknown	32.51			
16		ICAM-Control	Unknown	31.99			
17		B2M-LPS	Unknown	20.53			
18		IL1B-LPS	Unknown	24.68			
19		iNOS-LPS	Unknown	35.94			
20		TLR4-LPS	Unknown	NEG (NTC)			
21		TNFa-LPS	Unknown	NEG (NTC)			
22		TF-LPS	Unknown	23.90			
23		ESel-LPS	Unknown	19.79			
24		ICAM-LPS	Unknown	27.38			
25		B2M-LPS+HSS	Unknown	16.63			
26		IL1B-LPS+HSS	Unknown	19.53			
27		iNOS-LPS+HSS	Unknown	32.06			
28		TLR4-LPS+HSS	Unknown	30.05			
29		TNFa-LPS+HSS	Unknown	29.20			
30		TF-LPS+HSS	Unknown	19.50			
31		ESel-LPS+HSS	Unknown	16.21			
32		ICAM-LPS+HSS	Unknown	20.95			
33		Empty	Unknown	NEG (NTC)			
34		Empty	Unknown	NEG (NTC)			
35		Empty	Unknown	NEG (NTC)			
36		Empty	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	Joe HSS+LPS 02_02 2005 2006-02-08 (2)
Run Start	8/02/2006 11:35:05 a.m.
Run Finish	8/02/2006 1:45:47 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	81.5°c
Threshold	0.10453

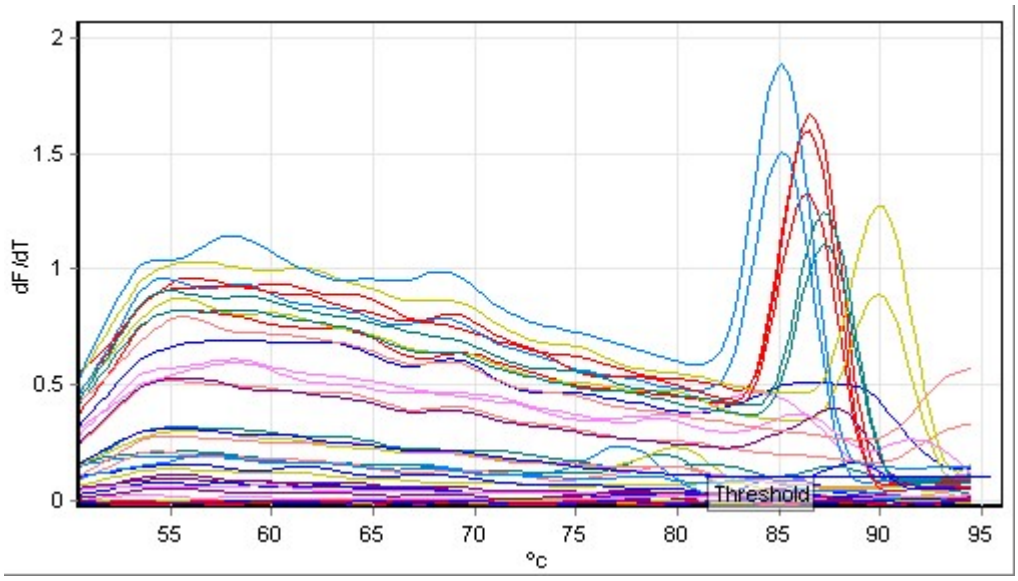
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	B2M-Blank			
2	IL1B-Blank			
3	iNOS-Blank		85.3	
4	TLR4-Blank			
5	TNFa-Blank			
6	TF-Blank			
7	ESel-Blank			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	ICAM-Blank			
9	B2M-Control		86.5	

10	IL1B-Control			
11	iNOS-Control		86.3	
12	TLR4-Control			
13	TNFa-Control		84.0	
14	TF-Control			
15	ESel-Control		87.7	
16	ICAM-Control			
17	B2M-LPS		86.5	
18	IL1B-LPS		90.0	
19	iNOS-LPS			
20	TLR4-LPS			
21	TNFa-LPS			
22	TF-LPS		85.2	
23	ESel-LPS		87.3	
24	ICAM-LPS			
25	B2M-LPS+HSS		86.5	
26	IL1B-LPS+HSS		90.0	
27	iNOS-LPS+HSS		88.5	
28	TLR4-LPS+HSS		87.7	
29	TNFa-LPS+HSS		86.0	91.5
30	TF-LPS+HSS		85.0	89.5
31	ESel-LPS+HSS		87.3	
32	ICAM-LPS+HSS			
33	Empty			
34	Empty			
35	Empty			
36	Empty			

Bin	Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (cord 4)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
9	B2M	Control	18.99	-	3.94E-07	1.7562	-	0.999762	253957.2	HK
17	B2M	LPS	20.53	-	1.45E-07	1.729365	-	0.990288	146353.6	HK
25	B2M	LPS+HSS	16.63	-	1.99E-06	1.723149	-	0.993131	1315743	HK
15	ESel	Control	32.51	-	0.000975	1.134163	-	0.635693	1.87E+08	736.1406
23	ESel	LPS	19.79	-	4E-07	1.69247	-	0.991273	336348.9	2.298194
31	ESel	LPS+HSS	16.21	-	3.33E-06	1.690181	-	0.990027	2261626	1.718896
16	ICAM	Control	31.99	-	0.002773	1.09298	-	0.434829	6.52E+08	2566.006
24	ICAM	LPS	27.38	-	7.34E-09	1.696327	-	0.942547	5824.313	0.039796
32	ICAM	LPS+HSS	20.95	-	3.98E-07	1.640939	-	0.978397	349165.9	0.265375
10	IL1B	Control	32.48	-	4.31E-08	1.47196	-	0.986898	39441.58	0.155308
18	IL1B	LPS	24.68	-	5.73E-08	1.661645	-	0.998611	40395.33	0.276012
26	IL1B	LPS+HSS	19.53	-	3.62E-07	1.713995	-	0.957488	301307.2	0.229002
11	iNOS	Control	29.73	-	1.43E-08	1.631108	-	0.997915	5396.455	0.021249
19	iNOS	LPS	35.94	-	5.2E-08	1.456074	-	1	15289.91	0.104472
27	iNOS	LPS+HSS	32.06	-	3.86E-10	1.709072	-	0.999995	386.2952	0.000294
14	TF	Control	33.83	-	0.003882	1.051144	-	0.103672	2.07E+09	8158.893
22	TF	LPS	23.9	-	2.52E-08	1.724263	-	0.995058	24796.67	0.16943
30	TF	LPS+HSS	19.5	-	9.36E-07	1.658458	-	0.997532	582078.7	0.442395
12	TLR4	Control	33.09	-	5.45E-10	1.640059	-	0.991642	870.0027	0.003426
20	TLR4	LPS		-	0.001585	1.094295	-	0.462626	0	0
28	TLR4	LPS+HSS	30.05	-	2.03E-08	1.591632	-	0.994594	9634.789	0.007323
13	TNFa	Control	30.75	-	2.65E-08	1.537948	-	0.619017	19987.74	0.078705
21	TNFa	LPS		-	0.007691	1.045466	-	0.294483	0	0
29	TNFa	LPS+HSS	29.2	-	1.9E-09	1.706186	-	0.966102	1879.529	0.001428

Data for cord 5

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F +61 2 9736 1364
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Quantitation Report

Experiment Information

Run Name	2006-02-07 (1)
Run Start	7/02/2006 5:43:17 p.m.
Run Finish	7/02/2006 7:54:30 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation Information

Threshold	0.00224
Left Threshold	17.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

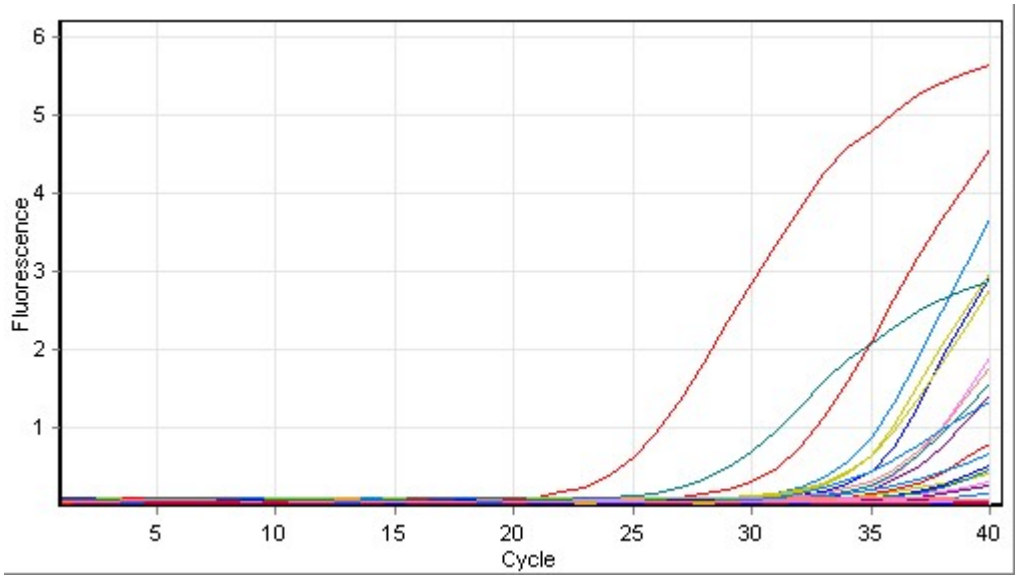
Messages

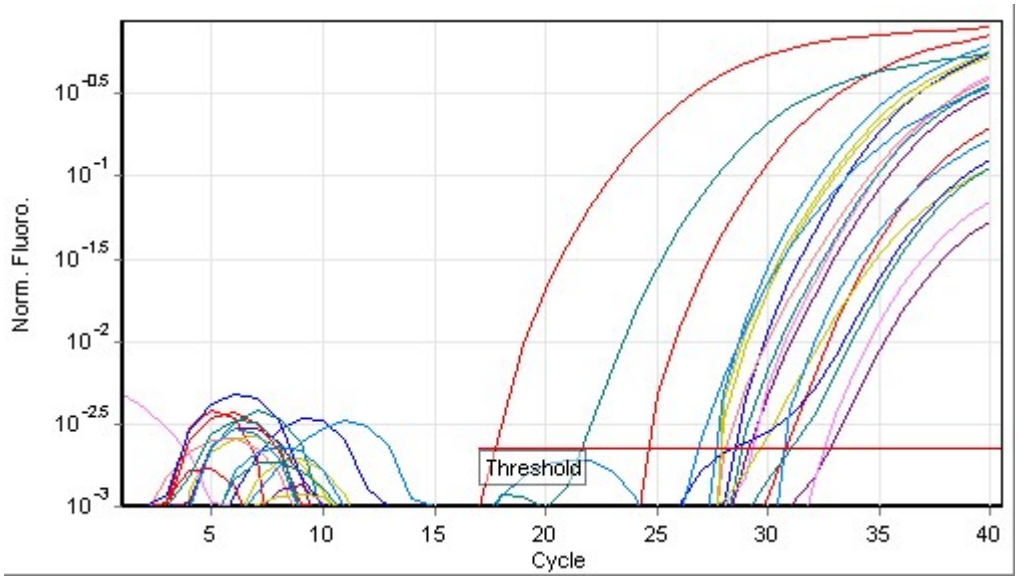
Message

Profile

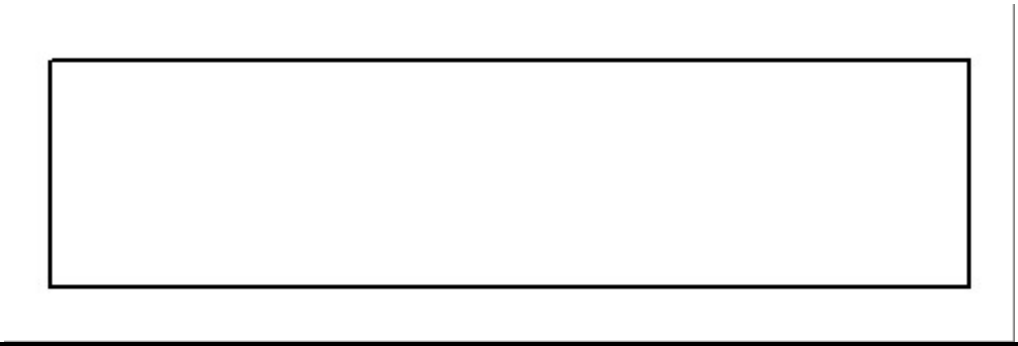
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (copies/ml)	Calc Conc (copies/ml)	% Var
1		B2M-Blank	Unknown	NEG (NTC)			
2		IL1B-Blank	Unknown	NEG (NTC)			
3		iNOS-Blank	Unknown	NEG (NTC)			
4		TLR4-Blank	Unknown	NEG (NTC)			
5		TNFa-Blank	Unknown	NEG (NTC)			
6		TF-Blank	Unknown	NEG (NTC)			
7		ESel-Blank	Unknown	NEG (NTC)			
8		ICAM-Blank	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (copies/ml)	Calc Conc (copies/ml)	% Var
9		B2M-Control	Unknown	17.81			
10		IL1B-Control	Unknown	27.57			
11		iNOS-Control	Unknown	28.44			

12		TLR4-Control	Unknown	29.15			
13		TNFa-Control	Unknown	29.23			
14		TF-Control	Unknown	27.49			
15		ESel-Control	Unknown	28.75			
16		ICAM-Control	Unknown	28.06			
17		B2M-Ringers	Unknown	30.82			
18		IL1B-Ringers	Unknown	29.67			
19		iNOS-Ringers	Unknown	28.32			
20		TLR4-Ringers	Unknown	32.75			
21		TNFa-Ringers	Unknown	32.45			
22		TF-Ringers	Unknown	30.61			
23		ESel-Ringers	Unknown	31.07			
24		ICAM-Ringers	Unknown	NEG (NTC)			
25		B2M-HSS	Unknown	24.46			
26		IL1B-HSS	Unknown	27.73			
27		iNOS-HSS	Unknown	NEG (NTC)			
28		TLR4-HSS	Unknown	NEG (NTC)			
29		TNFa-HSS	Unknown	NEG (NTC)			
30		TF-HSS	Unknown	26.85			
31		ESel-HSS	Unknown	21.68			
32		ICAM-HSS	Unknown	NEG (NTC)			
33		Empty	Unknown	NEG (NTC)			
34		Empty	Unknown	NEG (NTC)			
35		Empty	Unknown	NEG (NTC)			
36		Empty	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	2006-02-07 (1)
Run Start	7/02/2006 5:43:17 p.m.
Run Finish	7/02/2006 7:54:30 p.m.
Operator	Joe
Notes	
Run On Software Version	Rotor-Gene 6.0.27
Run Signature	The Run Signature is valid.
Gain Green	5.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	81°c
Threshold	0.21494

