Student Lab Guide

Athlete Gene PCR Experiment

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Scientific Background: Muscle Performance and the ACTN3 Gene

Why are some people naturally better at sprinting and jumping, while others perform better in endurance sports? Part of the answer may lie in a small genetic difference in your DNA!

How Your DNA can influence athletic performance

DNA is the instruction manual for every cell. It is made up of nucleotide bases (adenine, thymine, cytosine, and guanine) which are often represented as letters (A, T, C, and G). Segments of DNA called **genes** code for **proteins**, which perform most cellular functions. Genes can have different versions, called **alleles**. One way that alleles can differ is by single base pair changes that alter protein production: these are known as single nucleotide polymorphisms or **SNPs**.

The **ACTN3 gene (**also known as the "athlete gene" or the "sprinter gene"), codes for **α-actinin-3**, a protein in **fast-twitch muscle fibres**, which are important for sprinting and explosive movements. Within the *ACTN3* gene there is a SNP known as **R577X**, that allows or interrupts the production of **α-actinin-3**. Without α-actinin-3, fast-twitch fibres behave slightly more like slow-twitch fibres, and this may slightly favor endurance performance.

Investigating The Genetic Variation in ACTN3

This lab will allow you to investigate these genetic variants located in the *ACTN3* gene.

There are two possible variants:

- **R allele (C):** The SNP is a C, and this contributes to coding the amino acid arginine (**C**GA) which becomes incorporated into α-actinin-3 at that point in the protein synthesis. The protein α-actinin-3 is produced.

- **X allele (T):** The SNP is a T, which introduces a **nonsense mutation** (TGA) that results in a truncated (shortened) non-functional protein due to a premature **stop codon** (a sequence of three nucleotides that signals the ribosome to stop translation). As a result, no α-actinin-3 is produced.

Because we inherit two copies of the gene, three **genotypes** are possible:

- **RR / CC: Homozygous.** Full production of α-actinin-3 from two gene copies.

- **RX / CT: Heterozygous**. One gene can still produce α-actinin-3, and muscle structure and function remain largely normal.
  XX / TT: Homozygous. No gene copies can produce α-actinin-3.

It's important to note that people with the XX genotype are healthy because another protein compensates for the missing α-actinin-3. However, their fast-twitch fibres tend to behave slightly more like slow-twitch fibres, with slightly lower power output and potentially more endurance-like characteristics.

Additionally, this variation has only a small contribution towards predicting athletic performance. Athletic performance is determined by a very large number of factors, such as training, psychology, environment, and other genetic factors. Many sprinters have XX genotypes, and many endurance athletes have RR genotypes. However, RR or RX genotypes are extremely common among elite sprint athletes, suggesting that even this small effect is important at their level of performance.

Investigating the SNP using PCR

We can study this variation in the ACTN3 gene using a technique called bidirectional allele-specific **PCR (Polymerase Chain Reaction)**. Primers flank the SNP region from both directions, and the **polymerase enzyme** copies it repeatedly through the heating and cooling cycles. **Gel electrophoresis** separates the DNA fragments to reveal the genotype (RR, RX, or XX). This lab shows how a single DNA change can be connected to muscle physiology.

