

BC 367 Experiment 5

Forensic Analysis of Canine DNA Samples*

Introduction

The advent of the Polymerase Chain Reaction (PCR) two decades ago revolutionized forensic science. While older DNA typing techniques required a bloodstain the size of a dime, PCR allows a DNA profile to be generated from a single fingerprint.¹ The most common method of DNA analysis currently used by the forensic community is PCR at loci containing Short Tandem Repeats (“PCR-STR”).² Short tandem repeats, also called microsatellites, arise when a two-to-ten nucleotide sequence is repeated in the genome, much like a stutter. Following isolation of DNA from a sample of interest, one or more hypervariable regions containing short tandem repeats is amplified via PCR, leading to different fragment sizes across individuals. Amplifying several hypervariable regions simultaneously in the same tube (“multiplexing”) saves time, expense, and sample, as well as providing a highly discriminating test for identity.

In addition to nuclear DNA, cells also contain mitochondrial DNA (mtDNA). Both genomes can be useful forensically, depending on the amount of sample, its quality, and the nature of the identification.³ MtDNA contains a hypervariable noncoding “control” region of about a thousand bases that accumulates mutations approximately ten times faster than nuclear DNA. Furthermore, amplification is possible from many different types of samples (e.g., hair shafts, cheek cells, blood, teeth, and bone). Two other characteristics render mtDNA useful forensically: it is present in high copy number (several hundred genomes per cell) and it is maternally inherited (rendering individuals not only haploid, but also identical in sequence to their relatives along the maternal line). MtDNA is most useful for identification of human remains when the only reference material available is from relatives or when the remains are old and/or badly damaged. For example, the remains of the Romanovs, the Russian Imperial family executed in 1918 by the Bolsheviks, were excavated in 1991 and subsequently positively identified via mtDNA sequencing and comparison to England’s Prince Philip, whose maternal great-grandmother was the mother of Czarina Alexandra.⁴ More recently, mtDNA analysis confirmed mountaineer Reinhold Messner’s version of his brother’s fatal climbing accident on Nanga Parbat in 1970, disproving accusations that Reinhold had abandoned his brother in his own quest for first-ascent glory.⁵ Typically, mtDNA analysis is via sequencing of either the HV1 or HV2 subregion of the control region, both of which display an average 1-2% sequence variance in unrelated humans.⁶

Crime scene investigations often yield biological evidence from non-human sources such as domestic pets.⁷ With almost 40% of U.S. households containing one or more dogs⁸, many crime scenes contain traces of canine material such as hair. Simulations of burglary and assault have shown that contamination by animal hairs is virtually certain when a criminal enters the home of a pet owner.⁹ The recent development of methodology for genetic typing of animal hairs, which was previously limited to morphological comparisons, has greatly increased the specificity with which they can be compared to the known inhabitants of a crime scene or to suspect animals. In one landmark analysis, the composite STR genotype of a single cat hair contributed to a second-degree murder conviction.¹⁰

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Canine genomic DNA has many STR loci for DNA typing.^{11, 12} The probability of identity, a parameter that reflects the chance of a random match between two samples at a given locus, depends on the allelic variation at that site. Higher numbers of variants lead to more definitive identification. In this exercise, three hypervariable nuclear loci will be analyzed (Table 1). Two sex-specific loci will also be probed: SRY yields a 129 base pair (bp) product diagnostic for the y chromosome, while CHR.X yields a 183 bp product diagnostic for the x chromosome.

Table 1. Allelic variations at loci targeted by canine STR primers. P_{ID} is the probability of identity.

Locus	# of alleles	Size range	P_{ID}
PEZ 2	8	109-141	0.145
PEZ 15	20	183-249	0.043
VWF.X	7	151-187	0.319

Although highly distinguishing, nuclear DNA is experimentally challenging to amplify from samples containing very small amounts of DNA because each locus is present in only two copies per cell. Canine mtDNA has the advantage of containing a tandem repeat region in the control region not present in humans.^{13,14} Therefore, to increase your chances of a successful amplification of the canine samples in this exercise, you will also analyze a ~500 bp portion of the control region of mitochondrial DNA containing variable numbers of a 10-bp tandem repeat.¹³ Variation in the length of the control region has been found for many carnivores,¹⁴ providing a simpler method than sequencing for discriminating between individuals. Although some investigators report that the number of repeat units in this region varies across different samples from the same individual,¹⁵ the ease of amplifying mitochondrial DNA relative to nuclear DNA makes it worthwhile investigating this locus via PCR-STR analysis.