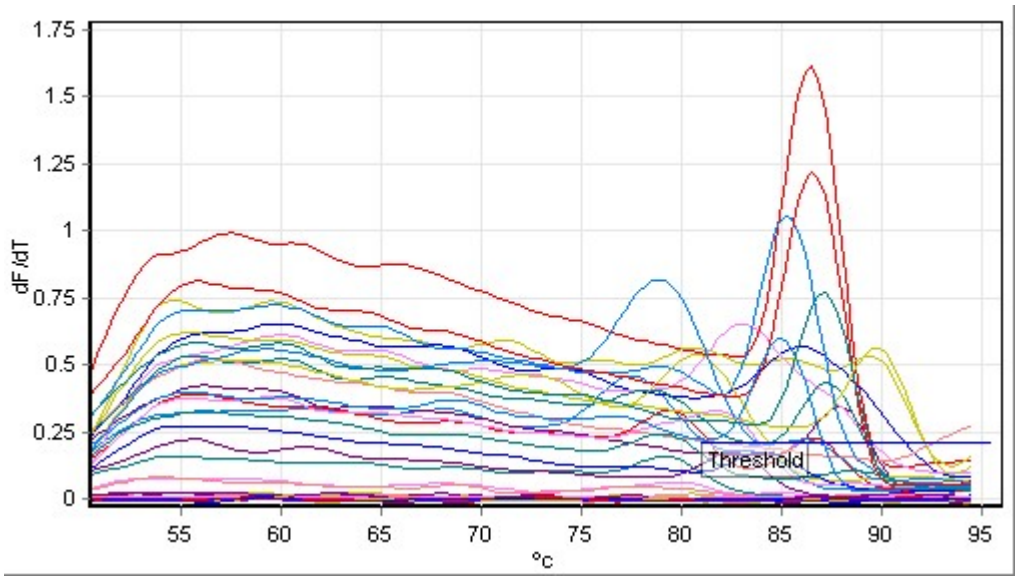
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 15 secs, acquiring to Cycling B([Green][1][1])
Melt (50-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	B2M-Blank			
2	IL1B-Blank			
3	iNOS-Blank			
4	TLR4-Blank			
5	TNFa-Blank			
6	TF-Blank			
7	ESel-Blank			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	ICAM-Blank			
9	B2M-Control		86.5	

10	IL1B-Control		85.3	89.5
11	iNOS-Control		86.0	
12	TLR4-Control		88.0	
13	TNFa-Control		83.0	
14	TF-Control		85.3	
15	ESel-Control		87.3	
16	ICAM-Control			
17	B2M-Ringers		86.5	
18	IL1B-Ringers			
19	iNOS-Ringers			
20	TLR4-Ringers			
21	TNFa-Ringers		81.7	
22	TF-Ringers		86.0	
23	ESel-Ringers			
24	ICAM-Ringers			
25	B2M-HSS		86.5	
26	IL1B-HSS		89.7	
27	iNOS-HSS			
28	TLR4-HSS			
29	TNFa-HSS			
30	TF-HSS		85.0	
31	ESel-HSS		81.5	87.0
32	ICAM-HSS			
33	Empty			
34	Empty			
35	Empty			
36	Empty			

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (Cord 5)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
9	B2M	Control	17.81	-	1.76E-07	1.817155	-	0.981978	268809.8	HK
25	B2M	HSS	24.46	-	6.19E-09	1.781602	-	0.98013	8210.902	HK
17	B2M	Ringers	30.82	-	5.71E-10	1.695236	-	0.98909	964.5948	HK
15	ESel	Control	28.75	-	4.23E-10	1.764214	-	0.999972	913.8329	0.0034
31	ESel	HSS	21.68	-	4.94E-08	1.72976	-	0.979593	77568.02	9.446954
23	ESel	Ringers	31.07	-	0.006231	1.048899	-	0.179867	2.54E+09	2634380
16	ICAM	Control	28.06	-	1.43E-09	1.716102	-	0.979824	2937.239	0.010927
32	ICAM	HSS		-	0.008431	0.998161	-	0.000546	0	0
24	ICAM	Ringers		-	0.015428	1.002578	-	0.002365	0	0
10	IL1B	Control	27.57	-	3.74E-10	1.829152	-	0.991189	659.1605	0.002452
26	IL1B	HSS	27.73	-	4.37E-10	1.82031	-	0.987545	684.5226	0.083368
18	IL1B	Ringers	29.67	-	0.002591	1.074767	-	0.25483	1.32E+09	1367010
11	iNOS	Control	28.44	-	1.44E-09	1.736738	-	0.9974	1702.85	0.006335
27	iNOS	HSS		-	0.015412	1.004069	-	0.007726	0	0
19	iNOS	Ringers	28.32	-	1.67E-09	1.626135	-	0.995635	11729.13	12.15964
14	TF	Control	27.49	-	3.58E-10	1.853491	-	0.995654	481.0175	0.001789
30	TF	HSS	26.85	-	4.36E-09	1.694996	-	0.891614	7871.165	0.958624
22	TF	Ringers	30.61	-	0.005627	1.084204	-	0.566989	9.43E+08	977525
12	TLR4	Control	29.15	-	1.7E-09	1.683511	-	0.993727	2851.153	0.010607
28	TLR4	HSS		-	0.019146	0.990066	-	0.02005	0	0
20	TLR4	Ringers	32.75	-	0.005739	1.034247	-	0.099425	3.72E+09	3854187
13	TNFa	Control	29.23	-	7.67E-10	1.730885	-	0.998424	1215.175	0.004521
29	TNFa	HSS		-	0.011109	0.984088	-	0.055493	0	0
21	TNFa	Ringers	32.45	-	4.69E-10	1.651856	-	0.988181	946.2747	0.981007

Data for cord 6

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Quantitation Report

Experiment Information

Run Name	Tumour conditoned cord 10.04.07
Run Start	29/04/2007 12:40:46 p.m.
Run Finish	29/04/2007 2:39:17 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	5.33

Quantitation Information

Threshold	0.0025
Left Threshold	23.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

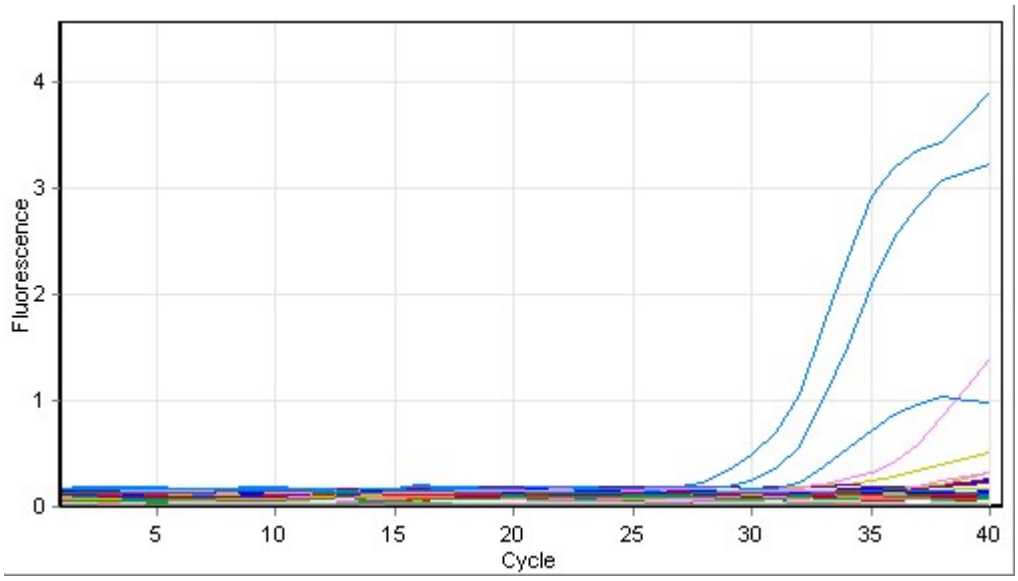
Messages

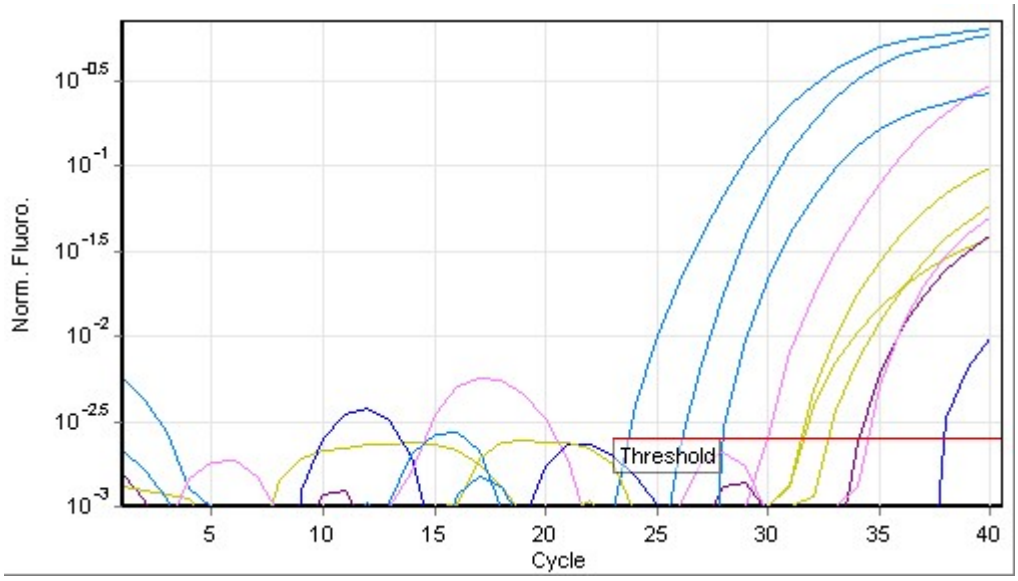
Message

Profile

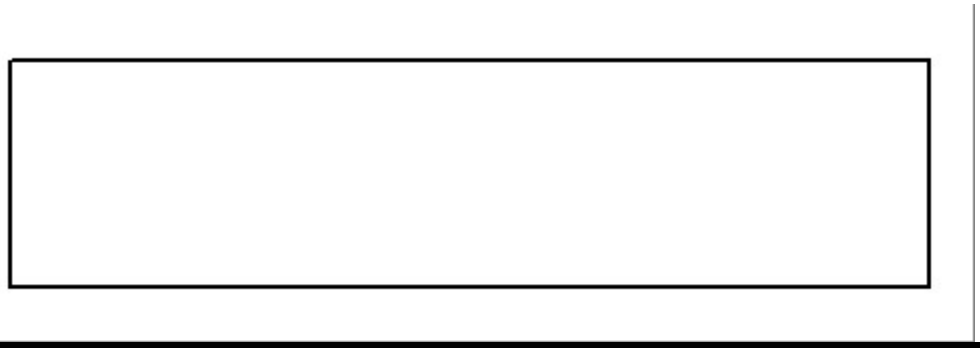
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-negative	Unknown	NEG (NTC)			
2		ESEL-Neg	Unknown	31.40			
3		ICAM-Neg	Unknown	37.76			
4		IL1B-Neg	Unknown	NEG (NTC)			
5		IL6-Neg	Unknown	29.99			
6		IL8-Neg	Unknown	23.58			
7		TNF-Neg	Unknown	NEG (NTC)			
8		TF-Neg	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	NEG (NTC)			
10		HSPY-TCM	Unknown	NEG (NTC)			
11		ESEL-TCM	Unknown	31.49			

12		ICAM-TCM	Unknown	NEG (NTC)			
13		IL1B-TCM	Unknown	34.04			
14		IL6-TCM	Unknown	NEG (NTC)			
15		IL8-TCM	Unknown	27.89			
16		TNF-TCM	Unknown	NEG (NTC)			
17		TF-TCM	Unknown	NEG (NTC)			
18		RNAPol-TCM	Unknown	NEG (NTC)			
19		HSPY-TCM	Unknown	NEG (NTC)			
20		ESEL-TCM	Unknown	32.56			
21		ICAM-TCM	Unknown	NEG (NTC)			
22		IL1B-TCM	Unknown	NEG (NTC)			
23		IL6-TCM	Unknown	34.38			
24		IL8-TCM	Unknown	26.09			
25		TNF-TCM	Unknown	NEG (NTC)			
26		TF-TCM	Unknown	NEG (NTC)			
27		RNAPol-TCM	Unknown	NEG (NTC)			
28			Unknown	NEG (NTC)			
29			Unknown	NEG (NTC)			
30			Unknown	NEG (NTC)			
31			Unknown	NEG (NTC)			
32			Unknown	NEG (NTC)			
33			Unknown	NEG (NTC)			
34			Unknown	NEG (NTC)			
35			Unknown	NEG (NTC)			
36			Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	Tumour conditoned cord 10.04.07
Run Start	29/04/2007 12:40:46 p.m.
Run Finish	29/04/2007 2:39:17 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	5.33

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	76°c
Threshold	0.13886

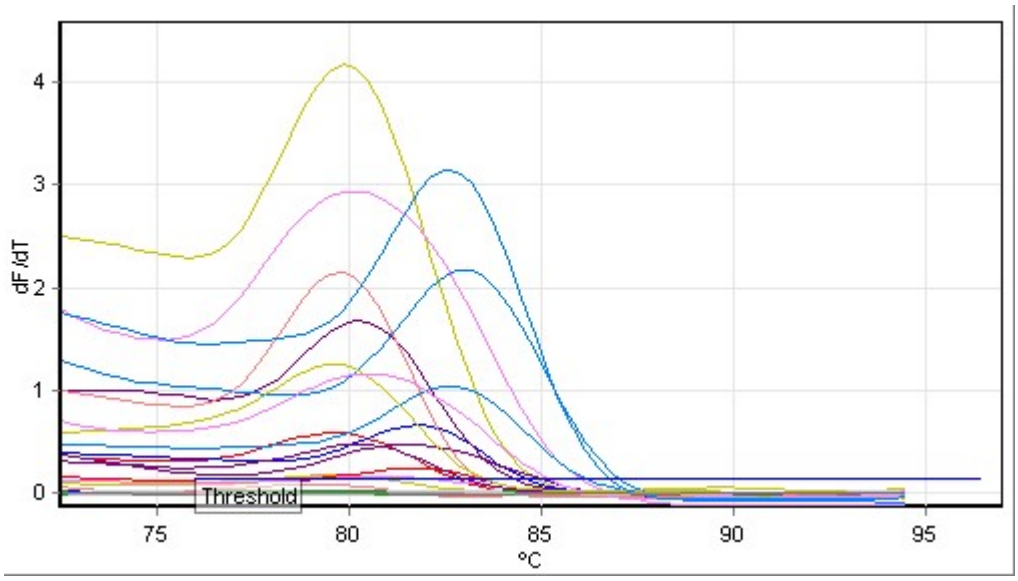
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1
1	HSPY-negative		79.5
2	ESEL-Neg		80.0
3	ICAM-Neg		81.7
4	IL1B-Neg		80.3
5	IL6-Neg		80.2
6	IL8-Neg		83.0
7	TNF-Neg		

(Continued on next page)...

