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# Development of Novel Allele Specific PCR Based Assays to Investigate the Contribution of Cortisol to Metabolic Syndrome and it's Potential Implications in Depression Treatment

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Development of Novel Allele Specific PCR Based Assays to Investigate the Contribution of Cortisol to Metabolic Syndrome, and it's Potential Implications in Depression Treatment.

By

Justin Robert Salm

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Submitted in partial fulfillment  
of the requirements for  
Honors in the Department of Biology

UNION COLLEGE  
June, 2017

## ABSTRACT

SALM, JUSTIN Development of Novel Allele Specific PCR Based Assays to Investigate the Contribution of Cortisol to Metabolic Syndrome, and it's Potential Implications in Depression Treatment.

Department of Biological Sciences, June 2017

ADVISOR: Brian Cohen

The shared circuitries of depression and the stress response, regulated by the Hypothalamic-Pituitary-Adrenal (HPA) axis, implies that major depression may reflect dysregulations of the HPA axis. Previous studies find that that melancholic depression is accompanied by hyperactive HPA activity, anxiety, insomnia etc. Adversely, patients with atypical depression present CRH deficiency accompanied by lethargy and fatigue. According to the corticosteroid receptor hypothesis, HPA axis dysregulation that causes these variable phenotypic depressive moods may be attributed to defective GR expression or function. Multiple GR polymorphisms that could impact its expression or function have been identified. Due to limitations, it was not feasible to study a depressed patient population at this time. Fortunately, the strong correlation between depression and obesity both being induced HPA dysregulation means that a similar study can be done by examining metabolic disorders.

A diagnosis of Metabolic Syndrome (MS) requires patients to present three or more of the following symptoms: elevated fasting blood glucose levels, elevated serum triglyceride levels, low serum HDL levels, elevated blood pressure, and truncal obesity. This pathology overlaps with Cushing's Syndrome (CS) except a diagnosis of CS requires hypercortisolemia. This similarity has led our lab and others to hypothesize that MS may be a Cushingoid-like state

caused by hypersensitivity of the glucocorticoid receptor (GR), a nuclear hormone receptor that is activated upon the binding of cortisol. Using an allele specific polymerase chain reaction (PCR) protocol, previous research in our lab found significant correlations between the BclI and N363S polymorphisms of the GR and altered patient metabolic profiles in a population of patients seeking bariatric surgery. This suggests that each contributes to obesity related diseases. Recent work in our lab has expanded our study to include the cortisol resistance polymorphism TthIII1 (rs10052957) to test the hypothesis that there would be a decreased prevalence of this polymorphism in our subject population. The TthIII1 allele is present at a lower frequency in our research population compared to reported frequencies, consistent with our hypothesis.

In addition to investigating the glucocorticoid receptor, we have also developed an allele specific PCR assay for previously identified single nucleotide polymorphisms (rs12086634 and rs846910) in the cortisol reductase enzyme known as 11beta-hydroxysteroid dehydrogenase (11b-HSD) type 1. The 11B-HSD1 enzyme converts inactive cortisone to cortisol, working primarily in adipose tissue. The polymorphisms listed above are associated with overactivity of the enzyme and may cause the Cushingoid-state observed in MS patients. In our patient population, the frequency of heterozygosity for the rs12086634 SNP was nearly 50% higher than the reported frequency in the general population, suggesting that this polymorphism may contribute to increased obesity and the MS metabolic profile, by increasing the localized cortisol in adipose tissue. Greater understanding of the interplay between these SNPs can help

physicians and patients make more informed decisions about treatment options for obesity and metabolic syndrome.

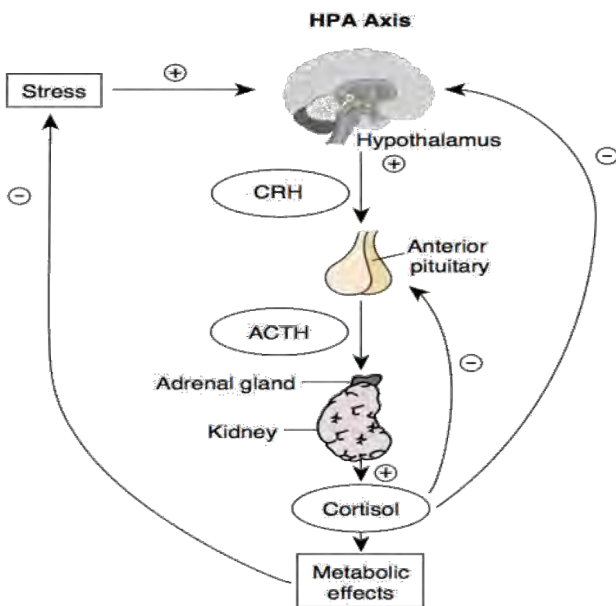
## Introduction

### Cortisol: an overview

Cortisol is a glucocorticoid, steroid hormone that has a variety of physiological and psychological effects throughout the body. Therefore, it is no surprise that cortisol and similar synthetic analogs are commonly used to treat numerous diseases, ranging from dermatological rashes to hematological malignancies<sup>0</sup>. With its viable receptors expressed in nearly all mammalian cells, cortisol influences many different functions<sup>1</sup>. The hormone contributes to the regulation and function of metabolic, cardiovascular, immune, and physiological and psychological stress responses-- all of which are prone to agitation and essential to restore following the stimulus of an acute stressor<sup>1,2</sup>.

Of its many target tissues, let's first investigate cortisol's role with respect to peripheral tissues. Cortisol helps maintain energy homeostasis by regulating metabolism in times of both health and disease. In the presence of a physiological stressor (famine, strenuous exercise etc.), cortisol acts on the liver to increase gluconeogenesis and glycogenolysis, thereby restoring glucose levels through the synthesis of smaller derivatives or the breakdown of glycogen<sup>0</sup>. Cortisol impacts the catabolism of both lipids and proteins. By increasing insulin resistance, the synthesis of very low-density lipoproteins, free fatty acid production, and the circulating levels of very low-density lipoprotein and low-density lipoprotein, triglyceride and cholesterol levels are increased<sup>0</sup>. Insulin resistance coupled with increased glucose levels induces greater insulin levels, leading to greater fat storage. Cortisol influences the distribution and accumulation of these increased fat levels in the abdominal region, often leading to obesity related diseases when cortisol levels are high<sup>3,4</sup>.

Glucocorticoids are released from the adrenal gland in response to a stressor activating the regulatory hypothalamic-pituitary-adrenal (HPA) axis. The role of the HPA axis is to maintain normal glucocorticoid secretion. This neuroendocrine system is regulated by stress responses, glucocorticoid signaling/feedback loops, and circadian rhythms<sup>2,5</sup>. In response to a perceived stressor, afferent neurons send a stimulus to the paraventricular nucleus of hypothalamic neurons, inducing the release of the hypothalamic hormones: corticotropin-releasing hormone (CRH), vasopressin, and oxytocin<sup>2,6</sup>. These hormones control the release of ACTH from corticotroph cells in the anterior pituitary<sup>6</sup>. Although vasopressin and oxytocin don't induce large secretions of ACTH independently, when in combination, the hypothalamic hormones



**Figure 13.** The Hypothalamic-Pituitary-Adrenal (HPA) axis. The numerous positive and negative feedback loops within this neuroendocrine system are mapped out in a simplified manner. This represents how stress initiates the pathway resulting in the production of cortisol and other glucocorticoids.

elicit more ACTH than CRH could on its own<sup>2</sup>. ACTH then binds to receptors on the adrenal cortex, where it induces the synthesis and secretion of glucocorticoids from the zona fasciculata<sup>6</sup>.

Glucocorticoids are involved in classic, multi-variable endocrine feedback loops (Figure 1), which allow for increased regulation of HPA stress

response and circulating glucocorticoid levels. CRH and ACTH stimulate an increase in glucocorticoid levels, while glucocorticoids negatively feedback on the hypothalamus and

anterior pituitary to reduce the synthesis and secretion of CRH, vasopressin, and ACTH respectively<sup>6</sup>. Interestingly, glucocorticoids have no feedback inhibition on oxytocin<sup>2</sup>. The circadian rhythm is determined by the activity of the hypothalamic suprachiasmatic nucleus<sup>2,5</sup>. Glucocorticoid secretions begin to spike just before waking, due to the presence of CRH and an increase in the adrenal response to ACTH<sup>2</sup>. This phenomena, known as the cortisol awakening response, leads to peak cortisol levels within a half hour of waking<sup>5</sup>. Cortisol levels then decrease relatively steadily in a diurnal rhythm, hitting a trough in the late evening<sup>2,5</sup>.

90-95% of secreted glucocorticoids are bound to corticosteroid-binding globulin (CBG), a carrier protein, increasing its solubility in water, allowing glucocorticoids to travel effectively through the circulatory system<sup>1</sup>. When cortisol arrives at a target tissue, disassociation with the CBG occurs, leaving behind a biologically available, lipid soluble steroid that can cross the cell's bilayer, bind to its receptor, enter the nucleus, and bind to its specific DNA region. Cortisol binds to both the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), though its affinity for the MR is ten times stronger than its affinity for the GR<sup>7</sup>. The GR and MR share many similarities both in structure and in expression, explaining why cortisol acts as a ligand for both receptors<sup>8</sup>. Structurally, their DNA binding domains are 94% homologous<sup>9</sup>. While the MR is expressed in peripheral tissues, it is highly expressed throughout the limbic regions of the brain, specifically in the hippocampus, amygdala and prefrontal cortex<sup>2,7</sup>. The GR is expressed more ubiquitously throughout the limbic system, especially in the hippocampus, the prefrontal cortex, the paraventricular nucleus of the hypothalamus, the dentate gyrus, and the amygdala<sup>7</sup>. GRs are also present in corticotrophs and other peripheral tissues<sup>7</sup>.



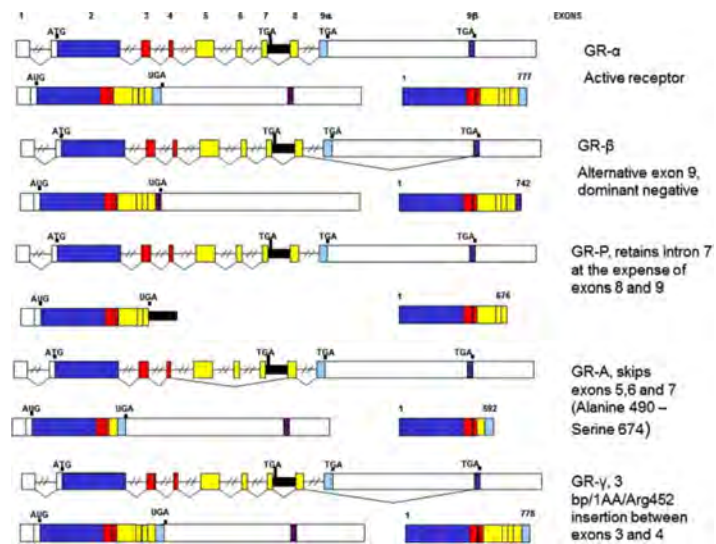
Considering that cortisol has a higher affinity for the MR, when at low levels, cortisol binds to MRs. Therefore, MRs are responsible both for feedback inhibition of the HPA axis at resting cortisol levels, and for starting the stress response<sup>2,8</sup>. As cortisol levels rise above the resting rate, GR binding becomes more common<sup>8</sup>. The GR terminates the stress response and promotes memory storage to better prepare the body to respond to that same stimulus in the future<sup>8</sup>. At these higher cortisol levels, though the MR is involved, it is the GR that is the primary contributor to HPA axis feedback inhibition<sup>8</sup>. In a study from 2011 investigating the relationship between glucocorticoids and the immune system, Zen et al., found that both the MR and the GR shared related immunosuppressive functions<sup>9</sup>. Though sample size was cited as a primary limitation in a similar study performed by Cheng et al., (2016) they found that during low dose cortisol treatments, the blocking effects on cortisol-mediated inflammation regulation were similar regardless of whether monocytes' GRs or MRs were inhibited<sup>8</sup>.

## The Glucocorticoid Receptor

The cortisol-MR interaction establishes an individual's basal response and control of the HPA axis. In addition, glucocorticoids have a lessened effect through the MR because MRs, in most tissues excluding the brain, are shielded by 11 $\beta$ -Hydroxysteroid dehydrogenase II (11 $\beta$ -HSDII), which converts the active cortisol into its inactive metabolite, cortisone<sup>0</sup>. Therefore, researchers are mostly interested in investigating how GRs, which play a larger role in the stress response, impact HPA axis regulation<sup>0-4</sup>.

The GR is a nuclear receptor encoded by *NR3C1*<sup>0</sup>. Located on chromosome 5, it has nine exons, and follows a normal nuclear receptor composition: there is an N-terminus transactivation domain, a C-terminus ligand binding domain, with a DNA binding domain in between<sup>7</sup>.

The 9 exons create the opportunity for multiple splice variants which, combined with alternative translation, result in functional diversity within the GR<sup>7</sup>. GR $\alpha$  is the biologically active isoform<sup>7</sup>. GR $\beta$ , (a result of the splice variant lacking helix 12) cannot bind glucocorticoids, but can still regulate other genes<sup>7</sup>. GR $\gamma$  is still biologically active, but has significantly less transcriptional activity compared to GR $\alpha$ <sup>7</sup>.



