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Genetic Determination of ABO Genotype from Buccal Cells: Incorporating PCR, Gel Electrophoresis, and ABO Genotyping into Undergraduate Study

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GENETIC DETERMINATION OF ABO GENOTYPE FROM BUCCAL CELLS:

INCORPORATING PCR, GEL ELECTROPHORESIS, AND ABO

GENOTYPING INTO UNDERGRADUATE STUDY

By

Brian M. Ginn

Honors Scholarship Project

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Olivet Nazarene University

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GRADUATION WITH UNIVERSITY HONORS

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BACHELOR OF SCIENCE

in

Biology

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ABSTRACT

We streamlined a protocol for isolating chromosomal DNA from buccal cells for the purpose of producing restriction fragment length polymorphisms (RFLPs) and identifying ABO genotype. This protocol involves DNA isolation, polymerase chain reaction, restriction enzyme digestion, and gel electrophoresis. Buccal cells are a viable source of chromosomal DNA for amplifying the ABO gene segments of interest. The collection of buccal cell samples is a less invasive method than collecting blood samples, which requires venipuncture. The DNA isolated from buccal cells was comparable in quality to the DNA isolated from blood cells. A complete protocol was produced for use in an undergraduate level laboratory.

Keywords: biology, genetics, ABO, genotyping, restriction enzyme, blood typing, electrophoresis, polymerase chain reaction, buccal cell

INTRODUCTION

The human genome consists of a complete set of DNA that is found in almost every cell type present in the human body. The information contained in this genome is packaged into chromosomes. On these chromosomes are segments of expressed DNA known as genes. Among the many important genes is the one that codes for the histo-blood group ABO system transferase – a protein essential in determining ABO blood type of an individual.

The ABO system of blood typing was first described by Karl Landsteiner in 1900 (Yamamoto, 2000). There are four phenotypes (A, B, AB and O) and six genotypes ($I^A I^A$, $I^A i$, $I^B I^B$, $I^B i$, $I^A I^B$, ii) that determine those phenotypes. The ABO system is interesting in that it features both a codominant and a dominant/recessive inheritance pattern. A and B can be expressed together, while O is masked by either A or B. Basic serological testing can determine an individual's phenotype, but the genotype of individuals with type A or type B blood is unable to be determined using this method.

In order to determine the genotype, DNA must be extracted from cells in the body. Two of the specific segments that code for the histo-blood group ABO system transferase can be located by use of specific DNA primers. These segments are located on chromosome 9. These sequences are then amplified by polymerase chain reaction (PCR), which increases the actual number of DNA segments from very few to many millions (Seltsam et al., 2003). The amplified DNA segments are then incubated with restriction enzymes, which bind and cleave the DNA at specific recognition sequences. The presence or absence of these sequences will determine whether or not the restriction enzyme binds and whether or not the segment is cleaved. These sequences in the segment of interest only vary by a single

nucleotide, and are thus referred to as single nucleotide polymorphisms (SNPs) (Doi et al., 2004).

The resulting DNA can then be subjected to gel electrophoresis alongside markers of determined length in order to determine the length of specific segments of the DNA that was cut (Crouse & Vincek, 1995). Because of the precise and known sequences of where these segments are cut, the resultant lengths of certain segments are found in predicted patterns that indicate the ABO genotype of any individual.

Research has already established the effectiveness of isolating DNA and locating the genes responsible for ABO blood typing (Fukumori et al., 1995). However, this protocol utilizes human white blood cells (WBCs) because of the ease of obtaining a large sample. Because the entire genome is found in every somatic cell (except red blood cells), buccal cells – the epithelial cells lining the oral cavity – obtained from a mouth rinse can be used as an alternative to WBCs for the purpose of isolating the genes that determine ABO. This research demonstrates that buccal cells are extremely adequate sources of DNA for the purpose of ABO genotyping and that no differentiation can be made between DNA obtained from buccal cells versus DNA obtained from WBCs. Using buccal cells is also a noninvasive method of obtaining the DNA, which makes it more practical for use in an undergraduate laboratory or clinical setting.

The fruits of this research have been adapted into a procedure that is designed for use by undergraduates at Olivet Nazarene University in Biology 440: Advanced Genetics. Through performing this experiment, students will learn important laboratory techniques including PCR, gel electrophoresis, and centrifugation, as well as the foundations of ABO blood types, which are important in human physiology and immunology. This protocol

provides a very detailed materials and methods section, explaining how to replicate the results of the research.