No.	Name	Genotype	Peak 1
8	TF-Neg		
9	RNAPol-Neg		

10	HSPY-TCM		
11	ESEL-TCM		79.7
12	ICAM-TCM		
13	IL1B-TCM		81.5
14	IL6-TCM		80.3
15	IL8-TCM		82.7
16	TNF-TCM		
17	TF-TCM		79.7
18	RNAPol-TCM		
19	HSPY-TCM		81.7
20	ESEL-TCM		79.5
21	ICAM-TCM		
22	IL1B-TCM		80.2
23	IL6-TCM		80.5
24	IL8-TCM		82.5
25	TNF-TCM		
26	TF-TCM		
27	RNAPol-TCM		
28			
29			
30			
31			
32			
33			
34			
35			
36			

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (TCM cord 6)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
11	ESEL	TCM	31.49	32.025	3.53E-14	2.148814	1.903401	0.907601	12.51001	2.84E-09
20	ESEL	TCM	32.56	32.025	3.82E-10	1.657988	1.903401	0.966717	12.51001	2.84E-09
10	HSPY	TCM	40	40	0.01093	1.003365	1.023565	0.004595	4.41E+09	HK
19	HSPY	TCM	40	40	0.003481	1.043764	1.023565	0.26127	4.41E+09	HK
12	ICAM	TCM	40	40	0.015959	0.977959	1.515104	0.028607	678.4448	1.54E-07
21	ICAM	TCM	40	40	2.33E-13	2.05225	1.515104	0.779946	678.4448	1.54E-07
13	IL1B	TCM	34.04	37.02	4.4E-09	1.535843	1.34792	0.936597	177439.3	4.02E-05
22	IL1B	TCM	40	37.02	0.003453	1.159997	1.34792	0.827689	177439.3	4.02E-05
14	IL6	TCM	40	37.19	0.008246	1.021085	1.432685	0.287702	17457.12	3.96E-06
23	IL6	TCM	34.38	37.19	6.94E-12	1.844285	1.432685	0.985744	17457.12	3.96E-06
15	IL8	TCM	27.89	26.99	2.49E-24	4.862226	3.466066	0.98279	3.01E-05	6.83E-15
24	IL8	TCM	26.09	26.99	2.62E-11	2.069906	3.466066	0.99122	3.01E-05	6.83E-15
18	RNAPol	TCM	40	40	0.016631	0.982478	1.283653	0.072747	514418.1	0.000117
27	RNAPol	TCM	40	40	1.28E-05	1.584827	1.283653	0.872758	514418.1	0.000117
17	TF	TCM	40	40	1.84E-19	4.123758	2.68446	0.583222	7.85E-08	1.78E-17
26	TF	TCM	40	40	1.67E-05	1.245161	2.68446	0.715686	7.85E-08	1.78E-17
16	TNF	TCM	40	40	1.51E-10	1.950443	1.723103	0.985099	3.951538	8.96E-10
25	TNF	TCM	40	40	0.000737	1.495764	1.723103	0.736823	3.951538	8.96E-10

Data for cord 6

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Quantitation Report

Experiment Information

Run Name	TCM-DMXAA cord 10.4.07
Run Start	29/04/2007 3:04:45 p.m.
Run Finish	29/04/2007 5:00:58 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.00314
Left Threshold	6.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

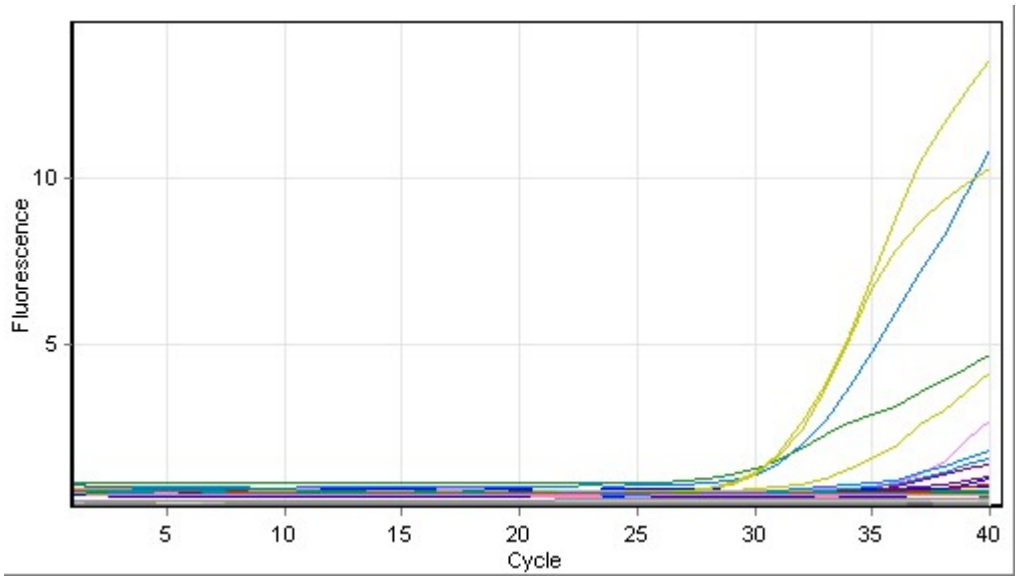
Messages

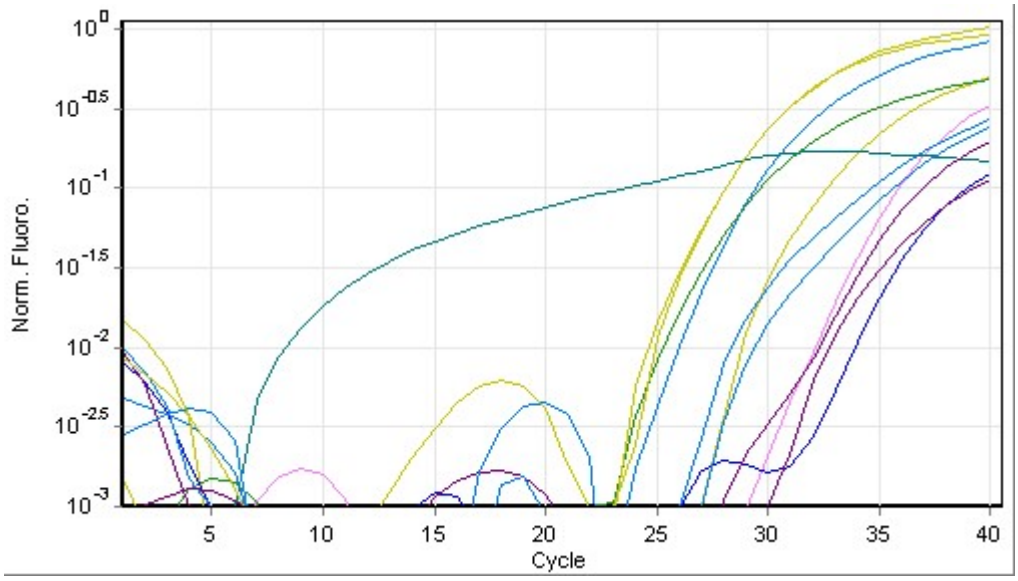
Message

Profile

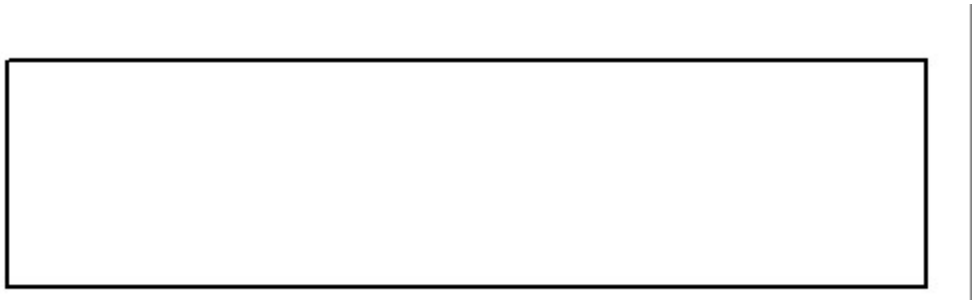
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-negative	Unknown	NEG (NTC)			
2		ESEL-Neg	Unknown	NEG (Multi Ct)			
3		ICAM-Neg	Unknown	NEG (NTC)			
4		IL1B-Neg	Unknown	NEG (NTC)			
5		IL6-Neg	Unknown	30.53			
6		IL8-Neg	Unknown	24.64			
7		TNF-Neg	Unknown	NEG (NTC)			
8		TF-Neg	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	23.84			
10		HSPY-TCM/DMXAA	Unknown	NEG (NTC)			
11		ESEL-TCM/DMXAA	Unknown	23.55			

12		ICAM-TCM/DMXAA	Unknown	32.22			
13		IL1B-TCM/DMXAA	Unknown	31.28			
14		IL6-TCM/DMXAA	Unknown	NEG (NTC)			
15		IL8-TCM/DMXAA	Unknown	NEG (Multi Ct)			
16		TNF-TCM/DMXAA	Unknown	6.61			
17		TF-TCM/DMXAA	Unknown	NEG (NTC)			
18		RNAPol-TCM/DMXAA	Unknown	NEG (NTC)			
19		HSPY-TCM/DMXAA	Unknown	NEG (NTC)			
20		ESEL-TCM/DMXAA	Unknown	24.10			
21		ICAM-TCM/DMXAA	Unknown	NEG (NTC)			
22		IL1B-TCM/DMXAA	Unknown	29.86			
23		IL6-TCM/DMXAA	Unknown	NEG (NTC)			
24		IL8-TCM/DMXAA	Unknown	27.95			
25		TNF-TCM/DMXAA	Unknown	NEG (NTC)			
26		TF-TCM/DMXAA	Unknown	NEG (NTC)			
27		RNAPol-TCM/DMXAA	Unknown	NEG (NTC)			
28			Unknown	NEG (NTC)			
29			Unknown	NEG (NTC)			
30			Unknown	NEG (NTC)			
31			Unknown	NEG (NTC)			
32			Unknown	NEG (NTC)			
33			Unknown	NEG (NTC)			
34			Unknown	NEG (NTC)			
35			Unknown	NEG (NTC)			
36			Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	TCM-DMXAA cord 10.4.07
Run Start	29/04/2007 3:04:45 p.m.
Run Finish	29/04/2007 5:00:58 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	77°c
Threshold	0.24167

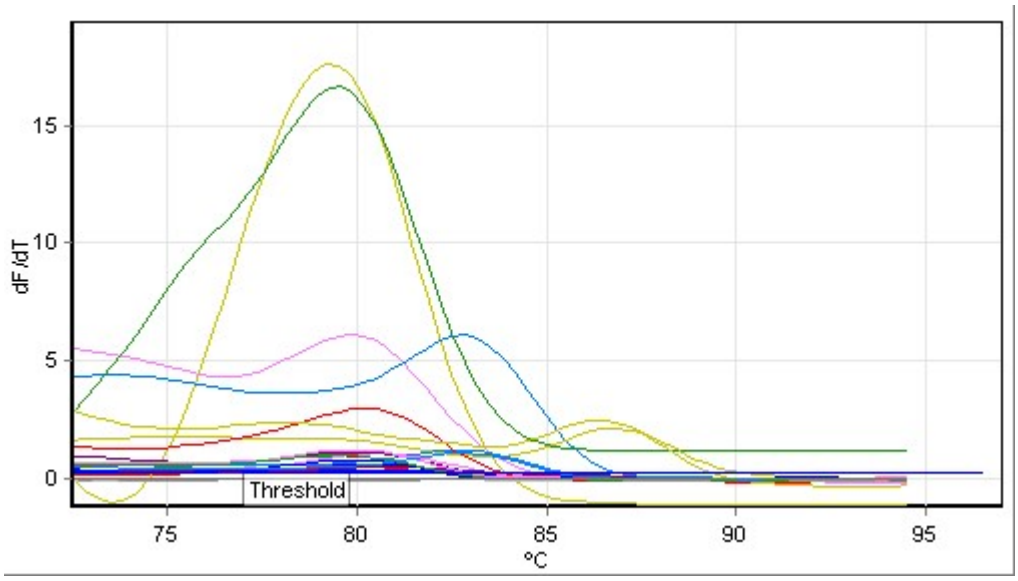
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2	Peak 3
1	HSPY-negative		80.0		
2	ESEL-Neg		79.3		
3	ICAM-Neg		80.5		
4	IL1B-Neg				
5	IL6-Neg		79.8		
6	IL8-Neg		82.7		
7	TNF-Neg				

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2	Peak 3
8	TF-Neg				
9	RNAPol-Neg		79.5	88.5	94.0

10	HSPY-TCM/DMXAA		79.3		
11	ESEL-TCM/DMXAA		78.7	86.8	
12	ICAM-TCM/DMXAA		80.5		
13	IL1B-TCM/DMXAA		80.8		
14	IL6-TCM/DMXAA		80.5		
15	IL8-TCM/DMXAA		82.5		
16	TNF-TCM/DMXAA				
17	TF-TCM/DMXAA				
18	RNAPol-TCM/DMXAA		79.5		
19	HSPY-TCM/DMXAA		80.2		
20	ESEL-TCM/DMXAA		78.0	86.5	
21	ICAM-TCM/DMXAA		80.0		
22	IL1B-TCM/DMXAA		80.0	85.0	
23	IL6-TCM/DMXAA		80.0		
24	IL8-TCM/DMXAA		82.7		
25	TNF-TCM/DMXAA				
26	TF-TCM/DMXAA				
27	RNAPol-TCM/DMXAA		79.5		
28					
29					
30					
31					
32					
33					
34					
35					
36					

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (TCM+DMXAA cord 6)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
11	ESEL	TCM/DMXAA	23.55	23.825	4.42E-10	1.998925	1.83035	0.999403	6227.876	128.3869
20	ESEL	TCM/DMXAA	24.1	23.825	1.58E-07	1.661775	1.83035	0.97975	6227.876	128.3869
10	HSPY	TCM/DMXAA	40	40	0.019301	0.986577	1.618397	0.157757	48.50865	HK
19	HSPY	TCM/DMXAA	40	40	2.68E-15	2.250217	1.618397	0.798706	48.50865	HK
12	ICAM	TCM/DMXAA	32.22	36.11	2.27E-20	3.153533	2.246777	0.966377	0.002261	4.66E-05
21	ICAM	TCM/DMXAA	40	36.11	7.23E-07	1.340021	2.246777	0.928804	0.002261	4.66E-05
13	IL1B	TCM/DMXAA	31.28	30.57	1.87E-11	1.823823	1.79331	0.927223	197.23	4.065873
22	IL1B	TCM/DMXAA	29.86	30.57	2.53E-10	1.762797	1.79331	0.993672	197.23	4.065873
14	IL6	TCM/DMXAA	40	40	0.009012	1.059099	1.349435	0	69685.2	1436.552
23	IL6	TCM/DMXAA	40	40	0.00082	1.639771	1.349435	0.684768	69685.2	1436.552
15	IL8	TCM/DMXAA	40	33.975	7.01E-09	1.683603	1.657239	0.989131	394.1011	8.124346
24	IL8	TCM/DMXAA	27.95	33.975	4.38E-09	1.630875	1.657239	0.953473	394.1011	8.124346
18	RNAPol	TCM/DMXAA	40	40	0.007405	1.020714	1.025332	0.26322	4.12E+09	84881841
27	RNAPol	TCM/DMXAA	40	40	0.004533	1.029951	1.025332	0.206485	4.12E+09	84881841
17	TF	TCM/DMXAA	40	40	0.046825	0.763657	1.586236	1	108.2707	2.231987
26	TF	TCM/DMXAA	40	40	2.23E-11	2.408814	1.586236	0.74503	108.2707	2.231987
16	TNF	TCM/DMXAA	6.61	23.305	0.211885	0.334199	0.723299	1	2.13E+13	4.39E+11
25	TNF	TCM/DMXAA	40	23.305	0.008147	1.1124	0.723299	0.701517	2.13E+13	4.39E+11

Data for cord 8

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Quantitation Report

Experiment Information

Run Name	control cord 2007-03-05
Run Start	5/03/2007 1:09:54 p.m.
Run Finish	5/03/2007 3:06:52 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.01015
Left Threshold	14.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