**Figure 2<sup>0</sup>**. Glucocorticoid Receptor Isoforms, formed by multiple promoter sequences in the 9 exons, alternative splicing, different translational start signals, and post translational modifications.

has

The GC-GR complex modulates target gene transcription through both genomic and non-genomic pathways<sup>7</sup>. After ligand binding, the GR-GC complex traffics towards the nucleus and mediates transcription in one of many different ways. It can promote GR-GR dimerization on the glucocorticoid response element leading to chromatin remodeling, which induces transactivation<sup>7</sup>. If the glucocorticoid binds to a negative GC response element (located in the promoter of a target gene), gene transcription will be inhibited. Glucocorticoids' nongenomic actions include influencing T-cell receptor signaling, increasing cellular energy metabolism, modulating apoptosis, and changing behavior<sup>7</sup>.

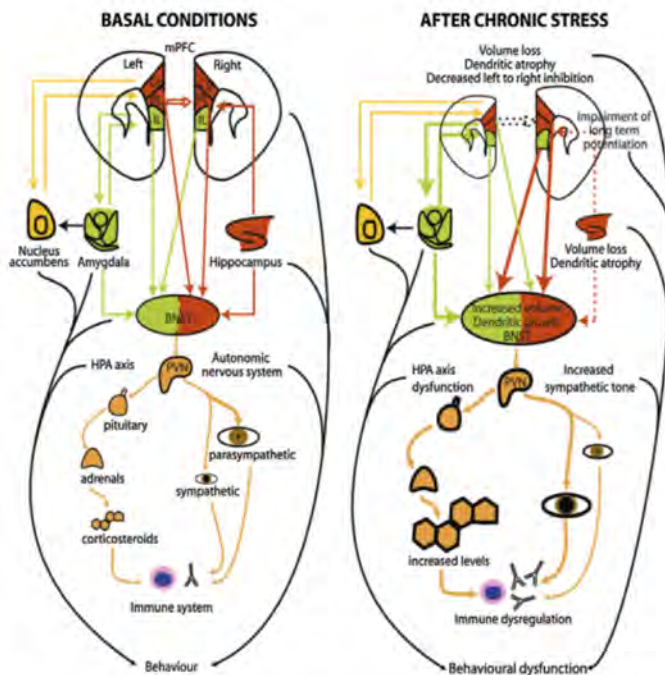
#### HPA Axis dysregulation and its implications on cortisol, stress and depression

As mentioned previously, the numerous variables and regulatory combinations in the HPA axis and its control system increase its ability to maintain equilibrium when activated, as there are multiple checkpoints where homeostasis can be restored. However, this also means that there are more places for things to go awry. Aberrant HPA activity, or HPA dysregulation in general, is linked with various psychiatric disorders as well as obesity<sup>8</sup>. HPA axis dysregulation is measured through a Dexamethasone suppression test (DST) or a Dexamethasone-CRH combined test (DEX/CRHT). In a DST, up to 2 mg of dexamethasone (an exogenous glucocorticoid) is administered to measure the adrenal negative feedback on the pituitary<sup>7</sup>. This provides information on an individual's baseline feedback levels<sup>0,2</sup>. DEX/CRHT is a refined DST that is a bit more accurate. In this test, 1.5 mg of dexamethasone is administered at around 11:00 PM, when an individual's natural cortisol levels should be low<sup>7</sup>. At around 3:00 PM the

following day, 100 µg of CRH is administered<sup>7</sup>. Meanwhile, cortisol and ACTH levels are collected every 15 minutes from 2:00 to 6:00 PM<sup>7,8</sup>. This test provides information on how effective the positive and negative feedback loops of an individual's HPA axis are, and is the most sensitive measure of HPA overactivity associated with depression<sup>2</sup>.

The association between HPA axis dysregulation and depression, especially unipolar and bipolar depressive disorder, has been established for decades<sup>7</sup>. Spijker et al., (2009) found that 80 percent of patients with a major depressive episode were non-suppressors in response to the DEX/CRHT, further highlighting the linkage between HPA axis dysregulation and depression<sup>7</sup>. This dysregulation, leading to Major Depression Disorder (MDD) and Bipolar Depression (BD) is connected to anatomical and neuroendocrine alterations in brain function--including changes in catecholamines and serotonergic neurotransmission<sup>7</sup>. Specifically, glucocorticoids were found to intensify the extent that dopamine affects the brain's reward system<sup>7</sup>. Patients with depression were also found to have anatomical changes and altered metabolic activity in their prefrontal cortex, amygdala, and hippocampus<sup>7</sup>. Patients with depression have hypometabolism in frontal regions, and hypermetabolism in the right hippocampus. Interestingly, the metabolic levels normalize with antidepressant treatment.

Stress, which activates the HPA axis, can be defined as an actual (physical) or perceived (psychological) threat to the well being of an organism<sup>2</sup>. A stressed stimulus can be acute (brief)



**Figure 3**<sup>23</sup>. The Neuropathology of stress. Comparing the interactions between the prefrontal cortex, the stress response, and the immune system normally (left) vs. during chronic stress (right). Note the HPA axis dysfunction on the left.

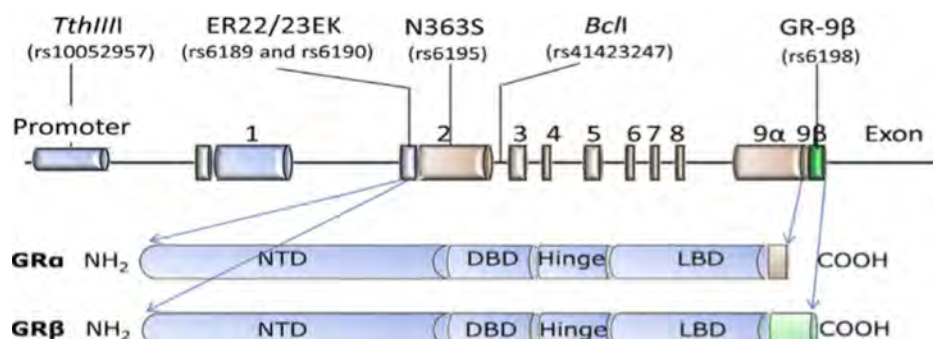
or chronic (long term). Physical and mental health is dependent on the HPA's ability to regulate stress. HPA axis dysregulations are associated with different neuropsychiatric diseases. HPA overactivity is associated with typical or melancholic depression, panic disorder, OCD and schizophrenia<sup>0,1,2,8</sup>. These are often characterized by elevated 24-hour free cortisol, and enlarged adrenal

glands<sup>6</sup>. Low HPA axis activity is characteristic of atypical depression<sup>8</sup>.

Chronic stress induces GR dysfunction or resistance (Figure 3), which often leads to the synthesis and secretion of more cortisol in an effort to overcome the receptor resistance<sup>7</sup>. However, the HPA response has been shown to be habituated or sensitized, after repeated exposure to a stressful stimulus<sup>11</sup>. The scientific community believes that the observed inter-individual variability of HPA axis response to repeated stimuli may be caused by GR variants<sup>11</sup>. Depression, therefore, may be a result of defective GR expression or function in the brain and corticotroph, based on different GR variants or isoforms in the tissue of interest<sup>2</sup>. This idea lead to the formation of the Corticosteroid receptor hypothesis, which explains how depression and the subsequent treatment impact HPA activity<sup>12</sup>. This can be broken down into two parts. The first part identifies the connection between depression and defective GR

expression or function, which results in a loss of inhibitory feedback, represented by dexamethasone resistance, causing basal HPA activity to rise. This, in turn, results in the increased production of ACTH-releasing factors leading to an increased ACTH response to DEX/CRHT<sup>13</sup>. Additionally, post mortem evidence of individuals diagnosed with MDD suggests they have fewer cerebral GRs and MRs. The second part identifies that proper treatment can overcome receptor deficits<sup>2</sup>.

Due to the fact that the GR isoforms all originate from the same gene, NR3C1, GR function is greatly impacted by mutations in that gene. Numerous single-nucleotide polymorphisms (SNPs) of the NR3C1 gene have been identified, and are associated with neuropsychiatric diseases as well as altered lipid and carbohydrate profiles<sup>14</sup>. While many studies have examined the phenotypic SNPs with respect to the corticosteroid receptor hypothesis, they all contain discrepancies due to differences in drug effects and treatment times, indirect autoregulatory effects from differences in glucocorticoid secretion, heterogeneity of study populations, and the fact that subtypes of depression have varying types of HPA dysfunction<sup>15</sup>.



**Figure 4**<sup>15</sup>. Location of Single Nucleotide Polymorphisms (SNPs) of the NR3C1. While there are other identified SNPs of the Glucocorticoid Receptor, TthIII1, ER22/23EK, N363S, BclI, and GR-9β are the best studied.

The  
BclI SNP  
is caused  
by a  
C→G

nucleotide change in intron 2, 646 nucleotides downstream of the respective intron junction<sup>0</sup>. BclI polymorphisms are associated with glucocorticoid hypersensitivity<sup>0</sup>. Studies have found that unfavorable metabolic characteristics including insulin resistance, diminished salivary cortisol responses, and increased abdominal visceral fat, body mass index (BMI), waist to hip ratio (WHR), and leptin and cortisol responses to a standardized lunch are all associated with the G-allele carriers<sup>11</sup>. Three different studies looked at the prevalence of homozygous mutant BclI carriers in a depressed group compared to a control group<sup>7</sup>. All three studies found that depressed group had roughly 15.5% homozygous mutant, while the control group had 9.9% express the mutant phenotype homozygously<sup>7</sup>. These studies established a relationship between the SNP, MDD, and obesity.

N363S is another GR hypersensitivity polymorphism that is the result of an asparagine to serine (N→S) amino acid change in codon 363 of exon 2, which is the result of an AAT→AGT nucleotide change<sup>7</sup>. A meta-analysis combining 12 studies and containing 5,909 individuals aged 25 or older, found that N363S carriers had increased BMI (+0.51 kg/m<sup>2</sup>) compared to non-carriers<sup>0</sup>. In addition to increased BMI, the SNP is associated with higher WHR, increased cortisol suppression, and increased salivary cortisol in response to an acute psychosocial stressor<sup>11</sup>. Additional GR hypersensitivity SNPs of interest are RS7701443 and RS2963156<sup>24</sup>.

The ER22/23EK SNP is a linked GAG.AGG→GAA.AAG nucleotide change in codons 22 and 23 of the transactivation domain<sup>7</sup>. The first nucleotide change is silent, as both forms code for glutamic acid (E)<sup>16</sup>. However, the second nucleotide change switches the amino acid from arginine (R) to lysine (K)<sup>16</sup>. This variant is associated with GC resistance, as carriers have a

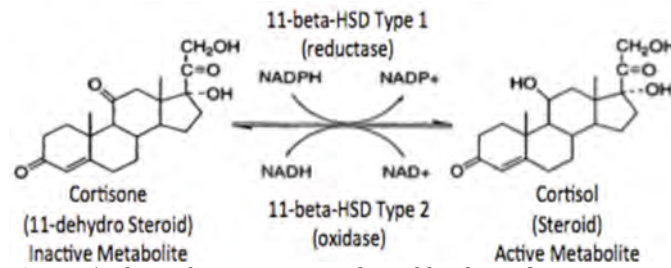
decreased response to DST<sup>7,11,15,16</sup>. They also had lower fasting insulin levels, and lower lipid profiles than noncarriers<sup>16</sup>. The polymorphism is associated with recurrent depression and improved responses to antidepressant medication<sup>7</sup>. Multiple studies comparing GR SNP haplotype frequencies in depressed versus normal populations found that the unipolar MDD group carrier frequency was 8-11% compared to a healthy control group where only 4% carried the polymorphism<sup>7</sup>.

Another GR resistance polymorphism is Tth///1, which is an SNP caused by a C→T change 6,305 base pairs upstream of the first initiation codon<sup>0</sup>. Initial findings suggested that Tth///1 carriers had higher basal cortisol levels, due to altered promoter activity, however this is still inconclusive, as other research suggests that Tth///1 only has effects when expressed in combination with other SNPs including ER22/23EK or Bcll<sup>0</sup>.

Glucocorticoids have numerous, differential effects based on an individual's glucocorticoid sensitivity. This sensitivity, and glucocorticoids subsequent action on target tissues, is, in part, determined by genetics and the functional NR3C1 polymorphisms listed above. In addition, steroid access to MRs and the enzyme 11β-HSD also play critical roles in regulating glucocorticoid concentrations.



The 11 $\beta$ -HSD I and II isoforms regulate how much active metabolite is available to bind to receptor<sup>24</sup>. Through conversion, 11 $\beta$ -HSDII the active metabolite, into the inactive metabolite, cortisone, and adipose tissue<sup>19</sup>. This reservoir of potential cortisol stored away in fat. The type I isoform works to access this stored cortisone by converting it into cortisol<sup>25,26</sup>. 11 $\beta$ -HSD is localized in adipose, liver, and lung tissue<sup>2</sup>. 11 $\beta$ -HSDII often guards MRs, converting cortisol into its inactive metabolite before it can act on the receptor<sup>2</sup>. This is why GR polymorphisms are more strongly associated with HPA dysregulation, even though glucocorticoids have greater affinity for MRs than GRs. Therefore, SNPs leading to 11 $\beta$ -HSD I overactivity are also of special interest as they are pertinent to HPA axis regulation, and can cause dysregulations that promote neuropsychological disease, metabolic syndrome, and related comorbidities<sup>25,26</sup>. Two Identified SNPs that induce 11 $\beta$ -HSD I overactivity are RS12086634 and RS846910<sup>25,26</sup>.



