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Dexamethasone- and age-sensitive genes in neonatal small intestine

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ABSTRACT

DEXAMETHASONE- AND AGE-SENSITIVE GENES IN NEONATAL SMALL INTESTINE

by
Carla Cerqueira

Regulation of GLUT5 is dependent on the presence of its substrate, fructose, but it is also correlated with the developmental (aging) process of the intestine. To identify fructose-responsive genes whose expression also changes with age, intestines of 10 and 20 d old pups were perfused, by Ferraris et al., with fructose and then compared by microarray analysis. From this, a gene clustering analysis revealed that some age- and fructose-specific genes are regulated by corticosterones, which normally increase in pups $\geq$ 14 d old. Subsequent work indicated that priming the gut with Dexamethasone (Dex, a glucocorticoid analog) allowed fructose to precociously stimulate GLUT5 even in suckling pups < 14 d old.

This suggests that the effect of Dex on GLUT5 is similar to the effect of age; both allow fructose to stimulate GLUT5. It is not known: ① if this similar effect of Dex and age is specific to GLUT5 or if it could be extended to a larger family of genes, and ② is Dex and age are acting through the same or different signaling mechanisms. In this study I tested the hypothesis that Dex allows fructose to stimulate GLUT5 by inducing the same genes as those induced by age. I therefore, determined by microarray analysis in 10 old suckling pups, the identity of genes that are regulated solely by fructose, solely by Dex and by Dex under fructose conditions. Genes regulated by Dex under fructose conditions were compared to genes regulated by age in the presence of fructose, which were identified in a previous microarray experiment by Douard et al.

Microarray results revealed 29 genes up-regulated by Dex, 14 by fructose, and 10 by Dex under fructose conditions. There were 64 genes down-regulated by fructose, 18 by Dex and 2 by Dex under Fructose conditions. Of these 12 Dex- under fructose conditions- sensitive genes there were no genes that were also age and fructose sensitive. There were however, four genes that were regulated by both age and Dex, independently of fructose. Hence, while there are common regulatory factors between age and Dex, the different populations of Dex- and age- sensitive intermediates, under fructose influence, suggest that there are alternative signaling pathways leading to the same outcome: an earlier onset of GLUT5 in the brushborder membrane of the small intestine.

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by
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DEXAMETHASONE- AND AGE-SENSITIVE GENES IN NEONATAL SMALL INTESTINE

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"Vanadate but not tungstate prevents the fructose-induced increase in GLUT5 expression and fructose uptake by neonatal rat intestine". Kirchner S, Kwon E, Muduli A, Cerqueira C, Cui XL, Ferraris RP. J Nutr. 2006 Sep;136(9):pp.2308-13.

"Fructose-induced increases in neonatal rat intestinal fructose transport involve the PI3-kinase/Akt signaling pathway". Cui XL, Schlesier AM, Fisher EL, Cerqueira C, Ferraris RP. Am J Physiology, Gastrointestinal Liver Physiology. 2005 Jun;288(6):pp.G1310-20. Epub 2005 Feb 3.

"Effect of Epidermal Growth Factor on induction of fructose transport in the neonatal rat intestine." Cerqueira C, Cui XL and Ronaldo Ferraris. Work presented at the 2004 Experimental Biology Conference, Washington, DC, 2004.

To my loved ones for all the support and encouragement.

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CHAPTER 1

INTRODUCTION

In the past few decades, there has been a tremendous increase in the consumption of fructose in the daily diet of Americans. For thousands of years, the daily human intake of fructose has been 16-24 g, mainly through the consumption of fresh fruits [2, 3]. Through the westernization of diets, today the daily consumption of fructose ranges from 80-100 g [2]. This high increase is primarily from the usage of high-fructose corn syrup in soft drinks and the infusion of sweeteners into convenient pre-packaged foods [2]. Soft drink consumption in children alone has increased 26 % in the last two decades. Adult studies have shown that there is a direct relationship between high fructose consumption and increase in incidents of obesity and type II diabetes [2]. Moreover, there is a strong correlation between childhood obesity and soft drink consumption in the past two decades.

The effects of the increase in daily fructose consumption are still fairly new and poorly understood especially during early childhood. The short term effects are slowly unfolding as more research is being dedicated to this purpose, however, the long term and chronic effects of fructose are yet to be discovered [2]. Currently, efforts have revealed the metabolism of fructose in the liver, but there is a lack of knowledge when it comes to the metabolism of fructose in the gastrointestinal tract. The long term goal of this project is to thoroughly understand the timing and signaling mechanisms that regulate sugar, particularly fructose uptake, in the small intestine.

1.1 Physiological Background

In the mammalian small intestine, there are two main developmental transitions due to functional demands that come with a natural change in diet. In the first transition, at birth, the intestine must absorb the nutrients from the mother's milk. At this point, the small intestine synthesizes the appropriate carriers to transfer these nutrients from the lumen into the blood. During weaning, the next transition, the intestine further develops through the addition of more carriers to accommodate a marked increase in variability of the diet.

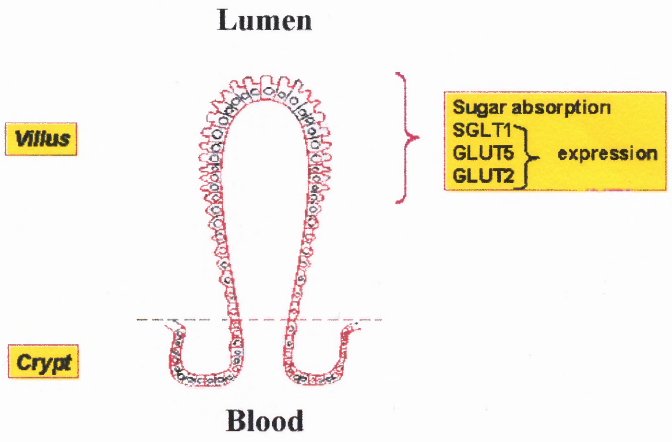


Figure 1.1 Model of Crypt-Villus axis in adult rat small intestine.

As fructose is consumed and taken into the intestine, it must cross from the intestinal lumen into the bloodstream and eventually the liver where it will be metabolized and turned into some form of energy. There are 3 major sugar transporters in the absorptive small intestinal cells (enterocytes) the sodium-dependent co-transporter SGLT1, the fructose transporter GLUT5 and glucose transporter isoform 2, GLUT2. SGLT1 transports mainly glucose/sodium/galactose, GLUT5 transports fructose and GLUT2 transports both fructose and glucose into the bloodstream [5]. GLUT5 and SGLT1 are located in the apical membrane, while GLUT2 is found in the basolateral

membrane of the enterocytes[5]. Enterocytes are located on the villus of the absorptive intestine (Figure 1.1).

During weaning, when rats are 14 – 28 d old, GLUT5 mRNA expression level and activity can be stimulated through the introduction of fructose into the diet, suggesting GLUT5 is regulated by both age and its substrate [5] [4]. Other sugars, when perfused do not have an effect on GLUT5 expression, while fructose does not affect other sugar transporters, with the exception of GLUT2 [6]. Fructose has been shown to have some influence on GLUT2 regulation, a fructose and glucose basolateral transporter; however, GLUT2 regulation is not specific to fructose since galactose and glucose have been shown to regulate this transporter as well [6, 7].

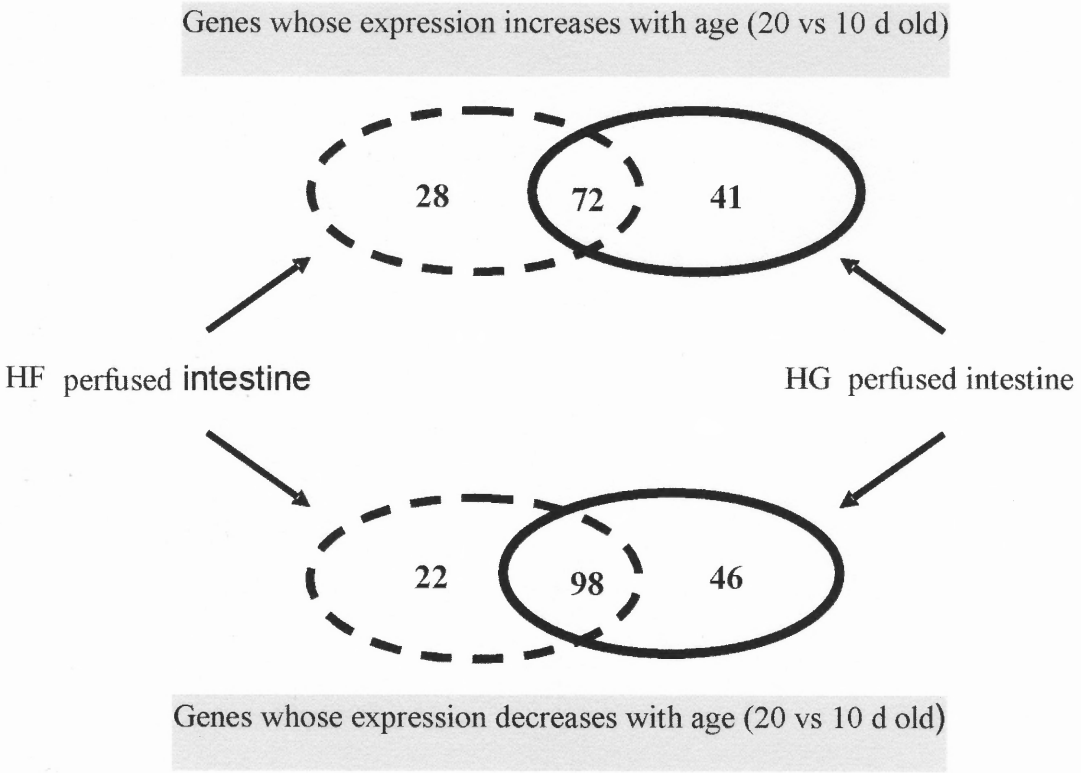


Figure 1.2 Age study results performed by Douard et al. (2008). The left side of the figure shows genes that were up and down-regulated by fructose and age; the right side reflects the genes that are regulated by glucose and age[8]. The genes in the middle reflect the genes that are regulated by age[8].

During the suckling stage, less than 14 d old, fructose alone does not stimulate GLUT5 expression or activity. Douard et al. used this physiological observation to mine for genes that were age and fructose specific; for the purposes of this paper their experiment will be referred to as the age study. Briefly, sets of 20 d and 10 d old rats were perfused with fructose or glucose solutions, through the small intestine [8]. The intestinal tissue collected was analyzed for changes in gene expression between both age groups with the purpose of segregating genes that were simultaneously fructose and age sensitive [8]. To remove genes that changed their expression with age but were not fructose sensitive, 10 d and 20 d old intestines perfused with glucose were used as a

negative control. The results showed that there were 28 genes that were up-regulated and 22 genes that were down-regulated with age under fructose perfusion; 41 genes were up-regulated and 46 were down-regulated with age under glucose condition; and 72 were up-regulated and 98 were down-regulated with age under both glucose and fructose perfusion (Figure 1.2) [8]. When the age and fructose-sensitive genes were clustered, it was discovered that most of these genes were regulated by corticosterones, and this was when Dexamethasone (Dex), a glucocorticoid analog was chosen as a substrate in the following experiments [8]. Dex was administered to neonatal rats (400 ng/g body weight) 48 h before fructose perfusions were done at 10 d of age [8]. The presence of Dex along with luminal fructose enhanced the GLUT5 expression and fructose uptake (Figure 1.3) similar to the enhancements experienced at 20 d intestine when exposed to fructose [8].

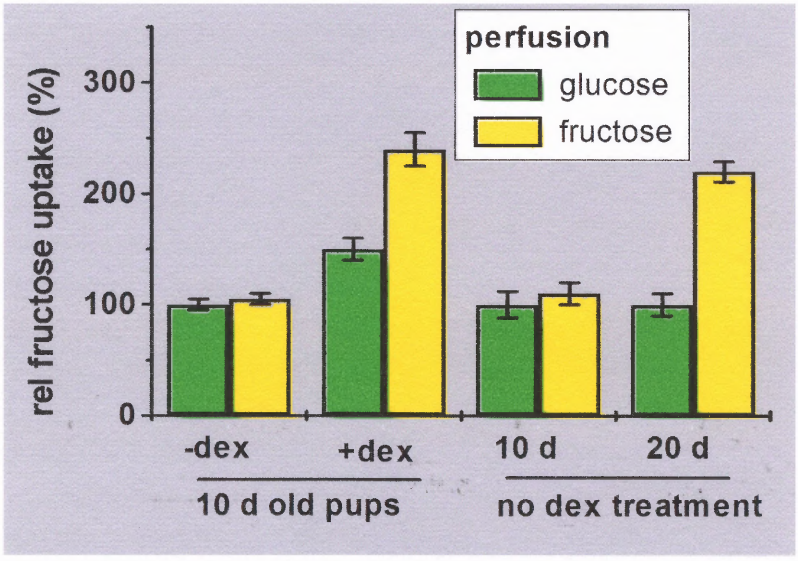


Figure 1.3. Relative fructose uptake in the small intestine at 10 d and 20 d with or without the Dex treatment. When Dex is administered fructose can up-regulate GLUT5 in 10 d old pups. Without any Dex, fructose uptake in 10 d old does not increase with fructose perfusion, however, in 20 d old pups it does increase with fructose perfusion [8].

Glucocorticoids seem to mimic an age effect, but its signaling factors need to be identified to answer the question, "Is the intestinal maturation seen at 10 d in the presence of Dex and fructose, being regulated by the same (Figure 1.4A) or by different signaling factors (age factors) (Figure 1.4 B) as those identified at 20 d being regulated by Age and fructose?"

Microarray technology was used to answer this question. Past experiments were performed using microarrays to identify genes that are regulated by fructose [4, 8]. Cui et al. performed an experiment on 20 d old small intestine, where he identified fructose (F) regulated genes under glucose (G) conditions (20 d F/ 20 d G) [4]. The second set of microarray experiments were performed by Douard et al. to identify genes that are age regulated under fructose (20 d F/ 10 d G) or glucose conditions (20 d G/ 10 d G), and genes that are fructose regulated at 10 d old under glucose conditions (10 d F/ 10 d G) [8]. The third set of microarray experiments were performed to identify genes regulated by Dex in 10 d old small intestine, under fructose conditions. The purpose of this paper was to take the Dex regulated genes in 10 d small intestine under fructose conditions (HFD/HFS) and compare them to the age regulated genes under fructose conditions (20 d F/ 10 d F) from the second set of microarray experiments, performed by Douard et al. [8]; the goal of this comparison is to reveal a common cell signaling pathway that is responsible for the regulation of GLUT5.

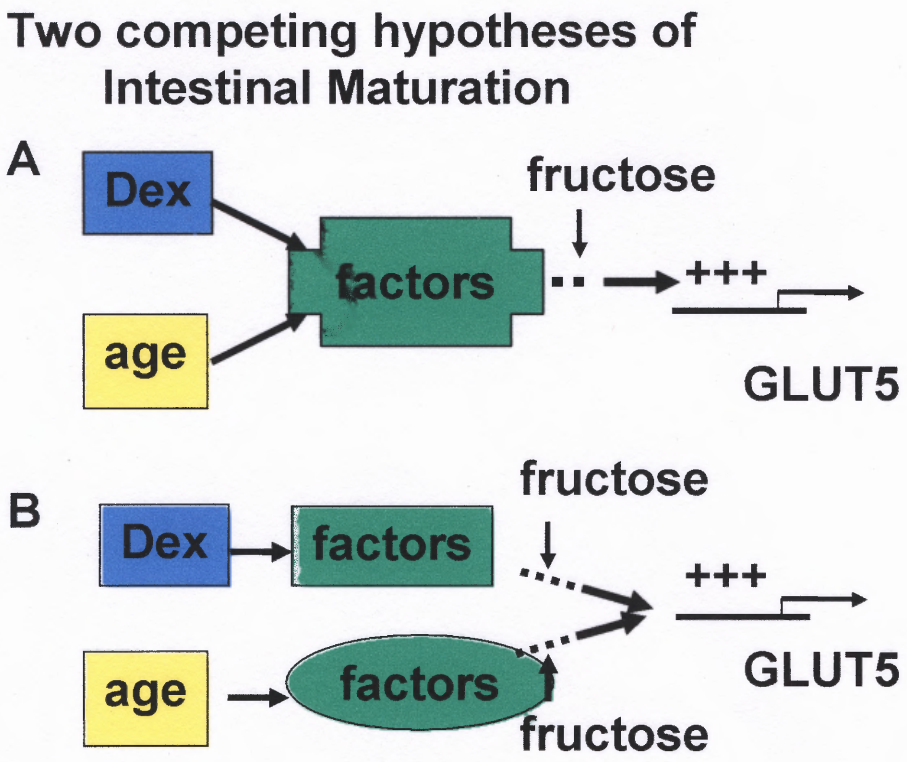


Figure 1.4 Model for the two hypotheses on how the signaling pathways are related between the 10 d Dex treated fructose perfused and the 20 d fructose perfused small intestine maturation is occurring. Hypothesis A states that this maturation is acting through the same signaling factors (pathway). Hypothesis B states that the maturation is acting through different signaling factors.

1.2 Microarray Background

Microarray technology uses a glass microscope slide, with oligonucleotide (DNA) segments that correspond to a specific gene bonded to the surface of the glass. Due to the composition of glass it is difficult to have DNA attach to a glass surface, therefore the glass slides are usually cleaned and coated with another substance to have a good adhesion with the segments of oligonucleotide sequences. The microarrays used in this experiment were coated with Poly-l-Lysine (Center for Applied Genomics, PHRI, Newark, NJ). The Poly-l-Lysine becomes an easy way to coat the glass surface because of the glass' adsorptive nature (glass has layers of adsorbed water [9]) toward polymers [9]. The interaction between the two surfaces forms H-bonds, coulombic interactions and van der Waal's force, thereby creating a strong bond between the glass and the coating [9]. The Poly-l-lysine coating is a polycationic surface which when it comes in contact with the polyanionic DNA (during printing), it forms a bond through coulombic interaction [9] making it possible to bind the oligonucleotide gene sequences onto the surface of the glass slide. The single stranded DNA is locked to the surface by ultraviolet radiation or baking [9]. The single DNA strand is about 60-70 oligonucleotides long, the whole gene sequence is not printed, only a small sequence that is uniquely representative of the gene of interest [9]. The length of each oligonucleotide sequence (more specifically the G-C %) is also adjusted in order for all the genes that are placed on the microarray to be hybridized at the same temperature (Tm) [9].

The printing of the genes onto the surface of the chip is done in an array format so that the genes are oriented in rows and columns that allows for their identification based upon their position after hybridizing the chip. Approximately 1 nanoliter of

oligonucleotide sequences are deposited per spot onto the surface of the poly-l-lysine coated glass chip by a microchanneled pin [9]. Every spot is approximately 100 microns in size and printed 30 microns apart [9]. To make this process as homogeneous as possible and minimize any cross-contamination of samples, the printing is robot operated; the pins pick up the oligonucleotide samples from a 96 or 384 well source plates, after the pins move over the glass slide and they touch the chip's surface to deposit the gene sequences [9]. Once the oligonucleotides are deposited the pins are sonicated, rinsed with distilled water, vacuum dried and returned to the original position to pick up more oligonucleotides from the well-plate to continue with the printing [9]. This cycled process makes the procedure uniform to minimize variation in size for the gene spots; therefore, when the comparison is made amongst the genes, the variations will be due to activity and not because of variation in the quantity of genetic material printed onto the chip [9].

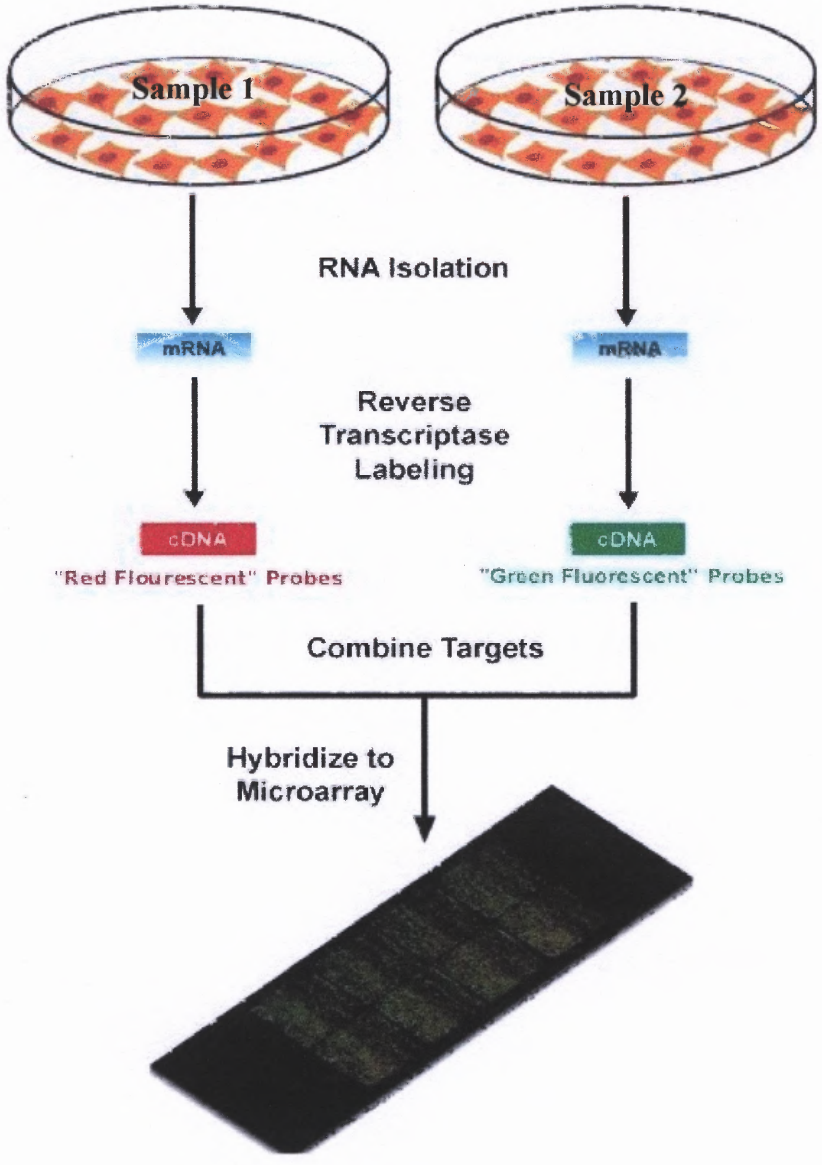


Figure 1.5 Overview of the preparation done to the tissue samples that are used in a microarray experimentation. Figure was taken from <http://upload.wikimedia.org/wikipedia/en/c/c8/Microarray-schema.jpg>.

mRNA samples are usually the common form of experimental samples that are tested using microarray technology because mRNA is a representation of the active genes in the nucleus; if the genes were not active they would not transcribe mRNA from the start. The mRNA is reverse-transcribed to a single strand of DNA called cDNA. The two cDNA samples are usually labeled with two different fluorescent dyes Cy5 (whose

fluorescent emission is collected at 670 nm, part of the red emission spectrum) and a Cy3 (whose fluorescent emission is 570 nm, part of the green emission spectrum) [9, 10]. The labeling is done during the reverse transcription process when a complementary DNA strand to the mRNA is synthesized using Cy3 or Cy5 bound oligonucleotide or a capture arm sequence (Genisphere technology, this sequence is bound to the oligo-dT and then binds to the proprietary "ball of fluophores", refer to Figure 2.3, 2.4). The labeled cDNA (from the two samples that are being compared) is now combined and the original RNA template is degraded (refer to Figure 1.5). The cDNA is denatured to ensure linearity and breakage of 3-D structures and any interactions that may have formed between all the single stranded cDNA and it is added to the microarray. The cDNA binds to the complimentary oligonucleotide sequences that are located on the surface of the microarray [10]. As the single-stranded cDNA is binding to the genes on the surface of the chip, there is a binding competition occurring between the Cy5-cDNA and the Cy3-cDNA to bind to their available complementary sequences. For each gene (spot) on the chip, there will be Cy5 and Cy3 labeled cDNA that will bind to it. To measure the relative differences between Cy5 and Cy3 labeled cDNA, the chips are placed in a scanner that excite the dye within the spots using two lasers (two different wavelengths, one for each dye) and records the fluorescence emitted from each dye [10]. These two measurements represent the relative expression level of that particular gene in the samples.

To analyze the change in expression of these genes, a ratio is calculated between the red and the green (Cy5/Cy3) intensities. There are various mathematical manipulations of this relationship that are used to deduce data from this relationship, but the basis of it all

is dependent on this ratio. The purpose of the ratio is to give an idea if a gene is up or down regulated (a fold change) though the comparison of the two cDNA samples, representing for example two different treatments [10]. Usually one of the samples is a control, a base level, while the other sample is the condition or treatment being studied. An example would be to look at genes changing as a result of the onset of Alzheimer's, one cDNA sample would be normal human tissue, and the other of the diseased tissue. The genes whose expression differ between normal and disease tissues may be linked to the Alzheimer's disease process (either upstream or downstream).

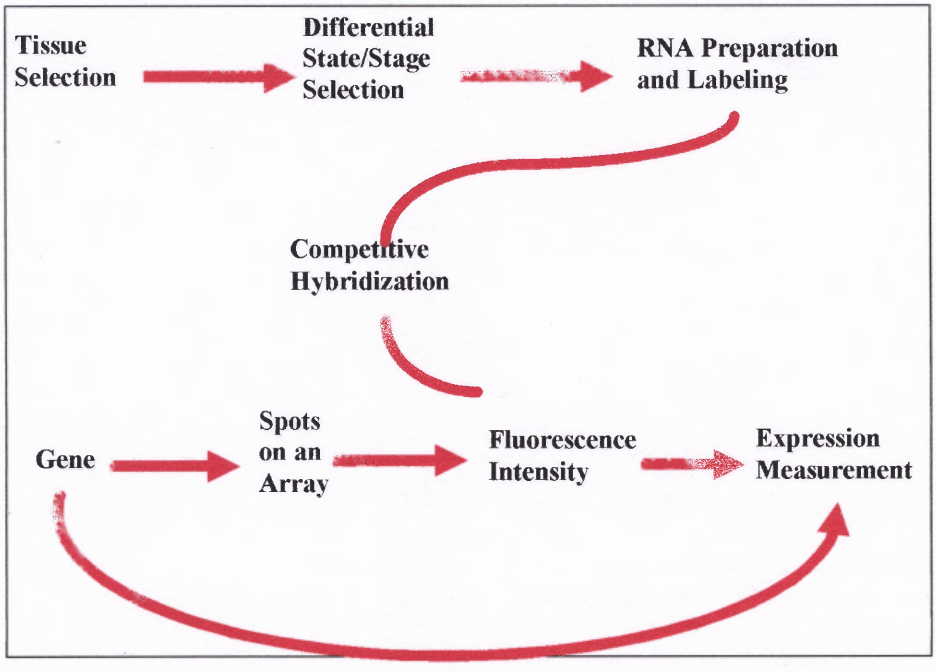


Figure 1.6 Summary of the steps in a Microarray experiment.

CHAPTER 2

MATERIALS AND METHODS

2.1 The Dexamethasone Study

To determine the effect of glucocorticoids on the maturation of the small intestine, in 10 d old pups four different treatment groups were made. Two were given a single intraperitoneal injection of Dex (400 ng/g body weight) at 8 d and 9 d of age (once daily). The other two were given 9 % saline also at 8 d and 9 d of age (negative control). At 10 d of age the Dex and saline treated animals were continuously perfused for 4 h with 100 mM fructose or glucose Ringer following the method of Jiang et al. [11].Therefore, the four pups were Dex-injected fructose perfused (HFD), Dex-injected glucose perfused (HGD), saline-injected fructose perfused (HFS) and saline-injected glucose perfused (HGS). The dose and the time course were chosen based on previous work that clearly demonstrated that a Dex injection 48 h previous to the perfusion induced GLUT5 expression in the small intestine of 10 d old fructose-perfused pups.

2.1.1 Animals and Treatment

Sprague-Dawley pregnant rats were purchased from Taconic (Germantown, NY) and housed in the UMDNJ Animal Facility under procedures approved by the Institutional Animal Care and Use Committee of the UMDNJ- New Jersey Medical School. They were kept under a 12 L:12 D photoperiod and temperature controlled (22-24 °C) room. They were fed an ad libitum diet from Purina Mills (Richmond, NJ). After parturition, the

pups were kept with the biological mother, and their day of birth was recorded as day 0 of age. The pups were injected at day 8 with Dex or saline, marked for identification purposes, and kept with the biological mother until the day of the perfusion.

2.1.2 Intestinal Perfusion

The pups were taken at 10 d of age from the biological mother, and were anesthetized through an intraperitoneal injection (0.2-0.4 ml/100 g body weight) of a ketamine anesthetic cocktail (ketamine 20mg/ml and 12.5 % xylazine in 0.9 % Nacl). Following this, each abdominal cavity was opened and exposing the small intestine with attached blood vessels and nerves. A small incision was made at the beginning of the small intestine and at the end (2 cm above the seccum). A small catheter was inserted into the intestinal lumen at the incision done to the small intestine and secured with suture thread. Ringer solution was used to flush out the content of the intestine. The catheter was then attached to Tygon tubing and perfused through a pump at 30 ml/h at 37°C. The fluid perfused was the glucose or fructose 100 mM solution prepared with Ringer. This sugar concentration used is physiological; it was found that after feeding on fructose pellets, the rats luminal concentration of the sugar peaked at 100 mM [12]. The pups were kept under anesthesia for 4 h by the continuous addition of a 25 % diluted Ketamine cocktail onto the liver. Temperatures of the intestinal cavity and the perfusion solutions were maintained at 37 °C by heat lamps and water baths. After the 4 h perfusion, intestinal tissue was frozen at -72 °C for future analysis.

2.1.3 mRNA Extraction and DNase Treatment

Total mRNA was extracted from the frozen tissue using the TRIzol Reagent (Invitrogen, Carlsbad, CA). Briefly, approximately 100 mg of tissue was homogenized in 1mL of TRIzol solution and processed according to the Invitrogen's usage Instructions. The total mRNA concentration was found using a spectophotomer (Beckmann DU^{R} 640, Fullerton, CA) and mRNA quality was characterized by 1 % Agarose electrophoresis gel with ethidium bromide staining. To remove any possible genomic contamination RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) prior to reverse transcriptase (RT). For more detail on this procedure, please refer to Appendix B.

2.2 Microarray Experiment

2.2.1 Experimental Design

The experimental design, analysis and interpretation of the microarray experimentation followed a similar procedure done by Cui et al.[4]. Twelve 8,000-oligonucleotide rat microarrays [Rat 8k Oligo Array; Center for Applied Genomics, PHRI, Newark, NJ] were used for this experiment.

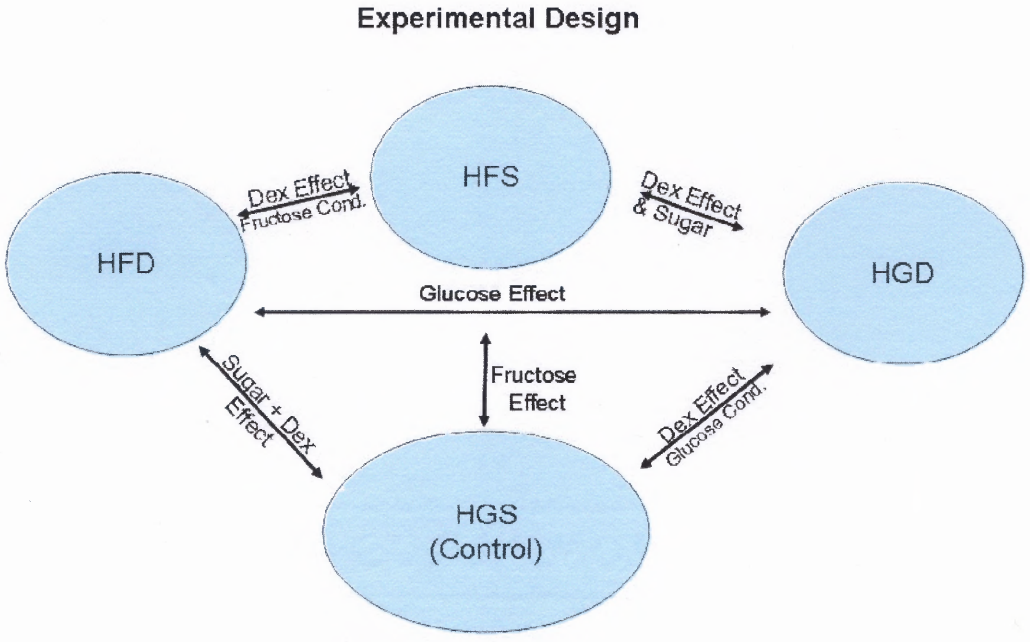


Figure 2.1 Experimental Design of the treatment groups and featured comparisons. The purpose is to look for genes under Dex and Fructose conditions at 10 d that mimic the age effect seen at 20d under fructose conditions.

There were four treatments of 10 d rats that were used for the microarray experiment, and four rats per treatment were used (n=4); High Glucose Saline (HGS), High Glucose Dexamethasone (HGD), High Fructose Saline (HFS) and High Fructose Dexamethasone (HFD). Microarray technology allows for the direct comparison of two treatment groups; if more treatments are to be compared a common treatment (control group) is necessary amongst all the experiments [9]. The mRNA for the HGS treatment was pooled and used as a master mix, subsequently designated as the control group (being the one treatment common amongst the 12 microarrays). The HGS was used as the treatment control since previous experiments in the lab have shown that perfusing glucose does not affect the activity or expression of GLUT5 in weaning rats [4]. The HGD/HGS microarray (Appendix A.1) revealed the Dex regulated genes in the small

intestine at 10 d; the HFS/HGS chip (Appendix A.2) revealed the fructose regulated genes in the small intestine at 10 d under saline conditions; and the HFD/HGS chip (Appendix A.3) reveals the genes regulated by both fructose and Dex in the small intestine at 10 d.

The HFD/HGS dataset (Appendix A.3) has two factors (Dex and fructose) that are affecting the results; therefore it was not possible to decipher which factor was specifically responsible for the regulation of each gene. Therefore, because both of these factors can have an affect on regulation, it was not possible to conclude if the changes seen are due to a Dex influence solely, fructose influence solely, or a combined effect by both Dex and fructose. To be able to distinguish between these 3 different cases, the HFD/HGS genes were compared with the HFS/HGS (Appendix A.2) genes in a 2-way SAM (explained further in Microarray Analysis section 2.2.3). Due to HFD/HGS and HFS/HGS having a common denominator, these sets of genes can be compared to each other, through a 2-way SAM (refer to Figure 2.1), to yield genes that are solely Dex regulated and genes that are Dex regulated under fructose conditions. At this point any genes that were solely fructose regulated have been removed from the HFD/HGS results (Appendix A.3); however, there are still some genes in the dataset that are regulated by Dex under glucose conditions along with genes that are Dex regulated under fructose conditions (Appendix A.4).

To remove these Dex regulated genes under glucose conditions, the genes from Appendix A.4 were compared with HGD/HGS (Dex regulated genes under glucose conditions) (Appendix A.1) to look for common genes between these two datasets. The common genes found were regulated solely by Dex independently of fructose or glucose

being present (noted in Appendix A.4 by *); so these genes were subtracted from the dataset to compile a new dataset, HFD/HFS, that only lists genes solely regulated by Dex under fructose conditions (Appendix A.5).

The Dex study microarray results (found in Appendix A) were compared to the Age Study results (20 d/10 d) that was performed by Douard et al. [8]. The common genes found were identified and compiled in Appendix A.6 (refer to Figure 2.2 for analysis overview). The genes of interest for this paper are any common genes between the 10 d Dex Regulated under fructose conditions (Appendix A.6) and the Age regulated genes after fructose perfusion (20 d F/ 10 d F). These common genes will prove that Dex is mimicking an Age effect when fructose is perfused, and that these genes are part of the signaling mechanism is responsible for the regulation of GLUT5 transcription and activity in the small intestinal cells.

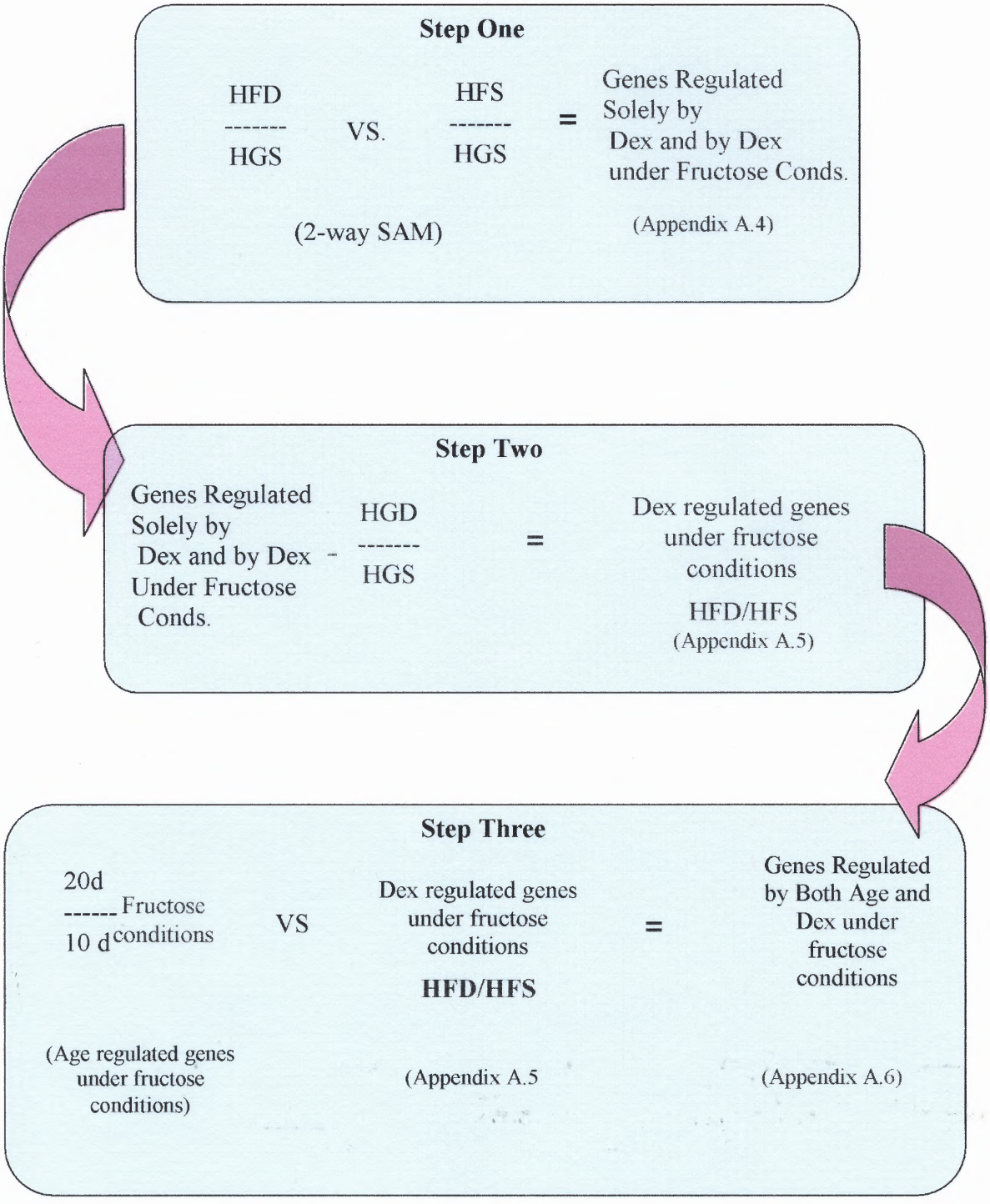


Figure 2.2 Gene Analysis Workflow done to identify the genes that are regulated by both age and Dex under fructose conditions in rat small intestine.

2.2.2 Microarray Experimental Protocol

Microarray experiments were performed using the Genisphere 3DNA Expression Array 350 Detection Kit for Microarrays (Genisphere, Hatfield, PA). Briefly, the mRNA samples were reverse transcribed with the Cy5 or Cy3 oligo-dT primers. The use of the Cy3 and Cy5 primers are the fundamental building blocks of Genisphere Microarray detection system. These primers have an extra sequence at the 5'end referred to as the 3DNA dendrimer arm (see Figure 2.3). There are two dendrimer arms used in this kit, one for the Cy5 (red) and one for the Cy3 (green) fluorescence tails (dyes). The dendrimer arm serves as the "capture sequence" for the fluorescent tails that are added in the 2nd hybridization step. Each fluorescent tail has a complementary sequence that binds to its corresponding dendrimer arm (that is at that point already bound to the cDNA), which in turn is bound to the complimentary oligonucleotide sequences, of the cDNA, on the microarray. When the Cy3 and the Cy5 tagged (has dendrimer arms bound to it) cDNA is added to the chip, the two treatment samples compete with each other to bind to the corresponding complimentary genes on the chip. This is a reflection of quantity in terms of regulation, because if a particular gene is more up-regulated in one treatment it will have more Cy5 (red) than Cy3 (green) fluorescence dye molecules therefore when it binds to the chip's complementary sequence there will be more Cy5 fluorescence tails bound to that gene, the vice versa is true for down-regulated genes.

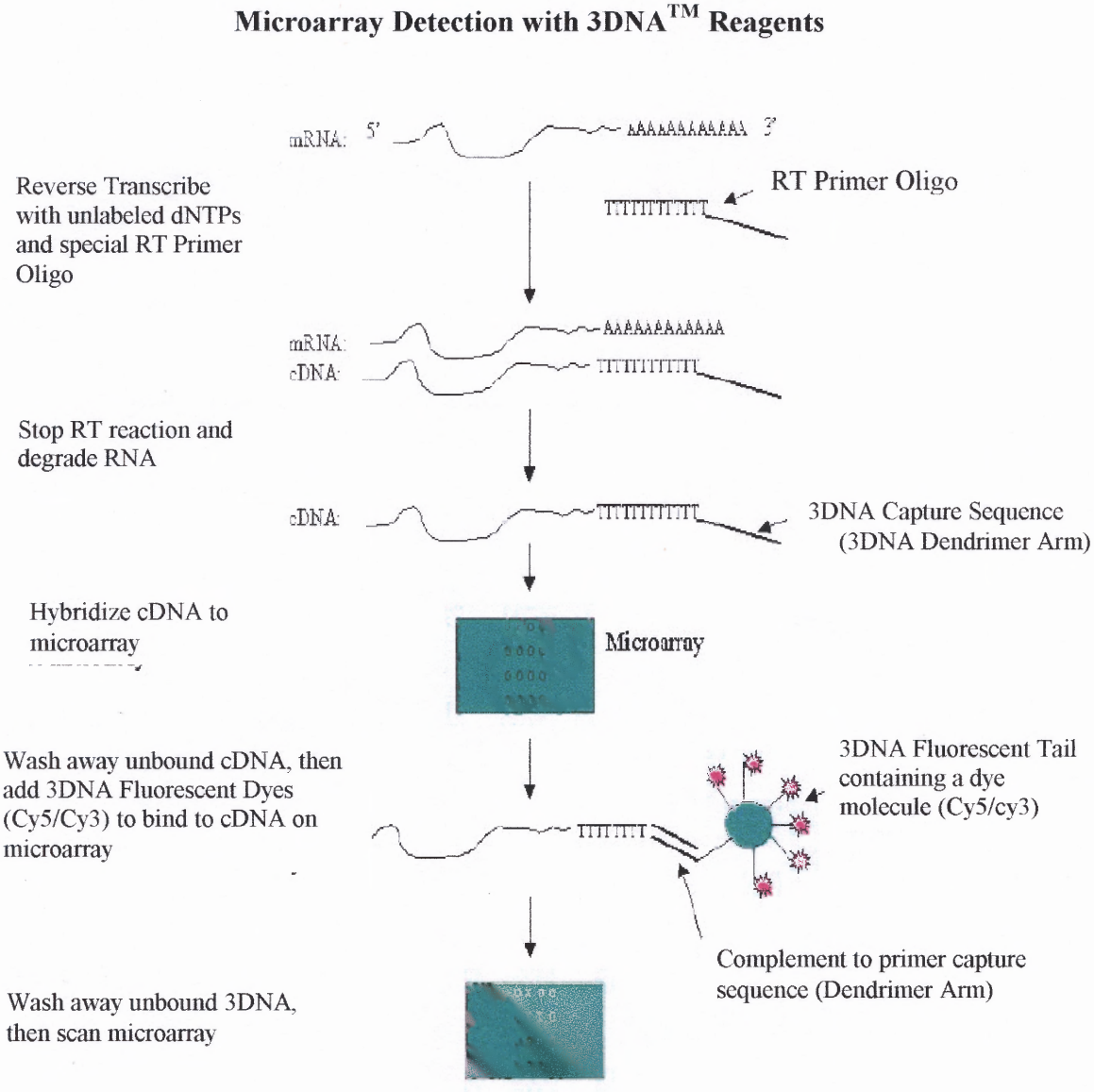


Figure 2.3 Model for the overall process of microarray detection using a 3DNA Reagents kit [1].

