

A RACE WITHOUT A FINISH LINE: USING GENETICS TO DETECT HOMOLOGOUS
BLOOD DOPING

by

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ABSTRACT

Blood doping refers to any illicit means used to increase the erythrocyte count in the body; thereby, increasing oxygen delivery to the muscles. Anti-doping authorities strongly oppose blood doping, and they have prohibited transfusions and the use of substances that increase hemoglobin levels. Athletes now avoid using erythropoiesis-stimulating agents and have reverted to older doping methods, such as blood transfusions. As blood transfusion become more prevalent, current tests for blood transfusions become more problematic. Homologous blood doping is the transfusion of another person's blood to increase one's own hemoglobin levels. The current method for detecting homologous blood doping is flow cytometry; however, this method is invasive, costly and requires proper conditions for storage of the blood samples.

This study outlines a test that uses genetic differences among individuals in order to detect doping. The proposed anti-doping test eliminates major limitations of other tests. This study also investigates the feasibility and sensitivity of using genetic variations among individuals to detect homologous blood doping.

Blood required for doping must be matched for ABO blood type, but because it comes from another individual it will be mismatched for genetic information (i.e. DNA). Given that an individual carries two copies of any chromosome and consequently two copies of most genes, foreign cell detection can be made possible by the discovery of a third or fourth copy of certain genes. Thus, this detection method identifies the presence of genes that do not belong to the athlete. In this study, doping conditions were simulated by mixing bloods at various ratios, including the 90:10 ratio that is commonly observed in athletic doping. PCR based methods of genotyping were used to detect donor DNA in the recipient's sample. Genetic markers (D1S80, D1S11, D17S30, APO-B and ACE I/D) were used to detect the heterogeneous blood samples. The test successfully identified a 10% allogeneic cell population using a finger prick of blood (50 μ L). As that dilution involved a heterozygous donor, this result demonstrates that the assay is sensitive enough to detect a 5% dilution. Overall, this genetic approach allows for the development of an efficient, sensitive and inexpensive test.

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GLOSSARY

Allele: An alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome.

Autologous: In blood transfusion and transplantation, a situation in which the donor and recipient are the same person.

Bases: The individual nucleotides that comprise DNA (A, C, T and G).

Chimera: having two or more different populations of genetically distinct cells that originated in different zygotes.

Chromosomes: Long segments of DNA on which genes are encoded. Humans have 23 distinct chromosomes and every cell (except sex cells) has two complete sets (one from mother, one from father).

Erythropoeisis: The formation or production of red blood cells.

Erythropoietin: A hormone produced by the kidney that promotes the formation of red blood cells in the bone marrow.

Flow cytometry: A technique used for counting and examining microscopic particles, such as cells, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous analysis of the physical and/or chemical characteristics of up to thousands of cells per second.

Gene: A protein-encoding segment of DNA. Genes are located on chromosomes.

Genotype: The combination of alleles at a particular locus.

Homologous: In blood transfusion, the transfer of blood taken from one person into the circulation of another.

Microchimerism: The presence of two genetically distinct and separately derived populations of cells, one population being at a low concentration.

PCR: Polymerase chain reaction, in the context of this study PCR is a method that allows amplification of a specific DNA segment via varied cycled temperatures.

Polymorphism: A common variation in DNA in which alternate sequences occur in populations.

Polymorphic locus: The location in the DNA of a polymorphism.

VNTR: Variable number of tandem repeats occurs when a segment of the gene sequence is repeated a variable number of times.

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INTRODUCTION

OVERVIEW

In sports, the term “doping” refers to the use of a substance (anabolic steroid or erythropoietin) or unsanctioned technique (blood doping, which is sometimes referred to as “blood boosting or blood building”) to improve athletic performance. The practice of enhancing athletic performance through the use of stimulants and other artificial means is as old as competition itself: ancient Greek athletes, for example, consumed special diets to increase strength. In sports, the term “transfusion” indicates an act, process, or instance of adding fluid (such as blood) into a vein or artery. As far as modern sports are concerned, the 20th century is rife with examples of performance enhancing drug use across many disciplines. Recently, in the 2007 Tour de France, Alexander Vinokourov and Andrej Kashechkin tested positive for homologous blood doping. In 2008, professional hockey player Alexei Cherepanov collapsed, leading to his death, during a game in Russia. Russian anti-doping authorities later reported the cause of death to be blood doping. Although homologous blood doping did not first appear on the world stage until 1970, it has recently become more prevalent. As more testing becomes available for other forms of doping; such as, anabolic steroids, blood stimulates, and other drugs, athletes revert back to more tradition methods of doping. In fact, since the introduction of the urine-based recombinant erythropoietin test in 2001, transfusion based doping practices have regained athletes’ interests (Giraud *et al.*, 2008). Therefore, the resurgence of blood doping has also triggered much interest among anti-doping authorities. This has attracted the World Anti-Doping Agency (WADA), the body whose chief responsibility is to enforce the World Anti-Doping Code, in funding researchers to investigate new methods of detection.

In 1972, the efficacy of blood transfusions on performance in sport was demonstrated to be due to an increase in the volume of red blood cells (RBCs) and therefore increase in oxygen

carrying capacity (Ekblom *et al.* 1972). Another study found that blood doping methods can provide up to a 10% enhancement of performance, making it an effective way to manipulate results in endurance sports (Ekblom and Berglund *et al.* 1991, Birkeland *et al.* 2000). The practice arose during the early 1960's and was commonly used at the 1968 Olympic Games to compensate for altitude effects of competing in Mexico City (2,240 meters). In the 1972 Olympic Games, Finnish distance runner and four-time Olympic gold medalist Lasse Viren won the 5,000m and 10,000m races, setting a world record for the latter. Viren's achievement led the world to believe that he had doped to drastically boost his performance. Long after the event, it was determined that Viren's performance was aided by blood doping, at the time not prohibited under the Olympic rules (World of Sports Science 2008; Retrieved 2010). Blood doping was banned in 1986.

THE ATHLETE'S STRIVE FOR THE HIGHEST MAXIMAL OXYGEN CAPACITY

The ergogenic effects of blood doping are to increase maximal oxygen uptake during endurance activities, by increasing the amount of circulating hemoglobin. Athletes use blood doping as a method to temporarily increase the quantity of red blood cells (RBCs) in the blood; therefore, increase the oxygen carrying capacity of the body. RBC levels can be increased via several methods: hypoxia exposure, the use of natural or synthetic erythropoietin (Epo), and blood transfusions. For the purpose of this study, I will discuss these methods in detail as they are more frequently used in comparison to other forms of blood boosting. All methods of blood boosting are effective; however, some are easier to administer and others more readily accessible to the athlete. It is important to mention that all methods are dangerous and athletes risk their lives every time they participate in any doping activity. Therefore, it is necessary to develop efficient anti-doping tests in order to deter athletes.

A) *HYPOXIA EXPOSURE*

Exposure to high altitudes (> 2500 m) stimulates the body to produce more RBCs in response to the lower oxygen availability (i.e. hypoxia). When exposed to hypoxic conditions the body struggles to produce the required energy needed to perform. This struggle initiates physiological changes that enhance the efficiency of the respiratory, cardiovascular, and oxygen transport systems. The “live high-train low” concept is widely used by athletes to improve their performance. Traditional altitude training had athletes train at high altitudes; however, the benefits were lost by the reduced ability to train hard with low oxygen availability (Levine *et al.*, 1997). Full benefits of high altitude training occurs when an athlete sleeps at high altitude and by prolonged exposure to altitude but does high intensity training at sea level where oxygen is more readily available (Hahn *et al.*, 2001, Werhlin *et al.*, 2006). The “live high-train low” concept allows for the best of both worlds. Simulated altitude environments can be created at sea level in order to have the same effect on the body. In 2001, a study of the HiLo paradigm reported significant performance improvements in response to a 27-day high altitude training camp (Stray-Gundersen *et al.*, 2001). The mechanism of improvement was described as stimulation of erythropoiesis leading to a near doubling of plasma erythropoietin concentration and, among other biological changes, an increase in hematocrit. Living high-training low does not presently contravene anti-doping practices set by anti-doping authorities such as the International Olympic Committee (IOC) and WADA.

B) ERYTHROPOIETIN

The body increases RBC production through a process known as erythropoiesis. This occurs constantly to replace cells lost to naturally occurring cell senescence but is increased when the partial arterial O₂ pressure drops (e.g. in a hypoxic environment). A feedback loop exists involving erythropoietin (Epo), a 165-amino acid glycoprotein produced by the kidneys, the liver and the adult humerus which stimulates RBC production in the bone marrow.

The development of recombinant human Epo (rHuEpo) in the 1980s replaced other methods of doping and soon became the preferred method of blood doping for endurance athletes (Rossi *et al.*, 1998; Gambrell and Lombardo 1994; Deacon and Gains 1995). In rHuEpo administration trials, 8% increase in RBC mass led to a 2-5% increase in endurance performance (Ekblom and Berglund *et al.* 1991; Birkeland *et al.* 2000).

The prototype drugs used in blood doping are epoetin (e.g./ Procrit®, Epogen®, Binocrit®, Eprex®, NeoRecormon®, Dynepo®), darbepoetin (e.g./ Aranesp®) and Continuous Erythropoietin Receptor Activators (CERAs) (e.g./ Mircera®). Epoetin and darbepoetin are all variations of the endogenous Epo; CERAs, however, continuously interact with the Epo receptor producing longer lasting effects. All are manufactured by recombinant DNA technology and all contain the same (or a similar) amino acid sequence to Epo. The effects of these drugs are the same as those achieved by HiLo training except they do not require an athlete to travel far from home; in fact, they can be used at home as they are injected subcutaneously. The administration of these drugs is another approach to increasing an athlete's RBC count. Once injected, these drugs act in the fashion as Epo by stimulating the body's RBC production. Consequently, Epo too was very attractive to athletes until reliable tests to detect it were developed in 2000. In the

2002 Winter Olympics, three cross-country skiers tested positive for Darbepoetin (CNNSI, February 24, 2002). The use of Epo in competition is currently prohibited by the IOC and WADA.

C) BLOOD TRANSFUSIONS

Athletes can always revert to the simplest and oldest method of blood doping by performing blood transfusions. There are two types of blood transfusions: autologous and homologous. For the purpose of doping, autologous transfusions requires that the athlete draw his or her own blood four to six weeks prior to competition, harvest it and reintroduce it to their circulation. Homologous doping is similar except that the athlete uses blood from another person and is not required to draw blood several weeks in advance. In either case, the blood is transfused into the athlete's circulation hours before competition. Homologous transfusion requires less planning and lends itself to a last resort doping method. Athletes tend to favour using homologous doping for several reasons. First, training ability does not decrease with homologous doping, as opposed to autologous doping where after initial blood withdrawal athlete cannot train at maximal level due to loss of blood for a period of time. Second, athletes can revert to homologous doping in unforeseen circumstances (e.g. illness, lack of training or injury) days or even hours prior to competition; no prior planning is required. The incredible advantage of homologous doping is that of quickly improving athletic performance and serving as a "last resort" cheating method.

Anti-doping authorities currently only perform random testing for blood doping. Athletes know to stay away from the use of steroids and blood stimulants since testing is so extensive. Although the use of blood transfusions for blood doping dates back several decades, its recent resurgence is likely due to the introduction of efficient Epo detection methods. Two

recent high profile doping cases - Tyler Hamilton (2004) and Alexander Vinokourov (2007) - are seen as evidence for a resurgence of homologous blood doping.

A test for homologous blood transfusions was implemented in 2004 (Giraud *et al.*, 2010). WADA is currently funding research projects aimed at developing a test for autologous transfusions.

SUMMARY

All three methods (hypoxia exposure, use of Epo, and blood transfusions) allow more oxygen to reach the muscles at peak performance; consequently, allowing the athlete to undergo less fatigue. All three methods are extremely effective performance-enhancers but are dangerous. The dangers of doping are highlighted in a later section, but first let's examine the procedure and physiology underlying blood doping.

HOMOLOGOUS BLOOD DOPING: THE PROCEDURE

Blood consists of approximately (by volume): 55% plasma, 44% RBCs, and 1% leukocytes (white blood cells or WBCs) and platelets (Schumacher *et al.*, 2000). The circulatory system of an adult contains approximately 5000 mL of blood. During a blood transfusion, an athlete will commonly transfuse one unit (500 mL) of blood. The transfusion of one unit will result in the admixture of approximately 10% allogeneic erythrocytes in the circulation. Ten percent of the athlete's circulating RBC will be from donor blood. A 10% increase in RBCs will benefit the athlete by increasing the athlete's oxygen-carrying capacity, thereby increasing endurance during sport (Archer *et al.*, 1982). After transfusion, the athlete will experience polycythemia, an increase in the proportion of blood volume that is occupied by red blood cells

(Martini *et al.*, 2003). The increase in blood volume after transfusion increases blood pressure; the body reacts by filtering the excess fluid (plasma) in order to decrease blood pressure. As plasma gets filtered, the concentration of RBC to plasma in the blood increases which causes polycythemia.

MAXIMAL OXYGEN CARRYING CAPACITY

RBCs contain hemoglobin, an iron-rich protein that carries oxygen within the blood stream. Maximal oxygen carrying capacity is the maximum amount of oxygen that hemoglobin can carry in circulation. During endurance exercise, the body relies primarily on aerobic energy. The amount of oxygen that can be transported is positively correlated to the efficiency of the energy conversion process of the body. Oxygen acts as the final electron carrier in the breakdown of glucose into energy; therefore more oxygen being carried in the blood allows for an increase in the release of energy. Accordingly, the most important factor influencing endurance performance is maximum oxygen carrying capacity (Noakes 1991). Meeting muscle oxygen demand is dependent on the cardiovascular system's capacity for oxygen delivery, which is determined by the product of cardiac output and oxygen extraction (Lindstedt *et al.* 2001). As stated earlier, oxygen is delivered to muscles via hemoglobin. Therefore increasing the hemoglobin concentration will allow an athlete to increase endurance. Consequently, athletes who use blood doping as a mean to improve their performance are endurance athletes. Typically, they participate in sports such as cross-country skiing and cycling.

The intensity of exercise is limited by factors such as: pulmonary diffusing capacity, skeletal muscle characteristics, cardiac output and hemoglobin concentration (Clausen 1976, Lindstedt *et al.* 2001, Bassett and Howley 1999). The prevailing view is that $\text{VO}_{2\text{max}}$ is limited primarily by the rate of oxygen delivery, not the ability of the muscles to take up oxygen from the blood. There has been a long history of research devoted to investigating the factors that

limit maximum oxygen uptake. These usually focus on a single bottleneck that set the upper limit to oxygen flow. In general, there are three categories of bottlenecks: oxygen uptake by mitochondria; oxygen uptake by the lungs; and lastly, oxygen delivery by circulation (Ferretti and di Prampero, 1995). Maximal oxygen uptake is referred to as an individual's $\text{VO}_{2\text{max}}$. During exercise, when 85–95% of $\text{VO}_{2\text{max}}$ is reached, there is an excessive release of CO_2 relative to the rate of oxygen uptake due to a limitation in the rate of delivery of oxygen (Coates 2004). This leads to the onset of anaerobic muscular metabolism with lactic acid production, which produces arterial acidosis and stimulates chemoreceptors to elicit hyperventilation (Coates 2004). Coates also noted that training experience and some genetic factors are determinants of overall fitness to exercise.

Looking at the factors limiting the intensity of exercise, we notice that all can be influenced by training experience and genetics. However, increasing one's hemoglobin concentration is the simplest way by which to improve performance. As noted above, maximal oxygen carrying capacity is limited by hemoglobin concentration; thus, increasing hemoglobin concentration will increase one's maximum oxygen carrying capacity.

THE PREVALENCE OF DOPING

The use of performance enhancing drugs and blood doping has had a long history in sport. The first documented use of drugs to improve an athlete's performance was Thomas Hicks during the 1904 Olympic Games. By 1967, the IOC had banned the use of performance enhancing drugs in Olympic competition and introduced the first drug tests at the 1968 Winter Games.