MATERIALS AND METHODS

A certified phlebotomist used 6 milliliter (mL) Vacutainer to draw blood samples from volunteers through brachial venipuncture. Approximately 5 milliliters of blood were obtained from each volunteer. These samples were centrifuged at 3000 rpm for 4 minutes, which yielded separation of plasma and red blood cells (RBCs), separated by a thin "buffy coat" of WBCs. In order to maximize the yield of WBCs, the entire buffy coat, as well as some plasma and RBCs, was extracted from the Vacutainer tube using a 9-inch Pasteur pipette and inserted into a Wintrobe tube of smaller diameter. The Wintrobe tube was centrifuged at 3000 rpm for 4 minutes. The sample again separated into the three layers. The buffy coat was then easier to extract because the smaller diameter of the Wintrobe tube gave a thicker buffy coat. One milliliter of DNAzol (Life Technologies) was added to lyse the cells and release the DNA. One-half milliliter of 100% ethanol was added to the DNAzol solution to precipitate the DNA, which was collected by centrifuging at 10,000 rpm for 5 minutes in a microcentrifuge. The DNA was then resuspended and washed twice in one milliliter of 75% ethanol, with centrifugation at 10,000 rpm for 5 minutes in a microcentrifuge between each wash. The DNA pellet was resuspended and dissolved in 50 microliters (μL) of 8 mM sodium hydroxide and the pH was stabilized with the addition of 5 μL of 0.1 M HEPES reagent.

Buccal cell samples were obtained from the same volunteers that gave blood samples, to ensure precision in results. Buccal cells were collected using a 10 mL rinse of 0.9% saline. This sample was centrifuged at 3000 rpm for 4 minutes. The supernatant was removed and discarded. DNAzol was then added to the cell samples in the same manner as was used with WBC samples. Precipitation, washing, centrifugation, resuspension, and stabilization of the buccal cell DNA was performed in the exact same way as the blood samples.

The series of polymerase chain reactions (PCR) consisted of preparing an Enzyme/PreMix/DNA solution, which contained 18.5 μL of sterile water, 1.0 μL of the isolated DNA template, 0.5 μL FailSafe PCR enzymes (Epicentre Biotechnologies) mix (2.5 units/ μL), and 25 μL of FailSafe PCR 2X Premix H. The quantities of each reagent in this solution are listed per one reaction.

Two primer premixes were made. The G Primer Premix consisted of 2.0 μL of sterile water, 1.5 μL of GA16 primer (20 μM) and 1.5 μL of GA17 (20 μM) primer. The F Primer Premix consisted of 2.0 μL of sterile water, 1.5 μL of FA31 primer (20 μM), and 1.5 μL of FY47 primer (20 μM). The Enzyme/PreMix/DNA solutions were placed in the appropriate PCR tubes, which were then placed in the thermocycler. A hot start protocol was employed to begin the PCR reaction; the open tubes remained in the thermocycler until it reached 72.0 $^{\circ}\text{C}$. Then the F and G Primer Premixes were loaded into the appropriate tubes, which were then capped. The PCR cycle consisted of initial denaturation for 3 minutes at 94 $^{\circ}\text{C}$, denaturation for 30 seconds at 94 $^{\circ}\text{C}$, annealing for 30 seconds at 58 $^{\circ}\text{C}$, extension for 30 seconds at 72 $^{\circ}\text{C}$, final extension for 10 seconds at 72 $^{\circ}\text{C}$. The middle three steps ran for 30 cycles.

Eight μL of the GA16-GA17 reaction products were combined with 1.0 μL of Kpn I restriction enzyme and 1.0 μL Kpn I buffer. Likewise, 8 μL of FY31-FY47 were combined with 1.0 μL of Alu I restriction enzyme and 1.0 μL Alu I buffer. Both restriction enzymes and buffers were supplied by Life Technologies, Inc. These mixtures were incubated in a 37 $^{\circ}\text{C}$ water bath for two hours, followed by ten minutes incubation in a 65 $^{\circ}\text{C}$ water bath, and finally, the reactions were held briefly in an ice bath.

