Forensics PCR (identification using genetic information)

Introduction

In this module, we are going to see an application of Polymer Chain Reaction (PCR) in the lab. We will be identifying people by their genetic information. This process includes PCR, Restriction Digestion Analysis (RDA) and Sanger DNA sequencing. Through this session, you will extract your own DNA from your cheek cell, use PCR to amplify the parts we are interested in DNA. With the amplified DNA sample, first we will use restriction enzymes which cut specific sequence in the DNA, to see whether your DNA has single nucleotide mutation, and will perform DNA gel electrophoresis to visualize the difference. Also, you will use Sanger sequencing to see how many repeats of certain short sequence you have.

DNA (Deoxyribonucleic acid)

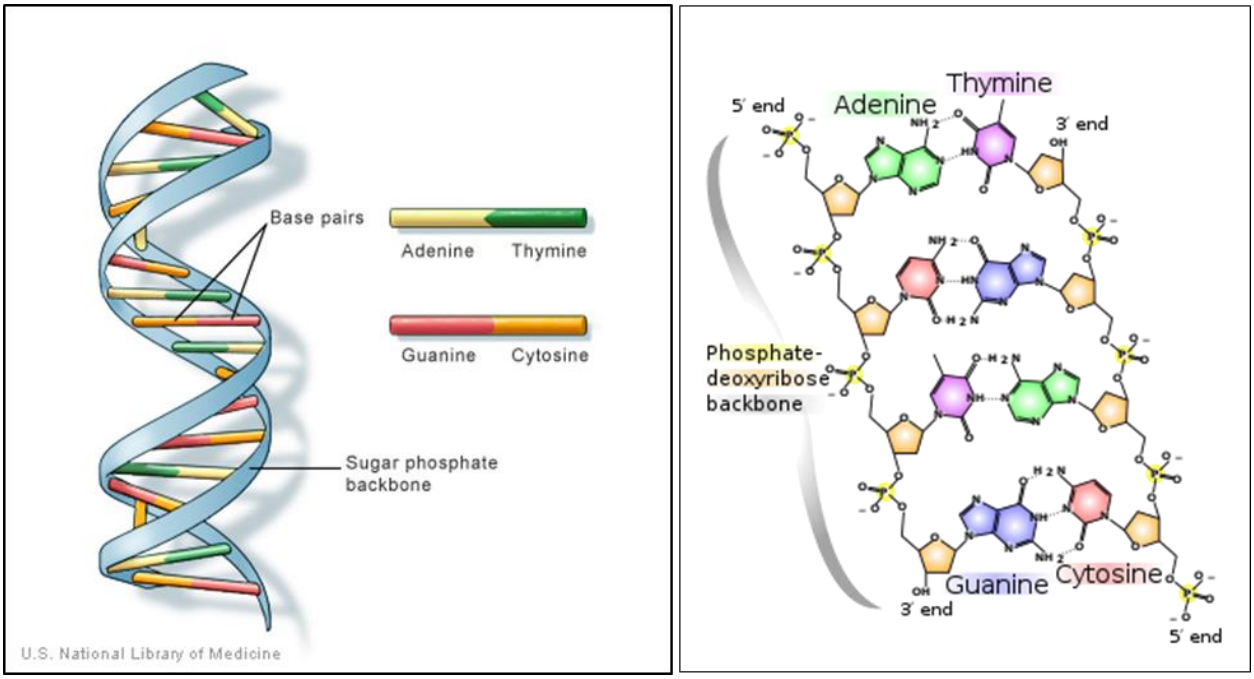


Figure 1. Structure of DNA (left). Chemical structure of DNA (right).A GC base pair with three hydrogen bonds, and AT base pair with two hydrogen bonds. Directionality of backbone. Two strands have opposite directionality to each other (5' to 3'/3' to 5').