Blood doping is believed to have entered sport in the late 1960s but was not made illegal until 1986. Blood doping was rife in the 1968 Games. The host city, Mexico City, is situated at

an elevation of 2240 metres. The high altitude prevented athletes from being able to compete at maximal level; to compensate many athletes doped and were caught. It was not until 1986 that blood doping was made illegal (Giraud *et al.*, 2010). Before then, blood doping was suspected among long-distance runners, notably Lasse Viren, winner of the 5,000m and 10,000m in the 1972 Munich and 1976 Montreal Olympics. As well, it was also used by the US cycling team in the 1984 Los Angeles Games. More recent cases include: Swedish cyclist Niklas Axelsson who was caught for doping in 2001; Tyler Hamilton who tested positive for homologous doping in the 2004 Athens Olympic Games; Alexander Vinokourov and Andrej Kashechkin, of the Astana Team, tested positive for homologous doping in the 2007 Tour de France; New York Rangers prospect and Russian hockey player Alexei Cherepanov died due to blood doping in 2008 (Canadian Press, TSN 2008, retrieved 2010); and German speed skater and five time Olympic gold medalist Claudia Pechstein was banned from the 2010 Olympics for abnormal blood parameters.

A study by Stray-Gundersen *et al.* (2003), found that in international cross-country ski racing, the top 50 athletes finish within 10% of the best time. The study investigated the prevalence of abnormal hematologic profiles in cross-country skiers. Using flow cytometry, erythrocyte and reticulocyte indices were analyzed from blood samples taken as part of routine International Ski Federation blood testing procedure from participants in the World Ski Championships. Sixty-eight percent of all skiers and 92% of those finishing in the top ten places were tested. The results showed that 17% of skiers had highly abnormal hematologic profiles; 19% had abnormal values; while only 64% had normal values. Fifty percent of medal winners and 33% of those finishing 4th to 10th place had highly abnormal hematologic profiles. In contrast, only 3% of skiers finishing 41st to 50th place had highly abnormal values. All reference values used were from the 1989 Nordic Ski World Championships data set and the

International Olympic Committee Epo 2000 project. This data suggest that the current blood doping testing programs are ineffective. Ramifications of doping not only affect the general public but can also pose radical danger to athletes. Some dangers and some already established modes of detections are presented below in order to demonstrate the necessity of better blood doping detection technique.

PERFORMANCE ENHANCEMENT BY BLOOD DOPING COMES AT A COST

The abuse of blood doping has many potential health risks (Table 1). Blood doping not only sheds a negative a light on the reputation of the sport but is also dangerous to the health of the athlete. By explaining the physical risks and dangers to athletes, we demonstrate the importance of new developments in detection techniques.

Health Risk	Explanation	Ramification	Doping Method
Anemia	A decrease in normal number of RBCs or less than the normal quantity of hemoglobin in the blood	Lack of adequate oxygen to the tissues. The body may compensate by increasing cardiac output causing palpitations, angina (if preexisting heart disease is present), intermittent claudication of the legs, and symptoms of heart failure.	Autologous
Thromboembolism	Formation of a clot in a blood vessel	Clot may plug a vessel in the lungs (pulmonary embolism), brain (stroke or cerebral embolism), gastrointestinal tract, kidneys, or leg.	Autologous and Homologous
Dehydration	Loss of bodily fluid is more than the intake	Heat injuries, cerebral edema, seizures, and kidney failure	Autologous
Hypertension	High blood pressure or tension in the arteries	Damage of the arteries, aneurysms, heart failure and ruptured blood vessels in the brain	Autologous and Homologous
Viral/Prion Infections	Infectious agents can survive in blood and infect the person receiving the blood transfusion	Contracting HIV, Hepatitis B and C and Creutzfeldt-Jakob disease	Homologous
Bacterial Infection	Blood can provide an excellent medium for bacterial growth, and can become contaminated after collection while it is being stored.	Risk of severe bacterial infection interfering with body's normal functions	Autologous and Homologous
Heart attack	The interruption of blood supply to part of the heart, causing some heart cells to die.	This is the leading cause of death in men and women who dope. (Beaglehole <i>et al.</i> , 2004)	Autologous and Homologous
Transfusion-associated graft versus host disease	Immune attack by transfused cells against the recipient	Recipient's immune system is not able to destroy the donor lymphocytes, leading to graft-versus-host disease.	Homologous
Acute Immune Hemolytic Reaction	Results from the rapid destruction of the donor red blood cells by host antibodies, usually related to ABO blood group incompatibility	Dyspnea, fever, chills, and severe pain. Shock may develop; low BP; and nausea and vomiting. Jaundice may follow acute hemolysis.	Homologous

Table 1 Potential health risks associated with blood doping (Ting P.H., 2005. Retrieved 2010).

In summary, although blood doping increases performance levels, it comes at a significant cost (Popovsky *et al.*, 1995). Increasing one's blood hemoglobin concentration enhances the body's oxygen delivery to muscles but may lead to a plethora of health risks. In both autologous and homologous blood doping, the most frequent cause of health complications is the increase in blood viscosity after transfusion (Bacher *et al.*, 2005, Dintenfass 1980). Thus there is for each individual an optimal hemoglobin value that is a trade off between oxygen carrying capacity and excess viscous drag to blood flow. Specific disadvantages to autologous blood doping include the likelihood of anemia resulting from the removal of blood. The body may take up to a few weeks to recover from this loss of blood which would make continued high-level training very difficult during this time. Moreover, the likelihood of dehydration, caused by the removal of blood and high intensity exercise, makes the signs and symptoms of high blood viscosity more pronounced. Another disadvantage includes limited storage time of blood, which requires precise timing of blood withdrawal before competition. The main disadvantages of homologous blood doping are the chance of blood-borne infections causing serious illnesses and improper screening of donor blood leading to the body rejecting donor blood.

THE RACE BEGINS

As control of traditional doping agents becomes more effective, athletes wanting to dope seek other means to enhance performance. Doping controls previously relied on detecting exogenous substances in urine; an obvious step then was to use endogenous substances that could not be detected in urine. The application of blood doping soon became more prevalent. In homologous doping, the transfusion of one unit of blood results in the admixture of approximately 10% allogeneic erythrocytes in the circulation. To detect blood doping by such

transfusions, methods capable of detecting 10% or less of allogeneic blood must be applied. In search for the perfect strategy to detect doping, the development of a reliable blood test must be used, together with urine testing.

Many methods of detecting blood doping have already been developed and put into use. The general approach to detecting blood doping is to use indirect methods for screening. These methods vary between anti-doping agencies but they usually involve using multiple hematological parameters. Commonly used parameters include hemoglobin level, hematocrit and absolute and percentage reticulocyte (immature RBCs) counts (Giles 2005).

Currently, all types of doping are initially screened by measuring levels of hematocrit. Hematocrit (HCT) is the proportion of blood volume that is occupied by RBCs. The normal values for HCT are: 46% for men and 38% for women (Purves *et al.* 2004). In some sports, such as cycling and skiing, the maximum value of HCT for competition is 50% in both male and female competitors. Governing bodies established this cut off value in order to prevent health risks; however, this does not stop athletes from doping, they dope to reach values just under the cut off value. Athletes normally dope with one unit of blood in order to stay under this threshold. Even though, more than one unit may help the athlete perform better, it will quickly be detected as a 10% increase in blood volume. This would result in a proportional 10% increase in HCT pushing a male competitor whose initial HCT is 46% over the 50% HCT limit.

Although autologous doping is difficult to detect, anti-doping agencies can use basic screening tests to suggest doping has occurred. For example, a hematology panel can detect elevated iron and bilirubin levels from hemolysis that has occurred from the retransfusion of fragile RBCs. Also, retransfusion results in higher hemoglobin levels and thereby in oxygen carrying capacity, suppressing endogenous Epo production, low circulating Epo can be suggestive of doping.

In order to address the use of homologous doping, a test was designed to obtain direct proof of homologous blood transfusions. The test, based on flow cytometry, more commonly known by its trademark name, FACS™ (Fluorescence Activated Cell Sorting) is currently being used. Every red blood cell has a specific set of blood group antigens. When these antigens are tagged with fluorescent dyes (antibodies), a flow cytometer can differentiate cells with different set of antigens (Nelson *et al.* 2003). The cells go through a detection tube where a laser illuminates the fluorescent tags and sensors sort the cells depending on which antigens fluoresce. The RBC antigens detected are major blood group antigens (ABO), Rh and a few minor blood group antigens. A graphical picture of a single spike will represent a specific person's inherent antigen. If a person has received even a small amount of transfused blood, a second, smaller spike will appear on the graph. The second spike represents the appearance of another antigen, and suggests that blood doping has occurred.

Flow cytometry testing is a good method for detecting homologous doping; however, there are some deterring factors. For example, it requires storage and transportation of athletes' blood samples; samples must be tested soon after collection; it is invasive (requires venipuncture), and the equipment is expensive. Consequently, the test is preformed randomly and thus may allow many athletes guilty of doping to remain undetected. However, the greatest problem with flow cytometry is the lack of suitable antibodies directed against the minor blood group antigens (Nelson *et al.*, 2003). In addition, the antibody concentration for blood group quantification is critical and optimal dilution for each batch of antiserum must be tested (Nelson *et al.* 2003). The number of antigen sites expressed on RBCs varies from antigen to antigen; for example, there are 10,000-33,000 Rh (D) sites per RBC (Szymanski *et al.*, 1989; Reid and Lomas-Francis 1997), while some minor antigens have only 1,000 sites per RBC (Reid and Lomas-Francis 1997). The large discrepancy in antigen sites and the lack of optimization of

antibody concentration can cause a prozone effect, in which a high antibody: antigen ratios result in small, undetectable agglutination complexes.

In addition, there are differences between similar antibodies from different suppliers; moreover, Nelson *et al.* (2003) reported that variation from batch-to-batch (from the same supplier) is even more startling. Currently, there are no standardized antibodies for use in flow cytometry and until serological companies develop them, no standard control for the detection of blood doping exists. Inconsistencies in testing lead to false results and shed a negative light on anti-doping testing. It is crucial that any tests developed follow a set standard protocol internationally.

PICKING THE BEST GENES

Humans have two sets of 23 chromosomes that carry genetic information contributing to the anatomy and function of the body. One set of chromosomes is passed to the offspring from each parent. The chromosomes carry approximately twenty-five thousand protein-encoding entities called “genes” (Consortium, 2004). At the DNA level, humans are 99.9 % the same (Collins and Mansoura, 2001), and variations that exist between individuals are in part due to the relatively small, 0.1%, differences in the genome. Variations within the genome that are common in a population are called “polymorphisms”, whereas new variations are referred to as “mutations”. Every individual has a unique genome, where some regions of the genome are more variable than others. To test for homologous doping, it is informative to look at these variable regions; in fact, forensic analysis often uses these variable regions to generate a DNA profile of an individual (Devlin and Risch 1992). These variable regions are referred to as variable number tandem repeats (VNTRs), also known as minisatellites. These are the most

polymorphic loci known in the human genome (Harding 2005). They are a class of highly variable tandemly repeated DNA sequences. These repeating units range from 10 to more than 100 base pairs (bp) in length, depending on the locus (Debrauwere *et al.* 1997). Figure 1 illustrates a heterozygous VNTR gene. Due to their highly polymorphic nature, minisatellites are useful genetic markers for forensic purposes, linkage analysis, population structure analysis, and chimerism detection (Pena and Chakraborty 1994; Wang *et al.* 2002).

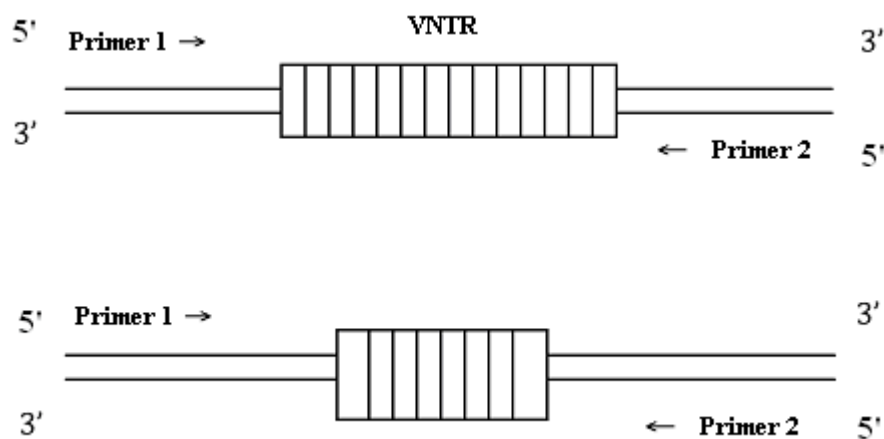


Figure 1 Heterozygous VNTR locus.

Every individual has two copies of every locus (excluding those found on the sex chromosomes), one inherited from each parent. A person can be homozygous or heterozygous for a locus; a variation of a locus is called an allele. The very nature of having only two copies of each locus is the reason that genetic testing is a good tool in detecting homologous blood doping. For example, the appearance of a third or fourth allele in a DNA profile implies that more than one blood population exists and is suggestive of homologous blood doping. However, it is possible for an athlete to have doped and have DNA profile that shows less than 3 alleles. This can occur if the athlete is homozygous for one allele and the donor homozygous for the

same (which would result in seeing only one allele in the DNA profile) or homozygous for another allele (which would result in seeing two alleles in the DNA profile). In this case, a secondary DNA test can be performed comparing the athlete's blood sample to their buccal (cheek) sample. If blood and buccal samples are mismatched, it is suggestive that blood doping has occurred. Therefore, buccal samples can be used to prepare non-blood cell DNA to use as an independent sample to corroborate the blood genotype.

This study's genetic detection method is qualitative, not quantitative. The test will confirm doping in two circumstances: if the DNA profile shows more than two alleles or if there is a mismatch between blood and buccal DNA profiles. A quantitative detection method is more costly and is discussed in the Future Directions portion of this paper.

This study identified a few of the most informative markers. The criteria for choosing informative VNTRs are to select allele-rich VNTRs (those with a large number of repeats) and those demonstrating multiple common alleles within the population. Those used in this study are: D1S80, D1S111, D17S30 and APO-B. These have been found to have an array of repeats in different individuals. Details of the VNTR loci can be found in Table 2.

VNTR loci	Found on Chromosome #	Repeat length (bp)	Common alleles	Size range (bp)	Num. of repeats	Reference
D1S80	1p36.32	16	18, 24	200-775 398-775 354-850	3-29 17-40 14-42 23 14-39 13-44	Budowle <i>et al.</i> 1995 Alonso <i>et al.</i> 1992 Nakajima <i>et al.</i> 1996 Chvatovic <i>et al.</i> 2003 Das <i>et al.</i> 2002 NCBI (UniSTS)
D1S111	1q22-q23	37	Unavailable	270-1047	6-27	Jeffreys <i>et al.</i> 1990
D17S30	17p13.3	70	4, 2, 3, 9	170-1080	14 1-11	Chvatovic <i>et al.</i> 2003 Horn <i>et al.</i> 1989
APO-B	2p24-p23	13-15 15	37,36, 38	510-1100	25 25-52 31-49	Chvatovic <i>et al.</i> 2003 Ludwig <i>et al.</i> 1989 Das <i>et al.</i> 2002
Gene locus	Found on Chromosome #	Length (bp)	Num. of alleles	Size range (bp)	Reference	
ACE	17	In= 65bp Del= 84bp	2	64-372	Rupert <i>et al.</i> 1999	

Table 2 VNTR and gene loci used in detecting homologous doping.

In addition to having selected informative VNTRs, we also selected an Alu element. Alu elements are classified as short interspersed nuclear elements (SINEs) amongst the class of repetitive DNA. It is estimated that approximately 10% of the human genome consists of Alu sequences; however, less than 0.5% is polymorphic (Roy-Engel *et al.*, 2001). The angiotensin-converting enzyme (ACE) is located on chromosome 17, contains an Alu insertion and is known to be polymorphic (Barbalić *et al.*, 2004). This locus is highly informative as both alleles are common in many populations, so there is a relatively high probability that the ACE I/D genotypes of any two unrelated individuals will differ.