The products of the enzyme digestion were combined with bromophenol blue and xylene cyanol loading dyes and subjected to electrophoresis on a 2% LE agarose gel

containing 4 mg/mL ethidium bromide in 1x TBE buffer at 110 millivolts for approximately 60 minutes alongside standardized PCR markers purchased from Promega, Inc. The gel was then transilluminated and photographed using ultraviolet light so that the results could be evaluated.

RESULTS

Expected banding patterns for the six ABO genotypes are listed below, followed by three photographs of electrophoresis gels showing banding patterns from both blood and buccal cell samples.

Table 1: Expected fragment size of digested PCR products of transferase A gene for the GA16-GA17 primer pair.		Banding Pattern for each Genotype					
Primer Pair (and restriction enzyme)	Fragment Size (base pairs)	AA	AO	BB	BO	OO	AB
GA16-GA17 (Kpn I)	231	+	+	+	+		+
	121		+		+	+	
	109		+		+	+	
<p>In Table 1, plus signs indicate the presence of a band of the expected size for a genotype. Absence of a plus sign (blank box) indicates the lack of a band of the expected size for a genotype.</p>							

Table 2: Expected fragment size of digested PCR products of transferase A gene for the FY31-FY47 primer pair.		Banding Pattern for each Genotype					
Primer Pair (and restriction enzyme)	Fragment Size (base pairs)	AA	AO	BB	BO	OO	AB
FY31-FY47 (Alu I)	275	+	+		+	+	+
	190			+	+		+
	85			+	+		+
<p>In Table 2, plus signs indicate the presence of a band of the expected size for a genotype. Absence of a plus sign (blank box) indicates the lack of a band of the expected size for a genotype.</p>							

In the following figures (Figures 1-3) of agarose gel electrophoresis products, each well is identified by a number, the ABO genotype of the sample, the primers used in the

sample, and the source of the sample. GA16-GA17 samples were digested with Kpn I restriction enzyme and FY31-FY47 samples were digested with Alu I. Samples that are the same genotype are from the one person.

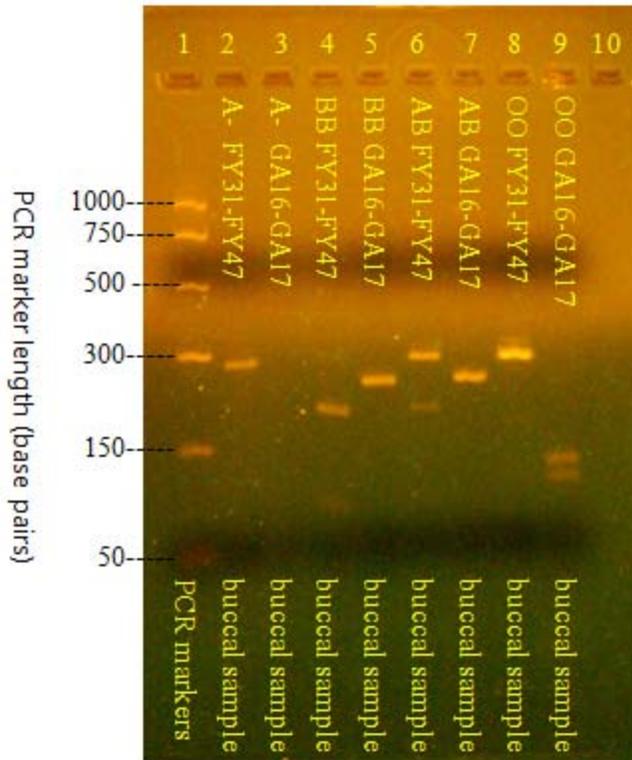


Figure 1: Restriction enzyme-digested fragments from buccal cell DNA samples from four individuals. The buccal cells of a known type A individual produced the sample seen in lane 2. The other sample did not appear in lane 3, and thus the genotype, AA or AO, cannot be determined.