**Figure 5.** The Redox reaction conducted by the 11 $\beta$ -HSD isoforms, in order to peripherally convert cortisone into cortisol, and vice-versa

The big picture is that the GR plays a vital role in mammalian function. The GR and its associated polymorphisms impact HPA axis control and cortisol levels. A study by Tronche et al., (1998) found that GR knockout mice were unviable, dying within minutes after birth due to collapsed lungs as a result of uncontrolled inflammation<sup>3</sup>.

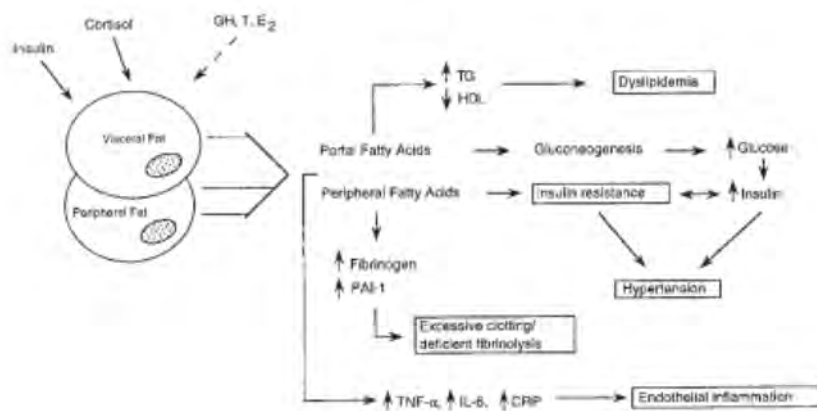
Neuropsychiatric treatments and their effects on HPA regulation

Numerous antidepressants currently on the market have been associated with decreasing the pituitary-adrenal hyperactivity common in typical depression. These include tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), Bupropion, Mirtazapine (alpha<sub>2</sub>-adrenergic antagonist), and electroconvulsive shock therapy<sup>2</sup>. Longitudinal studies where the aforementioned treatments were provided individually or in combination showed increased ACTH response to the CRH/DST as well as the normalization of HPA hormone secretion to circadian patterns and a reduction in adrenal gland volume if enlarged<sup>2</sup>. However, these findings are not universal across other similar studies.

From these treatments, it is understood that good treatment can overcome receptor deficits, by increasing/improving either the function or expression of the receptors, however we do not yet understand how antidepressants mechanistically normalize HPA activity<sup>2</sup>. However, it has recently been found that antidepressants affect GR expression differently in different parts of the brain<sup>10</sup>. Atypical depression, for example, could be successfully treated with either glucocorticoid interference or glucocorticoid supplementation. Glucocorticoid interference would be necessary if the GR is overactive or over-stimulated by high levels of glucocorticoids. Glucocorticoid supplementation would be necessary if the receptor is hyposensitive to cortisol or if cortisol levels are just too low. Therefore, in order to properly treat an individual, we need to know their cortisol levels in combination with their GR and 11β-HSD haplotype, meaning the SNPs they carry. Understanding and accounting for patient heterogeneity within the context of HPA regulation is the largest obstacle to overcome in order to successfully treat depression.

HPA Axis Overactivity and Obesity

Factors in major depression are directly linked to metabolic dysfunction, and even promote susceptibility to heart disease<sup>27</sup>. Since cortisol is essential to lipid and carbohydrate homeostasis, HPA overactivity often induces metabolic dysfunctions leading to, for example, Cushing’s syndrome (CS), which is characterized by hypercortisolemia<sup>18,27</sup>. For this reason, CS is associated with MDD, anxiety disorder, other psychological diseases, and even reduced hippocampal volume<sup>7</sup>. Classically, CS patients present obesity related symptoms including dyslipidemia, hypertension, glucose intolerance, and increased visceral adiposity<sup>1,18,19</sup>.



**Figure 7**<sup>27</sup>. Factors in major depression that promotes metabolic dysregulation and related comorbidities.

The World Health Organization reports that worldwide obesity has more than doubled since 1980<sup>20</sup>. With the increased rates in obesity comes a rise in obesity related symptoms including insulin resistance, high triglycerides, high blood pressure, low HDL-cholesterol and central adiposity<sup>19</sup>. Having three out of the five stated symptoms is sufficient in order to diagnose Metabolic Syndrome (MS)<sup>19</sup>. Patients with CS and MS share all of the same symptoms, except while CS is characterized by hypercortisolemia, MS is associated with normal

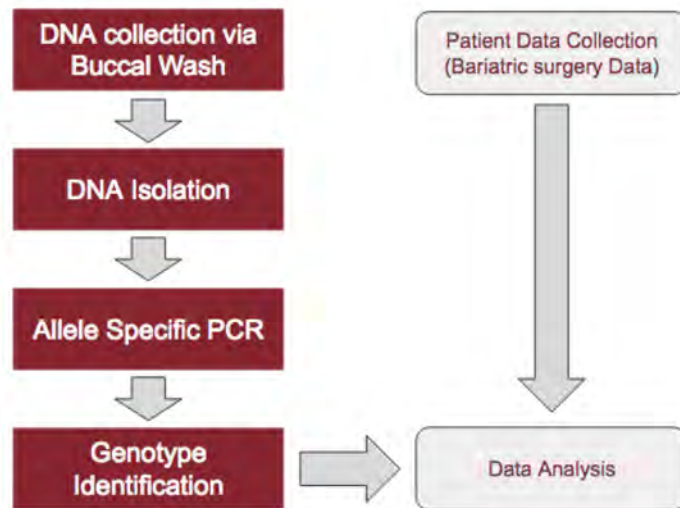
or even low cortisol levels. The lack in a mechanistic understanding of MS has led to the following hypothesis: MS may be a cushingoid-like state with normal cortisol levels, accompanied by GR hypersensitivity and/or overactive 11 $\beta$ -HSD I.

The increased rate of obesity is linked with the popularization of bariatric surgery-- a method to treat morbid obesity. Currently there are three main forms of bariatric surgery: the Roux-en-Y gastric bypass, the sleeve gastrectomy and the lap-band. Studies show that bariatric surgery can greatly alter metabolic pathways, inducing HPA axis dysregulation, which affects weight loss and leads to comorbidities including hypertension, hyperlipidemia, and depression, through mechanisms explained previously<sup>22</sup>. Therefore, the post-bariatric population works as an experimental group representative of MS.

The purpose of this study is to first determine the prevalence of GR hypersensitivity SNPs (BclI, N363S, RS7701443, RS2963156), GR resistance SNPs (TthIII1, ER22/23EK), and 11 $\beta$ -HSD SNPs (RS12086634, RS846910), in an obese, post-bariatric population compared to a control or random population composed of college students. In addition, we want to see how the presence of individual SNPs and haplotypes correlate with metabolic parameters, including BMI, blood glucose, serum triglycerides, HDL and LDL cholesterol, and systolic and diastolic blood pressure in the bariatric patient population. We also hope to determine whether there is a correlation between the presence of certain SNPs or haplotypes and the success of specific bariatric surgeries. We hope that these findings can aid both healthcare providers and potential bariatric patients when determining how to best treat or manage their weight.



## Methods



**Figure 8.** Summary of Research Approach

### DNA Collection – Ellis Bariatric Center

DNA was collected from bariatric patients at Ellis Bariatric Center in Schenectady, NY. Ellis Hospital’s Institutional Review Board approved the study prior to collection beginning (Appendix A). Prior to saliva collection, all potential participants were instructed on the purpose of the study and provided with the option to sign an informed consent form (Appendix B). If consent was granted, the participant would pour ~10 mLs of a 0.1% saline solution in their mouth, swish it around for 20-30 seconds, and spit it out into a collection cup. The wash product was transferred into a 15 mL centrifuge tube and labeled with the participant’s medical record number (MRN).

### DNA Isolation – Ellis Bariatric Patients

Chelex 100 beads by Bio-Rad© protocol was used to isolate the DNA (Appendix C).

### Isolated DNA collection—Union College

Union College's Human Subjects Review Committee approved this study prior to DNA collection (Appendix D). The Union students who participated in this study had previously collected and isolated their DNA using the same methods as described above. After instructing them on the purpose of the study, Union students were given an instruction sheet with a random sample number and a few questions (Appendix D), and an informed consent form (Appendix B). If they consented, they filled out the questions and transferred the contents of their remaining isolated DNA into a 1.5 mL centrifuge tube, which they then labeled with the random number on their instruction sheet.

### Data Collection – Ellis Bariatric Center

The relevant surgery and metabolic parameters of participating patients was collected by searching the individuals MRN in Ellis Bariatric Center's electronic medical records (EMR). Type of surgery, surgery date, pre-surgery weight (PSW), PSW date, original weight, height, starting BMI, lowest weight (LW), LW date, lowest BMI, current weight (CW), CW date, current BMI, fasting blood glucose, serum triglycerides, Serum LDL, Serum HDL, systolic blood pressure and diastolic blood pressure was collected. Using that information, ideal body weight, excess body weight (EBW), weight lost, and %EBW lost, was calculated.

### Allele-specific PCR

By entering the known DNA sequences of desired SNP's into Web Based Allele Specific Primer (WASP), wild-type (WT) and mutant (MT) primers were designed to bind specifically to a target polymorphism. Based on annealing temperatures, a desired primer set, consisting of common primer that ran in opposition to both the WT and MT primer, was selected (See Appendix E for various WASP results and selected primer information).

PCR tubes had a total volume of 50  $\mu$ L, which was broken down into the component reagents according to Table 1 below.

**Table 1.** Reagent Quantities per PCR Tube

Reagent	Quantity
1) Common Primer	1 $\mu$ L
2) WT or MT Primer	1 $\mu$ L
3) DNA Template	8 $\mu$ L
4) dNTPs	0.5 $\mu$ L
5) Taq polymerase	0.5 $\mu$ L
6) Buffer	5 $\mu$ L
7) DI Water	34 $\mu$ L

**Table 2.** Tested Oligonucleotides

Primer	Common	Wild Type	Mutant



BclI	BclI.down.new	BclI.up.WT.new	BclI.up.MUT.new
N363S	N363.2/4F	N363S.down.WT.new	N363S.down.MUT.new
RS120	rs12086634.com.f	rs12086634.wt.r	rs12086634.mut.r
RS84	rs846910.com.r	RS846910.wt.f	RS846910.mut.f
RS770	rs7701443.gr.com.r	rs7701443.gr.wt.f	rs7701443.gr.mut.f
RS296	rs2963156.gr.com.r	rs2963156.gr.wt.f	rs2963156.gr.mut.f
TthIII1	rs10052957.TthIII1.com.f	rs10052957.TthIII1.wt.r	rs10052957.TthIII1.mut.r
ER/EK	rs6190.EREK.com.f	rs6190.EREK.wt.r	rs6190.EREK.mut.r

### Gel Electrophoresis

Gel electrophoresis was used to analyze primer-binding efficiency, in order to first optimize the primer sets, and then subsequently genotype patients. The PCR reactions were processed through a 3% gel at 100 V, which ran for roughly 30 minutes. The gel was stained with ethidium bromide so bands could be detected under the UV light from G:BOX SYNGENE (Appendix G).

## **Results**

### PCR optimization

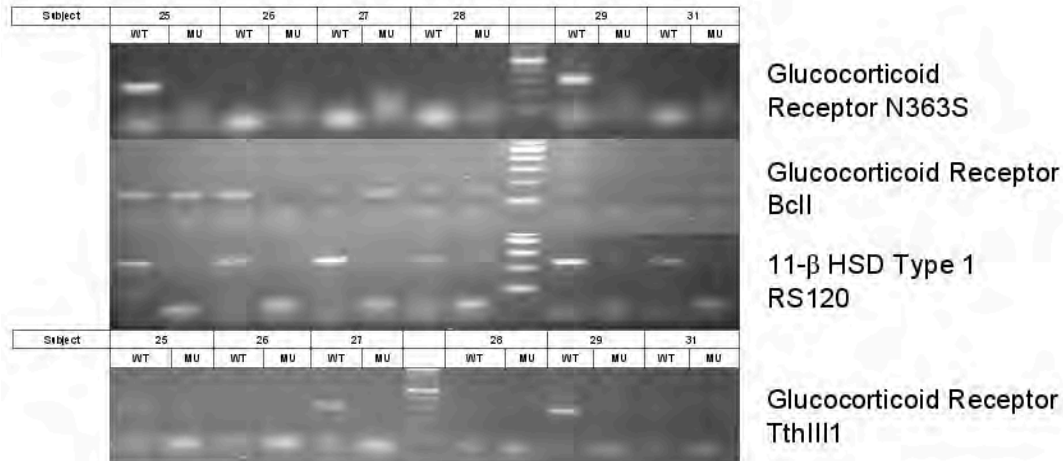
Using the annealing temperature calculated by the selected WASP result as a starting point, various PCR trials were run for each SNP until an Optimized annealing temperature was identified. PCR reactions were run in a Bio Rad C1000 Thermal Cycler. As seen in Table 3 below, six of the eight target SNPs were originally optimized.