A scan is taken of the microarray, where the fluorescence tags (Cy3 and Cy5) are excited at their respective wavelengths (532/635 nm) and the fluorescence that is given off is quantified. A ratio is taken of the fluorescence measurements (Cy5/Cy3). The quantitative comparison of one dye to the other, is the same as comparing one treatment to the other because each dye represents a different treatment group; ie: if Cy3 was used for HGS and Cy5 was used for HFS, the ratio of the Cy5 vs. Cy3 will reflect how the HFS genes are behaving in comparison to HGS. Therefore if a gene has more Cy5 (red) bound to it, we know it was up regulated in HFS in comparison to the HGS group and if the gene has more Cy3 (looks green) bound to it, that means that it was down-regulated in HFS when compared to HGS. There is a third possibility where if the gene is regulated the same in both treatments, meaning it is not up or down-regulated in HFS when compared to HGS, the fluorescent color is yellow, the Cy5/Cy3 ratio is equal to 1; sometimes black spots are encountered, which means that particular gene is not being expressed in either sample being tested, it is a black spot because there is no fluorescent signal.

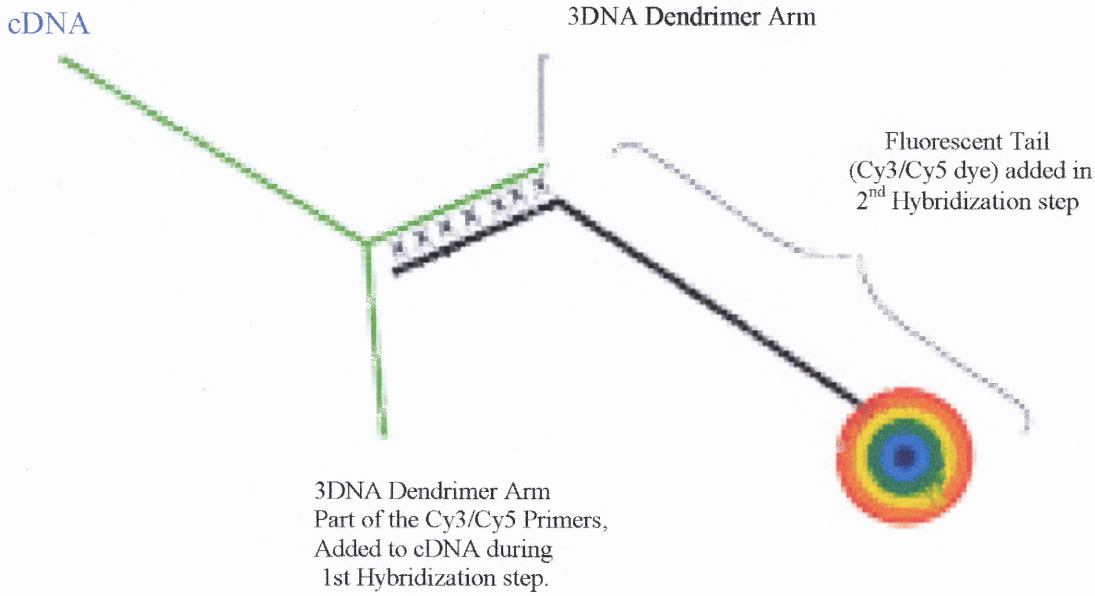


Figure 2.4 Model of the 3DNA Dendrimer Arm and its complimentary bind to the Cy3 or Cy5 fluorescent tail [1].

In the microarray procedure, as mentioned earlier each treatment group was reverse transcribed with one of the primers, Cy3 or Cy5, ie: if HGS was transcribed with Cy3, the other treatment (HFS for example) was transcribed with Cy5. In the experimental replications, labeling of the two dyes was alternated between the sample groups as suggested per Dudoit et al. [13]. This is called dye-flipping and it is done as a control method to do away with dye bias. Sometimes the dyes have a higher affinity for a particular gene and may bind heavily to it and in turn off-set the fluorescence ratio (Cy5/Cy3) and reflecting inaccurate gene activity. An example of dye-flipping is when the Cy3 primer is used on HGS and Cy5 primer is used on the specific treatment, in the next replication of this experiment the primers are flipped, so Cy5 is used on HGS and Cy3 is used on HFS. An important step to remember is that once the calculations of the

ratios are performed, Cy5/Cy3, this is not really reflective of the intial experimental design. The initial goal was to look at treatment/control and see how they were similar or differed. However, when dye-flipping is performed the treatment was not always tagged with Cy5, it was also tagged with Cy3. Therefore to look at the right comparisons, treatment/control, the experiments that had Cy5 as the control need to be flipped back. Flipped back meaning the reciprocal of that ratio must be performed so that all the ratios of the replicated experiments are reflecting the treatment/control and not Cy5/Cy3. The actual step procedure followed for this experiment is briefly described below.

After the reverse transcription the remaining mRNA was degraded; the two reactions were combined (the Cy3 and the Cy5 tagged samples (dendrimer arm bound to it) were placed into one tube, and the cDNA was concentrated using the Millipore Microcon YM-30 Centrifugal Filter Devices (Millipore, Billerica, MA). A hybridization mix was created to resuspend the cDNA, in accordance to the Genisphere 3DNA Array 350 Protocol (refer to Appendix C for more detail). The cDNA was denatured at 75°C and loaded onto the microarray for it to bind to its complimentary oligonucleotide sequences. The chip was left overnight in a dark humidified chamber at 53°C. The excess hybridization mix was washed off the next day through a series of washes and concentrations of SSC buffer solution.

The next step was to bind the Cy3 and Cy5 fluorescent tails to the cDNA-dendrimer arm molecules (referred to as the Capture Sequences in Figure 2.3). Remember from the last step, that the cDNA is at this point bound to its complimentary genetic sequences on the microarray (refer to pervious paragraph). Therefore, a 2^{nd} hybridization mix was created Cy3 and Cy5 fluorescent tails (dyes) were suspended in it.

The mix was denatured and loaded onto the chip to have the fluorescent tails (also referred to as the 3 DNA Capture Reagent with fluorescent dyes in Figure 2.3) bind to the corresponding dendrimer arms (refer to Figure 2.3 for Overall procedure details). The chip was again incubated in a dark and humidified chamber at 62°C for 3 hours. The chip went through a second washing procedure with the 2XSSC buffers and stored in a dark chamber until it was ready to be scanned.

Scanning of the chips was done using a GenePix 4000B Microarray Scanner (Molecular Devices, Downington, PA) and the software used was Genepix Pro 5.1 (Molecular Devices Corp., Downingtown, PA). The images were saved as .tif files, to be used in the future for analysis. For a more procedure details please refer to the protocol used located in Appendix C.

2.2.3 Microarray Data Analysis

To identify the genes present on the microarray and their intensities, the images were opened along with the gene array list (a gene map that labels the corresponding spots with the Gene ID) onto the GenePix Pro 5.1 (Molecular Devices, Downington, PA) software. The array list represented by a grid was aligned with the spots on the scanned image of the chip. Each spot on the array list was centered and adjusted in diameter to fit around each gene from the image of the chip; the white circle in Figure 2.5 represents the array list spots. Each gene was observed to make sure that it was uniform; meaning that each gene was not missing sections, such as the gene on the bottom right hand-side of Figure 2.5. An ideal gene is like the red spot found in the top- middle of figure 2.5, it is uniformly round, mostly of one color (red in this case) and it is not missing any large

sections of its surface area. If a gene was missing parts or sections it was flagged. Flagging a gene means that it would be marked with a strike through the middle, such as the gene on the bottom right and top left of Figure 2.5 below.

Once it was time to calculate the ratios of each gene, the GenePix Pro 5.1 recognized the flagged genes and would not incorporate them into the ratio calculations. If you refer to Appendix D, there are specific details on how to work with the software to load the array gene list, how to align the list spots to the genes on the chip and how to flag genes.

As the ratios were calculated by the GenePix Pro 5.1, the software option to do a global normalization (normalize to 1) on the whole chip was selected. This assumes that overall most gene expressions do not change based upon the treatment and the average ratio is equal to 1. Once the calculations and the global normalization were done the results were saved as a .gpr file for use in the next step.

The one issue that global normalization could not adjust for was non-homogeneity. It is very rare to have a chip that is perfectly hybridized across all of its sections. Many times, there are spatial differentials that a global normalization cannot adjust for, because these non-conformances are present in small areas of the surface and not across the whole chip. To normalize for these spatial differences, a local regression normalization was performed in addition to the global normalization.

The calculated ratios done by GenePix are actually a Log_2 (Cy5/Cy3) calculation. The intensities of these genes are calculated as $Log_2(x)$ values to makes the intensities symmetrical as far as numeric value. An Example of this would be Cy5 = 2 and Cy3 = 1 for sample 1; sample 2, Cy5=1 and Cy3=2. If a simple ratio is taken of these samples

Cy5/Cy3 for sample 1= 2 and sample 2= 0.5; but, if a Log_2(Cy5/Cy3) is calculated sample 1 = 1 and sample 2 = -1, therefore representing the intensities for up- and down-regulated genes symmetrically and focused around zero. For the simple ratios if the two samples were the same, meaning no change in regulation, (Cy5/Cy3 = 1/1 = 1) these genes are focused around 1, this also being the reason why the global normalization was done to 1. For the Log_2(Cy5/Cy3) = Log_2(1) = 0, therefore, the genes that are not regulated by the treatments at hand, are focused around zero. The quantitative regulation values are not being changed, they are just being displayed in a form that most people are used to.

Figure 2.5 A section of a microarray. This figure shows the alignment of the gene list onto the scan of the chip and the removal (flagging) of genes that are not homogeneous.

A Lowess normalization (Print-tip Lowess) was performed on all the calculated result files, obtained and saved as described in the previous section. A Lowess normalization is a local normalization (local regression). The Lowess normalization works by taking a group of genes (a subarray on the chip) and finding the weighted average of the spot intensities; it then normalizes (adjusts) the individual intensities, of

the corresponding subarray, by that calculated amount [9, 13]. A weighted average is performed by taking all the intensities in a select area, and given more weight (percent influence) to the intensities that are close together and the ones that are farther away are less influential to the calculated intensity average [9]. This way the genes that are closely related will be pulled into a tighter range, while the genes that were not closely related (different intensities) will remain as such but somewhat more correlated with other points. The select area on which the weighted average is taken is usually no more than 40 % of the whole parameter, it has been found through research that this percentage results in the smoothest scatter plots [13].

The Lowess normalization was done on every subarray of genes found on the chip; where every chip has 32 subarrays and each subarray has 250 genes. This local normalization reduces any dye and print-tip bias that might be present as well as any spatial differences that maybe present due to: uneven washing; bubbles trapped during hybridization; uneven placement of hybridization mix onto the chip; unleveled placement of the chamber during the hybridization steps; drying of the hybridization mix during hybridization; or a differential gradient in the background [9]. In general the red intensities tend to be lower than the green intensities [13] as well as pin specific differences in oligonucleotide content from the printing procedure, local normalizations have been more successful at removing these bias that global normalizations [13]. The Print-tip Lowess software used for the local normalization was designed by the Center of Applied Genomics (PHRI, Newark, NJ) based on the local normalization methods described by Dudoit et al. [13].

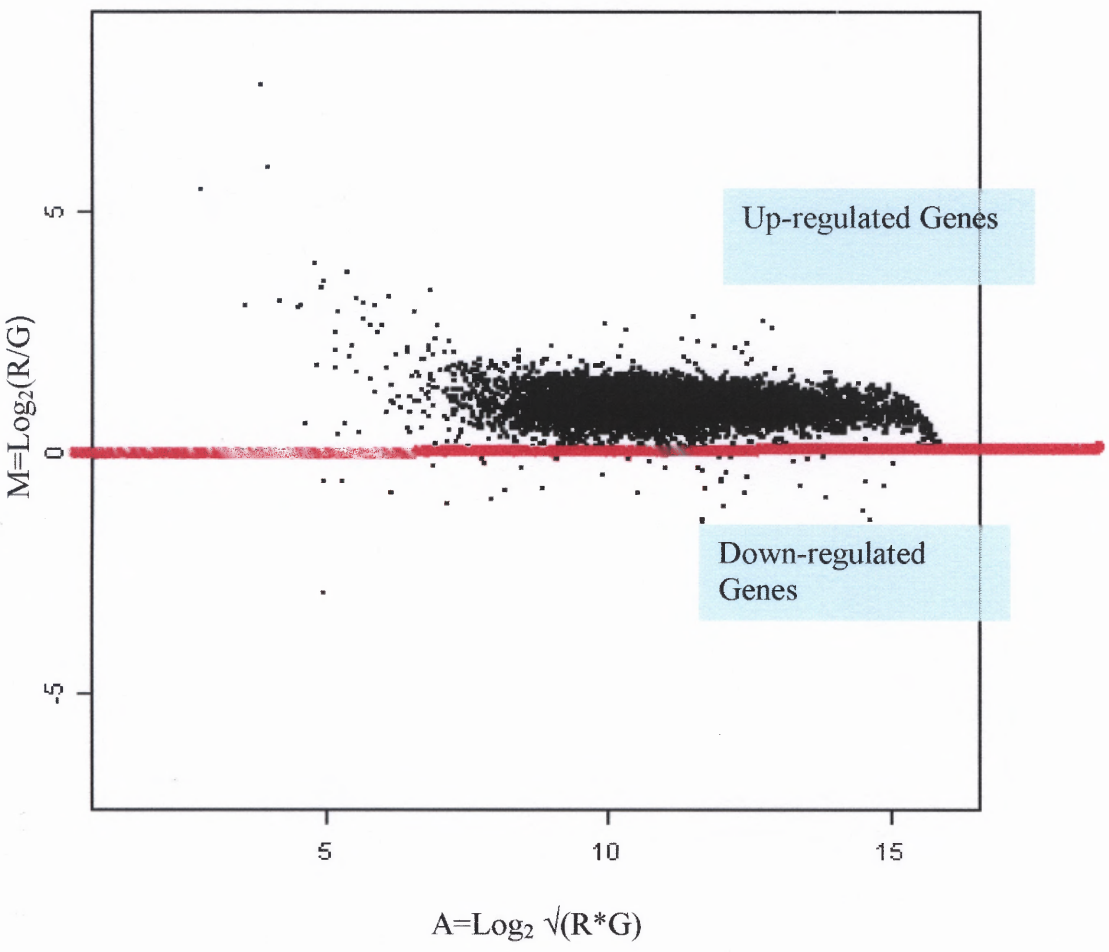


Figure 2.6 M-A plot showing the HFS/HGS data before the Lowess Normalization was performed. Notice how the data is not centered about zero and the backend of the data points are dropping toward the negative side and the front is turning up toward the positive side of the graph. These shifts at the ends are due to print-tip variance and dye bias in the results, while the whole data shift above 0 is due to a background gradient.

To check that the local normalizations done were working, an M vs. A plot is was calculated (Figure 2.6) [9], where $A=\log_2 \sqrt{(RG)}$ and $M=\log_2 R/G$ (R is the Cy5 intensity and the G is the Cy3 intensity) [13]. An M-A plot can be very informative in that it can give some insight into how the data is behaving. Lets take Figure 2.6 as an example, here the data is completely shifted above zero, which suggests that there is a common shift in all intensity ratios, such as a background gradient would do [9]. The two tails (up and

down) on the left and right side of the graph suggest that there is print-tip or dye bias in the intensity results [9]. After the local regression normalization, another M-A plot is calculated; refer to Figure 2.7, where the normalization adjusts the data values for these biases encountered. The normalization takes all of the intensities and readjusts the intensity values by the weighted average that is calculated [9], and in doing so the data points are all focused around zero; which is where they should be if the genes are not being regulated by the treatment (remember no change means: $Log_2(1/1)=0$).

The M-A plot, however, can also be used to determine the cut-off point for regulated genes (the genes that are regulated are calculated through a SAM test, which is explained a bit later in this section). The cut-off point, of the M-A plot, is where the genes above a certain fold change (for up-regulated) and below that same negative number (for down-regulated) are being regulated by the treatment [3]. The genes that are not changing in an M-A plot are clustered around zero (genes that are not changing their ratio Cy5/Cy3 =1, if the $Log_2(1) = 0$), therefore the genes with differences in gene expression in an M-A plot are the outlier points and these are the genes we are interested in.

Let's look at Figure 2.7 as an example. The red line shows where the +/- 0.8 line should be, which is the point where the outlier points begin. If one was to declare the cut-off point for the fold change below + 0.8, it would be incorrect because below this point is where all the genes are clustering together because they are not being regulated by the treatment. Therefore, even though an individual gene may have a high Cy5 quantity when compared to its complimentary Cy3 content, it does not mean that it is significant because its overall intensity is just like all the other genes. Therefore, it is vital to choose

the cut-off point after looking at where the data-points are clustered together and where the outliers are located [3]. Once the cut-off point is chosen, it is reverse-calculated to a fold change [fold change = anti-$\log_2$(cut-off point)] and this is applied to the analysis of the SAM results. This is another method used, besides statistical methods, to identify any genes that are false positives present in the data results as a result of a non-conservative FDR (False Discovery Rate); FDR is discussed further in the next paragraphs when discussing the SAM test.

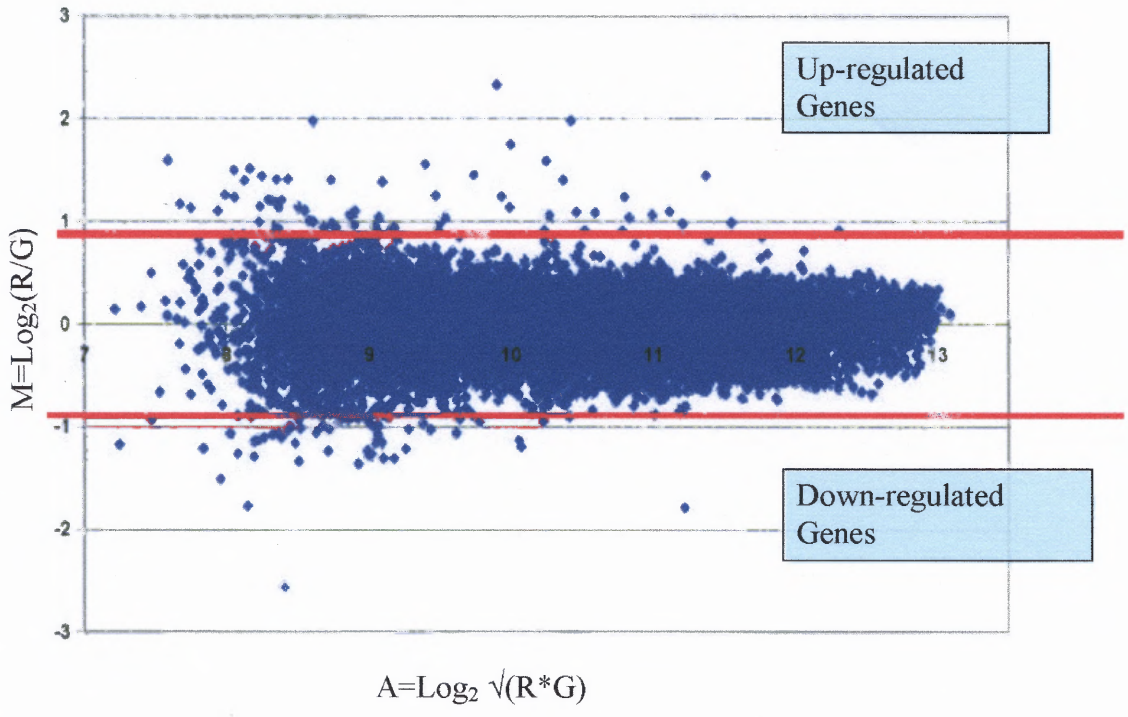


Figure 2.7 M vs. A Plot of the HFs/HGS after local regression normalization; the fold change cut-off is at +/- 0.8. The outlier points, above/Below the red line, are up or down regulated genes. The majority of the genes are clustered about zero ($\log_2$(Cy5/Cy3) =0, where Cy5/Cy3=1) because they are not changing with treatment.

Once the Lowess Local Regression Normalization was performed the data was compiled into an Excel file, and the flagged spots (described in the previous section)

were filtered out along with spots that had a diameter smaller than 60μm using a nomadic algorithm described in Appendix D, section B.3. Once filtered, the TIGR Multiexperiment Viewer (MeV) 4.0 software was used to further analyze the data by conducting a Significant Analysis of Microarrays (SAM) test to look for the genes in the results that were being regulated by the treatment.

A SAM test is a modified version of the T-Test adapted for the use in microarrays. With microarray data, low intensity spots have very low variance when compared to high intensity spots and tend to come up significant with the t-test whereas their signal is not deemed reliable. SAM D_i value is similar to the t-test t value with the exception of the addition a safety factor to the denominator (Figure 2.5)[3] which is added to eliminate spurious low variance. To calculate the safety factor, the data is binned into 100 sets by intensities and the safety factor is evaluated for the variance of the bins. SAM also estimates the FDR (false discovery rate) of the results by taking random permutations of the genes from the replicated treatment experiments to estimate how many genes would be expected significant just by chance and not due to the treatment [3].

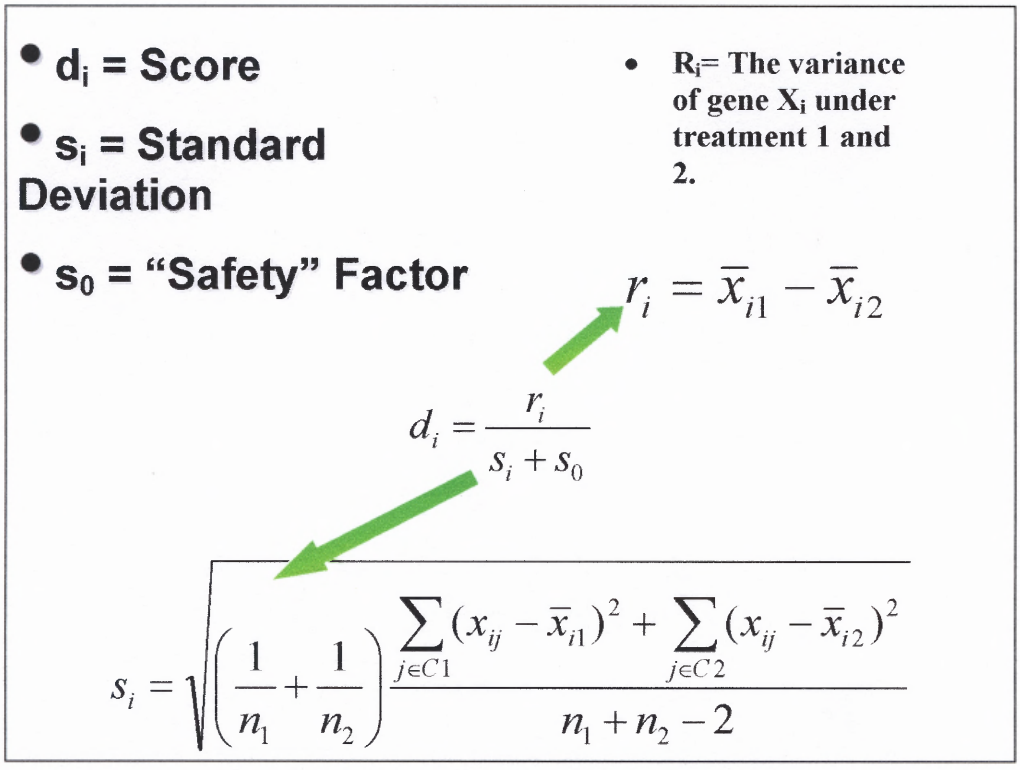


Figure 2.8 SAM Statistic Test is a t-test with the addition of a safety factor to normalize the standard deviation.

A SAM test is calculated as per the equation in Figure 2.8. D_i is the result of the calculation that dictates if the two samples being compared are significantly differentiated as a result of the treatment. Ri is the variance, the value difference between the two samples, the difference between the same gene but under the two treatments being compared. SI is the standard deviation factor found between the two genes being tested. The standard deviation equation is also described in the figure, where n stands for the number of samples, X is the value of the sample, 1 and 2 stand for the two treatments being compared. S_o is the safety factor that is calculated from the replicated microarray experiments for the same treatment (N=4).

A 2-way SAM, works the same way as the 1-way SAM, described above, except that a 2-way SAM is comparing two treatments directly after each one has been

compared to a treatment control individually, in the case of this experiment the control is HGS. Therefore, in a 2-way SAM, the two treatments; ie: HFS and HFD were first compared to HGS (ratios taken from the microarray experiments). In the first step the significantly regulated genes are calculated with SAM the same way as the one-way; but there is a second step where these two datasets (HFD/HGS and HFS/HGS) of significant genes are compared to each other and SAM calculates the significant genes between the two datasets and yields the HFD/HFS genes. This is a real advantage presented by the statistical software, because you can conclude the regulation between two treatments and not have to run a separate microarray experiments with the HFD and HFS treated tissues.

Another great feature about the SAM test is the ability to control the confidence level of the test results; meaning the amount of false-returns present in the data can be adjusted with the False Discovery Rate (FDR) [3]. The FDR is a feature of the SAM test, where by adjusting the DELTA, it is possible to control the percent of false negative or positive results that is to be expected at the end of the test (refer to Appendix D for specific steps on how to change DELTA) [3]. For this experiment, the DELTA was set to 10% FDR. It was decided to be less conservative with the results because it was possible to pick up some of the false negative genes; which really means that they are significantly regulated but would not be part of the results otherwise. Some of the reasons for false positive and negative genes are described in chapter 4, section 4.3. For more details on the specific steps used, please refer to Appendix D.

Once the SAM tests were finished, the results were all shuffled and needed to be sorted as up- or down-regulated. The results were imported into Excel once again, and the corresponding ratios ($\text{Log}_2(\text{Cy5/Cy3})$) were taken from the original data calculated by

GenePix Pro 5.1. To visualize the data in relative terms, the anti-Log_2 was applied to the ratios to get a fold change, meaning to see how much the gene has increased or decreased when compared to the control HGS. The fold-changes will be from +1 and higher for up-regulated and from -1 and lower for down-regulated genes. To estimate what the cut-off point should be for the fold change an M vs. A plot is consulted.

2.2.4 Sorting the SAM Results/ Design Primer

The gene results were now sorted by fold change and per treatment. The next step was to sort the genes by function (ie: metabolism, cell communication, regulation, etc.). The gene id was taken and searched for in www.pubmed.com. Various journals were consulted and the functions determined based on their known activities.

Once sorted into functional groups, genes with the largest fold change were identified to confirm their microarray results with realtime PCR. Primers were designed using Primer3 software (http://primer3.sourceforge.net/). Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Please refer to Appendix E for the protocol used for primer design.

2.2.5 Realtime PCR Confirmation

Due to the 10% FDR chosen during the SAM test, realtime Polymerase Chain Reaction (PCR) was used to confirm the regulation of the genes found in the microarray analysis. It was decided to accept 10% false positives and/or negatives because if a more conservative approach was chosen (FDR= 0%) there was a large possibility that some genes would never be identified and this data would be missed. Therefore, it is better to

have some false results and identify them as such through PCR, than to miss on identifying genes that are being regulated due to treatment. The PCR confirmation takes a bit longer to perform than a microarray because genes are confirmed one at a time.

The realtime PCR was performed using the MX 3000P ™ (Stratagene, La Jolla, CA) with the iTaq SYBR Green Supermix with Rox (Bio-Rad Laboratories, Hercules, CA) [8]. Briefly, a reverse transcriptase was performed primers were designed and purchased for the specific genes that were being confirmed. A PCR was performed on all the treatment samples used in the microarray experiment. The PCR process uses the cDNA samples, created from the reverse transcriptase, and primers to replicate the gene of interest's cDNA sequence through various cycles. In a cycle the cDNA is denatured to a single strand and the primer anneals to the cDNA. A DNA polymerase molecule takes the primer (RNA and DNA sequence of the gene of interest) and simultaneously reads the sequence of the single stranded cDNA and synthesizes its complimentary sequence from free oligonucleotides (dNTPs). The complementary sequences of the single-strand cDNA and DNA bind together to reform a double-stranded DNA molecule, this process of binding is called elongation. Therefore the steps in realtime PCR are denaturation, annealing and elongation. These steps occur various times throughout the PCR experiment as more and more DNA is exponentially replicated.

The MX 3000P is equipped with its own software and it outputs the quantity of cDNA present by detecting the amount of fluorescence given off by the iTaq SYBR Green in relative comparison to the Rox reference dye. The iSYBR Green, fluorescence tag, intercalates itself to DNA molecules and in turn gives the PCR user the ability to quantify the amount of DNA present in the experiment by the amount of fluorescence

that is detected. The DNA quantity amplified is directly related to the number of cycles that are performed, for the more cycles that are done, the more DNA is amplified and the more iSYBR Green fluorescence is present. Therefore, the amount of DNA that is amplified is given by the number of cycles that have occurred and the number of cycle counts used to calculate the relative expression, of the gene of interest, is where the fluorescence given off by iSYBR Green is first detected by the MX 3000P; this is what is referred to as the cycle threshold count (C_t). For details on the steps involved in this procedure please refer to Appendix F.

Once the counts are obtained from the MX 3000P, the relative expression of the target (ratio) gene is calculated using the following equation[8]:

$$\text{Ratio} = (E_{target})^{\Delta Ct_{target(control-sample)}} / (E_{ref})^{\Delta Ct_{ref(control-sample)}} \quad [8]$$

This calculation is based on the Real-time PCR efficiencies ($E= [10^{(1/slope)}]^{-1}$ and the cycle threshold count (Ct) difference between the unknown sample (target gene) and a control gene [8]. For this experiment the control gene was elongation factor 1α (EF1α), because this gene is not affected by Fructose or glucose perfusions, nor affected by age of the pups [8] [4]. The EF1α was used to normalize the PCR data results. The ratio that is calculated is actually of cDNA amount replicated during the PCR; but this is a direct reflection of the amount of mRNA that was present originally present in the tissue sample, and in turn this reflects the gene of interests' activity as a result of the treatment it was given. The next step was to take a relative measurement of the gene activity by normalizing the mRNA ratios (quantities) by the control treatment, HGS. The relative measurement is a way to look at the gene activity in terms of fold change. Please refer to figures for these results.

CHAPTER 3

RESULTS

The microarray experiments were done with a total of n=4, per treatment group. Three 1-way SAM tests were performed (HFS/HGS, HFD/HGS, HGD/HGS) and results are summarized in Appendix A.1-3; a 2-way Sam tests was performed on (HFD/HGD) and (HFD/HFS); the results are summarized in Appendix A.4. The function of each gene was researched on www.pubmed.com, and grouped according to its ontology. The datasets were analyzed and compared to decipher the Dex, fructose, and the Dex under fructose conditions regulated genes at 10 d, please refer to section 2.2.1 for more details about this evaluation. The results are compiled in Appendix A. Once the Dex regulated genes under fructose conditions (Appendix A.5) were identified, all the genes from this study were compared with the age study (20 d/10 d) results performed by Douard et al., and a list was compiled of common genes found between the two studies (Appendix A.6).

3.1 Dex Regulated Genes under Glucose Conditions at 10 d (HGD/HGS)

The Dex regulated genes under glucose conditions (HGD/HGS), refer to Figure 2.1, revealed 29 up-regulated and 18 down-regulated genes (Appendix A.1). The majority of the up-regulated genes (52%) were involved in metabolism (Figure 3.1); while most of the down-regulated genes were involved in cell communication (27%) and metabolism (22%) (Figure 3.2).

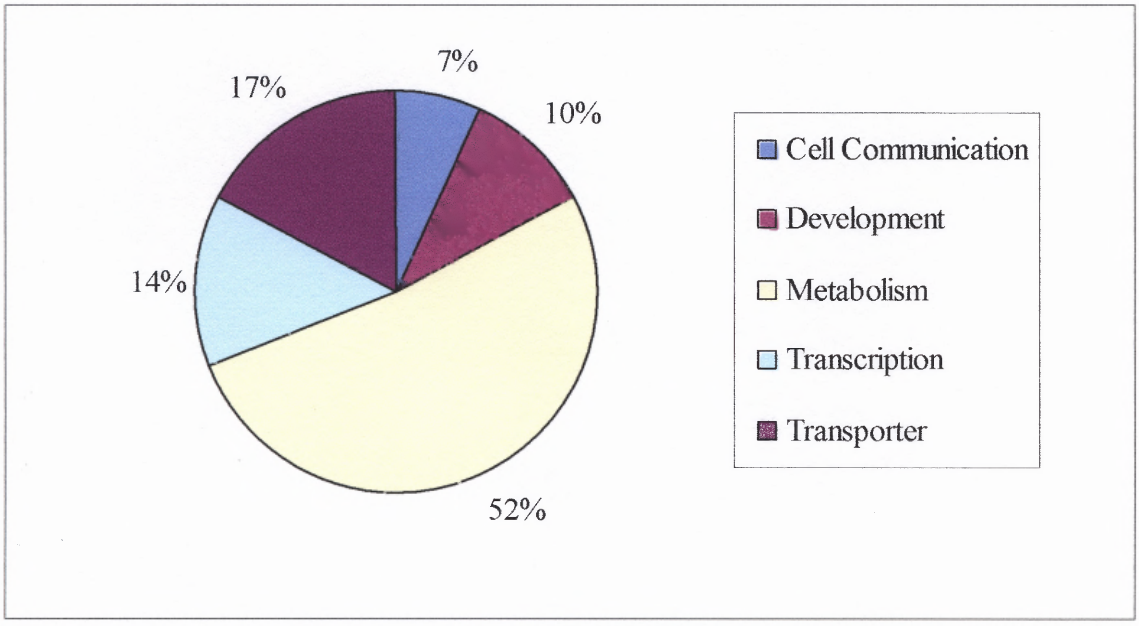


Figure 3.1 HGD/HGS up-regulated genes in 10 d small intestine.

The fold change amongst the up-regulated genes varied from to 1.51 (peroxisomal 2, 4-dienoyl CoA reductase) to 8.39 (Sucrase-isomaltase, SI). For the down-regulated genes the fold-change varied from –15.75 (pancreatitis associated protein, pap) -1.50 (WNT7A- like protein).

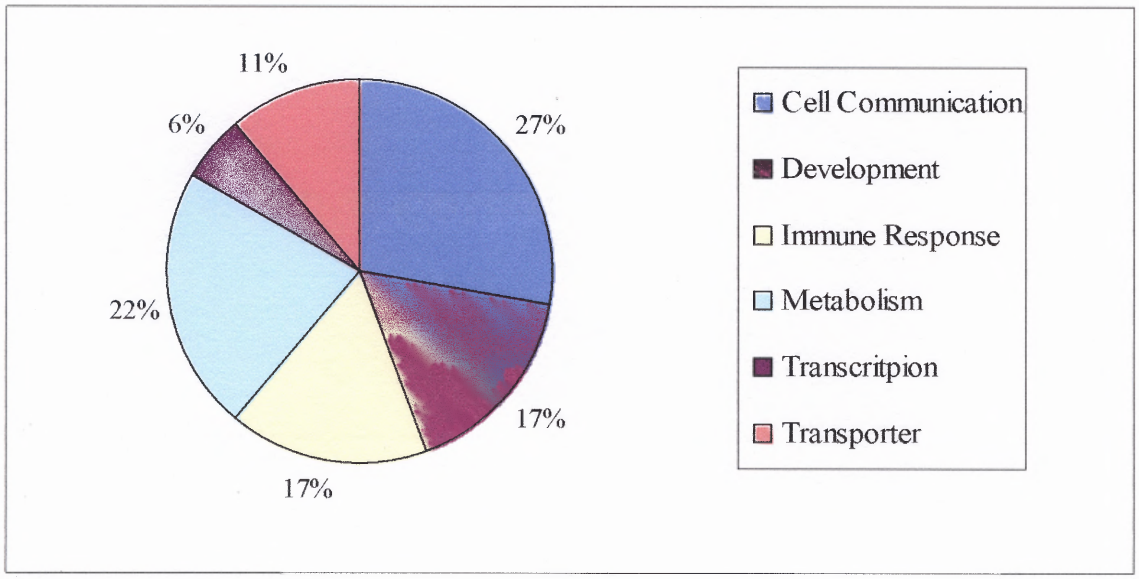


Figure 3.2 HGD/HGS down-regulated genes in 10 d small intestine.

This group represents genes that are Dex regulated under glucose conditions. These genes were compared with the results of the 2-way SAM (HFD/HGS vs. HFS/HGS) that yielded the genes that are Dex regulated independently of fructose or glucose and genes that are Dex regulated specifically under fructose conditions (refer to Figure 2.2 Step two for analysis overview). There were three common genes: Sucrase-isomaltase, fetal intestinal lactase-phlorizin hydrolase precursor and solute family carrier 3, that were labeled with an * in Appendix A.4. These three genes are regulated by Dex at 10 d under sugar conditions but independently of the nature of the sugar used.

3.2 Fructose Regulated Genes under Glucose Conditions at 10 d (HFS/HGS)

Fructose regulated genes under saline conditions (HFS/HGS), (Figure 2.1) revealed that there were 14 up-regulated genes and 63 down-regulated genes in the small intestine at 10 d (Appendix A.2).

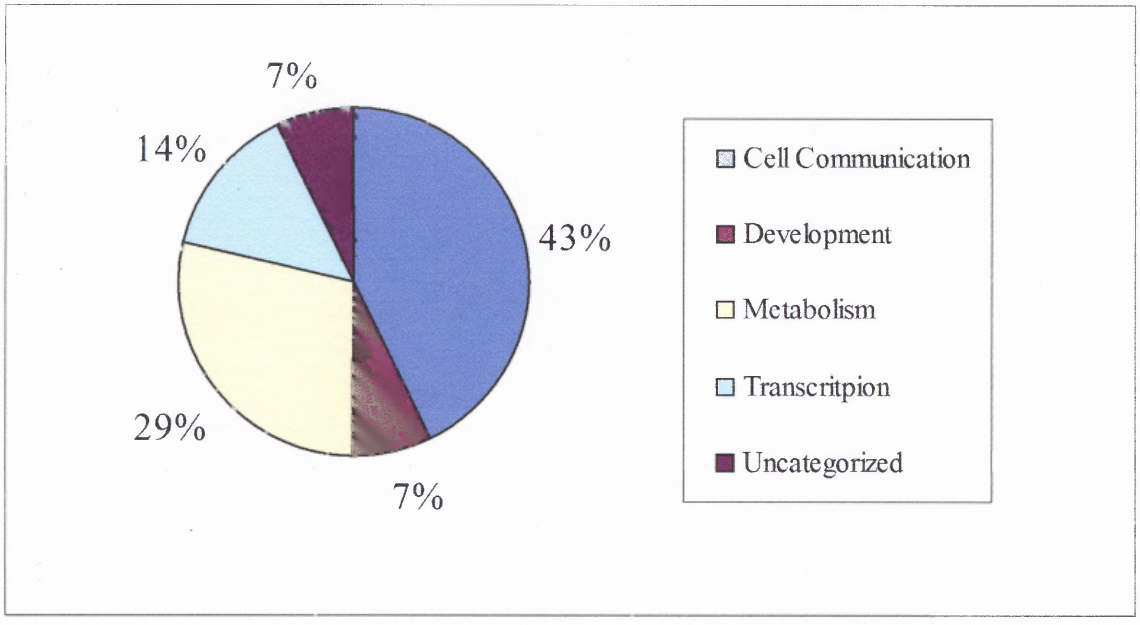


Figure 3.3 HFS/HGS up-regulated genes in 10 d small intestine.

The majority of the up-regulated (43%) (Figure 3.3) and the down-regulated (32%) genes were involved with cell-communication (Figure 3.4). The fold-change for the up-regulated genes varied from 1.50 (peroxisomal enoyl - CoA) to 2.01 (alpha 2-adrenergic receptor); while the fold-change for the down-regulated genes varied from -4.97 (pancreatitis associated protein III, papIII) to -1.50 (creatine kinase (brain), Ckb). There is a much larger down –regulation (almost 5-fold) when compared to the fold-change for the up-regulated genes (~2 fold). In Chapter 4, there is a brief explanation on this observation that ties in with development.

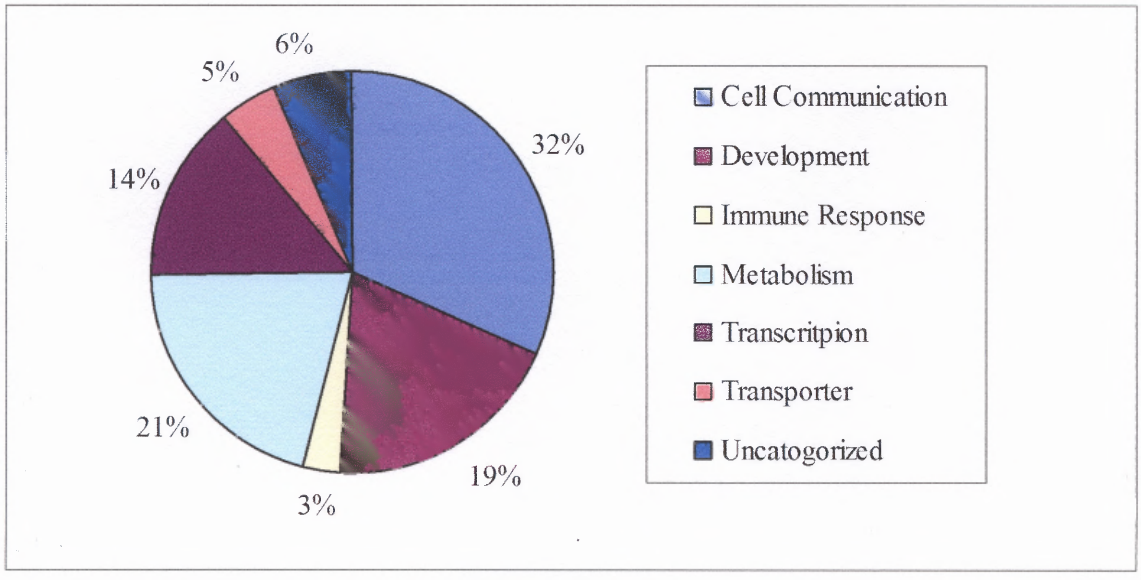


Figure 3.4 HFS/HGS down-regulated genes in 10 d small intestine

3.3 Fructose plus Dexamethasone Regulated Genes at 10 d (HFD/HGS)

This dataset is inconclusive since there are two factors (fructose and Dex) that are influencing the genomic regulation. It is difficult to decipher if the transcriptional changes seen are due to the presence of both Dex and fructose, Dex independently of fructose or fructose independently of Dex. The influence of both factors (Dex and fructose) can actually be seen in some of the gene results, if one compares them with the datasets that only have one factor that is influencing regulation, e.g. HFD/HFS. If one takes the regulation results for the glutamate receptor as an example, it was up-regulated 2.55 X in the Dex under fructose conditions (HFD/HFS) while in the fructose and Dex regulated results (HFD/HGS) it was up-regulated 3.21X. Dex is responsible for 2.55X change of the glutamate receptor and fructose (HFD/HGS) is responsible for the other 1.26X fold increase. This, however, is speculated and not proven because the two

treatments, Dex and Fructose, can be creating synergistic effect making this additive effect incorrect.

Regardless of the two changing factors, the genes in this dataset were organized into their respective ontology functions. The Dex and Fructose (HFD/HGS) revealed that there were 30 up-regulated and 29 down-regulated genes (Appendix A.3). About 55% of the up-regulated (Figure 3.5) genes were part of metabolism, while the majority of the down-regulated genes were part of cell communication (36%) and development (34%) (Figure 3.6).