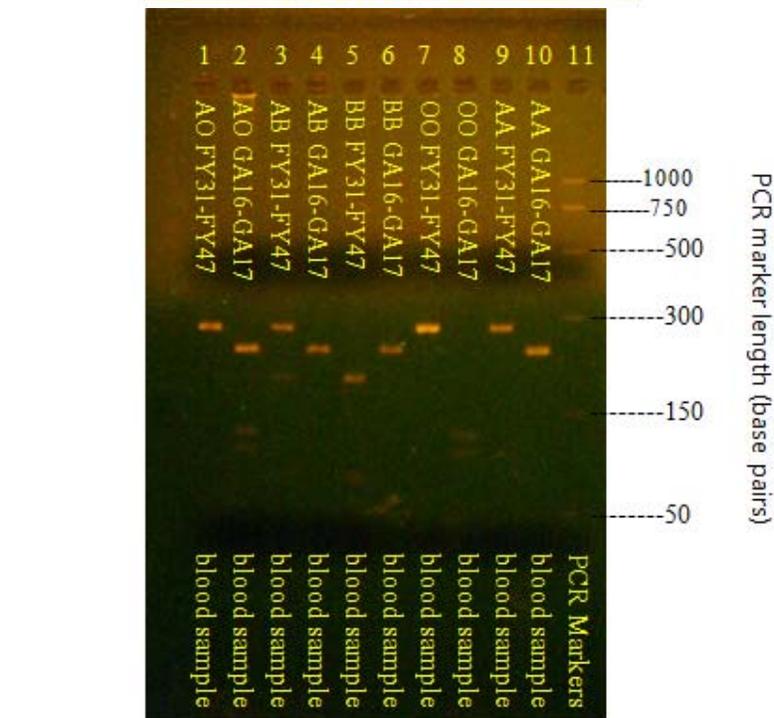


Figure 2: Restriction enzyme-digested fragments from blood DNA samples from five individuals.

Type O, found in Figure 1 lanes 8 & 9 (buccal sample) and in Figure 2 lanes 7 & 8 (blood sample) yields the same expected DNA segment patterns from both cell sample types. Both segments treated with restriction enzyme Kpn I and primers GA16 and GA17 yielded segments of 109 and 121 base pairs (bp), while the segments treated with restriction enzyme Alu I and primers FY31 and FY47 yielded a segments of 275 bp (uncleaved). Similarly, genotype BB, found in Figure 1 lanes 4 & 5 (buccal sample) and Figure 2 lanes 5 & 6 (blood sample) gives identical expected patterns. The samples treated with GA16-GA17 remained uncleaved, whereas the samples treated with FY31-FY47 were both cleaved, yielding bands of 190 bp and 85 bp.

Figure 2 depicts the differences in genotypes between a heterozygous and a homozygous individual with type A blood. Lanes 9 and 10 show the homozygous (AA) samples, which, when treated with both restriction enzymes in conjunction with the appropriate primer sets, failed to cleave, as expected. Lanes 1 and 2 show the heterozygous (AO) samples. The heterozygous sample treated with Alu I restriction enzyme and primers FY31 and FY47 failed to cleave, as expected. However, some of the heterozygous sample treated with Kpn I reaction enzyme and primer GA16 and GA17 did cleave while some remained uncut, due to presence of both alleles. The resulting bands of lengths 231, 121, and 109 bp corroborated the expected lengths listed in Table 1.

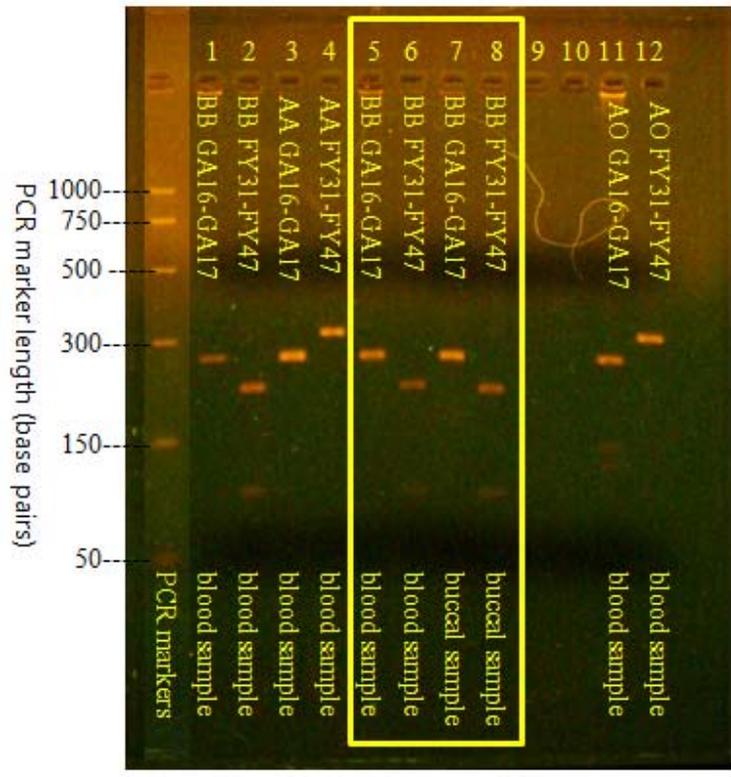


Figure 3: Restriction enzyme-digested fragments from blood DNA samples from four individuals and buccal DNA sample from one individual. This gel is included to give a side by side comparison of a buccal sample and a blood sample of the same genotype (lanes 5-8).