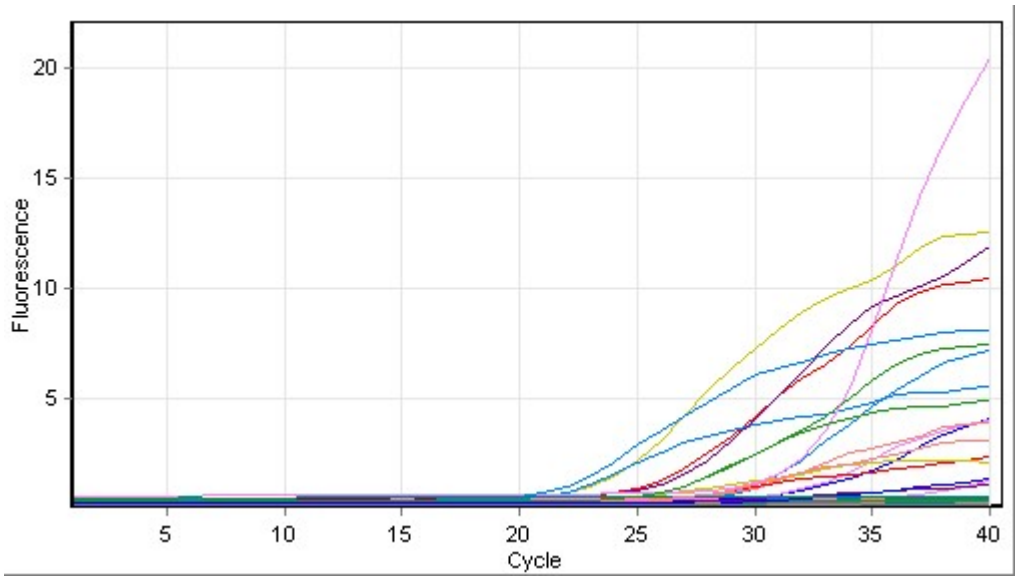
Messages

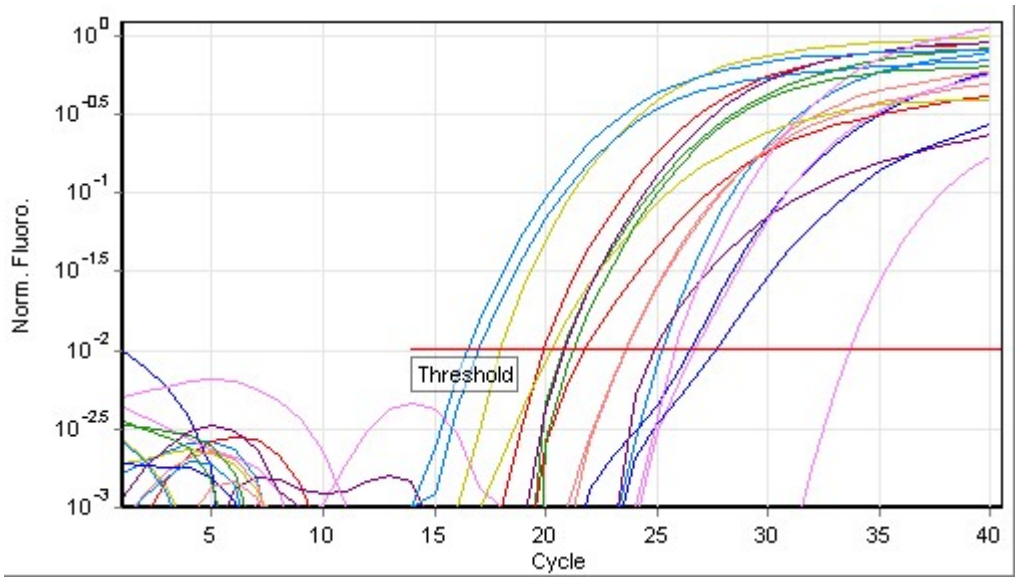
Message

Profile

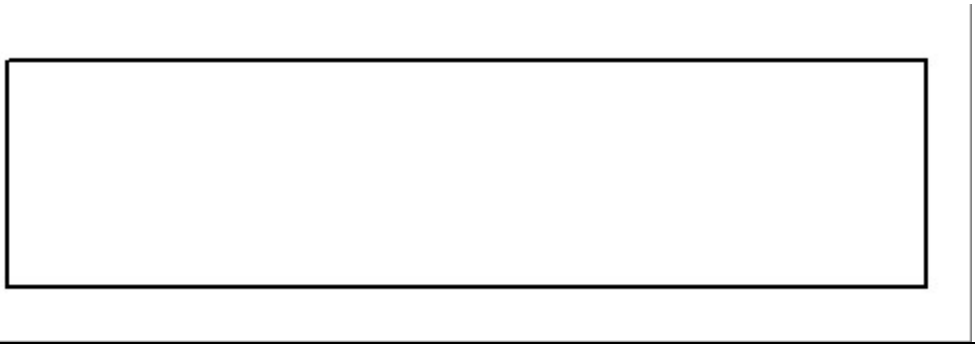
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-negative	Unknown	NEG (NTC)			
2		ESEL-Negative	Unknown	NEG (NTC)			
3		ICAM-Negative	Unknown	NEG (NTC)			
4		IL1B-Negative	Unknown	NEG (NTC)			
5		IL6-Negative	Unknown	33.64			
6		IL8-Negative	Unknown	25.22			
7		TNF-Negative	Unknown	NEG (NTC)			
8		TF-Negative	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Negative	Unknown	NEG (NTC)			
10		HSPY-PBS	Unknown	19.91			
11		ESEL-PBS	Unknown	17.93			

12		ICAM-PBS	Unknown	26.50			
13		IL1B-PBS	Unknown	24.88			
14		IL6-PBS	Unknown	26.73			
15		IL8-PBS	Unknown	16.95			
16		TNF-PBS	Unknown	NEG (NTC)			
17		TF-PBS	Unknown	23.58			
18		RNAPol-PBS	Unknown	20.77			
19		HSPY-PBS	Unknown	21.77			
20		ESEL-PBS	Unknown	20.26			
21		ICAM-PBS	Unknown	27.68			
22		IL1B-PBS	Unknown	20.85			
23		IL6-PBS	Unknown	25.76			
24		IL8-PBS	Unknown	16.44			
25		TNF-PBS	Unknown	NEG (NTC)			
26		TF-PBS	Unknown	23.54			
27		RNAPol-PBS	Unknown	21.26			
28		Blank	Unknown	NEG (NTC)			
29		Blank	Unknown	NEG (NTC)			
30		Blank	Unknown	NEG (NTC)			
31		Blank	Unknown	NEG (NTC)			
32		Blank	Unknown	NEG (NTC)			
33		Blank	Unknown	NEG (NTC)			
34		Blank	Unknown	NEG (NTC)			
35		Blank	Unknown	NEG (NTC)			
36		Blank	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	control cord 2007-03-05
Run Start	5/03/2007 1:09:54 p.m.
Run Finish	5/03/2007 3:06:52 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	80°c
Threshold	0.10241

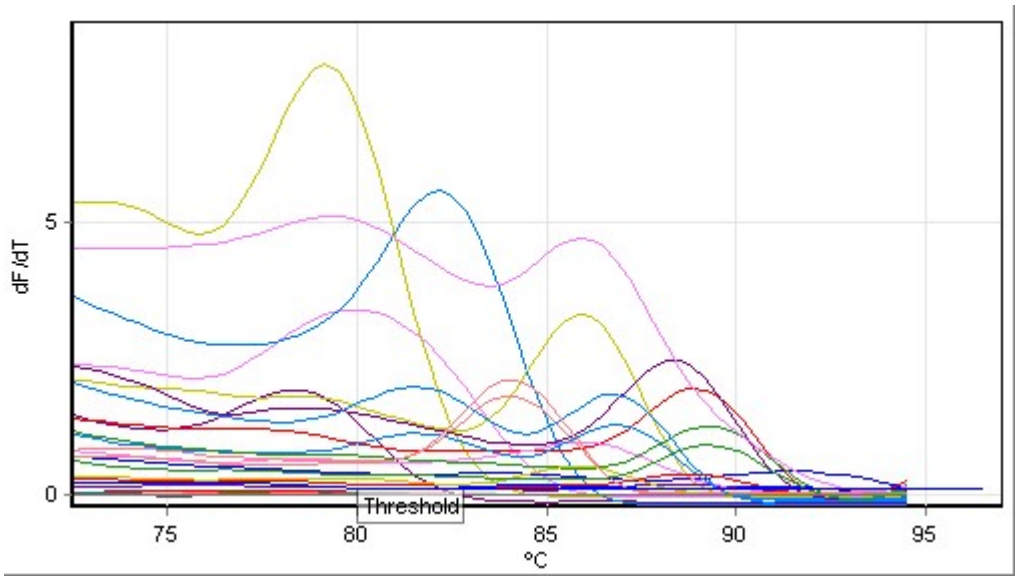
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	HSPY-negative			
2	ESEL-Negative			
3	ICAM-Negative			
4	IL1B-Negative			
5	IL6-Negative			
6	IL8-Negative		82.2	
7	TNF-Negative			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	TF-Negative			
9	RNAPol-Negative			

10	HSPY-PBS		88.8	
11	ESEL-PBS		86.0	
12	ICAM-PBS		83.5	91.5
13	IL1B-PBS		88.5	
14	IL6-PBS		86.0	
15	IL8-PBS		81.5	86.8
16	TNF-PBS			
17	TF-PBS		84.0	
18	RNAPol-PBS		89.3	
19	HSPY-PBS		88.7	
20	ESEL-PBS		85.8	
21	ICAM-PBS		84.5	91.2
22	IL1B-PBS		88.3	
23	IL6-PBS		86.0	
24	IL8-PBS		81.5	86.7
25	TNF-PBS			
26	TF-PBS		84.0	
27	RNAPol-PBS		81.0	89.3
28	Blank			
29	Blank			
30	Blank			
31	Blank			
32	Blank			
33	Blank			
34	Blank			
35	Blank			
36	Blank			

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (PBS Cord 8)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
11	ESEL	PBS	17.93	19.095	1.54E-06	1.739152	1.712735	0.98587	386283	1.57525
20	ESEL	PBS	20.26	19.095	5.52E-07	1.686317	1.712735	0.893893	386283	1.57525
10	HSPY	PBS	19.91	20.84	1.6E-06	1.660688	1.673371	0.997106	245220.1	HK
19	HSPY	PBS	21.77	20.84	1.55E-07	1.686054	1.673371	0.986496	245220.1	HK
12	ICAM	PBS	26.5	27.09	6.81E-08	1.633209	1.574158	0.977819	51425.86	0.209713
21	ICAM	PBS	27.68	27.09	2.36E-07	1.515106	1.574158	0.999929	51425.86	0.209713
13	IL1B	PBS	24.88	22.865	9.81E-06	1.387226	1.524627	0.993785	726414.7	2.962296
22	IL1B	PBS	20.85	22.865	1.04E-06	1.662028	1.524627	0.999362	726414.7	2.962296
14	IL6	PBS	26.73	26.245	4.33E-09	1.771743	1.753397	0.996124	4452.285	0.018156
23	IL6	PBS	25.76	26.245	3.49E-08	1.73505	1.753397	0.999384	4452.285	0.018156
15	IL8	PBS	16.95	16.695	1.17E-07	1.962544	1.948927	0.994419	162584.7	0.663015
24	IL8	PBS	16.44	16.695	2.73E-07	1.935311	1.948927	0.999735	162584.7	0.663015
18	RNAPol	PBS	20.77	21.015	1.58E-07	1.761302	1.804885	0.984133	45701.53	0.186369
27	RNAPol	PBS	21.26	21.015	3.67E-08	1.848468	1.804885	0.971414	45701.53	0.186369
17	TF	PBS	23.58	23.56	1.21E-07	1.671715	1.694399	0.967036	45041.33	0.183677
26	TF	PBS	23.54	23.56	5.74E-08	1.717082	1.694399	0.93655	45041.33	0.183677
16	TNF	PBS		0	28.0127	0.820429	0.92936	0.758613	0	0
25	TNF	PBS		0	0.023061	1.038291	0.92936	0.41115	0	0

Data for cord 8

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Quantitation Report

Experiment Information

Run Name	Tumour conditoned cord 2007-03-06 (1)
Run Start	6/03/2007 12:03:20 p.m.
Run Finish	6/03/2007 1:59:31 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.00419
Left Threshold	15.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

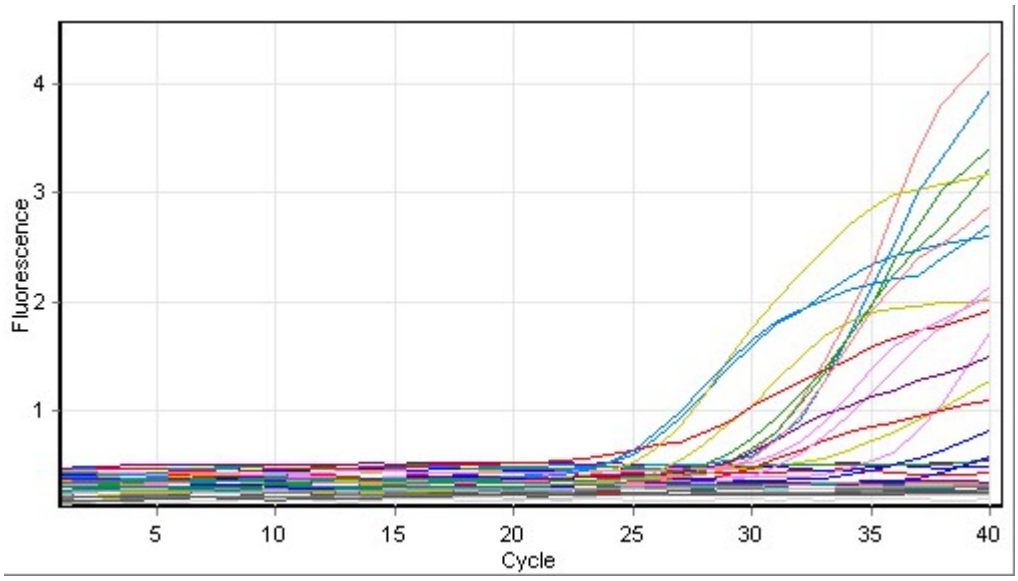
Messages

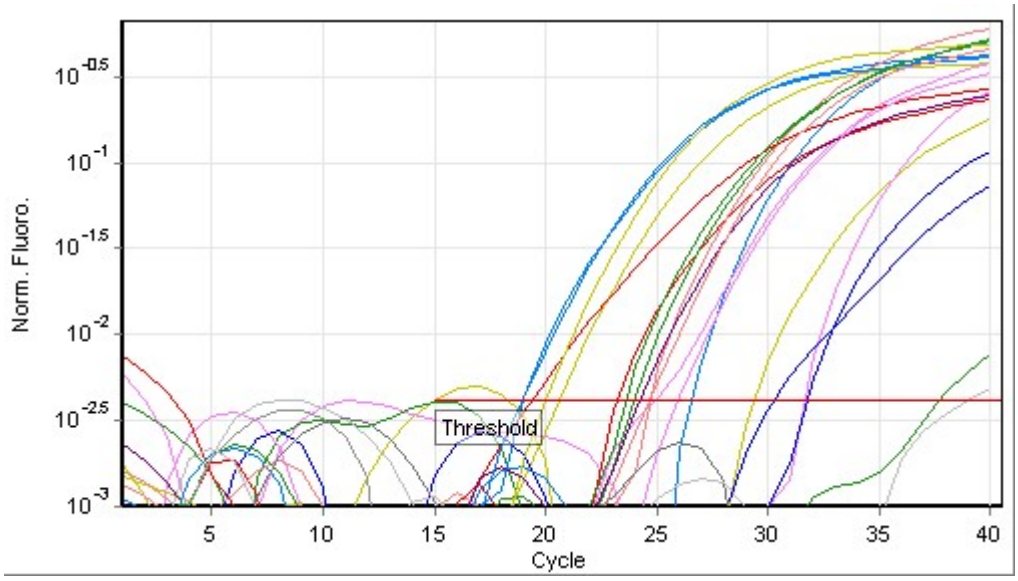
Message

Profile

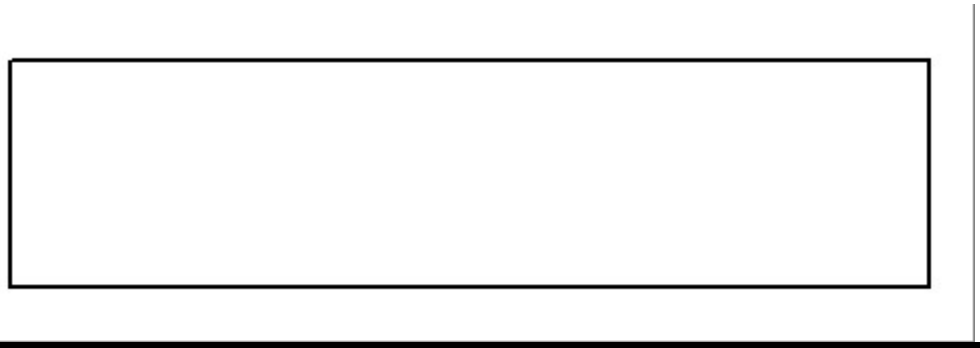
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-Neg	Unknown	NEG (NTC)			
2		ESEL-Neg	Unknown	NEG (Multi Ct)			
3		ICAM-Neg	Unknown	NEG (NTC)			
4		IL1B-Neg	Unknown	NEG (NTC)			
5		IL6-Neg	Unknown	31.52			
6		IL8-Neg	Unknown	26.52			
7		TNF-Neg	Unknown	NEG (NTC)			
8		TF-Neg	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	37.72			
10		HSPY-TCM	Unknown	23.16			
11		ESEL-TCM	Unknown	20.63			