**Table 3. Optimized PCR Annealing Temperature for each SNP**

Polymorphism	Annealing Temp (°C)
Bcl1	52°
N363	55°
RS12086634	52°
RS846910*	-
RS7701443	52°
RS2963156	55°
TthIII1	61°
ER/EK*	-
* Signifies failure to optimize	

Unfortunately, due to reasons still not fully understood, *BclI*, N363S, TthIII1, and RS12086634 were the only primers that continued to work throughout the entire genotyping process.

## Genotyping of Participants



**Figure 9.** An aggregation of the gel electrophoresis results for each of the four fully optimized SNPs. This figure includes subjects 25, 26, 27, 28, 29 & 31 from the Union College student population

Genotyping was determined based on the method described in the Gel Electrophoresis section.

Appendix G has additional agarose gel electrophoresis results. Figure 9 demonstrates what the genotyping looked like. A complete account of individual genotype and relevant clinical information is in appendix H.

## Prevalence of the SNPs

Based on the genotyping results, the total genotypic and allelic frequencies of both the random and the bariatric populations could be compared for each optimized SNP.

**Table 4.** The genotypic and allelic break down for all successful Ellis patients and Union students

Gene Polymorphism			Genotype Frequency				Allele Frequency							
Name	Name	Phenotype of mutant	Random population				Bariatric population				Random population		Bariatric population	
			WT	Hetero	MT	n	WT	Hetero	MT	n	WT	MT	WT	MT
GR	RS41423247 (BclI)	Hypersensitivity to cortisol	47	44.2	9.3	43	37	48.1	15	79	68.6	31.4	60.8	39.2
GR	RS6195 (N363S)	Hypersensitivity to cortisol	93	4.9	2.4	41	91	9.3	0	54	95.1	4.9	95.4	4.6
GR	RS10052957 (TthIII1)	Resistance to cortisol	92	2.8	5.6	36	97	3.4	0	29	93.1	6.94	98.3	1.7
11b-HSD Type 1	RS12086634	Type II Diabetes	75	21.9	3.1	32	61	30.4	8.7	23	85.9	14.1	76.1	23.9

There was no significant difference

between the random and bariatric population for the genotypic and allelic frequencies of NR3C1 hypersensitivity SNPs (*BclI* and N363S). However, *TthIII1*, a GR resistance SNP, was present at a much lower allelic frequency in the bariatric population compared to the random population tested and the frequency listed in the literature. In addition, the RS12086634 SNP, associated with overactive 11 $\beta$ -HSD I enzyme activity, was present at a much higher frequency in the bariatric population compared to the random population.

## Discussion:

In this study, we hypothesized that metabolic syndrome may be a cushingoid-like state with normal cortisol levels, accompanied by GR hypersensitivity and/or overactive 11 $\beta$ -HSD I. Based on our collected allelic frequencies, we found that the bariatric population not only had increased peripheral conversion of cortisone into the active metabolite cortisol, but it also lacked cortisol resistance polymorphisms. These allelic frequencies support our hypothesis that cortisol signaling and regulation is a contributing factor to morbid obesity, and can potentially bridge the mechanistic gap between Cushing's Syndrome and Metabolic Syndrome.

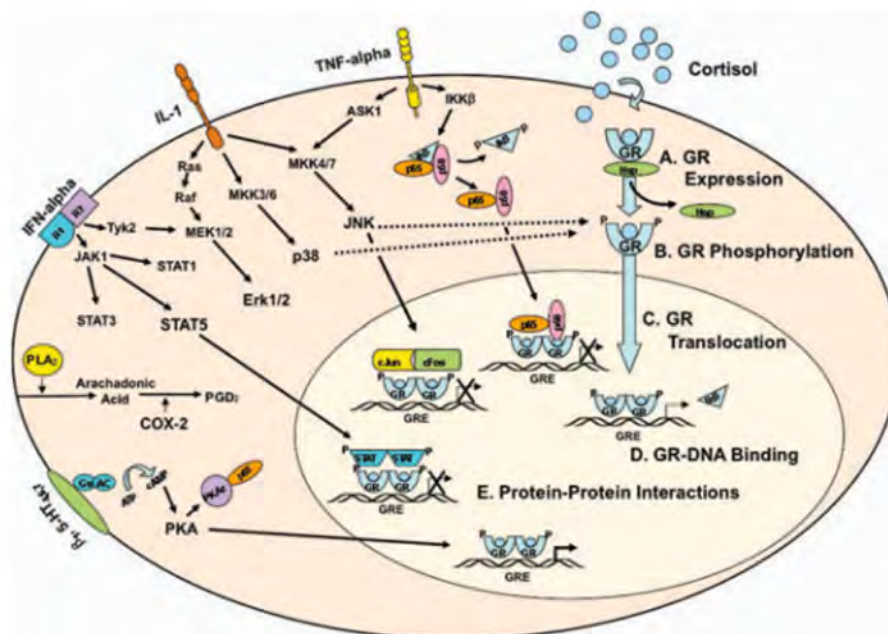
There is plenty of statistical analysis that still needs to be done to the collected data, which will aid in strengthening conclusions. First, haplotype analysis must be performed extensively. As of now, all that is known is the overall allele frequencies of the four optimized SNPs. Haplotype analysis is necessary in order to examine whether certain SNP combinations have larger impacts on HPA axis dysregulation than they would independently. It will be helpful to compare allelic frequencies with metabolic parameters and bariatric surgery success.

In addition, to adding haplotype analysis, we must broaden our approach by not just examining more SNPs within the current areas of study (NR3C1 and 11 $\beta$ -HSD I), but looking to see how other receptors and genes and signaling factors play a role. The MR is very much impacted by glucocorticoid signaling and plays a role in HPA axis regulation. Studies by Spijker et al., (2009) suggest that there is a connected pathophysiology between the GR and the MR involved in glucocorticoid signaling.

The MR becomes even more relevant when this research project begins genotyping depressed individuals, for cerebral MRs are not guarded by  $11\beta$ -HSD II, and therefore play a stronger role impacting neuropsychology compared to metabolism.

There are two other interesting areas to explore that could reveal relevant information about HPA axis regulation. Considering that only the free, unbound glucocorticoids can have a biological effect, levels of CBG, may influence cortisol bioavailability<sup>1</sup>. Severe CBG mutations have been associated with both chronic fatigue syndrome (CFS) and increased fat distribution<sup>28,29</sup>. There was also a correlation with hypocortisolism, a classic culprit for CFS<sup>29</sup>.

The second new area to introduce, which would improve this study, is to look more in depth at glucocorticoid receptor signaling. Clearly, a variety of factors including glucocorticoid concentrations, local glucocorticoid availability, HPA regulation, genetics/relevant polymorphisms, and NR3C1 receptor affinity all affect glucocorticoid sensitivity. Another interesting factor are disruptions in glucocorticoid signaling. Major depression is often characterized by HPA axis overactivity and high levels of cytokines<sup>17</sup>. In fact, inflammation is a key feature in some depression and obesity cases. A study with 35 healthy men and women identified a shared pathophysiology of immune dysregulation in both depression and obesity<sup>8</sup>. How is this possible if cortisol, in addition to cortisol's many effects mentioned earlier, is also acts as a strong anti-inflammatory? It has to do with the way cytokine signals access the brain and interact with pathophysiologic pathways (i.e. disrupt GR activity) relevant to mood regulation<sup>17</sup>.



**Figure 6<sup>17</sup>.** Cytokine-Glucocorticoid Receptor signaling pathways interactions These simplified signal transduction interactions demonstrates the complex inhibitory and induction relationships between cortisol, the GR and cytokines.

Cheng et al., (2013) discovered that inflammation regulated by GC-GR is the pathophysiological link between depressive symptoms and obesity. They identified a peripheral loop of bidirectional association within the GR signaling pathway when in the presence of inflammation. The peripheral blood monocytes of stressed individuals' contained lower levels of glucocorticoid response element expression, but an increase in inflammatory transcription factors<sup>8</sup>. Varying cytokines impacted the HPA axis, disrupting GR secretion, GR translation and GR posttranslational modifications. Therefore, depression induces inflammation dysregulation via GR insensitivity to glucocorticoids on immune cells, which results in an inflammation dysregulation that in turn promotes further glucocorticoid insensitivity<sup>8</sup>.

Due to the feasibility of acquiring a metabolic syndrome patient population compared to a depressed patient population, this study was first initiated on a bariatric population. Because of the connectedness of depression, specifically MDD, with dyslipidemia, insulin resistance and hypertension, we can extend this exact research method to depression. The Institutional Review Board at Albany Medical College has approved that very project, which will hopefully begin the summer of 2017.



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## Appendix

A- Ellis IRB Form and approval

**B- ELLIS HOSPITAL**  
**C- INSTITUTIONAL REVIEW BOARD**  
**D- APPLICATION FOR RESEARCH PROJECT**

E-

F- Project:

G- Prevalence of Glucocorticoid Receptor Polymorphisms in Morbidly Obese Patients

H-

I- Name, Title and Position of Principal Investigator (s):

J- Anne Jones, RN

K-

L- Co-Investigators:

M- Brian D. Cohen, PhD, Senior Lecturer, Union College Dept. of Biological Sciences

N-

O- Purpose of Project:

P- To characterize the presence of glucocorticoid receptor polymorphisms associated with obesity in patients undergoing bariatric surgery procedures.

Q-

R- Describe Population for Study:

S- The population for the study will be patients who are preparing for or have undergone bariatric surgery procedures for treatment of morbid obesity.

T-

U- Has IND # been obtained, if applicable: \_\_\_\_\_ IND# \_\_\_\_\_

V- Has NDE # been obtained, if applicable: \_\_\_\_\_ NDE# \_\_\_\_\_

W-

X-

Y- Probable Time Project May Take:

Z- Initial studies are projected for 5 months but may extend depending on enrollment rate

AA-

BB- Probable Starting Date :

CC- 1/5/14

DD-

EE- Potential Hazards and Precautions:

FF- There are no potential hazards or precautions for the subjects or the investigators

GG-

**HH- Potential Inconveniences or Discomforts to Subjects and Other Pertinent Clinical Information:**

**II- Subjects will be asked to swish a sterile saline solution in their mouths for 30 seconds which some subjects may find unpleasant.**

**JJ-**

**KK- Estimated Number of Human Subjects Required:**

**LL- We are currently targeting 100 subjects as a starting group, although we are hoping for a larger subject pool if possible**

**MM-**

**NN- Estimated Duration of Study:**

**OO- Initial study is projected for 5 months, although extension of the study may be requested depending on the rate of enrollment of subjects.**

**PP-**

**QQ- Have You Discussed This Protocol With Other Practitioners Affected?**

**RR- YES\_\_\_ NO\_X\_\_ If Yes, with Whom?**

**SS-**

**TT-**

**UU- Approval Must be Obtained from the Director of Pharmacy for Protocols**

**VV- Involving Investigational New Drugs Before IRB Review.**

**WW-**

**XX- Not Applicable**

**YY-**

\_\_\_\_\_  
**Director of Pharmacy**

**ZZ-**

**AAA- Approval Must be Obtained from the Director of Materials Management (Purchasing) for Protocols Involving Non-Approved Devices or the Purchase of New Devices Before IRB Review.**

**BBB-**

**CCC- Not Applicable**

**DDD-**

\_\_\_\_\_  
**Director of**

**Materials Management**

**EEE-**

**FFF- Source of Funds:**

**GGG- Materials for isolating and analyzing patient DNA will be provided from the Union College Student Research Grant Fund and the Union College Faculty Research Fund**

**HHH-**

**III- Facilities to be Utilized:**

**JJJ- DNA sample, demographic, and clinical information collection will be done at the Ellis Bariatric Offices**

**KKK- DNA analysis will be done at Union College in Dr. Cohen's research lab.**

LLL-

MMM- Have you or are you in the process of undertaking training or certification in the area of Human Subject Research?

NNN- Yes \_\_\_\_\_ No

OOO-

PPP- If Yes, Please provide a copy of the certification with this application.

QQQ-

RRR-

SSS- Do you have any Relationship with the Study Sponsor, Manufacturer of the Device or Medication in which you are investigating that should be disclosed?

TTT- Yes \_\_\_\_\_ No

UUU-

VVV- If Yes, please explain:

---

WWW-

XXX- *Note: Research that has been approved by the IRB may be subject to further appropriate review by Hospital administration if Ellis Hospital's resources are to be utilized in this study.*

YYY-

ZZZ- Signature of Department Chair/Division

Chief: \_\_\_\_\_

AAAA-

BBBB- *Ten (10) copies of the Protocol and/or Informed Consent need to be delivered (electronic copy preferred) to the IRB Chair, Michael Pasquarella, A-Ground, Ellis Hospital, at least One Week prior to the meeting.*

CCCC-

**Ellis**  
MEDICINE

Bellevue Woman's Center  
Ellis Health Center  
Ellis Hospital

المنارة للاستشارات

December 23, 2014

Anne Jones RN

Brian D Cohen PhD

Ellis Medicine Bariatric Care Center

2125 River Road Suite #302

Niskayuna, NY 12309

Re: Prevalence of Glucocorticoid Receptor Polymorphisms in Morbidly Obese Patients

Dear Ann and Dr. Cohen

Thank you for attending the December 18, 2014 meeting of the Institutional Review Board. Under full board review the protocol, informed consent (with changes) and other materials presented for the above mentioned study were approved. The study was approved for 2 years with an interim report due at the June 2015 IRB meeting. As a reminder, no additional changes may be made to this project without first submitting the changes to the IRB for review. Any inquiries or unanticipated problems must also be promptly reported.