A side-by-side comparison is made in Figure 3, lanes 5-8, where a buccal cell sample was run adjacent to a blood sample of identical genotype. Both of these samples yielded the same result, segments of 190 and 85 bp when treated with restriction enzyme Alu I and primers FY31 and FY47, and a single segment 231 bp in length when treated with restriction enzyme Kpn I and primers GA16 and GA17, which was expected. Similarly, the buccal sample for type O found in Figure I lanes 8 and 9 matches the blood sample for type O found in Figure 2 lanes 2 and 6.

DISCUSSION

By simply comparing the digestion products (see Figures 1 and 2), it is clear that the buccal cell DNA extraction method was successful in isolating DNA. This DNA, after being amplified by PCR, had a substantial enough concentration to clearly appear on a 2% agarose gel. The identical results of the same genotypes in Figure 3 confirm the success of the buccal cell DNA extraction method.

DNA isolation from was first performed using blood samples as a benchmark in order to verify successful isolation. There have been multiple methods of ABO genotype determination that have used blood samples as DNA sources (Fukumori et al., 1995; Doi et al., 2004; Crouse & Vincek, 1995; Seltsam et al., 2003; Olsson et al., 2005; Nishimukai et al., 1996; Beiboer et al., 2005; Rust, Funk, & Assmann, 1993; Fregel et al., 2005; Gassner et al., 1996; Hosseini-Maaf et al., 2007; Rozman, Dovc, & Gassner, 2000). One very beneficial step that I added to the protocol was the use of the Wintrobe tube for easier and more refined extraction of WBCs. When blood is centrifuged in a Vacutainer tube, the buffy coat is extremely thin and it is therefore difficult to avoid aspirating red blood cells (RBCs) and serum when removing the white buffy coat. Red blood cells and serum lower the purity of the WBC sample because, while all of the DNA may still be present, there is also much more cellular and extracellular debris from the RBCs and serum. Red blood cells do not contribute to the DNA isolated from a blood sample because they are anucleate – meaning that they contain no nucleus and therefore no DNA. The smaller diameter of the Wintrobe tube gives a more sizeable buffy coat that allows for easy extraction of most of the WBCs while lowering the risk of aspirating RBCs or serum. As far as our literature search indicated, this method of using a Wintrobe tube to substantially enrich WBC yield for the purpose of ABO genotyping

had never been done, and therefore contributes to an original component of this research project.

Another advantage of using the Wintrobe tube to enrich WBC samples was the sample size itself. The DNAzol protocol calls for samples of approximately 100 μ L. This quantity is easily exceeded if additional serum and RBCs are aspirated in the sample. By using the Wintrobe tube, I was able to utilize the DNAzol extraction method.

The concept of using buccal cells for the purpose of ABO genotyping is an original concept, as far as our literature search indicated. The protocol developed for the isolation of DNA from buccal cells is also original. Life Technologies, the manufacturer of DNAzol, lists many different protocols for isolating DNA from different sample types, such as whole blood, cell nuclei, tissues such as liver or muscle, and cell cultures, but buccal cells are not found among these sample types. Furthermore, none of the research found in our thorough search of ABO genotyping literature cites buccal cells as a DNA source. This may be because when the buccal cells are obtained using a saline rinse, the amount of extracellular debris makes for a comparatively low yield of DNA and a relatively unpurified sample.

My method of isolating DNA from buccal cells by using DNAzol reagent was shown to be not only successful, but also comparable to whole blood samples as a source of DNA. This means that future studies could utilize this noninvasive protocol for collecting DNA samples instead of collecting blood samples. Obtaining blood samples for research purposes typically requires the services of a licensed phlebotomy technician and subjects the donor to venipuncture, which can cause discomfort and bruising around the insertion site. My method of obtaining buccal cells using a saline rinse is completely noninvasive, requires no trained personnel, and carries no risk of infection or tissue damage with it. Even if the saline were

accidentally swallowed, the isotonic solution is such a small volume that it would not be harmful.