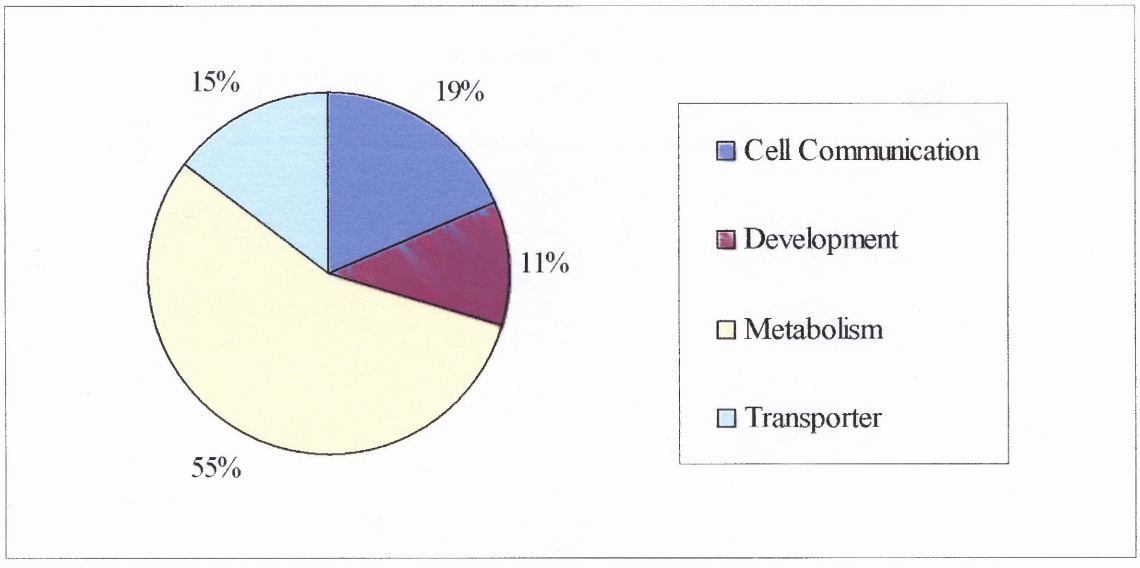


Figure 3.5 HFD/HGS up-regulated genes in 10d old small intestine.

The up-regulated genes varied from 1.46 (Proline- rich protein, Prpb) and 6.51 (Sucrase-isomaltase, SI); while the down-regulated genes ranged from -4.04 (Vascular cell adhesion molecule 1, Vcam1) to -1.51 (Aortic preferentially expressed gene 1, Apeg1).

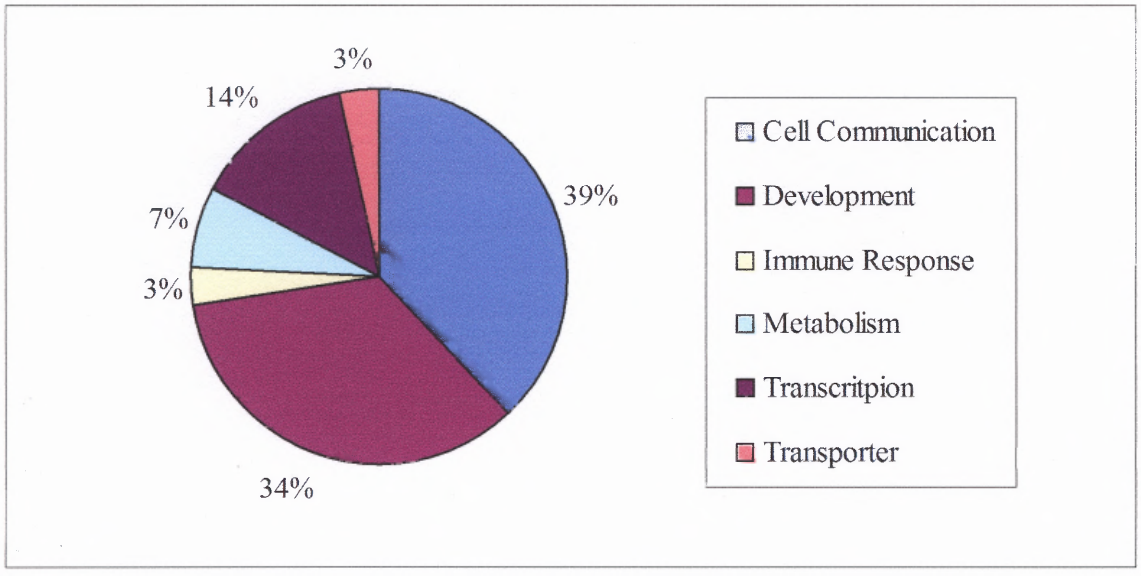


Figure 3.6 HFD/HGS down-regulated genes in 10 d small intestine.

3.4 Dex Regulated Genes under Fructose Conditions at 10 d (HFD/HFS)

A 2-way SAM was performed between the HFD/HGS and the HFS/HGS microarray to yield Dex regulated genes under fructose and glucose conditions (Appendix A.4). The 2-way SAM was performed between these two test results, because HFD/HGS contained genes that were regulated by both fructose and Dex, by fructose independently of Dex and by Dex independently of fructose. To remove the fructose regulated genes from the list, the 2-way SAM was performed. There were however, still genes in the 2-Way SAM results (Appendix A.4) that were regulated by Dex under fructose and glucose conditions. To remove these Dex regulated genes under glucose conditions, Appendix A.4 was compared with HGD/HGS and three genes were found to be common (noted by an * in Appendix A.4). This means that these three genes: Sucrase-isomaltase, fetal intestinal lactase-phlorizin hydrolase precursor and solute family carrier 3, are regulated by Dex

independently of fructose or glucose at 10 d. The rest of these genes are regulated by Dex under fructose conditions in 10 d small intestine.

The SAM revealed 7 up-regulated genes, and two down-regulated genes (Appendix A.5) by Dex under fructose conditions, and there were 3 up-regulated genes by Dex independently of fructose or glucose. The majority of the up-regulated genes are involved in cell communication (40%) and metabolism (50%) (Figure 3.7).

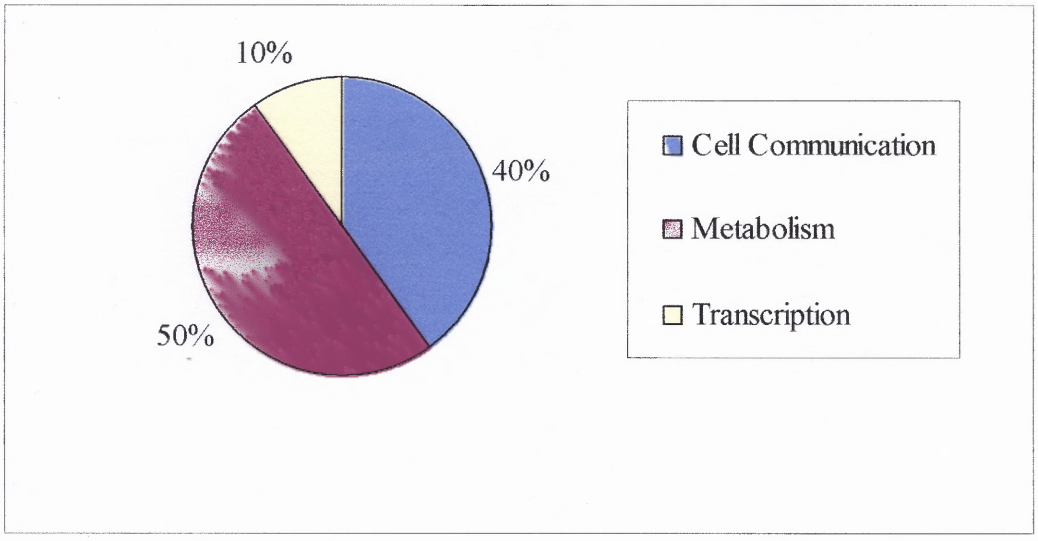


Figure 3.7 HFD/HFS up-regulated genes in 10 d small intestine.

For the down-regulated genes, both are regulated by Dex under fructose conditions; one is involved in Cell Communication (G-protein beta-2 subunit) and the other is involved in transcription (BCL2-related protein A1). The fold changes for the up-regulated genes varies from 1.53 (Pancreatic Trypsin 1, Prss1) to 3.98 (Sucrase-isomaltase, SI). The fold change for the G-protein beta-2 subunit is -1.91 and the BCL2-related protein A1 is -1.60.

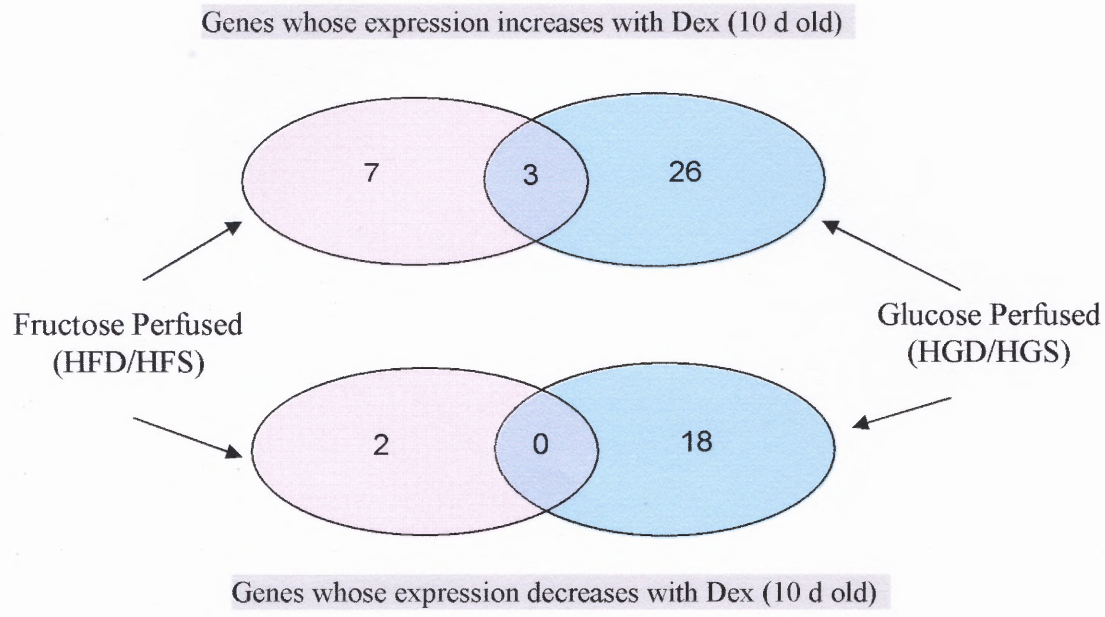


Figure 3.8 Number of Dex regulated genes in the 10 d rat small intestine when the intestine is primed with Dex 48h prior to being perfused with fructose or glucose. The genes in the middle, where the circles overlap, are the genes that are regulated by Dex at 10 d independently of the sugar perfused.

Figure 3.8 was created to compile the results described above. The left side of the figure describes the number of genes found to be regulated by Dex after a fructose perfusion; the right side describes the number of genes regulated by Dex after a glucose perfusion; the middle of the figure describes the number of genes that are regulated by Dex independent of the sugar perfused through the intestine. One of the three genes, Sucrase-isomaltase, that was up-regulated by Dex independent of the perfusion solution used has also been found to be age sensitive and independent of the sugar that was perfused in the Douard et al. age study [8].

3.5 Common Genes Between Age (20 d) and Dex (10 d) Regulated Genes

Once the genes of this study were identified and classified, they were compared to the age regulated genes isolated from the Age Study (20 d/10 d) performed by Dr. Douard et al. [8] Appendix A.6 is a synopsis of the common genes found between the two studies. The list shows the Gene ID, Gene Symbol used, the study which is was present if the gene was up or down regulated (noted by the direction of the black arrow).

There were no genes that were found common between the age regulated genes under fructose conditions (20 d F/10 d F) and the 10 d Dex regulated genes under fructose conditions. There were, however, four genes that were found to be common between the age regulated genes under fructose or glucose perfusion (20 d F/10 d F and 20 d G/10 d G) and the 10 d Dex regulated genes under glucose perfusion (HGD/HGS). Further analysis into these common genes will be discussed in chapter 4.

In total there were 15 genes common between the Age study (20 d/ 10 d) and the Dex study (Appendix A) conducted in 10 d old pups. Nine of these genes were down-regulated in the Age study while in the Dex study they were up-regulated. The genes were classified into their respective ontology groups and the majority of the genes (53%) genes (Figure 3.) were part of metabolism, showing that there is indeed a similarity in gene ontology between age and Dex regulation.

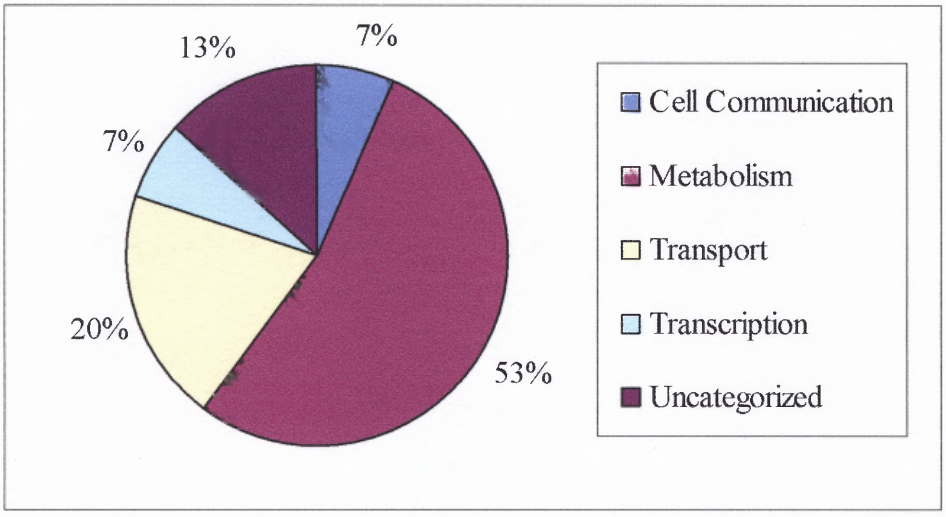


Figure 3.9 Common genes between 20d/10 d Age Study and the 10 d Dex Study of the rat small intestine.

3.6 Primer Design for Gene Confirmation

To start with gene confirmation, primers had to be designed to measure the expression levels for the specific genes. The way that the genes were selected for confirmation was based on their fold change and relationship to fructose regulation. Many of the genes in this study have been encountered before in fructose regulation at 10 d and/or 20 d (H-604 – MSB, UMDNJ –Newark, NJ 07103); therefore, there are a large number of primers that have already been designed and tested in other studies, please refer to Douard et al. [6]. Appendix A.7 lists the primers that were used to confirm genes GLUT5, PAP, G6P, SI, pfkfb-4 for this experiment in particular. The procedure used to design these primers can be found in Appendix E.

3.7 PCR Confirmation

Realtime Polymerase Chain Reaction (PCR) was done on five genes that are related to the fructose regulation in the small intestine; Pfkfb4, PAP, G6Pase, SI, and GLUT5 (fructose transporter). The house keeping gene commonly used for rat PCR experiments is the elongation factor 1 alpha (Efl a). This gene is known not to change during fructose and glucose experiments, making it a good negative control for sugar regulation experiments. It should be noted that Figure 3.10-14 are relative mRNA abundance levels due to the data normalization to HGS, the same control used in the microarray experimentation. Therefore, the numbers seen represent a percent change for that treatment group when compared to HGS. For the explanation of the results below, relative abundance levels of mRNA were used, for the percent change makes it easier to understand how much each gene is changing when there is a base level to compare it to; HGS is always 1 and the other groups are changing relative to it ie: below 1 are down-regulated and above 1 are up-regulated genes.

For SI (Figure 3.10) there was a tremendous increase in mRNA levels for the Dex experimental groups, both in the Glucose and the Fructose groups – approximately 600X increase. The PCR results (Figure 3.11) revealed that for PAP was down-regulated in HGD by about 1.5 X, but it was up-regulated in HFS (1.5 X) and HFD (3.25 X). For G6Pase (Figure 3.12) a gene that is up-regulated by fructose as early as 10 d old, had increasing levels of mRNA for HFS (2X), HGD (2.5X) and HFD (6X). This shows that even though fructose is known to regulate this gene, it can also be up regulated by Dex.

Pfkfb-4 (Figure 3.13) showed down regulation, a decrease in mRNA levels in HGD (1.5X), HFD (2X) and for HFS (2.8X). The last gene that was tested was GLUT5, and the results were consistent with Dr. Douard et al. (Figure 1.3), described in the introduction (Chapter 1) of this paper. The GLUT5 mRNA levels (Figure 3.14) showed no change in regulation by HFS at 10 d, but there was a 5.5 X increase for HGD and a 15.5X increase for HFD.

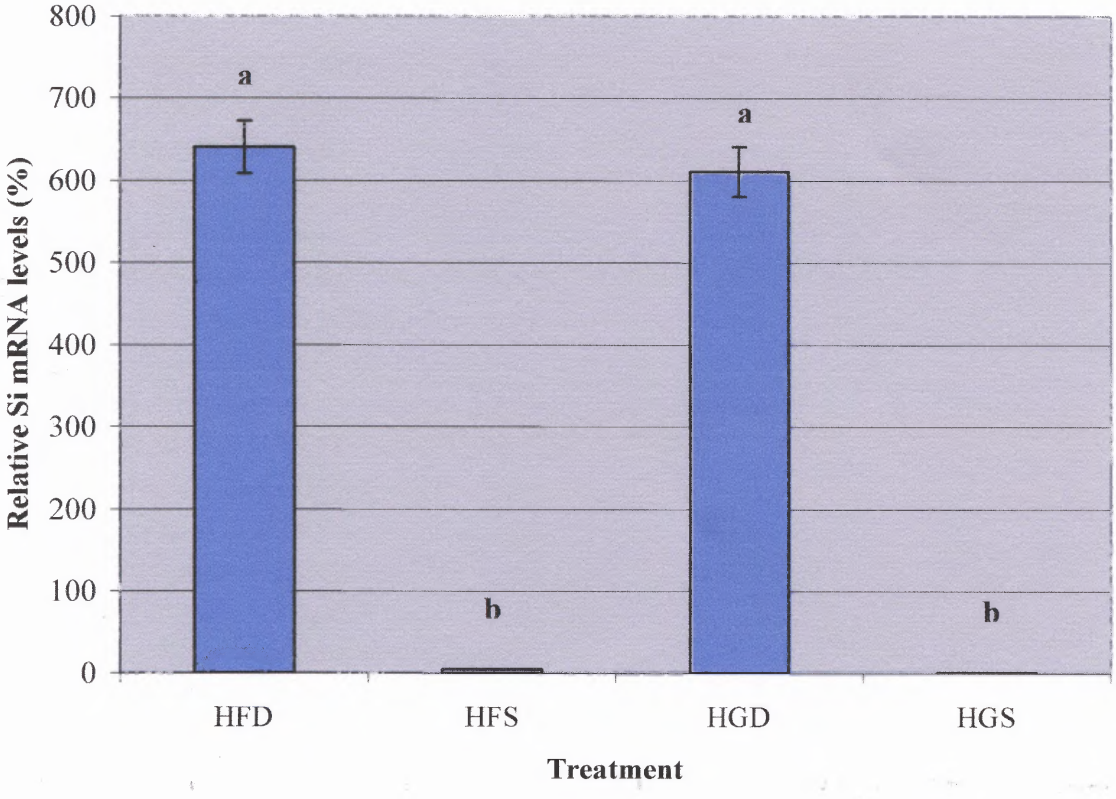


Figure 3.10 Sucrase-isomaltase (SI) relative mRNA levels (n=4) in 10 d small intestine. Letters indicate significant defference ($P < 0.05$).

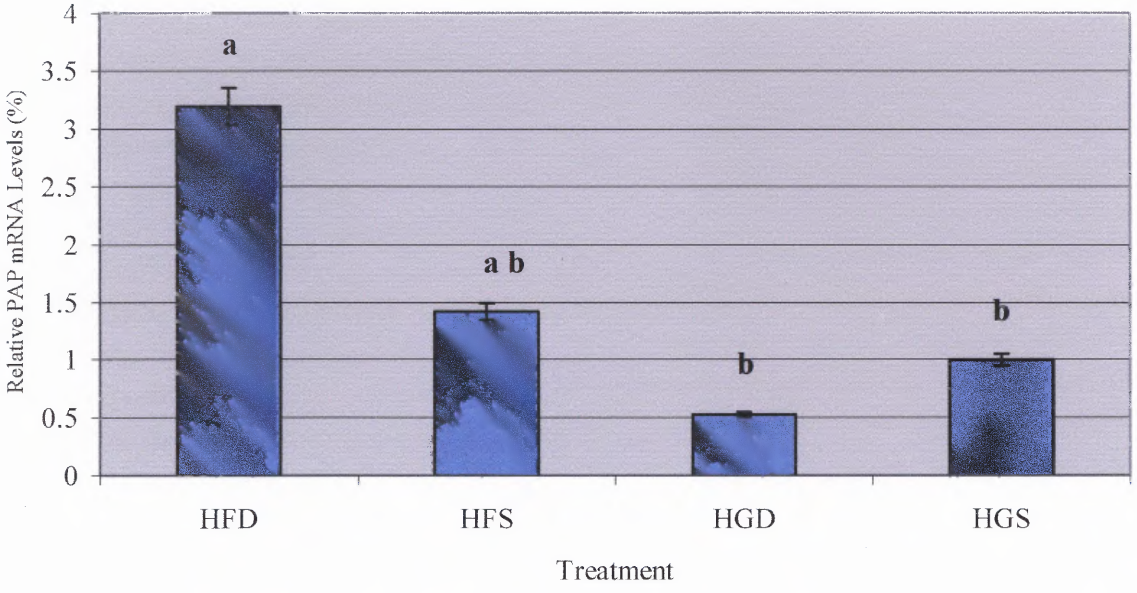


Figure 3.11 Pancreatitis associated protein (PAP) relative mRNA levels in 10 d small intestine. Letters indicate significant difference (P<0.05).

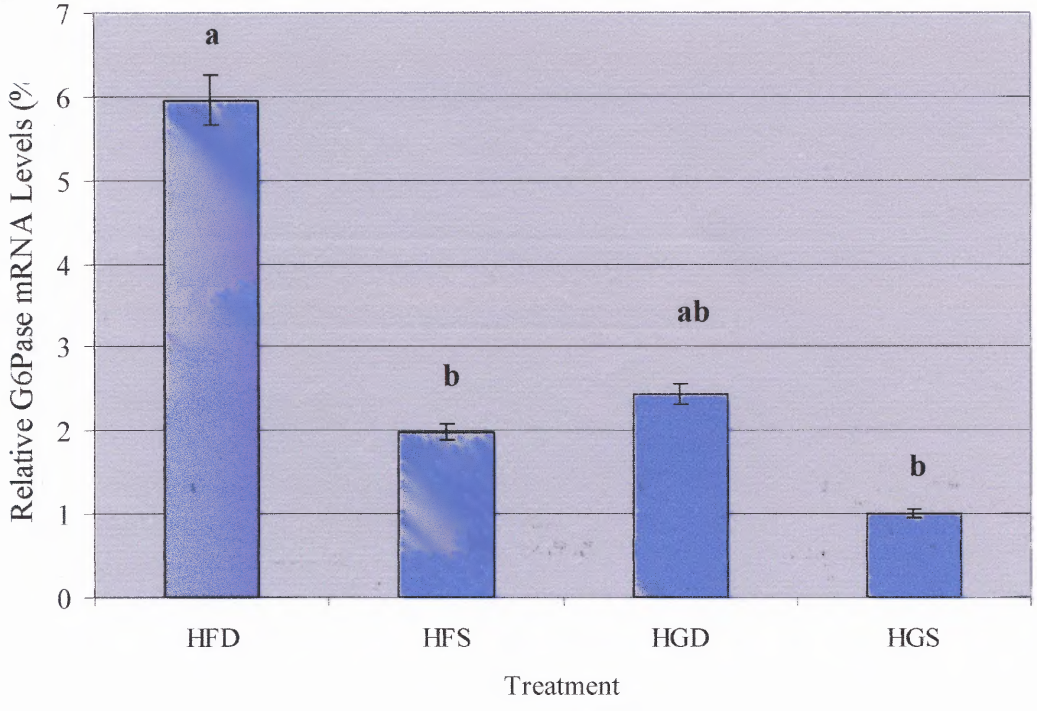


Figure 3.12 Glucose-6-phosphotase(G6Pase) relative mRNA levels (n=4) in 10 d small intestine. Letters indicate significant difference (P<0.05).

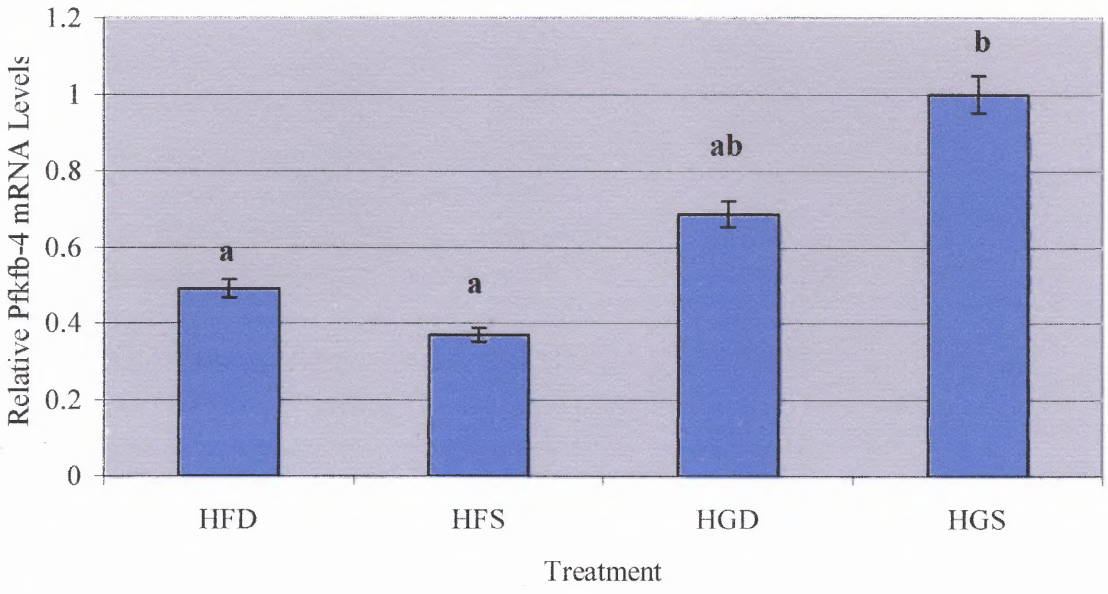


Figure 3.13 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (pfkfb4) relative mRNA levels (n=4) in 10 d small intestine. Letters indicate significant difference (P<0.05).

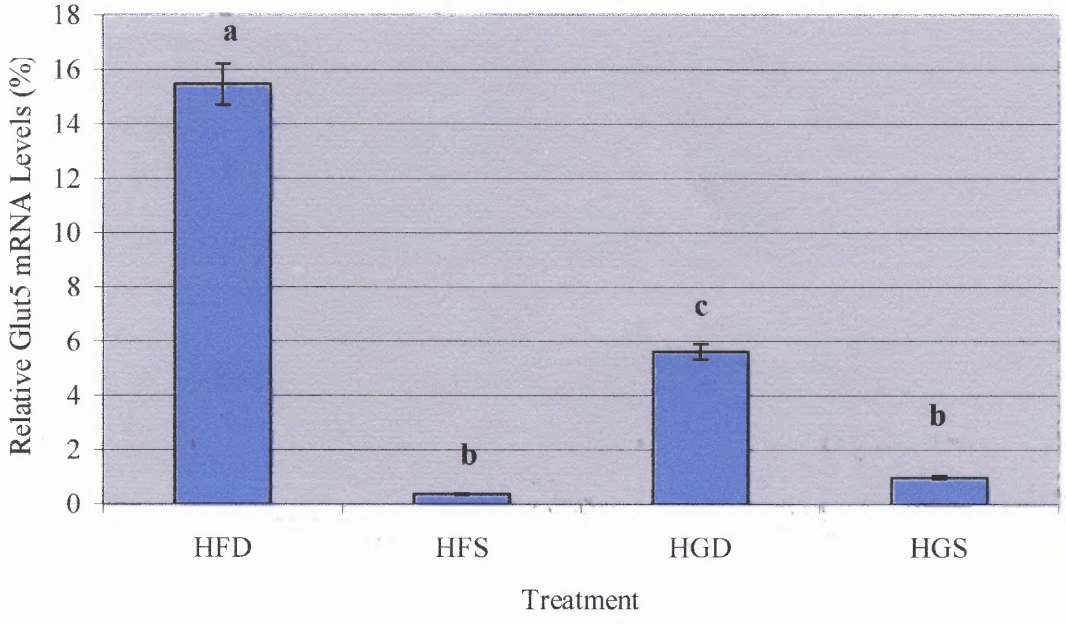


Figure 3.14 GLUT5 relative mRNA levels (n=4) in 10 d small intestine. Letters indicate significant difference (P<0.05).

CHAPTER 4

DISCUSSION

The purpose of this study was to compare the Dex effect seen at 10 d and relate it to the age effect seen at 20 d when the small intestine is perfused with fructose, and prove which one of the two hypotheses described in Figure 4.1 is true. It was speculated that the signaling pathway of these two scenarios, Dex at 10 d and age at 20 d both under fructose conditions, would be regulated by the same factors leading to the same outcome, an increase in GLUT5 mRNA and activity levels (Hypothesis A in Figure 4.1). The results of this study, however, revealed that there were no equivalent genes between the Dex regulated genes at 10 d old under fructose conditions and the age regulated genes at 20 d old when fructose perfused. This means that the use of Dex at 10 d old is not mimicking the Age effect seen at 20 d old when fructose is perfused through the intestine (see Figure 4.1). At 10 d old, Dex under fructose conditions, regulates a different set of genes than those that are regulated at 20 d old by fructose. Therefore, the precocious increase of GLUT5 mRNA and activity seen in these two ages is being regulated through different signaling factors. The interesting factor here is that even though different genes are being regulated by different factors at these two developmental stages, 20 d by age and 10 d by Dex, the outcome is the same, a precocious increase of GLUT5.

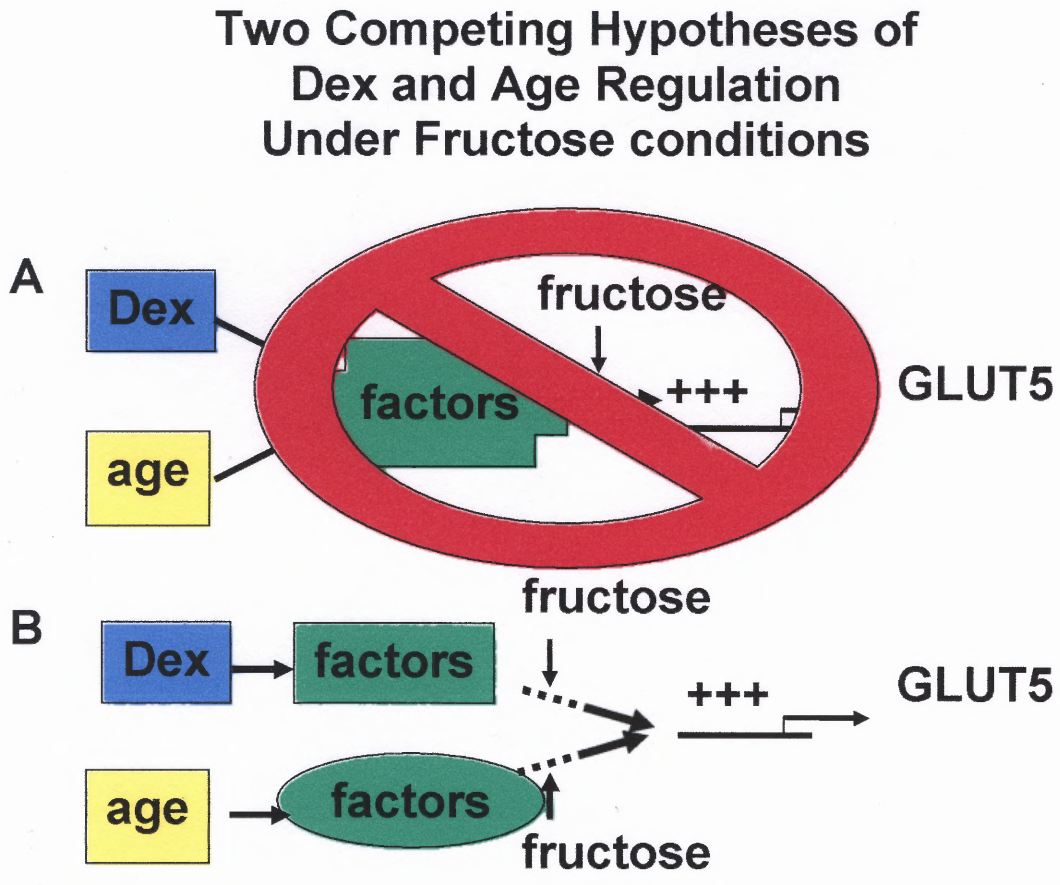


Figure 4.1 Models of the two competing theories that led to this study. The question was "When the small intestine is fructose perfused, the affect seen with Dex at 10 d and with age at 20 d, are due to a signaling cascade that acts through the same intermediate signaling factors or through separate intermediates that lead to the same outcome?" The answer is that Dex and Age are acting through different signaling factors when it comes to GLUT5 regulation in the small intestine.

Interestingly enough however, there were 4 genes that were found in common with Dex regulation at 10 d and age regulation at 20 d old in the small intestine. The common genes are Sucrase-isomaltase (SI), Glutathione-S-transferase (Gsta2), Deoxyribonuclease I (Dnase1) and Solute Carrier family 3- member 1(Slc3a1). SI and Gsta2 are part of metabolism, while Dnase1 is part of transcription and Slc3a1 is an amino acid transporter. Gsta2 is a detoxifying enzyme in the liver [14] and SI is a

glucosidase enzyme that breaks down sucrose into its monosaccharide components glucose and fructose.

Dex seems to up-regulate gene transcription in the nucleus with the up-regulation of Dnase1. Dnase1 likely plays an important role in the transcriptional changes related to development of the fructose transporter, GLUT5. The up-regulation of SI, is regulated by the presence of sucrose in the intestine [15]. This SI up-regulation however, is not specific to fructose but rather it is due to common metabolites between fructose and glucose metabolic pathway.

The up-regulation of GLUT5 in the intestine, with Dex at 10 d and age at 20 d old under fructose conditions, was the inspiration for this microarray experiment. The microarray results, however, did not characterize GLUT5 as a gene that was up-regulated at 10 d by Dex under fructose conditions (refer to Appendix A.4). PCR confirmation of this gene (Figure 3.14), after the microarray experiment however, revealed GLUT5 is indeed up-regulated by Dex at 10 d old under fructose conditions; hence it should be present in Appendix A.5 as an up-regulated gene. This scenario, where GLUT5 is missing is called a false negative result, this is further explained in Section 4.2. Since GLUT5 did not show up as an up-regulated gene in the microarray results, SI was chosen as a second marker gene to check that the microarray experiment was indeed working as the experiments were being conducted. SI is as a marker gene because it is known to change with fructose and/or glucose, and indeed it is regulated by age and Dex treated tissues. The technical issues encountered, and the possible reasons for the false negative and false positive results are described further in section 4.2, under Technical Difficulties.

With Slc3a1, a subunit of the cystine transporter, nothing is known in the literature about its regulation by Dex. Gsta2, the liver enzyme, is biphasic in its regulation by glucocorticoids for it is concentration dependent [16]. Falkner et al. found that at high (10mM) concentrations of glucocorticoids, this gene was repressed while at lower concentrations (1mM) it was activated (up-regulated) [16]; since a moderate dose of Dex was used for this experiment, it explains why Gsta2 was up-regulated in these results. It can be speculated, that these genes are up-regulated by Dex and are part of a signaling pathway that is paralleling the regulation of GLUT5. Glucocorticoids, such as Dex, are known to regulate a broad range of signaling pathways, so it not uncommon to see parallel effects of Dex in different tissues or signaling pathways.

10 of the 15 genes that were found to be common between the 20 d Age Study and this 10 d Dex study (Appendix A.6), are regulated differently, meaning that these genes are up-regulated in the Dex study, while in the Age study they are down-regulated. A possible reason for this occurrence is stated in a study conducted by Falkner et al., where it was found that some genes have a biphasic response to the concentration of glucocorticoids administered. It is possible that these genes are being regulated differently at 10 d with Dex administration compared to the 20 d Age regulation due to different glucocorticoid concentrations. But these differences in regulation can also be a side-effect of Dex's affects on tissues. Glucocorticoids, like Dex, are activated as a stress response and will create many responses throughout the body like gluconeogenesis in the liver, amino acid mobilization into the liver, increase fat breakdown and inhibit glucose uptake in muscle tissue in an attempt to restore and/or maintain glucose homeostasis.

This non-developmental maturation affects of Dex maybe a contributing factor to the difference in the signaling pathways seen at 10 d with Dex and at 20 d without Dex.

It should be noted, that there are some interesting patterns encountered in the data. The results for the 10 d Dex regulated genes under fructose conditions (Appendix A.5), show a large number of up-regulated genes involved in metabolism and a majority of down-regulated genes are involved in cell communication/Signal Transduction and Development (Appendix A.5). This same pattern was detected in the age study with genes that were differentially expressed at 20 d by age after being fructose perfused [8]. In the age study the majority of down-regulated genes were a part of development/proliferation while the majority of up-regulated genes were part of metabolism [8]. This pattern shows that at some point Age and Dex are affecting the same family of genes in the small intestine of the neonatal rat, even though they are affecting each individual gene differently.

4.1 Fructose, Dex and Dex plus Fructose Sensitive Genes at 10 d

4.1.1 Fructose Regulated Genes at 10 d under Glucose Conditions (HFS/HGS)

The results for fructose regulated genes at 10 d under glucose conditions (HFS/HGS), were compared to fructose regulated genes (20 F/20 G) in 20 d old small intestine, performed by Cui et al. [4], and there were no genes in common between both experimental groups. This is suggestive that there is a very different regulating mechanism for fructose between these two ages, since 10 d old intestine probably has not encountered fructose before. Some of the up-regulated genes were unexpectedly related

to fatty acid metabolism; Ornithine Aminotransferase, 3-Oxoacyl-CoA Thiolase, peroxisomal enoyl-CoA, ferritin light chain subunit and Cytochrome P450 (refer to Appendix A.2). Ornithine Aminotransferase is a key enzyme in the synthesis of Citrulline (an intermediate in the Urea Cycle) in the small intestine [17]. 3-Oxoacyl-CoA Thiolase is an enzyme that catalyzes the last step of the fatty-acid β-oxidation cycle [18]. Peroxisomal enoyl- CoA is an enzyme part of fatty acid metabolism and is also a part of the secretory pathway of the cells. The Ferritin Light chain subunit (Ftl1) and the Cytochrome P450 are essential elements to the electron transport chain reaction that occurs in the mitochondria during the synthesis of ATP [19]. Ftl1 is also involved in the regulation of ferritin, an iron storing molecule that is also responsible for the transportation of iron across the mucosal cells of the gastrointestinal tract [20]. It is interesting to note that a recent study was published by Mojiminiyi et al., it was found that higher body indices of iron were significantly correlated with diabetes complications [21]. Because of increased usage of fructose and its link to the development of insulin resistance that can leads Type II Diabetes [22], it would be beneficial to study ferritin and decipher its contribution to this diseased state.

It is known that in adults, fructose stimulates lipogenesis in the liver and adipose (fat) tissue, that eventually lead to hepatic and extrahepatic insulin resistance in rats [22]. This fructose effect however, was not known for neonates and intestinal tissue. It is really interesting to see that even though neonates cannot transport fructose, except for small amounts through the SGLT1, fructose is still able to highly stimulate the fatty acid synthesis pathway.

There was a much larger number of down-regulated (64), than up-regulated (14) genes that are fructose sensitive in 10 d old intestine. The majority of these genes are involved in development and cell communication. Epithelial cell differentiation was studied by Tadjali et. al. and they found there was a much larger number of genes that were down-regulated during cell proliferation than up-regulated [23]. The exact reason for this was not clearly known but it was speculated that this down-regulation was a result of a single transcription factor or a co-activator that decreases the transcription of a large number of genes at once during cell proliferation. This is suggestive that the large number of down-regulated genes, seen during fructose regulation, is part of the development process in the small intestine.

4.1.2 Dex Regulated Genes under Glucose Conditions (HGD/HGS)

The Dex regulated genes (Appendix A.1) under glucose conditions revealed a large number of up-regulated genes involved in protein, lipid and sugar metabolism. This is not a surprising since glucocorticoids, such as Dex, have been known to trigger metabolic function [24]. Other Dex regulated genes, besides the four Dex regulated genes discussed previously (Gsta2, SI, Dnase1 and Slc3a1), are: 3- Alpha Hydroxysteroid Dehydrogenase, Very-Long-Chain Acyl-CoA Synthetase, Fetal Intestinal Lactase Phlorizin Hydrolase Precursor, and 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4 (Pfkfb4). 3- Alpha Hydroxysteroid Dehydrogenase and Very-Long-Chain Acyl-CoA Synthetase are part of fatty acid metabolism. Fetal Intestinal Lactase Phlorizin Hydrolase Precursor is involved in the break down of milk [25] and is a gene that is normally up-regulated during the suckling developmental stage mainly because the

pups diet is mainly milk [26]. Pfkfb4 is an activator of glycolysis and it maintains the cellular level of fructose 2, 6-bisphosphatase, a key enzyme part of glycolysis and gluconeogenesis [27]. Glucocorticoids stimulate various genetic responses in the cell in attempt to maintain the blood-glucose levels, therefore it is part of their general function to stimulate and maintain metabolic functions in the cell.

Cui et. al. created a model of the potential influences of fructose on the gluconeogenises pathway of the 20 d old small intestine (Figure 4.2) [4]. SI and Pfkfb4 are linked to this model through the regulation of intermediate factors that effect the substrates marked with * on the model, Figure 4.2. Sucrase-isomaltase metabolizes sucrose to supply the entereocytes with fructose and glucose, while Pfkfb4 maintains levels of fructose 2, 6-bisphosphatase enzyme. The interesting factor is that these genes are regulated by Dex at 10 d old without the presence of fructose, and the model found below was designated for 20 d old intestine under fructose perfusion conditions (where glucose was used as a control). Thus it is evident that there is a correlation, in cellular metabolism, between Dex at 10 d and age at 20 d. There is however, something unique about the development of fructose transport that develops differently between 10 d and 20 d old.

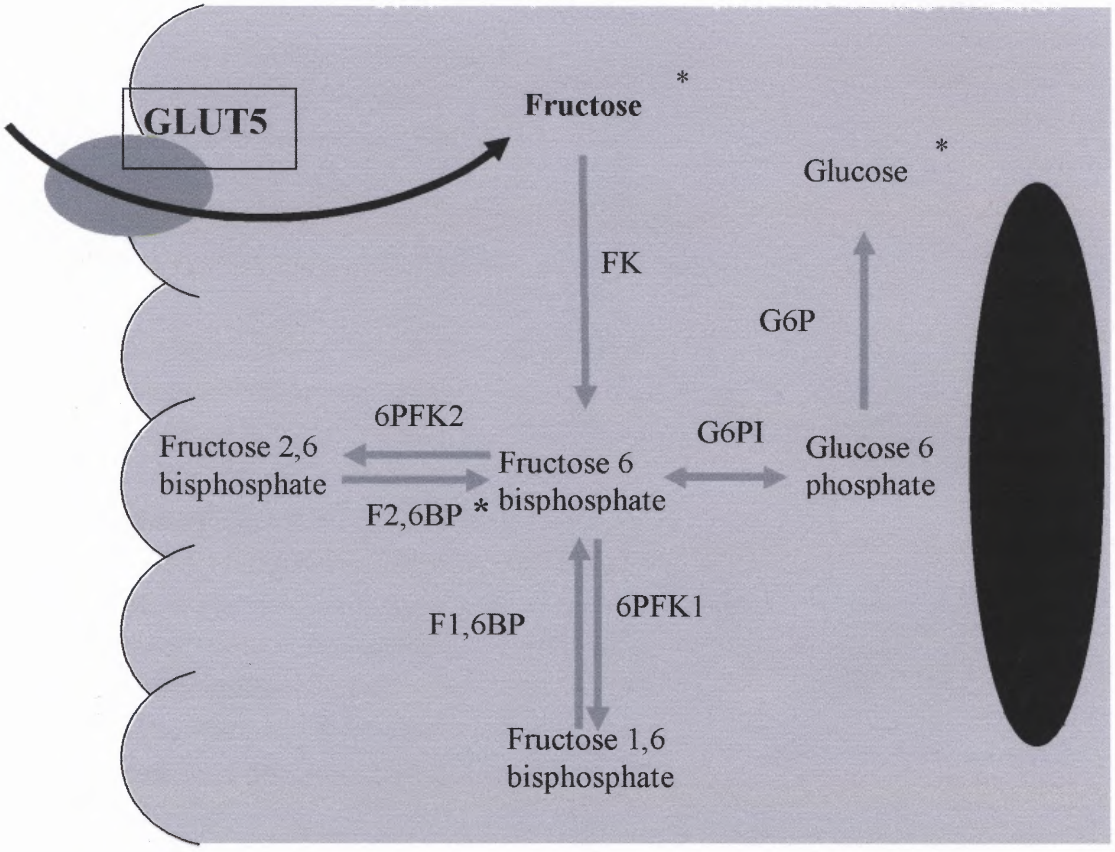


Figure 4.2 Model showing the potential influences of fructose perfusion on the gluconeogenesis pathway of neonatal rat intestinal cells [4]. * factors that are indirectly up-regulated by Dex at 10 d.