Thank you for your continued interest in medical research.

Sincerely,

*Michael V. Pasquarella*

Michael V. Pasquarella Pharm.D., R.Ph.

Chairperson

Institutional Review Board

cc: Pat Biggica

## B- Ellis Participant Informed Consent

### Informed Consent Form

The purpose of this research project is to look for a correlation between polymorphisms of the glucocorticoid receptor (variations in the DNA sequence of the natural receptor for the hormone cortisol) and clinical manifestations of obesity and related comorbidities such as elevated blood sugar, blood pressure, increased waist/hip circumference ratio, and altered serum lipid profiles.

Agreeing to participate in this study means that you will allow the testing of your DNA for the purposes of identifying receptor variations and you are agreeing for relevant medical data to be provided to the researchers to compare with the receptor variations. You will not receive information about your individual results from this study. Your DNA will not be used for any other purpose or analyzed in any other way. After 2 years, your DNA sample will be destroyed.

Your decision about whether or not to participate will not affect your treatment by Ellis Bariatric Medicine. You will not be compensated for your participation in the study.

If you decide to participate you will be asked to perform a mouthwash with sterile saline that will allow us to recover cells from inside your cheek. Some people find the salty taste of the saline wash a little unpleasant but should provide no significant discomfort or risk for you.

Identification of which genetic variant(s) of this gene you have will not affect your treatment in any way. As information is gathered about the relationship between variations of the cortisol receptor gene and obesity (and related diseases), it may eventually help identify opportunities for complimentary therapies for obesity, but this is beyond the scope of the present study. Currently we are strictly interested in determining if there is a connection between this gene and obesity (and related diseases) and any discovery will not affect your treatment plan.

Your DNA sample will be coded with your patient identification number instead of your name before it is given to researchers at Union College for DNA analysis. The researchers will also be given access anonymously to relevant medical information from your records required to complete the study. Records given to Union College researchers will only have your patient ID number and not your name. Relevant medical information will include but not strictly be limited to:

1. Your weight before you began treatment
2. Your height
3. Your waist to hip ratio or waist circumference (where available) before you began treatment
4. Fasting blood sugar
5. Serum triglycerides



6. Serum LDL and HDL
7. Blood pressure
8. Related medications that you are taking that might affect these measures
9. Treatments you receive at Ellis Bariatric such as medications and surgical procedures

Your DNA sample will be kept in the laboratory of Dr. Brian Cohen at Union College and will only be accessible to him or to his student researchers working on this research project. Similarly, relevant medical information made available to Dr. Cohen will only be available to him or his student researchers and will not be accessible to anyone else at Union College.

Although we do not anticipate making any discoveries that would alter your care or cause you to wish to drop out of the study, if any such discoveries are made the medical staff of Ellis Bariatric will contact you and give you the option to withdraw from the study. Choosing to withdraw from the study will in no way affect your care as a patient of Ellis Bariatric. We anticipate that more than 100 patients will be a part of the study and the more patients that are included, the more significant any findings will be.

If you have any questions, please ask Anne Jones, RN, CBN, at Ellis Bariatric or Dr. Brian Cohen, Union College. Their contact information can be found below. You will be asked to sign one copy of this informed consent form and will be given a copy to keep for your records.

The choice to be in this study and to stay in this study is strictly voluntary. Refusal to participate will involve no penalty or loss of benefits which you are otherwise entitled. You may discontinue your participation at any time with no penalty or loss of benefits which you are otherwise entitled. If you wish to leave the study, please contact Ms. Anne Jones and inform her that you wish to withdraw from the study. You will be asked to sign a written form indicating your desire to withdraw. Ellis Bariatric will then inform Dr. Cohen of your ID number and your DNA sample will be destroyed and your relevant medical information will be removed from the data set.

Thank you for considering being a part of this research study. If you have any questions before participating or at any time during the study, please do not hesitate to contact us.

Anne Jones, RN, CBN  
Ellis Bariatric Medicine  
[jonesa@ellismedicine.org](mailto:jonesa@ellismedicine.org)  
518-831-7030

Brian D. Cohen, PhD  
Union College  
[cohenb@union.edu](mailto:cohenb@union.edu)  
518-388-8018

Michael Pasquarella, PharmD, RPh  
Chair, Institutional Review Board  
Ellis Hospital  
[pasquarellam@ellismedicine.org](mailto:pasquarellam@ellismedicine.org)  
518-243-1818

1. I have been informed about the purpose of this genetic test and experimental research project.
2. I understand that I will not receive the results from the testing.
3. I have been informed as to who may have access to my biological sample, and that the laboratory may retain any leftover sample until the end of the study, at which point it will be destroyed.
4. I have read the material provided and this consent form in full. My questions have been answered to my satisfaction.
5. I consent to provide a sample for genetic testing and to have relevant medical data provided anonymously to the researchers.
6. I understand that my participation is completely voluntary and I may withdraw at any time without affecting my care as a patient of Ellis Bariatric.

---

*Signature*

*Date*

## C – DNA Extraction Protocol

### Isolation of Crude Human Genomic DNA samples suitable for PCR

The source of DNA that you will use for PCR reactions next week will be your cheek cells, which will be obtained by a sterile saline mouthwash. The cells are collected by centrifugation and resuspend in a solution containing the resin "Chelex", which binds metal ions that would otherwise inhibit the PCR reaction. The cells are then lysed by boiling, and centrifuged to remove cell debris and the Chelex resin. The result is a crude genomic DNA prep that is "good" enough for PCR!

You should have the following materials available **before** you start:

#### **Materials:**

Microfuge tubes and racks; micropipettors and tips  
One 15 ml plastic "Blue-top" tube  
One Non-sterile (but unused) plastic "Dixie" cup  
One plastic transfer pipette  
Labelled Waste container for "spit" waste  
Microfuge tube containing 1.2 ml of 10% Chelex (labeled "Chelex")

#### **Shared Materials:**

Clinical Table-top Centrifuge for pelleting cells  
Boiling water baths with "floatie" for boiling samples  
Microfuge

- 1) Get a boiling water bath ready for use in step 8. Fill up an ice bucket halfway using crushed ice obtained from the Biology "autoclave room" on the third floor.
- 2) Label a 15 ml tube (blue-top) with your initials on the top and the side of the tube, and pipette (or carefully pour) 10 ml of sterile saline into the tube. Label a new microfuge tube with your initials, also on the top and side of the tube.
- 3) Pour all of the saline solution into your mouth (don't swallow it!), and vigorously swish for 15 seconds. Carefully expel the fluid into a plastic Dixie cup, and then pour the liquid back into the 15 ml tube and reclose the cap.
- 4) Place your tube in a clinical centrifuge balanced against another tube opposite it in the rotor. Try and fill up the rotor if you can with tubes from other members of the class. Centrifuge for 10 minutes at 1,000 X g for 10 minutes to pellet the cells.
- 5) After the spin is over, use a transfer pipette to pipette off as much of the supernatant as possible into the labeled waste container (we'll autoclave this later). Pipette off the liquid so that the cells remain with only a minimal amount of liquid (approx 200  $\mu$ L). You might have to briefly (1 min.) spin the tube again if the cells get dislodged from the side/bottom of the tube during this process. Resuspend the cells in the minimal volume of remaining supernatant by vortexing, and then transfer the whole volume using the same plastic transfer pipette to a new, labelled microfuge tube. Vortex this tube containing the cells for 5-10 seconds, making sure that there are no clumps of cells remaining.
- 6) Resuspend the Chelex beads (in the microfuge tube labeled "10% Chelex") by vortexing for 10 seconds. Before the beads have

had a chance to settle, pipette 500  $\mu$ L of Chelex solution into your microfuge tube containing the cells.

7) Resuspend the cells in the Chelex by vortexing for 10 seconds. Make sure that no cell clumps remain.

8) Incubate the microfuge tube containing the cells/Chelex mixture in the boiling water bath for 10 minutes, using a plastic "floatie". Other peoples' tubes will also be boiled at the same time in the water bath, so make sure that your tube is labelled well!

9) Remove the floatie carefully from the boiling water bath, take your tube and incubate it on ice for two minutes. Spin the tube in a microfuge (max. speed) for 1 minute to pellet the Chelex and cell debris to the bottom of the tube.

10) Using a 200  $\mu$ L pipette, carefully transfer 200  $\mu$ L of the supernatant (pipette from the top) to a new, labeled (with your initials) microfuge tube. Avoid transferring any of the pelleted cells or the Chelex. Discard the tube containing the pellet. YOUR PREP IS DONE AT THIS POINT! You should store your genomic DNA in the fridge in a labeled box or tube rack.

# UNION COLLEGE HUMAN SUBJECTS REVIEW COMMITTEE

## 2016-2017 APPLICATION TO ENGAGE IN RESEARCH INVOLVING HUMAN SUBJECTS

For full instructions, please visit <http://bit.ly/hsrc>

Please type your responses in the fields provided. The grey fields will expand if more space is needed. When completed, please print and deliver to **Joshua Hart**, Chair of the Human Subjects Review Committee, Bailey Hall 302. Emailed, hand-printed, or unsigned applications will be returned unreviewed. Blue links can be followed by holding the “control” key and clicking.

- 1a. Name of student researcher (if applicable) > Justin Salm  
Box number > 1826  
Email address > [salmj@union.edu](mailto:salmj@union.edu)  
Major > Biology
- 1b. Name of student researcher (if applicable) > Brianna Godlewski  
Box number > 703  
Email address > [godlewsb@union.edu](mailto:godlewsb@union.edu)  
Major > Biology
2. Name of faculty researcher or sponsor > Brian Cohen  
Office location > Wold 220  
Email address > [cohenb@union.edu](mailto:cohenb@union.edu)  
Department > Biology
3. Title of project > Prevalence of Polymorphisms in the stress related genes in an Undergraduate Population
4. Is this research funded? > SRG funds requested for materials  
If yes, by whom? >
5. Approximate number of participants: > 64  
Approximate age range of participants: > 18-21  
Other important characteristics of participants (e.g., prisoners, minors, participants in poor mental or physical health, etc.) > None
6. Where will the data be collected? > Union College
7. If data will be collected at a location other than Union College’s campus, identify how you secured permission from the appropriate individuals at the other location(s). Include this individual’s name and contact information. > N/A
8. How will participants be sampled, recruited, or otherwise enlisted? > Participants will be BIO 225 Students

9. What rewards, payment, or other credit will be provided for participation, if any? > None

10. How will the anonymity of participants and/or the confidentiality of the data be ensured? > The DNA samples will be given a random number and students will be asked to transfer the DNA into the new tube so that the tubes will not have any other identifying marks. The number on the DNA sample will be correlated with the form containing information on whether the student has previously been diagnosed with depression and the students height and weight. The form will have no identifying information other than the matching number for the DNA sample. Once filled out, the student will fold the form, tape it shut, and hand it to their lab professor. The lab professor will then give the completed forms and related saliva samples to the researchers.

For the following items, indicate YES or NO. If the answer is YES, please explain.

11. Is it reasonably possible that any of the participants will be placed at risk with regard to physical pain or discomfort, psychological stress or discomfort, or social injury (e.g., diminished reputation or damaged social or personal relationships)? > No

12. Will information that might affect participants' willingness to participate be withheld from them prior to securing informed consent to take part in the research? > No

13. Will there be any coercion or penalties that might negate a participant's freedom to refuse to participate in the study or withdraw from participation? > No

14. Will any of the researchers who will be conducting the study be placed at risk with regard to physical or psychological pain, discomfort, or harm? > No

15. Will any deception be involved? If so, explain the nature of the deception, the need for the deception, and how risks from that deception will be mitigated. > No

16. Will topics or questions about depression or about thoughts of or attempts to engage in of self-injury or suicide be included? > Yes. For part a of this project, the goal is to establish a correlation between specific gene polymorphisms in an undergraduate student population and depression. For this research, it is imperative to know whether the DNA sample belonged to an individual with depression. However, due to the precautions explained in question 10 above, this information can only be related back to the DNA sample, not the student.

For the following items, indicate YES or NO. If the answer is NO, please explain.

17. Will all promises and commitments made to the participants regarding their participation be duly honored by the researcher? > Yes

18. Will it be made clear from the onset of the study that participants are free to withdraw from the study at any time? > Yes

19. Immediately following their participation, will all participants be provided with a complete explanation (debriefing) of the nature of the study so as to eliminate any possible misconceptions about its purpose and to eliminate any stress or discomfort experienced by participants? > Yes

20. If payment is offered, immediately following their participation, will all participants be provided with payment as promised (e.g., credit for course, gift certificate, etc) ? > Yes

21. The United States Government, via [45CFR46.116\(d\)](#), states that informed consent must be obtained from participants unless, among other criteria, it is not practicable to do so.