PCR amplification is dependent on a delicate balance of reagent and template concentrations, meaning that optimal results often require lengthy experimental adjustments of reaction conditions such as the concentrations of $MgCl_2$ and primers. Fortunately, we have done this for you already...

Experimental Background

When the impeccably punctual Dr. Millstone, professor of geology, doesn't show up for class one day, her students notify campus security. An officer drives to her house to investigate, finds the professor dead at the bottom of the cellar stairs with a broken neck, and alerts local police. Examination of the professor's body yields a collection of dog hairs, unusual for a woman who disliked animals, and what looks suspiciously like a dog bite on her hand. Neighbors report that they heard barking and then saw a man in a blue car leaving the house on the previous night. The professor's email shows she had an evening meeting scheduled with a student disgruntled about failing Millstone's course. When questioned, the student reports that the meeting never took place. He was late for the appointment, found Dr. Millstone's office locked, and assumed that she gone home. When asked about dogs, he readily admits that his parents, who live nearby, recently purchased a purebred male puppy.

Further investigation into Professor Millstone's affairs reveals that Professor Rock Hedd, fellow geologist and show dog owner, drives a blue car. Rumor has it that Professor Hedd blames Professor Millstone for his recent tenure denial. The two are known to have had a longstanding mutual dislike.

Police now suspect that Professor Millstone's death was no accident! The DNA scientist at the Maine State Crime Laboratory is out sick, so our class has been asked to assist in the case. The samples that you will analyze include dog hairs found on the victim's clothing, a possible saliva sample from the bite on her hand, hair and saliva samples from Dr. Hedd's dog (a female),

hairs found on the dirty laundry in the student's dorm room, and a saliva sample from his parents' puppy. Coincidentally (but then again, Waterville is a small town), Dr. Hedd's dog and the disgruntled student's dog are not only of the same breed but are also from the same breeder, which could complicate your analysis. You will also have control samples from a known female and a known male dog. Your assignment is to extract DNA from each of these samples and then amplify your samples using PCR with different primer sets. You will analyze your PCR products via gel electrophoresis to create a DNA profile for each piece of canine evidence associated with the case. Careful analysis of the resulting data may give you some insight into the untimely demise of Professor Millstone.

Experimental Procedure

Week 1. Isolation and Amplification of DNA from Canine Samples

Working cooperatively with the other scientists in your laboratory, catalogue and divide up the evidence to be tested. (We recommend that you carefully document this information.) Each student should be responsible for isolating the DNA from one sample.

Part I. Canine Total DNA Extraction

A. Extraction from Hairs

You will use a commercial kit (the "DNA IQ" Kit from Promega) to carry out the DNA extraction from the canine hairs. This system uses a magnetic resin to purify total DNA after disrupting the sample tissue with a combination of proteinase K and other reagents.

1. You need to collect about ten hairs for DNA isolation in a microcentrifuge tube. If the hairs are short, simply use the whole hair, root-side down. Otherwise, use adhesive tape to gather the hairs together. Hairs should be stuck onto the tape with the root end and about 1 cm of hair protruding off the bottom. You should be able to identify the root end by the visible bulbous structure at its base. The far end of the hair is sharp and pointy. Dark hairs are easier to visualize if placed on white paper; white hairs can be viewed against the lab bench. Use tweezers to handle the hairs.
2. Place the hairs root down in a microcentrifuge tube with the tape outside the tube. Cut off the hairs with a razor blade and discard the tape with its hair remnants.
3. Add 100 μL freshly prepared Incubation Buffer/DTT/Proteinase K (see below) and incubate at 56°C for one hour.
 - To make 100 μL of Incubation Buffer/DTT/Proteinase K: combine 80 μL Incubation Buffer, 10 μL 1 M DTT, and 10 μL stock proteinase K solution.
4. Add 200 μL freshly prepared Lysis Buffer/DTT (see below) and 7 μL DNA IQ resin (vortex well to resuspend the resin first).
 - To make Lysis Buffer/DTT, combine 2 μL 1 M DTT with 200 μL Lysis buffer.
5. Vortex briefly and incubate at room temperature for five minutes. Hair remaining after the first incubation should disintegrate during this step.
6. Vortex again and place immediately on magnetic stand. The magnet attracts the resin, which is binding the DNA.
7. Avoiding the pelleted resin, remove and dispose of supernatant.
8. Add 100 μL Lysis Buffer/DTT (made fresh; see above) and repeat steps 6 & 7.
9. Add 100 μL 1X Wash Buffer and repeat steps 6 & 7.