4.1.3 Dex Regulated Genes under Fructose Conditions (HFD/HFS)

More specific analysis of the Dex regulated genes under fructose conditions (Appendix A.5) was conducted to investigate the specific function of each gene. It turns out that three of the cell communication/ signal transduction genes are neural receptors or factors that are sensitive to stress. The Glutamate receptor (Grin2a) is mainly localized in the brain that mediates the body's stress response [28], and it is also involved in neural

development in postnatal life [29]. Because of the effect of Dex on this receptor in the intestine it can be hypothesized that Grin2a is also involved in the ontogeny of the small intestine under Dex conditions. The corticotrophin-releasing factor binding protein (CRF-BP) is regulated by corticotrophin-releasing factor (CRF), which is a key mediator of the behavioral, autonomic and hormonal response to stress in the central nervous system [30-32]. Interestingly, even if CRF-BP is not sensitive to Corticosteroids, CRF, one of its activators is sensitive to corticosteroids [30]. This suggests that the CRF-BP up-regulation seen in this experiment by Dex (a corticosteroid analog) could be a secondary signaling cascade of Dex in the small intestine. The third gene is the neuronal nicotinic acetylcholine receptor alpha 2 subunit which is an ligand gated ion channel that creates excitatory responses in reaction to acetylcholine, a neurotransmitter that controls voluntary muscle movement [33]. This receptor plays an important role in chemical signaling throughout the nervous system [34] and it was shown to be up-regulated in the presence of Dex by Maestrone et al. in the neuromuscular system [35]. These three genes have been identified in a stress response that is a part of brain and muscle cells. However, their existence in the intestine in response to fructose and Dex may suggest that they play a role in the signaling cascade leading to the induction of the GLUT5 transporter.

Four genes are up-regulated by the Dex, under fructose conditions and involved in metabolism are: pancreatic trypsin, very-long-chain acyl-CoA synthetase, Aldolase B, and Ivd. Pancreatic Trypsin is part of digestion (it is a serine protease) and it hydolyzes proteins to small peptides (amino acid chains). Very-long-chain acyl-CoA synthetase and Isovaleryl Coenzyme A dehydrogenase (Ivd) [36], both metabolize fatty acids. Liver Aldolase B is an enzyme, also known as fructose-1-phosphate aldolase, that is part of

gluconeogenesis in the liver (synthesis of glucose) and also a part of fructose metabolism through glycolysis [37]. It is thus interesting, that while Dex and fructose together are able to stimulate GLUT5 and increase fructose absorption into the entereocytes, these cells are also being stimulated to activate lipogenesis, a condition that was seen stimulated by fructose [38] in section 4.1.1. An important factor to note is that genes regulating the fatty acid metabolism were not the same between the fructose regulated genes under glucose conditions (Appendix A.2) and the Dex regulated genes under fructose conditions (Appendix A.5). It is speculated that this up-regulation of lipogenesis is done through a common family of genes, however the individual genes are different and this difference is part of the puzzle as to why GLUT5 is regulated through one treatment (Dex under fructose conditions)but not through the other (fructose under glucose conditions).

The two down-regulated genes, G-protein Beta-2 subunit and BCL2a1 are part of cell communication/signal transduction and transcription, respectively. BCL2a1 is involved in tissue development and homeostasis through its manipulation of apoptosis [39]. It becomes pro-apoptotic or anti-apoptotic depending on its interaction between other family members of the BCL2 family and other select proteins [39]. It was found by Madsen-Bouterse et al. that the administration of glucocorticoids creates an anti-inflammatory affect and as a result delays spontaneous apoptosis [40], therefore the down-regulation seen here to BCL2a is a result of the Dex effect on the cell but its relationship to Dex is not known. The G-protein Beta-2 subunit is responsive to catecholamines (stress-released hormones), and in turn it (G-protein) activates the adenylate cyclase/ cAMP signaling pathway that stimulates glycogenolysis in muscle and

liver tissue [41]. Takahashi et al. found G-protein beta subunit is up-regulated by Dex, under normal conditions in fetal rat [42]. In this study however, G-protein beta-2 subunit is down-regulated by Dex because of the fructose conditions. Since Fructose can be used as form of energy, this must inhibit G-protein activity through a negative feed-back loop, for it does not need to activate glycogenolysis since fructose can go straight into glycolysis without the need to break down glycogen to create glucose.

After the analysis of the microarray results there are very few genes found to be regulated by Dex under fructose conditions in the small intestine. Such a small number made it difficult to identify a specific signaling pathway involved in the regulation of these genes. A possible explanation for the few regulated genes can be the response rate of the cells to the glucocorticoids. In this experiment, the long term affects of Dex (after 48h) was studied but not the short-term effects. Agbemafle et al. found that after two hours of glucocorticoid treatment there was precocious increase in regulation at the cellular level, however with continuous Dex treatment, after a few hours, certain genes stopped responding [43]. It would be beneficial to look at a short term effect of Dex on the intestine since it can be that the intermediate factors, which do not seem to be regulated similarly after 48h or Dex treatment, are part of an early cascade of cell signaling but have slowed down or become deactivated. To fully understand Dex regulation and observe its signaling factors (short and long-term), a time course should be performed where gene activity can be monitored and be related to dose of Dex. There is also a possibility that a large amount of Dex present in the bloodstream can activate a negative feedback loop, where genes start to be regulated differently, in opposite

directions, after a period of time. Therefore, it would be recommended that in future studies a Dex bioassay be performed as the experiment is being conducted.

4.2 Technical Difficulties

It is common in microarray-based experiments to have false negative and/or positive results. The positive fructose controls used in the lab are GLUT5, G6Pc and SI, while the negative fructose control gene is SGLT1. Naturally one would expect the fructose positive controls to show up on the treatment groups with fructose perfusion, and the fructose negative control not to show up on any of the fructose treatments. The levels of SGLT1 and G6pc mRNAs were not significantly changed in the microarray results however, PCR confirmation of G6pc revealed that this gene is in fact up-regulated in HFD treated pups in comparison to HFS and HGS (Figure 3.12). This means that G6Pc was a false negative in the microarray results, for it should have been part of the results for the Dex regulated genes under fructose conditions results (Appendix A.5). SI was significantly up-regulated according to the microarray results and was confirmed by PCR for HFD and HGD treatment groups confirming that SI is mainly regulated by age and Dex and not specific to the presence of sugar (Figure 3.10). GLUT5 was not present in any of the microarray results but it was confirmed by PCR to be significantly up-regulated in HFD (15 fold) and HGD (5 fold) but in the HFS treatment (Figure 3.14), which is an expected result for 10 d old pups [6, 8, 44].

Discrepancies in microarray data can happen for various reasons. There were some set-backs that were encountered during the microarray experiment that could have

lead to false positive and negative results as mentioned above. An issue that was noticed was the Poly-l-lysine background on the microarrays and its high affinity for the Cy3 dye [13]. There was a pronounced background that possibly shifted the ratios, regardless of the normalizations described in chapter 2. It was also noticed that the washing procedure described in Appendix C, was not 100% efficient and many times extra washing steps had to be taken to remove streaking from the chip's surface.

Non-homogeneous hybridization of the chip, where there was more binding on one side of the chip compared to the other side, was often encountered. Other causes for color shifts in spot intensity could have been caused by the placement of the hybridization mix on the surface of the chip; the hybridization mix dried up on the sides of the chip when left overnight; air bubbles being present under the cover slip; or the orientation of the chamber (flat) when left overnight in incubation (refer to Appendix C for details). When there was a problem with a certain section of the chip, the area was flagged and excluded from analysis, meaning that those genes were removed from the results. When removing these flagged genes it is possible to be removing genes that are only present on the chip once and this creates a situation where genes will be missed in the results because physically the gene spot was compromised.

The main two issues with the spotting and non-homogenous hybridizations is that some genes do not repeat more than once on the chip and the gene intensity becomes shifted, either too green or too red can create false positives; while shifts toward yellow creates spot intensities that looks as if there is no change and these are false negatives. Due to genes just repeating once on every chip, it is very easy to have a mislabeling, especially since it is one spot amongst 7,999 other spots. There are many steps in the

microarray experiment process that have been incorporated into the procedure to increase the odds and decrease the need for guess work. One such method was the dye-flip step for treatment repetitions, this does away with one dye binding heavily to a particular gene; the key step with this method is to remember to switch the ratios around before beginning the analysis. The other methods come in forms of normalizations; global normalization can do away with a background gradient and the local regression normalization can do with spatial variances on the chip and any variances in the oligonucleotide quantities originally printed onto the chip. It is important to keep in mind however, that local regression normalization is based on the weighted average of the ratios of each array- block, and every gene counts toward that average making it very easy to shift the ratios since a whole block can be part of a spatial difference. Therefore, it is important to use different tools that check on all of these normalization steps to make sure that the results are not becoming skewed and remain congruent.

Future improvements should be done to the Cy5 and Cy3 labeling system, where affinity should be equivalent between both dyes, both toward the gene sequences as well as the different coating on the microarray chips. A possibility would be to study the affinity of one dye to the other to balance the competitiveness between both dyes during the hybridization step. The hybridization chamber should be kept constant and not be moved between experiments to maintain consistency. For the application of the hybridization mix and the cover slide of the chip should be automated, a small fixture that will indicate where the mix should be placed and a lever that can drop the cover slip on the chip without adding air bubbles to the procedure. These are all improvements that can be done to the procedure with the hopes of achieving homogeneity throughout the chip.

However, biologically maybe a new coating for the chips should be considered, where only the oligonucleotides on the chip should be competing for the labeled primers.

4.3 Conclusion of Study

In summary, there are false negative and positive results present in the data however, false positives can be easily identified through PCR. False negatives are not identifiable because there is no way to know that a gene is not present in the results unless it is expected to be there, such as what happened with GLUT5 and G6pc in this experiment. Creditability to this experiment was confirmed by SI and its consistency across all treatment groups in the microarray experiments. GLUT5 was not present in the microarray results, where most likely it was due to its remote location on the chip and the different problems encountered with the hybridization process. The exact location of GLUT5 was in block 14, column one row five, which so happens to be in an area that often was non-homogenous and genes were removed from analysis with flagging. The low number of responsive genes, can be part of an early response to Dex and/or be part of a negative feedback response due to high levels of Dex in the bloodstream; therefore to confirm their regulation under Dex conditions, a time course should be performed where gene activity and a bioassay of Dex is performed. These experiments will possibly identify more genes that are regulated by Dex under fructose conditions, so that a pathway building tool can be used to identify a signaling pathway. Ultimately, the relationship seen in this experiment between age and Dex treatment will be understood better as the regulating factors and the signaling pathways are identified to explain the

early onset of fructose regulating genes on the brushborder membrane when the substrate is introduced into the small intestine.

APPENDIX A

MICROARRAY GENE RESULTS AND PRIMERS DESIGNED

The contents of Appendix A are the genes found to be regulated by: **A.1** Dex under Glucose Conditions (HGD/ HGS), **A.2** Fructose under Saline Conditions (HFS/HGS), **A.3** Fructose and Dex (HFD/HGS), **A.4** Solely by Dex independent of the sugar and by Dex under Fructose (HFD/HFS + HGD/HGS), **A.5** Dex under fructose conditions (HFD/HFS). **A.6** is the list of genes that were found to be common between Age Study (20 d/ 10 d) and the 10 d Dex study. **A.7** is a list of primers that were used for PCR confirmation of some of the gene results from the microarray experiment.

A.1 HGD/HGS Dex Regulated Genes at 10 d Under Glucose Conditions

Up Regulated Genes

Name	ID	Fold Change	Stdv ratio
Cell Communication/ Signal Transduction			
coronin, actin binding protein 1B (Coro1b)	NM_019222	1.6213	0.1454
NMDA receptor glutamate-binding subunit (Grina)	S61973	1.5264	0.0654
Development			
T-kininogen (Kng).	NM_012696	3.0583	0.4408
GADD153	U36994	1.9504	0.2698
perchloric acid soluble protein	AF015949	1.5494	0.0900
Metabolism			
Sucrase-isomaltase (SI)	NM_013061	8.3988	0.8226
Fatty acid binding protein 1, liver (Fabp1)	NM_012556	3.4930	0.3654
fetal intestinal lactase-phlorizin hydrolase precursor	X56747	3.4042	0.4467
acetyl-coenzyme A carboxylase	J03808	2.2358	0.3267
kidney Zn-peptidase aminopeptidase N	M25073	2.0027	0.0903
aminopeptidase B	U61696	1.9666	0.2187
Glutathione-S-transferase (Gsta2)	NM_017013	1.9584	0.1980
3-alpha hydroxysteroid dehydrogenase	AF159099	1.7454	0.0421
Meprin 1 alpha (Mep1a)	NM_013143	1.7427	0.1076
acetyl-CoA carboxylase	AB004329	1.7209	0.2226
putative glycogen storage disease type 1b protein	AF080468	1.6230	0.0691
cytosolic malate dehydrogenase (Mdh)	AF093773	1.5882	0.1877

A.1 HGD/HGS Dex Regulated Genes at 10 d Under Glucose Conditions (continued)

UDP-glucuronosyltransferase (UGT2B12) kidney variant	U06274	1.5575	0.1408
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (Pfkfb4)	NM_019333	1.5344	0.1513
peroxisomal 2,4-dienoyl CoA reductase px-2,4-DCR#1	AF021854	1.5140	0.1181
Transcription			
Deoxyribonuclease I (Dnase1)	NM_013097	5.3384	0.4765
selenoprotein P, plasma, 1 (Sepp1)	NM_019192	2.7142	0.1537
small glutamine-rich tetratricopeptide repeat (TPR)	AJ222724	1.9288	0.2147
isoleucyl tRNA synthetase	U75922	1.7738	0.2249
Transporter			
solute carrier family 3, member 1 (Slc3a1)	NM_017216	2.5491	0.3037
Aquaporin 5 (Aqp5)	NM_012779	2.3001	0.2130
Glut 2 (glucose transporter, type 2) (Slc2a2)	NM_012879	1.9878	0.2309
Lectin, galactose binding, soluble 9 (Galectin-9) (Lgals9)	NM_012977	1.8367	0.0833
copper transporter 1	AF268030	1.6454	0.1401

Down-Regulated Genes

Name	ID	Fold Change	stdv ratio
Cell Communication/ Signal Transduction			
Vascular cell adhesion molecule 1 (Vcam1)	NM_012889	-7.1731	0.7978
reggie1-1	AF023302	-2.0464	0.1574
kringle-coding gene marking the eye and the nose (Kremen)	NM_053649	-1.6180	0.2449
RL/IF-1	X63594	-1.5459	0.1301
Shal-related potassium channel Kv4.3	U42975	-1.5423	0.1091
Development			
Tclone15	U31867	-2.4095	0.3524

A.1 HGD/HGS Dex Regulated Genes at 10 d Under Glucose Conditions (continued)

glia-derived nexin/protease nexin-1	X71010	-1.5722	0.1569
WNT7A-like protein	AF481945	-1.5010	0.1349
Immune Response			
CC chemokine CCL28 (Scya28)	NM_053700	-1.9963	0.3144
interferon, alpha-inducible protein 27-like (Ifi27l)	NM_130743	-1.7070	0.1832
E-3 epididymal fluid protein (LOC171412)	NM_134391	-1.6782	0.1984
Metabolism			
pancreatitis associated protein (pap)	M55149	-15.759	0.7273
Thioredoxin	X14878	-1.7178	0.1634
Peptidylprolyl isomerase A (cyclophilin A) (Ppia)	NM_017101	-1.7082	0.1869
Dihydroorotase	AB007768	-1.6753	0.1453
Transcription			
Guanine nucleotide-binding protein G-s, alpha subunit, Genbank no U51565 (Gnas)	NM_019132	-1.8818	0.1616
Transporter			
chromaffin granule amine transporter (CGAT)	M97380	-2.4413	0.2471
peptide/ histidine transporter	AB000280	-1.5702	0.1309

A.1 Shows genes that are significantly ($P<0.05$) up and down-regulated, by more than 1.5 fold change, by Dex under glucose conditions in 10d old small intestine. Values are fold change ± SE for each gene; n=4 experiments.

A.2 HFS/HGS Fructose Regulated Genes at 10 d Under Saline Conditions

Up Regulated Genes

Name	ID	Fold Change	stdv
Cell Communication/ Signal Transduction			
alpha 2-adrenergic receptor	S67316	2.0155	0.3708
neuropilin-2	AF016297	1.8926	0.3117
protocadherin-T3 (pcdh-T3)	AF177696	1.8568	0.2851
protocadherin-T4 (pcdh-T4)	AF177698	1.8465	0.2988
rat metallothionein-i (mt-1)	J00750	1.7763	0.1940
ferritin light chain subunit	L01122	1.5871	0.1383
Development			
Drosophila hairy gene homologue	L04527	1.7125	0.1022
Metabolism			
3-oxoacyl-CoA thiolase	X05341	1.7530	0.1967
ornithine aminotransferase	M11842	1.6545	0.1236
Cytochrome P450, subfamily IVB, polypeptide (Cyp4b1)	NM_016999	1.6451	0.1939
peroxisomal enoyl-CoA: hydrotase-3-hydroxyacyl-CoA bifunctional enzyme	K03249	1.5024	0.1123
Transcription			
selenoprotein P, plasma, 1 (Sepp1)	NM_019192	1.9265	0.3027
TIP120-family protein TIP120B, short form	AB029342	1.8235	0.2392
Uncategorized			
proline-rich acidic protein	AF214733	1.7139	0.1865

A.2 HFS/HGS Fructose Regulated Genes at 10 d Under Saline Conditions (continued)

Down-Regulated Genes (HFS)

Name	ID	Fold Change	Stdv
Cell Communication/ Signal Transduction			
Vascular cell adhesion molecule 1 (Vcam1)	NM_012889	-3.1703	0.1857
Caldendrin	Y17048	-2.9106	0.0858
ubiquitin C (Ubc)	NM_017314	-2.2808	0.0830
heat shock protein 22	AF314540	-1.9204	0.2819
Reversion induced LIM gene (Ril)	NM_017062	-1.8867	0.2255
G-protein-linked receptor kinase (beta adrenergic receptor kinase 1) (Adrbk1)	NM_012776	-1.8114	0.2521
a disintegrin and metalloproteinase domain (ADAM) 15 (metargidin) (Adam15)	NM_020308	-1.7889	0.0817
vanilloid receptor-like protein 1 (Vrl1)	NM_017207	-1.7546	0.3112
putative GTP-binding protein	AF329824	-1.7373	0.2246
hsp70.2 mRNA for heat shock protein 70	Z75029	-1.7076	0.3194
Chloride channel 1 (skeletal muscle) (Clcn1)	NM_013147	-1.6812	0.1799
thymosin beta-4	M34043	-1.6579	0.1597
glucagon-like peptide-2 receptor precursor (GLP2R)	AF105368	-1.6462	0.3127
Prolactin-like protein A (Prlpa)	NM_017036	-1.6318	0.0946
CaM-KII inhibitory protein	AF041854	-1.6252	0.2419
Kallistatin	U51017	-1.6090	0.1623
cytoplasmic beta-actin (Actx)	NM_031144	-1.5914	0.1807
neuronal calcium channel alpha 1E subunit	AF057029	-1.5559	0.2442
Glutamate receptor, ionotropic, N-methyl D-aspartate 2B (Grin2b)	NM_012574	-1.5512	0.1170
Dynamin 2 (Dnm2)	NM_013199	-1.5109	0.1008

A.2 HFS/HGS Fructose Regulated Genes at 10 d Under Saline Conditions (continued)

Development			
NRAGE	AF217964	-2.2264	0.4556
lamina-associated polypeptide 1C (LAP1C)	U19614	-2.0949	0.1953
Bcl2-associated X protein (Bax)	NM_017059	-1.7868	0.2234
Tyrosine hydroxylase (Th)	NM_012740	-1.7954	0.2913
tropoelastin	M60647	-1.7652	0.1285
spongiotrophoblast specific protein	AB009890	-1.7001	0.3318
Bcl-2-related ovarian killer protein (Bok)	NM_017312	-1.6841	0.3172
lamin C2	X99257	-1.6425	0.2275
WNT7A-like protein	AF481945	-1.6212	0.2548
cofilin 1, non-muscle (Cfl1)	NM_017147	-1.6016	0.2481
Nestin (Nes)	NM_012987	-1.5905	0.1490
Profiling	X96967	-1.5152	0.1024
neuronal nitric oxide synthase	AF008912	-1.5054	0.1777
Immune Response			
palate, lung, and nasal epithelium carcinoma associated (Plunc)	NM_172031	-1.8179	0.2561
interferon-alpha 1 (IFN-alpha1)	X00336	-1.5139	0.1108
Metabolism			
pancreatitis associated protein III (PAPIII0)	L20869	-4.9700	0.4381
pancreatitis associated protein (pap)	M55149	-3.8815	0.9349
glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	X02231	-2.2761	0.6098
Peptidylprolyl isomerase A (cyclophilin A) (Ppia)	NM_017101	-2.1819	0.3712
short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD), nuclear gene encoding mitochondrial protein	AF069770	-2.0586	0.2896
Obesity (murine homolog, leptin) (Lep)	NM_013076	-1.9547	0.4707
endoplasmin (GRP94)	S69315	-1.8421	0.2570

A.2 HFS/HGS Fructose Regulated Genes at 10 d Under Saline Conditions (continued)

Lactate dehydrogenase A (Ldha)	NM_017025	-1.7883	0.3908
serine/threonine protein kinase	D26178	-1.6606	0.1237
homocysteine respondent protein HCYP2	AF036537	-1.6177	0.2923
Glycogenin	AF021343	-1.6157	0.2317
mitochondrial ATP synthase beta subunit	M19044	-1.5320	0.2275
Creatine kinase, brain (Ckb)	NM_012529	-1.5032	0.1122
Transcription/ Translation			
R-RCD1	AB016069	-3.1292	0.6912
nucleosome assembly protein	AF062594	-1.9133	0.2268
RNA polymerase II	AB017711	-1.8666	0.2179
scaffold attachment factor B	AF056324	-1.6943	0.3134
acidic ribosomal phosphoprotein P0	X15096	-1.6633	0.3228
ribosomal protein L34	X14401	-1.6066	0.2375
mutY homolog (E. coli) (Mutyh)	NM_133316	-1.5924	0.2148
splicing factor 1 homolog	AF079873	-1.5745	0.0956
Brain factor-3 (HFH-BF-3)	L13193	-1.5154	0.2229
Transport			
FXYD domain-containing ion transport regulator 5 (Fxyd5)	NM_021909	-1.8234	0.0681
novel golgi-associated protein GCP360	D25543	-1.8086	0.2807
Beta-galactoside-binding lectin (Lgals1)	NM_019904	-1.7261	0.1933
Uncategorized			
Rat drs (a gene down-regulated by v-src)	D78359	-2.4326	0.2301
Carcinoembryonic antigen gene family (CGM3) (Cgm3)	NM_019126	-1.7137	0.1837
Set beta isoform	S68987	-1.6790	0.1941
hypothetical protein LOC56728 (LOC56728)	NM_019908	-1.5189	0.0431

A.2 Shows genes that are significantly ($P<0.05$) up and down-regulated, by more than 1.5 fold change, by Fructose under saline conditions in 10 d old small intestine. Values are fold change ± SE for each gene; n=4 experiments.

A.3 HFD/HGS Fructose and Dex Regulated Genes at 10 d

Up Regulated Genes

Name	ID	Fold Change	stdv
Cell Communication/ Signal Transduction			
Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (Grin2a)	NM_012573	3.2158	0.3508
triosephosphate isomerase	L36250	2.3868	0.3486
ERM-binding phosphoprotein	AF154336	2.2835	0.3254
corticotropin-releasing factor binding protein (CRF-BP)	X58023	2.0105	0.1351
myelin-associated glycoprotein (Mag)	NM_017190	1.6139	0.1821
Development			
epidermal growth factor receptor related protein	AF187818	2.7775	0.3263
myosin heavy chain	L24897	1.5307	0.0638
Hydroxymethylbilane synthase (Hmbs)	NM_013168	1.5002	0.0614
Metabolism			
Sucrase-isomaltase (SI)	NM_013061	6.5179	0.6384
fetal intestinal lactase-phlorizin hydrolase precursor	X56747	4.6456	0.8262
liver aldolase B	M10149	3.2036	0.3104
cytoplasmic aminopeptidase P (Abp1)	AF038591	2.4197	0.4560
amiloride binding protein (long form)	X73911	2.2150	0.3608
kidney Zn-peptidase aminopeptidase N	M25073	2.1851	0.1823
Glucose-6-phosphatase (G6pc)	NM_013098	2.1107	0.2970
3-alpha hydroxysteroid dehydrogenase (Acaa2)	AF159099	1.9954	0.2291
3-oxoacyl-CoA thiolase	X05341	1.9794	0.2518
Fructose-1,6- biphosphatase (Fbp1)a	NM_012558	1.9170	0.2117
very-long-chain acyl-CoA synthetase	D85100	1.8639	0.1651
cathepsin B	X82396	1.7713	0.1466

A.3 HFD/HGS Fructose and Dex Regulated Genes at 10 d (continued)

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (Pfkfb4)	NM_019333	1.7046	0.1686
ornithine aminotransferase	M11842	1.6098	0.1343
fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase	L27084	1.5007	0.1898
Translation/Transcription Regulation			
3-methylcholanthrene-inducible truncated UDP-glucuronosyltransferase	J05132	1.9984	0.2580
Proline-rich protein, salivary (Prpb)	NM_012632	1.4625	0.1324
NEFA precursor (Nucb2)	AF250142	1.7286	0.0998
Transporter			
solute carrier family 3, member 1 (Slc3a1)	NM_017216	2.6607	0.4154
amino acid transporter (Slc7a7)	AB020520	2.0282	0.3380
cell adhesion regulator (Slc39a1) (iron-regulated transporter-1)	U76714	1.9881	0.1905
glut8 mRNA for glucose transporter 8	AB033418	1.5144	0.1411

Down Regulated Genes

Gene Name	**ID**	**Fold Change**	**STDV**
Cell Communication/ Signal Transduction			
Vascular cell adhesion molecule 1 (Vcam1)	NM_012889	-4.0434	0.2669
Peptidylprolyl isomerase A (cyclophilin A) (Ppia)	NM_017101	-2.7355	0.5268
ribosomal protein S2	U92698	-2.4588	0.3664
protocadherin alpha 10 homolog	AF177684	-2.2193	0.3268
G-protein beta-2 subunit	AF277892	-2.1047	0.2939
protocadherin-T5 (pcdh-T5)	AF177699	-1.9405	0.2671
Statin-like protein (Stnl)	NM_012660	-1.8727	0.3046
Chloride channel 1 (skeletal muscle) (Clcn1)	NM_013147	-1.7712	0.2353
ribosomal protein L7a	X15013	-1.6052	0.1577
Dopamine receptor D2 (Drd2)	NM_012547	-1.5554	0.0608

A.3 HFD/HGS Fructose and Dex Regulated Genes 10 d (continued)

kringle-coding gene marking the eye and the nose (Kremen)	NM_053649	-1.5364	0.0961
Development			
protein kinase C-binding protein NELL2	U48245	-2.4325	0.4667
tropoelastin	M60647	-2.2577	0.1760
alpha-1 type I collagen	M12199	-2.1709	0.3725
NRAGE	AF217964	-1.8178	0.0164
lamin C2	X99257	-1.7345	0.1578
cytoplasmic beta-actin (Actx)	NM_031144	-1.7092	0.1612
collagen type III alpha-1	M21354	-1.6709	0.2135
Tyrosine hydroxylase (Th)	NM_012740	-1.6434	0.2194
dnchc2 mRNA for cytoplasmic dynein heavy chain	AB041881	-1.6272	0.2142
Aortic preferentially expressed gene 1 (Apeg1)	NM_012905	-1.5120	0.1639
Immune Response			
CC chemokine CCL28 (Scya28)	NM_053700	-1.6351	0.1172
Metabolism			
pancreatitis associated protein III (PAPIII0)	L20869	-4.0802	0.2457
short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD), nuclear gene encoding mitochondrial protein	AF069770	-1.5685	0.1549
Translation/Transcription Regulation			
ribosomal protein L32 (Rpl32)	NM_013226	-2.1200	0.2329
nucleosome assembly protein	AF062594	-2.0409	0.0668
Basic helix-loop-helix transcription factor (bHlH) (Mist1)	NM_012863	-1.9053	0.2448
RNA polymerase II	AB017711	-1.6803	0.2152
Transporter			
FXYD domain-containing ion transport regulator 5 (Fxyd5)	NM_021909	-1.6896	0.2071

A.3 Shows genes that are significantly ($P<0.05$) up and down-regulated, by more than 1.5 fold change, by Dex and Fructose in 10d old small intestine. Values are fold change ± SE for each gene; n=4 experiments.

A.4 [HFD/HFS + HGD/HGS] = Genes Solely Dex Regulated and Dex Regulated under Fructose Conditions at 10 d

Up Regulated Genes

Gene Name	Access No.	Fold Change	Stdv
Cell communication/ Signal Transduction			
Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (Grin2a)	NM_012573	2.5572	0.8166
corticotropin-releasing factor binding protein (CRF-BP)	X58023	1.9399	0.5249
neuronal nicotinic acetylcholine receptor alpha 2 subunit	L10077	1.6285	0.4006
Pancreatic trypsin 1 (Prss1)	NM_012635	1.5367	0.3489
Metabolism			
Sucrase-isomaltase (SI)*	NM_013061	3.9873	1.1829
fetal intestinal lactase-phlorizin hydrolase precursor*	X56747	3.6861	1.1890
liver aldolase B	M10149	2.3283	0.7496
very-long-chain acyl-CoA synthetase	D85100	1.8517	0.4921
Isovaleryl Coenzyme A dehydrogenase (Ivd)	NM_012592	1.5409	0.3758
Transporter			
solute carrier family 3, member 1 (Slc3a1)*	NM_017216	2.3848	0.7341

Down Regulated Genes

Gene Name	Access No.	Fold Change	Stdv
Signal Transduction/ Cell Communication			
G-protein beta-2 subunit	AF277892	-1.9189	0.5461
Transcription Regulation			
BCL2-related protein A1 (Bcl2a1)	NM_133416	-1.6027	0.4098

A.4 Shows genes that are significantly ($P<0.05$) up and down-regulated, by more than 1.5 fold change, solely by Dex independent of the sugar present and by Dex under Fructose conditions in 10 d old small intestine. Values are fold change ± SE for each gene; n=4 experiments. The * notes genes that are specifically regulated by Dex independently of fructose or glucose, while the other genes are Dex regulated under fructose conditions.

A.5 Dex Regulated Genes Under Fructose Conditions at 10 d (HFD/HFS)

Up Regulated Genes

Gene Name	Access No.	Fold Change	Stdv
Cell communication/ Signal Transduction			
Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (Grin2a)	NM_012573	2.5572	0.8166
corticotropin-releasing factor binding protein (CRF-BP)	X58023	1.9399	0.5249
neuronal nicotinic acetylcholine receptor alpha 2 subunit	L10077	1.6285	0.4006
Pancreatic trypsin 1 (Prss1)	NM_012635	1.5367	0.3489
Metabolism			
liver aldolase B	M10149	2.3283	0.7496
very-long-chain acyl-CoA synthetase	D85100	1.8517	0.4921
Isovaleryl Coenzyme A dehydrogenase (Ivd)	NM_012592	1.5409	0.3758

Down Regulated Genes

Gene Name	Access No.	Fold Change	Stdv
Signal Transduction/ Cell Communication			
G-protein beta-2 subunit	AF277892	-1.9189	0.5461
Transcription Regulation			
BCL2-related protein A1 (Bcl2a1)	NM_133416	-1.6027	0.4098

A.5 Shows genes that are significantly ($P<0.05$) up and down-regulated, by more than 1.5 fold change, by Dex regulated genes under fructose conditions (HFD/HFS) in 10d old small intestine. Values are fold change ± SE for each gene; n=4 experiments.

A.6 Common Genes between Age (20d/10d) and Dexamethasone (10d) under Fructose Conditions

Gene ID	Symbol	(20d vs. 10d) Age + Fructose	Dex (D) + Fructose(F) Array
Cell Communication		AGE EFFECT	DEX EFFECT
S61973	Grina*	↓ 20dF/10dF & 20dG/10dG	↑ HGD/HGS
Metabolism			
M10149	Aldolase B	↑ 20dG/10dG	↑ HFD/HFS
NM_013061	Si*	↑ 20dF/10dF & 20dG/10dG	↑ HGD/HGS & HFD/HFS
NM_017013	Gsta2*	↑ 20dF/10dF & 20dG/10dG	↑ HGD/HGS
NM_017025	Ldha	↑ 20dF/10dF & 20dG/10dG	↓ HFS/HGS
NM_016986	Acadm	↓ 20dG/10dG	↑ HFD/HFS
X05341	Acaa2	↓ 20dG/10dG	↑ HFS/HGS
NM_019333	Pfkfb4	↓ 20dF/10dF	↑ HGD/HGS
D85100	Slc27a2	↓ 20dF/10dF & 20dG/10dG	↑ HFD/HFS
Transcription			
NM_013097	Dnase1*	↑ 20dF/10dF & 20dG/10dG	↑ HGD/HGS
Transport			
NM_017216	Slc3a1*	↑ 20dG/10dG	↑ HGD/HGS, HFD/HFS
NM_012977	Lgals9	↓ 20dG/10dG	↑ HGD/HGS
AB033418	Glut 8	↓ 20dF/10dF & 20dG/10dG	↑ HFD/HFS
Uncategorized			
L01122	Ftl1	↓ 20dF/10dF	↑ HFS/HGS
AF214733	Upa	↓ 20dF/10dF & 20dG/10dG	↑ HFS/HGS

A.7 Genes from Microarray Results Confirmed by PCR

Gene Name	Symbol	Gene ID	Direction	Primer Sequence (5' →3')	Probe Size, bp	Temp.
Glucose-6-phosphatase	G6Pc	NM_013098	Forward	GGCTCACTTTCCCCATCAGG	146	59°
			Reverse	ATCCAAGTGCGAAACCAAACAG		
Sucrase-isomaltase	SI	L25926	Forward	GCCTTACCCTGCCTTTGA	216	55°
			Reverse	ATTTCTCTTGCCCACCACTC		
Pancreatitis Associated Protein	PAP	J05132	Forward	ATGTCTTCTCCTTTGGTGGC	408	57°
			Reverse	TGGTGGTAGTGGTGCTGTTC		
Elongation factor 1α	EF1 α	NM_1758378	Forward	CTCCACTTGGTCGTTTTGCTGT	165	59°
			Reverse	AGACTGGGGTGGCAGGTGTT		
Facilitated Glucose Transporter	GLUT5	D13871	Forward	TGCAGAGCAACGATGGAGAAA	220	59°
			Reverse	ACAGCAGCGTCAGGGTGAAG		
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	Pfkfb4	NM_019333	Forward	TGGATGAAGAACAGGACAGG	154	55°
			Reverse	CGGCAGAGGTAGATGGAG		

APPENDIX B

RNA EXTRACTION AND DNASE TREATMENT PROCEDURE

This appendix contains the procedure that was used for mRNA extraction and DNAse treatment of the rat intestinal samples in preparation for the microarray procedure.

RNA EXTRACTION AND DNASE TREATMENT PROCEDURE

1.0 RNA Extraction

1.1 Tare sterile tubes on a balance

1.2 Put sample into tube

1.3 Add 1ml Trizol/100 mg (0.1 g sample)

1.4 Homogenize

1.5 Put on ice

1.6 Clean mixer (special h2O in sterile tubes, then ethanol, then kimwipes)

1.7 Transfer 1 ml of sample into eppendorf tubes (if 2 ml sample, use 2 tubes)

1.8 Label accordingly

1.9 Centrifuge: 11500 rpm 4C 10 mins

1.10 Transfer supernatant into eppendorfs

1.12 Incubate 10 min on ice

1.13 Add 0.2 ml chloroform / 1 ml trizol

1.14 Shake/vortex 20 sec each

1.15 Centrifuge 11500 rpm 20 min 4C

1.16 Transfer supernatant into eppendorf tubes (the clear liquid)

1.17 Add 0.5 ml (500 ul) isopropanol

1.18 Vortex 10 sec each

1.19 Incubate on ice for 15 minutes

1.20 Centrifuge 11500 rpm 10 min 4C

1.22 Discard supernatant, save pellet

1.23 Add 1 ml ethanol (70%) per sample --- 602 fridge

1.24 Centrifuge 9000 rpm 5 min 4C

1.25 Remove ethanol

1.26 Dry pellets (about 10 mins using fan) --- make gel while drying

1.27 Suspend pellets in 100 ul RNAase free h2O, break up pellet, pipet in-out repeatedly

2.0 Prepare electrophoreses gel:

2.1Weigh agarose (604 cabinet)

2.1.1 500 mg agarose/50 ml TAE 1X

2.1.2 Use Erlenmeyer flask to mix solution

2.2 Microwave for about 1 min or till big bubbles form

2.3 Tape tray with autoclave tape

2.4 Use big wells of combs

2.5 When agarose is cold, add 2 ul ethidium bromide

2.6 10X Buffer: (blue violet color)

2.7 Samples for gel

2.7.1 2 ul h2O

2.7.2 2 ul buffer

2.7.3 6 ul sample

2.7.4 mix

3.0 Prep for spectrophotometer mRNA quantification:

3.1 sample dilution: 99 ul h2O + 1 ul sample

3.2 Use nucleic acid application (UV on)

4.0 Running the Agarose gel

4.1 Add TAE 1X to the electrophoreses chamber make sure level is high

4.2 Place gel in the chamber, remember the samples run to the red connection side

4.3 Load 10 ul of sample in each well

4.4 Turn on the chamber

4.5 Let the gel run for about 20 mins

4.6 Check under fluorescent light that mRNA bands are of good quality for going to the next step.

5.0 DNAse TREATMENT

5.1 Get samples, store in ice to defrost

5.2 Label microtubes

5.3 Make the following mix per sample:

+ 39 ul DEPC H2O

+10 ul DNase buffer 10x

+ 50 ul of each sample, vortex samples

+ 1.5 ul DNase, vortex

5.4 Incubate for 10 mins at 37 C (using a BioRad cycler)

5.5 Retrieve samples from cycler, immediately incubate in ice for 5 mins

5.7 While incubating:

5.8 Prepare the following mix per sample:

+ 100 ul NaDaC ammonium acetate

+ 10 ul EDTA pH 8

+ 390 ul DEPC H2O

5.9 Centrifuge above mix and incubated samples for about 7 sec using minispin

5.10 Add the samples from microtubes (transfer entire volume) into the tube with the mix

5.11 Add 700 ul phenol, chloroform, isoamyl alcohol mix; vortex 20 sec each sample tube

5.12 Centrifuge at 11500 rpm for 20 mins at 4 C

5.13 Remove aqueous phase and transfer to another eppendorf tube

5.14 Add 700 ul 100% ethanol to each tube, mix for 5 secs

5.15 Incubate at -20 C for minimum of 2 hours

5.16 Take samples from fridge and centrifuge at 11500 rpm for 30 mins at 4 C

5.17 Discard supernatant and save pellet

5.18 Wash the pellet with 300 ul 70% ethanol

5.19 Centrifuge for 10 mins 11500 rpm at 4C

5.20 Discard supernatant and save pellet

5.21 Air dry the pellets

5.22 Once dry, suspend pellets in 20 ul DEPC H2O

5.23 Run an agarose gel and spectrophotometer (to measure quality and quantity)

APPENDIX C

GENISPHERE MANUAL 2-STEP PROTOCOL USING 3DNA ARRAY 350 KIT

This Appendix contains the protocol that was used to hybridize all of the microarray up to the point where the chip was ready to be scanned. This protocol was adapted from the Genisphere website < www.genisphere.com> by personnel from the Center for Applied Genomics, Newark, NJ.

GENISPHERE MANUAL- 2-STEP PROTOCOL USING 3DNA ARRAY 350 KIT

1.0 cDNA synthesis and labeling:

Reaction mix:

For one reaction, prepare one tube with Cy3 primer and another with Cy 5 primer:

1-10ul RNA (3.0ug Total rat RNA)

1.0ul Cy3 or Cy5 RT primer (1 pmol/ul) (Vial 2)

0-10ul RNase Free water (Vial 10)

---

11 ul Total Mix Volume

- mix and microfuge briefly.
- place tubes at 80°C for 10 min.
- snap cool on ice for 2-3 min.

Mix separately in a tube:

1ul Superase-In (Vial 4)

4ul 5X Super Script First Strand buffer(in SuperScript kit)

1ul dNTPs (10mM each for dATP, dCTP, dGTP, dTTP) (Vial 3)

2ul 0.1M DTT(in SuperScript kit)

1ul Super Script II RT Enzyme (200 units)

9ul Final Reaction Volume (keep on ice until used).

- mix gently(do not vortex), and spin contents to bottom of tube.
- add the reaction mix to the 12ul of RNA-RT primer mix from above
- gently mix and incubate at 42°C for 2 hours.
- stop reaction with 3.5ul 0.5M NaOH/50mM EDTA,
- incubate at 65°C for 10 min.
- neutralize with 5ul 1M Tris-HCl, pH 7.5.
- combine Cy3 and Cy5 reactions.
- Rinse the original tubes with 73µl of 10mM Tris, pH 8.0, 1mM EDTA (TE).
- Combine wash with the cDNA mixtures into one tube for a total volume of 130µl.

2.0 Concentration of the cDNA with Millipore Microcon YM-30 Centrifugal Filter Devices

cDNA samples may be concentrated using the Millipore Microcon YM-30 Centrifugal Filter Devices (30,000 molecular weight cutoff, Millipore catalog number 42409). These devices are capable of reducing the volume of the cDNA synthesis reaction from 130µl to 3-10µl in as little as 8-10 minutes. The procedure below reiterates the manufacturer's directions with minor adaptations for the 3DNA Array 350 Kit.

Important: Users of the MicroconYM-30's should evaluate their own centrifuge settings to determine the optimal time and speed settings to yield final volumes of 3-10µl.

1. Place the Microcon. YM-30 sample reservoir into the 1.5mL collection tube.
2. Pre-wash the reservoir membrane by adding 100µl TE pH 8.0 to the Microcon. YM-30 sample reservoir.

3. Place the tube/sample reservoir assembly into a fixed angle rotor tabletop centrifuge capable of 10-14,000g.
4. Spin for 3 minutes at 12,000g.
5. Add all 130µl from the cDNA reaction to the Microcon. YM-30 sample reservoir. Do not touch the membrane with the pipet tip.
6. Centrifuge for 9 minutes at 12,000g.
7. Remove the tube/sample reservoir assembly. Separate the collection tube from the sample reservoir with care, avoiding spilling any liquid in the sample reservoir.
8. Add 5µl of 1X TE buffer (10mM Tris-HCI, pH 8.0 / 1mM EDTA) to the sample reservoir membrane without touching the membrane. Gently tap the side of the concentrator to promote mixing of the concentrate with the 1X TE buffer.
9. Carefully place the sample reservoir upside down on a new collection tube. Centrifuge for 2 minutes at top speed in the same centrifuge.
10. Separate the sample reservoir from the collection tube and discard the reservoir. Measure the volume collected in the bottom of the tube (3-9.5µl total volume). The cDNA sample may be stored in the collection tube for later use.

3.0 Successive Hybridization of cDNA and 3DNA to Microarray (2-Step Protocol)

Add RNase-free deionized distilled water to the concentrated cDNA preparation to achieve a total volume of 10µl.