The ACE I/D gene polymorphism is described as consisting of an insertion/deletion in intron 16. In the absence of the insertion, the primer pair ACE-1/ACE-3 amplifies an 84 base product. In the presence of the insertion, the primer pair ACE-2/ACE-3 amplifies a 65 base product. Both products are amplified in heterozygotes. Competition of primers limits amplification of the large (372 bp) ACE-1/ACE-3 product (shown in Figure 2).

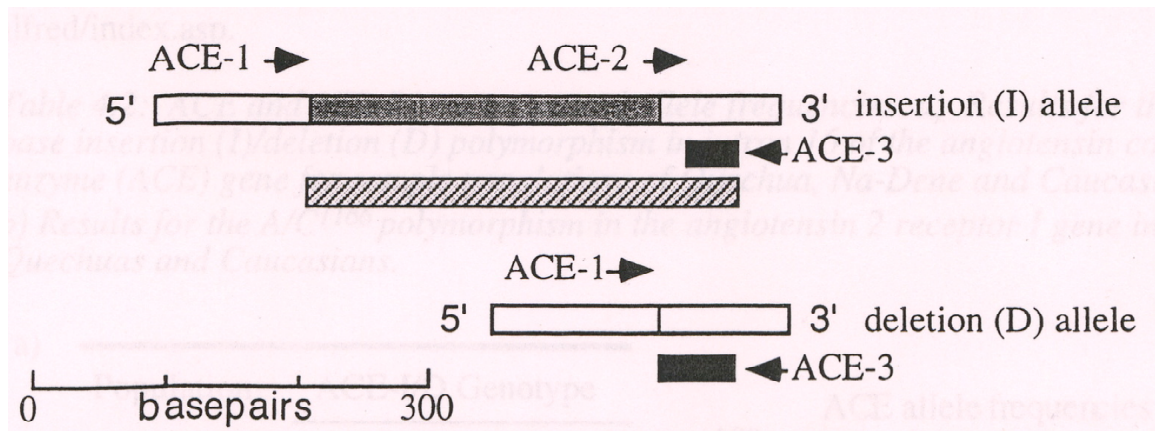


Figure 2: PCR based assay for the insertion/deletion polymorphism in the angiotensin converting enzyme (ACE) gene. The *Alu* insertion is shown in grey. ACE-1, ACE-2 and ACE-3 are primers. Diagnostic amplification products are shown in black. The large product potentially amplified by ACE-1/ACE-3 (striped) in the presence of the insertion is rarely seen. Figure from Rupert (2000).

OBJECTIVE OF THIS STUDY

There is widespread concern regarding fairness in sports. Blood doping undermines fairness; but, most importantly, it places the athletes' health at risk. The primary focus of this study is to reduce athletes' risks of developing serious complications associated with blood doping through the development of an improved blood doping test. We hope that this genetic-based test will facilitate the banning of athletes who dope by providing strong evidence of doping. Consequently, the prevalence of doping should decrease in light of detection methods which meet scientific standards of technical accuracy; inexpensive; and allow for easy, non-invasive testing. This study has addressed all of these criteria by developing a test that is non-invasive, cost efficient, having a set standard protocol that can be used universally, not requiring temperature-controlled transportation or storage of blood samples. These attributes allow testing to be done widely and more frequently, permitting the banning of athletes who decide to cheat.

RESEARCH QUESTION

Can informative gene loci be used for the detection of homologous blood doping?

MATERIALS AND METHODOLOGY

SAMPLE COLLECTION

Blood and buccal cell samples were collected from five healthy unrelated individuals. Subjects were recruited from the University of British Columbia faculty and staff (Consent Form and Recruitment Poster in Appendix G and H). The participants' ages ranged from 25 to 50 years (mean = 37.8). The ethnic groups represented included one Latin America, one East Asian, and one Middle Eastern and two European. Exclusion criteria included recent illness or injuries to minimize variation in white blood cell count and for reasons of lab biosafety. Ethical approval was obtained from the University of British Columbia, Clinical Research Ethics Board (CREB) (Appendix B). All participants provided written consent prior to participating in the study.

BLOOD SAMPLE PREPARATION

Blood was drawn from the antecubital vein of participants on January 23, 2009. Approximately 3mL of blood was collected from each participant. Upon blood collection, a complete blood count (CBC) was performed using a Hemavet HV950FS Multispecies Hematology System (Drew Scientific, Oxford, UK). It was particularly important to identify the WBC count as the DNA in blood is only found in WBCs. Upon identifying the WBC count for every participant (Table 3), the samples were normalized for WBC counts to ensure that each sample contains the same concentration of DNA (Table 4). All mixtures were normalized to

1000 WBC cells/ul per tube. Blood doping was simulated by mixing the samples in the following: 100: 0 and 0:100 (“undoped”), 90:10 (typical doping), 50:50 (a positive control to make sure than both polymorphisms amplify equally) and the reciprocal 10:90 (Table 5). Genomic DNA was extracted from blood using PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). DNA was stored at -4°C for the majority of the experiment and then frozen at -20°C for long-term storage. Blood collection and DNA extraction were performed on the same day to avoid WBC cell death that would lead to changes in DNA concentration.

Subjects	WBC Count (1000cells/ul)
Y1	5.72
X1	4.52
X2	8.10
X3	5.80
X4	4.38

Table 3 Subjects' WBC count after blood collection. The first letter of each code represents gender (Y is male; X is female).

Mix	Ratios				
	Y1		X1		
Y1 x X1 (ul)	100% 174.83	90:10% 157.3 : 22.1	50:50% 87.4 : 110.6	10:90% 17.5 : 199.1	100% 221.24
	Y1		X2		
Y1 x X2 (ul)	100% 174.80	90:10% 157.3 : 12.3	50:50% 87.4 : 61.7	10:90% 17.5 : 111.1	100% 123.50
	Y1		X3		
Y1 x X3 (ul)	100% 174.80	90:10% 157.3 : 17.2	50:50% 87.4 : 86.2	10:90% 17.5 : 155.2	100% 172.40
	Y1		X4		
Y1 x X4 (ul)	100% 174.80	90:10% 157.3 : 22.8	50:50% 87.4 : 114.2	10:90% 17.5 : 205.5	100% 228.30
	X1		X2		
X1 x X2 (ul)	100% 221.20	90:10% 199.1 : 12.3	50:50% 110.6 : 61.7	10:90% 22.1 : 111.1	100% 123.50
	X1		X3		
X1 x X3 (ul)	100% 221.20	90:10% 157.3 : 17.2	50:50% 87.4 : 86.2	10:90% 17.5 : 155.2	100% 172.40
	X1		X4		
X1 x X4 (ul)	100% 221.20	90:10% 199.1 : 22.8	50:50% 110.6 : 114.2	10:90% 22.1 : 205.5	100% 228.30
	X2		X3		
X2 x X3 (ul)	100% 123.50	90:10% 111.1 : 17.2	50:50% 61.7 : 86.2	10:90% 12.3 : 155.2	100% 172.40
	X2		X4		
X2 x X4 (ul)	100% 123.50	90:10% 111.1 : 22.8	50:50% 61.7 : 114.2	10:90% 12.3 : 205.5	100% 228.30
	X3		X4		
X3 x X4 (ul)	100% 172.40	90:10% 155.2 : 22.8	50:50% 86.2 : 114.2	10:90% 17.2 : 205.5	100% 228.30

Table 4 Quantity (in μ L) of participants' blood mixed to create doping scenario. Doping ratios (columns); μ L of blood mixed per subject (rows).

Mixtures	Ratios				
Y1 x X1	100%	90:10%	50:50%	10:90%	100%
Y1 x X2	100%	90:10%	50:50%	10:90%	100%
Y1 x X3	100%	90:10%	50:50%	10:90%	100%
Y1 x X4	100%	90:10%	50:50%	10:90%	100%
X1 x X2	100%	90:10%	50:50%	10:90%	100%
X1 x X3	100%	90:10%	50:50%	10:90%	100%
X1 x X4	100%	90:10%	50:50%	10:90%	100%
X2 x X3	100%	90:10%	50:50%	10:90%	100%
X2 x X4	100%	90:10%	50:50%	10:90%	100%
X3 x X4	100%	90:10%	50:50%	10:90%	100%

Table 5 Artificial doping chart, showing blood mixtures and ratios for each subject.

BUCCAL DNA PREPARATION

The subjects provided buccal samples by brushing the inside of their mouths with a ‘cytobrush’ (Medscand Medical AB, Malmö, Sweden). The samples were stored in paper envelopes at room temperature, allowing them to air dry. DNA was isolated from the cytobrushes using techniques described by Saftlas *et al.* (2004), which are described here. The brushes were incubated at 55°C overnight (or for at least 8 hrs) in a 700 µL mixture of lysis buffer (Recipes, Appendix C) and proteinase K (0.11 mg/mL) to breakdown cellular proteins and remove the cells from the bristles. After incubation, the tubes were centrifuged for 2 minutes at 15,900 g at 4 °C. The brushes were discarded, and RNase (0.03 mg/mL) was added to the supernatant which was then incubated for 60 minutes at 55°C to denature RNA. 320 µL of 5M potassium acetate precipitation buffer was added and the tubes were stored on ice for 10 minutes and then centrifuged (15,900 g) for five minutes. The supernatants were transferred to new tubes and the pellets that had precipitated out of solution were discarded. Glycogen (0.025 mg/mL) and 510 µL of isopropanol were added to the solution and the tubes were stored on ice for 20 minutes, glycogen acts as a DNA carrier to pull the DNA out of solution (as described in the Invitrogen Catalogue, 2008). The tubes were centrifuged (15,900 g) for 10 minutes and the supernatants were discarded leaving DNA pellets. The DNA pellets were rinsed with 70%

ethanol (200 μL) followed by a one-minute centrifuge to remove remaining salts. The ethanol was carefully discarded from each tube, and the DNA pellets were air dried and re-suspended in 90 μL TE buffer (10 mM Tris/Cl, 1 mM EDTA pH 8.0) for future use. DNA was stored at -4°C for the majority of the experiment and then frozen at -20°C for long-term storage.

Buccal DNA samples can be used for persons demonstrating heterozygosity at all tested loci. The buccal DNA can confirm whether an individual has doped by comparing blood and buccal DNA profiles. An indication of doping is a mis-match of DNA profiles identified in blood and buccal DNA from the same person. This implies that there is an additional source of DNA in the person's blood.

DNA QUANTIFICATION

To ensure that DNA was properly extracted, all samples were quantified for DNA. The concentration of DNA was analyzed using a spectrophotometer. The machine was calibrated to zero absorbance using samples of distilled water and TE buffer. The absorbance of a 1 μL aliquot of each sample was analyzed at two wavelengths (260 nm and 280 nm) and the concentration of DNA was calculated based on the constant: 1 OD (optic distance) is equal to 50 $\mu\text{g/mL}$ of DNA. Each amplification sample contained 50-100ng of DNA.

POLYMERASE CHAIN REACTIONS (PCR)

PCR was performed in total volumes of 25 μL containing: 18.5 μL -19.0 μL of sterilized water, 0.7 μL of MgCl_2 solution, 2.5 μL of 10X PCR buffer, 0.2 μL of 10mM of each dGTP, dATP, dTTP, dCTP, 1 μL of 25 μM of each primer (forward and reverse), 0.1 μL of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 0.5 μL -1.0 μL of genomic DNA. The list of

PCR ingredients can be found in Appendix D. Amplification of VNTR loci Apo-B, D17S30, D1S111, D1S80 and gene locus ACE I/D were carried out using the primers described by Boerwinkle *et al.* (1989), Horn *et al.* (1989), Ugozzoli *et al.* (1991), al-Nasser *et al.* (1996), and Rupert J. (2000), respectively. The primer sequences and PCR conditions are described in Appendix E and F.

GEL ELECTROPHORESIS

PCR products were separated by polyacrylamide gel electrophoresis (PAGE). PAGE gels (10cm x 8cm x 0.75mm) that were 8% Acrylamide/Bis (Recipe, Appendix C) were inserted in a BioRad vertical gel electrophoresis chamber and the chamber was filled with tris-borate EDTA buffer at pH 8.5 (1 x TBE, see Appendix C). PCR products (11 µL) were loaded into individual wells with approximately 1 µL bromophenol blue loading buffer (30% glycerol, 10% TE). Following electrophoresis, the gels were soaked in a dilute solution of ethidium bromide (0.5 µg/mL) to stain the DNA. The DNA bands were visualized using ultra-violet light and the gels were digitally photographed with a Cannon Power Shot A620 with Canon LA-DC58F Lens adapter. The size of the DNA bands were determined based on their location in the gel compared to a standard 100 bp reference ladder (Invitrogen, Carlsbad, CA, USA).

RESULTS

DNA isolated from subjects was screened for four VNTR loci (D1S80, D1S111, APO-B and D17S30) and one gene locus (ACE I/D). Once the genotype of the subjects for the 5 loci were identified, the DNA was mixed in different ratios simulating blood doping. The experiments were repeated several times to ensure reliability of assay.

Each lane of the polyacrylamide gel represents the genomic DNA of a different individual. The alleles were classified by size using 100 bp ladder and were designated according to the number of repeat units. The distribution of VNTR allelic frequencies for each subject is listed in table 6:

	Y1	X1	X2	X3	X4
ACE I/D	Heterozygous I/D	Heterozygous I/D	Heterozygous I/D	Homozygous Deletion D/D	Homozygous Insertion I/I
D1S80	12/18	18/18	10/18	16/24	18/21
D17S30	8/13	4/13	1/13	2/2	6/6
D1S111	3/3	3/5	7/11	1/5	1/1
APO-B	37/37	37/37	37/37	37/37	37/37

Table 6: The distribution of VNTR and gene allele frequencies for each subject.

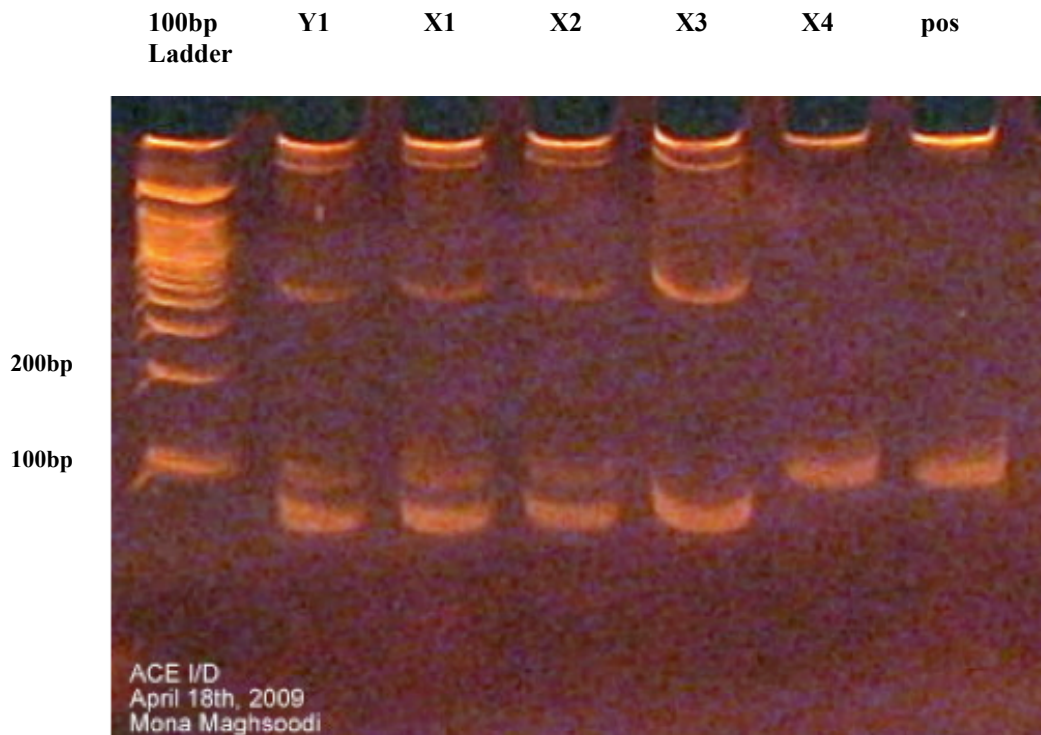


Figure 3 *ACE I/D polymorphism* on an 8% polyacrylamide gel. Genotypes shown are of subjects in a non-doping situation. Columns from left to right: 100bp ladder, subject Y1, subject X1, subject X2, subject X3, subject X4, positive control DNA. Subjects Y1, X1, and X2 are heterozygous for insertion and deletion alleles. Subject X3 is homozygous for the insertion alleles (65bp). Subject X4 is homozygous for the deletion alleles (84bp). The positive control DNA is also homozygous for the deletion alleles. A larger band (372bp) is seen in the columns Y1, X1, X2 and X3. This upper band is a product of the primer pairs for the ACE I/D polymorphism and appears in all columns where the subject displays an insertion allele.