DNA is a molecule composed of two chains that coil around each other to form a double helix carrying genetic information (Figure 1 left). A DNA strand is a polymer composed of the backbone and 4 different nucleotides; **C**ytosine, **G**uanine, **A**denine, and **T**hymine. The sequence, which is called a gene, of these 4 nucleotides contains genetic information of organisms. A nucleotide on one strand forms hydrogen bond to the complementary base (C and G, A and T) on the other strand. This is called complementary base pairing (Figure 1 right). Complementary two DNA strands form double-stranded DNA (dsDNA). As hydrogen bonds are not covalent, the two strands of DNA can be pulled apart by a mechanical force or high temperature and rejoined. The backbone of the DNA strand is made from alternating phosphate and sugar groups. The sugar in DNA is 2-deoxyribose, which is a pentose (5 carbons) sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth

carbon atoms of adjacent sugar rings. These are known as the 3’ end and 5’ end carbons. This orientation of the 3’ and 5’ carbons along backbone confers directionality to each DNA strand. The direction of the nucleotides in one strand is opposite to their direction in the other strand (Figure 1 right).

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular biological technique that can amplify the number of copies of a specific DNA segment. We can replicate short sections of DNA several millionfold in just a few hours by using PCR.

PCR requires a DNA template, where the target sequence that will be amplified is located—see Figure 2. Heat is used to separate (denature) the template DNA into single strands. This allows oligonucleotide (short single-stranded DNA molecule) primers, chemically synthesized, 20-30 nucleotide long pieces of single-stranded DNA that are designed by the researcher to amplify a specific region of DNA to base pair with complementary sequences on the template DNA. Researchers use primers to control the site from which DNA replication will begin. Two different primer sequences are used to amplify each region of the target dsDNA. One primer is complementary to the sequence on one strand of the target DNA, and the other primer is complementary to the sequence on the other strand of the target DNA. The 3’ end of both primers must face the target DNA. DNA polymerase then adds nucleotides to the 3’ end of the primers, doubling the number of target DNA sequences. The cycle of heat denaturation, primer binding (annealing), and DNA polymerization (extension) is typically repeated 15-35 times.

Kary Mullis came up with the idea for PCR in 1983. Mullis originally used E. coli DNA polymerase, which denatures at the high temperatures used to separate the DNA strands. As a result, Mullis needed to add new enzyme for each of cycle of the reaction. Mullis also needed to move the tubes to a different water bath for every temperature change. Thus, while PCR was very powerful, it was also very labor intensive and did not always yield reproducible results. The solution to this problem was to use DNA polymerase from a bacterium, Thermus aquaticus, that lives in the hot pools at Yellowstone National Park. This heat-stable polymerase (referred to as Taq polymerase, from the name of the bacterium, Thermus aquaticus) does not denature at 90 °C, so it only needs to be added once. The development of thermal cyclers, machines that automatically cycle through specific reaction temperatures for specified periods of time, further simplified the process. The use of PCR spread quickly and revolutionized molecular biology, as for the first time it allowed researchers to amplify literally any
gene (so long as the sequences of flanking regions are known) to sufficient copies (in the millions) for further manipulation. In 1993, Kary Mullis was awarded the Nobel Prize in Chemistry for the invention of PCR.

To perform the reaction, a small quantity of template DNA is combined with buffer, Taq DNA polymerase, two oligonucleotide primers, the four deoxynucleotide building blocks of DNA (dATP, dCTP,

dGTP, dTTP), and the cofactor MgCl2. A typical PCR cycle consists of:

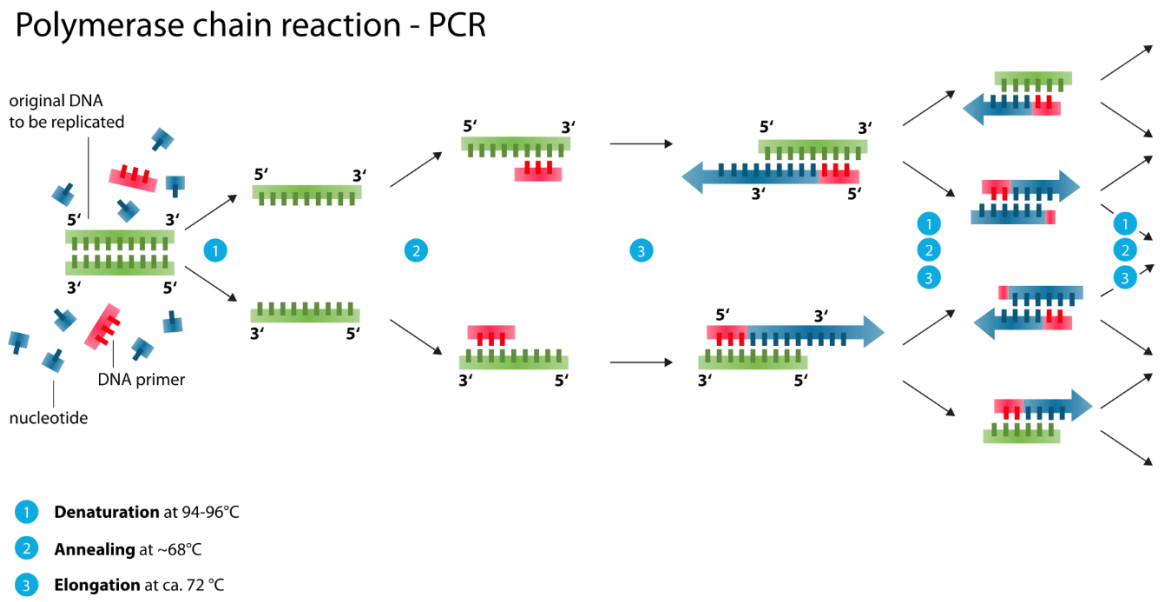


Figure 2. Schematic for Polymerase chain reaction.

1. 10 seconds at 98°C. DNA is denatured into single strands.
2. 30 seconds at the annealing temperature for the particular primer pair used in the reaction. At the annealing temperature, which is lower than the denaturation temperature in step 1, strands of DNA anneal (hybridize). Because the primers are present at much higher concentrations than the template DNA, the template DNA will anneal to the primers instead of reforming the original double helix. The annealing temperature is calculated from the base composition of both primers to ensure proper annealing of the primers to the template DNA. The annealing temperature will therefore vary depending upon the sequences of the primers (e.g., a primer pair rich in Gs and Cs will have a higher annealing temperature than ones that have relatively more Ts and As). In this course, we will use primers that have annealing temperatures that range between 55 and 68°C.
3. 30 seconds / 1000 bps at 72°C. Taq polymerase extends the primers, resulting in a new strand of DNA.

Each cycle doubles the number of target DNA sequences, so they increase by geometric progression in subsequent cycles. Assuming 100% efficiency, 33 cycles will result in an amplification factor of one billion! Even with realistic efficiencies (<100%), a single copy of DNA can be amplified to the quantities necessary for restriction enzyme digestion and/or viewing after a gel run, or cloning and sequencing, in just a few hours.

Restriction Digestion Analysis (RDA)

Restriction enzymes recognize and cleave (cut) specific DNA sequences. The specific sequences are known as restriction sites, of which there are many sites which are cut by individual restriction enzymes. These enzymes are first found in bacteria, where the enzymes are used as a defense mechanism against viruses. The enzymes recognize and cut up viral DNA, preventing an infection.

Each restriction enzyme recognizes a specific DNA sequence and cuts the sequence between specific bases. For example, AhdI, one of the restriction enzymes used in this module, recognizes and cuts the following sequence.

5'…C$^{\downarrow}$T N A G…3'

3'…G A N T$_{\uparrow}$C…5' (where N indicates any DNA nucleotide)

This can be used to see if there is a single nucleotide polymorphism (SNP) in the sequence. If one allele of a SNP includes the sequence 5'-C**T**GAG-3', AdhI will recognize and cut it. If the other allele changes that to 5'-C**C**GAG-3', AdhI will not cut it. In this way the two alleles can be distinguished from each other by gel electrophoresis.