12		ICAM-TCM	Unknown	31.70			
13		IL1B-TCM	Unknown	NEG (NTC)			
14		IL6-TCM	Unknown	24.96			
15		IL8-TCM	Unknown	18.98			
16		TNF-TCM	Unknown	NEG (NTC)			
17		TF-TCM	Unknown	24.60			
18		RNAPol-TCM	Unknown	23.93			
19		HSPY-TCM	Unknown	19.41			
20		ESEL-TCM	Unknown	20.01			
21		ICAM-TCM	Unknown	30.29			
22		IL1B-TCM	Unknown	24.20			
23		IL6-TCM	Unknown	25.92			
24		IL8-TCM	Unknown	18.95			
25		TNF-TCM	Unknown	NEG (NTC)			
26		TF-TCM	Unknown	24.66			
27		RNAPol-TCM	Unknown	23.53			
28		Blank	Unknown	NEG (NTC)			
29		Blank	Unknown				
30		Blank	Unknown				
31		Blank	Unknown				
32		Blank	Unknown	NEG (NTC)			
33		Blank	Unknown	39.05			
34		Blank	Unknown	NEG (NTC)			
35		Blank	Unknown	NEG (NTC)			
36		Blank	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	Tumour conditoned cord 2007-03-06 (1)
Run Start	6/03/2007 12:03:20 p.m.
Run Finish	6/03/2007 1:59:31 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	76°c
Threshold	0.03331

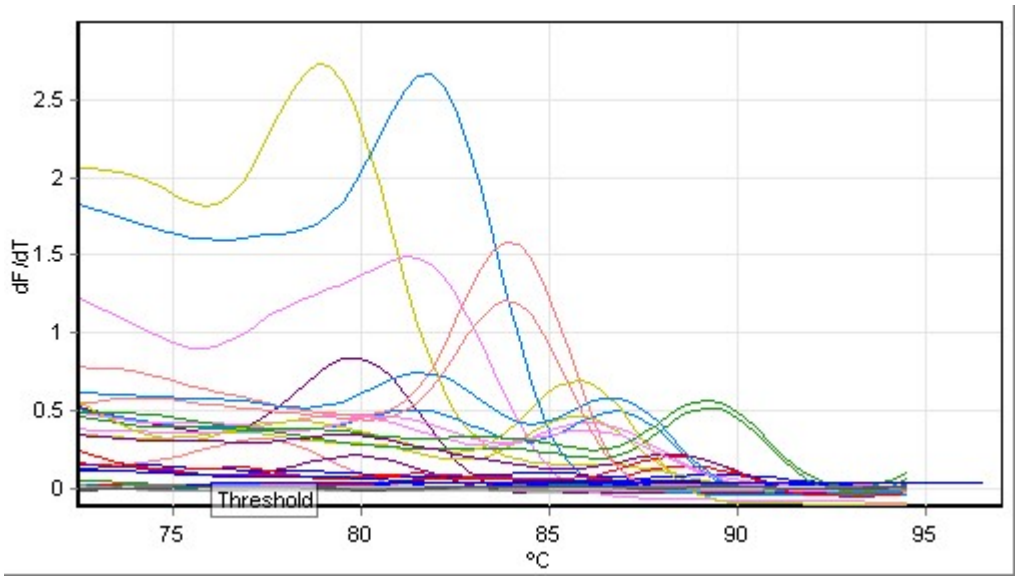
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2	Peak 3
1	HSPY-Neg				
2	ESEL-Neg		79.0	86.3	
3	ICAM-Neg		79.2		
4	IL1B-Neg		79.8		
5	IL6-Neg		81.3		
6	IL8-Neg		81.7		
7	TNF-Neg				

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2	Peak 3
8	TF-Neg		77.3		
9	RNAPol-Neg				

10	HSPY-TCM		88.7		
11	ESEL-TCM		76.7	85.7	
12	ICAM-TCM		84.7	89.5	
13	IL1B-TCM		79.8		
14	IL6-TCM		79.5	86.0	
15	IL8-TCM		81.3	86.7	
16	TNF-TCM				
17	TF-TCM		84.0		
18	RNAPol-TCM		76.2	83.5	89.2
19	HSPY-TCM		76.5	81.5	88.5
20	ESEL-TCM		78.3	85.7	
21	ICAM-TCM		77.8	85.8	
22	IL1B-TCM		79.5	88.2	
23	IL6-TCM		80.5	86.0	
24	IL8-TCM		81.5	86.5	
25	TNF-TCM				
26	TF-TCM		83.8	91.5	
27	RNAPol-TCM		83.0	89.2	
28	Blank				
29	Blank				
30	Blank				
31	Blank				
32	Blank				
33	Blank				
34	Blank				
35	Blank				
36	Blank				

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for Normalized ICN (TCM cord 8)

No.	Gene	Sample	Ct	Avg Ct	No	PCR eff	Avg Eff	R2	ICN	GOI/B2M
11	ESEL	TCM	20.63	20.32	1.67E-08	1.865021	1.839834	0.990516	46656.74	0.000915
20	ESEL	TCM	20.01	20.32	5.02E-08	1.814648	1.839834	0.998467	46656.74	0.000915
10	HSPY	TCM	23.16	21.285	2.47E-06	1.454046	1.288286	0.974374	51004438	HK
19	HSPY	TCM	19.41	21.285	0.019735	1.122526	1.288286	0.99761	51004438	HK
12	ICAM	TCM	31.7	30.995	4.43E-10	1.713912	1.80064	0.992937	135.5996	2.66E-06
21	ICAM	TCM	30.29	30.995	4.41E-11	1.887368	1.80064	0.99495	135.5996	2.66E-06
13	IL1B	TCM	24.2	24.2	0.00982	0.971315	1.151935	0.179718	3.65E+08	7.162358
22	IL1B	TCM	24.2	24.2	4.4E-05	1.332554	1.151935	0.965509	3.65E+08	7.162358
14	IL6	TCM	24.96	25.44	3.03E-09	1.767732	1.667141	0.992458	25248.78	0.000495
23	IL6	TCM	25.92	25.44	1.53E-07	1.56655	1.667141	0.991629	25248.78	0.000495
15	IL8	TCM	18.98	18.965	2.9E-07	1.705652	1.711061	0.996677	422023.6	0.008274
24	IL8	TCM	18.95	18.965	3.32E-07	1.716471	1.711061	0.994601	422023.6	0.008274
18	RNAPol	TCM	23.93	23.73	1.01E-06	1.520943	1.519166	0.99996	549193.6	0.010768
27	RNAPol	TCM	23.53	23.73	1.35E-06	1.517389	1.519166	0.99935	549193.6	0.010768
17	TF	TCM	24.6	24.63	0.00047	1.268197	1.501996	0.998948	498731.6	0.009778
26	TF	TCM	24.66	24.63	1.34E-08	1.735795	1.501996	0.995724	498731.6	0.009778
16	TNF	TCM		0	8.29E-05	1.431257	1.421308	0.794202	0	0
25	TNF	TCM		0	2.1E-06	1.411359	1.421308	0.603733	0	0

Data for cord 8

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Quantitation Report

Experiment Information

Run Name	DMXAA cord 2007-03-06 (1)
Run Start	6/03/2007 2:08:49 p.m.
Run Finish	6/03/2007 4:04:55 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.00315
Left Threshold	20.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

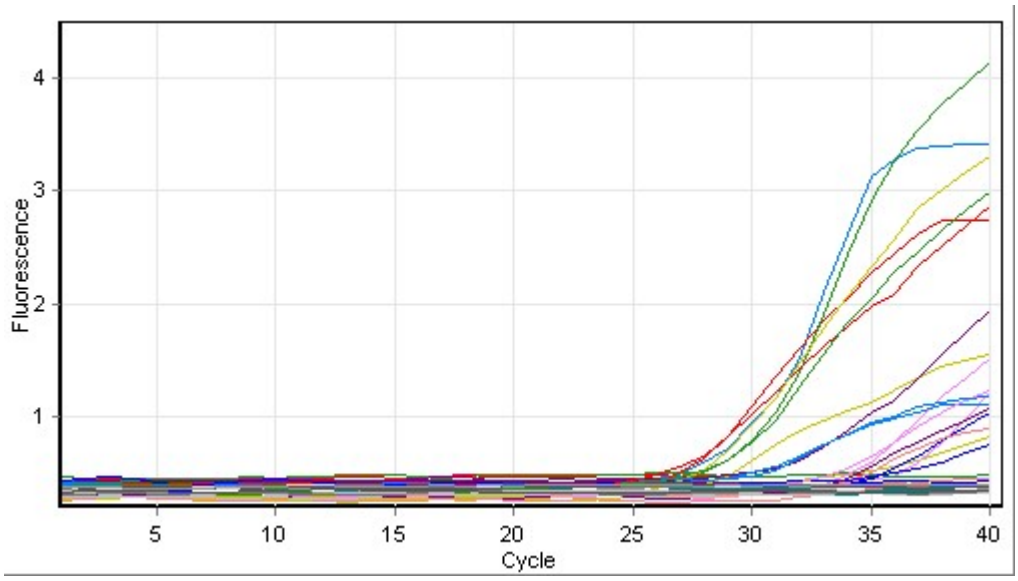
Messages

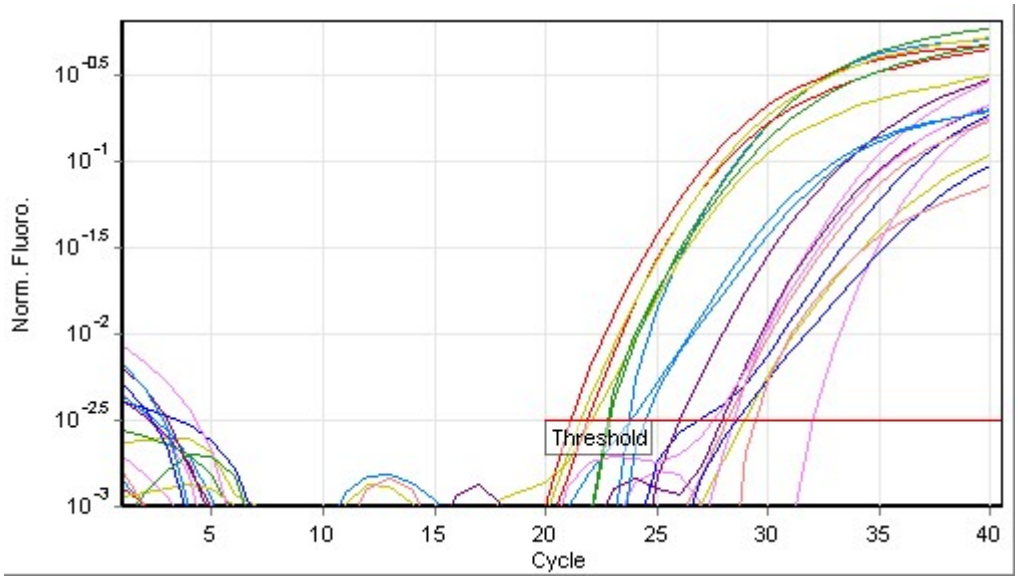
Message

Profile

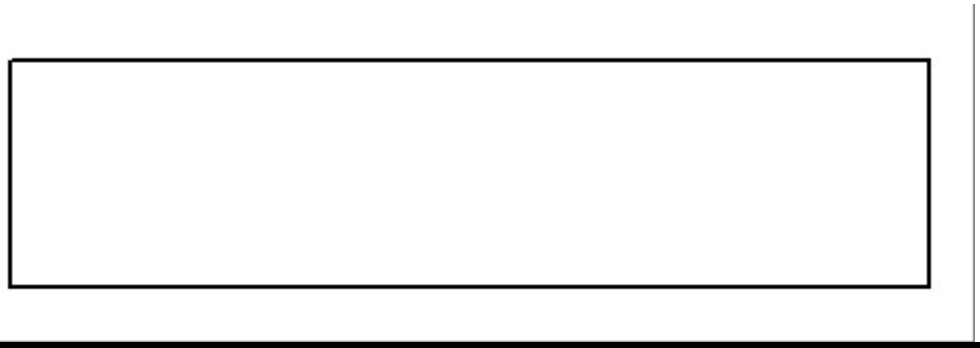
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-Neg	Unknown	NEG (NTC)			
2		ESEL-Neg	Unknown	28.93			
3		ICAM-Neg	Unknown	NEG (NTC)			
4		IL1B-Neg	Unknown	NEG (NTC)			
5		IL6-Neg	Unknown	31.94			
6		IL8-Neg	Unknown	23.58			
7		TNF-Neg	Unknown	NEG (NTC)			
8		TF-Neg	Unknown	NEG (NTC)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	NEG (NTC)			
10		HSPY-DMXAA	Unknown	21.13			
11		ESEL-DMXAA	Unknown	22.08			