10. Repeat Step 9 two more times (3X total).
11. Air-dry for 10 minutes. DO NOT over dry.
12. Add 100 μL Elution Buffer and vortex briefly.
13. Incubate at 65°C for 5 minutes.
14. Remove from heat. Vortex briefly and place on magnetic stand. NOTE: It is crucial to vortex and separate the resin while the solution is still hot. DNA recovery will decrease as the solution cools.
15. Remove supernatant to clean microcentrifuge tube. (Don't discard this time!) Sample is now ready for PCR amplification. This is your "template DNA."

B. Extraction from Saliva Swabs

Salivary DNA from bite wounds can unequivocally identify the attacker.¹⁶ You will use the same commercial kit (the "DNA IQ" Kit from Promega) as for the hairs to carry out DNA extraction from saliva samples from the presumed dog bite. This system uses a magnetic resin to purify total DNA after disrupting the sample tissue.

1. Shave the outer layer of cotton off both ends of the swab into a 1.5-mL microcentrifuge tube.
2. Add 250 μL freshly prepared Lysis Buffer/DTT (see below) and incubate at 70°C for 30 minutes.
 - To make Lysis Buffer/DTT, combine 2.5 μL 1 M DTT with 250 μL Lysis buffer.
3. Transfer the Lysis Buffer and sample to a spin filter apparatus. Spin at 3500 rpm for 3 minutes. Discard the contents of the basket and transfer the eluant to a fresh 1.5-mL microcentrifuge tube.
4. Add 7 μL DNA IQ resin (vortex well to resuspend the resin first).
5. Vortex briefly and incubate at room temperature for five minutes. Vortex mixture for 3 seconds once every minute during this incubation.
6. Vortex again and place immediately on magnetic stand. The magnet attracts the resin, which is binding the DNA.
7. Avoiding the pelleted resin, remove and dispose of supernatant.
8. Add 100 μL Lysis Buffer/DTT (made fresh; see above) and repeat steps 6 & 7.
9. Add 100 μL 1X Wash Buffer and repeat steps 6 & 7.
10. Repeat Step 9 two more times (3X total). Make sure that all liquid has been removed after the last wash.
11. Air-dry for 10 minutes. DO NOT over dry.
12. Add 70 μL Elution Buffer and vortex briefly.
13. Incubate at 65°C for 5 minutes.
14. Remove from heat. Vortex briefly and place on magnetic stand. NOTE: It is crucial to vortex and separate the resin while the solution is still hot. DNA recovery will decrease as the solution cools.
15. Remove supernatant to clean microcentrifuge tube. (Don't discard this time!) Sample is now ready for PCR amplification. This is your "template DNA."

Part II. PCR Amplification

For the male and female control samples, amplify *only* the x- and y-chromosome loci (CHR.X and SRY, respectively), and make sure that you amplify *both* loci for each control dog (both positive and negative controls are important).

A. Preparation of Master Mixes

When working with many samples it can be useful to create a master mix of the solution(s) needed for amplification. This mix can then be aliquoted into your PCR tubes along with the template DNA from your extraction. This not only makes for more consistent conditions but also can reduce the need for pipetting tiny volumes.

See below for the master mix recipes, good for one reaction as written. Multiply by the number of samples you will be amplifying, then add 1/2 volume more of each reagent. For instance, if you have 4 samples to amplify, you would make a 4.5X master mix. To prepare the master mix, add each reagent to the side of a microcentrifuge tube then centrifuge briefly to mix. Keep the reagents and the master mix itself on ice at all times.

Note that the SRY primer pair targets the y chromosome specifically, while the CHR.X pair targets the x chromosome. Samples from a known female and a known male dog will be available as “controls” for you to ensure that these sex-specific amplifications worked.

Each pair should prepare a Master Mix for at least one locus, scaled up according to the total number of canine samples to be tested for that locus (recipe below). Make sure that between all analysts, you have prepared all six canine master mixes.

Canine Master Mix Recipes (good for a single sample; scale up according to the number of samples + 0.5 samples)

For PEZ 2 or SRY

2.5 μ L dNTPs (at 2 mM)
2.5 μ L PCR buffer (10x stock)
2.0 μ L MgCl₂ (at 25 mM)
0.5 μ L EACH of the forward and reverse primers (PEZ 2 or SRY at 10 μ M)
6.5 μ L dH₂O
0.5 μ L Taq polymerase (add last, just prior to thermocycling)

For PEZ 15, CHR.X, or VWF.X

2.5 μ L dNTPs (at 2 mM)
2.5 μ L PCR buffer (10x stock)
2.0 μ L MgCl₂ (at 25 mM)
0.25 μ L EACH of the forward and reverse primers (PEZ 15, CHR.X, or VWF.X at 10 μ M)
7 μ L dH₂O
0.5 μ L Taq polymerase (add last, just prior to thermocycling)