Hybridization Mix:	
Concentrated cDNA (from step 1)	10 µl
dT Blocker (Vial 9)	2.0 µl
RNase Free water (Vial 10)	8.5 µl
Hybridization Buffer (Vial 6)	21 µl
C0T-1 DNA (denatured at 95-100°C for 10 minutes)	0.5 µl
Final Hybridizaton Mix Volume	42 µl

3.1 Gently vortex and briefly microfuge the Hybridization Mix after addition of all components.

3.2 Incubate the Hybridization Mix first at 75-80°C for 10 minutes and then keep at 53°C until loading on the array.

3.3 Vortex and microfuge briefly.

3.4 Add the Hybridization Mix to a pre-warmed microarray .

3.5 Apply a glass coverslip to the array and incubate overnight in a dark humidified chamber at 53°C.

4.0 Post cDNA Hybridization Wash

4.1 Prewarm the 2X SSC, 0.2%SDS wash buffer to 42°C.

4.2 Remove the coverslip by washing the array in 2X SSC, 0.2%SDS for 2-5 minutes at room temperature (or until coverslip floats off).

4.3 Wash for 10-15 minutes at 42°C with 2X SSC, 0.2%SDS.

4.4 Wash for 10-15 minutes at room temperature with 2X SSC.

4.5 Wash for 10-15 minutes at room temperature with 0.2X SSC.

4.6 Immediately transfer the array to a dry 50mL centrifuge tube. Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide. Avoid contact with the microarray surface.

5.0 3DNA Hybridization Preparation for 2nd Hybridization

5.1 To prevent any possible formation of aggregates follow the steps below:

5.1.1 Thaw (room temp.) the 3DNA Array vial 1 Reagants in a dark room for 20 minutes

5.1.2 Vortex at maximum setting for 3 secs and microfuge briefly.

5.1.3 Incubate at 50-55°C for 10 minutes.

5.1.4 Vortex at the maximum setting for 3-5 secs and microfuge the tubes briefly.

5.2 Thaw and resuspend the 2X Hybridization Buffer (Vial 6) by heating it to 70°C for 10 minutes. Vortex the vial at maximum settings and microfuge for 1 minute.

5.3 Thaw (room temperature) Anti-Fade Reagant (Vial 8).

5.4 Preparation of the Antifade Stock (helps reduce fading of the fluorescent dyes during hybridization):

a. 1µl of Anti-Fade Reagant (Vial 8).

b. 100µl of 2X Hybridization Buffer (Vial 6)

c. Vortex and briefly microfuge mix.

d. Cover tube with aluminum (it is light sensitive).

5.5 Follow recipe below for Preparation of 2nd Hybridization Mix:

Hybridization Mix 2.

Anti-Fade Stock	21 µl
3DNA Capture Reagent #1 (Vial 1, e.g. Cy3)	2.5 µl
3DNA Capture Reagent #2 (Vial 1, e.g. Cy5)	2.5 µl
Nuclease Free Water (Vial 10)	16µl
Final Hybridizaton Mix Volume:	42µl

5.5.1 Gently vortex and briefly microfuge the Hybridization Mix after addition of all components.

5.5.2 Incubate the Hybridization Mix first at 75-80°C for 10 minutes, and then keep at 62°C until loading on the array.

5.5.3 Add the Hybridization Mix to the pre-warmed microarray.

5.5.4 Apply a glass coverslip to the array and incubate 3-3.5 hours in a dark humidified chamber at 62°C .

6.0 Post 3DNA Hybridization Wash

6.1 Prewarm the 2X SSC, 0.2%SDS wash buffer to 60-65°C

6.2 Remove the coverslip by washing the array in 2X SSC, 0.2%SDS for 2-5 minutes at room temperature (or until coverslip floats off).

6.3 Wash for 10-15 minutes at 60-65°C with 2X SSC, 0.2%SDS.

6.4 Wash for 10-15 minutes at room temperature with 2X SSC.

6.5 Wash for 10-15 minutes at room temperature with 0.2X SSC.

6.6 Immediately transfer the array to a dry 50mL centrifuge tube.

6.7 Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide. Immediately transfer the array to a light-protective slide box, taking care not to touch the array surface.

7.0 Materials for experiment

7.1 Buffers:

Buffer	Mix	Total Volume
2X SSC, 0.2% SDS	100ml of 20x SSC + 20ml of 10%SDS + 880ml ddH_2O	1000 mL
2x SSC	100ml of 20x SSC + 900ml ddH_2O	1000 mL
0.2x SSC	10ml of 20x SSC+ 990ml ddH_2O	1000 mL

7.2 Reagents

Name	Stock Concentration	Source	Catalog #
Genisphere 350 kit (Cy3)		Genisphere	W300130NL
Genisphere 350 kit (Cy5)		Genisphere	W300140NL
0.1M DTT	From Kit	Invitrogen	Superscript Kit: 18064-014
Superscript II RT	From Kit	Invitrogen	Superscript Kit: 18064-014
5X First-Strand Buffer	From Kit	Invitrogen	Superscript Kit: 18064-014
SSC	20X	Ambion	9763
SDS	10%	Ambion	9822
Milipore YM30 Microcon centrifugal columns		Millipore	42409
TE	TE pH8.0	Ambion	9849
COT 1 DNA	1ug/ul	Invitrogen	15279-011
NaOH (pellets)	1 M	EMD	SX0593-1
EDTA (powder)	100 mM	Invitrogen	15632-011
Tris-HCl	1 M	Sigma	T-2788

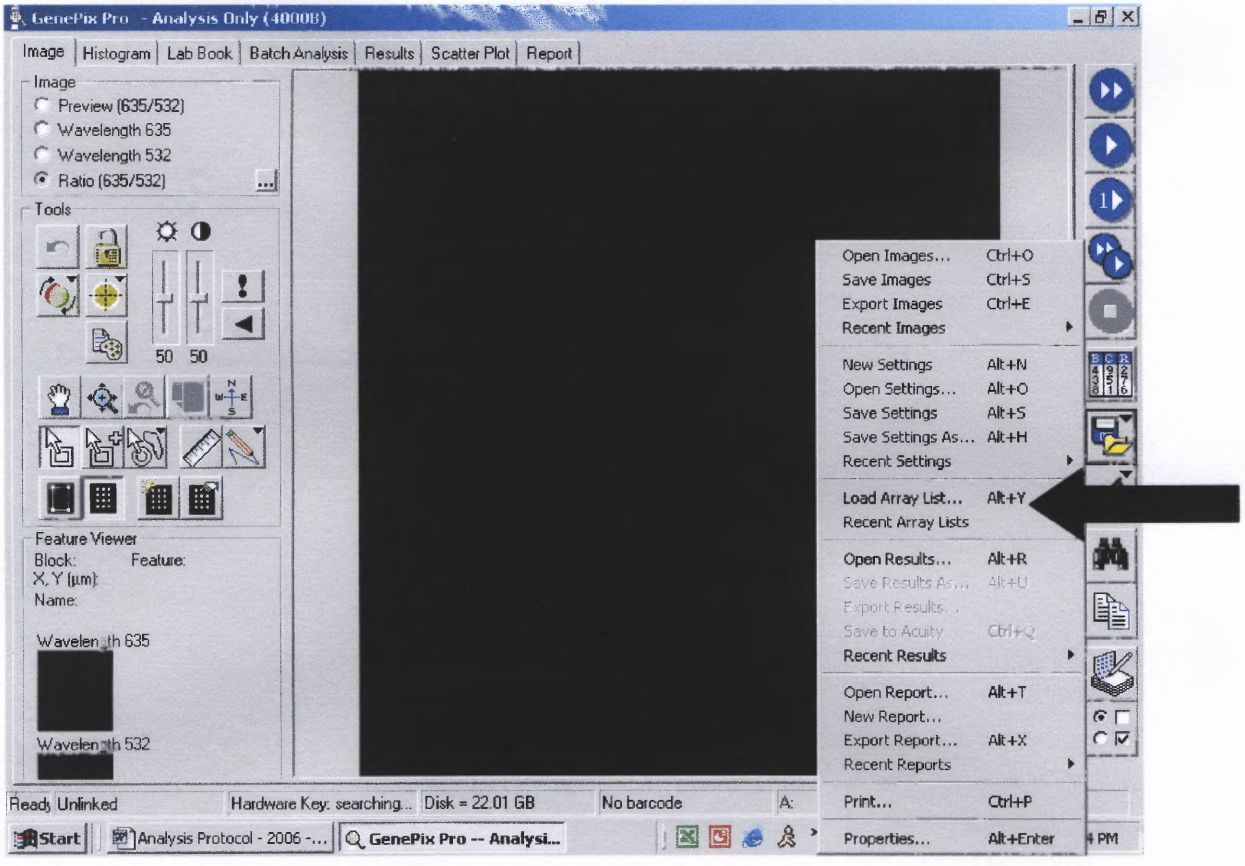
APPENDIX D

MICROARRAY IMAGE AND STATISTICAL ANALYSIS PROCESS

This Appendix contains the step by step procedure on how to scan a microarray chip; analyzed the data output by GenePix 5.1; the steps for global and local regression normalization; and how to do a SAM test on the data results.

A. Gridding

1. Open up the Geneprix 5.1 software and select the "Analysis Only" option.
2. Open the Microarray (chip) image (.tiff file) by clicking on the diskette icon on the right hand menu and select the "Open Images" option.

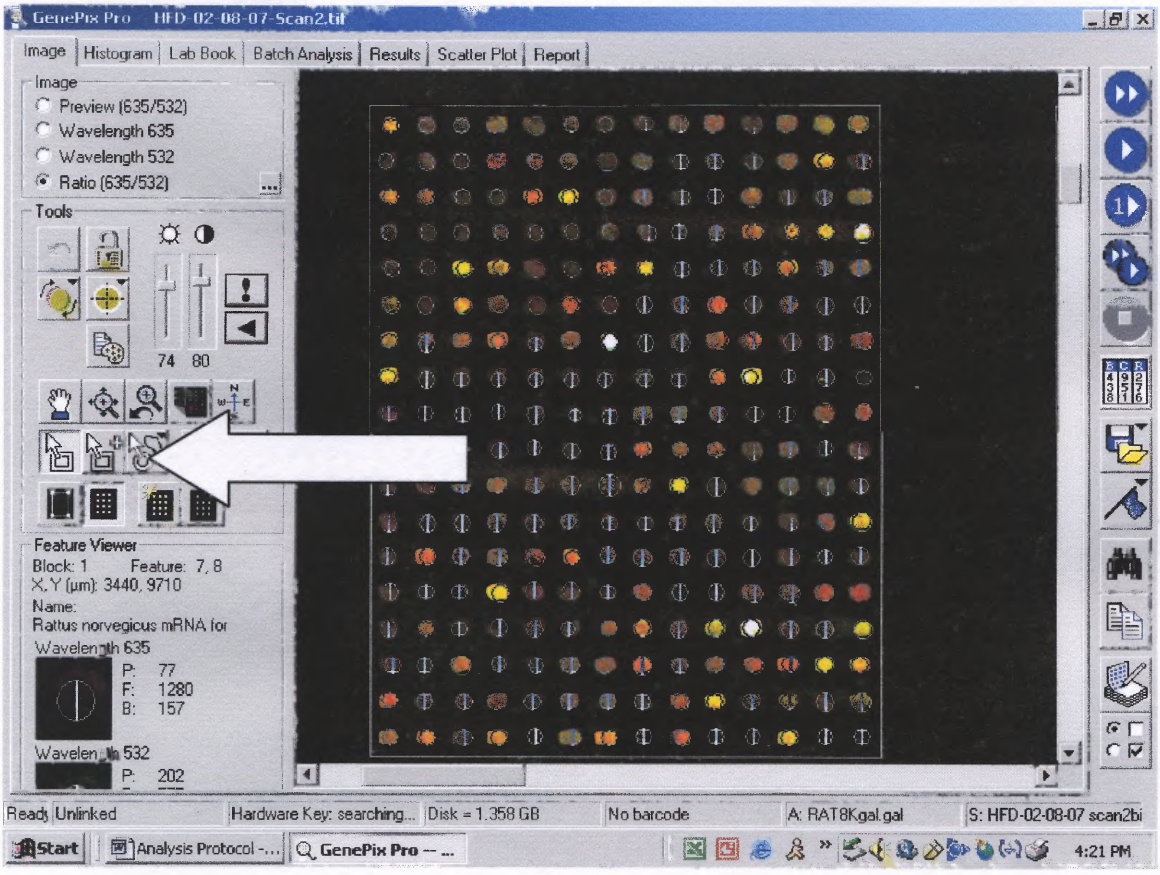


3. Under the same icon select "Load array List". This is the gene list (.gal file) that corresponds to your chip ie: Rat8k.gal.
4. Select the list and align it with your blocks as best you can. To align left click and drag the list over the spots on the whole image.

5. On the left menu click on the eyeloop (next to the blue hand) to zoom in on the blocks and do last minute adjusting over the spots.
6. Hit the "F5" key for the software to do an automatic alignment.
7. The settings for the list can be saved and should be saved periodically throughout this alignment. To save the settings, on the right menu select the diskette icon. Scroll down to find "Save Setting as". Select this option and name the file the same as the Microarray image, this will save as a .gps file.

NOTE: This is very important because if something happens to the computer while in middle of the alignment process one can always open up the settings and they will be the same as the last save.

8. On the left menu click on the icon that has a square with an arrow. This allows you to select each individual list for the corresponding block of genes.

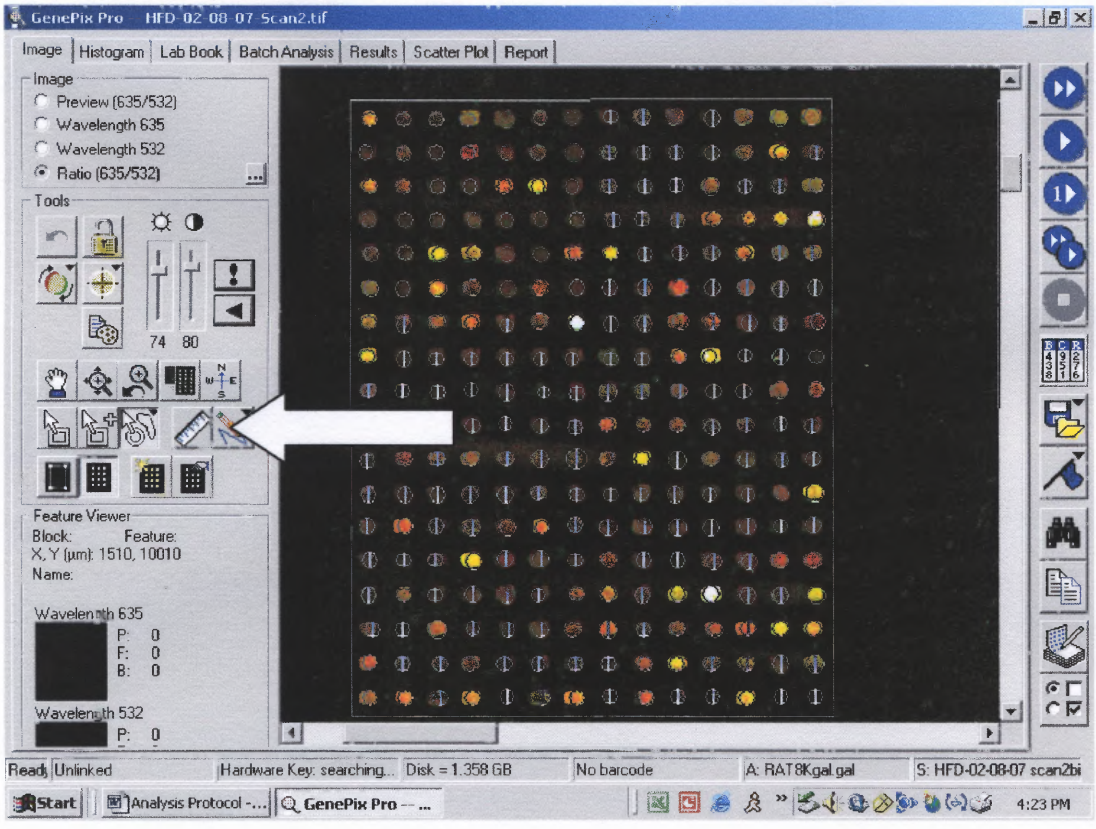


9. Adjust each block by left clicking and dragging. Once aligned (for the most part) hit the "F5" key – this will further adjust all the spots in that particular group of genes (The block alignments should be done for the whole microarray). To move to the next block just hit the ">" and it will take you to the next block automatically.
10. Once all the blocks are adjusted individually the next step is to flag and adjust spots individually for each block of genes.

NOTE: Flagging means when a particular spot is damaged in someway or not there it should be marked.

11. Start from the most top left block.

12. On the left menu select the icon with the circle (sometimes it looks like a foot shape- as below) and a white arrow across it. This will allow you to left click on a gene spot and manipulate it.



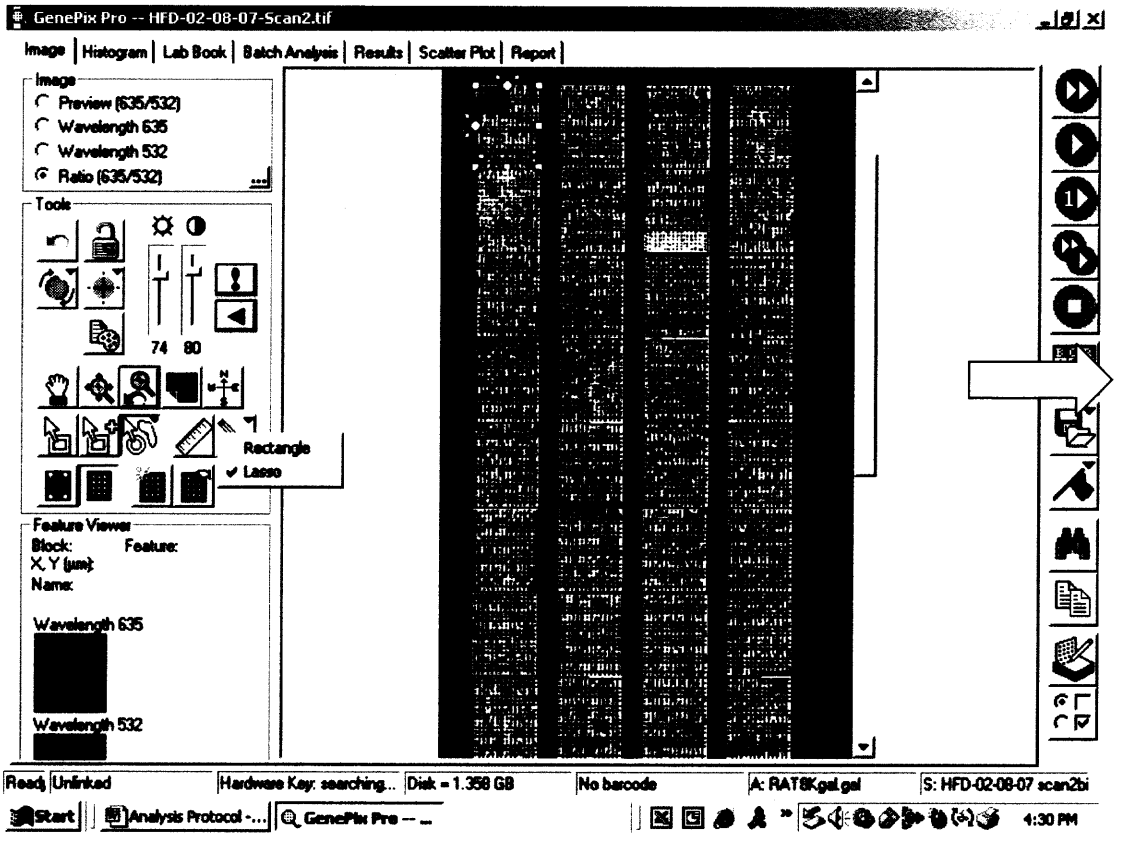
13. With this icon selected you can adjust the array list circle over the image (gene) spot to perfect align the two; you can also adjust the size of the circle to hug the gene spot on the microarray image.

14. To change the size of a circle you select the spot hold down the "Ctrl" key and use the up and down arrow keys to adjust the size.

15. If a spot needs to be flagged, be marked because of its appearance, you select the gene spot and hit the "N" key. This will place a vertical slash across the gene spot, where later on it can be filtered out of the analysis if so desired.

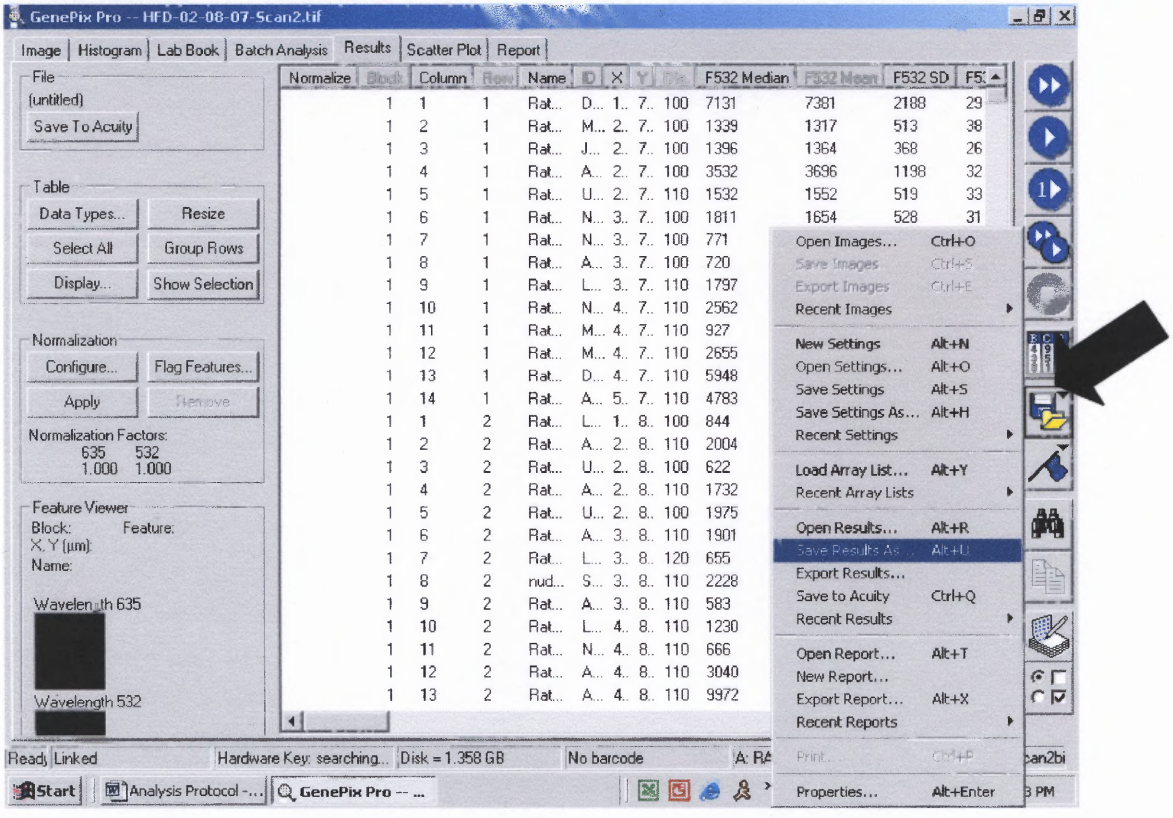
16. The list circles should all be adjusted individually, if needed, across each gene spot to make sure that only the gene spots will be measured for intensity and not the background.

17. Once all the spots have been adjusted from all the blocks, Save your settings one last time. To calculate the intensities of your genes click on the icon on the right hand side that has numbers in a block (see image below).



NOTE: This icon will take all the spots, measure the intensity and release a table with the corresponding gene name, the mean, median and the background – intensities of your gene spots for both the 635 (cy5) and your 532 (cy3) wavelengths. At the very end all the way at the right handside of the table it will also give you the log ratio of the cy5/cy3. This ratio is the number that will be used later on for statistical analysis with the flags column and the diameter size of your gene spot.

18. This table with the results should be saved. On the right handside click on the diskette icon and scroll down to the "Save results as" option. This file will save as a .gpr and it should be named the same as your original Microarray image. (Refer to image below)



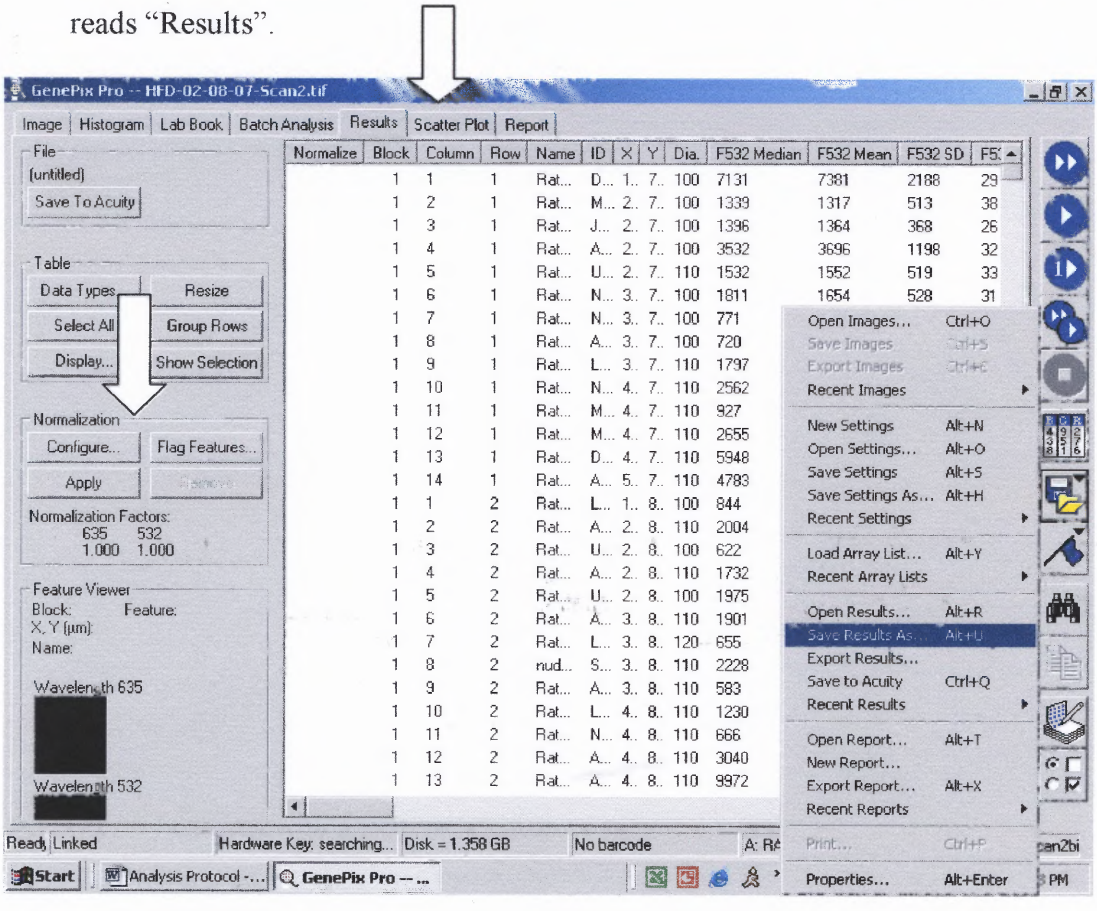
19. To analyze this data you will need to use Microsoft Excel.

B. Normalization and Filtering of Data

The first step is to make a decision as to what kind of normalization one should do. There is global normalization and Winprint BS normalization. The Global Normalization is done, where you are normalizing the entire chip to one. With the Winprint Normalization what you are doing is to move the cloud of intensities down to zero so that you end up with a few samples that are over and some are under analyzed with this method.

B.1 Global Normalization

1. For global normalization this can be done through the usage of GenePix.
2. Open up your .gpr file with the results. Click on the tab, at the top of the page that reads "Results".



3. When looking at the table with the results, on the left menu there is the option to "configure" the data.
4. Select configure and it should say that it will normalize all of the data to 1.
5. Click "Normalize" and save the new results as "...gnorm.gpr" file.

B. 2 Winprint Normalization

1. The Winprint Icon should be located on the desktop of the computer.
2. Open your folder with all of the .gpr files.
3. Left click on the .gpr file to drag it over the winprint icon on the desktop. This should automatically create a ...norm.gpr file that can be put back in the folder.
4. The normalization is done.

B.3 Filtering the Data Results

1. Click the .gpr files of the data results, right click and open it with Microsoft Excel.
2. On this new worksheet copy the name and gene ID columns along with the columns that read "Logratio", "Flags" and "diameter".
3. Open a new worksheet of excel and paste these columns in the spreadsheet.
4. Open the other microarray results (all of your .gpr files) and copy the logratio, the flags and the diameter and paste these in the same worksheet. When you are done you should have all of your microarray data, for all of your experiments, in one worksheet.
5. Save this file as arraydata.xls.

6. The next step requires that you flip your numbers for the logratio, depending on the labeling that was done with the cy5 and cy3 during the experimentation.
    1) When the labeling was done, there were times that the control group (the pooled mix) was labeled with cy3 and other times it was labeled with cy5 and the second treatment group was labeled with the corresponding dye. This was done to normalize for dye bias.
    2) The log analysis done always takes the $\log_2$(cy5/cy3), which for our experiment it corresponds to $\log_2$(treatment/pooled mix), but this is not the case for the experiments that the dyes were flipped.
    3) To correct this, one needs to look at the labeling that was done and check to see if the mix was labeled with cy3 or cy5. The experiments with the mix labeled with cy3 do not need to be changed, this ratio is accurate. However, for the other half of the experiments where the mix was labeled with cy5, the logratios on the arraydata.xl file need to be multiplied by a -1. What this does is that it inverts the logratio so that you are now looking at the same $\log_2$(treatment/mix) and not the $\log_2$(mix/treatment).
7. Once the ratios have been corrected and flipped, the data now needs to be prepared for filtering.
8. To filter the data means that you are now selecting only your good gene spots for analysis.
9. Your data needs to be setup properly to avoid any confusion.

1) Select all of your columns, minus the Gene Name and ID –column A and B, and use "Ctrl +C" to copy them.
2) Scroll to the right of your worksheet, skip a column, and paste the data columns, scroll to the right of the second set of columns, skip a column and paste again. This way you should have three sets of data.
3) In the first set you will, leave all the columns with the diameters and delete the other columns with the logratio and the flags. Make sure to label your columns with the corresponding date and treatment.
4) The second set of data you will delete the diameter and the logratios and leave the flags' columns. Make sure to label your columns with the corresponding date and treatment.
5) For the last set delete the columns with the diameter and the flags and keep the columns with the logratios. Make sure to label your columns with the corresponding date and treatment.
6) You are now ready to begin the filtering.

10. The best way to do this is to write a numeric logic expression in excel where it will only take the spots that have a particular diameter and have not been flagged during the gridding.
    a. Such an equation can be …=IF(AND(A1>60,B2=0),C3,"NA")… type this in a new column all the way to the right of your data sets.

b. The A1 refers to the column in the arraydata that has the diameter, the B2 is the column with the flags and the C3 refers to the logratio column.

c. The significance of this equation is that if the diameter is larger than 60 and it has not been flagged it should output the logratio into this particular cell (that you are writing the formula in), if it has been flagged or is smaller than 60, the output should be NA and it will not be a part of the analysis.

d. This filter is to be applied to all the microarray datasets.

e. At the end of this filtration, you should have new columns of data (just with the logratios) that need to be labeled with the treatment and date, so that it is know what experimentation the data belongs to.

f. Copy the columns with the gene name, gene ID and these new columns with the filtered log ratios.

g. Open a new Microsoft excel sheet and do a paste special, and select values.

h. This excel file should be saved as arraydata-filtered.xl.

i. Save a second file of this data as a text file ie: arraydata-filtered.txt

11. Your data is now ready to be statistically analyzed through the MeV 4.0 software.

C. Using TIGR Multiexperiment Viewer (MeV) for Statistical Analysis

C.1 Loading the Data File

a. Open up the MeV software.

b. Under the file menu → select load data.

c. From the Top drop down menu → select the Tab Delimited … option

d. Set up the path to where you saved your arraydata-filtered.txt file. Sometimes there is an issue creating this path if the files are not on the hard drive of the computer.

e. Once the pathway is set to your file location there is a white menu in the middle of the page where your file name should be present.

f. Select your file ie: arraydata-filtered.txt, and the software will load it so that you can see the name of the genes and the logratios.

g. Select the left top cell with the logratio number, do not select the other cells with the labeling, the program knows to use these as labeling.

h. Scroll to the bottom and select Load.

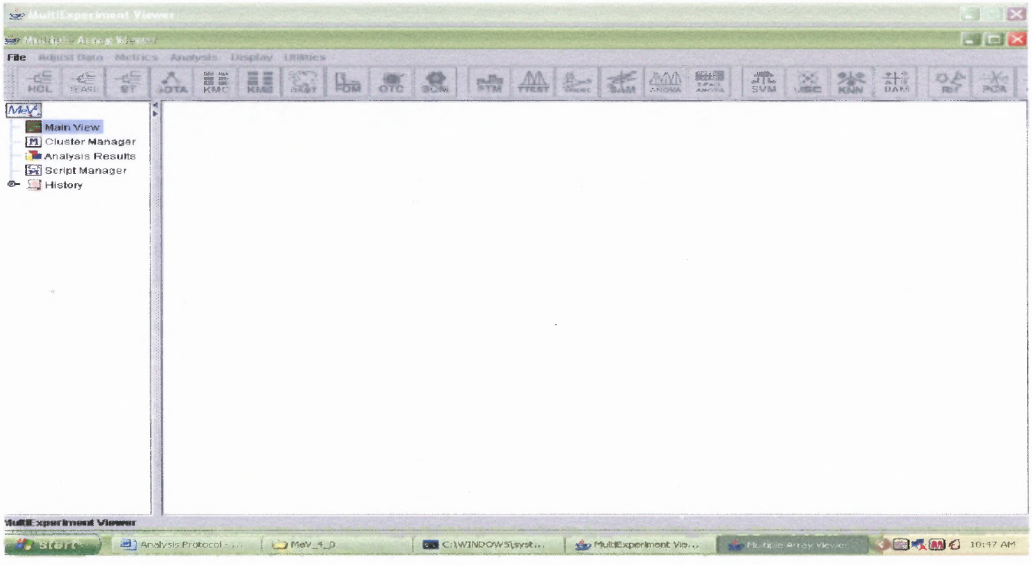
i. The data can now be filtered again before you proceed with the analysis.

j. From the top menu select Adjust data → select data filters → Select the Percentage cut-off.

Note: This percentage cut-off means that in order for the program to analyze a particular gene across all the treatments the gene needs to be present a certain percentage ie: if you use 80.0% this means that your genes need to be present across all of your treatments at least 80 % of the time, if it is present less than that – say that it was a spot flagged too many times across the treatments and it is only present in two treatments – the program will not incorporate this gene into the analysis. This method helps to have plenty of replicates per treatment to see a concrete pattern.

k. To view and sort the genetic data there are two unique options. On the left handside you can select the HCL (Hierarchical Clustering) icon, the software will create a cluster tree that relates the genes along with the treatments. There is also the option to create a PCA icon that will create a 3-D Graph that clusters all the genes so that you can see how they relate.

l. On the same menu bar your have all the different kinds of Statistical analysis test that you can perform with the data.

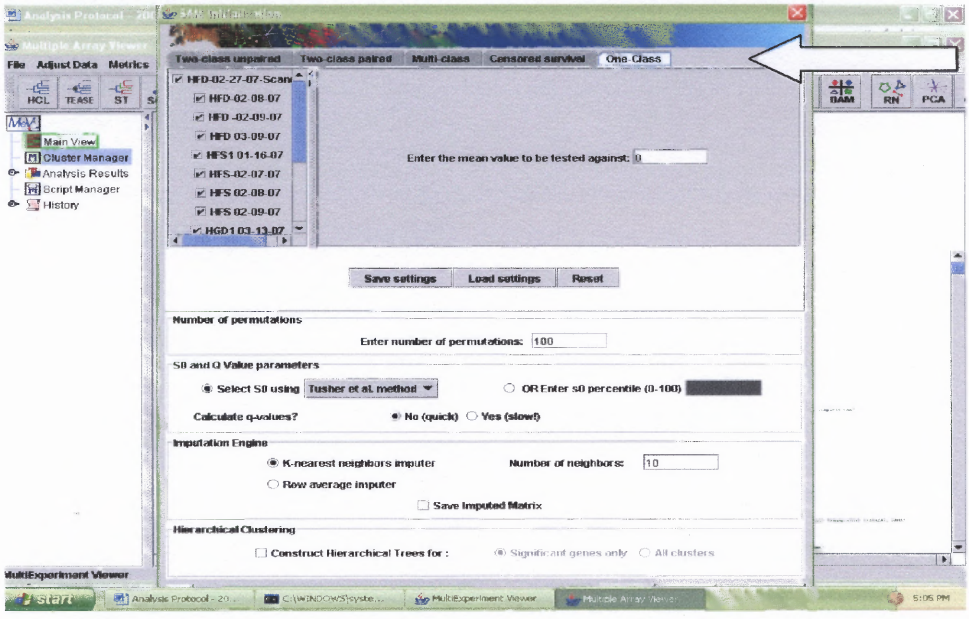


NOTE: There are icons for the SAM test, T-Test, Anova, etc. These are all different types of tests design to give you the (+/-) significant and non-significant changing genes in your data. There is the option of just looking at one treatment or comparing two treatments to see how the genes are behaving.

C.2 Running a Significance Analysis for Microarray (SAM) Test

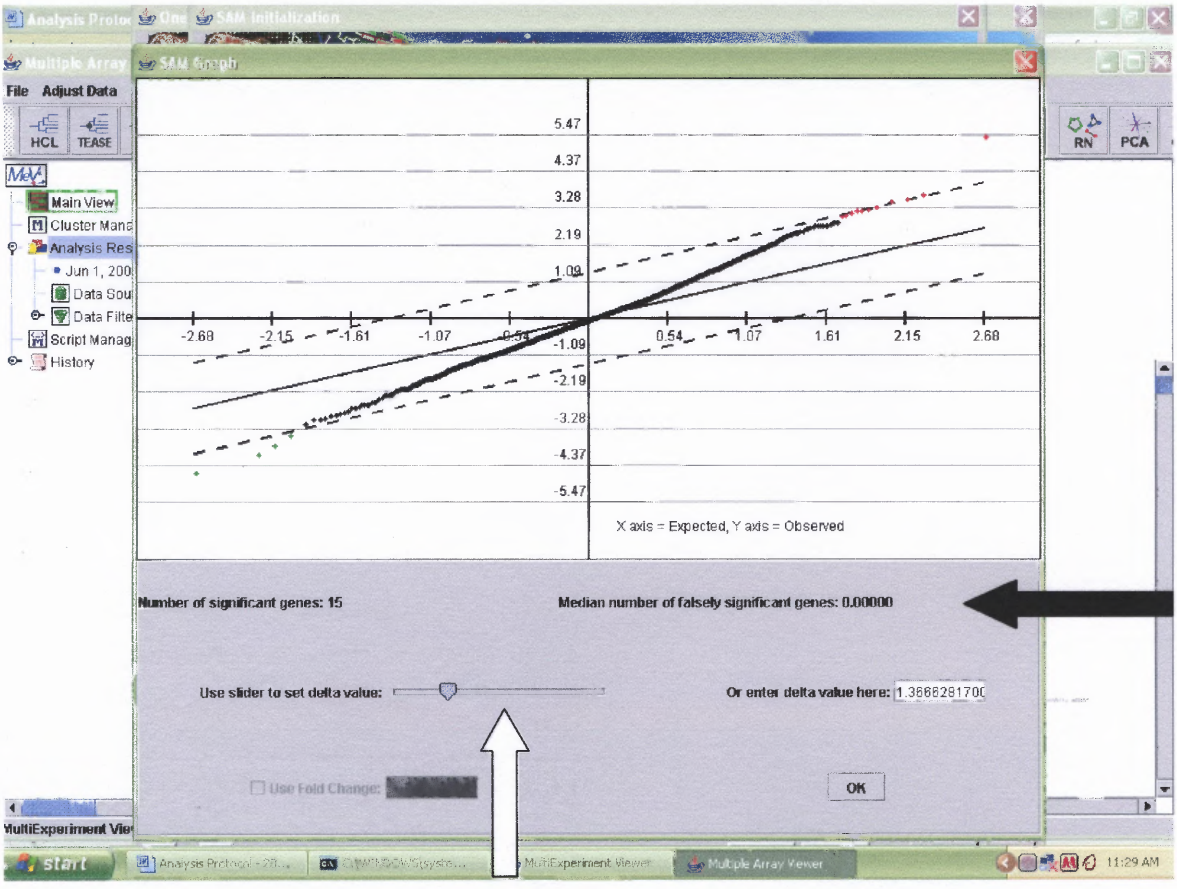
1. Click on the SAM icon from the top menu bar.

2. Select the New analysis option.
3. The next screen, seen below select the one-class tab (as shown below) if you are doing one treatment analysis, or if you are comparing two treatments select the two-class unpaired tab.



C.2.1 One Class Analysis

1. Select the one-class tab. On the left you will notice all of your treatment groups and they are all selected.
2. Keep the treatment that you are doing selected (the menu on the top left corner) and deselect the others that you are not going to analyze.
3. Scroll to the bottom of the page and select ok.
4. It will give you the unique permutations or ask if you should default to 100 permutations. Select "use all of them" and click ok. This will be done with the unique permutations and not the default 100.

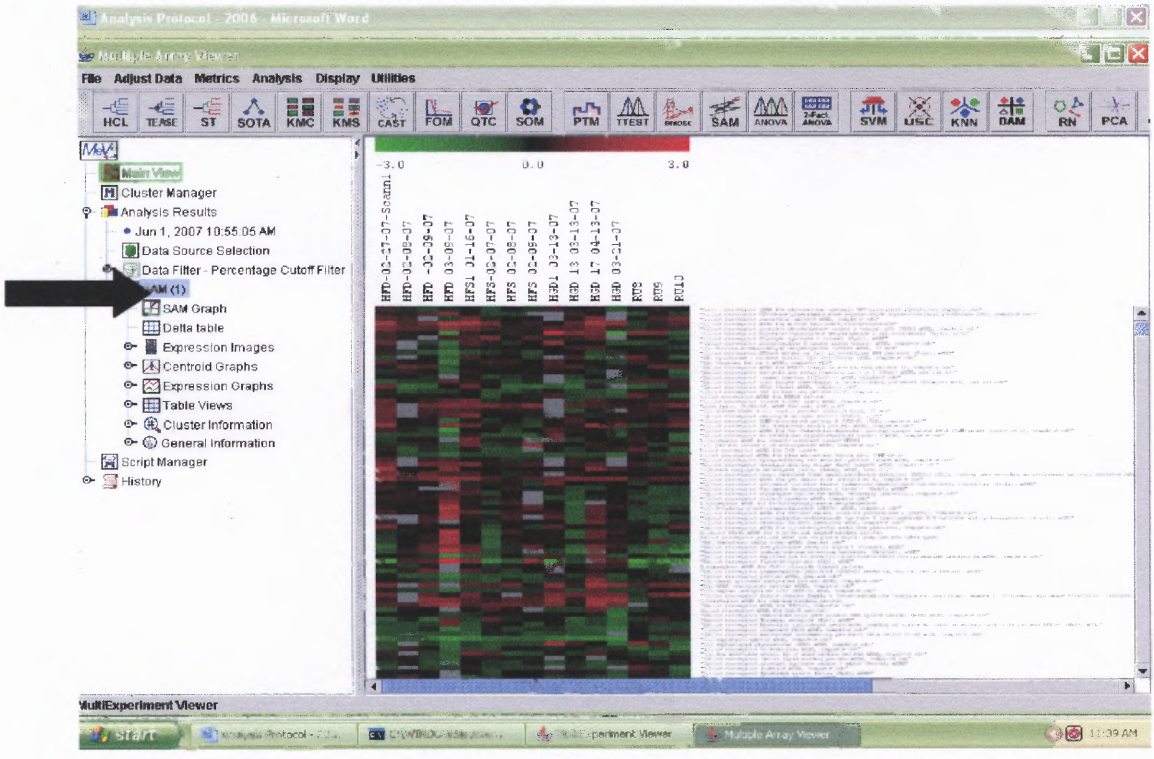


5. The SAM graph will appear in the next screen, as you can see from above.
6. Here you can adjust the percent error of your results. The option for the "Median number of falsely significant genes" can be adjusted with the change of the delta value.