Key Terms

ACTN3 gene: A gene that provides instructions for making the protein α-actinin-3.
Allele: A different version of the same gene. In this case, the ACTN3 gene has R and X alleles.
Allele-specific PCR: A type of PCR that can distinguish between different alleles of a gene.
Codon: A sequence of three DNA or mRNA bases that codes for an amino acid or a stop signal.
DNA (Deoxyribonucleic acid): The molecule that carries genetic information in cells. It is made of a sequence of bases (A, T, C, G).
DNA polymerase: The enzyme that builds new DNA strands during PCR.
Fast-twitch muscle fibres (also known as Type II): Muscle fibres adapted for fast, powerful movements such as sprinting. They fatigue more quickly.
Gel electrophoresis: A method used to separate DNA fragments by size using an electric current.
Gene: A section of DNA that contains the instructions to make a protein.
Genotype: The combination of alleles an individual has (RR, RX, or XX).
Heterozygous: Having two different alleles (RX).
Homozygous: Having two identical alleles (RR or XX).
Nonsense mutation: A mutation that changes a codon into a stop codon, resulting in a shortened, non-functional protein.
PCR (Polymerase Chain Reaction): A laboratory technique used to make many copies of a specific DNA sequence.
Phenotype: The observable characteristics that result from the interaction between genotype and environment.
Primer: A short DNA sequence that's used to define the starting point of DNA replication in PCR.
Protein: A molecule made of amino acids that performs important functions in cells.
R allele (C allele): The version of the gene that allows normal production of α-actinin-3.
R577X: A specific SNP in the ACTN3 gene. The R version produces a functional protein, while the X version introduces a stop signal.
Slow-twitch muscle fibres (also known as Type I): Muscle fibres adapted for endurance activities. They contract more slowly but are more resistant to fatigue.
SNP (Single Nucleotide Polymorphism): A variation in a single DNA base at a specific position in the genome.
Stop codon: A codon that signals the end of protein synthesis.
X allele (T allele): The version of the gene that creates a stop codon, preventing production of α-actinin-3.
α-actinin-3: A protein found mainly in fast-twitch muscle fibres and involved in powerful muscle contractions.

Experimental Plan

- You will be **performing** a basic human DNA extraction from your own cheek cells using a saline mouthwash sample to release the cells; centrifuging to concentrate the cells; and heating to break open the cells and release DNA.

- You will be **learning** the principles of Polymerase Chain Reaction (PCR) and how to apply it to detect tiny genetic differences (Single Nucleotide Polymorphisms or SNPs), by using a PCR technique called bidirectional allele-specific PCR.

- You will be **discovering** the concept of how a nonsense mutation in one gene (ACTN3) can act as a switch to stop the gene from a protein, and thereby affecting (in a small way) human athletic performance.

- You will be **interpreting** the results of gel electrophoresis to determine a person's genotype (RR: homozygous dominant, RX: heterozygous, or XX: homozygous recessive) for the ACTN3 gene.

The Three Stages of the Lab

#	Stage	Goal	What you will do
1	**DNA Extraction**	Isolate your DNA	You will collect cheek cells using a saline mouth rinse. You will then use a centrifuge to gather the cells and heat to release the DNA.
2	**PCR**	Copy your target gene	You will use your extracted DNA as a template. The thermocycler will make many millions of copies of a fragment of the ACTN3 gene region containing the R577 SNP, as well as fragments specific to the R and X alleles (if present).
3	**Gel Analysis**	Visualize your results	You will load your samples into an agarose gel prepared by your instructor. By comparing your DNA bands to a known ladder, you will determine your genotype.

Protocol 1: DNA Extraction Using a Saline Mouthwash

What are we trying to achieve in this part of the experiment?

The goal of today's experiment is to extract a DNA sample from cheek cells in your saliva using a 0.9% saline solution. The saline solution is isotonic (of the same salt concentration as your cells), which helps protect your cells and DNA during collection.

ⓘ **Note:** This protocol should take approximately 20 minutes to complete.

Before You Start

ⓘ **Important:** For best results, make sure it's been at least 30 minutes since you last ate food, drank liquids (other than water), or brushed your teeth with toothpaste. If you don't, these substances might carry through into your DNA extraction and PCR, and make DNA amplification more difficult.

⚠ **Safety:** Wear nitrile gloves, and eye protection if required. Handle only your own sample.

Step-by-Step Protocol

Step 1. Label your sample tubes

- ☐ Prepare and label a 1.5 mL microcentrifuge tube with a permanent marker (e.g. your initials and the date).

Step 2. Get a buccal cell sample by rinsing your mouth with 0.9% saline solution.

- ☐ Measure approximately 10 mL of 0.9% saline (salt water) into a plastic cup or shot glass and pour it into your mouth.
- ☐ Rinse your inner cheeks vigorously for 60 seconds, swishing the water from side to side and rubbing your cheeks against your teeth to dislodge cheek cells.
- ☐ Spit the saline solution back into the plastic cup or shot glass.