Due to insufficient sampling sizes, not all ABO genotypes were able to be collected. In Figures 1-3, there is a marked absence of any sample, buccal cell or blood, which verified a BO (heterozygous) genotype. Furthermore, buccal cell samples were not able to be obtained for all genotypes. As previously mentioned, the three genotypes that had both buccal cell and blood samples displayed identical results from both samples, verifying the effectiveness of using buccal cells in lieu of blood as a source of DNA. As with much research, more volunteers and a larger sample size would produce more significant results. This is certainly a factor upon which the project could be improved.

In order to improve upon this experimentation, one major change could be made to significantly enhance the results. Because there are segments of relatively similar lengths (i.e. 121 bp versus 109 bp – a difference of only 12 bp), separation is not consistently well-defined on the 2% agarose gel. This is evidenced in Figure 3 lane 11 in which primers GA16 and GA17 and restriction enzyme Kpn I were used to treat a sample from AO. Separation of the 121 bp and the 109 bp segment is visible, yet faintly so. More pronounced separation could be achieved by using a gel with a higher concentration of agarose (3% or 4% instead of the 2% that was used). Another alternative would be the use of SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), which would likely yield ever finer separation and improved visibility of the segments.

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APPENDIX A. UNDERGRADUATE LABORATORY PROTOCOL

DNA Amplification of ABO Primers (GA16-GA17 & FY31-FY47) with FailSafe PCR PreMix H – Generic reaction setup for 1 PreMix

Materials

1.5 mL microcentrifuge tubes	0.2 mL PCR tubes
Microcentrifuge tube racks	Electrophoresis equipment
SL 10 and tips	Microcentrifuge
SL 200 and tips	Vortex
SL 1000 and tips	Thermal cycler
15 mL centrifuge tubes	Clinical centrifuge
UV transilluminator	Digital camera
Microwave	37°C water bath
	65°C water bath

Reagents

FailSafe PCR Enzyme Mix (2.5 units/ μ L)	DNAzol®
FailSafe PCR PreMix H	0.1 M HEPES
Sterile dH ₂ O (sterilized using syringe filter)	8mM NaOH
0.9% NaCl solution	75% ethanol
LE agarose	100% ethanol
10 mg/mL ethidium bromide	Loading dyes (bromophenol blue & xylene cyanol)
PCR markers	
GA16 primer (20 μ M = 20 pmole/ μ l) - AGAAGCTGAGTGGAGTTCCAGGTG	
GA17 primer (20 μ M = 20 pmole/ μ l) - TGATGGCAAACACAGTTAACCC	
FY31 primer (20 μ M = 20 pmole/ μ l) - GAAATCGCCCTCGTCCTT	
FY47 primer (20 μ M = 20 pmole/ μ l) - TGCTGGAGGTGCGCGCCTAC	

Isolation and Resuspension of DNA from Buccal Cell Samples

Collection

1. Rinse mouth by vigorously swishing ~10 mL of 0.9% NaCl (isotonic saline) for 1 minute.
2. Carefully recollect saline into 15 mL centrifuge tube.

Homogenation

1. Centrifuge sample in clinical centrifuge at 3000 rpm for 4 minutes.
NOTE: When centrifuging, always make certain that your sample is counterbalanced.
2. Remove and discard the supernatant. Take care not to aspirate the cell pellet.
3. Transfer the cell pellet to a 2 mL microfuge tube.

4. Add 1 mL DNAzol reagent to the sample. Lyse the cells by gently pipetting solution through a SL 1000 pipette tip set to 1000 μ L.

DNA Precipitation

1. Add 0.5 mL of 100% ethanol to DNAzol solution.
2. Cap and gently invert the tube several times to mix. Overzealous shaking of the tube can break the DNA. Allow tube to sit at room temperature and DNA to precipitate.
3. Using the microcentrifuge, spin the samples at 10,000 rpm for 5 minutes.
NOTE: Place tubes into microcentrifuge with tube hinge facing upward. This way, when DNA is concentrated at the bottom, it will be more easily located if not visible.
4. Following centrifugation, remove and discard the supernatant. Take care not to dislodge or aspirate the DNA pellet.