12		ICAM-DXMAA	Unknown	26.76			
13		IL1BDMXAA	Unknown	25.99			
14		IL6-DMXAA	Unknown	28.41			
15		IL8-DMXAA	Unknown	23.78			
16		TNF-DMXAA	Unknown	NEG (NTC)			
17		TF-DMXAA	Unknown	28.16			
18		RNAPol-DMXAA	Unknown	22.78			
19		HSPY-DMXAA	Unknown	21.90			
20		ESEL-DMXAA	Unknown	21.59			
21		ICAM-DXMAA	Unknown	28.60			
22		IL1BDMXAA	Unknown	27.92			
23		IL6-DMXAA	Unknown	27.57			
24		IL8-DMXAA	Unknown	24.38			
25		TNF-DMXAA	Unknown	NEG (NTC)			
26		TF-DMXAA	Unknown	29.38			
27		RNAPol-DMXAA	Unknown	22.62			
28			Unknown	NEG (NTC)			
29			Unknown	NEG (NTC)			
30			Unknown	NEG (NTC)			
31			Unknown	NEG (NTC)			
32			Unknown	NEG (NTC)			
33			Unknown	NEG (NTC)			
34			Unknown	NEG (NTC)			
35			Unknown	NEG (NTC)			
36			Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	DMXAA cord 2007-03-06 (1)
Run Start	6/03/2007 2:08:49 p.m.
Run Finish	6/03/2007 4:04:55 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	77°c
Threshold	0.11868

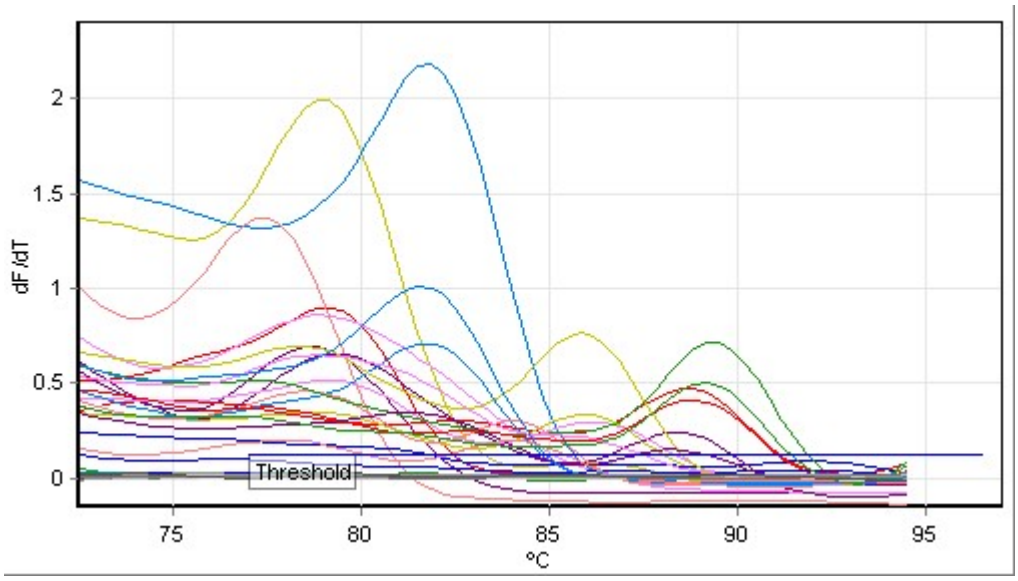
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2	Peak 3
1	HSPY-Neg		79.0		
2	ESEL-Neg		79.0		
3	ICAM-Neg				
4	IL1B-Neg		78.7		
5	IL6-Neg		79.0		
6	IL8-Neg		81.7		
7	TNF-Neg				

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2	Peak 3
8	TF-Neg		77.5		
9	RNAPol-Neg				

10	HSPY-DMXAA		82.5	88.7	
11	ESEL-DMXAA		78.7	86.0	
12	ICAM-DXMAA				
13	IL1BDMXAA		79.3	88.5	
14	IL6-DMXAA		79.2	86.0	
15	IL8-DMXAA		81.7		
16	TNF-DMXAA				
17	TF-DMXAA		78.5	84.0	
18	RNAPol-DMXAA		77.2	89.3	
19	HSPY-DMXAA		77.3	82.7	88.8
20	ESEL-DMXAA		78.5	85.8	
21	ICAM-DXMAA				
22	IL1BDMXAA		81.3	88.3	
23	IL6-DMXAA		78.8		
24	IL8-DMXAA		81.5		
25	TNF-DMXAA				
26	TF-DMXAA		78.0	84.0	
27	RNAPol-DMXAA		89.0		
28					
29					
30					
31					
32					
33					
34					
35					
36					

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for cord 10

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Quantitation Report

Experiment Information

Run Name	Control cord 2007-03-12
Run Start	12/03/2007 3:24:29 p.m.
Run Finish	12/03/2007 5:23:15 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.00169
Left Threshold	3.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

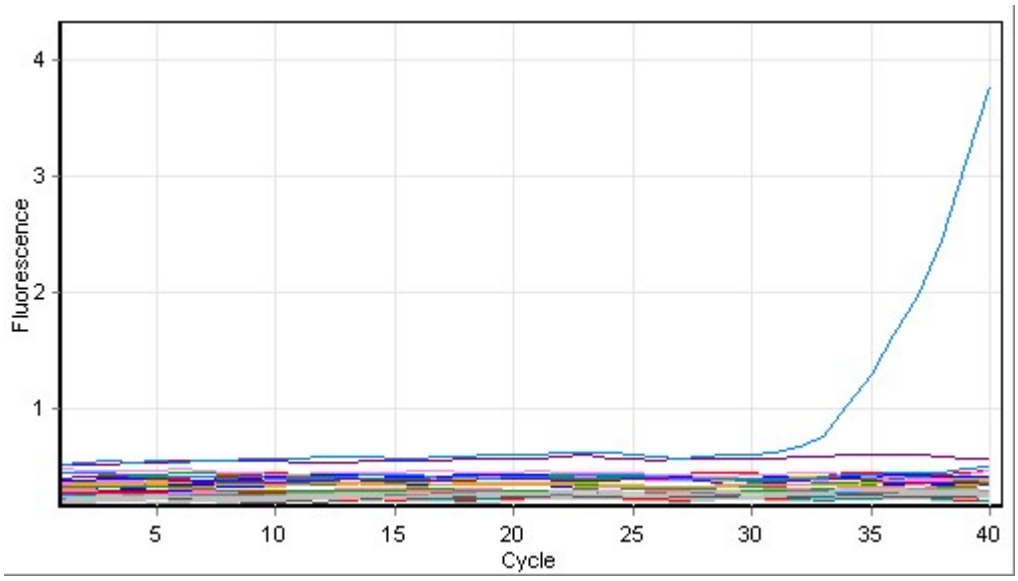
Messages

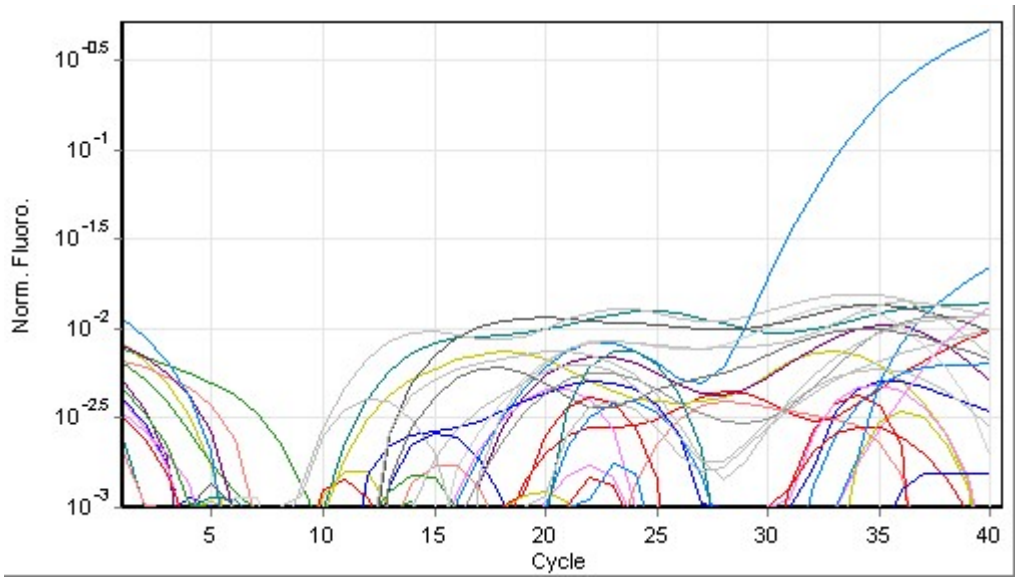
Message

Profile

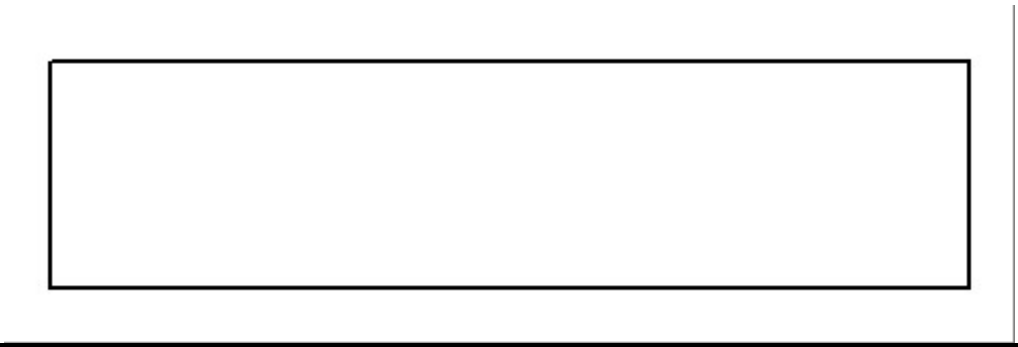
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-Neg	Unknown	19.49			
2		ESel-Neg	Unknown	NEG (Multi Ct)			
3		ICam-Neg	Unknown	NEG (Multi Ct)			
4		IL1B-Neg	Unknown	17.24			
5		IL6-Neg	Unknown	NEG (Multi Ct)			
6		IL8-Neg	Unknown	16.74			
7		TNF-Neg	Unknown	10.57			
8		TF-Neg	Unknown	NEG (Multi Ct)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	8.65			
10		HSPY-Control	Unknown	NEG (Multi Ct)			
11		ESel-Control	Unknown	5.02			

12		ICam-Control	Unknown	3.08			
13		IL1B-Control	Unknown	3.14			
14		IL6-Control	Unknown	NEG (Multi Ct)			
15		IL8-Control	Unknown	NEG (Multi Ct)			
16		TNF-Control	Unknown				
17		TF-Control	Unknown	6.45			
18		RNAPol-Control	Unknown	5.13			
19		HSPY-Control	Unknown	NEG (Multi Ct)			
20		ESel-Control	Unknown	NEG (Multi Ct)			
21		ICam-Control	Unknown	NEG (Multi Ct)			
22		IL1B-Control	Unknown	5.56			
23		IL6-Control	Unknown	3.51			
24		IL8-Control	Unknown	NEG (Multi Ct)			
25		TNF-Control	Unknown	NEG (Multi Ct)			
26		TF-Control	Unknown				
27		RNAPol-Control	Unknown	3.46			
28			Unknown	16.03			
29			Unknown	14.74			
30			Unknown	17.34			
31			Unknown	13.22			
32			Unknown	12.62			
33			Unknown	17.08			
34			Unknown	NEG (Multi Ct)			
35			Unknown	9.31			
36			Unknown	NEG (Multi Ct)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	Control cord 2007-03-12
Run Start	12/03/2007 3:24:29 p.m.
Run Finish	12/03/2007 5:23:15 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	76°c
Threshold	0.09738

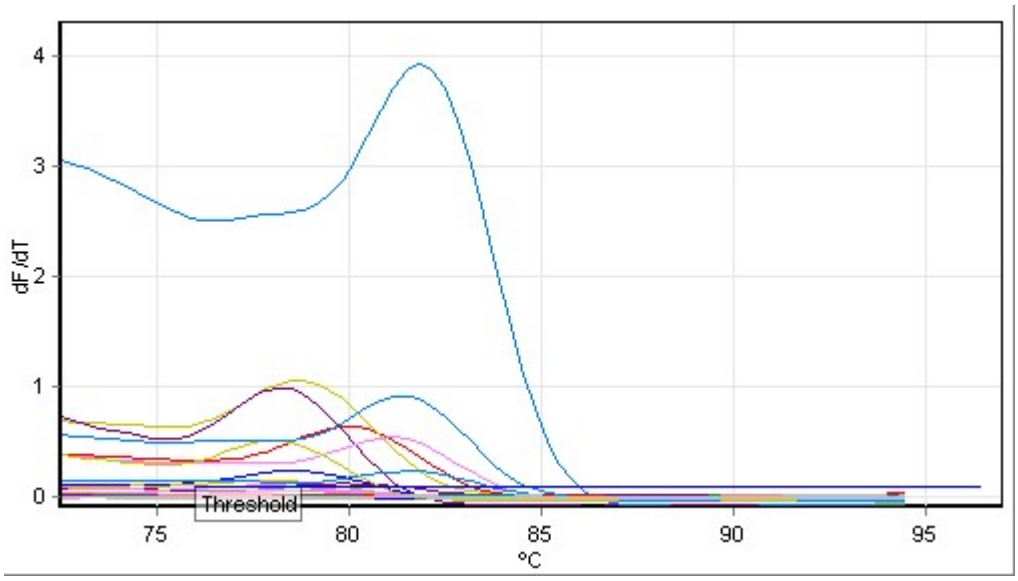
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	HSPY-Neg		80.0	
2	ESel-Neg		78.7	
3	ICam-Neg		78.5	
4	IL1B-Neg		78.2	
5	IL6-Neg		76.5	81.0
6	IL8-Neg		81.8	
7	TNF-Neg			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	TF-Neg			
9	RNAPol-Neg			

10	HSPY-Control			
11	ESel-Control		78.0	
12	ICam-Control			
13	IL1B-Control		80.0	
14	IL6-Control			
15	IL8-Control		76.5	81.5
16	TNF-Control			
17	TF-Control			
18	RNAPol-Control			
19	HSPY-Control			
20	ESel-Control		78.0	
21	ICam-Control			
22	IL1B-Control			
23	IL6-Control		77.8	
24	IL8-Control		77.2	81.5
25	TNF-Control			
26	TF-Control			
27	RNAPol-Control			
28				
29				
30				
31				
32				
33				
34				
35				
36				

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for cord 10

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Quantitation Report

Experiment Information

Run Name	DMXAA cord 2007-03-14 (1)
Run Start	14/03/2007 3:21:04 p.m.
Run Finish	14/03/2007 5:19:00 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Quantitation Information