ⓘ **Visual Check:** Your spit saline should appear slightly cloudy compared to fresh saline. This cloudiness is your cheek cells, as well as any other debris from your mouth.

Step 3. Transfer your sample into the microcentrifuge tube

- ☐ Use the transfer pipette to transfer your saliva sample from the glass into the labeled 1.5 mL microcentrifuge tube, filling it up to the 1 mL mark.

Step 4. Centrifuge your sample to form a cell pellet

- ☐ Place the tube with your saliva sample into the centrifuge.

⚠ **Safety warning:** Ensure that you balance the centrifuge using tubes with contents of equal weight before running the centrifuge.

- ☐ Close the lid and run the centrifuge at 4,000 xG for 120 seconds (or ~2,700 xG for 5 min on slower centrifuges).
- ☐ Once finished, check the tube for a pellet (of cheek cells) at the bottom or side of the tube. It should look like a small white ball or smear. Do not mistake air bubbles at the bottom of the tube for a pellet. The remaining liquid (supernatant) should be clear.

ⓘ **Visual check:** The pellet should be firmly attached to the bottom. If loose, centrifuge for the same duration again.

Step 5. Recover the pellet

- ☐ Carefully pour the supernatant back into your plastic cup or shot glass, making sure to retain the pellet in the tube.

⚠ **Safety Warning:** When pouring off the supernatant containing your biological cheek cells, pour gently to avoid splashing. Ensure all liquid goes directly into the designated container.

- ☐ Use a transfer pipette to remove any remaining liquid without disrupting the pellet.

Step 6. Wash the pellet

- ☐ Add 1 mL of fresh 0.9% saline solution to the tube with your transfer pipette, making sure you have some left for the next step.
- ☐ Close the tube and break up the pellet by flicking the tube.

ⓘ **Visual check:** After flicking the tube, the liquid should become cloudy again, and the solid white pellet should be completely broken apart into the saline.

Step 7. Recover the washed cells

- ☐ Centrifuge the sample tube again to reform a pellet (4,000 ×G for 120 seconds, or ~2,700 xG for 5 min on slower centrifuges).

⚠ **Safety warning:** Ensure that you balance the centrifuge using tubes with contents of equal weight before running the centrifuge.

- ☐ Carefully remove as much liquid as you can by pouring and/or pipetting into your plastic cup or container.

Step 8. Resuspend the washed cells

- ☐ Set your 200 µL micropipette to 100 µL. Using a new pipette tip, transfer 100 µL of fresh 0.9% saline into the sample tube.
- ☐ Close the tube and resuspend the pellet by flicking the bottom of the tube until the cells are entirely dispersed into the solution.

ⓘ **Visual check:** The liquid should look uniformly cloudy and milky. If you see large visible clumps, continue flicking until they break apart.

Step 9. Transfer the sample into a PCR tube for extraction

- ☐ Using the same pipette tip, transfer 100 µL of the cell mixture from the 1.5 mL tube into a new 200 µL PCR tube.

Step 10. Label the PCR Tube

- ☐ Click the lid closed and label the side of the PCR tube to identify the sample. Do not label the lid, as the heated lid might remove the ink.

Step 11. Heat the sample

- ☐ Place your PCR tube in the thermocycler block, in numerical order in case the labelling comes off.
- ☐ Set the thermocycler to heat the sample at 95 °C for 10 minutes to heat the cells and release the DNA, then 15 °C for 1 min to cool it.

⚠ **Safety Warning:** If you open the thermocycler before the cooling step finishes, the block and your 200 µL PCR tube will be extremely hot (95°C).

Step 12. Mix the sample

- ☐ Carefully remove the PCR tube (the block may still be hot).
- ☐ Flick the PCR tube for 5–10 seconds to mix the sample.