DNA Washing

1. Add 1.0 mL of 75% ethanol to the tube containing the DNA pellet.
2. Gently invert the tube 6 times.
3. Centrifuge in microcentrifuge at 10,000 rpm for 5 minutes.
4. Carefully remove the supernatant with a micropipette, taking care not to dislodge or aspirate the DNA pellet with the pipette tip.
5. Repeat DNA Washing steps 1-3.
6. Remove the supernatant and cap the tube, to prevent excessive desiccation of the DNA pellet.

DNA Resuspension

1. Resuspend the DNA pellet in 50 μ L 8mM NaOH at a concentration of 0.2-0.3 μ g/ μ L.
2. Dissolve the DNA by gently passing it through the pipette tip. Do this slowly to avoid shearing of the DNA.
3. Transfer the supernatant containing the resuspended DNA to a new, sterile 1.5 mL tube.
4. Add 5 μ L of 0.1 M HEPES in order to obtain a stable pH.
5. If not using the DNA immediately, store at -20 $^{\circ}$ C.
The DNA is stable at pH 7-8 for over one year at -20 $^{\circ}$ C.

Polymerase Chain Reaction

Mix Preparation

1. Prepare the Enzyme&PreMix&DNA Mix. Thaw & thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine all of the

following then deliver 45 µL of the mixture to the appropriate PCR tubes.

	<u>Volume per rxn</u>	<u>Total Volume</u>
Sterile water	(18.5 µL per rxn)	_____
Bld type ___ DNA template (10 ⁴ –10 ⁶ copies)	(1.0 µL per rxn)	_____
FailSafe PCR Enzyme Mix (2.5 units/µL)	(0.5 µL per rxn)	_____
FailSafe PCR 2X Premix H	(25 µL per rxn)	_____

Total volume = (45 µL per rxn)

NOTE: DNA template source = Human DNA extracted with DNAzol from buccal cells. Use undiluted DNA. Make a different Enzyme&PreMix&DNA mix for DNA from each blood type (A, B, AB, and O).

Multiply the "Volume per rxn" for each component times the number of samples + 0.2-0.4 (extra to accommodate pipetting error).

2. Prepare the Primer Premixes. Thaw & thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine all of the following:

<u>GA16-GA17 Primer Premix</u>	<u>Volume per rxn</u>	<u>Total Volume</u>
Sterile water	(2.0 µL per rxn)	_____
GA16 primer (20 µM)	(1.5 µL per rxn)	_____
GA17 primer (20 µM)	(1.5 µL per rxn)	_____
Total volume =	(5 µL per rxn)	

<u>FY31-FY47 Primer Premix</u>	<u>Volume per rxn</u>	<u>Total Volume</u>
Sterile water	(2.0 µL per rxn)	_____
FY31 primer (20 µM)	(1.5 µL per rxn)	_____
FY47 primer (20 µM)	(1.5 µL per rxn)	_____
Total volume =	(5 µL per rxn)	

Multiply the "Volume per rxn" for each component times the number of samples + 0.2-0.4 (extra to accommodate pipetting error)

Hot Start

1. Turn on Labnet MultiGene thermocycler.
2. Insert tubes without sealing caps into the thermal cycler.
3. Open the Ginn folder. Highlight the ABO GEN 1 program with the up/down keys and press "RUN" (F1). The mode should be set to "1: Tube control" and the Volume should be set to 50 µL.

4. Press "Start" (F1) to begin program. First the lid will heat up followed by the first segment of the program. It will heat to 72.0 °C and pause until the "Resume" (F1) key is pressed. This is designed to hold the temperature at 72.0 °C for a hot start until all reaction components are placed in the tube.
5. When the hot start temp reaches 72 °C, add the following solutions to the appropriate tubes, using a new pipette with each tube.
 - a. Add 5 µl of GA16-GA17 primer premix to appropriate PCR tubes.
 - b. Add 5 µl of FY31-FY47 primer premix to appropriate PCR tubes.
 Cap each tube, press forcefully with finger to insure tube is snapped shut.
6. After all the tubes are loaded, press "Resume" (F1).
 - Initial denaturation for 3 minutes at 94 °C
 - | | | | |
|---|--|---|--------------------|
| { | Denaturation for 30 seconds at 94 °C
Annealing for 30 seconds at 58 °C
Extension for 30 seconds at 72 °C | } | total of 30 cycles |
|---|--|---|--------------------|
 - Final extension for 10 seconds at 72 °C
 - Hold at 4 °C