For mtDNA control region

2.5 μ L dNTPs (at 2 mM)
2.5 μ L PCR buffer (10x stock)
2.0 μ L MgCl₂ (at 25 mM)
1.5 μ L EACH of the forward (WD3) and reverse (WD6) primers (10 μ M stock)
9.5 μ L dH₂O
0.5 μ L Taq polymerase (add last, just prior to thermocycling)

B. Amplification

1. For nuclear loci, add 15 μL Master Mix and 10 μL of your template DNA extract to a 0.2 mL PCR tube. Make sure your tube is firmly closed- you should hear a “snap.”
2. For the mitochondrial locus, the amount of template depends on its source. To a 0.2 mL PCR tube add 20 μL Master Mix and either
 - a) 2.5 μL of your HAIR template DNA extract and 2.5 μL sterile dH_2O .

OR

 - b) 5.0 μL of your SALIVA template DNA extract.

Make sure your tube is firmly closed- you should hear a “snap.”
3. Thermocycle under the following conditions: 95°C / 5 min; 10 cycles of (95°C / 30 sec, 65°C / 30 sec, 72°C / 30 sec); 30 cycles of (95°C / 30 sec, 55°C / 30 sec, 72°C / 30 sec); 72°C / 10 min; 4°C / ∞ .

Week 2- Analysis of PCR Products**Part III. Gel Electrophoresis of Canine PCR-STRs**

You will run two different types of gels to analyze your samples: polyacrylamide and agarose. Each has its own advantages and disadvantages. Polyacrylamide provides superior resolution for the PCR-STR analysis but quenches the fluorescence of ethidium bromide, commonly used to stain DNA. Agarose is easier to work with, yet cannot resolve fragments as well. You will therefore use polyacrylamide for your STR's and agarose for your sex-specific markers.

A. Preparation and Electrophoresis of 6% Polyacrylamide Gels

Prepare a total of three polyacrylamide gels in the lab for analysis of the STR's. One gel will be used for the mitochondrial DNA samples (hair found on victim; putative dog bite; two suspect dogs, hair and saliva samples for each). Two gels will be used for the nuclear STR's (hair found on victim; putative dog bite; two suspect dogs, hair and saliva samples for each). Note that with three hypervariable nuclear loci and six types of canine DNA, you cannot fit all the nuclear STR's on a single gel, which has ten wells. For each gel:

1. Add 8 mL 30% acrylamide (37:5:1) to 28 mL dH_2O in a small beaker.
2. Add 4 mL 10X TBE and mix well.
3. Carefully wash a pair of glass plates, rinsing with ethanol to remove water.
4. Assemble plates and spacers, using bulldog clips to hold the plates together.
5. Add 400 μL 10% APS to the acrylamide solution, then add 20 μL TEMED, mixing with the pipette tip as you do so.
6. Quickly pour solution into plate assembly, insert appropriate comb, then lay horizontal to cure. Gel will be set when solution remaining in beaker has polymerized.
7. Put the gel on the gel stand, add 1X TBE, and pre-run at 200 V for 1 hour.
8. Add 5 μL of native loading dye to each of your PCR products. (Note: you should have PCR products from three different nuclear loci and one mitochondrial locus for all canine samples except for the sex controls.)
9. Load molecular weight markers (a mixture of 2 μL marker + 8 μL dH_2O + 2 μL loading dye) in Lane 1 of each gel.

10. Load each canine sample into its own well. All mitochondrial DNA should be loaded onto a single gel and the nuclear samples should be split across two gels.
Make sure you know which lane on which gel corresponds to each sample. Confusing the evidence would be a miscarriage of justice as well as bad science.
11. Run the gels at 225 V until the dye front has traveled about $\frac{3}{4}$ of the way down the gel (~2 h). While the gels are running, you will watch Frontline: The Case for Innocence, which documents the importance of DNA testing in the criminal system.
12. Turn off the power supply and remove gels from apparatus.
13. Carefully separate the plates, making sure that the gel remains on the small plate. This plate (with the gel) should be placed gel-up in a container with 250 mL 1x TBE.
14. Add 38 μ L SYBR Gold stain concentrate to the solution, put the lid on the container, and incubate with shaking for 45 minutes.
Caution: The stock solution of SYBR Gold in DMSO is a potential mutagen that can be absorbed through the skin. It may also irritate the skin, eyes, mouth, and upper respiratory tract. Exercise caution when pipetting.
15. Cover the UV transilluminator on the GelDoc system with plastic wrap before moving the gel.
16. Remove the gel and plate from stain and cover the gel with plastic wrap. CAREFULLY remove the gel from the glass plate onto the plastic wrap. Place the gel wrap-side down on the transilluminator.
17. Photograph your results for later sample analysis. Remember that the ladder is in Lane 1, so if your gel flipped over, you will need to correct for this. Note that you are looking for the major band in each lane- use the information in the Introduction to assess expected product sizes. (Extra minor bands are not unusual when using nondenaturing gel electrophoresis; they arise from DNA heteroduplexes.) Post your annotated gel image (identifying lane assignments) on the FileServer within 24 hours after lab.