Threshold	0.0069
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

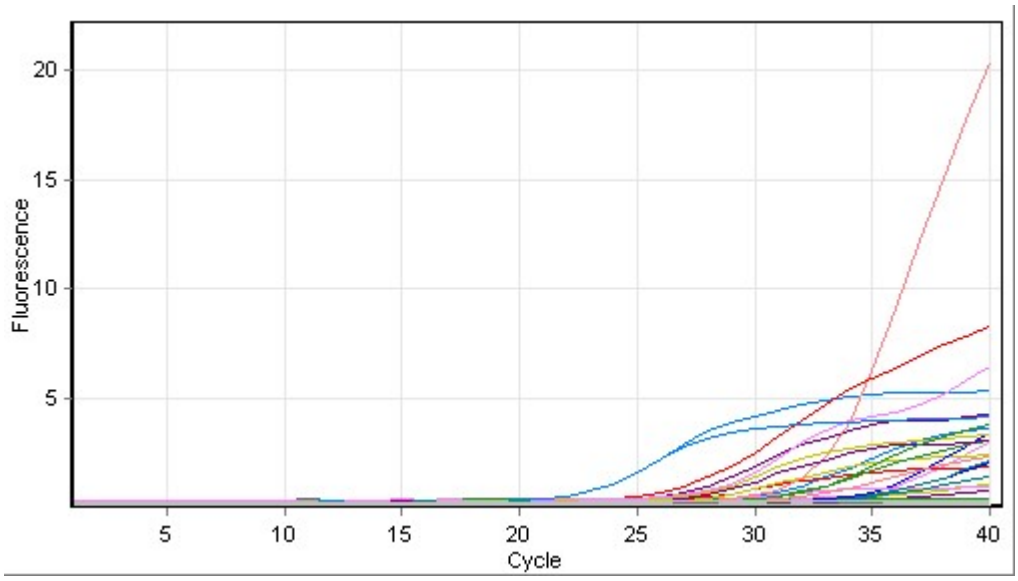
Messages

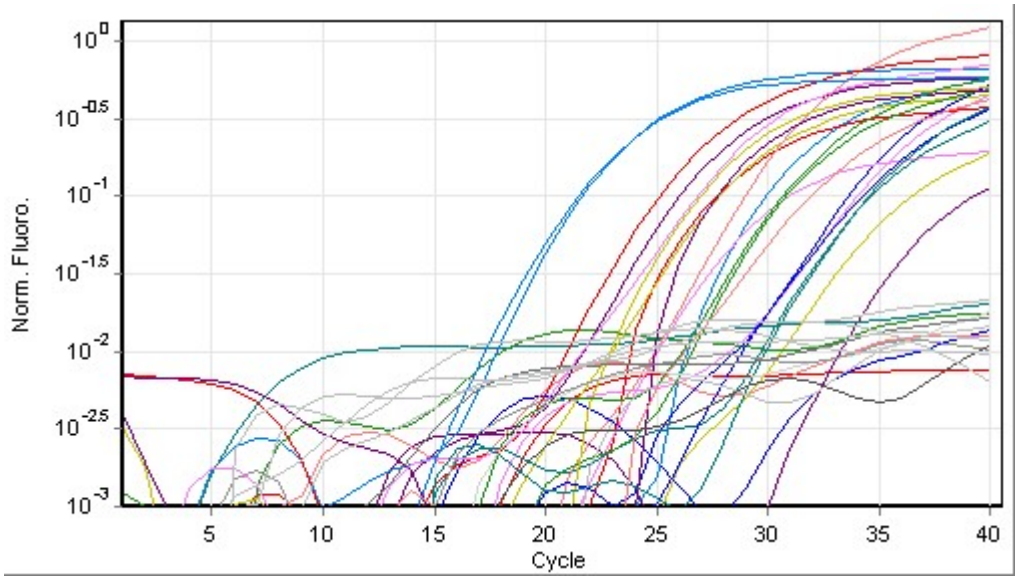
Message

Profile

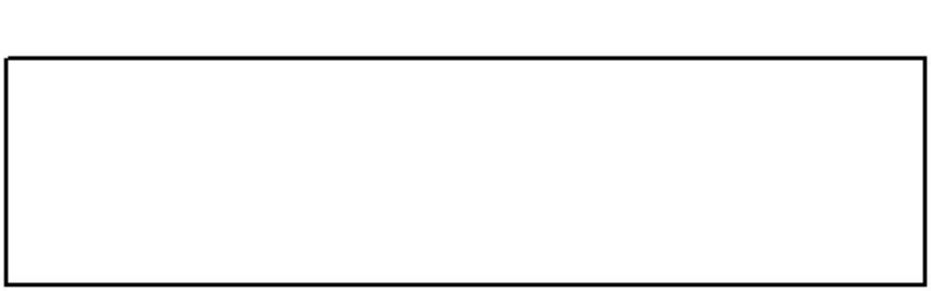
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HSPY-Neg	Unknown	NEG (Multi Ct)			
2		ESel-Neg	Unknown	29.86			
3		ICam-Neg	Unknown	33.06			
4		IL1B-Neg	Unknown	32.59			
5		IL6-Neg	Unknown	26.57			
6		IL8-Neg	Unknown	25.96			
7		TNF-Neg	Unknown	8.40			
8		TF-Neg	Unknown	NEG (Multi Ct)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-Neg	Unknown	16.57			
10		HSPY-DMXAA	Unknown	NEG (Multi Ct)			
11		ESel-DMXAA	Unknown	22.19			

12		ICam-DMXAA	Unknown	27.29			
13		IL1B-DMXAA	Unknown	21.06			
14		IL6-DMXAA	Unknown	24.37			
15		IL8-DMXAA	Unknown	17.29			
16		TNF-DMXAA	Unknown	29.04			
17		TF-DMXAA	Unknown	24.19			
18		RNAPol-DMXAA	Unknown	25.99			
19		HSPY-DMXAA	Unknown	20.44			
20		ESel-DMXAA	Unknown	22.41			
21		ICam-DMXAA	Unknown	28.13			
22		IL1B-DMXAA	Unknown	24.62			
23		IL6-DMXAA	Unknown	20.86			
24		IL8-DMXAA	Unknown	16.71			
25		TNF-DMXAA	Unknown	29.34			
26		TF-DMXAA	Unknown	25.31			
27		RNAPol-DMXAA	Unknown	25.29			
28		Blank	Unknown	NEG (Multi Ct)			
29		Blank	Unknown	33.15			
30		Blank	Unknown	22.79			
31		Blank	Unknown	18.23			
32		Blank	Unknown	37.54			
33		Blank	Unknown	18.27			
34		Blank	Unknown	14.33			
35		Blank	Unknown	20.20			
36		Blank	Unknown	NEG (Multi Ct)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	DMXAA cord 2007-03-14 (1)
Run Start	14/03/2007 3:21:04 p.m.
Run Finish	14/03/2007 5:19:00 p.m.
Operator	Kiri
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	8.

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	78°c
Threshold	0.29299

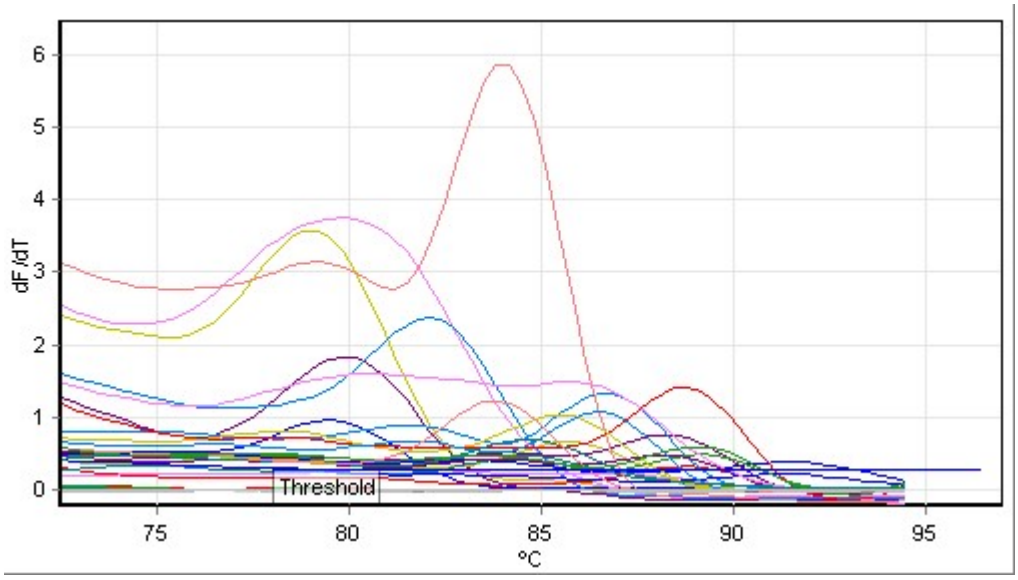
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
1	HSPY-Neg					
2	ESel-Neg		79.0			
3	ICam-Neg		79.5			
4	IL1B-Neg		80.0			
5	IL6-Neg		79.8			
6	IL8-Neg		82.0			
7	TNF-Neg					

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
8	TF-Neg					
9	RNAPol-Neg					

10	HSPY-DMXAA		88.8			
11	ESel-DMXAA		85.7			
12	ICam-DMXAA		83.0	91.5		
13	IL1B-DMXAA		79.0	84.2	88.3	
14	IL6-DMXAA					
15	IL8-DMXAA		81.5	86.7		
16	TNF-DMXAA		84.5			
17	TF-DMXAA		79.2	84.0		
18	RNAPol-DMXAA		83.8	89.2		
19	HSPY-DMXAA		78.2	82.0	84.2	88.7
20	ESel-DMXAA		78.2	85.5		
21	ICam-DMXAA					
22	IL1B-DMXAA		83.8	88.2		
23	IL6-DMXAA		80.5	85.7		
24	IL8-DMXAA		81.7	86.5		
25	TNF-DMXAA		84.5			
26	TF-DMXAA		83.8			
27	RNAPol-DMXAA		83.5	89.0		
28	Blank					
29	Blank					
30	Blank					
31	Blank					
32	Blank					
33	Blank					
34	Blank					
35	Blank					
36	Blank					

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for cord 11

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Quantitation Report

Experiment Information

Run Name	control cord 2007-03-01 (1)
Run Start	1/03/2007 12:03:43 p.m.
Run Finish	1/03/2007 2:11:37 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	9.33

Quantitation Information

Threshold	0.0069
Left Threshold	25.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	2%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

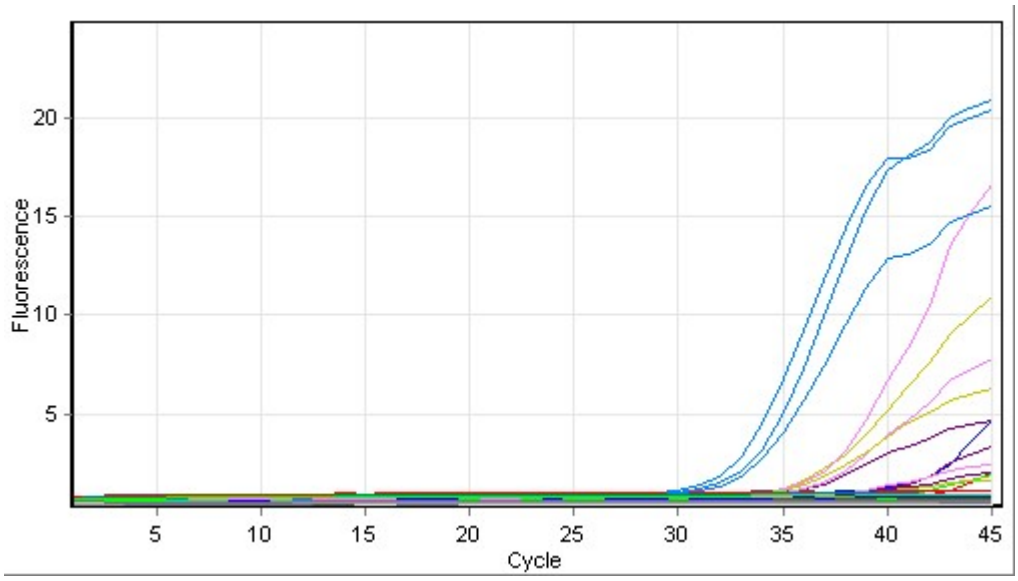
Messages

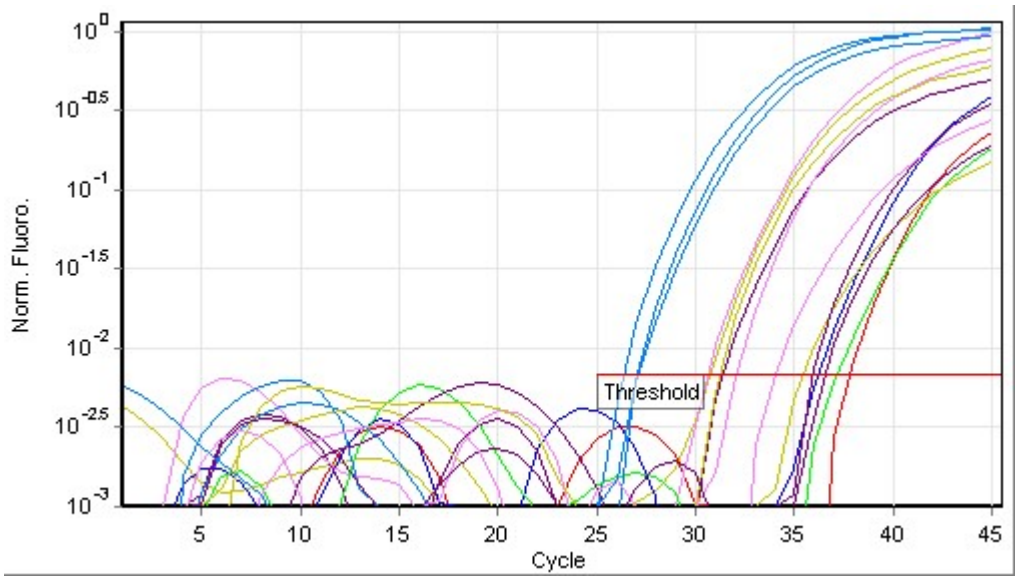
Message

Profile

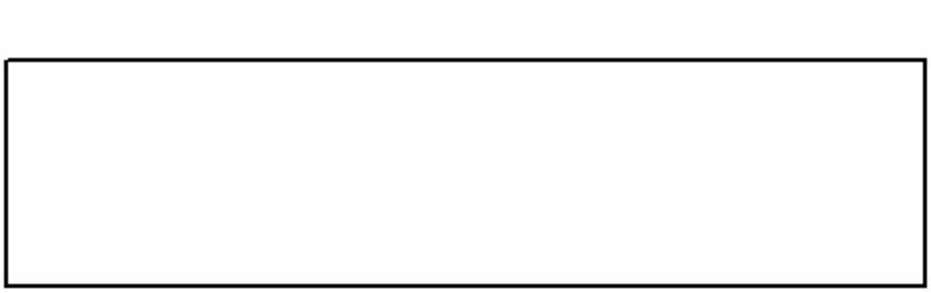
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (45 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HPSY-Neg	Unknown	37.80			
2		ESel-Neg	Unknown	30.75			
3		ICAM-Neg	Unknown	36.22			
4		IL1B-Neg	Unknown	31.34			
5		IL6-Neg	Unknown	30.55			
6		IL8-Neg	Unknown	26.32			
7		TNF-Neg	Unknown	NEG (NTC)			
8		TF-Neg	Unknown	37.18			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		HPSY-pbs	Unknown	NEG (NTC)			
10		Esel-pbs	Unknown	35.46			
11		ICAM-pbs	Unknown	NEG (NTC)			
12		IL1B-pbs	Unknown	35.93			
13		IL6-pbs	Unknown	32.02			
14		IL8-pbs	Unknown	27.07			
15		TNF-pbs	Unknown	NEG (NTC)			
16		TF-pbs	Unknown	NEG (NTC)			
17		HPSY-pbs	Unknown	NEG (NTC)			
18		Esel-pbs	Unknown	31.20			
19		ICAM-pbs	Unknown	NEG (NTC)			
20		IL1B-pbs	Unknown	36.54			
21		IL6-pbs	Unknown	34.00			
22		IL8-pbs	Unknown	27.13			