If you do plan to obtain informed consent, please indicate how, whether it be an informed-consent form, a clickable button on a Website, or via other documentable means: > An informed-consent form

If you do not plan to obtain informed consent, please explain how your study meets each of the four criteria for waiving this requirement as set forth by 45CFR46.116(d). > N/A

22 (*OPTIONAL*). In addition to the specific explanations that may have been provided with the responses to items #11 through #21, you are welcome to provide any further comments that might help the Committee determine whether the proposed research is likely to produce benefits so significant as to outweigh any questionable or risk-producing research procedures. >

PLEASE ATTACH THE FOLLOWING APPENDICES.

APPENDIX W: Briefly explain the purpose of the research and provide a general description of the methods to be employed (200 words should be sufficient).

The research attempts to examine the presence of specific polymorphisms located in the glucocorticoid receptor, the mineralocorticoid receptor and the enzyme 11- $\beta$  HSD1 to establish a relationship between the relevant polymorphisms and depression (part a) or obesity(part b). To do so, cells extracted from patients' cheek cells can be tested for the presence of the polymorphisms using a PCR method, and data can be collected on their height and weight to find the patients' BMIs. The student population represents a control group that could then be compared to a group of depressed patients (part a) and morbidly obese bariatric patients (part b) to establish a difference in the prevalence of the polymorphisms in the different populations. Because cheek cell samples are already being collected in BIO 225 as part of an experiment, we would simply ask the students consent to use their DNA samples for our experiment as well as provide whether they have previously been diagnosed with depression and their height and weight.

APPENDIX X: Provide a copy of the informed consent materials you plan to administer to participants (unless you have made a case for not using one in #21. Whether you use the sample form found [here](#) or the OHRP checklist found [here](#), please ensure that your form contains all the elements called for by OHRP.

### INFORMED CONSENT FORM

Our names are Brianna Godlewski and Justin Salm and we are students at Union College in Schenectady, NY. We are inviting you to participate in a research study. Involvement in the study is voluntary, so you may choose to participate or not. **Participation in the study will not affect your grade in BIO225. Your instructor will not know if you have participated or not.** A description of the study is written below.

Our research is interested in determining if there is a relationship between variations in sequences of genes associated with stress responses and the prevalence of obesity and depression. You will be asked to provide access to the DNA sample that you generated for your BIO225 lab and ask you to answer a few questions including your height and weight and if you have ever been diagnosed with depression. If you choose to participate in the study, we will ask you to put your DNA from lab into a new tube labeled with an arbitrary number and also to fill out a separate form marked with the same number. This will take approximately 5 minutes. There are no foreseeable risks to taking part in this study. If you no longer wish to continue, you have the right to withdraw from the study, without penalty, at any time.

**Your responses will be anonymous: the arbitrary number will not be connected to you in any way so we will not be able to identify who filled out each form or whose DNA is being analyzed.**

All DNA samples collected will be destroyed after analysis, no later than 7/1/17.

By signing below, you indicate that you understand the information above, and that you wish to participate in this research study.

---

Participant Signature

---

Printed Name

---

Date



APPENDIX Y: Provide a copy of **all materials** to be used in your study. If an interview procedure is to be used, a detailed list of the types of questions that will be asked should be described. If participants will be exposed to any stimuli, copies of those stimuli should be presented. In the case of oral presentations, a transcript is sufficient. **To be clear: anything a participant will read, see, be asked, or answer needs to be included here.**

Instructions:

1. If you choose to participate, please sign the informed consent form.
2. Regardless of your decision to participate, please fold over the informed consent form, tape it closed, and return it to your instructor. This way neither your instructor nor your peers will be able to tell if you have chosen to participate.
3. Please transfer your DNA to the new tube, regardless of whether or not you choose to participate in the study.
4. If you choose to participate in the study, please complete the form below. If you choose not to participate in the study, do not complete the form below.
5. Regardless of your decision to participate, please fold the form over, tape it closed and return this form and your DNA to your instructor. This way neither your instructor nor your peers will be able to tell if you have chosen to participate.
6. After class we will review the forms and keep only those samples where the form has been completed.

Sample Number: \_\_\_\_\_

Height (ft and in) \_\_\_\_\_ Weight (lbs) \_\_\_\_\_

Have you ever been diagnosed with depression? (Y/N) \_\_\_\_\_

APPENDIX Z: Provide a copy of the debriefing to be presented to participants, either in text or orally. A debriefing statement is a statement presented to participants after their participation. It should provide some information about the research study in which they just participated. It need not provide detailed discussions to include literature reviews or full hypotheses, but it should provide the participants with at least a basic understanding of what the research is about. If it is not feasible to provide a debriefing, please explain why.

Because we will not know which students have chosen to participate, we do not intend to provide a debrief.

CERTIFICATION: I/we certify that:

The statements herein are factual to the best of my/our knowledge;  
I/we have described our methods and materials accurately and completely;  
I/we have not begun data collection in any way and will not do so until given HSRC approval;  
If the proposal is approved, I/we will not make any modifications to the study until receiving additional HSRC approval;  
I/we understand that the approval, if granted, expires one year from the initial approval date.

\_\_\_\_\_  
Student researcher (if applicable)      date      Faculty researcher/advisor      date

\_\_\_\_\_  
Student researcher (if applicable)      date

## E- WASP Results

i. N363S

AS Primer Picking Result for SNP2015-01-22

Input sequence 5':

```
ATTAAGGATAATGGAGATCTGGTTTTGTCAAGCCCCAGTAATGTAACACTGCCCCAA
GTGAAAACAGAAAAAGAAGATTTTCATCGAACTCTGCACCCCTG
GGGTAATTAAGCAAGAGAAACTGGGCACAGTTTACTGTCAGGCAAGCTTTCCTGGA
GCAAATATAATTGGTAATAAAATGTCTGCCATTTCTGTTTCATGG
TGTGAGTACCTCTGGAGGACAGATGTACCACTATGACATGAATACAGCATCCCTTT
CTCAACAGCAGGATCAGAAGCCTATTTTTAATGTCATTCCACCA
ATTCCCGTTGGTTCCGAAA[A/G]TTGGAATAGGTGCCAAGGATCTGGAGATGACAAC
TTGACTTCTCTGGGGACTCTGAACTTCCCTGGTTCGAACAGTT
TTTTCTAATGGCTATTCAAGCCCAGCATGAGACCAGATGTAAGCTCTCCTCCATCC
AGCTCCTCAACAGCAACAACAGGACCACCTCCCAAACCTCTGCC
TGGTGTGCTCTGATGAAGCTTCAGGATGTCATTATGGAGTCTTAACCTTGTGGAAGCT
GTAAAGTTTTCTTCAAAGAGCAGTGAAGGACAGCACAATTA
CCTATGTGCTGGAAGGAATGATTGCATCATCGATAAAATTTCGAAGAAAAAACTGCC
AGCATGCCGCTATCGAAAATGTCTTCAGGCTGGAATGAACCTG
GAAGCTCGAAAAACAAAGAAAAA
```

Sequence Size: 720

Primer Picking Parameters:

Primer Size Opt: 20 Min: 18 Max: 36  
GC% Opt: 50.0 Min: 20.0 Max: 85.0  
Tm Opt: 55.0 Min: 40.0 Max: 65.0  
Max Tm Diff: 100.0  
Max Self Complementarity: 8.0  
Max 3' Complementarity: 3.0  
Max PolyX in Primer: 3  
Number Primer Return: 5  
Mismatch at the penultimate primer position: 'Yes'  
In-silico PCR Filtering: 'No'  
Mispriming Filtering: 'No'

Oligo 1 :

Any Self End

Wildtype Forward Primer 5':

ATTCCCGTTGGTTCCGAATA

	Pos.	Len.	Tm	GC%	Self
ATTCCCGTTGGTTCCGAATA	301	20	61.41	45.00	5.00 2.00
Mutant Forward Primer 5': ATTCCCGTTGGTTCCGAATG	301	20	64.11	50.00	5.00 2.00
Common Reverse Primer 5': GCTTACATCTGGTCTCATGC	441	20	55.83	50.00	4.00 2.00

Product Size: 141

---

Pos.	Len.	Tm	GC%	Self	Any	Self
Oligo 2 :						
End						
Wildtype Forward Primer 5':						
TTCCCGTTGGTTCCGAATA	302	19	61.21	47.37	5.00	2.00
Mutant Forward Primer 5':						
TTCCCGTTGGTTCCGAATG	302	19	64.06	52.63	5.00	2.00
Common Reverse Primer 5':						
TTACATCTGGTCTCATGCTG	439	20	54.65	45.00	4.00	2.00
Product Size: 138						

---

Pos.	Len.	Tm	GC%	Self	Any	Self
Oligo 3 :						
End						
Wildtype Forward Primer 5':						
TCCCGTTGGTTCCGAATA	303	18	59.85	50.00	5.00	2.00
Mutant Forward Primer 5':						
TCCCGTTGGTTCCGAATG	303	18	62.86	55.56	5.00	2.00
Common Reverse Primer 5':						
TTACATCTGGTCTCATGCTG	439	20	54.65	45.00	4.00	2.00
Product Size: 137						

---

Pos.	Len.	Tm	GC%	Self	Any	Self
<b>Oligo 4</b>						
<b>: N363S.new</b>						
<b>Any Self End</b>						
<b>Wildtype Reverse Primer 5':</b>						
<b>AGATCCTTGGCACCTATTCCATT</b>	<b>342</b>	<b>23</b>	<b>62.17</b>	<b>43.48</b>	<b>4.00</b>	<b>1.00</b>
<b>Mutant Reverse Primer 5':</b>						
<b>AGATCCTTGGCACCTATTCCATC</b>	<b>342</b>	<b>23</b>	<b>62.67</b>	<b>47.83</b>	<b>4.00</b>	<b>0.00</b>
<b>Common Forward Primer 5':</b>						
<b>ATACAGCATCCCTTTCTCAA</b>	<b>242</b>	<b>20</b>	<b>54.87</b>	<b>40.00</b>	<b>3.00</b>	<b>1.00</b>
<b>Product Size: 101</b>						

---

Pos.	Len.	Tm	GC%	Self	Any	Self
Oligo 5 :						
End						
Wildtype Reverse Primer 5':						
GATCCTTGGCACCTATTCCATT	341	22	61.38	45.46	4.00	1.00
Mutant Reverse Primer 5':						
GATCCTTGGCACCTATTCCATC	341	22	61.89	50.00	4.00	0.00
Common Forward Primer 5':						
AATACAGCATCCCTTTCTCA	241	20	54.87	40.00	3.00	1.00
Product Size: 101						

## ii. TthIII1

AS Primer Picking Result for rs10052957

Input sequence 5':

```
CTTACGGAATTTAATTGAAGAGATGAACTCCAGTGTGCCAGAAAGGACGTGAGATTTAGCATTATAATAATAACACAAT
TCTAACGAAAGTCAACATTTA
TTGCGCATCTGCTGTCTGACAGACACTATTGAAAGTGTTTTATGCACAGTATCCTAAGTCTTTAGGTATGGCATTACT
AATCTGTAACCCCTGGGAECTT
ATAATTAATAACAAGAAATAGGATGAATCCCTATCTGAGTGGGAAGGTATTTGATTGTCTTAGAAGCAGAGGTGGAATG
AAGGTGATGTATTCAGACTCA
[A/G]TCAAGGCAAGGACCTGATCTATCTTGTGCATAGCTTTATGTCCCACTCCTGGAACACAGCCCACTACATAGCAGG
CACCCAACAAATACATTACAG
GGTTATAATAACATTGAGTCAACTAGTTAACTAGATGTAAAATCACTTTTACTGGGTTATTGTTGTGGCCAAATGGGTT
AATAAAAAGTGTACAGATAAC
CCTCAAGATCCTCATTTTTATTGTTTTGTGTTTTAAACATCTGAGTGCTCCCCCACTTTTTTTTTAAATAAACTAGATT
AGATTCTTGATTGTATGTTCT
TATAG
Sequence Size: 601
```

Primer Picking Parameters:

```
Primer Size      Opt: 20 Min: 18 Max: 36
GC%              Opt: 50.0 Min: 20.0 Max: 85.0
Tm               Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff:    2.0
Max Self Complementarity: 5.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'Yes' Form Human Library
```

Oligo 1

	Pos.	Len.	Tm
GC% Self Any Self End			
Wildtype Reverse Primer 5':			
AGATAGATCAGGTCCTTGCCTTGTT	325	25	62.71
1.00		44.00	5.00
Mutant Reverse Primer 5':			
AGATAGATCAGGTCCTTGCCTTGTC	325	25	63.21
1.00		48.00	5.00
Common Forward Primer 5':			
TGAACTCCAGTGTGCCAGAAA	24	21	61.82
0.00		47.62	4.00

Product Size: 302

In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----  
-----

Oligo 2

	Pos.	Len.	Tm
GC% Self Any Self End			
Wildtype Reverse Primer 5':			
GATAGATCAGGTCCTTGCCTTGTT	324	24	61.98
1.00		45.83	5.00