NOTE: In this step by decreasing the delta value you increase the number of significant genes with this test. However, the delta value should be adjusted only to the point that you get 10% false positives. Therefore, the reading for the "Median… falsely significant genes" should be ≤ 0.10. If you want to be conservative and not have any false positives, just select OK and do not adjust the delta value.

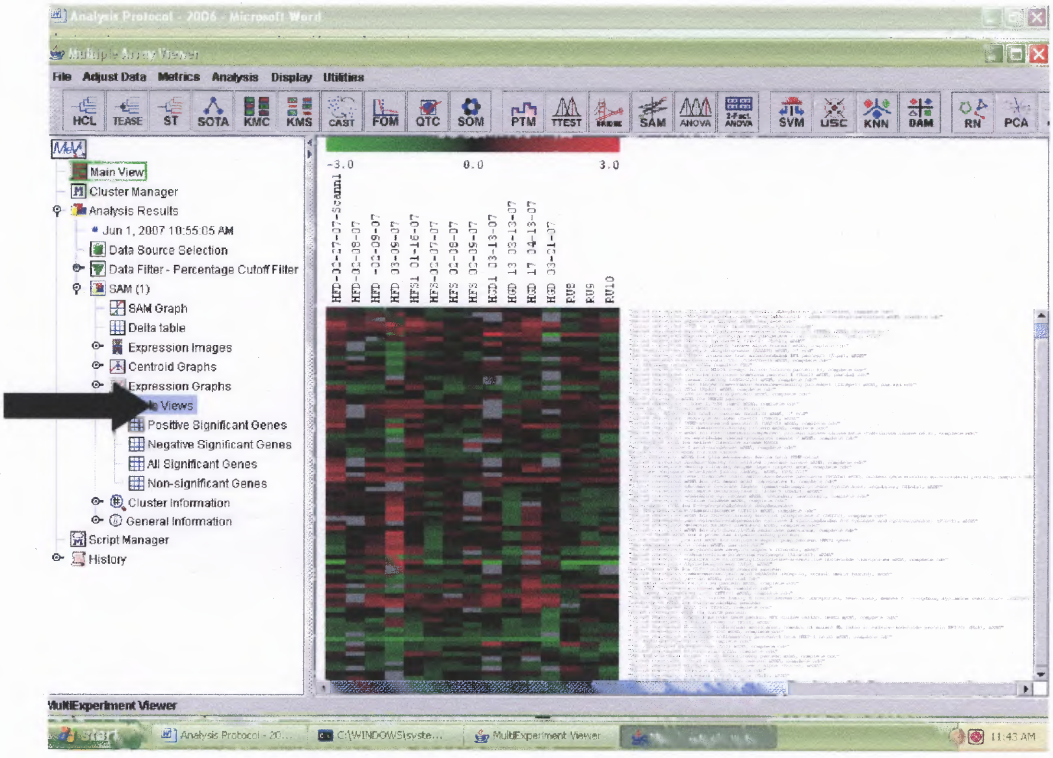
7. Once the delta value is adjusted, select OK.

8. You are now back on the original screen, but on the left hand-side menu there should be a SAM (1) that is highlighted in blue. This is your test and you can left click to open it.

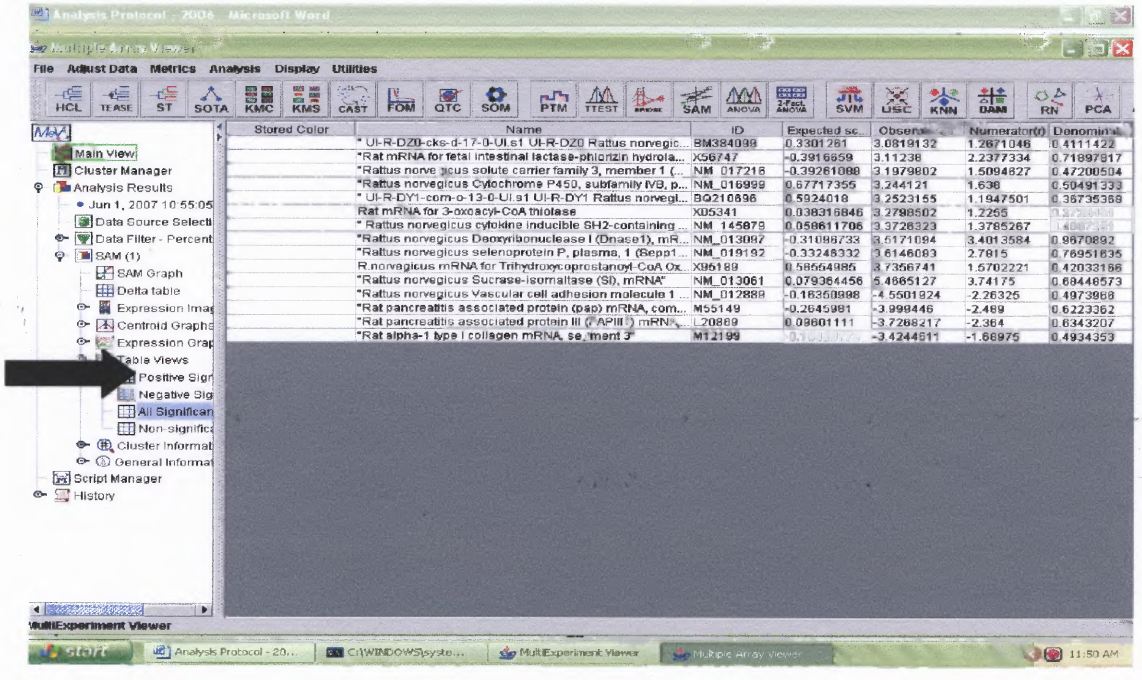


9. You have various options to select from and they all give you different forms of the data.

10. Click on the Table views, where this will give you your significantly changing genes that are up or down-regulated along with the non-significant genes.



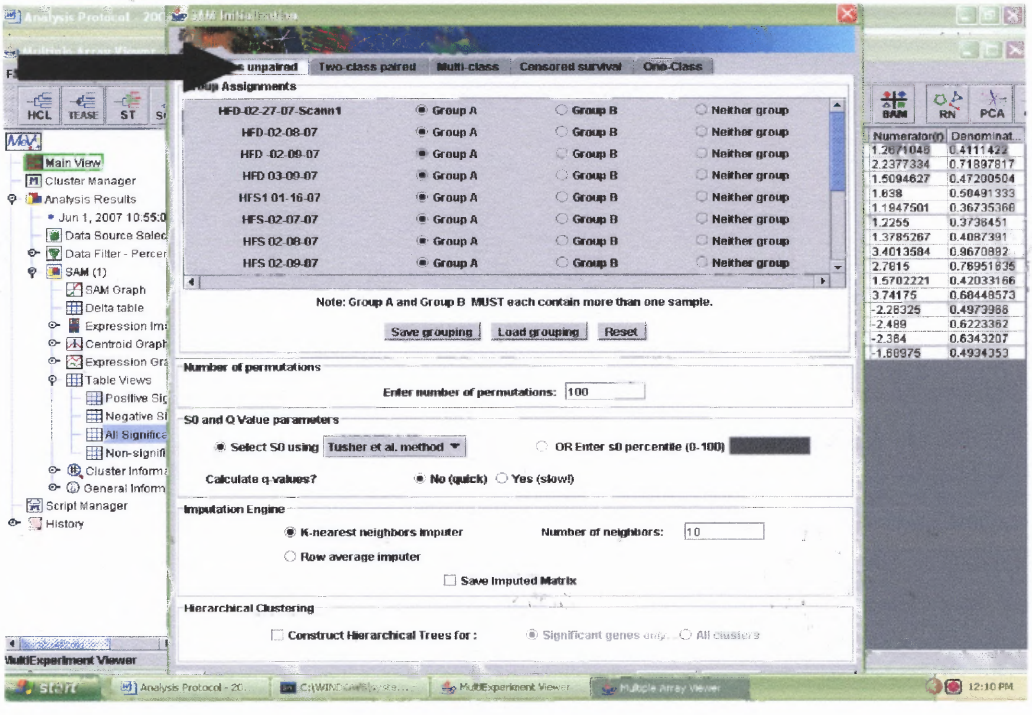
11. The table that you want to open is "All Significant". This will give you a list of the gene name, gene ID and the digital value change.



12. Now you want to save this table. Go to File → Save Analysis As.
13. Name your file appropriately- use the treatment groups and the test that was used; it should be saved as a .txt extension. Note where you are saving the file to on the hard drive. You will need to open it later on with Microsoft Excel.

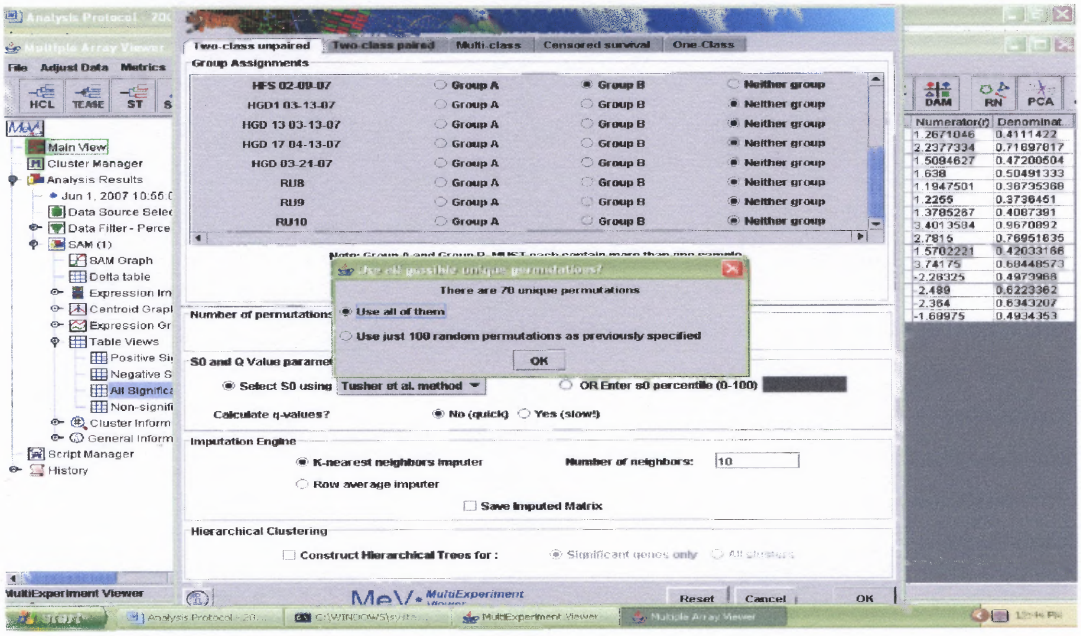
C.3 Two-Class Unpaired Analysis

1. Click on the SAM Icon.
2. Select New Analysis Option, click OK.
3. Select the Two-class Unpaired Tab. You should see a screen similar to the one found below.



4. Group A should be one treatment group and Group B is your second treatment that you are comparing.

5. The other treatment groups in your data, if any, should be selected as neither group.
6. Select Ok at the bottom of the page.
7. The next screen will as you about permutations. Select the "Use all of them" options and select OK.



For the rest of the protocol please follow steps 5-13 from the "One-Class Analysis" section of this protocol.

APPENDIX E

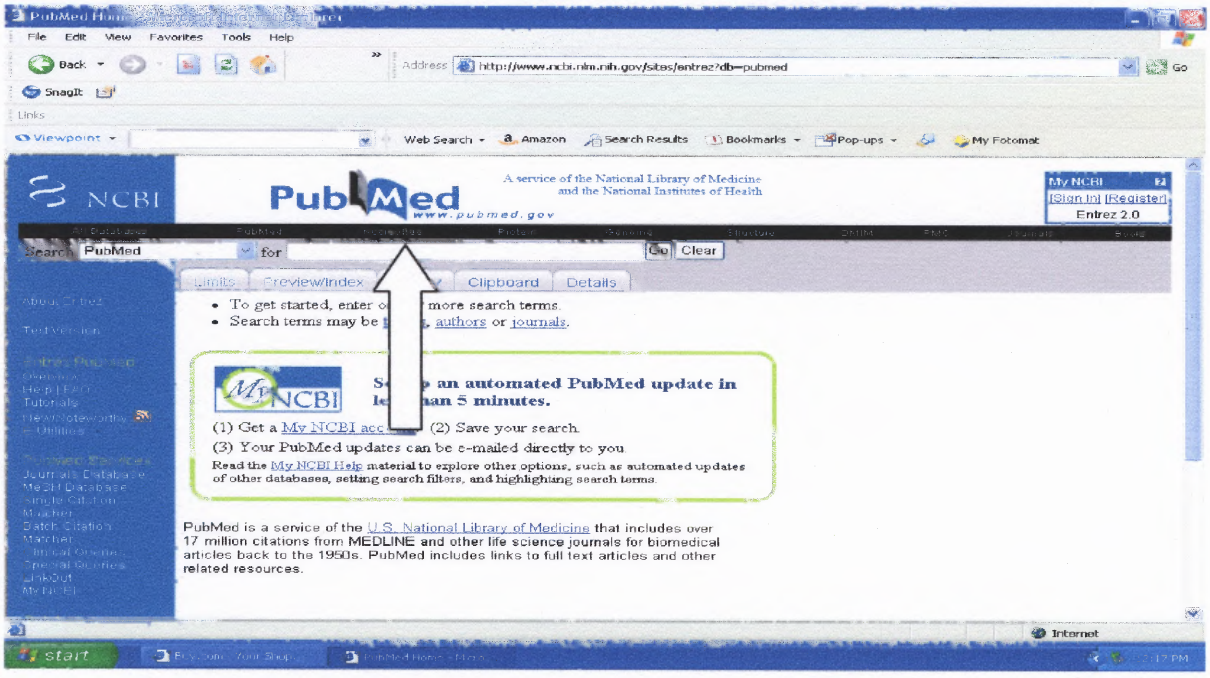
RATTUS NORVEGICUS PRIMER DESIGN PROTOCOL

This appendix contains a step by step procedure on how to design a primer for rat tissue to use on PCR or reverse-transcriptase.

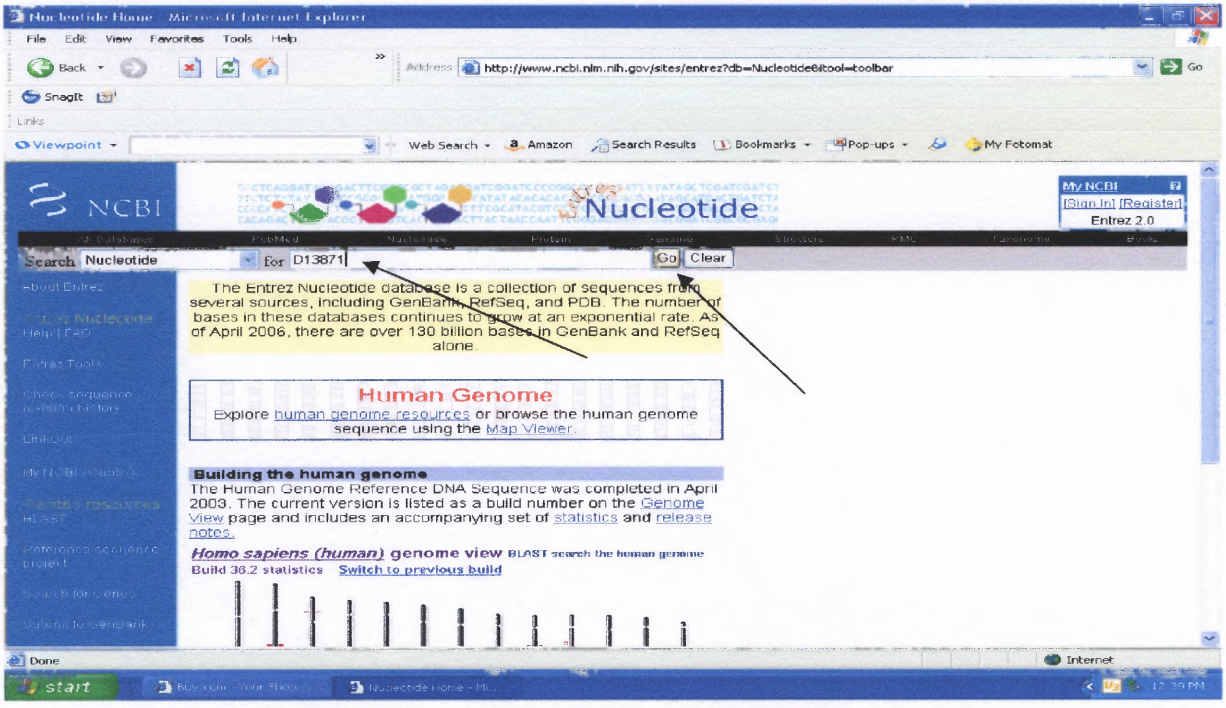
RATTUS NORVEGICUS PRIMER DESIGN PROCEDURE

A. Getting the Gene Sequence

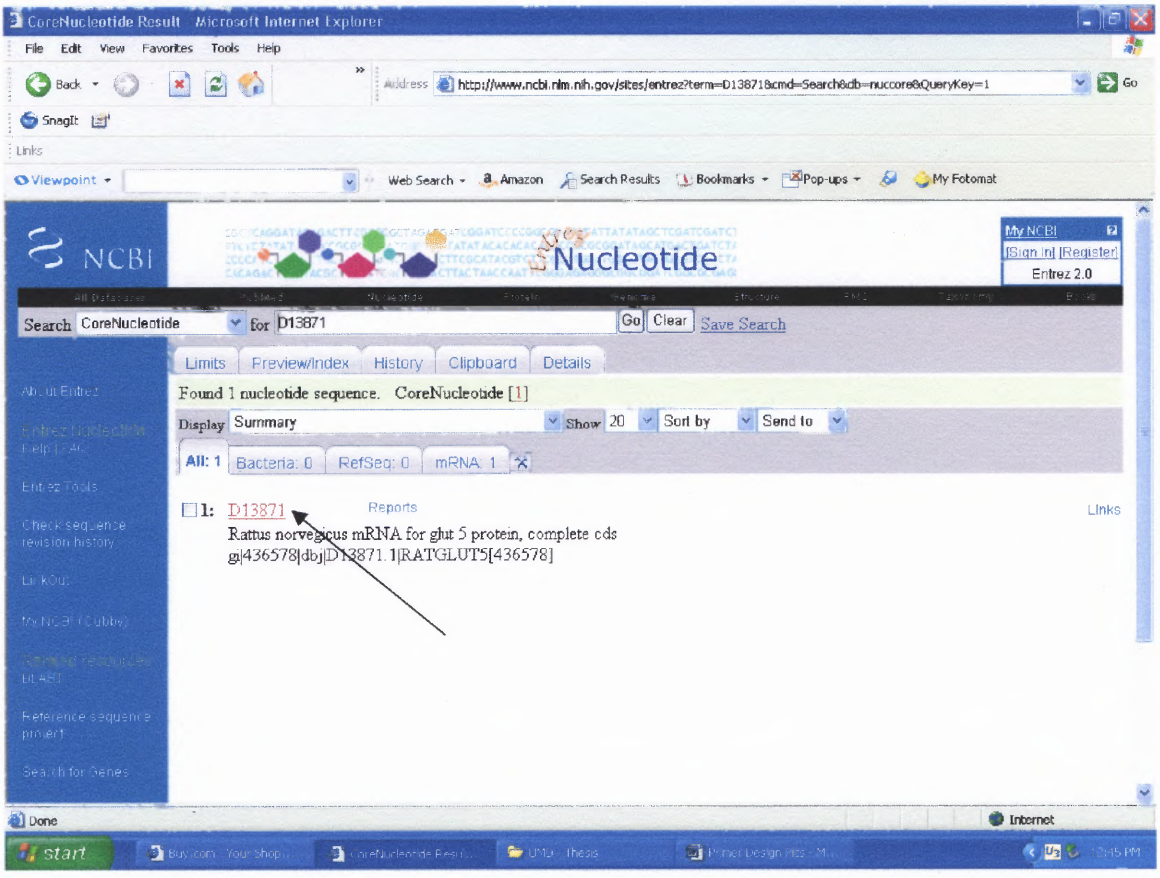
1. Obtain the Gene ID number (copy it by pressing "Ctrl" and "C" simultaneously) ie: D13871.
2. Go to www.pubmed.com
3. Select the Nucleotide tab.



4. Paste the ID number in the appropriate space and select "Go".



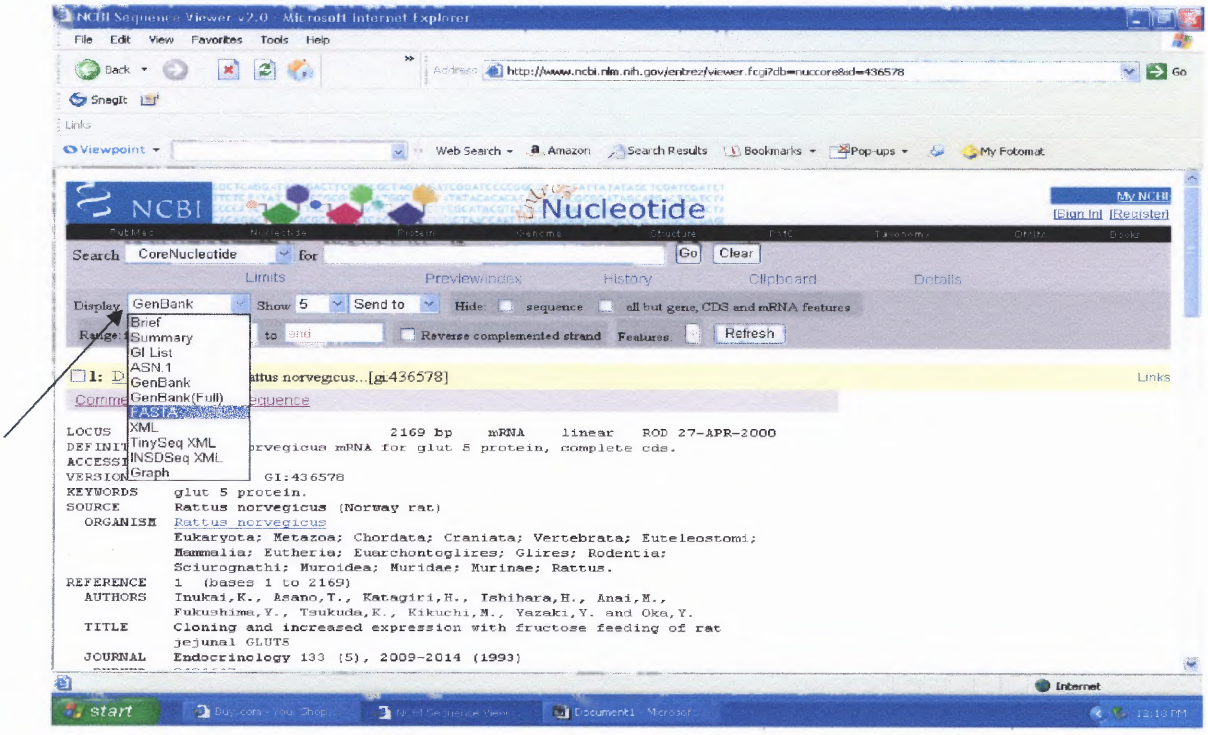
5. You should get a hit for your gene, select the ID Number that is highlighted in blue.



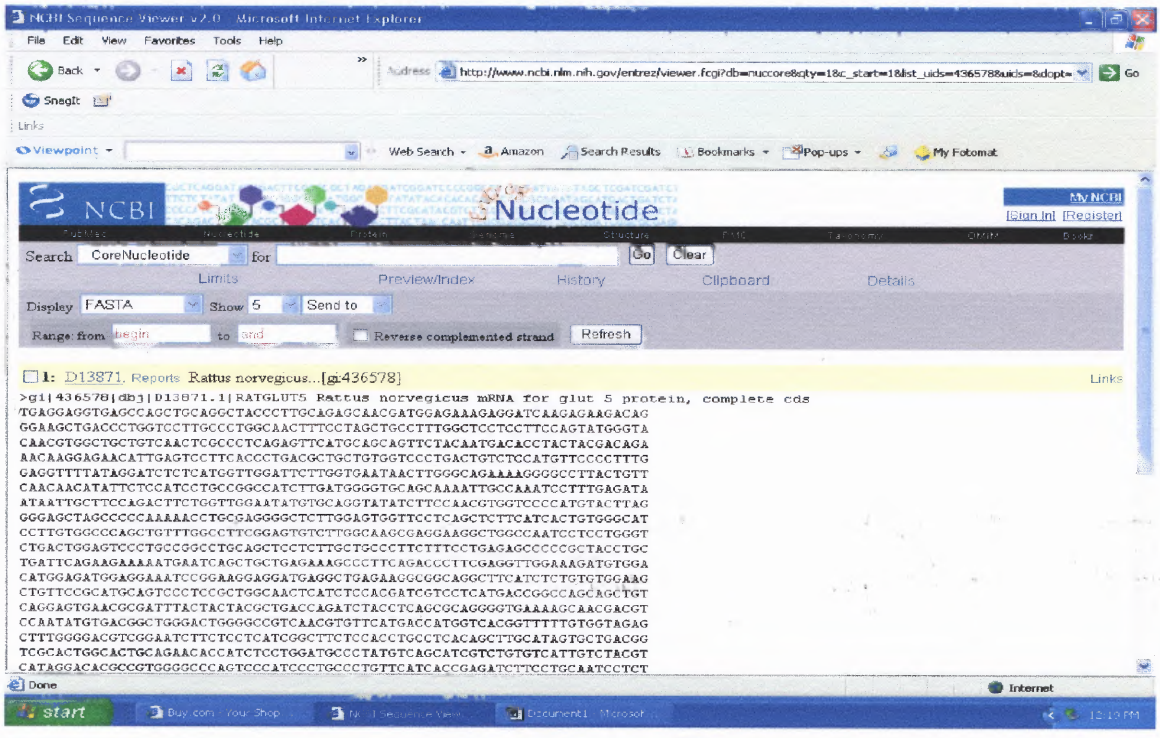
6. The next screen should be a history of the gene, along with some related literature articles. If you scroll to the bottom of the page, there should be a partial or a complete sequence of your gene.

NOTE: If the above statements are not true, your gene may go by another ID number and you would need to do a search for the alternative ID number. Reading the related articles may help you getting started on your search.

7. Scroll to the top of the page and on the menu bar where it reads “Genbank” click on it and select “FASTA” option.



8. This should give you the gene sequence.



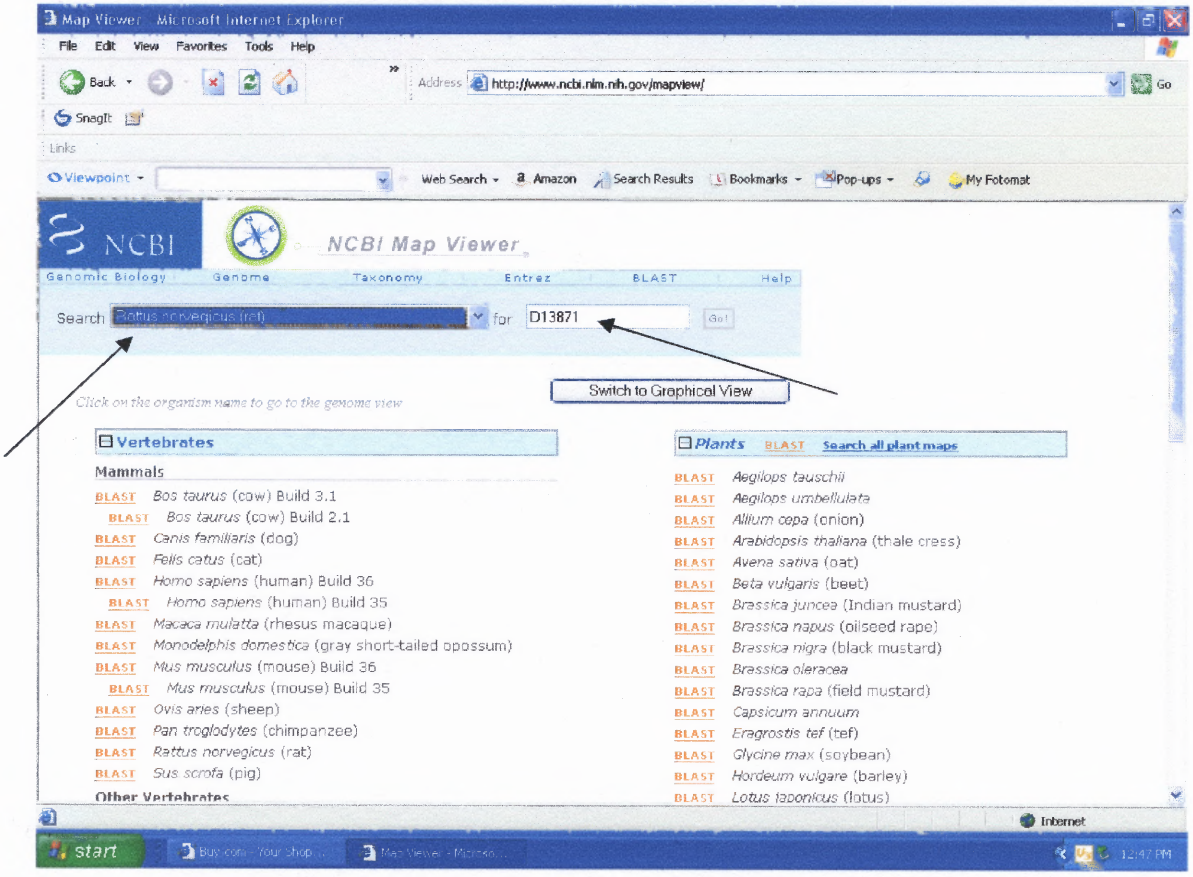
9. Copy and paste this sequence in a word document.

10. Save the word document with the gene name.

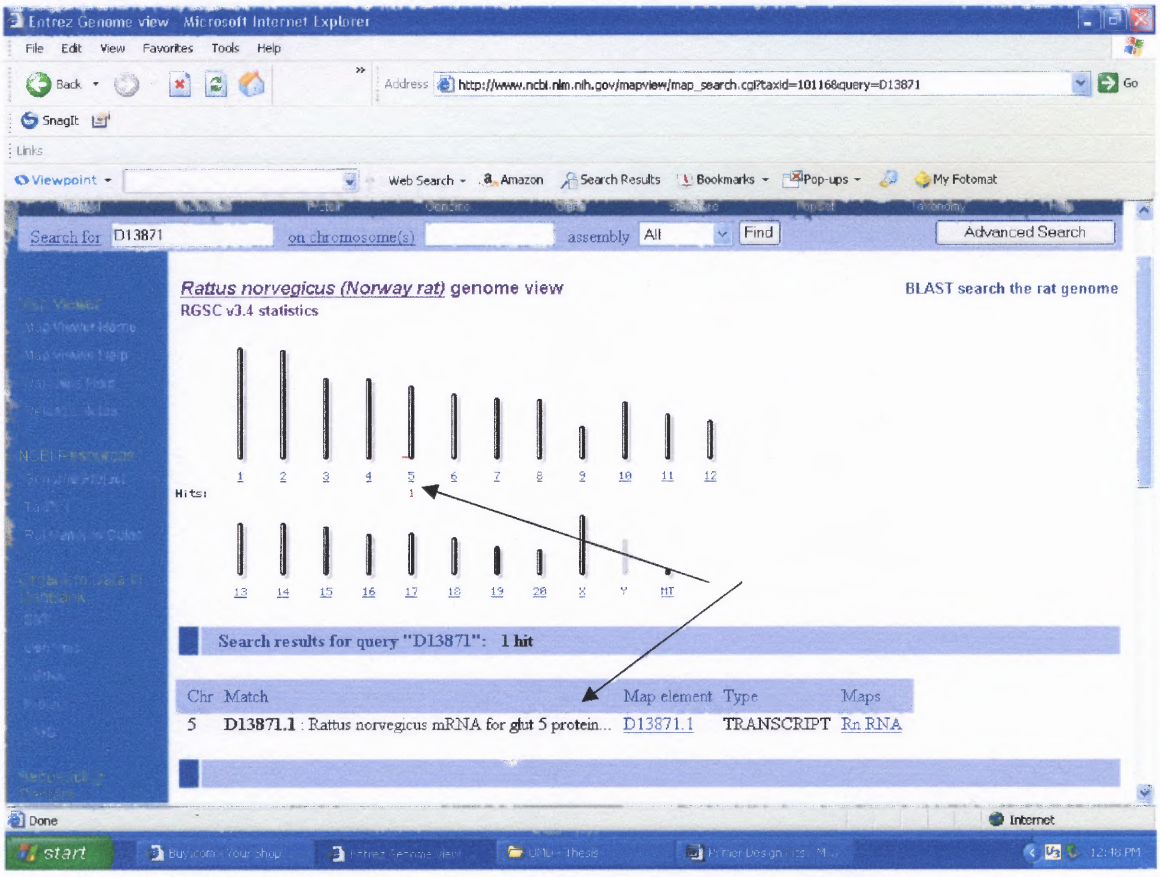
B. Identifying Your Exons

1. Go to **http://www.ncbi.nlm.nih.gov/mapview/.**

2. At the left hand side, where it asks for "Search", scroll down the menu to select "Rattus Norvegicus". In the next window type in the Gene ID # and select "Go"

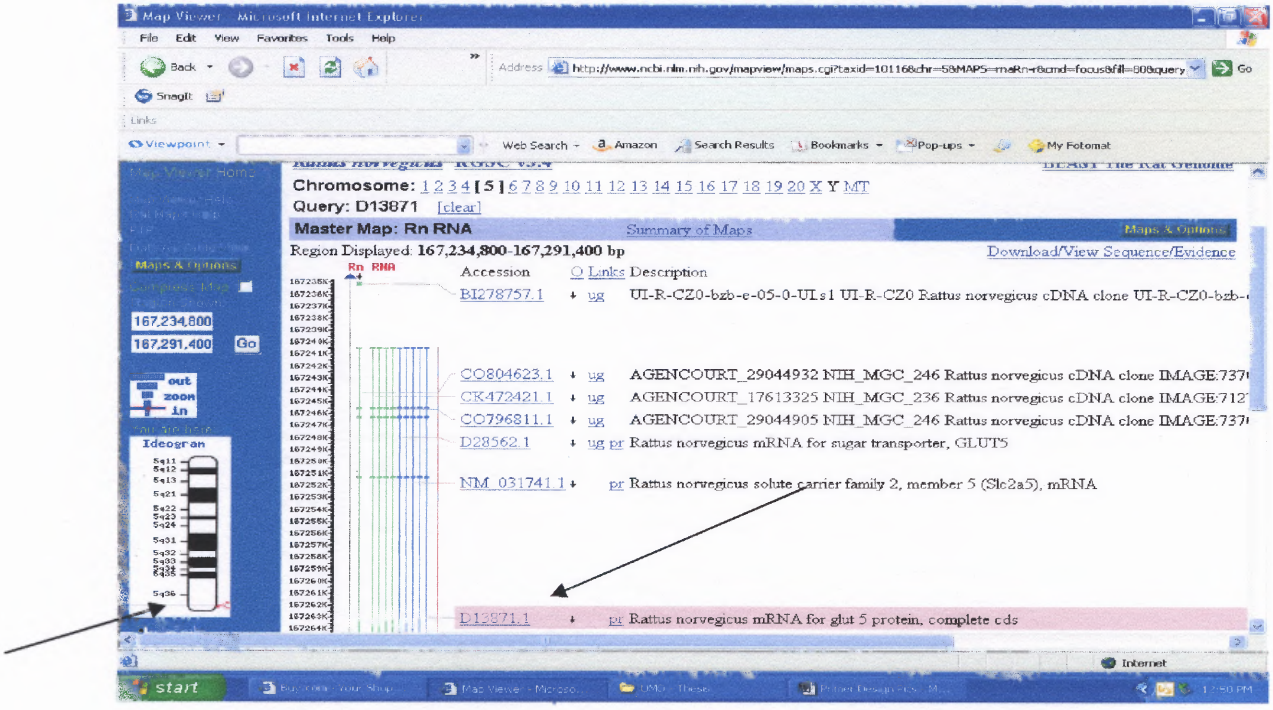


3. The next screen should show you the chromosomes where your gene is located. If you look at the screen below you see that our gene, D13871, is located on Chromosone 5.

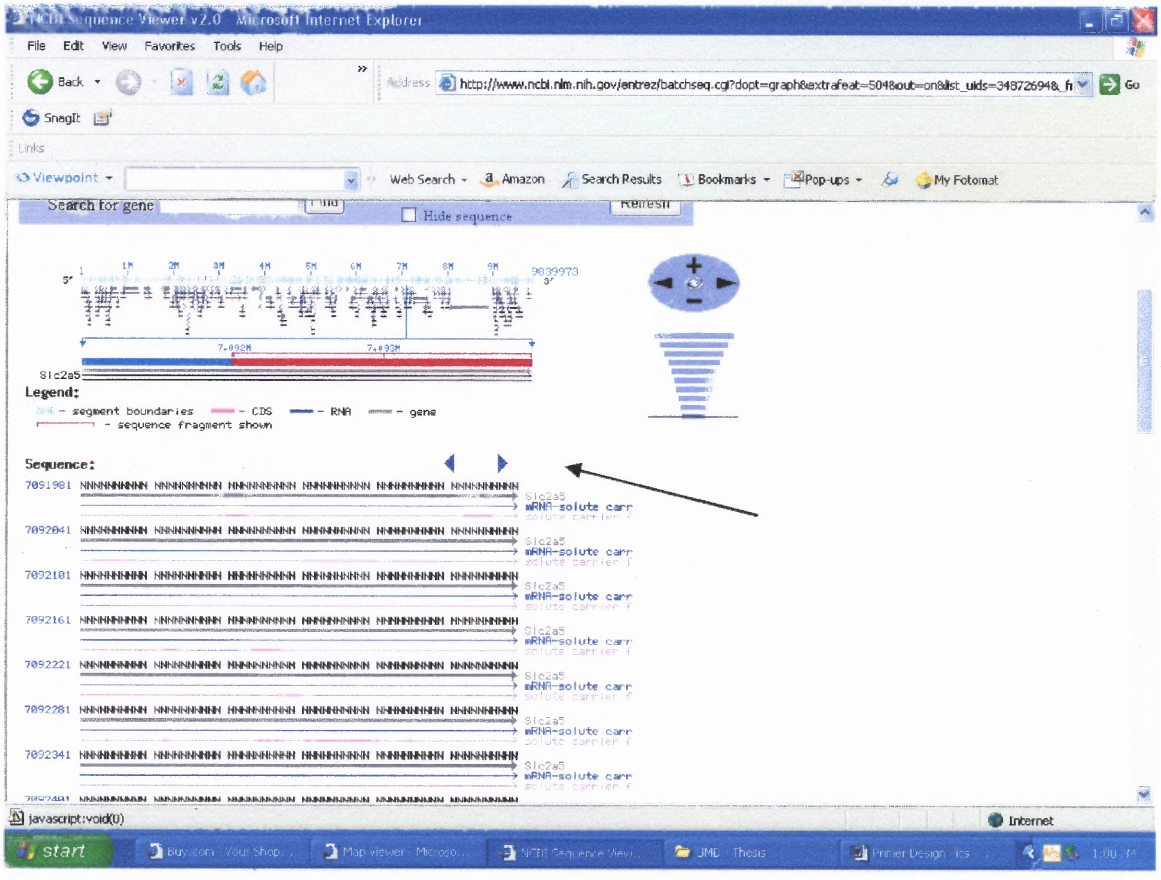


NOTE: Sometimes you will get more than one hit in the same and/or different chromosomes, depends on the gene family. At this point look at all the locations and try and decipher which one location you are interested in. This may require further research to determine the right location.

4. Click on the chromosome that your gene is located.



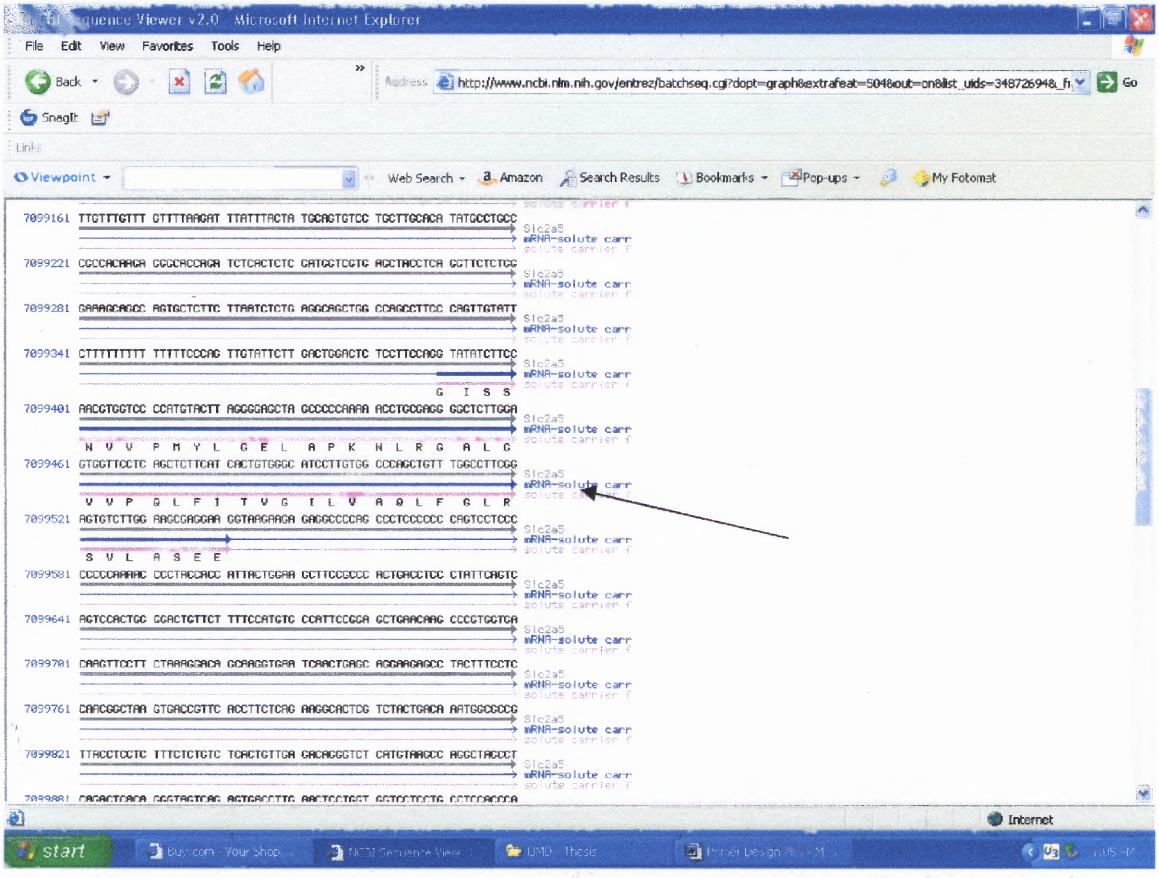
5. The next screen will have your gene highlighted in pink and it will point the exact location of the gene on the chromosome (left handside picture in above diagram).
6. Click on the blue letters that read "sv" right to the gene name. If the letters are not there, as is the case above, left click to the right of the gene ID and there will be a menu. On the menu select the option "Show Sequence".
7. At this point you are given the whole sequence of the gene, the exons and the introns.



Comment: The purpose of looking at the introns and the exons is that when building a primer you want the primer to bind across two different exons. This is helpful for if there is any genomic contamination it will be amplified between the two exons, on the intron area. The reason why we also have to look at the whole sequence of the gene is that we need to identify in our word file where each exon begins and ends so we may know where to design our primer. The FASTA sequence that we saved earlier is only of the genes exons, but we need to identify where each one is located and this is the reason we need to use Mapviewer and screen the whole sequence.