Electrophoresis

1. After thermal cycling is complete:
 - a. Remove 10 µl of PCR product from each tube. Place into new 1.5 mL tube and add 1 µL of loading dye. Vortex and centrifuge. This will be loaded into 2.0% LE agarose gel along PCR markers
 - b. Place 5 µL PCR markers in a new 1.5 mL tube and add 1 µL loading dye. All 6 µL of this mixture will be loaded into the agarose gel.
2. Prepare 2.0% LE agarose gel.
 - a. 0.8 g of LE agarose per 40 mL of 1X TBE buffer.
 - b. Boil in microwave until completely dissolved. Stir frequently.
 - c. Cool at room temperature until it can be handled in bare hands. It should be hot, just able to handle with bare hands. Then add 4 µL of 10 mg/mL ethidium bromide, stirring by swirling the solution being careful to avoid formation of bubbles.
NOTE: ethidium bromide is a carcinogen. Always use gloves when handling ethidium bromide or any gels containing it.
 - d. Pour into gel tray and allow gel to solidify.
 - e. Gel can be stored at 4 °C for 24 hours in sealed container with a few milliliters of 1X TBE in the bottom to keep the gel moist.
3. Once the gel is solid, remove the gel tray ends and the well comb. Submerge the gel in 1X TBE buffer in the electrophoresis apparatus. Load PCR products and DNA markers into appropriate lanes. Subject to electrophoresis at about 110 volts for about 1 to 1.5 hours. The bromophenol blue dye (dark blue) should have migrated to about 1 cm from the bottom of the gel.

4. Photograph gel using UV transilluminator and digital camera.

Restriction Digestion of the PCR Reaction

1. Label two 1.5 mL microfuge tubes and add the following reagents to those tubes.

Tube 1:	GA16-GA17 PCR rxn	8 μ L
	10X Kpn I buffer	1 μ L
	Kpn I restriction enzyme	1 μ L
	Total =	<hr/> 10 μ L

Tube 2:	FY31-Fy47 PCR rxn	8 μ L
	10X Alu I buffer	1 μ L
	Alu I restriction enzyme	1 μ L
	Total =	<hr/> 10 μ L

2. Incubate at 37 °C for about 2 hours. Then incubate at 65 °C for 10 minutes then about 5 minutes on ice. You can hold on ice while setting up agarose gel.
3. After incubation is complete:
 - a. Add 1 μ L of loading dye to each tube. Vortex and centrifuge.
 - b. Remove 5 μ L of GA16-GA17 PCR rxn and transfer it to a clean microfuge tube. Also remove 5 μ L of FY31-FY47 PCR rxn and transfer it to another clean microfuge tube. Add 1 μ L of loading dye to each sample. Save the remaining PCR product for subsequent experiments.
 - c. Place 5 μ L PCR markers in a new microfuge tube and add 1 μ L loading dye. All 6 μ L of this mixture will be loaded into the agarose gel.
4. Prepare 2.0% LE agarose gel.
5. Once the gel is solid, remove the gel tray ends and the well comb. Submerge the gel in 1X TBE buffer in the electrophoresis apparatus. Load PCR products and DNA markers into appropriate lanes. Electrophoresis at about 100 volts for about 1 to 1.5 hours. The bromophenol blue (dark blue) should have migrated to about 1 cm from the bottom of the gel.
6. Photograph gel using UV transilluminator and digital camera.

Expected Results:

Below is a table summarizing all the possible banding patterns that could be seen in these gels. Each banding pattern represents a different ABO genotype.

		Banding Pattern for each Genotype					
Primer Pair (and restriction enzyme)	Fragment Size (base pairs)	AA	AO	BB	BO	OO	AB
GA16-GA17 (Kpn I)	231	+	+	+	+		+
	121		+		+	+	
	109		+		+	+	
FY31-FY47 (Alu I)	275	+	+		+	+	+
	190			+	+		+
	85			+	+		+

Table 1: Expected fragment size of digested PCR products of transferase A gene.

The plus signs indicate the presence of a band of the determined size for that genotype. Absence of a band indicates lack of the band for that genotype.

PCR markers' sizes in a 2% agarose gel.