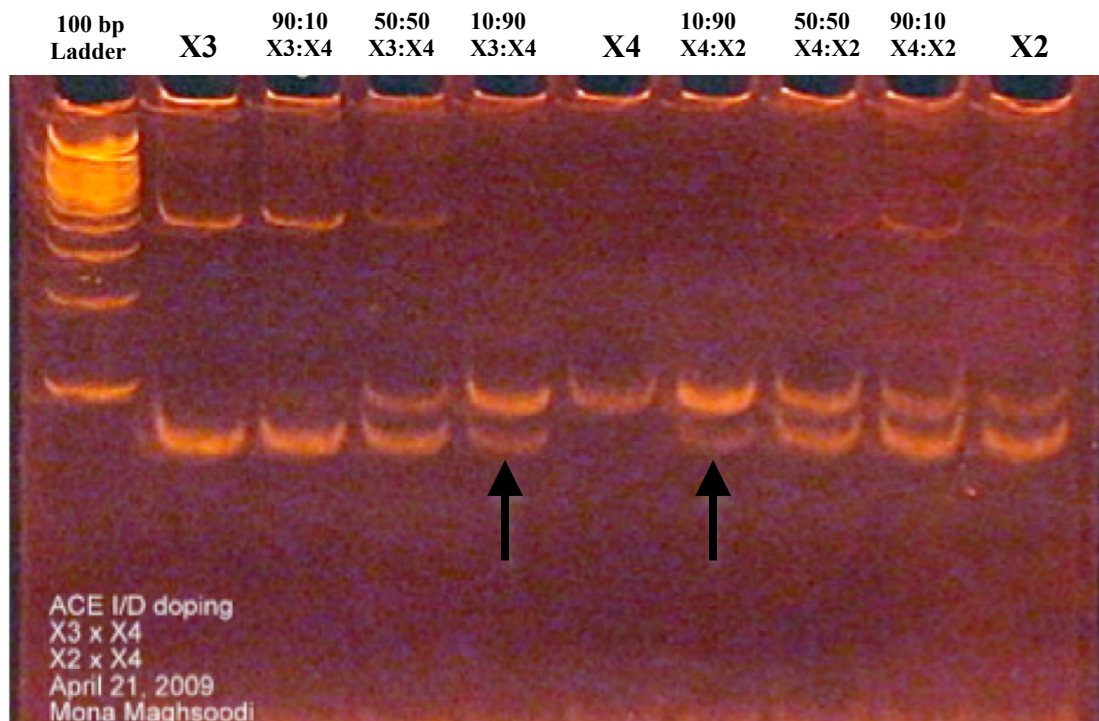


Figure 4 Doping: ACE I/D polymorphism on an 8% polyacrylamide gel. Genotypes shown are of subjects in a doping situation. Here we have artificially doped subjects X3 and X4; and subjects X4 and X2. Columns from left to right: 100bp ladder, 100% subject X3, 90% X3: 10% X4, 50% X3: 50% X4, 10% X3: 90%X4, 100% subject X4, 10% X4: 90% X2, 50% X4: 50% X2, 90% X4: 10% X2, 100% subject X2. Both arrows point to the bands contributed by the donor.

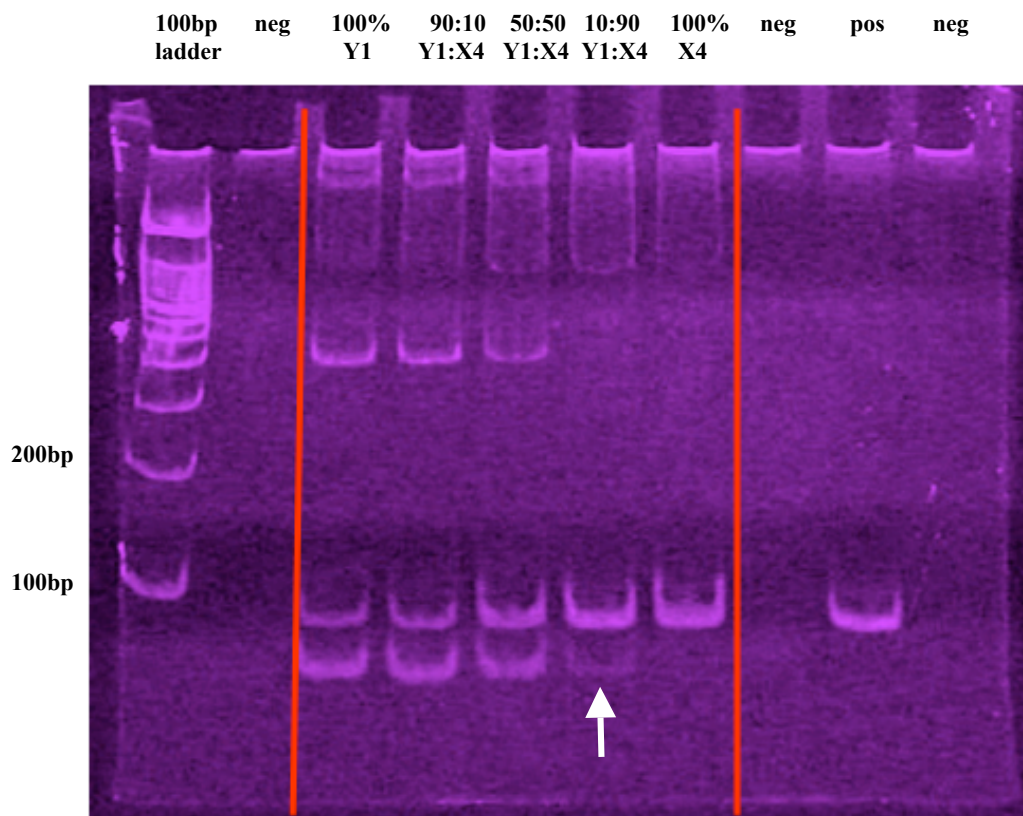


Figure 5 (Repeated assay for sensitivity) *ACE I/D* polymorphism seen on 8% polyacrylamide gel. Genotypes shown are of subjects in a doping situation. Here we have artificially doped subjects Y1 and X4. Columns from left to right: 100bp ladder, negative control (water), 100% subject Y1, 90% Y1: 10% X4, 50% Y1: 50% X4, 10% Y1: 90%X4, 100% subject X4, negative control (water), positive control of homozygous deletion, negative control (water). The arrow points to the band that is significant of doping.

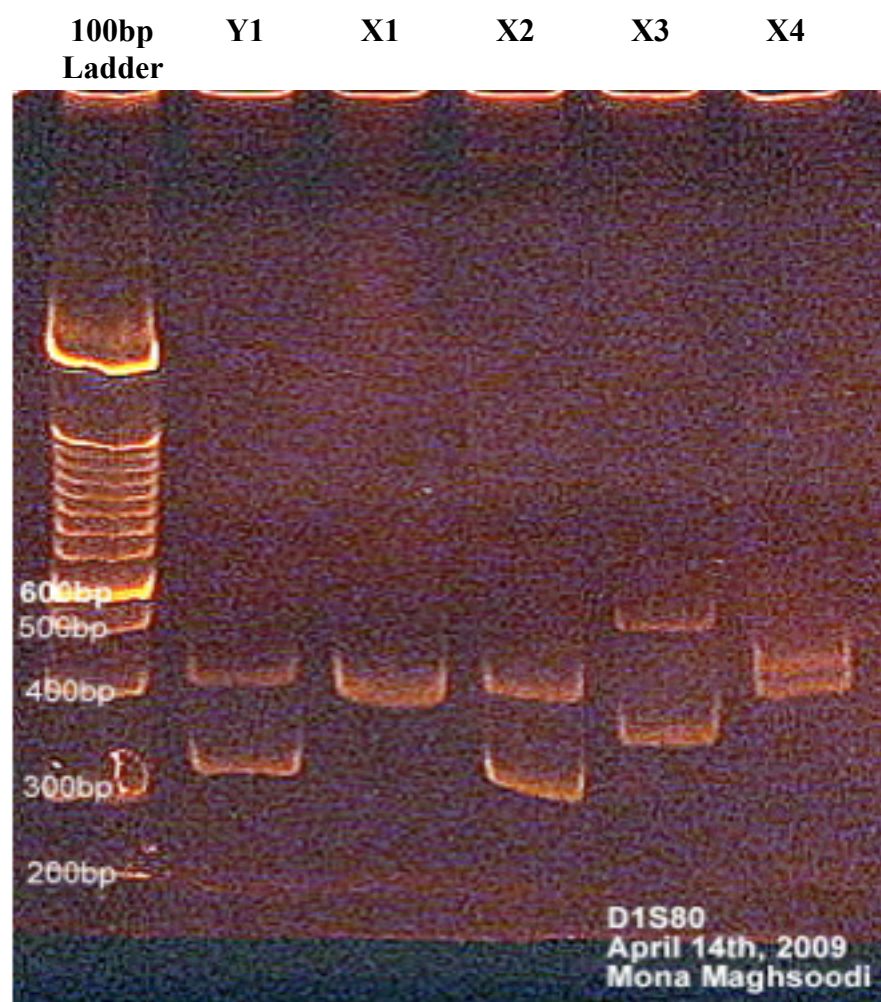


Figure 6 *D1S80 locus polymorphism* on 8% polyacrylamide gel. Genotypes shown are of subjects in a non-doping situation. Columns from left to right: 100bp ladder, subject Y1, subject X1, subject X2, subject X3, and subject X4.

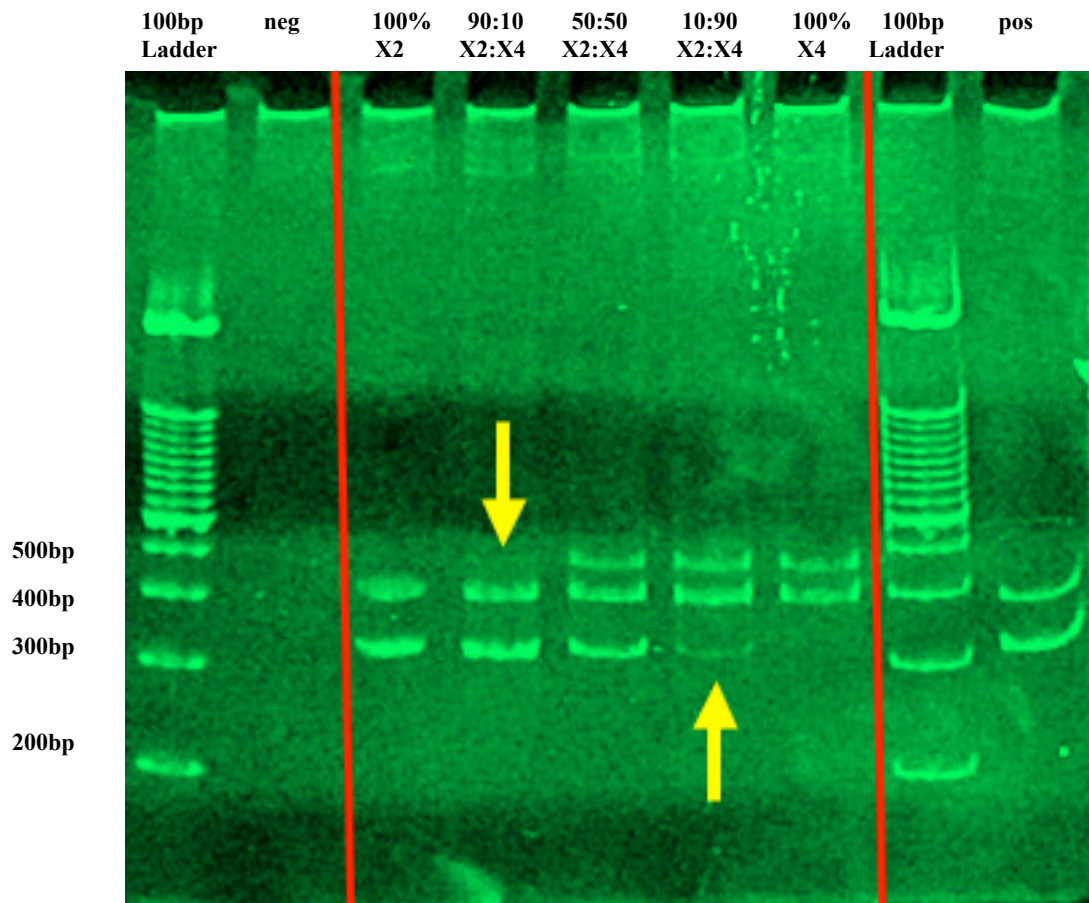


Figure 7 Doping: D1S80 locus polymorphism on an 8% polyacrylamide gel. Genotypes shown are of subjects in a doping situation. Here we have artificially doped subjects X2 and X4. Columns from left to right: 100bp ladder, negative control (water) 100% subject X2, 90% X2: 10% X4, 50% X2: 50% X4, 10% X2: 90%X4, 100% subject X4, 100bp ladder, positive control. Both arrows point to bands that are significant to doping.

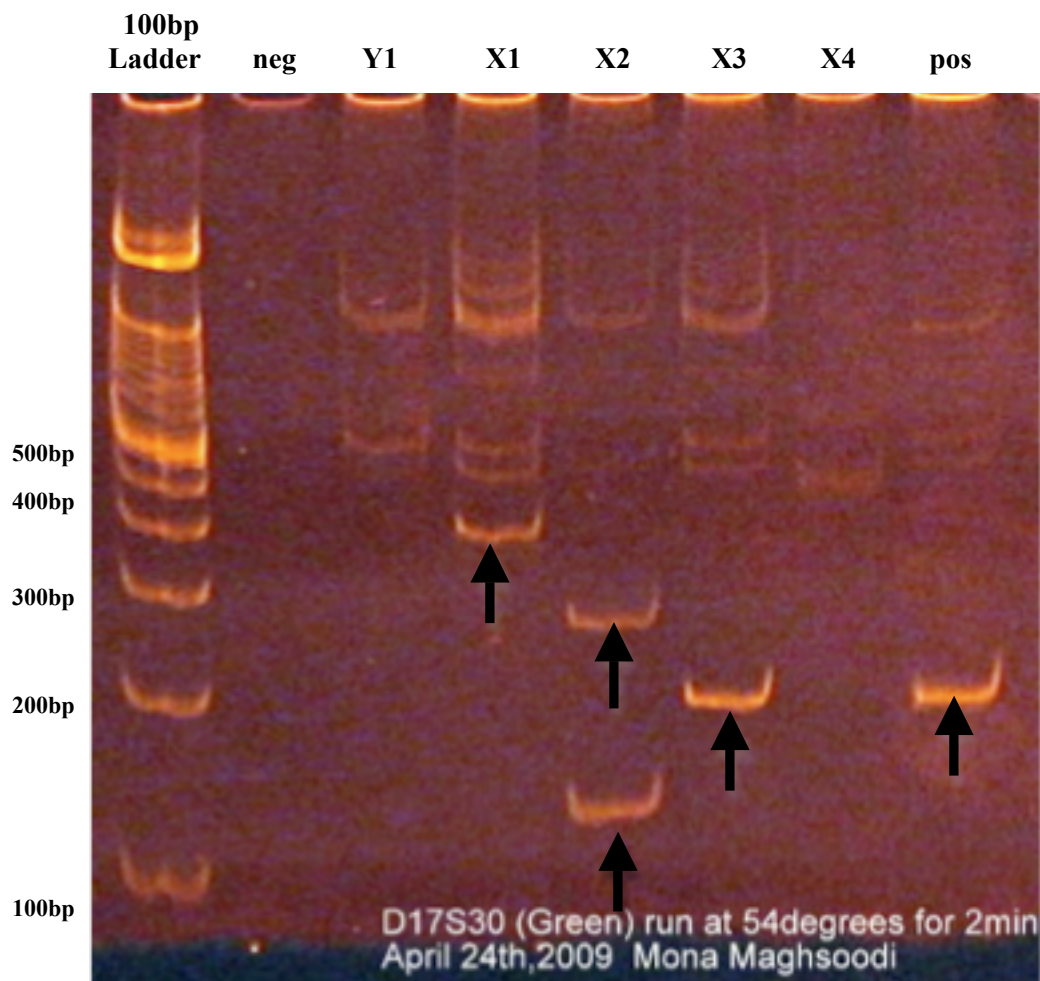


Figure 8 *D17S30 locus polymorphism* on 8% polyacrylamide gel. Genotypes shown are of subjects in a non-doping situation. Columns from left to right: 100bp ladder, negative control (water), subject Y1, subject X1, subject X2, subject X3, subject X4, positive control DNA. This locus was a very difficult locus to amplify and hence not all genotypes are clearly displayed. The genotypes of subjects Y1 and X4 are unclear. Here, arrows point to what we believe to be real bands. All other bands appear to be PCR artifacts, or nonspecific products.