Step 13. Clean up the sample

- ☐ Use the PCR tube adapter inside a centrifuge tube adaptor (a 500 µL tube inside a 1.5 mL tube, with the lids cut off) to fit the PCR tube into the centrifuge.

⚠ **Safety warning:** Ensure that the centrifuge is balanced, using another tube of equal weight (e.g., a water-filled balancing tube) if necessary.

- ☐ Run the centrifuge (4,000 ×G for 120 seconds, or ~2.7k ×G for 5 min for slower centrifuges).

ⓘ **Important:** The clear liquid (supernatant) at the top of the tube now contains your extracted DNA. Do NOT throw it away. Be extremely careful to only pipette this clear liquid, leaving the solid cell debris at the bottom undisturbed.

Step 14. Transfer the clean sample to a new tube

- ☐ Using a new pipette tip, transfer 40 µL of the clear supernatant into a new, clean 200 µL PCR tube using a 200 µL micropipette. Be careful to avoid the cell debris.
- ☐ Label the new tube containing the DNA template sample.
- ☐ If not proceeding immediately to PCR, store the template sample in the freezer at around -20°C.

Protocol 2: Set Up the PCR

What are we trying to achieve in this part of the experiment?

In this step, you will use PCR to amplify part of the *ACTN3* gene, producing a PCR product that can be visualized on an agarose gel to determine the result of the PCR assay.

The sequence of the gene fragment we are copying includes the C/T mutation that affects whether the protein α-actinin-3 is produced. This C/T mutation is also known as a single nucleotide polymorphism (SNP) with the code rs1815739.

The experiment uses a method called bi-directional allele-specific PCR, and it uses four primers. Two primers allow the copying of a larger DNA fragment in which the SNP occurs: these are called the outer primers. This larger fragment acts as an internal PCR control that will be amplified in all successful PCRs. The other two primers are located within this fragment and, together with the outer primers, allow the specific detection of either the C or T mutation of the SNP rs1815739: these are called the inner primers.

Before You Start

To set up the PCR, you will need to add four components to a PCR tube in specific volumes:

- PCR Grade Water
- 5x HOT FIREPol® Master Mix
- Athlete Gene primer mix
- Your DNA template.

The total volume for your PCR reaction will be 20 µL in an empty PCR tube.

Step-by-Step Protocol: Setting up the PCR

Step 1: Label your PCR tube

- ☐ Label or number your PCR tube on the side and top with a fine permanent marker.

Step 2: Add PCR Grade water

- ☐ Set your micropipette to 12 µL.

- ☐ Using a fresh tip, transfer 12 µL of PCR Grade Water into the PCR tube. Discard the tip.

Step 3: Add Master Mix

- ☐ Set your micropipette to 4 µL.
- ☐ Using a fresh tip, transfer 4 µL of the 5x PCR master mix into the PCR tube. Discard the tip.

Step 4: Add Primer Mix

- ☐ Set your micropipette to 2 µL.
- ☐ Using a fresh tip, transfer 2 µL of the primer mix (Athlete Gene PCR primers) into the PCR tube. Discard the tip.

Step 5: Add DNA Template

- ☐ Set your micropipette to 2 µL.
- ☐ Using a fresh tip, transfer 2 µL of your Template Sample into the Reaction Tube. Discard the tip, and close the tube securely.
- ☐ Mix the contents by gently flicking the tube.

Step 6: Make a Negative Control (one per group)

- ☐ Label a tube “NC” for negative control
- ☐ Repeat Steps 2 to 4 to make a PCR mix, but add an extra 2 µL of water instead of DNA template.
    - ☐ Add 14 µL of PCR grade water
    - ☐ Add 4 µL of PCR master mix
    - ☐ Add 2 µL of primer mix (Athlete Gene PCR primers)
- ☐ Mix the contents by gently flicking the tube.

Step 7: Run the PCR program

- ☐ Place your PCR tubes and negative control in the thermocycler block.
- ☐ Check that the PCR program has been set to the following program:
    - Initial Denaturation: 15 mins at 94°C (hot start PCR)
    - 35 Cycles of:
        - Denaturation: 30 seconds at 94°C
        - Annealing: 45 seconds at 67°C
        - Extension: 45 seconds at 72°C
    - Final Extension: 120 seconds at 72°C

- ☐ Start the PCR program.