Mutant Reverse Primer 5':  
 GATAGATCAGGTCCTTGCCTTGTC 324 24 62.48 50.00 5.00  
 1.00  
 Common Forward Primer 5':  
 GATGAACTCCAGTGTGCCAGA 22 21 61.28 52.38 4.00  
 3.00  
 Product Size: 303  
 In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----  
 -----  
 Oligo 3  
 : Pos. Len. Tm  
 GC% Self Any Self End  
 Wildtype Reverse Primer 5':  
 ATAGATCAGGTCCTTGCCTTGTT 323 23 60.37 43.48 5.00  
 1.00  
 Mutant Reverse Primer 5':  
 ATAGATCAGGTCCTTGCCTTGTC 323 23 60.85 47.83 5.00  
 1.00  
 Common Forward Primer 5':  
 ATGAACTCCAGTGTGCCAGA 23 20 59.26 50.00 4.00  
 3.00  
 Product Size: 301  
 In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----  
 -----  
 Oligo 4  
 : Pos. Len. Tm  
 GC% Self Any Self End  
 Wildtype Reverse Primer 5':  
 TAGATCAGGTCCTTGCCTTGTT 322 22 60.13 45.46 5.00  
 1.00  
 Mutant Reverse Primer 5':  
 TAGATCAGGTCCTTGCCTTGTC 322 22 60.63 50.00 5.00  
 1.00  
 Common Forward Primer 5':  
 ATGAACTCCAGTGTGCCAGA 23 20 59.26 50.00 4.00  
 3.00  
 Product Size: 300  
 In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----  
 -----  
 Oligo 5  
 : Pos. Len. Tm  
 GC% Self Any Self End  
 Wildtype Reverse Primer 5':  
 AGATCAGGTCCTTGCCTTGTT 321 21 60.13 47.62 5.00  
 1.00  
 Mutant Reverse Primer 5':  
 AGATCAGGTCCTTGCCTTGTC 321 21 60.64 52.38 5.00  
 1.00  
 Common Forward Primer 5':  
 GAACTCCAGTGTGCCAGAAA 25 20 58.85 50.00 4.00  
 0.00  
 Product Size: 297

In-silico PCR Accepted: There is only one PCR product in the current human genome.

iii. ER22/23EK

AS Primer Picking Result for rs6190

Input sequence 5':

```
GGATTGCATTGTACTTTTAAATGTGGCATGCTGAATGGGAGCAGGGGACATGGCTTTTTATTCTGGAAGATAGAAACTA
CTCTTCTGGTAACAAAGAATT
TGATTCGGAGTTAACTAAAAGGTTTCATTTAACAAGCTGCCTCTTACTAATCGGATCAGGAAGATAATGTGACTTTAGAG
CTTATGATGTTTTTCCCCCGT
TTTTGTTTTTTGTTTTGTAGTTGATATTCACCTGATGGACTCCAAAGAATCATTAACCTCTGGTAGAGAAGAAAACCCCA
GCAGTGTGCTTGCTCAGGAGA
[A/G]GGGAGATGTGATGGACTTCTATAAAACCCTAAGAGGAGGAGCTACTGTGAAGGTTTCTGCGTCTTCACCTCAC
TGGCTGTCGCTTCTCAATCAG
ACTCCAAGCAGCGAAGACTTTTTGGTTGATTTTTCCAAAAGGCTCAGTAAGCAATGCGCAGCAGCCAGATCTGTCCAAAGC
AGTTTCACTCTCAATGGGACT
GTATATGGGAGAGACAGAAACAAAAGTGATGGGAAATGACCTGGGATTCCCACAGCAGGGCCAAATCAGCCTTTCCTCG
GGGAAACAGACTTAAAGCTT
TTGGA
```

Sequence Size: 601

Primer Picking Parameters:

Primer Size Opt: 20 Min: 18 Max: 36  
GC% Opt: 50.0 Min: 20.0 Max: 85.0  
Tm Opt: 55.0 Min: 40.0 Max: 65.0  
Max Tm Diff: 2.0  
Max Self Complementarity: 5.0  
Max 3' Complementarity: 3.0  
Max PolyX in Primer: 3  
Number Primer Return: 5  
Mismatch at the penultimate primer position: 'Yes'  
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000  
Mispriming Filtering: 'Yes' Form Human Library

Oligo 1

	Pos.	Len.	Tm
GC% Self Any Self End			
Wildtype Reverse Primer 5': TAGAAGTCCATCACATCTCCAT 2.00	322	22	56.18
Mutant Reverse Primer 5': TAGAAGTCCATCACATCTCCAC 0.00	322	22	56.70
Common Forward Primer 5': AGGTCATTTAACAAGCTGC 3.00	120	20	54.71

Product Size: 203

In-silico PCR Accepted: There is only one PCR product in the current human genome.

Oligo 2

	Pos.	Len.	Tm
GC% Self Any Self End			
Wildtype Reverse Primer 5':			

AGAAGTCCATCACATCTCCAT 2.00	321	21	56.02	42.86	4.00
Mutant Reverse Primer 5': AGAAGTCCATCACATCTCCAC 0.00	321	21	56.55	47.62	4.00
<b>Common Forward Primer 5': AACAAAGAATTTGATTCGGA 3.00</b>	<b>90</b>	<b>20</b>	<b>54.86</b>	<b>30.00</b>	<b>5.00</b>

Product Size: 232  
In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----

Oligo 3  
: =ER/EK2

	Pos.	Len.	Tm		
GC% Self Any Self End					
Wildtype Reverse Primer 5': GAAGTCCATCACATCTCCAT 2.00	320	20	54.73	45.00	3.00
Mutant Reverse Primer 5': GAAGTCCATCACATCTCCAC 0.00	320	20	55.27	50.00	3.00
<b>Common Forward Primer 5': CAAAGAATTTGATTCGGAGT 3.00</b>	<b>92</b>	<b>20</b>	<b>54.44</b>	<b>35.00</b>	<b>5.00</b>

Product Size: 229  
In-silico PCR Accepted: There is only one PCR product in the current human genome.

-----

Oligo 4  
:

	Pos.	Len.	Tm		
GC% Self Any Self End					
Wildtype Reverse Primer 5': AGTCCATCACATCTCCAT 2.00	318	18	50.22	44.44	2.00
Mutant Reverse Primer 5': AGTCCATCACATCTCCAC 0.00	318	18	50.73	50.00	2.00
<b>Common Forward Primer 5': AGAATTTGATTCGGAGTTA 3.00</b>	<b>94</b>	<b>20</b>	<b>51.70</b>	<b>30.00</b>	<b>5.00</b>

Product Size: 225  
In-silico PCR Accepted: There is only one PCR product in the current human genome.

iv. BclI

AS Primer Picking Result for SNP2015-01-23

Input sequence 5':

ACCTCTGGAGGACAGATGTACCACTATGACATGAATACAGCATCCCTTTCTCAACA  
GCAGGATCAGAAGCCTATTTTAAATGTCATTCCACCAATTCCCG



TTGGTTCCGAAAATTGGAATAGGTGCCAAGGATCTGGAGATGACAACTTGACTTCT  
CTGGGGACTCTGAACTTCCCTGGTCGAACAGTTTTTTCTAATGG  
CTATTCAAGGTAAGATCAGTGTCTTTCTGTTTCTTAAGAATGGTACATTTAAGGTAGA  
TTAATAGATGTAAATCTTCATTGATTTATATGTGTTCTCTAA  
AGATTCATGTGCTTTTTTATATGAATAAGTTAAGTGGCCTTTTGAAAGTAGGAAAGG  
TAGACAACCTAAGTGACATCTGTACGTAACCATTTTCAGGTTT  
TTTTCTTAAATAGTGGTTTTTCAGTATCCCATTGGCCAACGGTGAGGATTTTATTTAAC  
ATTTTTAAAATAATGTTGCTCATTAAACAGATATCTTAAACGAA  
AAATTATATAAATTCAGGAGAGTATAATGTCTCATAATATCATATTGTGTTGTGCATG  
GTCATTCAGCTGTTTTAGAATATGTTCTTATATTACAATAAA  
TGATACCCTTACTTACATAGTCAAAAGTTGTGCTGCCTTATTTGTAATTCGTTAAGT  
GTTAGCTTGAGATTAAGAGTAAAGCAGAAGTACTAACA  
AGAGCCCTATTCTTCAAACCTGAATCTTCTGTTAAAGAATTTGAGTTTTGAAGTTGCTA  
AAGCAATGCAGTGAACAGTGTACCAGACCATAGTATTAGACA  
CAGGTCTTGCTCACAGGGTCTTGCCATAAAGTAGACAAGTTATGTCTGCTGAT[C/G  
]AATCTCTTTAAGAGAGGAATTGGTGTCAACATGGTGCAAAA  
CAAAATTTTACGTTCAAATGTTCTGCAAGTTCTCAAGTAGATAACTGATGGCCAAA  
ATTGTTAAGCTTCAATTTTCAGCTTTTCGTTTGATTTTTCTCTT  
TTTTTTACTCAGTCGTTTATAAGCATACTGATTTTTTTGTCTGACCCAAAAAGGTCAG  
AAAATGGAATTATCAGAAAAAGTTCTAAATGTAGATATACG  
TGTTGGTAGGGGTGAATTTCTTACCCCGTAACCTCATCCCCAATTCAGATAAATGC  
TAGGTTTTATATCCATTTTAGTTGTGAAGGAAAATATAAAAAT  
GTGGATTGTAGTGACACAAGATTGATTAATCAGCGGGTTTTTTTTAAAAGAAGACATG  
GTAGACAGTGATTTATTTGTATGTAACCTATTGAAGTTTTTTCT  
TAAATGTTAGTGATATTCATCGTTCCCATTAAGTATTTCAGATTTTTGAAAATCCT  
TTTTCTGTGAAAGCTATCCTAACCTGGAGGATGTCTCTTTT  
CTTTCCTCTGTACTTAAGAAGCTTTTCTTGTTAGGGAAATAATTTAGAATTAGATTTA  
GGCTATGTTCTGTTCTTCTAAAAGGCTTAGTTGTCAAAAAA  
AAAAAAAAAAAAACCAAAAAACCTTGGTTCTTACATGTCTTAATGTGAACTACCTCCTA  
ATCTATTGTTAAATAATTATCCTTTATTTAGAAGAACTA  
CTTCAACCTGAGTTGAAGTTTTAAATCTTTTCAGTAAGGAGATTTGAGATCTTTATT  
ATTGCATAAGCTGTTGTGTTTTAAATGCTAAAAGACATGCTG  
TGTTTTAAATTTTCAATTGCAAATTTTTGGCAATAGAATTCGCATACTTGGTTTTCTT  
AAAAGAGTTAAGTACGGTTGATTTGACTAAGCTATCTGTAG  
GAACTCTTAAATTGATTTATAAAACATGTAATTATACAAAGAAAAATAAAACATCTTA  
GGAAACTCTTGGGGATTATTAATGGATTTTGCCTGATAAT  
CATCATGGCATGGTTTTTCATTTTCTTACTATAAAGAAAAGGCAAGGGACAAAACCT  
ATT

Sequence Size: 1956

Primer Picking Parameters:

Primer Size Opt: 20 Min: 18 Max: 36  
GC% Opt: 50.0 Min: 20.0 Max: 85.0  
Tm Opt: 55.0 Min: 40.0 Max: 65.0  
Max Tm Diff: 100.0  
Max Self Complementarity: 8.0  
Max 3' Complementarity: 5.0  
Max PolyX in Primer: 3  
Number Primer Return: 5

Mismatch at the penultimate primer position: 'Yes'  
 In-silico PCR Filtering: 'No'  
 Mispriming Filtering: 'No'

Oligo 1 :	Pos.	Len.	Tm	GC%	Self		
Any Self End							
Wildtype Forward Primer 5':							
AGTAGACAAGTTATGTCTGCTGACC	831	25	58.13	44.00	8.00	4.0	
0							
Mutant Forward Primer 5':							
AGTAGACAAGTTATGTCTGCTGACG	831	25	59.10	44.00	8.00	4.0	
00							
Common Reverse Primer 5':							
AAATCAAACGAAAGCTGAAA	989	20	55.26	30.00	4.00	0.00	
Product Size: 159							

---

Oligo 2 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Forward Primer 5':							
GTAGACAAGTTATGTCTGCTGACC	832	24	57.14	45.83	6.00	4.00	
Mutant Forward Primer 5':							
GTAGACAAGTTATGTCTGCTGACG	832	24	58.16	45.83	6.00	4.00	
Common Reverse Primer 5':							
AAATCAAACGAAAGCTGAAA	989	20	55.26	30.00	4.00	0.00	
Product Size: 158							

---

Oligo 3 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Forward Primer 5':							
TAGACAAGTTATGTCTGCTGACC	833	23	56.23	43.48	6.00	4.00	
Mutant Forward Primer 5':							
TAGACAAGTTATGTCTGCTGACG	833	23	57.29	43.48	6.00	4.00	
Common Reverse Primer 5':							
AAATCAAACGAAAGCTGAAA	989	20	55.26	30.00	4.00	0.00	
Product Size: 157							

---

Oligo 4 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Forward Primer 5':							
AGACAAGTTATGTCTGCTGACC	834	22	56.07	45.46	6.00	4.00	
Mutant Forward Primer 5':							
AGACAAGTTATGTCTGCTGACG	834	22	57.17	45.46	6.00	4.00	

Common Reverse Primer 5':  
 TTGACACCAATTCCTCTCTT 884 20 54.73 40.00 4.00 0.00  
 Product Size: 51