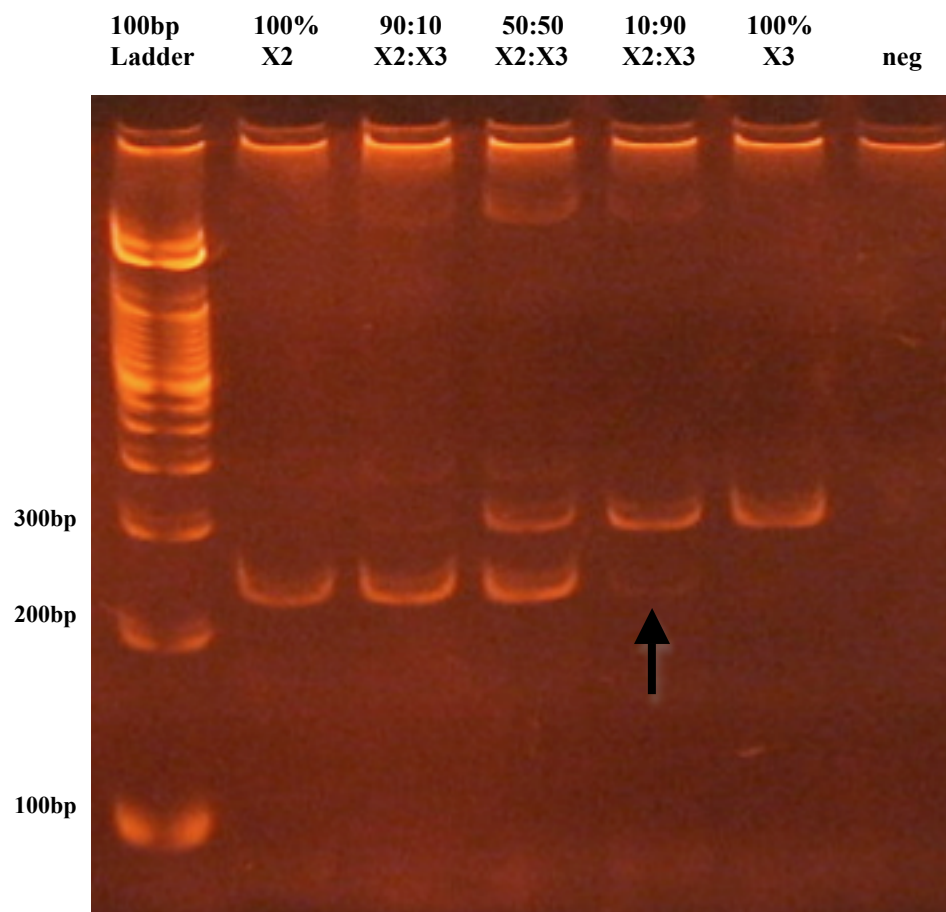


Figure 9 Doping: *D17S30* locus polymorphism on 8% polyacrylamide gel. Genotypes shown are of subjects in a doping situation. Columns from left to right: 100bp ladder, 100% subject X2, 90% X2: 10% X3, 50% X2: 50% X3, 10% X2: 90%X3, 100% subject X3, negative control (water). The arrow points to the band contributed by the donor.

The criteria for primer “failure” in this study is defined as those that produce profiles that exhibit the presence of PCR artifacts, nonspecific products, low signal, or no PCR product at all when the positive and negative worked. There was one primer pair out of a total of five that failed to consistently amplify. The D1S111 primers seldom amplified. We were able to obtain genotypes of each subject; however, doping resulted in low signaling or no PCR products. A figure of doping D1S111 locus is, therefore, not provided.

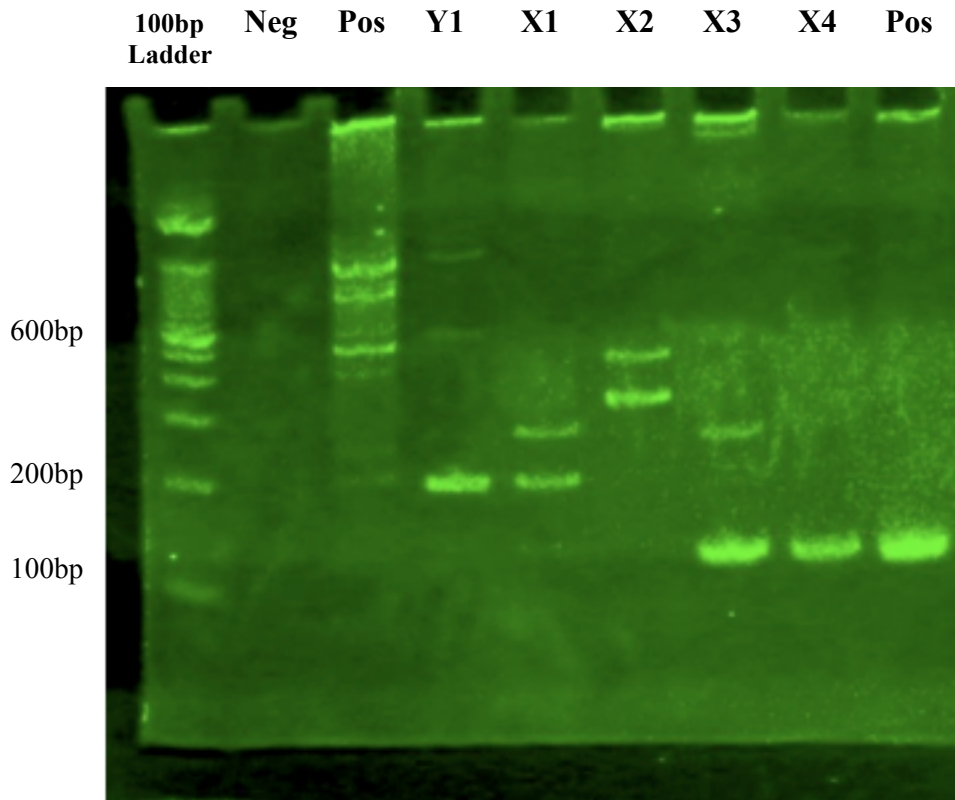


Figure 10 *D1S111 locus polymorphism* seen on 8% polyacrylamide gel. Genotypes shown are of subjects in a non-doping situation. Columns from left to right: 100bp ladder, negative control (water), positive control (tissue DNA), subject Y1, subject X1, subject X2, subject X3, subject X4, positive control DNA. This locus was a very difficult locus to amplify. The genotype of subject Y1 is unclear (three bands appear in this column).

The criteria for “informative” VNTR loci in this study are defined as those that produce profiles that exhibit variation in population. There was one VNTR locus that did not exhibit variation in our subjects. All our subjects displayed the same genotype in the APO-B locus. “Doping” of subjects would not be informative; therefore, we did not dope any subjects for this locus; as a result, a figure of doping APO-B locus is not provided.

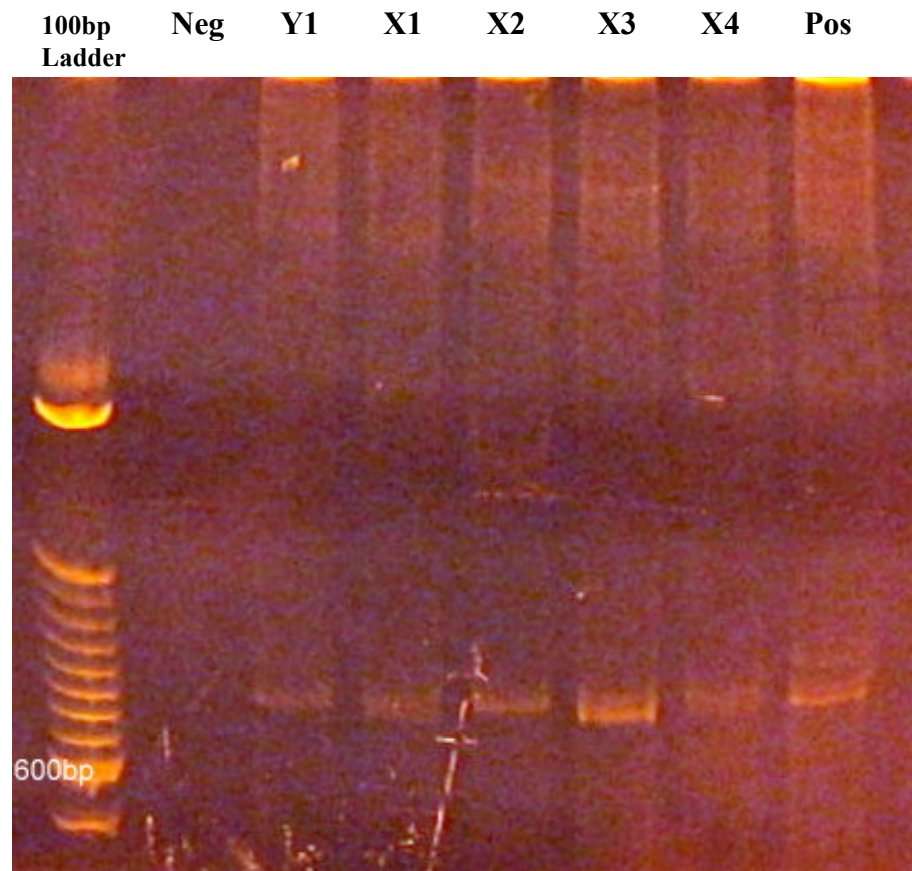


Figure 11 APO-B locus polymorphism seen on 8% polyacrylamide gel. Genotypes shown are of subjects in a non-doping situation. Columns from left to right: 100bp ladder, negative control (water), subject Y1, subject X1, subject X2, subject X3, subject X4, positive control DNA. All our subjects demonstrated the same genotype for this locus. We did not test this locus in any “doping” experiments, as results would not be informative.

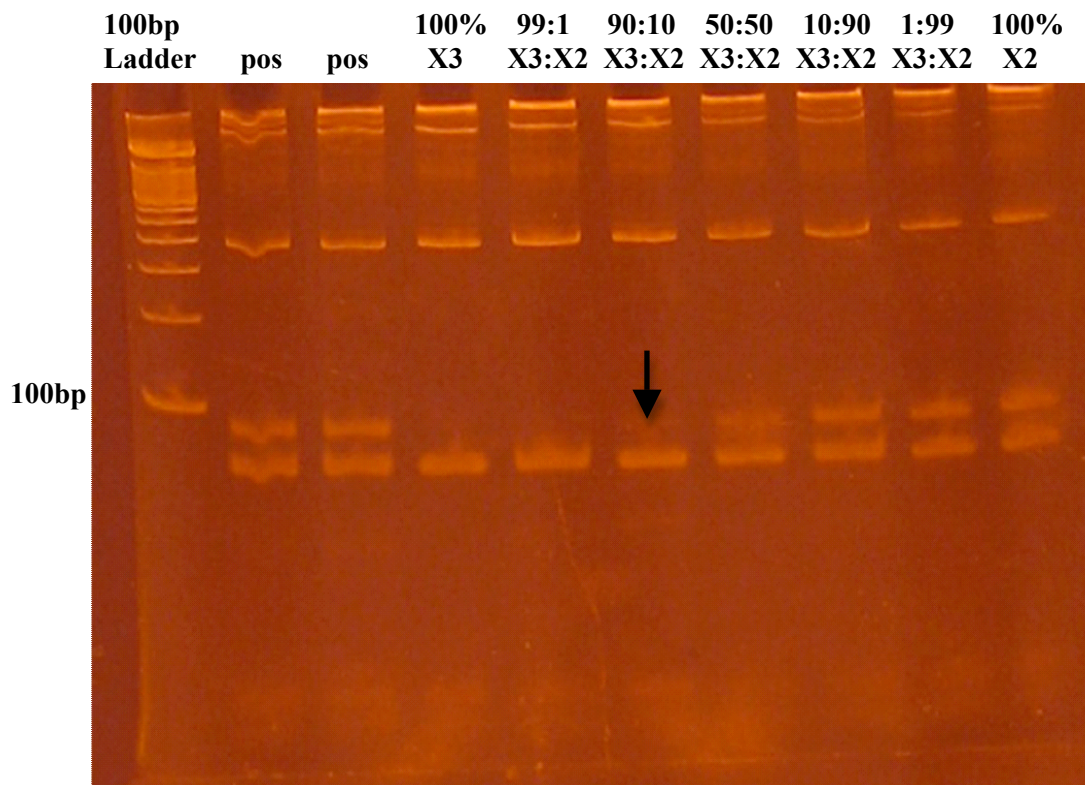


Figure 12 Gradient doping (using finger prick blood): ACE I/D polymorphism on an 8% polyacrylamide gel. Genotypes shown are of subjects in a doping situation using a finger prick of blood. Here we have artificially doped subjects X2 and X3. Columns from left to right: 100bp ladder, positive control, positive control, 100% subject X3, 99% X3: 1% X2, 90% X3: 10% X2, 50% X3: 50% X2, 10% X3: 90% X2, 1% X3: 99% X2, 100% subject X2. Arrow points to the band contributed by the donor. This represents a 10 in 90 dilution of donor blood to recipient; however, as the donor is heterozygous the allele indicated by the arrow represents only 50 % of the donor's genotype. For this reason, this figure demonstrates that this assay could detect a 5% dilution (i.e. if the donor was homozygous their genotype would be detected in a dilution of 5 in 95).

DISCUSSION

The technique of PCR-based detection of homologous blood doping successfully identifies mixed blood cell populations in a simulated doping scenario, as seen in Figures 4, 5, 7, 9 and 12. Figures 3, 6, 8, 10 and 11 show the genotypes of participants for the following markers: ACE I/D, D1S80, D17S30, D1S11 and APO-B (respectively). The resolution of this qualitative technique is dependent on optimal PCR conditions for each locus and clarity of gel

image. The lifespan of WBCs vary from a few hours to months (Kaiser G.E., 2007. Retrieved 2010). Therefore, blood samples should be collected immediately before or after competition. The advantage of using genetic testing is that DNA can be stored for years.

In this study, VNTR and gene loci were used to detect the different blood populations. Different transfusion ratios were performed in order to determine the sensitivity of the test. This test was easily capable detecting 10% of allogeneic blood. The capacity to detect small volumes of donor WBC DNA suggests this is as an effective and sensitive method for identifying athletes who have transfused donor blood.