Gel Electrophoresis

After a PCR reaction and/or RDA is done, you would need to confirm that the reaction was successful, estimate how much product was produced by PCR, determine the length of the product from PCR/RDA. Gel electrophoresis provides a quick inexpensive way to evaluate the DNA products.

In solution, the acidic phosphate backbone of DNA liberates hydrogen ions and gives the DNA molecule a net negative charge. Therefore, the DNA will migrate toward the positive pole of an electric field. Gel electrophoresis separates DNA molecules by allowing a DNA sample to migrate through an electric field while impeded by a neutral sieve-like substance such as agarose or polyacrylamide. The mesh-like matrix of the gel restricts the movement of molecules in proportion to their size.

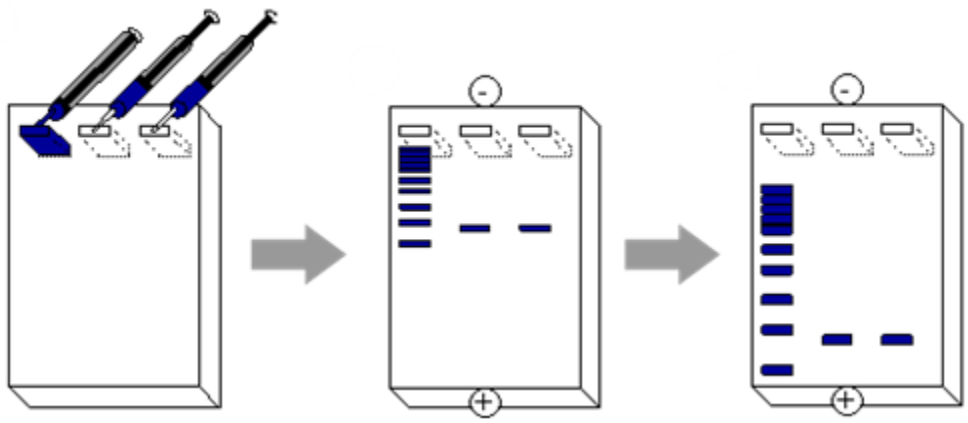


Figure 3. Agarose gel electrophoresis. DNA is loaded into wells at the top of a gel. A current is passed through the gel, pulling DNA towards the positively charged electrode. The DNA fragments are separated by size, with smaller fragments moving fastest towards the electrode.

Short Tandem Repeat (STR)

Tandemly repeated DNA sequences are widespread throughout the human genome and show sufficient variability among individuals in a population that they have become important in several fields including genetic mapping, linkage analysis, and human identity testing. These tandemly repeated regions of DNA are typically classified into several groups depending on the size of the repeat region. Minisatellites (variable number of tandem repeats, VNTRs) have core repeats with 9-80 bp, while microsatellites (short tandem repeats, STRs) contain 2-5 bp repeats. The forensic DNA community has moved primarily

towards tetranucleotide repeats, which may be amplified using the polymerase chain reaction (PCR) with greater fidelity than dinucleotide repeats. The variety of alleles present in a population is such that a high degree of discrimination among individuals in the population may be obtained when multiple STR loci are examined.