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Oligo 5 : End	Pos.	Len.	Tm	GC%	Self Any	Self
Wildtype Forward Primer 5': GACAAGTTATGTCTGCTGACC	835	21	54.85	47.62	6.00	4.00
Mutant Forward Primer 5': GACAAGTTATGTCTGCTGACG	835	21	56.01	47.62	6.00	4.00
Common Reverse Primer 5': ACTTGAGAACTTGCAGGAAC	935	20	54.53	45.00	4.00	2.00

Product Size: 101

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v. RS 12086634

AS Primer Picking Result for rs12086634

Input sequence 5':

TGAGCAATCTCTCATTAAAGCCCCCGTTACTTCAGAGACTACCCCCAAAAATCTG  
 CAGCTAAGACTGATGCCATTTCTGCTGTATCACTGCAGGTGGT  
 ATCCCACTGCCTGGAGCTTGGAGCAGCCTCAGCACACTACATTGCTGGCACCATG  
 GAAGACATGACCTTCGCAGAGCAATTTGTTGCCCAAGCAGGAAAG  
 CTCATGGGTGAGGCTGTTTCTCTTACCTCCTCCTCTGAACTTTGCCCTTGGGGTCA  
 CCAAGAGCTTTTGGGAGGAGAATGGGAAAGGTATCAACCCCAGA  
 [G/T]GATTTCTTAATATAGCCATCTCTTGCAGGAGGACTAGACATGCTCATTCTCAAC  
 CACATCACCAACACTTCTTTGAATCTTTTTTCATGATGATAT  
 TCACCATGTGCGCAAAGCATGGAAGTCAACTTCCTCAGTTACGTGGTCCTGACTG  
 TAGCTGCCTTGCCCATGCTGAAGCAGAGCAATGGAAGCATTGTT  
 GTCGTCTCCTCTCTGGCTGGTAAGTGGGACAGGGACATATGTGGAAGGTAGAAGA  
 AAAAAAAAAATGCCAGGGATGATGCCAGGCTCTGAAGTAGACATA  
 AATTT

Sequence Size: 601

Primer Picking Parameters:

Primer Size Opt: 20 Min: 18 Max: 36  
 GC% Opt: 50.0 Min: 20.0 Max: 85.0  
 Tm Opt: 57.0 Min: 40.0 Max: 65.0  
 Max Tm Diff: 100.0  
 Max Self Complementarity: 8.0  
 Max 3' Complementarity: 5.0  
 Max PolyX in Primer: 3  
 Number Primer Return: 5

Mismatch at the penultimate primer position: 'Yes'  
 In-silico PCR Filtering: 'No'  
 Mispriming Filtering: 'No'

Oligo 1 :	Pos.	Len.	Tm	GC%	Self		
Any Self End							
Wildtype Reverse Primer 5':							
AAGAGATGGCTATATTAAGAAATTC	325	25	53.90	28.00	5.00	5.00	
Mutant Reverse Primer 5':							
AAGAGATGGCTATATTAAGAAATTA	325	25	52.46	24.00	4.00	2.0	
0							
Common Forward Primer 5':							
TCTGCTGTATCACTGCAGGT	78	20	56.96	50.00	8.00	4.00	
Product Size: 248							

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Oligo 2 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Reverse Primer 5':							
AGAGATGGCTATATTAAGAAATTC	324	24	52.48	29.17	5.00	5.00	
Mutant Reverse Primer 5':							
AGAGATGGCTATATTAAGAAATTA	324	24	50.98	25.00	4.00	2.00	
Common Forward Primer 5':							
TCTGCAGCTAAGACTGATGC	54	20	56.88	50.00	6.00	3.00	
Product Size: 271							

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Oligo 3 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Reverse Primer 5':							
GAGATGGCTATATTAAGAAATTC	323	23	51.32	30.44	5.00	5.00	
Mutant Reverse Primer 5':							
GAGATGGCTATATTAAGAAATTA	323	23	49.76	26.09	4.00	2.00	
Common Forward Primer 5':							
TGATGCCATTTCTGCTGTAT	68	20	56.74	40.00	5.00	2.00	
Product Size: 256							

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Oligo 4 :	Pos.	Len.	Tm	GC%	Self	Any	Self
End							
Wildtype Reverse Primer 5':							
AGATGGCTATATTAAGAAATTC	322	22	49.40	27.27	5.00	5.00	
Mutant Reverse Primer 5':							
AGATGGCTATATTAAGAAATTA	322	22	47.81	22.73	4.00	2.00	

Common Forward Primer 5':  
TCTGCAGCTAAGACTGATGC                      54    20    56.88    50.00    6.00    3.00  
Product Size: 269

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Oligo 5 :	Pos.	Len.	Tm	GC%	Self Any	Self
End						
Wildtype Reverse Primer 5': GATGGCTATATTAAGAAATTC	321	21	48.01	28.57	5.00	5.00
Mutant Reverse Primer 5': GATGGCTATATTAAGAAATTA	321	21	46.36	23.81	4.00	2.00
Common Forward Primer 5': TGATGCCATTTCTGCTGTAT	68	20	56.74	40.00	5.00	2.00

Product Size: 254

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## F- Gel Electrophoresis Protocol

### Pouring an agarose gel

#### Assembling the gel box

- Turn the gel box so the electrodes are facing you
- Place the gel tray into the box perpendicular to the box so that the rubber gaskets on the side seal along the edge of the box (figure 1)
- There are 2 notches for the combs- make sure one set of notches is on the far side of the gel box and one set is closer to you in the middle (see figure 2)

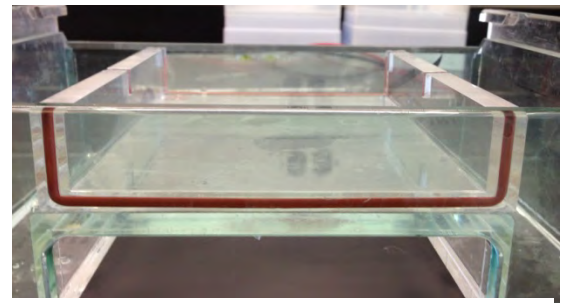


Figure 3

#### Making the gel

- Measure 100ml 1XPE buffer with a graduated cylinder and place in a 500ml Erlinmeyer flask
- Add 1g of agarose
- Swirl, then microwave (for the microwave in WLDC201 microwave for 1 minute and 15 seconds).

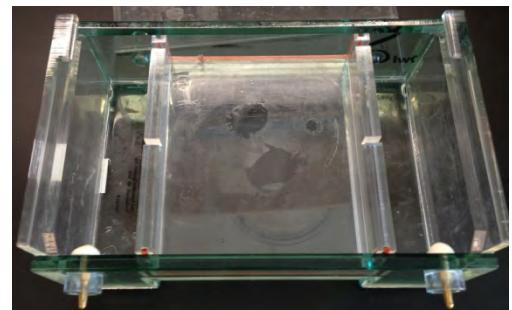


Figure 4

- Remove from microwave with a hot glove, hold up to the light and swirl to make sure everything is dissolved.

### Pouring the gel

- When the gel has cooled slightly, add 10ul of ethidium bromide solution to 100ml of gel, swirl, and then pour into the gel tray.

FROM THIS POINT FORWARD YOU MUST WEAR GLOVES WHEN TOUCHING THE GEL OR THE BUFFER

- Place combs into the tray- the 12 well comb should be at the top of the gel and the 20 well comb in the middle. Both should be pointing to 1.5mm
- Allow the gel to solidify
- Immediately wash the flask out with hot water. Failure to do so will result in residual agarose gelling in the bottom of the flask in which case you will be severely mocked at lab meeting.

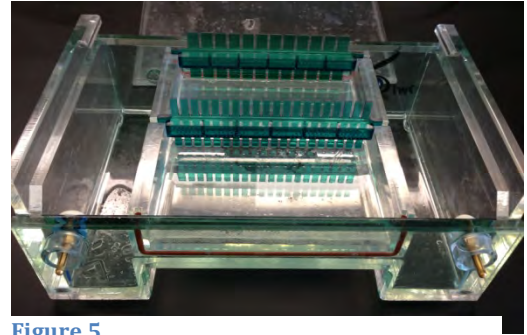


Figure 5

### Running the gel

- When the gel has solidified, turn the gel perpendicularly so that the 12 well comb is on the left.
- Add sufficient 1XPE to the gel box so that the gel is completely submerged
- Add 40ul ethidium bromide to the gel box on the right hand side.
- Load samples onto the gel and slide the lid on.
- Connect the electrodes to the power supply
- Press the set button and check the current (pun intended) settings. Make sure the dials for Watts and Milliamps are at the max and set the voltage with the dial.
- Press the start button and the light labeled DC should come on. A few seconds later you should hear a pop and a few seconds after that you should see bubbles in the gel box.
- If the DC light fails to stay on, you may have a bad connection somewhere so you should go back and recheck them all. Sometimes you might need to slide the lid off and then back on again.

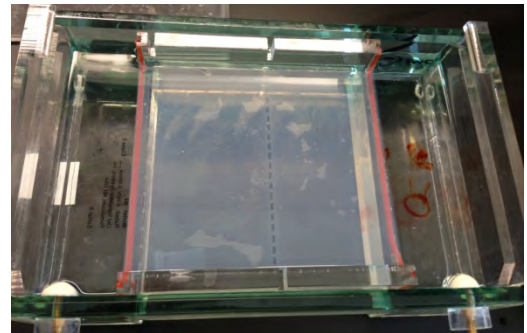


Figure 6

## Disposing of the gel

PUT ON GLOVES

Remove the gel from the tray and place it on a blue absorbent pad in the hood.

When the gel has dried, place it in the yellow waste can in the common room near the ice machine (WLDC207)

Buffer should be poured down the drain and the gel tray and box should be rinsed with DI water and placed back in the drawer

## G- Collected Patient Data

### i. Ellis

The image shows a large grid table, likely a data collection sheet. The table has many columns and rows. On the left side, there is a vertical bar with a color gradient from green at the top to red at the bottom. The table contains some faint, illegible text and numbers, but most of the cells are empty. The text appears to be organized into sections, possibly corresponding to different patient data points or time intervals.

ii. Union Students

Sample Number	12,222288 % MT			12,222288 % MT			12,222288 % MT			12,222288 % MT		
	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)	48.01% (48.01%)
1	63	100	100	63	100	100	63	100	100	63	100	100
2	64	100	100	64	100	100	64	100	100	64	100	100
3	64	100	100	64	100	100	64	100	100	64	100	100
4	65	100	100	65	100	100	65	100	100	65	100	100
5	65	100	100	65	100	100	65	100	100	65	100	100
6	65	100	100	65	100	100	65	100	100	65	100	100
7	65	100	100	65	100	100	65	100	100	65	100	100
8	65	100	100	65	100	100	65	100	100	65	100	100
9	65	100	100	65	100	100	65	100	100	65	100	100
10	65	100	100	65	100	100	65	100	100	65	100	100
11	65	100	100	65	100	100	65	100	100	65	100	100
12	65	100	100	65	100	100	65	100	100	65	100	100
13	65	100	100	65	100	100	65	100	100	65	100	100
14	65	100	100	65	100	100	65	100	100	65	100	100
15	65	100	100	65	100	100	65	100	100	65	100	100
16	65	100	100	65	100	100	65	100	100	65	100	100
17	65	100	100	65	100	100	65	100	100	65	100	100
18	65	100	100	65	100	100	65	100	100	65	100	100
19	65	100	100	65	100	100	65	100	100	65	100	100
20	65	100	100	65	100	100	65	100	100	65	100	100
21	65	100	100	65	100	100	65	100	100	65	100	100
22	65	100	100	65	100	100	65	100	100	65	100	100
23	65	100	100	65	100	100	65	100	100	65	100	100
24	65	100	100	65	100	100	65	100	100	65	100	100
25	65	100	100	65	100	100	65	100	100	65	100	100
26	65	100	100	65	100	100	65	100	100	65	100	100
27	65	100	100	65	100	100	65	100	100	65	100	100
28	65	100	100	65	100	100	65	100	100	65	100	100
29	65	100	100	65	100	100	65	100	100	65	100	100
30	65	100	100	65	100	100	65	100	100	65	100	100
31	65	100	100	65	100	100	65	100	100	65	100	100
32	65	100	100	65	100	100	65	100	100	65	100	100
33	65	100	100	65	100	100	65	100	100	65	100	100
34	65	100	100	65	100	100	65	100	100	65	100	100
35	65	100	100	65	100	100	65	100	100	65	100	100
36	65	100	100	65	100	100	65	100	100	65	100	100
37	65	100	100	65	100	100	65	100	100	65	100	100
38	65	100	100	65	100	100	65	100	100	65	100	100
39	65	100	100	65	100	100	65	100	100	65	100	100
40	65	100	100	65	100	100	65	100	100	65	100	100
41	65	100	100	65	100	100	65	100	100	65	100	100
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43	65	100	100	65	100	100	65	100	100	65	100	100
44	65	100	100	65	100	100	65	100	100	65	100	100
45	65	100	100	65	100	100	65	100	100	65	100	100
46	65	100	100	65	100	100	65	100	100	65	100	100
47	65	100	100	65	100	100	65	100	100	65	100	100
48	65	100	100	65	100	100	65	100	100	65	100	100
49	65	100	100	65	100	100	65	100	100	65	100	100
50	65	100	100	65	100	100	65	100	100	65	100	100