SENSITIVITY AND SPECIFICITY

Sensitivity and specificity are statistical measures of the performance of a binary test. Sensitivity measures the proportion of true positives (true positives divided by the sum of true positives and false negatives). Specificity measures the proportion of true negatives (true negatives divided by the sum of true negatives and false positives). A test with high specificity will have a low false negative rate (e.g. not letting people get away with doping); whereas, a test with high sensitivity will have a low false positive (e.g. not falsely accusing any athlete of doping). An ideal test will attempt to optimize both but there is often a trade off – the more sensitive a test, the more likely to get false positives, the more specific a test, the more likely to get false negatives. The sensitivity of this test is dependent on two factors: informative polymorphism selection and resolution of the photographed gel. The VNTRs used in this study are highly variable; that is to say, there are a number of common alleles in the population. Furthermore, the use of a high-resolution camera is very important as it allows detection of very faint DNA bands. Specificity was ensured using controls in every experiment. Two types of

controls were used; samples that contained no DNA (to test for contaminants that could lead to false positives) and samples with a known genetic profile (to ensure that the assay was working properly and characterizing the correct genotypes).

Possible causes of false positives and false negatives have been identified. In both cases, we noted that handling errors and contamination were the leading sources of inaccuracy. Contamination of laboratory equipment, blood samples, needles and other supplies may be a possible source of false results; however, this is true for any laboratory testing. Contamination of DNA samples by PCR product likely has the most pronounced impact on false results. This is a common problem when many samples are PCR amplified for the same regions. The PCR product is the perfect target for the primers, so a slight contamination is quickly amplified. PCR contamination would result in successful amplification of the allele in every sample tested. Another source of false-positive can occur during PCR amplification of repeats if the repeat unit is short and the polymerase “stutters” (i.e. reads the same region a second time before moving on). This would result in additional bands that would look like evidence for a second DNA in the sample. To avoid stutter, we chose longer repeats for this project.

Another potential source of false positives is mutations. This is quite unlikely as the mutation would have to occur in hematopoietic stem cell to be sufficiently common in the blood to amplify and would have to alter the sequence such that it would be mistaken for an allele (e.g. add or delete a repeat). Allele-rich VNTRs can be extremely informative; however, careful selection of allele repeat lengths must be considered.

Large differences in allele size can cause a problem because short products will amplify more quickly. This differential amplification can mask the larger product and make it appear as though a homozygous genotype exists where in fact the individual possesses a heterozygous genotype.

Mixed cell populations can arise naturally as a consequence of disease, blood transfusions and following bone marrow or blood stem cell transplantation (Shaiegan *et al.*, 2006; Sakai *et al.*, 2006; Mathé *et al.*, 1963; Carter *et al.*, 1998). These three conditions are the only cases in which a heterogeneous WBC population can arise in an individual, aside from special instances of hemorrhage between mother and fetus, intrauterine twin-twin transfusion and rare tetragametic chimeras (Verdiani *et al.*, 2009, Daniels 1999). The ability of PCR to detect foreign cells has been extensively studied (Alizadeh *et al.*, 2002). If an athlete has received a blood transfusion or has undergone bone marrow transplant anti-doping authorities must be notified. Moreover, if an athlete has ever been pregnant anti-doping authorities must also be made aware. This is not due to the fetal blood in the mothers blood stream per se, which is represented by a fetal bleed of 12 mL, or 0.25% fetal blood (Nelson 2003), but a fetal haemopoietic stem cell could have crossed into the mother's circulation and be contributing to her blood cell complement. Genotyping the child would address the problem if this was offered as a defense in a doping case. In the case of fetomaternal hemorrhage, our test would not detect such low levels of heterogeneous cells; in fact, we were unable to detect doping levels less than 5% (Figure 12). The lack of sensitivity at this level implies that our test will not detect naturally occurring foreign cells; the test will only detect foreign cells at doping levels. If an athlete has transfused more than one unit of homologous blood, the detection window would far exceed the requirements of this test.

This study is a qualitative test, not quantitative. Quantitative Polymerase Chain Reaction (QPCR) techniques could be used for quantitative testing. Poor amplification has little or no value to anti-doping agencies. Figure 10 (D1S11) depicts a locus that inconsistently amplifies; markers such as this are not valuable in doping tests. In fact, a study by Wang *et al.* (2002) used

similar markers and reported the frequency of informative VNTR loci to be ranked: D1S80>D17S30>APO-B>D1S111, with D1S111 being the least informative VNTR of all loci used. We found this ranking to be true in our study.

Athletes' lives are disrupted frequently by anti-doping agencies, even if they never test positive. Since a positive test will likely ruin an athlete's career, there should be no doubt when delivering a positive test verdict.

PICKING THE RIGHT MARKERS

It is important to pick informative markers in order to ensure high sensitivity of the test. As mentioned earlier, "informative" markers are defined as those that produce profiles that exhibit a variation in population. A large number of alleles mean that the number of possible genotypes is enormous. The following equation can be used to calculate the possible number of genotypes of each loci:

$n = \# \text{ of alleles}$

Homozygous genotypes = n

Heterozygous genotypes = $[n \times (n-1)]/2$

$\# \text{ of genotypes} = \text{homozygous genotypes} + \text{heterozygous genotypes}$

ACE I/D (2 alleles) = 3 genotypes

D1S80 (26 alleles) = 351 genotypes

APO-B (25 alleles) = 325 genotypes

D17S30 (14 alleles) = 105 genotypes

D1S111 (21 alleles) = 231 genotypes

It is important to use loci with evenly distributed allelic frequencies as genotypes will be

more variant within a population and the odds of detecting doping will be greater. Allele frequencies show the genetic diversity of a species population or equivalently the richness of its gene pool. The ACE I/D is characteristic of an evenly distributed marker in many populations. Let us consider the ACE I/D in the French-Canadian population. The allelic frequency is found to be: allele I = 0.42, allele D = 0.58 (Faldik *et al.* 2003) and the predicted genotypic distribution as seen if in Hardy-Weinberg equilibrium would be: I/I = 0.18, I/D = 0.5 and D/D = 0.32. In fact, ACE I/D has similar frequencies in many other populations such as Middle Eastern, European, African and South American (Salem and Batzer 2009; Nápoles *et al.*, 2006; Mello *et al.*, 2003). For the purpose of this study, a minor allele frequency of at least 0.4 (evenly distributed) is ideal. Let us consider an unevenly distributed gene, for example, a gene with a minor allele frequency of 0.10. Using the Hardy-Weinberg equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

(Where p^2 is the proportion of individuals in the population who are homozygous dominant; $2pq$ is the proportion of individuals who are heterozygous; and q^2 is the proportion of individuals who are homozygous recessive).

We determine the genotype frequencies to be: AA= 0.81, Aa= 0.18, aa= 0.01. Little variance is found within the population, as observed by 81% of the population being homozygous dominant for the gene. Therefore, this gene is not very informative; and consequently, not a good locus to be used in the detection of homologous blood doping. However, a polymorphism that is evenly distributed, such as the ACE I/D (Figure 4.0), will have a higher genotypic frequency among the different possible combinations. According to the Hardy-Weinberg equilibrium an allelic frequency of 50:50 would display genotypic frequencies of: BB= 0.25, Bb =0.50, bb =0.25 (Table 6). It would be more likely to detect homologous blood doping using the later frequencies as all 3 genotypes are frequently observed compared to the previous examples where only one genotype is dominantly seen in the population.

	I/I 0.25	I/D 0.50	D/D 0.25
I/I 0.25	0.0625	0.125	0.0625
I/D 0.50	0.125	0.25	0.125
D/D 0.25	0.0625	0.125	0.0625

Table 7: ACE I/D polymorphism genotype frequencies. Top horizontal row depicts donor blood; far left column depicts recipient blood. Green illustrates an informative result if doping occurred (37.5% informative results); Orange illustrates inconclusive results if doping occurred (62.5% inconclusive results).

It has been estimated that there are over 29,224 VNTRs in the genome (Naslund *et al.*, 2005). Multiple polymorphisms are needed for the test as individuals who are heterozygous would be uninformative at that particular locus (as any donor genotype would be masked) and there is a chance that at a single locus, the donor and the recipient have the same genotype. The chance of two unrelated individuals being genetically identical at 11 loci is about 0.002% (assuming allele frequencies 50:50). More polymorphisms could be incorporated if greater confidence (i.e. higher sensitivity) is required. We predict that any number of informative gene or VNTR loci can be used; however, only one informative locus is required to detect doping.

INTERNATIONAL ADOPTION

In order to further improve detection of abnormal blood profiles, WADA is leading the development of a strategy against doping in sport called the Athlete Passport which is based on following athletes' biological variables over time (WADA, 2009. Retrieved 2009). Detecting individual changes over time greatly increase the possibility of showing intrinsic abnormalities that disappear when comparing athletes with a normal reference group. The objective of this strategy is to detect abnormal variations of determined biological variables in order to better target testing and sanction those found with abnormal variations.

A study by Malcovati *et al.* in 2003, investigated the feasibility of a hematologic passport for athletes competing in endurance sports. Biological variations of hematologic parameters of professional athletes were analyzed and the possibility of defining subject-specific reference ranges between physiologic and abnormal variability was investigated. The analysis showed that despite the homogeneity of biological characteristics (i.e. age, sex) and sport discipline in the population of athletes, there were considerable variations in the hematologic parameters from subject to subject (Malcovati *et al.*, 2003). This highlights the importance of subject-specific reference ranges. The study demonstrated that hemoglobin and HCT values were higher at the beginning of the competitive season, and then declined in the well-trained athletes. This trend was observed in all the athletes, independent of their sport. In light of these data, the detection of increased values of hemoglobin and HCT during the competitive season should be considered abnormal.

It is important to implement the Athlete Passport but only if it can be used to catch cheaters; therefore, we must make sure that no loop holes exist. One way to enhance the Athlete Passport is to document genotypes. Not only should the passport have physiological information but it should also record genetic information, allowing anti-doping agencies to compare blood to the known genotype of the athlete. This can also lead to the identification of donor blood, if the donor is another athlete (most often blood is donated from team members). Furthermore, documenting genotypes can be useful for the positive identification of bagged blood frequently found in search of medical equipment.

THE ANTI-DOPING CHALLENGE: LIMITATIONS TO THE TEST

A limitation to this test is if an athlete seeks to transfuse blood from an identical twin. This test will not detect homologous doping between twins as all genetic information is identical. Furthermore, this test does not have the resolving power to detect blood that has been

leukoreduced. Leukoreduction is the removal (of the majority) of WBCs from the blood by filtration. Since, the majority of WBCs are removed (and WBCs contain DNA, mammalian RBCs do not contain DNA) the test can no longer genetically detect a foreign cell population. However, QPCR may be able to detect leukodepleted donor blood as it is more sensitive. The markers from this study can be used in a QPCR detection method.

CONCLUSION

This test successfully identified a 10% allogeneic cell population using a finger prick of blood (50 μ L). The test was found to be sensitive enough to detect 5% DNA (i.e. blood) dilution using the relatively low resolution gels and imaging equipment available. The D1S80, D1S111, D17S30, APO-B and ACE I/D loci, while they are easy to work with and very informative, they are by no means the only loci that can be used to genetically identify a heterogeneous blood cell population.

It is certain that the advancement of molecular biology techniques has changed and will continue to fundamentally change our original concept of doping. PCR genotyping of blood following transfusion has proved to have advantages over previous techniques. Identifying a technique that can detect homologous blood doping in a cost-effective, non-invasive manner without requiring storage of blood will allow more frequent testing. The ability to test more athletes regularly may encourage some to think twice about doping; benefiting both the sport and athletes who risk their lives and careers in so doing. However, as with all elements of effort from anti-doping regulatory agencies, for every test designed to detect the presence of illegal substances or illegal use of doping techniques, a parallel force is seeking a way to outwit the testers. Consequently, even using genetics to detect homologous blood doping is a race without a finish line.

FUTURE DIRECTIONS

If sport is to keep its standards and reputation in society, further action and support to develop efficient, sensitive and reliable methods for detection is required. These will most probably rely on analysis in blood samples. An advantage that blood samples provide is using modern DNA technology and performing quantitative measurements that are impossible in an untimed urine specimen. To test doping using a quantitative assay QPCR can be used.

QPCR is a laboratory technique based on regular PRC which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification of one or more specific sequences in a DNA sample. DNA should be prepared from pure blood and the mixtures using the same methods listed above. Genotyping should be done using a 96-well plate with either allele specific florescence primers (as done in Alizadeh *et al.*, 2002) or non-specific SyberGreen (which fluoresces when bound to double stranded DNA, as done in Lee *et al.*, 2006) will be used as a marker for amplification. Quantitative PCR machines measure the florescence after each reaction cycle, analyze the amplification curves and use this information to calculate the initial concentration of template DNA (Wilhelm *et al.*, 2003).

Each DNA preparation (pure and mixed) should be amplified in duplicate with each set of primers using the methods described in Alizadeh *et al.*, 2002 and Lee *et al.*, 2006. The recipient's DNA will be much more common than the donor's DNA and therefore amplify much more rapidly. The presence of a rapidly amplifying genotype (the recipient's) and a slowly amplifying genotype (the donor's) would be evidence of a mixture. The assays are based on those developed for detecting microchimerism in individuals who have received bone marrow transplants or blood transfusions Alizadeh *et al.*, 2002 and Lee *et al.*, 2006. Microchimerism arises when a transfused hematopoietic stem cell becomes established in the recipient and

replicates. The assays developed by Alizadeh *et al.*, 2002 and used in Lee *et al.*, 2006 are sufficiently sensitive to detect a second genotype from DNA present at less than 0.01% of the total DNA. This would be the equivalent of doping with 0.5 mL of blood – 1/1000 less than a typical “doping” transfusion.

REFERENCES

- Alizadeh, M.**, Bernard M., Danic B., Dauriac C., Birebent B., Lapart C., Lamy T., Le Prise. PY., Beauplet A., Bories D., Semana G., Quelvennec E., (2002) Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002. 99(12), 4618-25.
- Al-Nassar KE**, Mathew J, Thomas N, Fatania HR. (1996) Analysis of the D1S80 (pMCT118) VNTR locus polymorphism in a native Kuwaiti population by the polymerase chain reaction. *Forensic Science International* 1996; 78: 131–138.
- Alvarez A.**, Rives S., Montoto S., Sanz C., Pereira A., (2000) “Relative sensitivity of direct antiglobulin test, antibody’s elution and flow cytometry in the serologic diagnosis of immune hemolytic transfusion reactions.” *Haematologica*, February 2000, Vol: 85(2): 186-188.
- Archer G**, Parker G, editors. Walsh and Ward's A guide to blood transfusion, Pepperday Pty Ltd., Sydney. 1982.
- Bacher A.**, Eggensperger E., Koppensteiner R., Mayer M, Klimscha W., (2005) Pentoxifylline attenuates the increase in whole blood viscosity after transfusion. *Acta Anaesthesiologica Scandinavica* 2005 Jan; 49(1):41-46.
- Barbalić M.**, Perićić M., Škarić-Jurić T., Smolej Narančić N., (2004) ACE Alu Insertion Polymorphism in Croatia and its Isolates. *Croatian Scientific*, Vol 28 (2004), 2; 603-610.
- Bassett D.R, and Howley E.T.**, (1999) Limiting factors for maximum oxygen uptake and determinates of endurance performance. *Medicine & Science in Sports and Exercise*, pages 70-84.
- Birkeland, K.I.**, Stray-Gundersen, J. and Hemmersbach, P. (2000). Effect of rhEPO administration on serum levels of sTfR and cycling performance. *Medicine and Science in Sports and Exercise*, 32, 1238-1243.
- Boehnke M.** (1991) Allele frequency estimation from data on relatives. *American Journal of Human Genetics*, January; 48 (1): 22–25.
- Boerwinkle E**, Xiong W, Fourest E, Chan L. (1989) Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: application to the apolipoprotein B 3' hypervariable region. *Proc Natl Acad Sci USA* 1989; 86: 212–216.
- Canadian Press, TSN.** (2008) Russian investigators say Cherepanov was 'doping'. December 29, 2008. <http://www.tsn.ca/nhl/story/?id=261215>. Retrieved December 29, 2008
- Carter A.S.**, Bunce M., Cerundolo L., Welsh K.I., Morris P.J., Fuggle S.V., (1998) Detection of Microchimerism After Allogeneic Blood Transfusion Using Nested Polymerase Chain Reaction Amplification With Sequence-Specific Primers (PCR-SSP): A Cautionary Tale. *Blood*, Vol. 92 No. 2 (July 15), 1998: pp. 683-689

Castellon R, Hamdi HK (2007). "Demystifying the ACE polymorphism: from genetics to biology". *Current Pharmaceutical Design*, 13 (12): 1191–8.