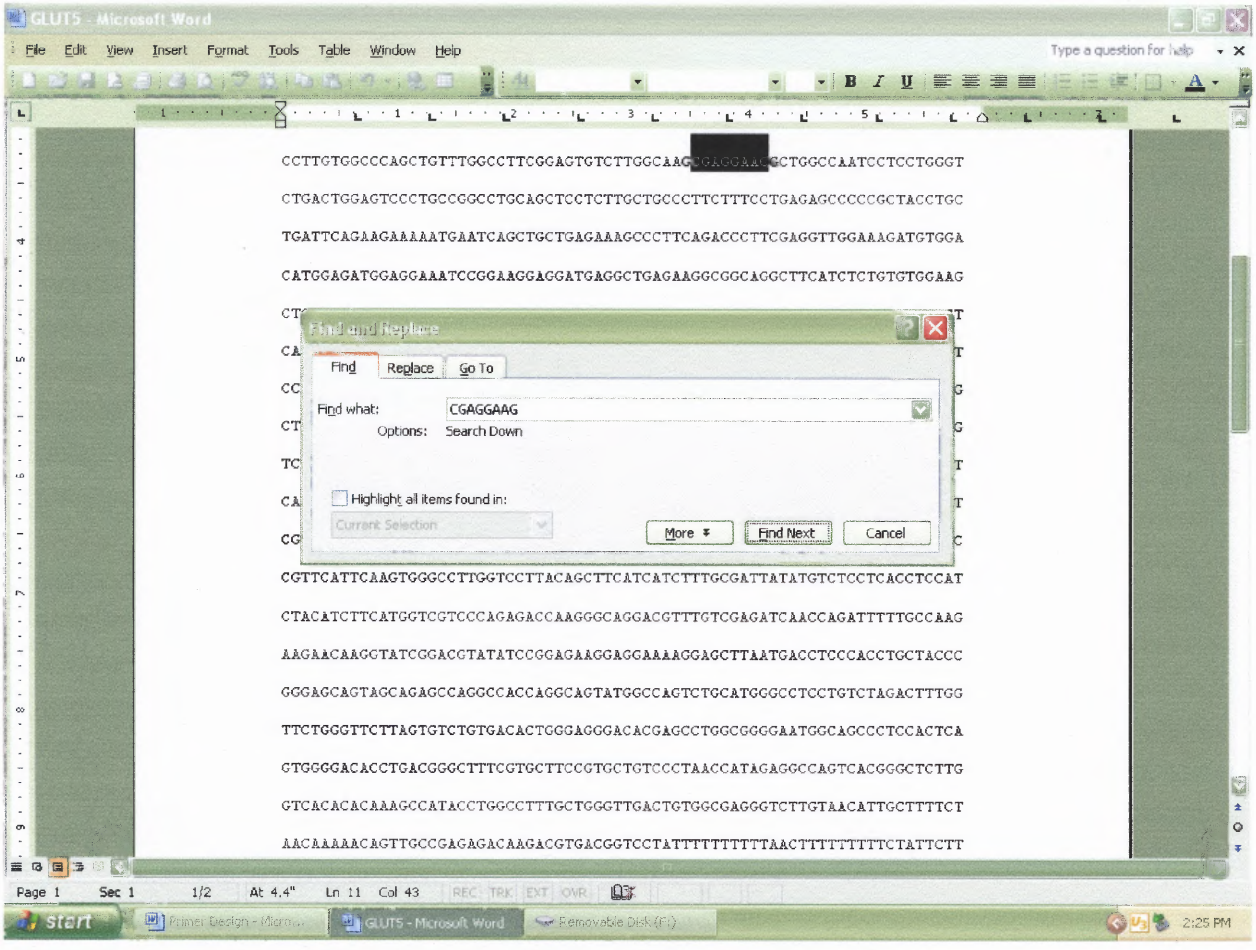
8. The screen above shows you the whole sequence, as seen above the "NNNNNNNNNNN" means that it is unknown. To scroll from one page to the next click on the blue arrows at the top or bottom of the screen.

9. The blue, pink and gray lines underneath the sequence tell you what direction you are going in and also tell you what kind of sequence you are looking at. The blue is the mRNA sequence, the Pink is the Protein sequence and the grey is used to show you were the gene begins and where it will end (it usually has a square end/beginning).
10. You are only interested in the blue line. The Exons are located when that line turns into a Bold Blue Line (Please see below).



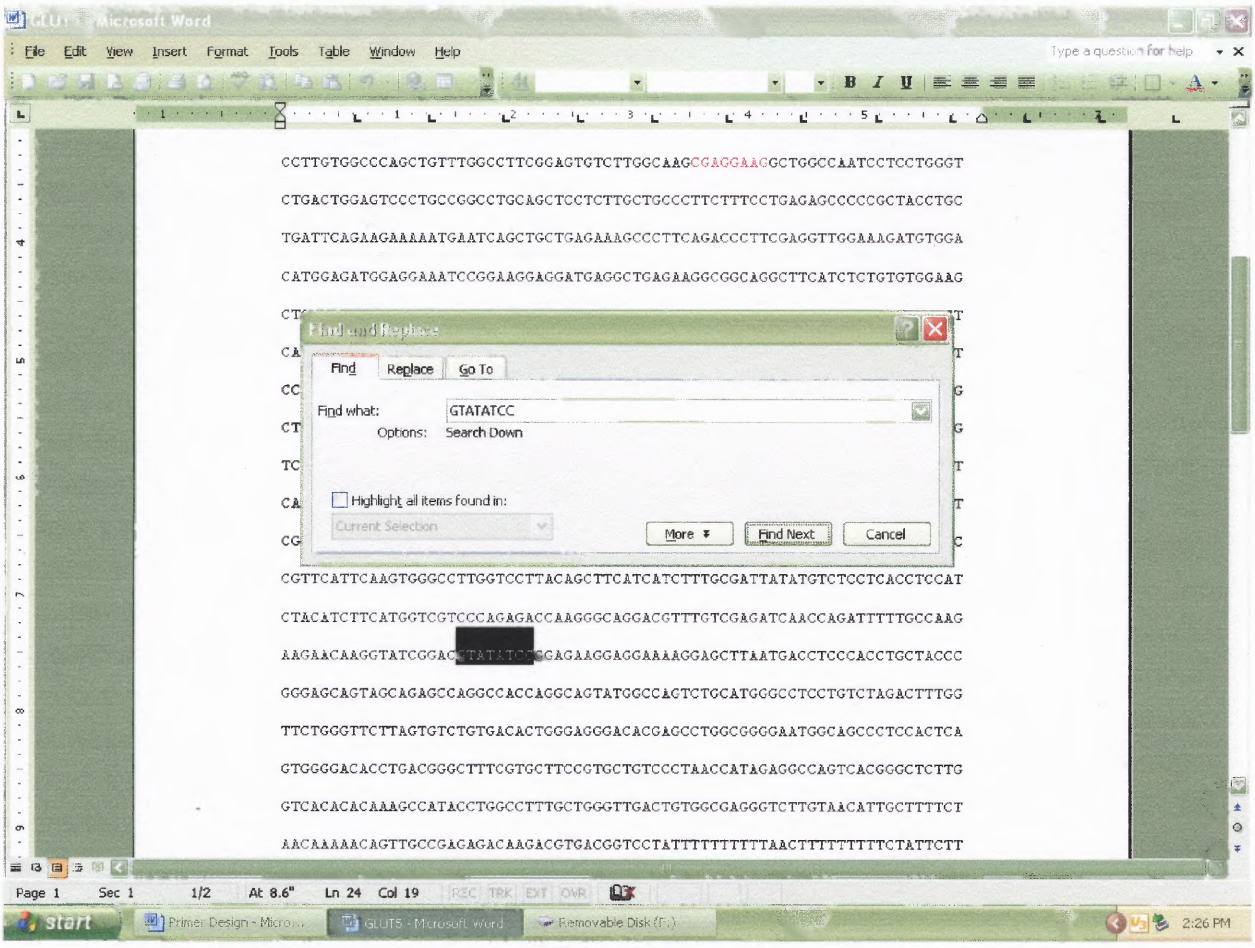
11. When you have located the exon sequence, note the first few (8) letters of the sequence and the 8 letters of the end sequence.
12. Open up your word file, with your FASTA sequence saved.

13. Do a word find for the beginning of the exon sequence (be careful for the sequence may be cut in half between two lines of you word file so you may need to cut your 8 letter sequence to 4 or 5 if word Find cannot locate it).



14. Once you find the beginning of the sequence, change the font color.

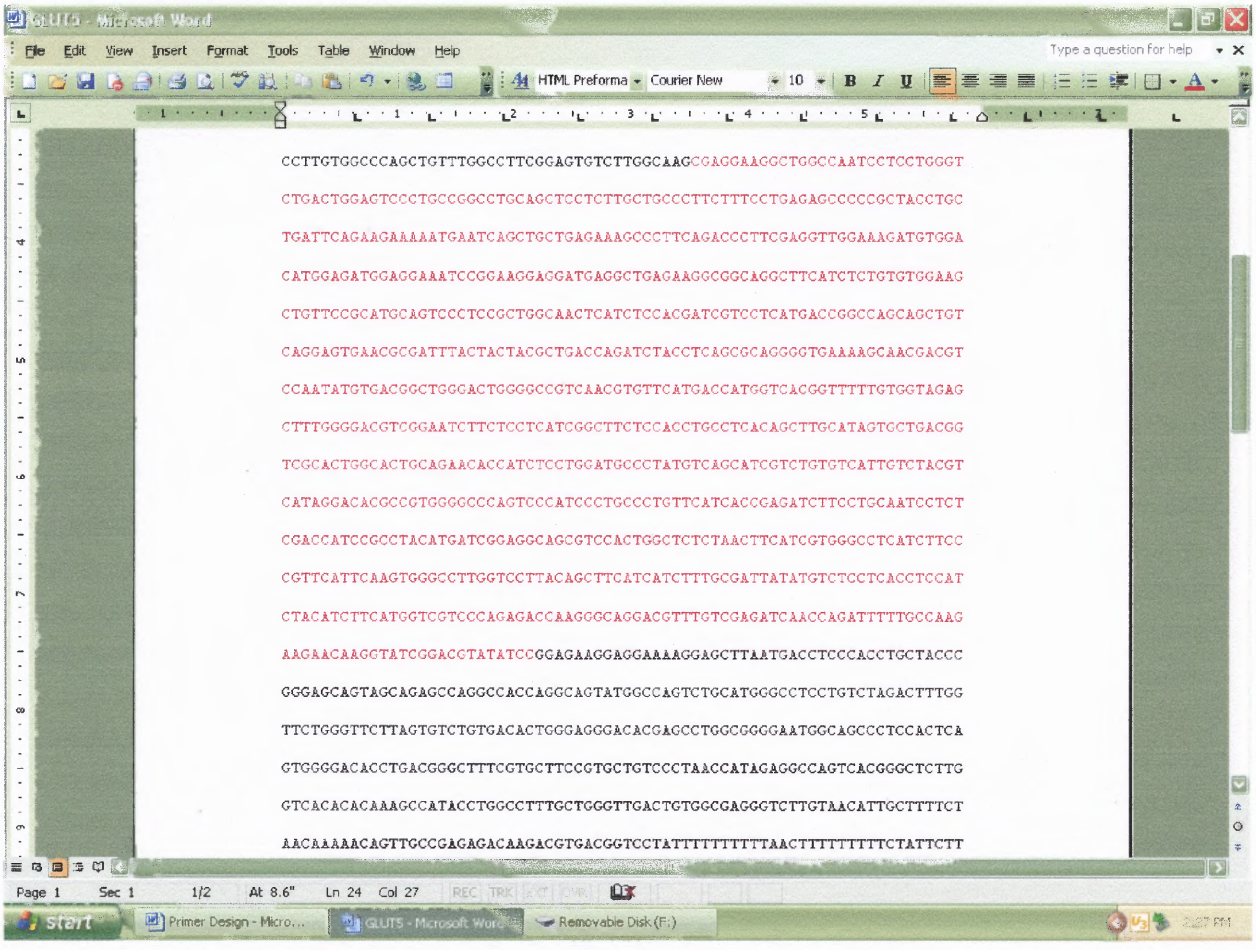
15. Use word find again and find the end of your exon (again be careful that your sequence is NOT cut between two line).



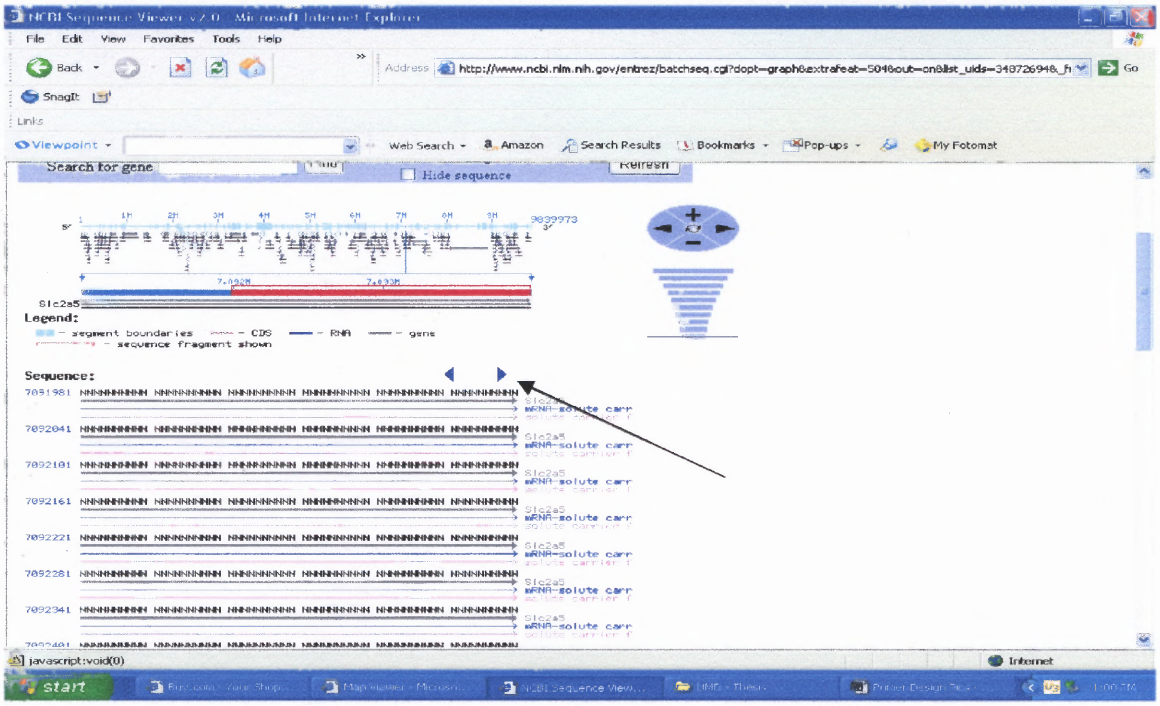
16. Change the end sequence to the same font color as the beginning of the exon.

17. Do a last minute check with mapviewer that you have the right sequence.

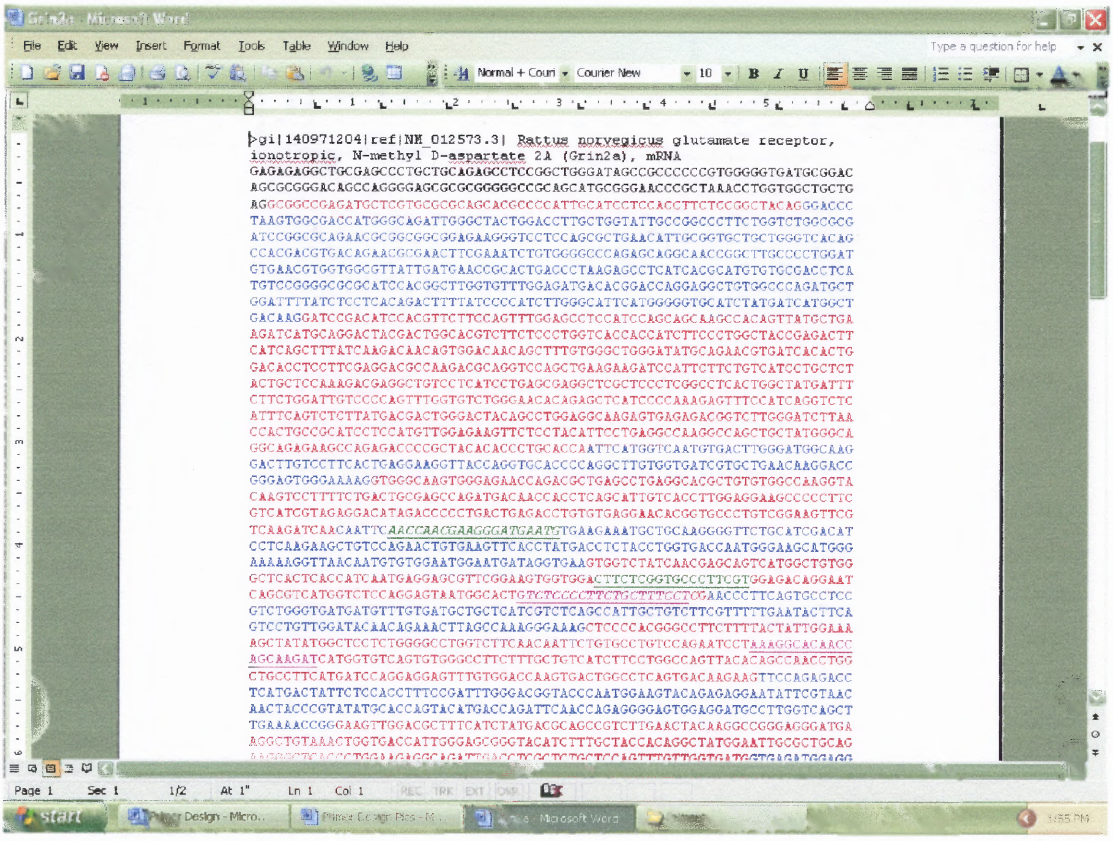
18. Once confirmed change all of your exon to the same color font.



19. You have identified one exon. You must go back to mapviewer and keep pressing the right blue arrow to scroll through the rest of the sequence to identify the rest of the exons.



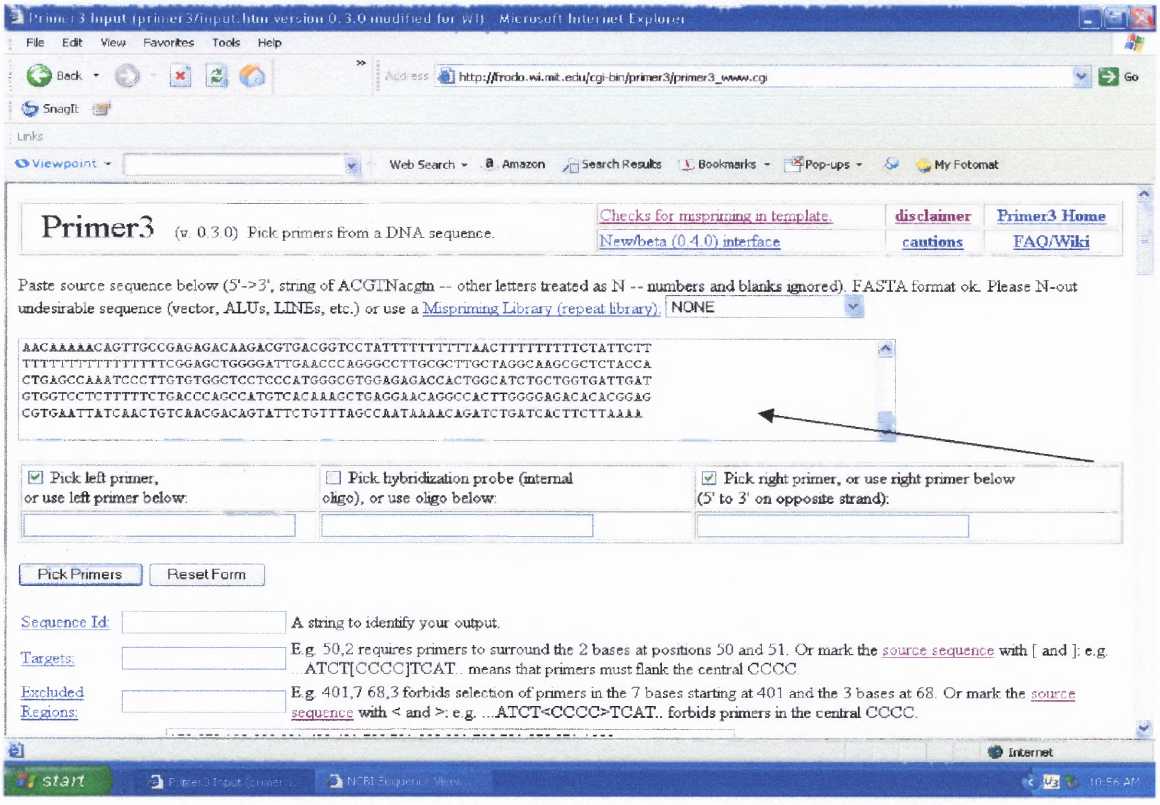
20. When you reach the end of the sequence, your grey line will end with a square and your word file will have all the sequence in different colors corresponding to the exons that were identified. See example below.



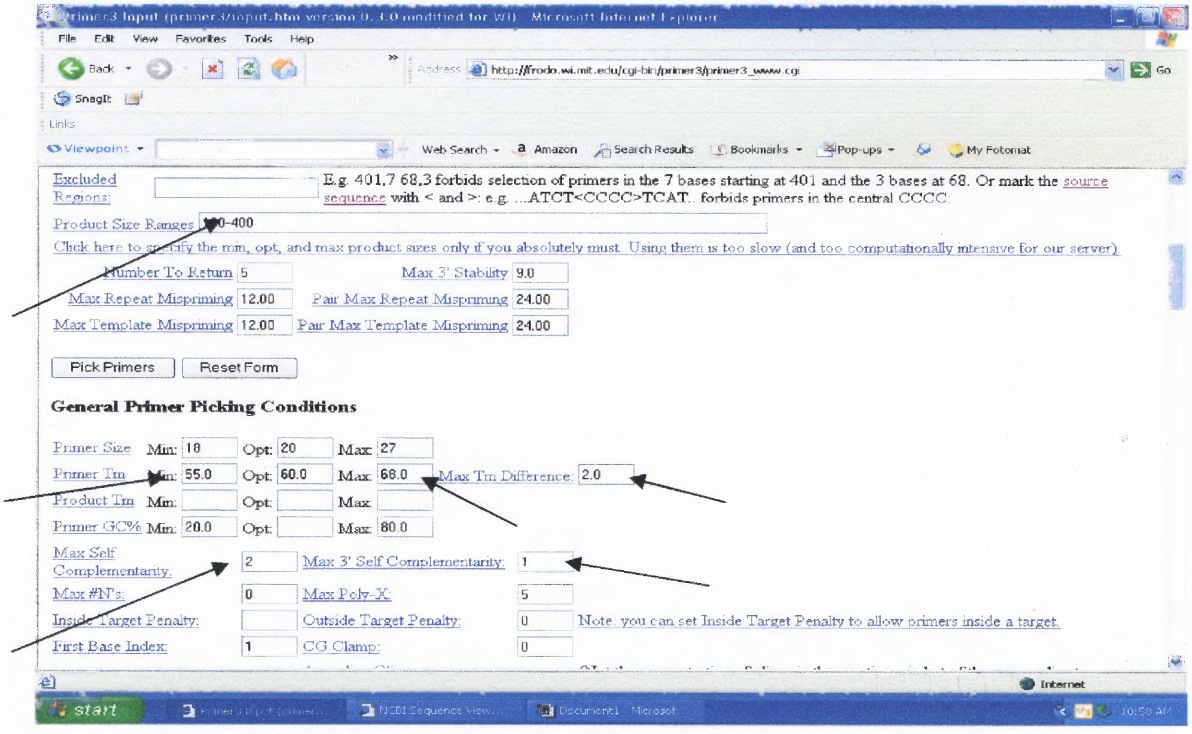
21. Save your word file with all the changes.

C. Primer3 – Primer Design

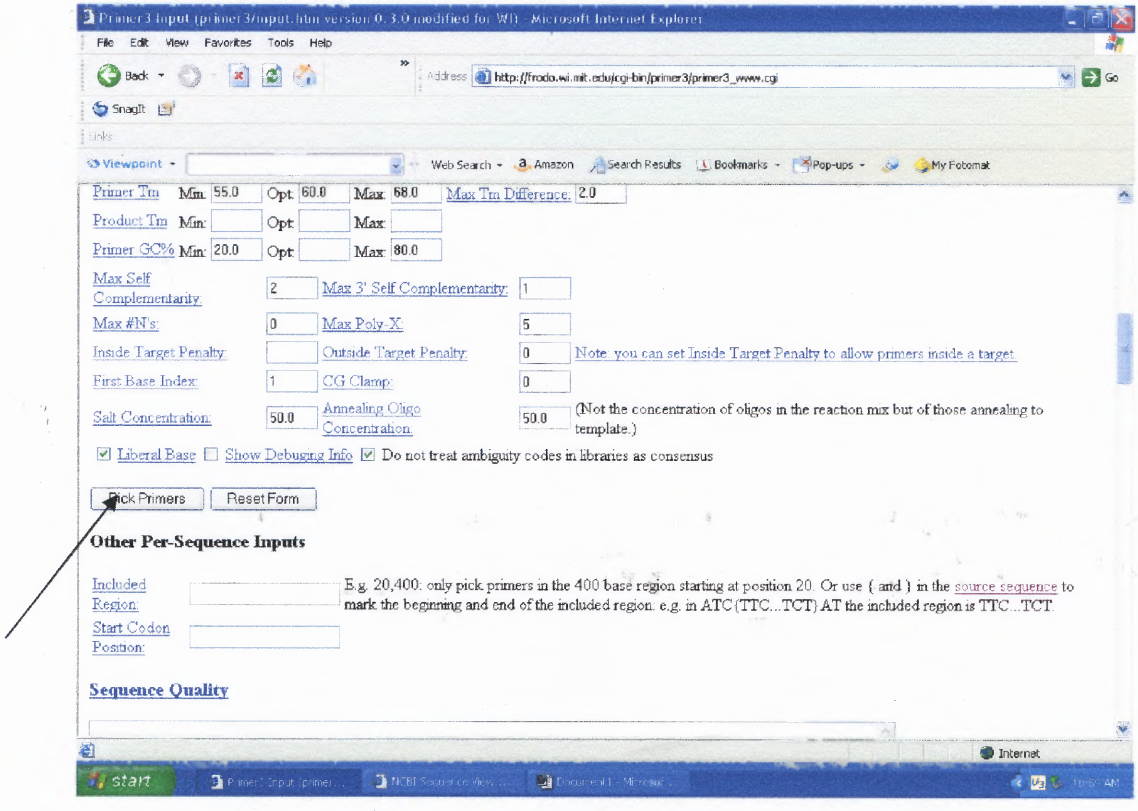
1. Go to http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
2. Open your word file with the gene sequence, and copy the whole sequence ("Ctrl + C).
3. Paste it in the Primer3 website in the allocated space.



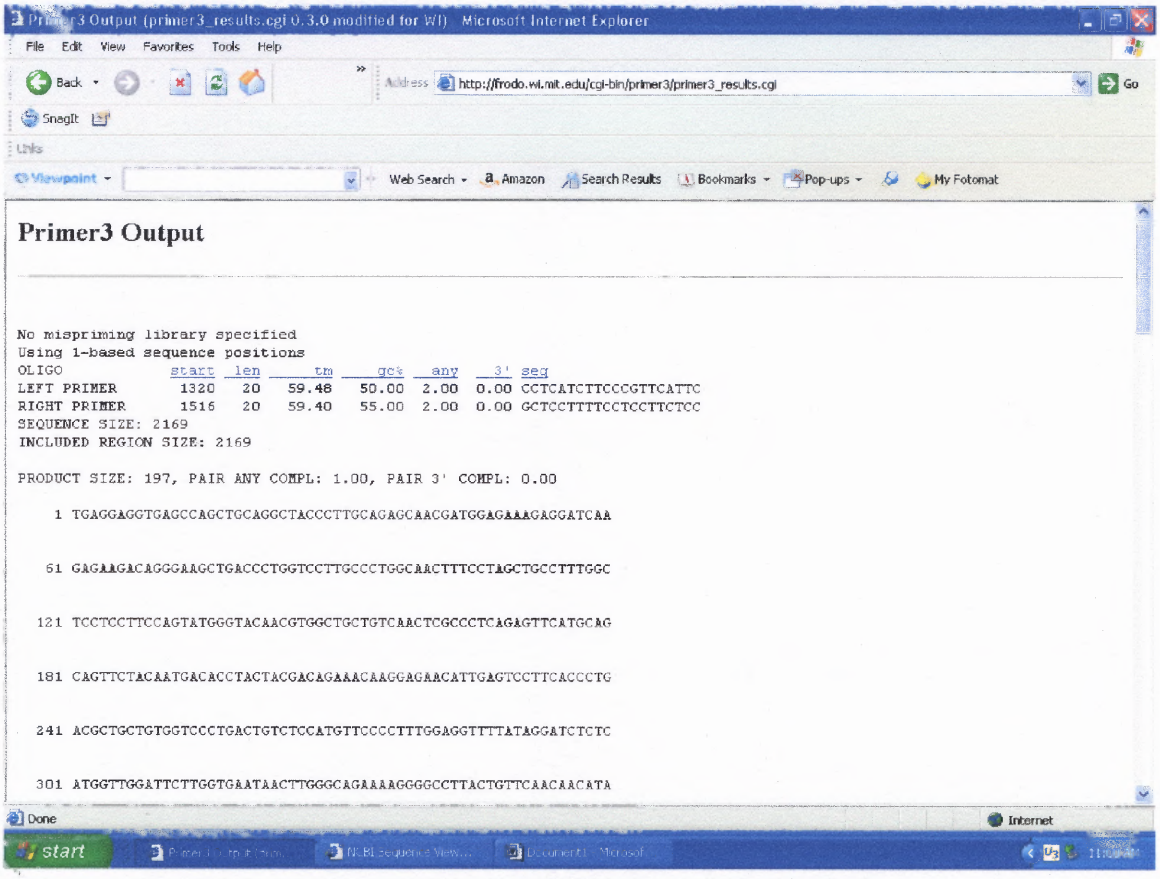
4. Select the options for the left primer and the right primer (see diagram below).
5. Under the "product size" select from 100-400 bases long.
6. For the "Primer Tm" put from 55°C - 68°C. For the "Max Tm Difference" put in 2.0.
7. For the "Max Self Complimentary" use 2.0.
8. For "Max 3' Complimentary" use 1.0 .



9. Select "Pick Primers".



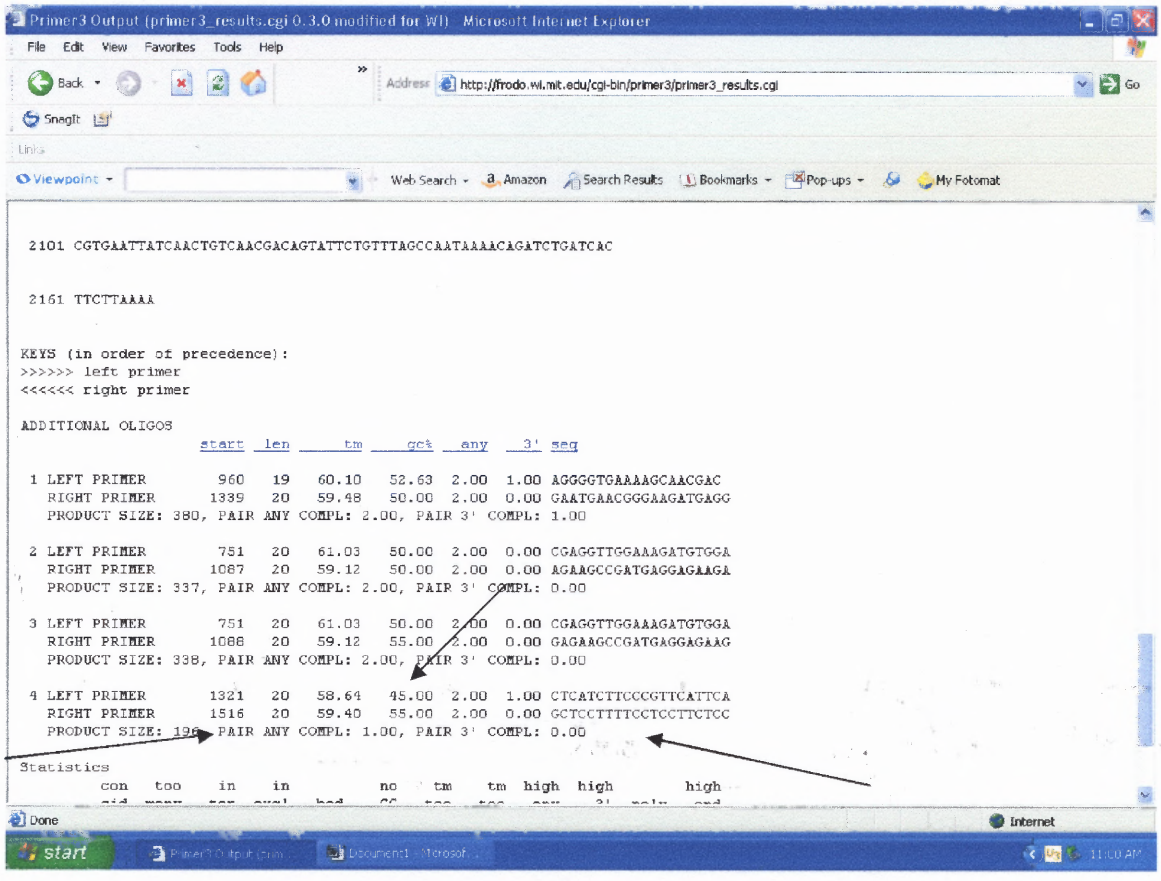
10. The software will give you a couple of options for the primers. One at the top of the page and a few at the bottom of the page. The primer is usually 20 bases long and it will give you two sequences both are 3' → 5'. The second sequence you need to find the reverse compliment to give you a 3' → 5', for the sequence given is for 5'→3' (this step will be done with Bioedit software).



NOTE: You must select at least two primers that can be used for your gene. You want two because when you do a final check to see that your primer is specific to your gene, you want to have a backup in case that your first primer is not specific enough. Sometimes the primers will pick up on a family of genes, so you must look for other primers because you want your gene to be tagged and not its whole family.

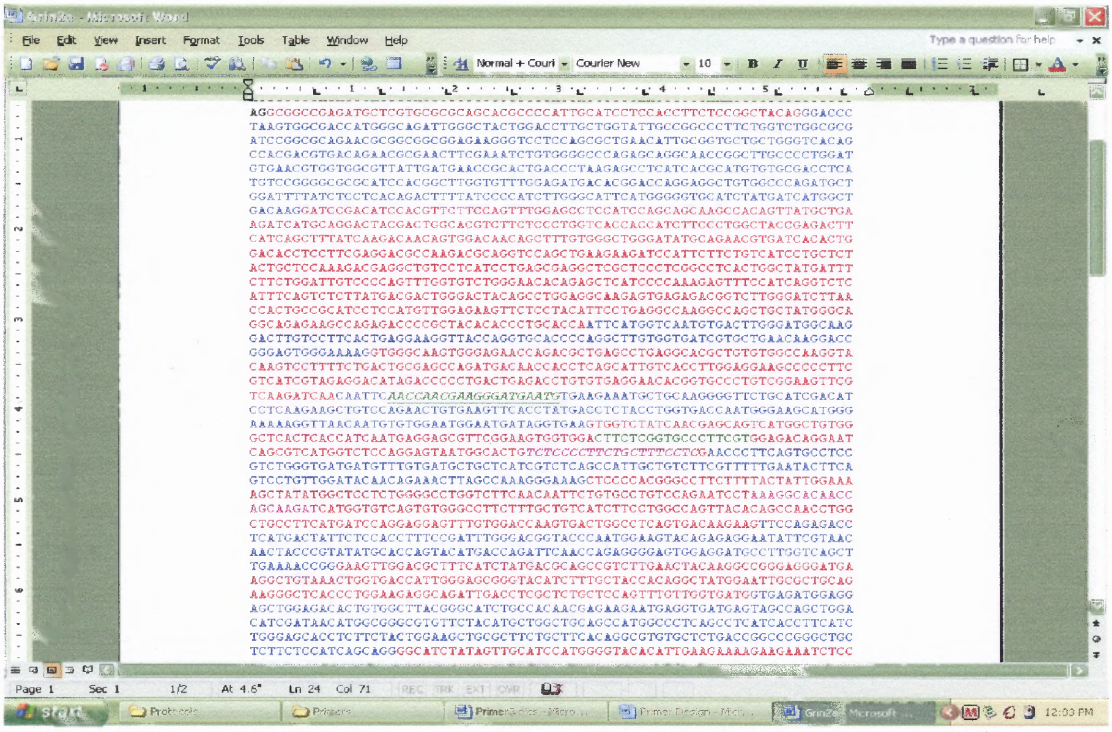
D. Using BioEdit, Locating Primer on Sequence

1. Open up your word file with the labeled exons.
2. To pick the right primers look at the primers given by the Primer3 software. You want to pick two primers that are:
    1. The shortest base length.
    2. Have the closest Tm to each other and around 60°C.
    3. Have the least amount of complements (want them to be 0.0 if possible). You do not want to have a complement because the primer may bind to itself and not your gene.

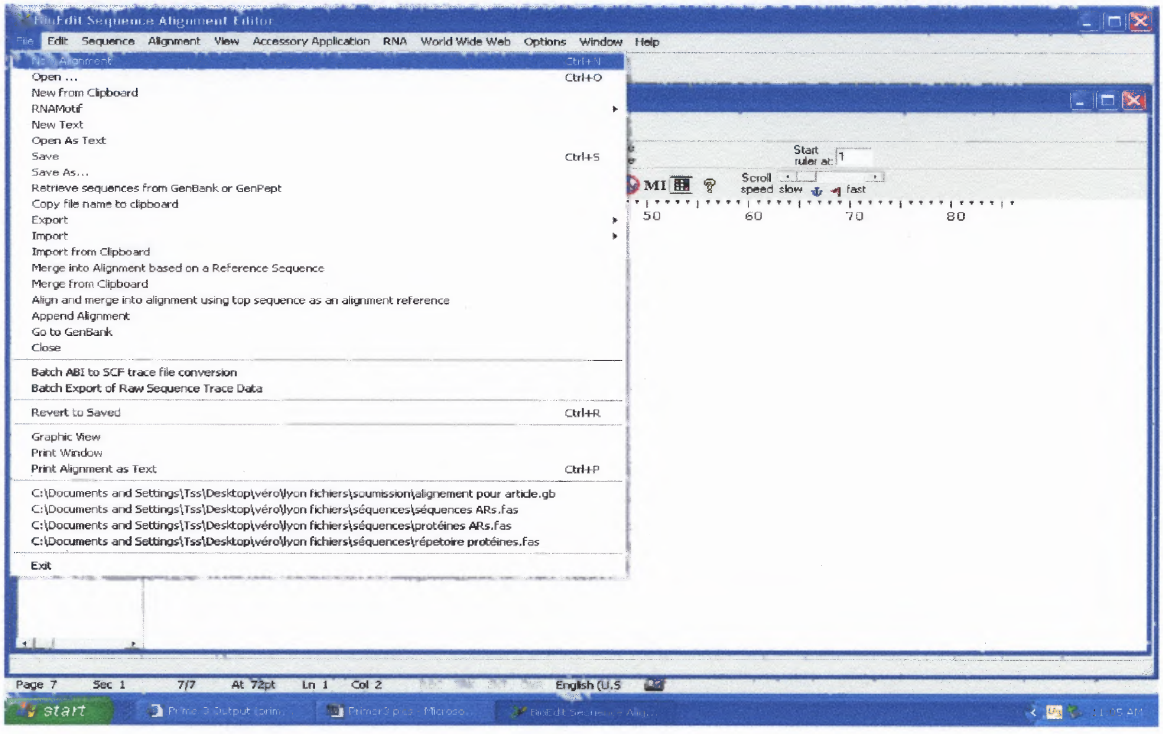


3. There are two sequences to each primer as can be seen from above. Copy the first sequence of the selected primer.

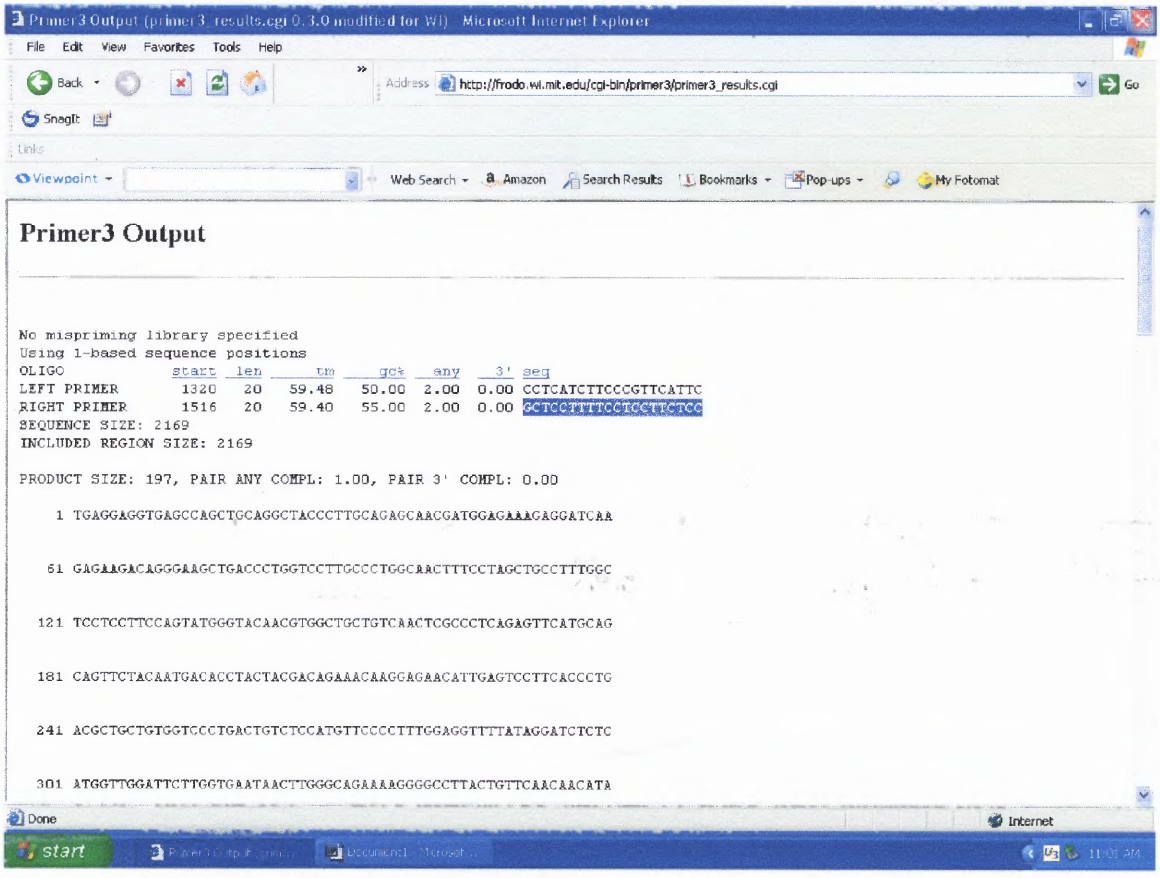
4. Open the word file with gene sequence, where the exons have been deciphered.
5. Open "word find" and paste the primer sequence. If this sequence falls between an exon-exon junction it is not usable. If it falls in the middle of an exon, it is usable.
6. If the first primer is ok, underline it on your gene sequence in the word file. See picture below.