ⓘ **Note:** The thermocycler will now run for **between 1.5 and 2.5 hrs** (depending on thermocycler model).

- ☐ When finished, store the PCR product in a fridge or freezer, or use it immediately for gel electrophoresis.

Protocol 3: Load, Run, and Visualize an Agarose Electrophoresis Gel

What are we trying to achieve in this part of the experiment?

The goal of this part of the experiment is to take the DNA fragments amplified in the PCR step, physically separate them by size, and then visualize them using a fluorescent DNA stain on an agarose electrophoresis gel prepared by your instructor.

Loading the amplified DNA into the gel wells and applying an electric current (electrophoresis) causes the fragments to migrate. Larger fragments move more slowly than smaller fragments through the gel's matrix, separating the original mixture into discrete bands based on their size.

To determine the exact size of these fragments, a 100 bp DNA ladder (a set of fragments with known sizes) is run in a parallel lane. The separated DNA is visualized by exposing the stained gel to blue light, causing the DNA bands to fluoresce.

To interpret your results, you can compare a photograph of your results with the example results on the next page.

Step-by-Step Protocol:

Loading and running an agarose gel

You will now load your DNA samples and the DNA ladder into the gel wells.

⚠ Safety warning: Before you start, ensure that your electrophoresis tank is disconnected from any power sources.

Step 1: Loading the DNA Ladder

- ☐ Set your micropipette to 5 µL.
- ☐ Using a fresh pipette tip, draw up the 100 bp DNA Ladder and carefully load it into the first well (usually Well 1). Discard the tip.

ⓘ **Visual Check:** As you dispense the DNA ladder, you should see the heavy, colored DNA ladder solution sink neatly to the bottom of the well. If it floats away into the surrounding buffer, your pipette tip may be positioned too high.

- ☐ Using a new pipette tip, repeat the process for the LAST well in the row of wells.

Step 2: Loading the PCR Product

- ☐ Record which PCR results you will be loading into the gel, and in what order.
- ☐ Set your micropipette to 5 µL.
- ☐ Using a fresh pipette tip, load 5 µL of the first PCR Product into the next empty well (e.g. Well 2). Discard the tip.

ⓘ **Visual Check:** As you dispense the PCR product, you should see the heavy, colored PCR product solution sink neatly to the bottom of the well. If it floats away into the surrounding buffer, your pipette tip may be positioned too high.

- ☐ Load the remaining PCR products, in order, into the remaining wells, using a new pipette tip each time.

Step 3: Running the gel

- ☐ Gently close the gel box and connect the leads to the power supply.
- ☐ Run the gel for 30-45 minutes at 50 V - 80 V (depending on your gel tank model).

⚠ Safety Warning: Before touching the power supply or connecting leads, ensure your lab gloves and the surrounding bench area are completely dry to prevent electrical shock.

Step 4: Turn off the power supply

- ☐ Once the run is complete, disconnect the gel box. Your gel is now ready for visualization.

Visualizing and Analysing Results

Step 5: Agarose gel results visualization

- ☐ Carefully place the gel box or agarose gel slab on the blue light transilluminator (Bento Lab or equivalent system).
- ☐ Place the Gel Imaging Hood, or the transilluminator orange filter, over the gel to filter out the blue light.
- ☐ Turn the blue light transilluminator on to visualize the DNA bands, which will fluoresce due to the DNA stain bound to the DNA.
- ☐ Take a photograph for documentation.