Clausen J.P., Circulatory adjustments to dynamic exercise and effect of physical training in normal subjects and in patients with coronary artery disease. *Progress in Cardiovascular Disease* May-Jun 1976; Vol 18, Issue 6, 459-95.

Coats A.J.S, Anaemia and heart failure. *Heart* 2004, Vol 90. pages 977-979.

Collins F.S. and Mansoura M.K, (2001) The Human Genome Project: Revealing the Shared Inheritance of All Humankind. *Cancer*, Vol: 91, 221-225.

Consortium (2004). Finishing the euchromatic sequence of human genome. *Nature* Vol 431, 931-945.

Cram, S., (2002) Flow cytometry, an overview. *Methods in Cell Science*, 24: 1-9.

Cui KH, Warnes GM, Jeffrey R, Matthews CD. (1994) Sex determination of preimplantation embryos by human testis-determining- gene amplification. *Lancet* Vol: 343: 79–82.

Daniels G., (1999) Functional aspects of red cell antigens. *Blood Review* Vol: 13; 14-35.

Deacon J., and Gains P., (1995) A phantom killer: doctors target a new performance-enhancing drug. *Maclean's*, Toronto, 108 (27 November), 48, p. 58

de Bruijn M.H. and Fey G.H., (1985) Human complement component C3: cDNA coding sequence and derived primary structure. *PNAS* Vol 82(3) :708-712.

Debrauwere H, Gendrel CG, Lechat S, Dutreix M., (1997) Differences and similarities between various tandem repeat sequences: minisatellites and microsatellites. *Biochimie*. Vol 79 (9-10): 577-86.

Devlin B., and Risch N., (1992) Ethnic differentiation at VNTR loci, with special reference to forensic applications. *American Journal Human Genetics*. Vol:51 (3): 534–548.

Dintenfass L., (1980) Autoregulation of Blood Viscosity in Health and Disease. *Vascular and Endovascular Surgery*, Vol. 14, No. 4, 227-237

Eklom, B., Goldbarg A.N., Gullbring B., (1972) Response to exercise after blood loss and reinfusion. *Journal of Applied Physiology* Vol. 33, No. 2.

Eklom, B. and Berglund, B. (1991). Effect of erythropoietin administration on maximal aerobic power. *Scandinavian Journal of Medicine and Science in Sports*, 1, pages 88- 93.

Ferretti G. and di Prampero P.E., (1995) Factors limiting maximal O₂ consumption: effects of acute changes in ventilation. *Respiratory Physiology*, February 1995, Vol 99, Issue 2, pages 259-271.

Faldik K., Deslauriers B., Pausova Z., Gaudet D., Tremblay G., Kotchen T.A., Hamet P. Tremblay J., (2003) P-113: Effect of ACE gene I/D polymorphism on hypertension-related traits in french-canadian families. *American Journal of Hypertension* (2003) 16: 279-286.

Gambrell, R.C., Lombardo, J.A., (1994) Drugs and doping: blood doping and recombinant human erythropoietin, Mellion, M.B. (ed.): Sports medicine secrets. Philadelphia: Hanley & Belfus, 1994, pp. 130-133

Giles I., Hematologyautomation aids in athletic testing. *Medical Laboratory Observer*, July 2005.

Giraud S., Robinson N., Mangin P., Saugy M., (2008) Scientific and forensic standards for homologous blood transfusion anti-doping analyses. *Forensic Science International*, July 2008, Vol. 18; 179 (1), pages 23-33.

Giraud S., Sottas P.E., Robinson N., Saugy M., (2010) Blood transfusion in sports. *Handbook of Experimental Pharmacology*. Vol 195, pages 295-304.

Hahn, A.G., Gore C., Martin D.T., Ashenden M.J., Roberts A.D., Logan P.A., (2001) An evaluation of the concept of living at moderate altitude and training at sea level. *Comparative Biochemistry and Physiology*, Vol 128, Issue 4, April 2001, Pages 777-789.

Harding R.M., (2005) VNTRs in Review. *Evolutionary Anthropology: Issues, News, and Reviews*. June 2005, Vol1, Issue 2, Pages 62-71

Horn GT, Richards B, Klinger KW. (1989) Amplification of a highly polymorphic VNTR segment by the polymerase chain reaction. *Nucleic Acids Res* 1989; 17: 2140.

Jeffreys AJ, MacLeod A., Neumann R., Povey S., Royle NJ (1990). Major minisatellite loci detected by minisatellite clones 33.6 and 33.15 correspond to the cognate loci D1S111 and D7S437. *Genomics*, 70, 449-52.

Kaiser D., The Innate Immune System, September 2007
<http://student.ccbcmd.edu/courses/bio141/lecguide/unit4/innate/bloodcells.html>

Lee, T.H., Chafets D.M., Reed W., Li W., Yunting Y., Chen J., Utter G.H., Busch M.P., (2006) Enhanced ascertainment of microchimerism with real-time quantitative polymerase chain reaction amplification of insertion-deletion polymorphisms. *Transfusion*, 2006. 46(11): p. 1870-8.

Levine B.D. and James Stray-Gundersen J., (1997) "Living high-training low": effect of moderate-altitude acclimatization with low-altitude training on performance, *Journal of Applied Physiology*, Vol. 83, No. 1, pages 102-112.

Lindstedt S.L., and Conley K.E., (2001) Human aerobic performance: too much ado about limits to O₂, *The Journal of Experimental Biology*, Vol. 204, 3195-3199.

Malcovati L., Pascutto C., Cazzola M., (2003) Hematologic passport for athletes competing in

endurance sports: a feasibility study. *Haematologica*, 88 (05): May 2003: 570-581.

Martini F.H., Timmons M.J, Tallitsch R.B. (2003) *Human Anatomy 4th ed*, Prentice-Hall, pp. 538-545.

Mathé G., Amiel J.L., Schwarzenberg L., Cattani A., Schneider M., (1963) Haematopoietic Chimera in Man After Allogenic (Homologous) Bone-marrow Transplantation, *British Medical Journal*, Dec 28, 1963, pages 1633-1635

Mello G., Parretti E., Gensini F., Sticchi E., Mecacci F., Scarselli G., Genuardi M., Abbate R., Fatini C., (2003) Maternal-Fetal Flow, Negative Events, and Preeclampsia Role of ACE I/D Polymorphism. *Hypertension*, 41: 932.

Napoles O., Castellanos M.S., Leal L., Casavilla R., Camacho H., Ferrer A., Cintado A., Villareal A., Benitez J.V., Nazabal M., Velasco J.G.P., Cabale B., Novoa L.I., Duenas M., (2007) ACE I/D polymorphism study in a Cuban hypertensive population. *Clinical Chimica Acta*. Vol 378, Issues 1-2, March 2007, Pages 112-116.

Naslund K., Saetre P., von Salome J., Bergstrom T.F., Jareborg N., Jazin E., (2005) Genome-wide prediction of human VNTRs. *Genomics*, Jan 2005, Vol 81 (1), 24-35.

Nelson M., Popp H., Sharpe K., Ashenden M., (2003) Proof of homologous blood transfusion through quantification of blood group antigens. *Haematologica*, 88 (11): November 2003: 1284-1295.

Niu T., Chen X, Xu X., (2002) Angiotensin converting enzyme gene insertion/deletion polymorphism and cardiovascular disease: therapeutic implications. *Drugs* 62 (7) 2002: 977-93.

Noakes, T., (1991) *Lore of Running Discovering the Science and Spirit of Running*, Leisure Press, 1991; pp. 18.

Pena, S.D.J. and Chakraborty, R., (1994) Paternity testing in the DNA era. *Trends Genetics* Vol.: 204-209.

Persson U., Truedsson L., Westman K.W.A., Segelmark M., (1999) C3 and C4 allotypes in anti-neutrophil cytoplasmic autoantibody (ANCA)-positive vasculitis, *Clinical and Experimental Immunology*. 1999 May; 116(2): 379-382.

Popovsky M.A., Whitaker B., Arnold N.L.,(1995) Severe outcomes of allogeneic and autologous blood donation: frequency and characterization. *Transfusion*, 1995, Vol 35, Issue 9, pages 734-737.

Purves, W. K.; Sadava D., Orians G.H., Heller H.C., (2004). *Life: The Science of Biology* (7th ed.). Sunderland, Mass: Sinauer Associates. pp. 954

Reid M.E. and Lomas-Francis C., (1997) The blood group antigen facts group. Academic

press, NY USA 1997.

Rossi R., Gambelunghe C., Parisse I., Lepri E., Rufini S.,(1998) Erythropoietin and doping, *Med. dello Sport*, Turin, 51, 2, pp. 191-195

Roy-Engel, A., Carroll M.L., Vogel E., Garber R.,K., Nguyen S.V., Salem A.H., Batzer M.A., Deininger P.L., (2001) Alu Insertion Polymorphisms for the Study of Human Genomic Diversity. *Genetics* 159: 279–290.

Rupert J.L., (2000) Genetic variation and adaptation in the Quechua, a high altitude native population. University of British Columbia 2000.

Sachdeva M.P., Mastana S.S., Saraswathy K.N., Elizabeth A.M., Chaudhary R., Kalla A.K.. (2004) Genetic variation at three VNTR loci (D1S80, APOB, and D17S5) in two tribal populations of Andhra Pradesh, India. *Annals Human Biology*. 2004 Jan-Feb;31(1):95-102.

Saftlas, A.F., Waldschmidt, M., Logsdan-Sackett, N., Triche, E., and Field, E. (2004). Optimizing buccal cell DNA yields in mothers and infants for human leukocyte antigen genotyping. *American Journal of Epidemiology* 160, 77-84.

Sakai M., Ohashi K., Yamashita T., Akiyama H., Sakamaki H., Ohta K., Kisida S., Kamata N., (2006) Immune-mediated myelopathy following allogeneic stem cell transplantation. *International Journal of Hematology*, June 2006, Vol.84; No.3; Pages 272-275.

Salem A.H. and Batzer M.A., (2009) High frequency of the D allele of the angiotensin-converting enzyme gene in Arabic populations. *BMC Research Notes* 2009, 2:99

Schumacher, H.R., Rock W.A., Stass S.A., (2000) Handbook of Hematologic Pathology. Information Health Care, p. 559.

Shaiegan M., Hadjati E., Aghaiipour M., Iravani M., David M., Bernard D., (2006) Flow Cytometric Evaluation of Red Blood Cell Chimerism after Bone Marrow Transplantation in Iranian Patients: A Preliminary Study. *Archives of Iranian Medicine*, Oct 2006; 9(4):406-9.

Stray-Gundersen J., Chapman R.F., Levine B.D., (2001) "Living high-training low" altitude training improves sea level performance in male and female elite runners. *Journal of Applied Physiology*, September 2001, Vol 91, Issue 3, pages 1113-1120.

Stray-Gundersen J., Videman T., Penttilä I., Lereim I., (2003) Abnormal Hematologic Profiles in Elite Cross-Country Skiers: Blood Doping or ? *Clinical Journal of Sport Medicine*: May 2003 - Volume 13 - Issue 3 - pp 132-137.

Szymanski I.O., Araszkievicz P., Odgren P., Snyder L.M., (1989) Decreased amount of the Rh antigen D in hereditary spherocytosis (HS). *British Journal of Haematology* 1989 Dec; 73(4): 537-540.

Ting P.H., (2005) AnesthesiologyInfo.com. 2002-2005. Retrieved 2010.

Ugozzoli L, Yam P, Petz LD., Ferrara G.B., Champlin R.E., Forman S.J., Koyal D., Wallace R.B., (1991) Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. *Blood* 1991; 77: 1607–1615.

Veridiani S., Bonsignore A., Casarino L, Ferrari G.M., Zia S.C., DeStefano F., (2009) An unusual observation of tetragametic chimerism: forensic aspects. *International Journal of Legal Medicine*, 2009, 123:431–435

WADA, World Anti-Doping Agency (2009), <http://www.wada-ama.org/en/Resources1/Q-and-A/Q-A-Blood-Doping/> Updated Oct 2009; Retrieved 2009.

Wang L., Chou P., Gonzalez-Ryan L., Huang W., Haut P., Kletzel M. (2002) Evaluation of mixed hematopoietic chimerism in pediatric patients with leukemia after allogeneic stem cell transplantation by quantitative PCR analysis of variable number of tandem repeat and testis determination gene. *Bone Marrow Transplantation*. January 2002, 29 (1), 51-56.

Wilhelm, J. and A. Pingoud, (2003) Real-time polymerase chain reaction. *Chembiochem*, Vol 4(11): p. 1120-8.

Wehrlin J.P., Zuest P., Hallén J., Marti B., (2006) Live high-train low for 24 days increases hemoglobin mass and red cell volume in elite endurance athletes, *Journal of Applied Physiology* 100: 1938-1945.

World of Sports Science (2008), <http://www.faqs.org/sports-science/Ba-Ca/Blood-Doping.html>, Updated 2008; Retrieved 2010.

APPENDICES

- Appendix A A Brief History of Blood Doping
- Appendix B Clinical Research Ethics Board Certificate
- Appendix C Recipes
- Appendix D PCR Ingredients
- Appendix E Primer Sequence
- Appendix F PCR Conditions
- Appendix G Subject Consent
- Appendix H Recruitment Poster

APPENDIX A: A BRIEF HISTORY OF BLOOD DOPING

1628 English physician William Harvey discovers the circulation of blood.

1795 Philip Syng Physick performs the first human blood transfusion, although he does not publish this information.

1818 James Blundell performs the first successful transfusion of human blood to a patient for the treatment of postpartum hemorrhage and publishes these results.

1900 Karl Landsteiner discovers the first three human blood groups, A, B, and C. Blood type C was later changed to O.

1902 Alfred Decastello and Adriano Sturli add AB, the fourth type.

1912 Roger Lee demonstrates that it is safe to give group O blood to patients of any blood group, and that blood from all groups can be given to group AB patients. The terms "universal donor" and "universal recipient" are coined.

1916 Introduction of a citrate-glucose solution that permits storage of blood for several days after collection.

1932 The first blood bank is established in a Leningrad hospital.

1939/40 Karl Landsteiner discovers the Rh blood group system.

1940 John Elliott develops the first blood container, a vacuum bottle extensively used by the Red Cross.

1943 The introduction of acid citrate dextrose (ACD) solution, which reduces the volume of anticoagulant, permits transfusions of greater volumes of blood and permits longer term storage.

1950 In one of the single most influential technical developments in blood banking, Carl Walter and W.P. Murphy, Jr., introduce the plastic bag for blood collection, replacing breakable glass bottles.

1959 Max Perutz deciphers the molecular structure of hemoglobin, the molecule that transports oxygen and gives red blood cells their color.

1967 Rh immune globulin is commercially introduced to prevent Rh disease in the newborns of Rh-negative women.

1979 A new anticoagulant preservative, CPDA-1, extends the shelf life of whole blood and red blood cells to 35 days, increasing the blood supply and facilitating resource sharing among blood banks.

1981 First Acquired Immune Deficiency Syndrome (AIDS) case reported.

1983 Additive solutions extend the shelf life of red blood cells to 42 days.

1904 Thomas Hicks ran to victory in the Saint Louis Olympics with the help of raw egg, injections of strychnine and does of brandy administered to him during the race.

1906 HuEpo discovered.

1960 First reported case of blood doping. That same year, Danish cyclist Knud Enemark Jensen dies during competition at the Rome Olympics. Autopsy results reveal traces of amphetamine.