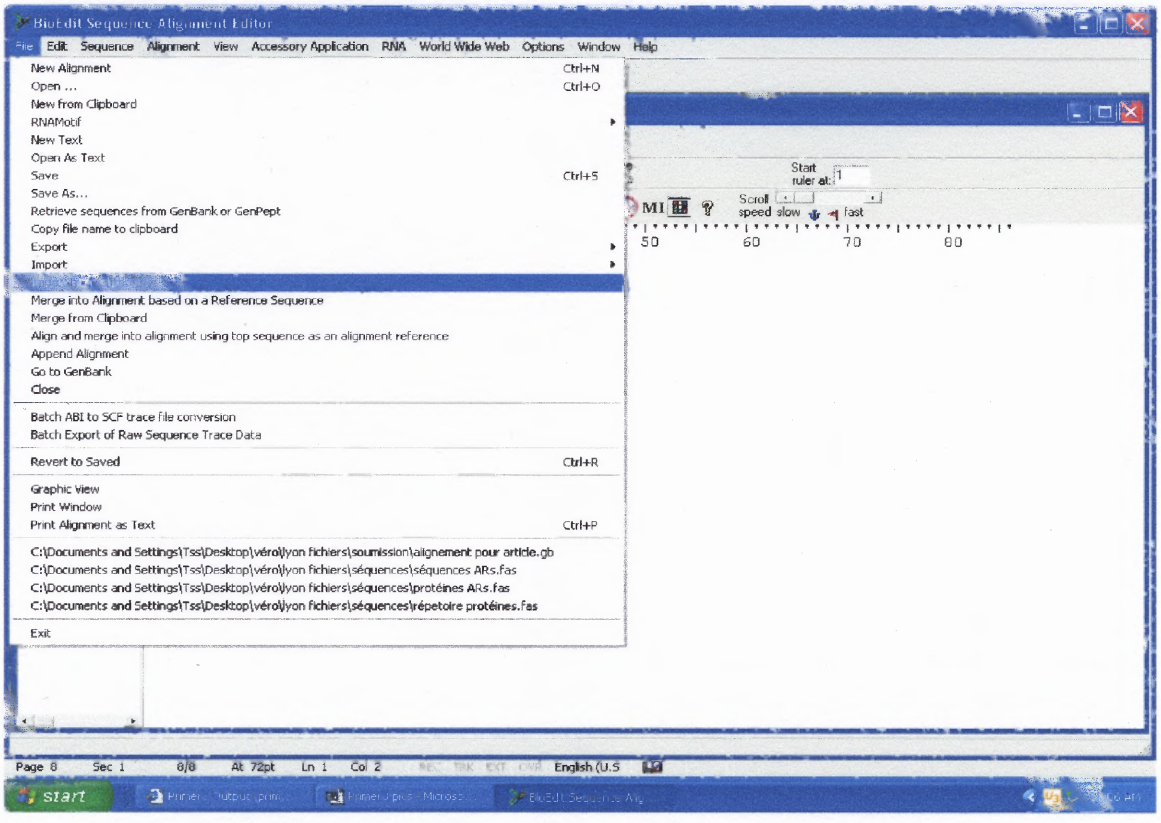
7. To check the second sequence of the primer, you need to find the reverse complementary of that sequence, for in your (word file) gene sequence you are dealing with mRNA and that is 3'→5' where the second sequence given by primer3 is 5'→3'. To do this you will use the Bioedit Sequence Alignment Editor software.
8. Open up the Bioedit Software.
9. Got to File → New Alignment.



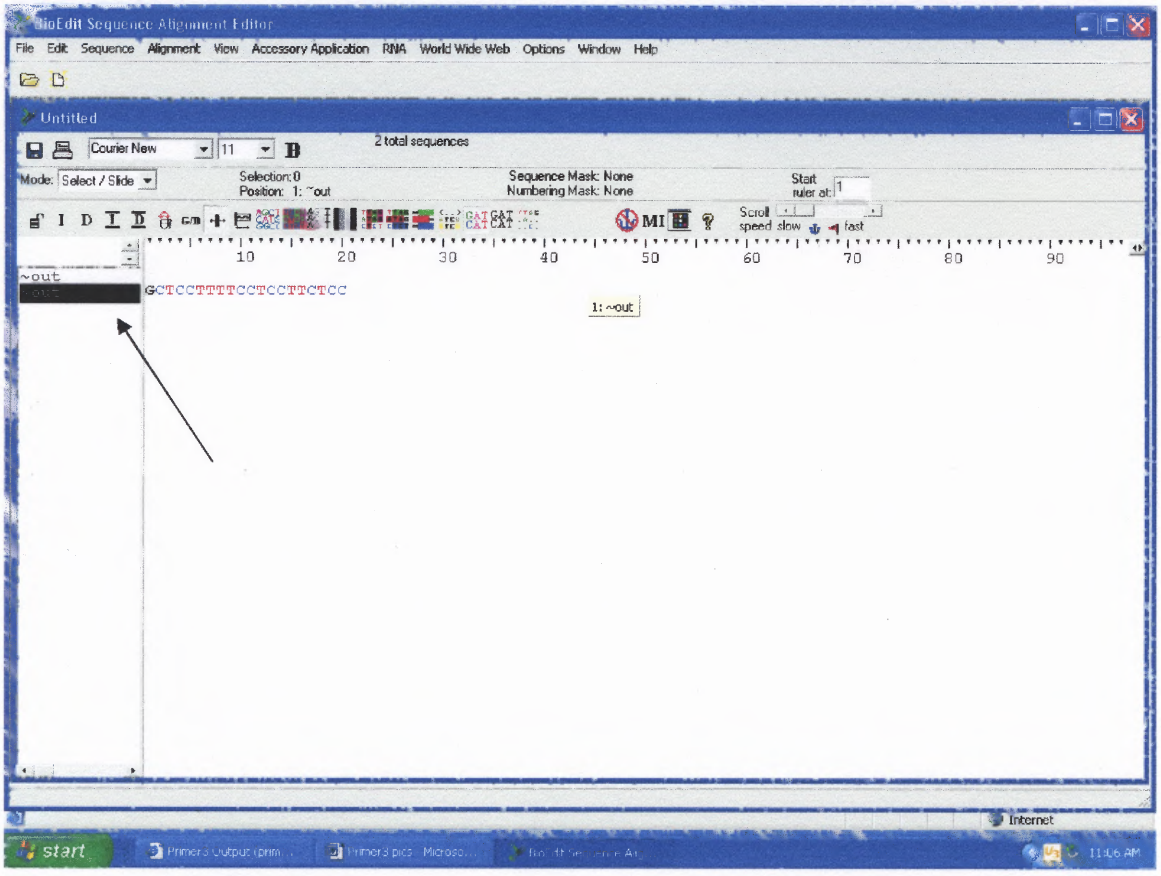
11. Copy the second primer sequence (highlight and use 'Ctrl+C').



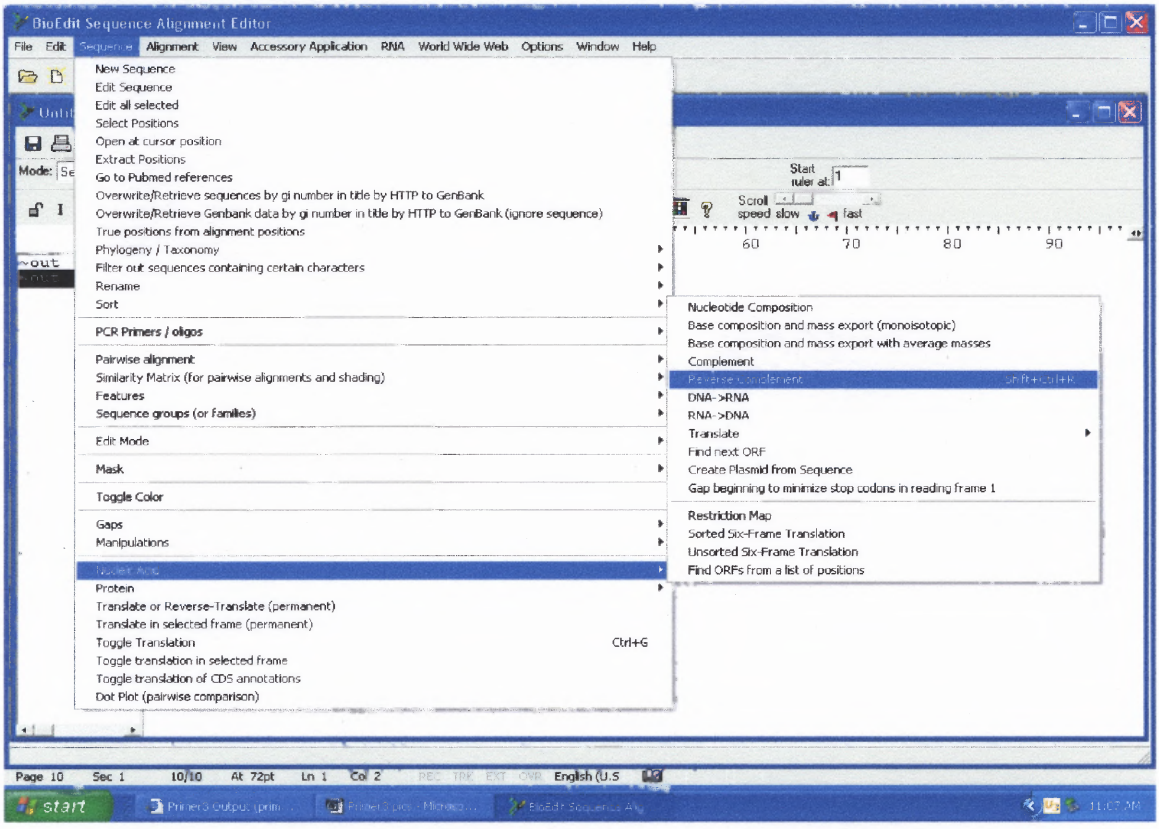
12. Go to the Bioedit software and under the "file" menu select "Import from Clipboard".



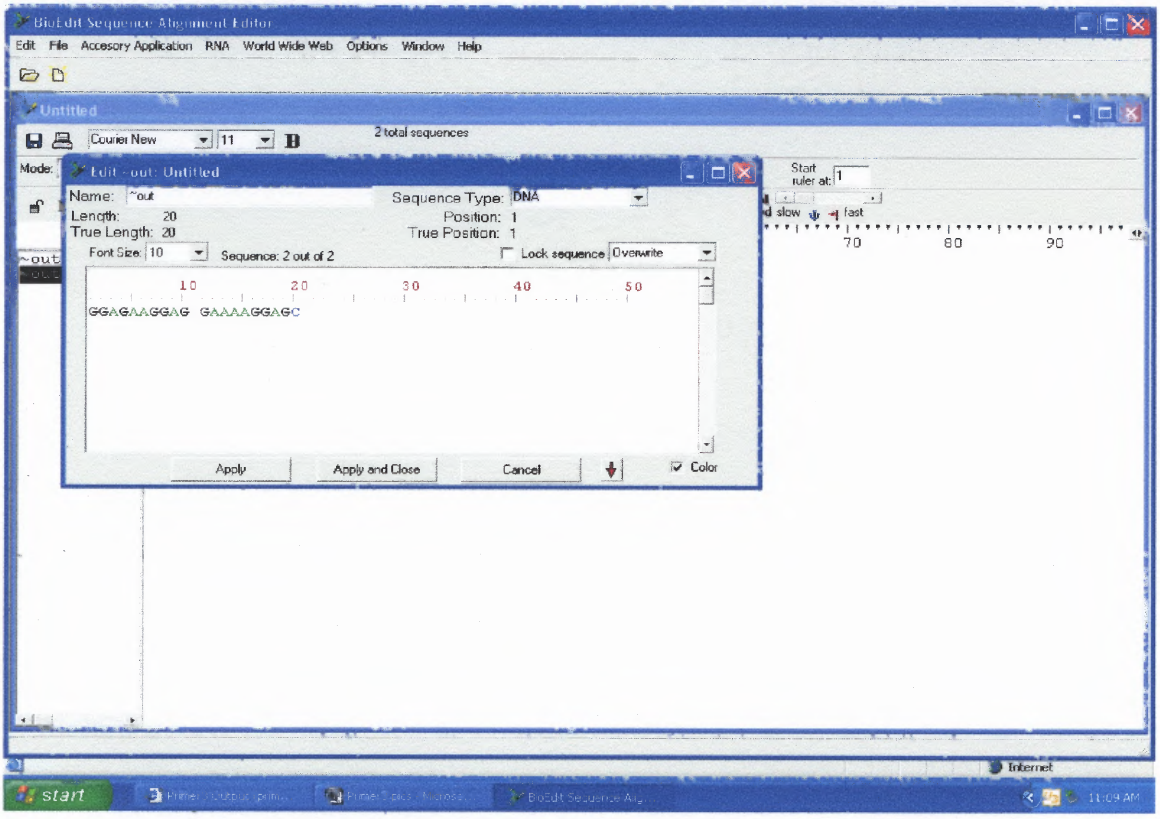
13. Your sequence should be imported as "~OUT".



14. Select the "~OUT" and go to Sequence→ Nucleic Acid → Reverse Complement.

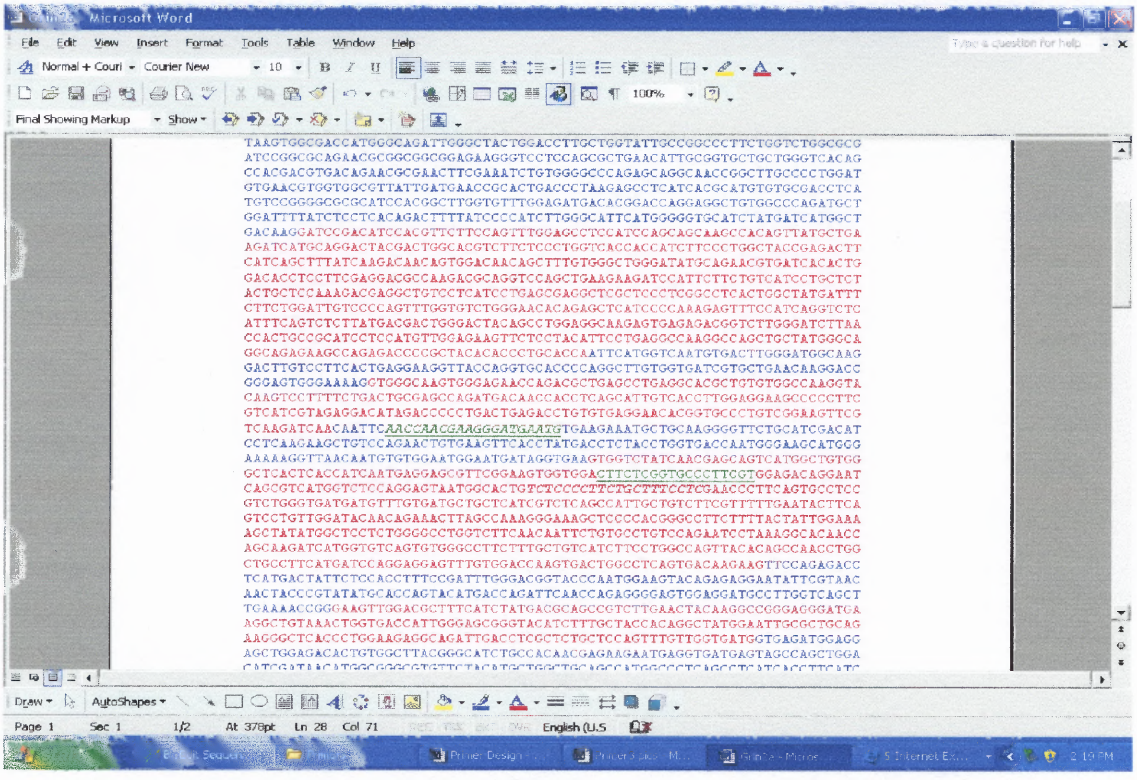


15. Double left click on the ~OUT.



16. Copy this new sequence from the pop-up screen.

17. Open the word file with your gene sequence. Use "word find" to look for the second sequence to the primer.



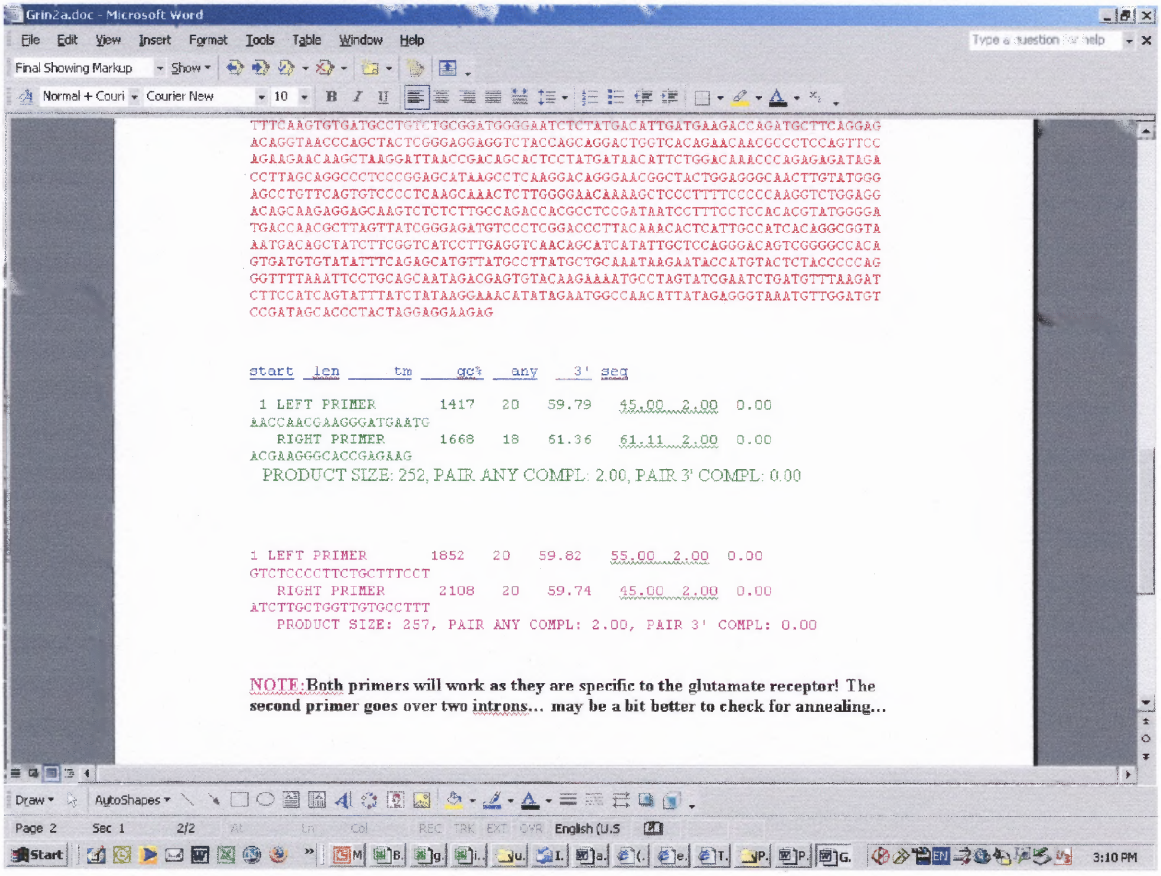
18. Once you find the complement sequence of the primer, underline it as was done for the first sequence earlier.

NOTE: Your two sequences should be located in different exons, as is the one in the above example. A primer that is in the same Exxon is useless because you will not be able to look for genomic contamination. The primer's forward and reverse sequences can be one, two, three exons away but definitely should not be in the same exon.

19. If your first and second sequence land in the same exon, you need to go back to your other primers and follow steps 3-17.

NOTE: If none of the primers work, the next step is to remove some of your gene sequence and follow Section C of this protocol until you find a suitable primer.

20. Once you determined the right primer, Copy all of its information from the Primer3 website and paste it to the bottom of your word file. This will be useful for ordering your primer and future experiments.

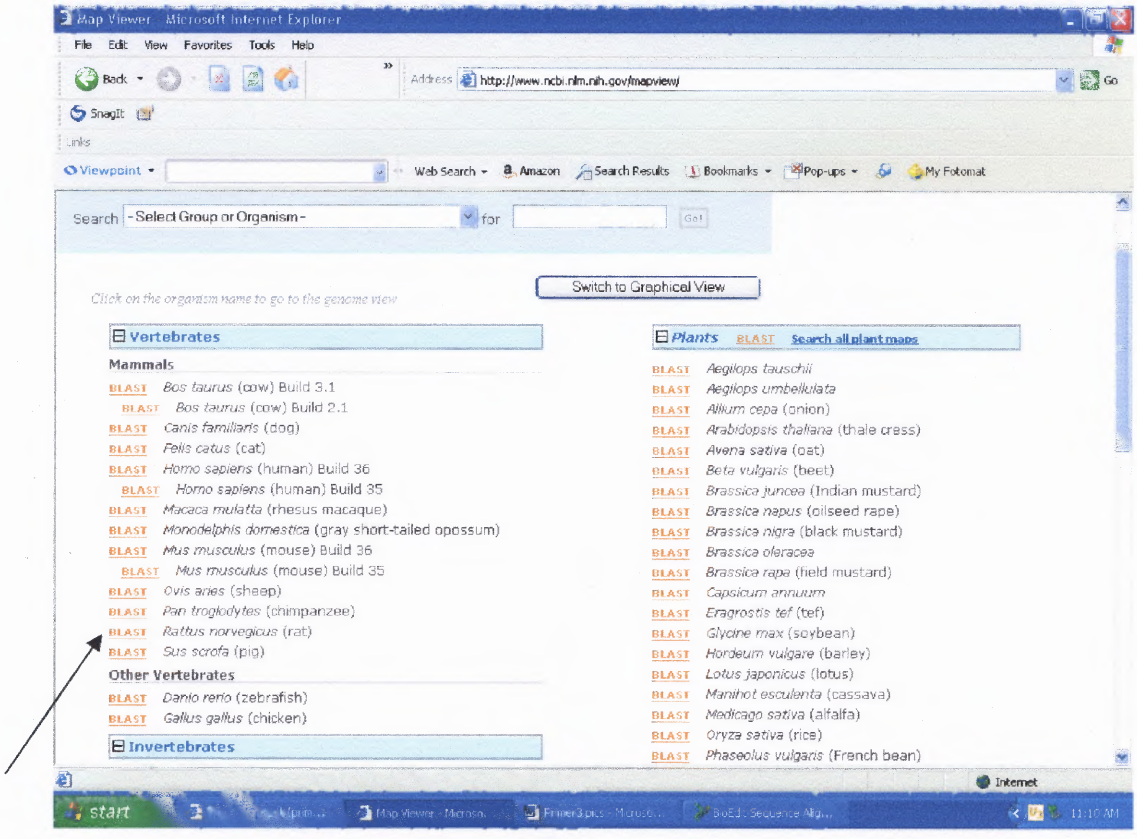


E. BLAST your Primer

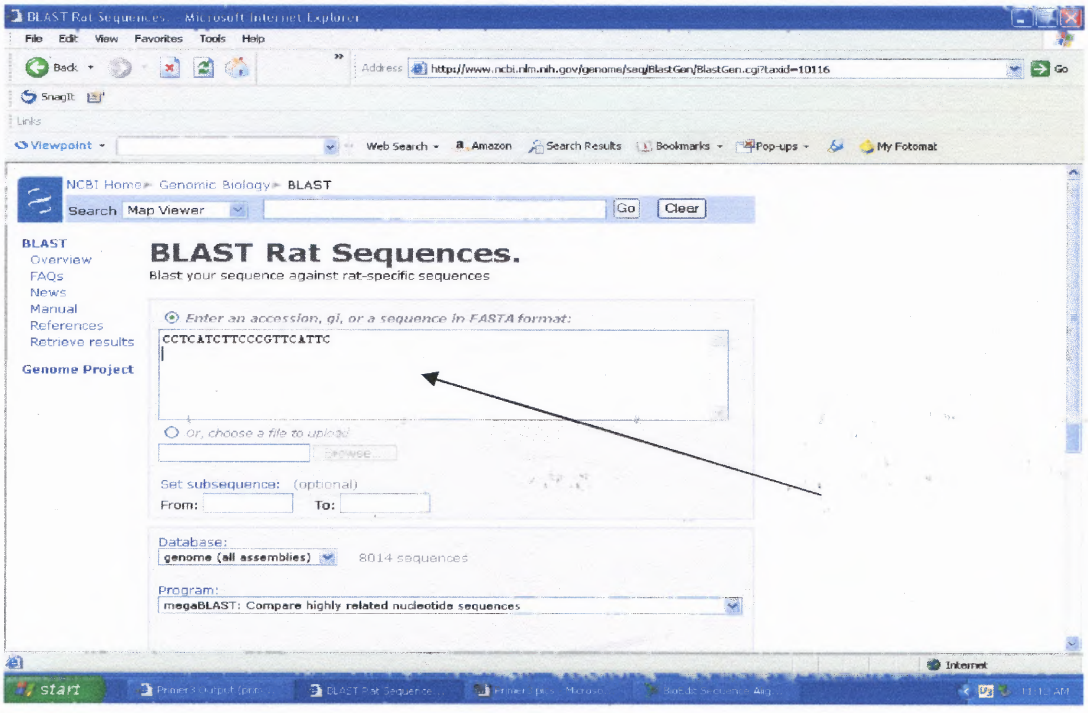
[This last step is done to confirm that your primer is specific to your gene of interest.]

1. Go to http://www.ncbi.nlm.nih.gov/mapview/.

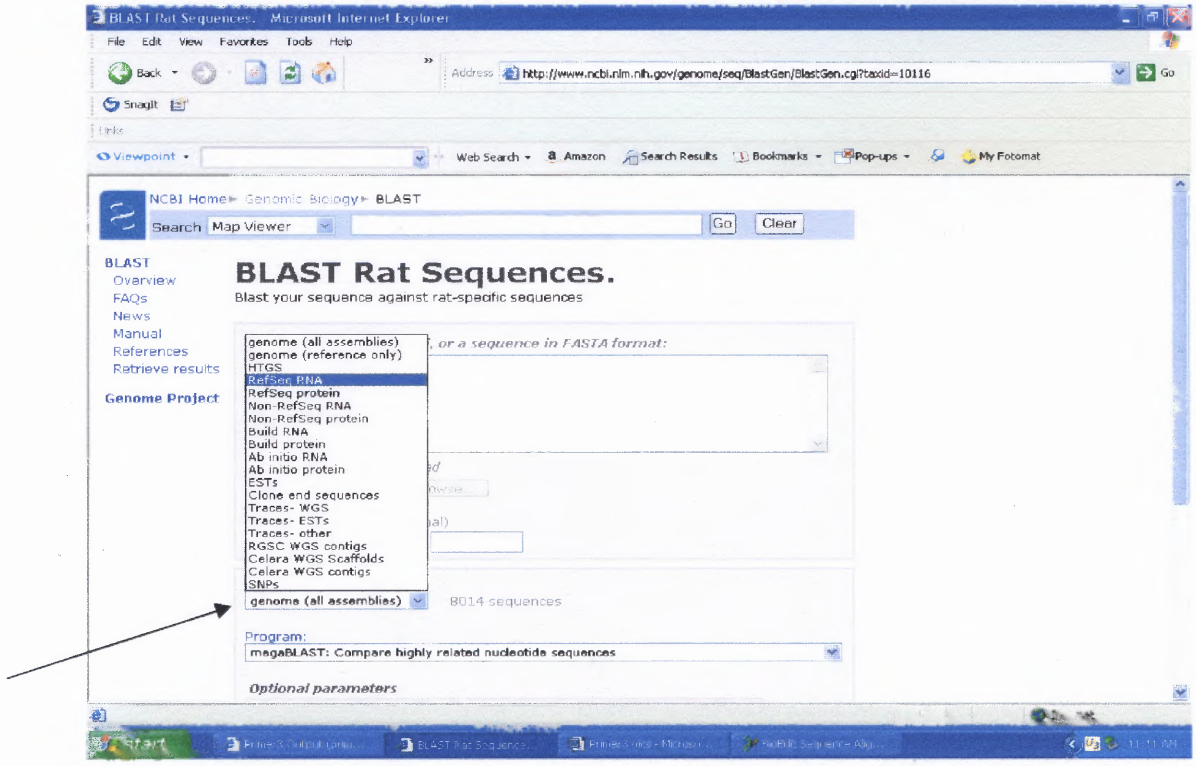
2. To the left of the Screen select the BLAST Rattus Norvegiccus option.



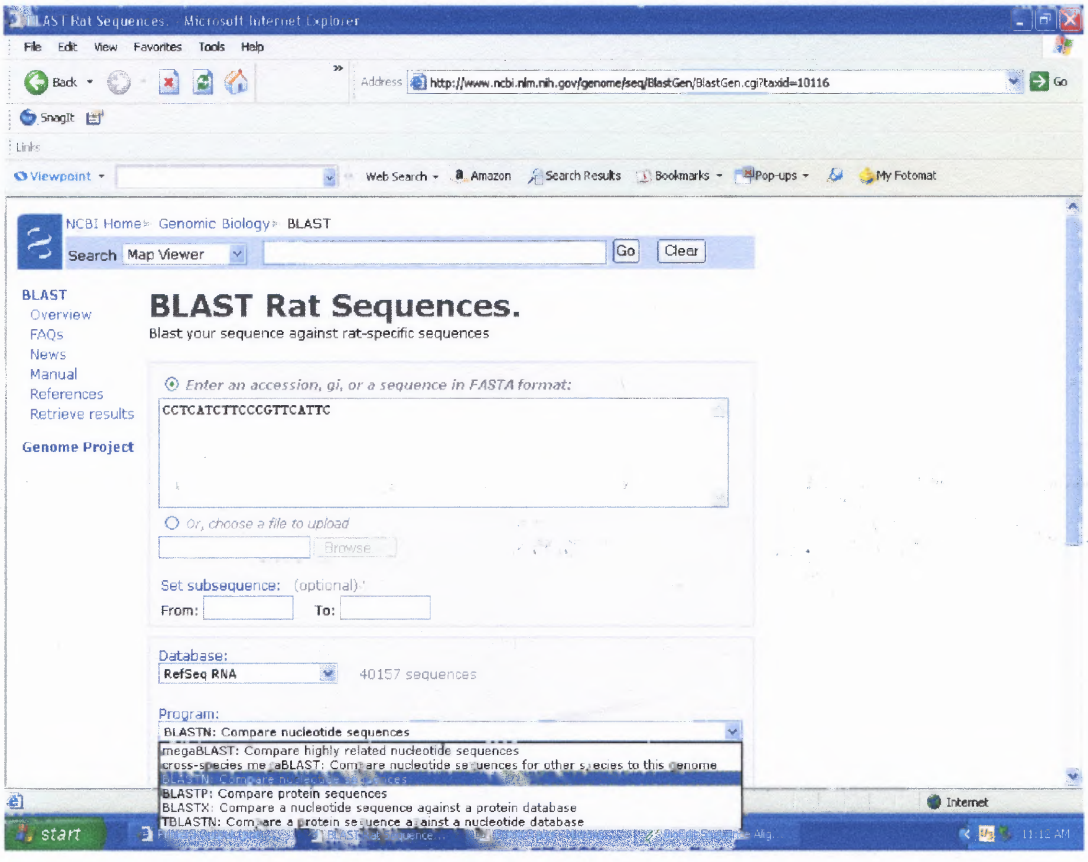
3. Copy your primer's forward sequence and paste it into the allocated space.



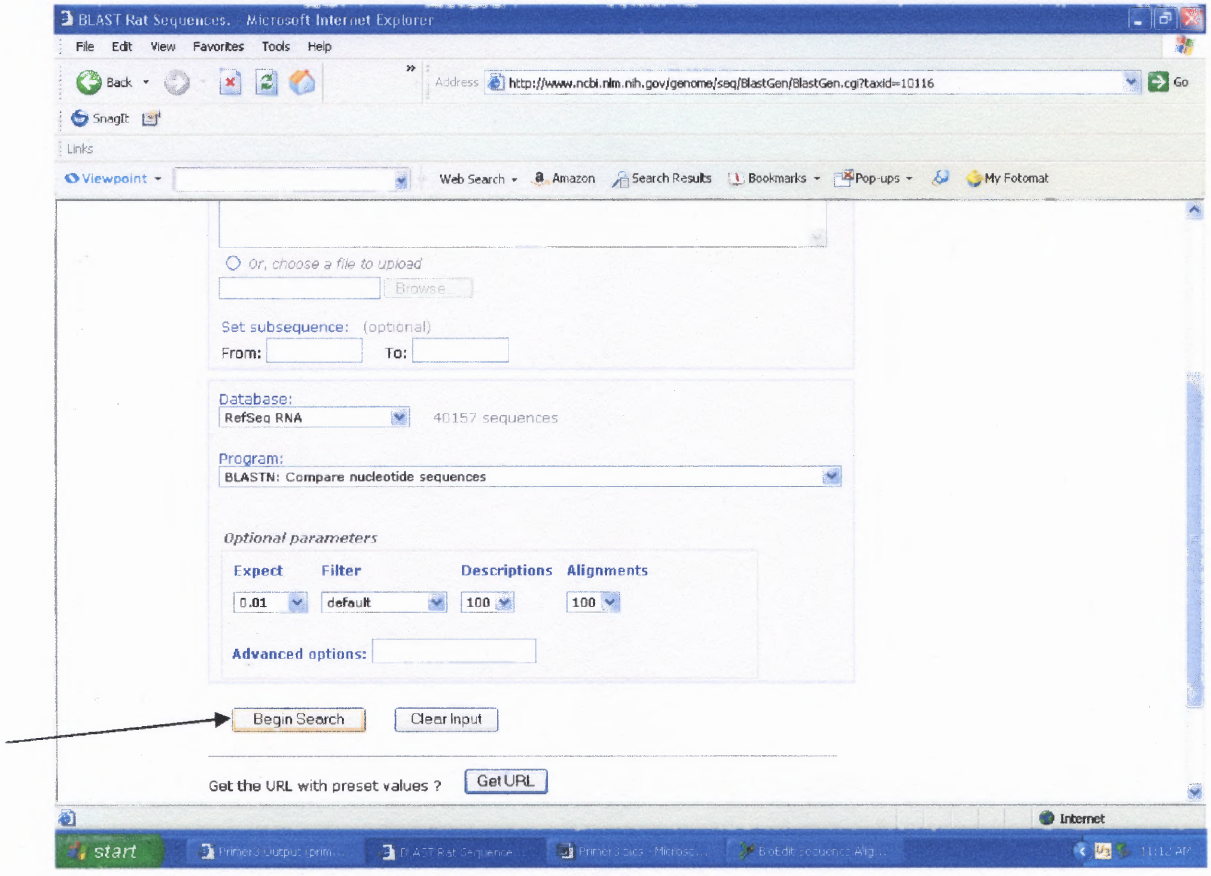
4. For the Database select the Refseq RNA.



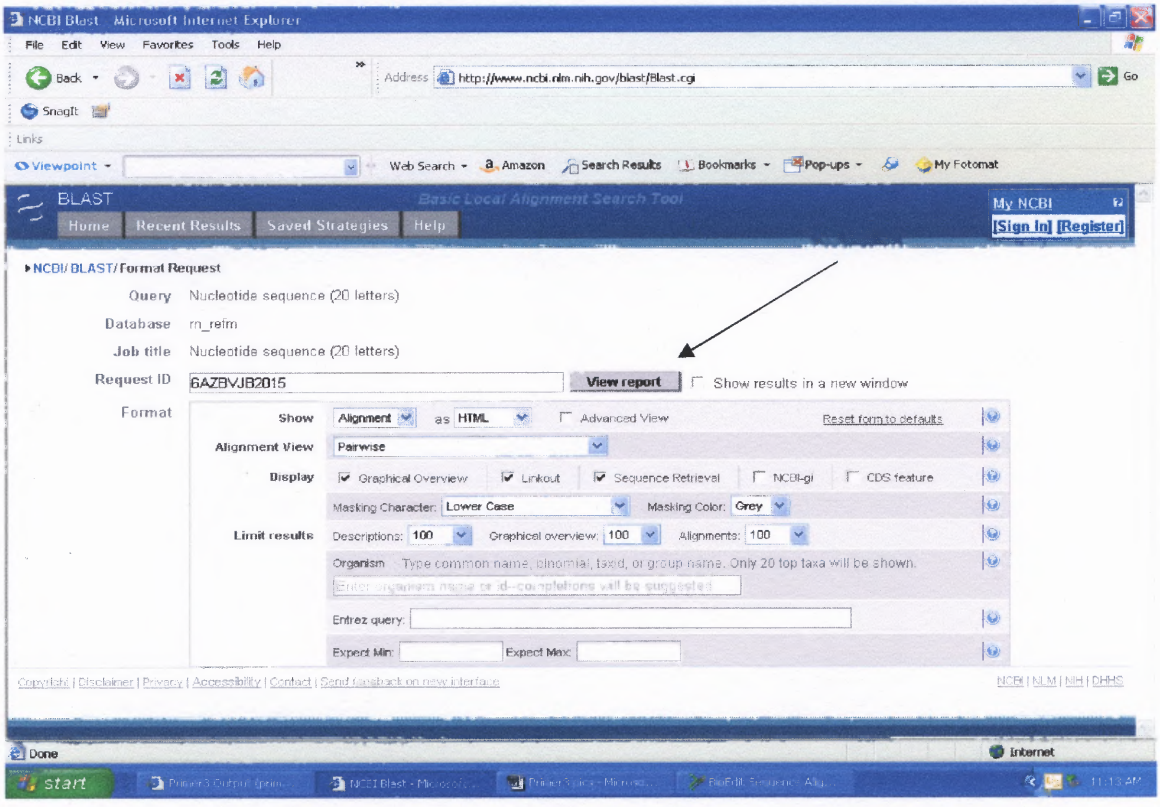
5. For the Program select The BLASTN (to compare nucleotides).



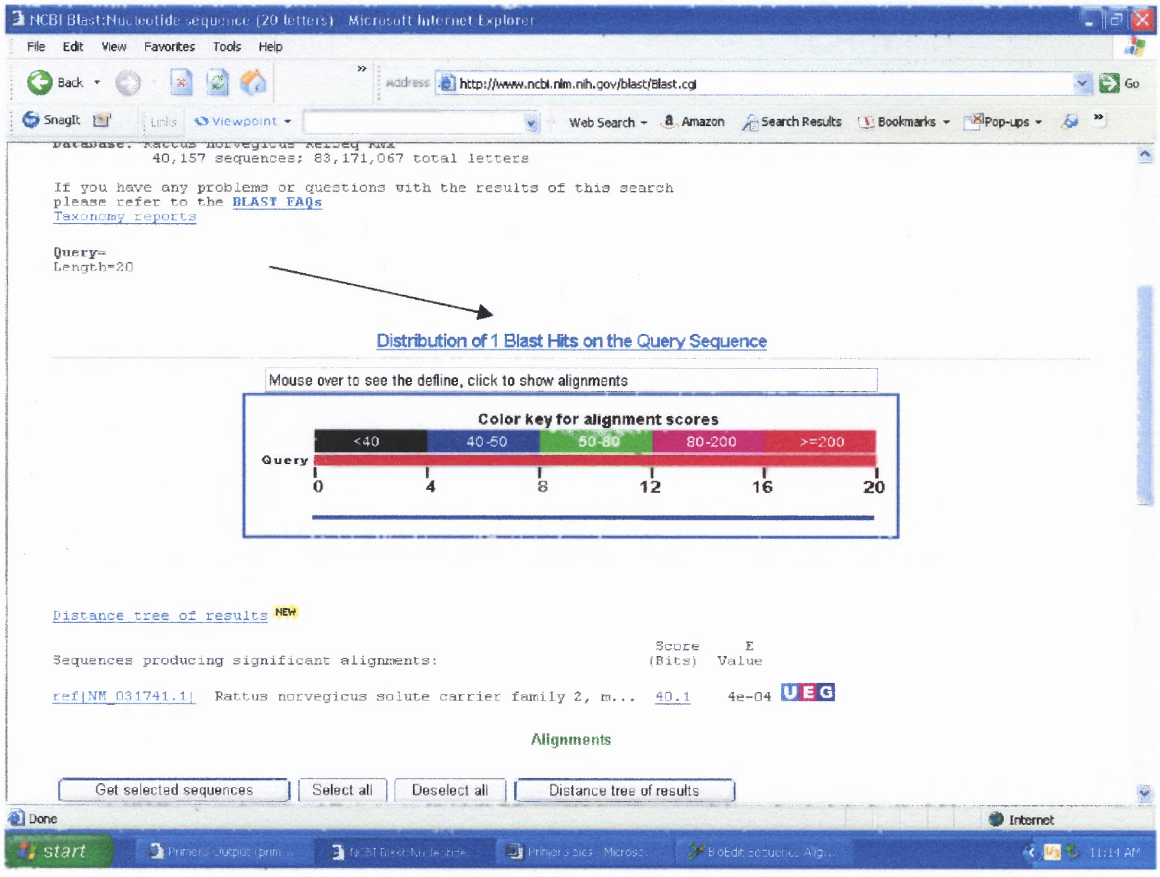
6. Select Begin search.



7. In the next screen select View Report.



8. The results should reveal that you have one BLAST Hit on your sequence and it should be specific to your gene.



NOTE: If you have more than one hit for your primer sequence, you need to go back to Section C and D of this protocol and redesign a primer.

9. Go to your word file and copy the second (reverse) Primer sequence. Follow section E steps to check that the second part of the primer only has one Hit as well when put through a BLAST.

Once the forward and reverse sequence of your primer is specific only to your gene (meaning there is only one BLAST Hit) you have designed your primer!

APPENDIX F

REALTIME PCR PROTOCOL

This appendix contains a step by step protocol on how to run a polymerase-chain reaction procedure using the iSYBR green and ROX – iTaq system.

REALTIME PCR PROTOCOL

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1. Dilution of cDNA (samples from reverse-transcriptase)

Take a 1.5 mL centrifuge for each sample and add:

1.1 124 µl Depc H_2O

1.2 Add 4 µl of each sample / per tube

2. Making a standard Curve

2.1 Pick random samples and you are going to pull them together

2.2 Add 5µL of each sample and add it to a 1.5 mL centrifuge tube

2.3 The number of samples that you pick determines the total volume

ie 10 samples = 50µL of samples pulled together. → place on ice

2.4 Add DEPC H_2O, you 3 fold the amount of sample that you have.

ie 50µL of pooled samples, you add 150µL of DEPC H_2O

2.5 You now dilute the samples two fold as described below:

S = 200µL of the pooled samples (diluted 3 fold)

S_1= 50µL of S + 50 µL of DEPC H_2O

S_2 = 50µL of S_1 + 50 µL of DEPC H_2O

S_3 =50µL of S_2 + 50 µL of DEPC H_2O

S_4 = 50µL of S_3 + 50 µL of DEPC H_2O

S_5 = 50µL of S_4 + 50 µL of DEPC H_2O → place them on ice

NOTE: These new samples S_1 – S_5 are your standard curve samples.

2.6 Remove the Sybr Green Master Mix and the reference dye from freezer and place on ice with samples. While these thaw, setup the real-time machine.

3. Real-time Machine Set-Up

Turn on the machine the button is in the back.

Click on the Mx3000P software on the desktop of the computer

Click on the option that says Sybr Green Analysis with dissociation curve.

It opens to the window with the plate setup:

Do duplicates for your standard curve – highlight ten wells

NOTE: to click you click and drag to highlight the wells

Under well type → standard

For fluorescence data → ROX and FAM

Reference Dye → ROX (click the "all wells" option)

Standard quality → -2X (dilution)

Click on the first standard sample and highlight both wells

Do the same for the second sample and so on, this should set-up a dilution number at the bottom of the wells, starting with 5

Standard Units → relative

Click on auto-increment → click on the wells to number them, the first standard sample (select the two wells at the same time to number it 1, the second sample 2 and so on).

For your samples do not do duplicates → select the number of wells to correspond with the number of samples that you have.

Under well type → unknown

Flourescence data → ROX and FAM

Click auto-increment and number them accordingly by clicking on each well (ie 6,7,8 ....)

For the Genomic DNA controls → do not do duplicates

Select the number of wells depending on the number of samples that you have.

3.4.15 Well type → NO-RT

3.4.16 Flourescence data → ROX and FAM

3.4.17 Click on auto-increment → select the wells to number them accordingly

3.4.18 Take the No Amplification Control – the sample with just with the enzyme → select the well

3.4.19 Well-type → NAC

3.4.20 Flourescence data → ROX and FAM

3.4.21 Click on auto-increment → click on the well to number it

3.4.22 Click one last well, this one is just DEPC H_2O (which you make when you set-up the plate for the machine)

3.4.23 well-type → NTC

3.4.24 Flourescence data → ROX and FAM

3.4.25 Click on auto-increment and number the well.

3.5 Set up the Thermal Profile as the following:

10 min → 95°C (activation)

30 sec → 95°C

30 sec → 59° C (Annealing step- this step varies (the temperature may not be 59) depending on the probe that is being used

1 min → 72° C (elongation step- this temp and/or time may vary depending on the probe)

Dissociation step – last step – stays the same no need to change it

4. Preparing the Master Mix

Below is the master mix per sample, what you do is multiply that by the number of samples that you have including the standard curve, your controls and the last sample of water.

6.25 µL of the 2X Sybr Green Master Mix

0.5µL of Forward Primer

0.5 µL of Reverse Primer

0.1875µL Reference Dye

0.0625µL of DEPC H_2O

5. Filling in the Plate

5.1 In each Well Add:

5.1.1 7.5 μL of Mix

5.1.2 5.0 μL of each sample

NOTE: the last well (the NTC sample) you add 5μL of DEPC H_2O

5.2 Place covers over the wells

5.3 Place the tray in the strategene machine and click on RUN

5.4 Click START

5.5 Save the file under the appropriate name and folder

NOTE: If you are going to leave the machine running overnight or unattended click on the option to turn off the lamp once the run is finished.

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