B. Preparation and Electrophoresis of 1.5% Agarose Gel

Prepare a single large agarose gel in the lab for analysis of the sex-specific markers (hair found on victim; putative dog bite; two suspect dogs, hair and saliva sample for each; male control; female control).

1. Add 250 mL 1X TBE buffer to 3.75 g agarose in an Erlenmeyer flask.
2. Microwave in 30 sec bursts to dissolve the agarose, being careful not to boil over.
3. Allow the dissolved agarose solution to cool on the benchtop.
4. When cool enough to handle, add 50 μ L stock ethidium bromide (0.5 mg/mL) to the solution and swirl to mix.
Caution: Ethidium bromide is a mutagen that can be absorbed through the skin. It is also an irritant to the skin, eyes, mouth, and upper respiratory tract. Exercise caution when handling and dispose of pipette tips and any contaminated gloves or tips in the appropriate solid waste container.
5. Pour the solution into the casting tray, being careful to minimize bubbles.
6. Set the comb in the tray to create the wells, and let gel solidify. Gel will be slightly cloudy when finally set.
7. Just prior to loading, place agarose gel and support deck into the electrophoresis chamber, submerging completely with 1X TBE Running Buffer.

8. Mix together 15 μL of the SRY and CHR.X PCR products from one canine sample on parafilm and add 6 μL of native loading dye. Repeat with additional canine samples.
9. Load molecular weight markers (a mixture of 2 μL marker + 8 μL dH_2O + 2 μL loading dye) in Lane 1.
10. Load each canine sample mixture into a separate well.

Make sure you know which lane corresponds to each sample. Confusing the evidence would be a miscarriage of justice as well as bad science.

11. Run the gel at 200 V until the dye front has traveled just less than halfway down the gel (~1.5 h). While the gels are running, you will watch Frontline: The Case for Innocence, which documents the importance of DNA testing in the criminal system.
12. Turn off the power supply and remove support deck and gel from apparatus.
13. Cover the UV transilluminator on the GelDoc system with plastic wrap before moving the gel.
14. One or two major bands should be visible in each sample lane. Photograph your results for further comparison, making sure to label each lane. Remember that the ladder is in Lane 1, so if your gel flipped over, you will need to correct for this. Post your annotated gel image (identifying lane assignments) on the FileServer within 24 hours after lab.

Analysis

For each gel, plot the log of the MW (in bp) versus the mobilities of the molecular weight size markers to generate a standard curve.

For each gel, determine the sizes of the major PCR products for each canine sample.

Calculate the expected frequency of the complete profile for the three hypervariable nuclear loci assuming no genetic linkage between each locus. That is, how unique is a given profile?

What do the results of the sex-specific probes suggest?

For the mitochondrial locus, do your findings support a constant profile independent of tissue type for a given individual or some variation? Does this agree with literature reports?

The number of 10-bp repeats within the mitochondrial control region varies from 25 to 38.

Calculate the likelihood that the mtDNA fragment found at the crime scene matches a particular dog by chance, assuming equal frequencies for each variant.

Overall, do your results implicate or exclude either of the suspects in the crime?

Write-up

Undoubtedly, law enforcement officials will want to have a concise report of your conclusions regarding the forensic samples. Therefore, instead of turning in your lab notebook this week, prepare a ONE-PAGE report describing your experiment, outlining and substantiating your results, and providing your analysis. As always, you should address all major points of the experiment, including, but not limited to, the objective, a description of your methods, your data, results, and analysis, a discussion of your conclusions, error analysis, and references as appropriate. Be sure to include some degree of confidence in your results by referring to control samples and the expected frequency of a given profile. Attach supporting tables or figures that are important to substantiate your case- these do not count towards the one page of text. (Note that all gel images should be included in your report. You should use results from the other lab sections if your lab section's results are incomplete.)

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