1963 France is the first country to enact an anti-doping legislation.

1966 UCI and FIFA introduce doping tests in their World Championships.

1967 IOC institutes a Medical Commission and sets up the first list of prohibited substances. Urgency of anti-doping highlighted by another tragic death, that of Tom Simpson during the Tour de France.

1968 Drug tests first introduced at the Olympic Winter Games in Grenoble and the Olympic Games in Mexico.

1974 Introduction of a reliable test method for anabolic steroids.

1976 IOC adds anabolic steroids to its list of prohibited substances.

1977 Purified Epo is isolated from human urine for the first time.

1980 Recombinant huEpo created.

1985 USCF and USOC banned doping. Epo gene is cloned.

1986 IOC bans blood doping.

1987 Recombinant Epo is first available in Europe.

1987-1990 A number of deaths of competitive Dutch and Belgian cyclists is linked to Epo use

1988 First cyclist banned for using rHuEpo. Ben Johnson tested positive for stanozolol (anabolic steroid) at the Olympic Games in Seoul. Johnson's case focused the world's attention to the doping problem.

1989 Recombinant Epo is approved by the FDA for manufacture.

1990s A remarkable drop in the level of top results. The main front in anti-doping rapidly shifted to blood doping as more effective test methods were being introduced.

1998 A large number of prohibited medical substances were found by police in a raid during the Tour de France.

1999 World Anti-Doping Agency is established.

2000 First test introduced for blood doping and Epo detection. Epo test, based on a combination of blood and urine analysis was first implemented at the Sydney Olympic Games in 2000.

2004 Tyler Hamilton tests positive for homologous blood doping.

2007 Andrej Kashkakin and Alexander Vinokourov test positive for homologous blood doping in the Tour de France.

APPENDIX B: CLINICAL RESEARCH ETHICS BOARD CERTIFICATE



The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF FULL BOARD APPROVAL

PRINCIPAL INVESTIGATOR: James L. Rupert		INSTITUTION / DEPARTMENT: UBC/Education/Human Kinetics		UBC CREB NUMBER: H07-02848	
INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:					
Institution		Site			
UBC		Vancouver (excludes UBC Hospital)			
Other locations where the research will be conducted: N/A					
CO-INVESTIGATOR(S): N/A					
SPONSORING AGENCIES: N/A					
PROJECT TITLE: Evaluating the potential of genotyping as a test for homologous blood doping in athletes					
THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: May 13, 2009 The full UBC Clinical Research Ethics Board has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.					
REB FULL BOARD MEETING REVIEW DATE: May 13, 2008					
DOCUMENTS INCLUDED IN THIS APPROVAL:				DATE DOCUMENTS APPROVED:	
Document Name	Version	Date	May 23, 2008		
<u>Protocol:</u>					
Protocol 1.1	1.1	May 21, 2008			
<u>Consent Forms:</u>					
Consent form v1.1	1.1	May 21, 2008			
<u>Advertisements:</u>					
Poster	1.1	May 21, 2008			
CERTIFICATION: In respect of clinical trials: 1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations. 2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices. 3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing. The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.					
Approval of the Clinical Research Ethics Board by one of:					
 Dr. Gail Bellward, Chair					

APPENDIX C: RECIPES

Lysis Buffer (DNA Isolation)

100 mM	NaCl
10 mM	TrisCl
25 mM	EDTA
0.5%	SDS

TBE Buffer (5x)

27 g	Tris base (s)
13.75 g	Boric Acid (s)
10 mL	EDTA (0.5 M)
490 mL	Water

8% PAGE Gel

6.3 mL	Water
2.4 mL	5 x TBE
3.2 mL	Acrylamid/Bis (30%)
80 µL	APS (10%, ammonium persulfate)
12 µL	TEMED

APPENDIX D: PCR INGREDIENTS

Standard Polymerase Chain Reaction (1 reaction, 25uL)

17.5 μ L	Water
2.5 μ L	10x PCR Buffer
0.7 μ L	MgCl ₂
0.2 μ L	dNTP
1.0 μ L	Forward Primer (20 <i>pmol</i> / μ L)
1.0 μ L	Reverse Primer (20 <i>pmol</i> / μ L)
0.2 U	<i>Taq</i> DNA polymerase
2.0 μ L	DNA template

APPENDIX E: PRIMER SEQUENCE

APO-B	5'ATG GAA ACG GAG AAA TTA TG3' 3'CAT AAA CGG TTC ACT CTT CC3'	Boerwinkle <i>et al</i> 1989
D1S80	5'GAA ACT GGC CTC CAA ACA CTG CCC GCC G3' 3'CGT TCC CCG TGC ACG TAG AGG TTG TTC TG3'	al-Nasser <i>et al</i> 1996
D17S30	5'CGA AGA GTG AAG TGC ACAG G3' 3'GCG ACT TCT TAT TTC TGA CAC3'	Horn <i>et al</i> 1989
ACE I/D	ACE-1: 5'CAT CCT TTC TCC CAT TTC TC3' ACE-2: 5'AAT TCA GAG CTG GAA TAA AAT T3' ACE-3: 5'TGG GAT TAC AGG CGT GAT ACA G3'	Rupert 2000
D1S111	5'TGT GAG TAG AGG AGA CCT CAC3' 3'AAA GAC CAC AGA GTG AGG AGC5'	Ugozzoli <i>et al</i> 1991

APPENDIX F: PCR CONDITIONS (USING THE G-STORM THERMOCYCLER GS1)

The PCR program for ACE I/D:

94°C for 30 sec,
35 cycles of:
94°C for 60 sec,
55°C for 60 sec,
72°C for 2 min.
Total runtime: 2:41.07

The PCR program for D1S80:

94°C for 1 min,
30 cycles of:
94°C for 15 sec,
68°C for 15 sec,
72°C for 15 sec,
The final synthesis chain at 72°C for 10 min.
Total runtime: 45.14

The PCR program for APO-B:

Heated lid 111°C
The first denaturation at 94°C 2 min
30 cycles of:
94 ° C for 1 min,
50 ° C for 3 min,
72 ° C for 1 min.
Total runtime: 2:51.56

The PCR program for D17S30 loci:

Heated lid 111°C
The first denaturation at 94°C 2 min
35 cycles of:
94°C for 1 min,
54°C for 30 sec,
72°C for 1.30 min,
The final synthesis chain at 72°C for 5 min.
Total runtime: 2:31.09

The PCR program for D1S111 loci:

The first denaturation at 95°C 2 min
40 cycles of
94°C for 30 sec,
57°C for 30 sec,
72°C for 30 sec,
The final synthesis chain at 72°C for 5 min.
Total runtime: 1:10.04

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SUBJECT INFORMATION AND CONSENT FORM

Project: Evaluating the potential of genotyping as a test for homologous blood doping in athletes.

Principal investigator: Jim Rupert, PH.D.

School of Human Kinetics
Room 346 Wesbrook Building, UBC
Vancouver, B.C., CANADA V6T 1Z2
Phone (604) 822-8462 Fax (604) 822-9451; E-mail: rupertj@interchange.ubc.ca

Other investigators:

Dr. Wendy Robinson, Ph.D.; UBC Medicine, Faculty of Medical Genetics

Dr. Maria Gallo, Ph.D.; UBC School of Human Kinetics,

Maghsoodi Mona, M.Sc.; UBC School of Human Kinetics

Sponsor: UBC Faculty of Education/School of Human Kinetics

Emergency Telephone Number: Dr Jim Rupert (as above) or cell (778) 386-0908 (24 hours/day; 7 days/week)

Introduction: Homologous blood doping is a form of performance enhancement prohibited by the World Anti-doping agency in which athletes inject a unit (about ½ liter) of blood from another person into their circulation to improve their oxygen carrying capacity. As aerobic performance depends on oxygen availability, this practice can improve athlete's endurance performance. The procedure can be risky as it puts a load on the heart and there is a risk of infection if the blood taken is contaminated. Current tests for homologous blood doping are expensive and sensitive to sample quality. We propose that a test based on genetic variation would be an easier and more efficient way to check for "foreign" blood cells in an athlete's circulation. This study, which does NOT involve blood doping or blood transfusions, investigates feasibility of DNA testing as a screen for homologous blood doping.

Your participation is voluntary: Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks and discomforts. Please read this form carefully.

If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision.

Who is conducting the study: This project is being conducted by researchers in the School of Human Kinetics at UBC. The investigators are not receiving any compensation for doing this research from any commercial organization. You are welcome to request any details concerning the funding arrangements from the Principal Investigator.

Objectives of the study: 1) To determine if small amounts of one individual's blood can be detected by DNA testing when mixed with a large volume of a second person's blood (both whole blood and red blood cells only), 2) to investigate the effect of storage time on the ability to make such a detection and 3) to evaluate the use of finger prick sampling as a source of blood DNA. Buccal cell DNA is used to confirm the blood cell DNA results.

Who can participate in the study?

Men and women over the age of 19.

Who should not participate in the study?

Individuals who feel that they may be unwell due to a communicable, blood born disease (the lab is set up to work with blood from well individuals only). Women who are or have been pregnant (this is to avoid the very small chance of circulating fetal cells interfering with the experiments).

What does the study involve?

Buccal DNA sample: A sample of cells from the inner cheek will be collected using a *cytobrush* (Med). This will feel similar to rubbing a firm toothbrush against your inner cheek.

Blood DNA sample An alternate DNA source is a small blood sample, either a finger prick (2-3 drops) and/or a blood withdrawal from the antecubital (inside elbow) vein of 30 ml (2 tablespoons). Venous blood will be drawn by a researcher trained in venipuncture (blood withdrawal).

Please Note: This study does not involve any form of “blood doping” or blood transfusions.

DNA banking (optional):

As the discovery of new genetic variants may inform further development of this potential anti-doping strategy, the investigators would like to keep the DNA samples obtained as part of this study for future genetic analysis. Declining to allow the investigators to “bank” your DNA for future anti-doping related studies, IN NO WAY affects your participation in the “Evaluating the potential of genotyping as a test for homologous blood doping in athletes ” part of the project. If you agree to allow DNA banking, the researchers will not contact you for further consent. All guarantees of confidentiality described in this consent form will apply to any future use of the DNA and you may ask to have your DNA sample destroyed at any time.

What are the possible side effects for participating in this study?

There may be a very slight tingling or irritation in the inner cheek after the buccal swabbing or a mild, brief pain at the site, bruising, lightheadedness and/or fainting, and slight swelling and redness where the blood was drawn. There is also a rare possibility of infection after blood-drawing. There is also small possibility of loss of confidentiality that could result in your genotype at the sites tested becoming known. To our knowledge, there is limited utility to this information (i.e. if an insurance company obtained the data); however, you should be aware that this risk exists.

What are the benefits of participating in this study?

There are no benefits for participating in this study; however, the final results for the study will be available to you if you indicate on the consent form that you would like follow-up information. There is no remuneration for participating.

What happens if I decide to withdraw my consent to participate?

Your participation in this research is entirely voluntary. You may withdraw from this study at any time without explanation. If you choose to enter the study and then decide to withdraw at a later time, all data collected about you during your enrolment in the study will be retained for analysis.

If you chose the option of allowing the researchers to bank your DNA sample for future studies on the use of genes to detect blood doping you may withdraw from the study at any time and request that your DNA sample be destroyed.

Compensation for Injury

Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else.

Will my taking part in this study be kept confidential?

Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of the UBC Research Ethics Board for the purpose of monitoring the research. No records which identify you by name or initials will be allowed to leave the Investigators' offices. Everything (e.g. DNA samples, results) are coded and the key stored separately, so even in the event of a security failure in the lab or the office, it is highly unlikely that anyone could link you to the data or the DNA.

Who do I contact if I want to know more about the study, or to discuss my results?

You are more than welcome to ask questions about the study at any time. Any of the investigators will be glad to discuss the results of the study with you. We welcome your comments about any aspect of the study. Please note that complex genetic traits are likely influenced by many genetic and environmental factors, and, while we hope the data from our study will tell us about the utility of our proposed anti-doping test, the data will not be very informative at the individual level and has no clinical utility or medical relevance.

Who do I contact if I have any questions or concerns about my rights as a subject during the study?

If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services at 604-822-8598.

SUBJECT CONSENT TO PARTICIPATE

Project: Evaluating the potential of genotyping as a test for homologous blood doping in athletes.

Principal investigator: Dr. Jim Rupert, School of Human Kinetics, UBC

I would like a copy of the overall results of the study (provide an E-mail address to which this can be sent)

To participate in the DNA banking component of the study, please read the box on the next page

- I understand that by signing this form, I am consenting to participate in the study.
- I have had sufficient time to consider the information provided and to ask for advice if necessary.
- I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the result will only be used for scientific objectives.
- I understand that my participation in this study is voluntary and that I am completely free to refuse to participate, or to withdraw from this study at any time.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I have read this form and I freely consent to participate in this study.
- I have been told that I will receive a dated and signed copy of this form.

Printed name of subject: _____ Signature: _____ Date _____

Printed name of witness: _____ Signature: _____ Date _____

Printed name of principal investigator/representative: _____ Signature: _____ Date _____

Optional Voluntary Donation of Tissue for Unspecified Uses: Banking DNA samples

There are millions of common genetic variations in humans. This initial goal of the project is to look at variants in two genes (ACE and Ush 1) but the researchers would like to keep the DNA samples that were made from your cheek sample and blood samples indefinitely so that they can test other variants for this application. This procedure is often called DNA banking and requires a separate consent. Please read the following, and if you are willing to allow the researchers to bank your DNA, sign below.

Storage and future use of your DNA:

Your DNA will be stored in a secure lab at UBC and will not carry any personal identifiers (*i.e.* it will be coded by a number). Any future use of the DNA will be overseen by the Principal Investigator (Jim Rupert) listed in the consent form (*i.e.* the DNA will not be provided to other researchers - if Dr. Rupert is working with other investigators, the DNA samples will remain in his lab and under his control). Before the DNA can be used for any future studies by Dr. Rupert, the proposed research will be evaluated and approved by the Clinical Research Ethics Board at UBC to confirm that the DNA is not being used for studies that differ from those to which you have consented. No attempt will be made to link your DNA to that of any member of your family. The researchers are interested only in general population categories, not in individuals, families or pedigrees.

Consent for DNA banking

I agree that the researchers may use the DNA sample obtained from me as part of this project in future similar studies of the role of genetics in **anti-doping studies**. I understand that the investigators will not contact me to request further consent for these studies and that the DNA may be kept indefinitely (unless I request that it be destroyed). I understand that allowing my DNA sample to be banked for future studies is completely optional, and that declining to do so does not effect my participation in the other components of this study. The DNA samples will not be used for commercial purposes.

Printed name of subject: _____ Signature: _____ Date _____

Printed name of witness: _____ Signature: _____ Date _____

Printed name of principal
Investigator/representative: _____ Signature: _____ Date _____

You are free to withdraw, without explanation, from the DNA banking component of the project anytime you wish. To do so, simply contact Dr. Rupert (604) 822-8462 or rupertj@interchange.ubc.ca and request that your DNA sample be removed from the bank and destroyed.

APPENDIX H: RECRUITMENT POSTER

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Volunteers needed for anti-doping research



Project: Evaluating the potential of genotyping as a test for homologous blood doping in athletes

Principal investigator: Dr. Jim Rupert, School of Human Kinetics, U.B.C.

The UBC School of Human Kinetics is looking for volunteers to take part in a study on the use of blood DNA in detecting "blood doping".

We are looking for males and females over the age of 18 who are not currently dealing with a blood-borne disease (to the best of their knowledge) or who are not, nor have been, pregnant. Subjects will be asked to provide 30 mls of blood (three small tubes), a finger prick blood sample, and a cheek swab sample (all to be used for DNA preparation). The total time commitment for participating in this study is approximately 22 minutes (one time only).

If you, or someone you know, meet all of the above criteria and would like to learn more about the study, please contact Mona Maghsoodi at: School of Human Kinetics, University of British Columbia
(604) 827-3411 or monamaghsoodi@yahoo.com

Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com	Blood samples for doping research Contact: Mona Maghsoodi monamaghsoodi@yahoo.com
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