Experiment

Forensics PCR

You need to obtain:

- A bucket of ice for your table
- A sterile sponge swab (Fisher Scientific: 22-025-192)
- A 1.5 mL microcentrifuge tube containing saline (clear tube, 0.90% w/v of NaCl in sterile water)
- A 1.5 mL microcentrifuge tube containing 500 µL of Chelex beads (Biorad: 143-2832, 100-200 mesh Chelex, 5-10% w/v in sterile water)
- An empty 1.5 mL microcentrifuge tube
- Four colored microcentrifuge tubes, each containing a different primer mix to be shared by everyone at your table (PER=yellow; TAS=pink; Actinin=blue, F13A1=green, concentration=0.5 µM of forward & reverse primers).
- Four PCR tubes per student, each containing a Ready-To-Go PCR bead (Fisher Scientific: 46-001-013)
- Table top centrifuge
- Heat block: preheat to 100°C

Information of genes and enzymes

Genes we are looking at in this section: Bold areas in the sequence are restriction enzyme binding sites

PER1: a clock gene that regulates circadian rhythms

AGCATCTGTAACTCAGGGGGGAGTTCTGGAGCTGGAGATGAAAATCTGGGATGTATTAATGTATTA
CCTGATATTTAAAGCCATGAAACTGGAAATGACCAAGGTAGTGGGTACAGATAGAGAAGAGGTTG
GAAAATTGAGCCTTGGGAACAGTCCAACATTTAGAGATCCAGAAGGTGAGGAACAGCAAAGAA
GTCTGAGAATAAGCTGCTGATGAAGTAGAAGGAATATCAGAAG**G[A/G]CGTAGTGTC**CTAGAAGG
CAAGCGAAGAGTGTTTCTAATGGAAGAGTGAGCATCGGTATCAGCTTCCACGAAGTAATGGTCAA
TAGATTTAGCAATGTGATGATCATTCGGCAACCTTAACAAGGGCAGCTTTGATGGGGGCGGTGGA
CACCTGATTAGCAAGGGTCCAAAGGAGAGT

Actinin3 (ACTN3): a gene important for muscle function

ACTGCTGCCCTTTCTGTTGCCTGTGGTAAGTGGGGGACACCAGCTGACACTTCCTGCCTGTCGTCC
CCAGAGCCTGCTGACAGCGCACGATCAGTTCAAGGCAACACTGCCCGAGGCTGA**C[C/T]GAG**
AGCGAGGTGCCATCATGGGCATCCAGGGTGAGATCCAGAAGATCTGCCAGACGTATGGGCTG
CGGCCCTGCTCCACCAATCCCTACATCACCCTCAGCCCGCAGGACATCAACACCAAGTGGGA

TAS2R38: a gene encodes for a bitter taste receptor

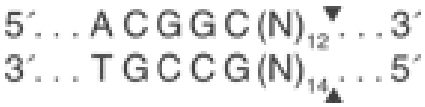
TGGAAGGCTTTGTGAGGAATCAGAGTTGTATTCCTGAAGAATCAGAGGCATATTTATGAAGACTCAC
AGGCGTATTAATGAAGAGCTCATTTCATGTCCATTCTCAGCACAGTGTCCGGGAATCTGCCTTGTGG
TCGGCTCTTACCTTCAGGCTGCTCTGAGCCCAGAGCAGAATGGTCATCACAGCTCTCCTCAACTT
GGCATTGCCTGAGATCAGG**A[C/T]GGCT**GCATGCCCAGAGGGACAAGCTGCCATTATCCCAAC
ACAAACCATCACCCCTATTTTGTCGCGCCACAGAATCAGTAGGGGCACAGAGATGAAGGCAGC
ACAGGATGATATCACAAAGAAGCAGAAAAAGGAGACAAGAGACTTGAGGGCTTTAATGTGGGC
CTCCAGGCTGGGGTCACGAGAGTTTCTGGTATAGACCTTCATTGTCCT

The restriction enzymes we use: the restriction enzymes below recognize and cut at a different DNA sequence (N indicates any base: A, C, G, or T)

AhdI:

DdeI:

BceAI:



I. DNA extraction from cheek cells (Day 1, 30 min)

1. Take a sterile spongy swab out of its wrapper. Swab the inside of each of your cheeks for 10 seconds (20 seconds total). Be sure to rub both sides. (Not too hard. If you see blood, let TA know.)
2. To release the cells from the sponge swab, twirl the swab several times in the microcentrifuge tube containing the saline solution (clear tube). Cap the tube and **label it with your initials**.
3. Now you will concentrate the cells into a pellet by centrifuging. Place your sample tube, together with others from your group, in a balanced configuration in the microcentrifuge. Spin them for 1 minute at 13,000 rpms.
4. There should be a cell pellet at the bottom of the tube. Carefully take the supernatant using a micropipette and discard into the sink. Be careful not to disturb the cell pellet at the bottom of the microcentrifuge tube. It is OK to leave a small amount of saline solution in the tube so as to avoid losing the pellet.
5. Next you will lyse the cells to release the DNA. Chelex beads are added prior to the lysis step to protect the DNA from being cut by DNA-digesting enzymes. Re-suspend the 500 µL sample of Chelex beads (purple tube) by carefully pipetting them up and down 2-3 times. Before the beads settle, pipet them into the tube containing your cheek cell pellet.
6. Re-suspend the cells in the solution with the Chelex beads by pipetting up and down several times. Make sure there are no visible cell clumps.

7. Boil the cell sample on a heat block (100°C) for 5 minutes. This will denature proteins and break open the cells and organelles, releasing the DNA into the solution.
8. **Slowly** pipet up and down the solution for a few times to further break up the cells, then place your tube, with those of other students, in a balanced configuration in a microcentrifuge. Spin your sample at 13,000 rpms for 1 minute.
9. Transfer 200 µL of the supernatant (which contains the DNA) from your sample to a clean 1.5 ml tube. Avoid disturbing the cell debris and Chelex beads at the bottom of the tube. Discard the tube containing the Chelex beads and cell debris.
10. **Label the tube containing the DNA with your initials**. This will be your template DNA for the PCR reactions. Store your DNA on ice until you are ready to add it to the PCR reactions.

II. PCR reaction (Day 1, 20 min + a few hours PCR reaction)

1. Label four PCR tubes with your initials and a number 1-4. **Label both the side of the tubes and the top**.
2. Use a 200 µL micropipette and a fresh tip to add 22.5 µL of each primer mix to one of the PCR tubes containing a Ready-To-Go PCR Bead. Be sure to add the correct primer mix to each tube, and change your tip before measuring out the next primer mix:
   1) PER1 forward and reverse primers (yellow)
   2) ACTN3 forward and reverse primers (blue)
   3) TAS2R38 forward and reverse primers (pink)
   4) F13A1 forward and reverse primers (green)
3. Use a 20 µL micropipette and a fresh tip to add 2.5 µL of DNA to each reaction tube. Don't forget to change the pipette tip before measuring out the DNA each time.
4. Tightly cap each PCR tube, make sure that all the reagents are at the bottom of the tube. (If needed, centrifuge the tubes briefly.)
5. Place your reaction tubes into the PCR machine. Your TA will set the PCR machine to hold at a low temperature until all students' reactions are ready (T_a=56.1°C, $t_{elongation}$ = 30 sec).
6. Once all students have finished setting up their reactions, your TA will start the PCR program. You will analyze your PCR products using RDA and DNA gel electrophoresis in next session.

- Before Day 2, TA need to make a 2% agarose gel (100 ml 1xTAE buffer+2 g agarose, add SYBR Safe for prestaining) with 40 lanes. Use 2 combs to make 2 rows of 20 lanes.

III. Restriction Digestion Analysis (Day 2, 1.5 hr)

1. From your TA, obtain:
   1) Your PER1, ACTN3 and TAS2R38 PCR products
   2) One tube of each enzyme buffer mix (Mix1: AhdI (blue), Mix2: DdeI (yellow), Mix3: BceAI (pink)) to be shared by the members of your lab group.
      - TA should mix 2 µL CutSmart Buffer + 1 µL enzyme + 9 µL MBG water per student.
   3) Three 1.5 mL microcentrifuge tubes
2. Label the three 1.5 mL microcentrifuge tubes with your initials and the name of each locus: PER1, ACTN3, or TAS2R38.