23		TNF-pbs	Unknown	NEG (NTC)			
24		TF-pbs	Unknown	NEG (NTC)			
25		Blank	Unknown	NEG (NTC)			
26		Blank	Unknown	NEG (NTC)			
27		Blank	Unknown	NEG (NTC)			
28		Blank	Unknown	NEG (NTC)			
29		Blank	Unknown	NEG (NTC)			
30		Blank	Unknown	NEG (NTC)			
31		Blank	Unknown	NEG (NTC)			
32		Blank	Unknown	NEG (NTC)			
33		Blank	Unknown	NEG (NTC)			
34		Blank	Unknown	NEG (NTC)			
35		Blank	Unknown	NEG (NTC)			
36		Blank	Unknown	NEG (NTC)			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

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Melt Report

Experiment Information

Run Name	control cord 2007-03-01 (1)
Run Start	1/03/2007 12:03:43 p.m.
Run Finish	1/03/2007 2:11:37 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	9.33

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	76°c
Threshold	1.13183

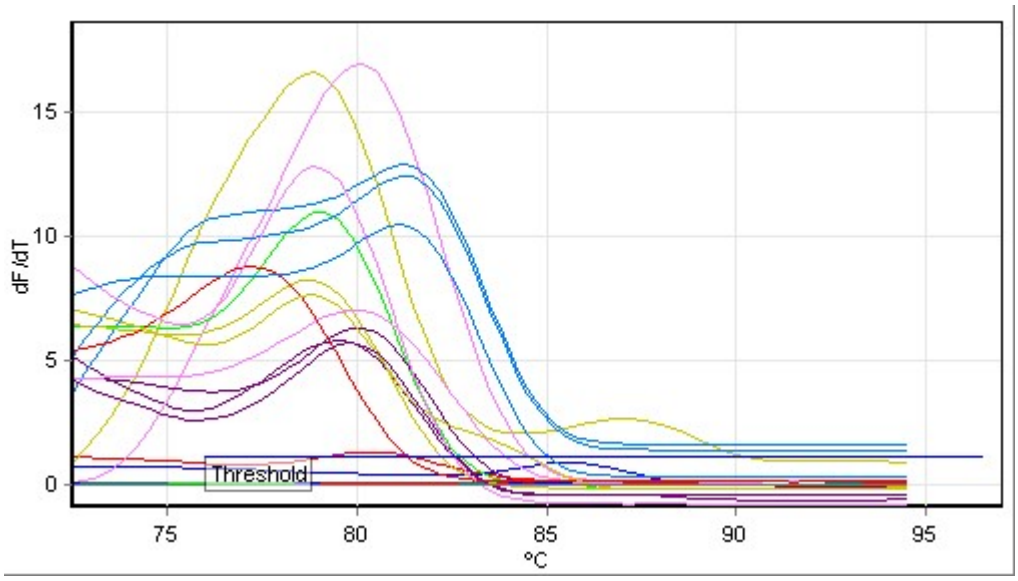
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (45 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2
1	HPSY-Neg		80.3	
2	ESel-Neg		78.8	87.0
3	ICAM-Neg			
4	IL1B-Neg		79.5	
5	IL6-Neg		80.0	
6	IL8-Neg		81.2	88.8
7	TNF-Neg			

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2
8	TF-Neg		79.0	
9	HPSY-pbs			

10	Esel-pbs		78.7	
11	ICAM-pbs			
12	IL1B-pbs		80.0	
13	IL6-pbs		80.0	
14	IL8-pbs		81.2	93.5
15	TNF-pbs			
16	TF-pbs			
17	HPSY-pbs		77.2	
18	Esel-pbs		78.8	
19	ICAM-pbs			
20	IL1B-pbs		79.8	
21	IL6-pbs		79.0	
22	IL8-pbs		81.0	
23	TNF-pbs			
24	TF-pbs			
25	Blank			
26	Blank			
27	Blank			
28	Blank			
29	Blank			
30	Blank			
31	Blank			
32	Blank			
33	Blank			
34	Blank			
35	Blank			
36	Blank			

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

Data for cord 11

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Quantitation Report

Experiment Information

Run Name	tumour conditioned cord 2007-03-01
Run Start	1/03/2007 4:47:36 p.m.
Run Finish	1/03/2007 6:43:35 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	9.33

Quantitation Information

Threshold	0.00487
Left Threshold	12.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

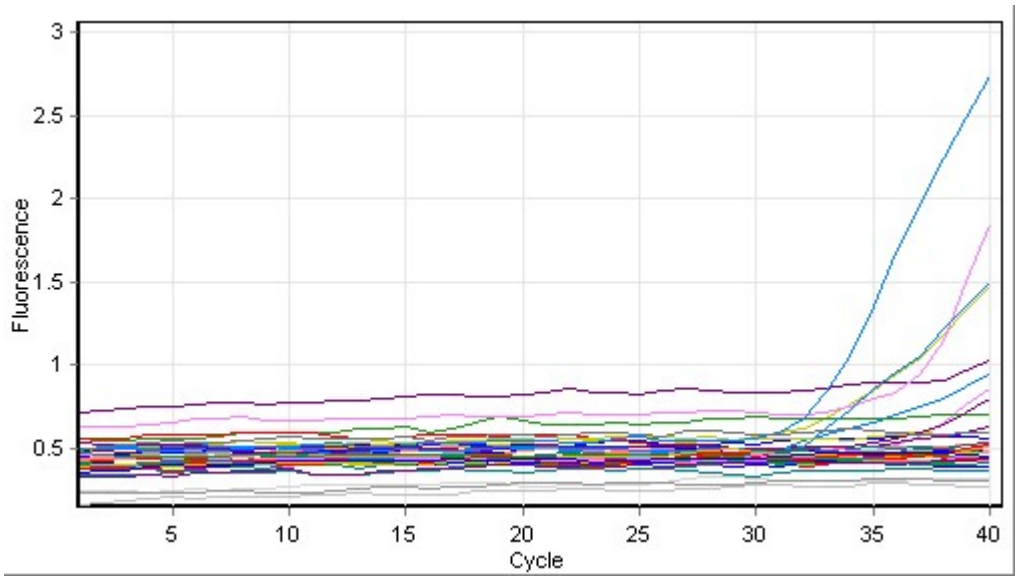
Messages

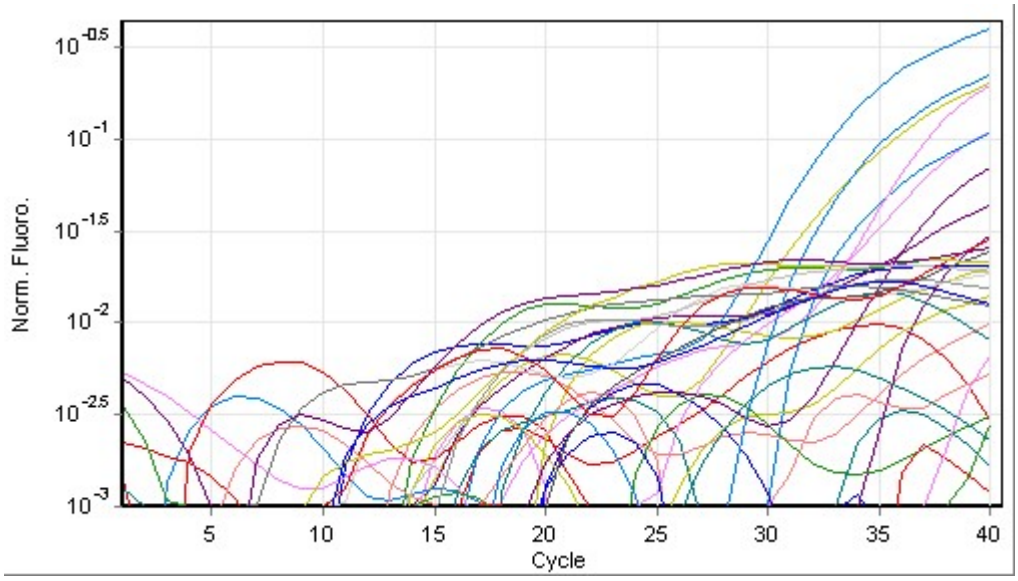
Message

Profile

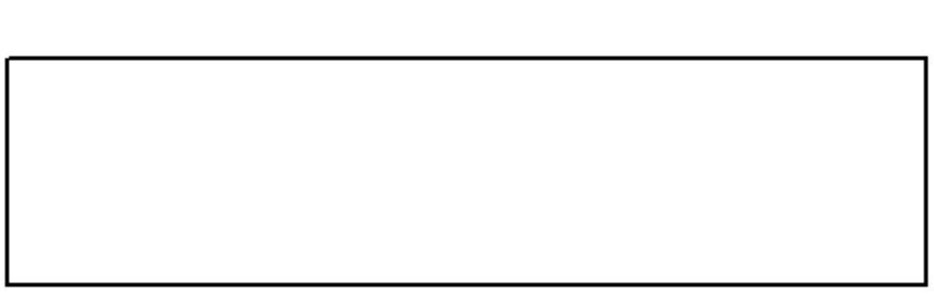
Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Raw Data For Cycling B.Green





Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		HPSY-negative	Unknown	NEG (Multi Ct)			
2		ESel-negative	Unknown	NEG (Multi Ct)			
3		ICam-negative	Unknown	13.15			
4		IL1B-negative	Unknown	18.49			
5		IL6-negative	Unknown	27.15			
6		Il8-negative	Unknown	30.93			
7		TNF-negative	Unknown				
8		TF-negative	Unknown	NEG (Multi Ct)			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
9		RNAPol-negative	Unknown	15.71			
10		HPSY-TCM	Unknown				
11		ESel-TCM	Unknown	21.19			

12		ICam-TCM	Unknown				
13		IL1B-TCM	Unknown	35.52			
14		IL6-TCM	Unknown	23.83			
15		Il8-TCM	Unknown	19.97			
16		TNFTCM	Unknown	20.41			
17		TF-TCM	Unknown	39.33			
18		RNAPol-TCM	Unknown				
19		HPSY-TCM	Unknown				
20		ESel-TCM	Unknown	33.42			
21		ICam-TCM	Unknown				
22		IL1B-TCM	Unknown	32.47			
23		IL6-TCM	Unknown	39.26			
24		Il8-TCM	Unknown	29.48			
25		TNFTCM	Unknown	NEG (Multi Ct)			
26		TF-TCM	Unknown				
27		RNAPol-TCM	Unknown				
28		Blank	Unknown	14.20			
29		Blank	Unknown	17.71			
30		Blank	Unknown	17.65			
31		Blank	Unknown	12.77			
32		Blank	Unknown	23.35			
33		Blank	Unknown	NEG (Multi Ct)			
34		Blank	Unknown	17.52			
35		Blank	Unknown	15.80			
36		Blank	Unknown	14.47			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold.
NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

corbett RESEARCH
A Division of Corbett Life Science

14 Hilly Street Mortlake NSW 2137 Australia
T +61 2 9736 1320
F +61 2 9736 1364
W www.corbettlifescience.com

Melt Report

Experiment Information

Run Name	tumour conditioned cord 2007-03-01
Run Start	1/03/2007 4:47:36 p.m.
Run Finish	1/03/2007 6:43:35 p.m.
Operator	KM
Notes	
Run On Software Version	Rotor-Gene 1.7.61
Run Signature	The Run Signature is valid.
Gain Green	9.33

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	0°c
Threshold	0.

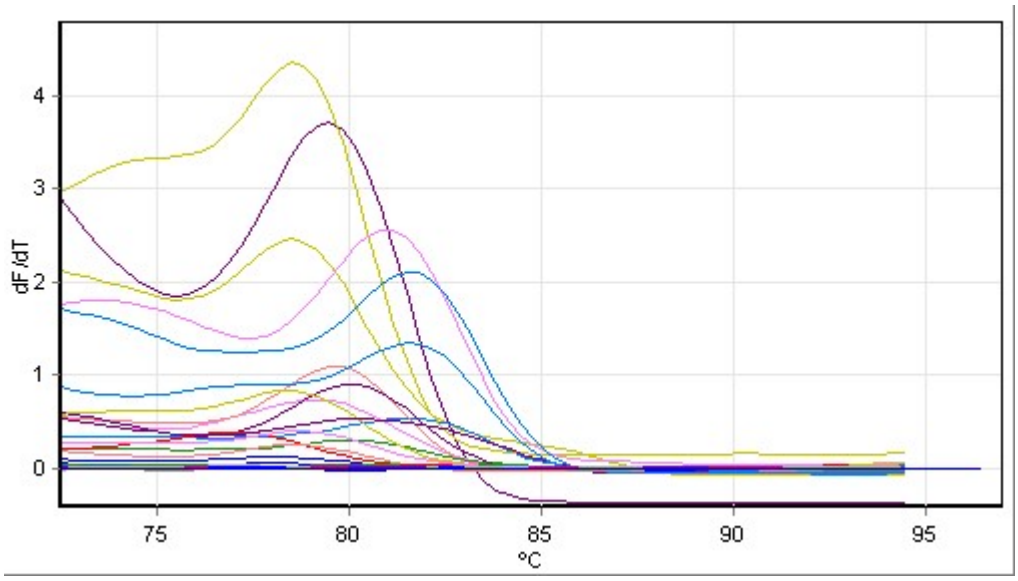
Messages

Message

Profile

Cycle	Cycle Point
Hold @ 95°c, 15 min 0 secs	
Cycling (40 repeats)	Step 1 @ 94°c, hold 20 secs
	Step 2 @ 55°c, hold 20 secs
	Step 3 @ 68°c, hold 30 secs, acquiring to Cycling A([Green][1][1])
	Step 4 @ 80°c, hold 10 secs, acquiring to Cycling B([Green][1][1])
Melt (72-95°c) , hold secs on the 1st step, hold 5 secs on next steps, Melt A([Green][1][1])	

Melt data for Melt A.Green



No.	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
1	HPSY-negative		77.5	82.5	88.0	93.5	
2	ESel-negative		78.5				
3	ICam-negative		78.0	84.3	89.0	92.5	
4	IL1B-negative		79.5				
5	IL6-negative		79.2	87.0	90.0		
6	Il8-negative		81.5	90.7			
7	TNF-negative		76.8	82.5	89.5		

(Continued on next page)...

No.	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
8	TF-negative		79.7				
9	RNAPol-negative		74.0	80.0	87.5	92.2	

10	HPSY-TCM		78.2	82.0	87.0		
11	ESel-TCM		78.5	90.3			
12	ICam-TCM		78.0	85.0			
13	IL1B-TCM		80.0				
14	IL6-TCM		73.5	81.0			
15	Il8-TCM		81.5				
16	TNFTCM		75.7	78.0	85.3	91.0	
17	TF-TCM		78.0	84.0	91.5		
18	RNAPol-TCM		75.0	83.5	89.0		
19	HPSY-TCM		77.0	87.3	91.7		
20	ESel-TCM		78.5	86.5			
21	ICam-TCM		76.5	83.0	90.2		
22	IL1B-TCM		80.2				
23	IL6-TCM		73.8	78.7	88.3		
24	Il8-TCM		81.5				
25	TNFTCM		73.8	80.0	82.8	87.7	90.5
26	TF-TCM		78.5				
27	RNAPol-TCM		75.5	80.7	88.2		
28	Blank		75.0	79.7	84.2	87.5	
29	Blank		77.2	82.7	87.7	92.8	
30	Blank						
31	Blank		77.7	82.3	88.0		
32	Blank		75.3	79.7	84.0	88.3	
33	Blank		75.5	81.5	86.7	92.5	
34	Blank		72.8	78.2	83.0	87.5	92.8
35	Blank		77.5	82.5			
36	Blank		81.0	88.5			

Bin Name	Temperature	Sample No.	Sample Name	Peak

(Continued on next page)...

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