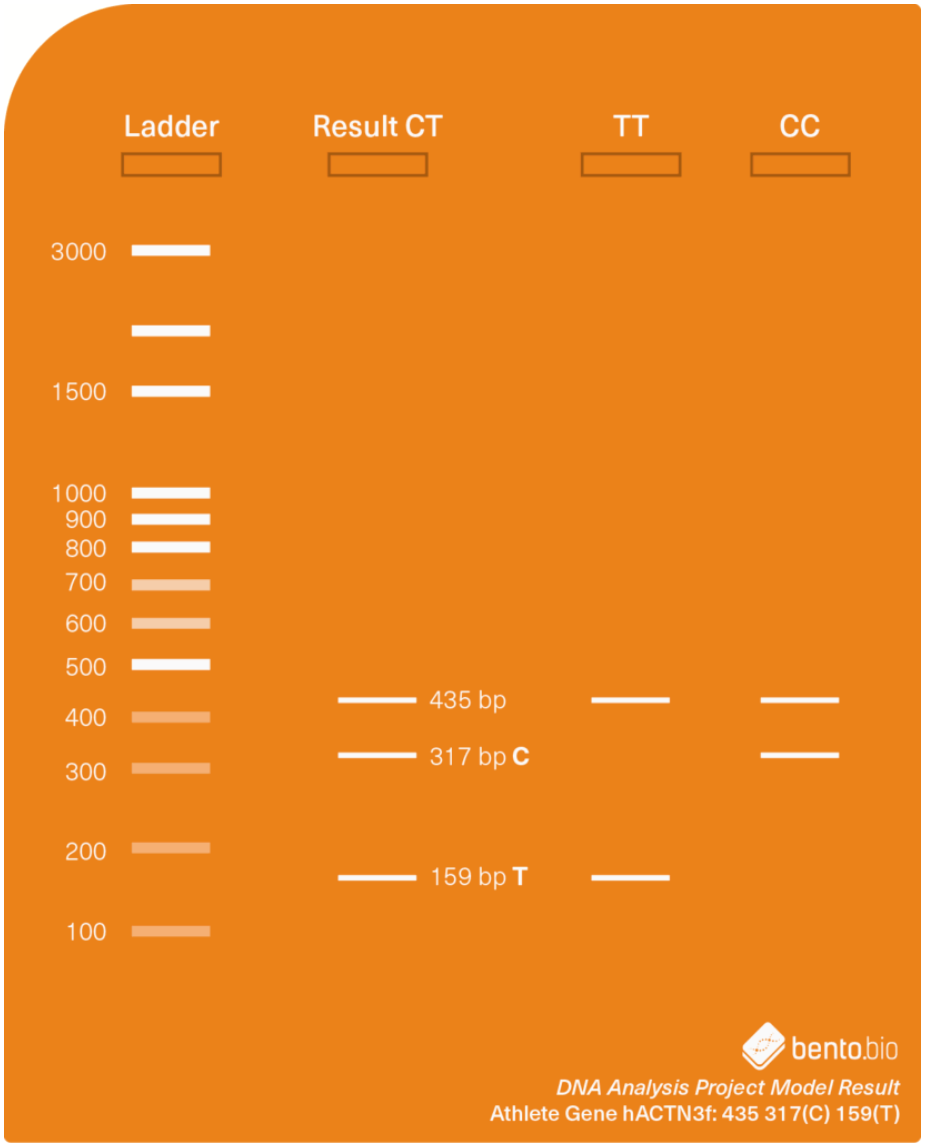
Step 6: Agarose gel results interpretation

- ☐ Compare the bands in your sample lanes to the expected results for each genotype in the table below and the model gel results on the next page.

Note: The control band (435 bp) should be present in all positive PCR results. The presence of the other two bands determines the genotype according to the table below.

Genotype	Definition	Bands on Gel	Typical Phenotype
CC	Homozygous dominant	435 bp + 317 bp	Produces α-actinin-3, may be slightly better adapted for speed/power athletics
CT	Heterozygous	435 bp + 317 bp + 159 bp	Produces some α-actinin-3, may be slightly better adapted for speed/power athletics
TT	Homozygous recessive	435 bp + 159 bp	Produces no α-actinin-3, may be slightly better adapted for endurance athletics

- ☐ After documentation, dispose of the agarose gel in the regular trash.



Model agarose gel results for the Athlete Gene PCR assay