3. Add enzyme-buffer mix and your PCR product to each tube using the amounts listed in the table below.

Reaction	Enzyme-Buffer Mix	PCR product
PER1 + AhdI	12 µL Mix 1 (blue, AhdI)	8 µL PER1
ACTN3 + DdeI	12 µL Mix 2 (yellow, DdeI)	8 µL ACTN3
TAS2R38 + BceAI	12 µL Mix 3 (pink, BceAI)	8 µL TAS2R38

4. Vortex your tubes briefly to mix the reaction components, then put them in a balanced configuration in a microcentrifuge and spin them for 10 seconds at maximum speed to collect the liquid at the bottom of the tube.
5. Place your reactions into a 37°C heat block for one hour to allow the restriction enzymes to cut the DNA.
6. At the end of the hour, remove your reactions from the heat block. If you notice that any liquid has condensed on the lids of the tubes, put them in a balanced configuration in a microcentrifuge and spin them for 10 seconds at maximum speed to collect the liquid at the bottom of the tube.

IV. DNA gel electrophoresis (Day 2, ~ 1 hr)

1. Place the agarose gel into the electrophoresis chamber. Add 1x TAE buffer until it covers the surface of the gel. Make sure no bubble is trapped in the well.
2. Add 4 µL 6 x Loading buffer (purple) into your RDA products. Pipet several times to completely mix.
3. Load 12 µL of each RDA product in each lane.

Lane 1	2	3	4	5	6	7	8	9	…
100 bps ladder	PER1 student 1	PER1 student 2	PER1 student 3	ACTN3 Crime Scene	ACTN3 student 1	ACTN3 student 2	ACTN3 student 3	TAS2R38 student 1	…

4. Start applying voltage to start electrophoresis. (200 V for 50~60 min.)
5. During the running go to session V for PCR purification of F13A1 PCR product.
6. Image gel on blue light transilluminator. Take pictures of the gel.

V. PCR purification for Sanger sequencing for counting STR (Day 2, < 30 min)

1. This is for sequencing your F13A1 product to count the number of short tandem repeat. Sanger sequencing requires PCR purification since the product has impurities such as primers, polymerase, dNTPs, etc. Qiagen PCR purification kit will be used for this session.
2. Add 5 volues of Buffer PB to 1 volume of the PCR product. (e.g. add 100 µL of Buffer PB to 20 µL PCR product) The color of the mixture will be yellow for the pH indicator added.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the mixture to the QIAquick column and centrifuge for 30 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
6. To wash, add 750 µL Buffer PE to the QIAquick column and centrifuge for 30 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min to completely remove the wash buffer.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µL Buffer EB to the center of the QIAquick membrane and centrifuge the column for 1 min. Now purified PCR product is collected in the tube.

10. Label the tube. On the top of the tube write down “F13A1-your initial” on the side of the tube write down today’s date.
11. TA will send the product to sequencing facility and you will receive the sequence result of the product by email.
12. Go back to IV-6 to finish the experiment.

Lab Report Questions (include the questions for how to use pipettes)

1. Why do you need to perform PCR on DNA obtained from a crime scene and from you?
2. After 30 cycles of PCR, how many times the target will be amplified?

PCR+RDA

3. For each of the genes, which allele would be cut by the restriction enzyme?
   PER-1 (A or G):
   Actinin 3 (C or T):
   TAS2R38 (C or T):
4. How many different results (genotypes) can you get from each gene?
5. For each gene, what bands do you see? According to the band observed, what is your genotype?
6. Is the result of PCR+RDA enough to identify people? What is the probability of having the same genotypes of three genes. (Assume that each genotype appears with equal probability, 1/3.)?

PCR+STR

7. In sequencing results, how many STR does your DNA have? If your sequence failed why does it happen? (Usually failure happens when there are multiple kinds of DNA molecules are mixed. Note that chromosomes have 2 alleles (a pair) except sex chromosome.)