GenoSensor Corporation

GenoSensor Marine Biology: Shark Week II

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User's Manual

GenoSensor Marine Biology: Shark Week Kit II Manual

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Literature Citation

When describing a procedure for publication using these products, please refer to them as the *GenoSensor Marine Biology: Shark Week Kit II*.

Notes for Instructors

Kit Components and Storage Conditions:

Component	Storage
2X PCR Master Mix	-20ºC
Sample A	-20ºC
Sample B	-20ºC
Sample C	-20ºC
Unknown DNA	-20ºC
Negative control	-20ºC
DNA ladder	-20ºC

Preparation for PCR (for 6 teams)

1. Set up thermal cycler and the PCR program.
2. Thaw 2X PCR Master Mix on ice.
3. Spin Master Mix for 10 seconds, then vortex for 10 seconds, spin for another 10 seconds.
4. Label 6 microcentrifuge tubes "MM" and aliquot 40 µL of 2X PCR Master Mix into three tubes and 50 µL into the other three tubes. KEEP ON ICE.
5. Label 6 tubes (24 total) each "A, B, C, and U" and aliquot 10 µL of each DNA sample, store on ice.
6. Label 3 tubes "N" and aliquot 10 µL of negative control, store on ice.
7. In class, distribute 1 each "MM, A, B, C, U" tubes to all teams and 1 "N" tube to three teams (they will share later for electrophoresis). Be sure to give the 50 µL "MM" tubes to the teams that also have the "N" tube.
8. Students will use 10 µL of 2X PCR Master Mix with 10 µl sample DNA for a final PCR volume of 20 µL.

Electrophoresis

- Electrophoresis reagents are not provided in the kit. Please refer to the Additional Required Materials list, on page 4.
- Best results are obtained by adding DNA dye (i.e., Gel Red, Sybr® Safe) to molten agarose.
- For light sensitive DNA dyes, avoid exposing the agarose gel to light. It is best to store and run the gel in a dark room, or cover the gel with a box during gel polymerization and the whole electrophoresis process.
- DNA ladder supplied is enough for 3 lanes with 10 µL each.
- Negative control supplied is enough for 6 lanes with 10 µL added after PCR.
- DNA samples "A, B, C, and U" supplied is enough for up to 20 µL / lane for 6 teams.

Shipping, Storage and Safety

Shipping and Storage

GenoSensor Marine Biology: Shark Week kits are shipped on blue ice. Components should be stored at temperatures shown in the above table. At proper storage conditions, components are stable for 1 year from the date received. Expiration dates are also noted on product labels.

Safety Warnings and Precautions

This product is intended for research use only. It is not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes: if contact should occur, wash immediately with water and follow your laboratory safety protocols. Safety Data Sheets for products are available upon request.

GenoSensor Marine Biology: Shark Week Kit II Overview

The GenoSensor Marine Biology: Shark Week Kit II introduces common techniques used in DNA research and in marine biology. The kit creates a scenario in which a shark washes up on shore and local marine biologists cannot identify it out of over 400 known species. They are excited to determine the shark is a new, never before, discovered species and are eager to begin learning all they can through research to help their marine taxonomist colleague classify the unknown shark species. The biologists decide to begin by analyzing the shark's stomach contents to determine it's main food source. The stomach sample collected is labeled "Unknown Sample", and it is represented by a plasmid. They narrow possible stomach contents down to three local food sources. They are labeled "Sample A, B, and C" and are represented by three different plasmids. One of the samples will match the unknown stomach sample. The goal of the experiment is to identify which of the samples matches the unknown by performing a polymerase chain reaction (PCR) on the four samples. After completing the experiment students will understand the concepts behind PCR, gel electrophoresis, and the genetic concepts driving the experiment.

Kit Components and Storage Conditions

Materials for 6 teams

Component	Amount (27 rxns)	Storage
2X PCR Master Mix	270 µL	-20ºC
Sample A	60 µL (6 rxns)	-20ºC
Sample B	60 µL (6 rxns)	-20ºC
Sample C	60 µL (6 rxns)	-20ºC
Unknown DNA	60 µL (6 rxns)	-20ºC
Negative control	30 µL	-20ºC
DNA ladder	30 µL	-20ºC

Additional Required Materials

- Thermal Cycler
- Heat Block or (heat plate, Beaker with de-ionized water; water bath, Tube floater; Thermometer)
- Ice
- Microcentrifuge
- Microcentrifuge tubes (36)
- Vortexer (optional)
- Micropipettes (p10, p200, p1000)
- Pipette tips
- PCR tubes (27)
- Tube Racks
- Ethanol or ethanol wipes
- Electrophoresis equipment

- Electrophoresis supplies: agarose, TBE buffer, DNA loading buffer, running buffer, gel dye (e.g., SYBR® safe, Gel Red)
- UV light box or “Gel Doc” equipment and program
- Permanent marker

Student Guide

Objective overview

1. Understand how DNA is responsible for genotypic differences between food sources of interest.
2. Investigate techniques used in DNA technology: DNA sequence diversity and uniqueness, PCR, gel electrophoresis.
3. Investigate and understand the process for gel electrophoresis including analyzing band pattern data.

In this lab you will examine an abridged version of a DNA analysis – PCR. During the exercise you will learn to analyze and compare a number of DNA fragments to determine whether or not they are from the same food source organism. These fragments can be visualized through a process known as "gel electrophoresis."

DNA is long double helix polymer that uses deoxyribose rings (sugars) and phosphate molecules as support in its backbone. Attached to the backbone are unique sequences of nucleotides which are often referred to as base pairs. There are two different types of nucleotides: purines and pyrimidines. Adenine (A) and Guanine (G) are both purines because they have two rings in their structures. Meanwhile, Thymine (T) and Cytosine (C) are pyrimidines because they have only a single ring in each of their structures. These nucleotides form a bond with their complementary base pair on the other strand of DNA. This is how the double helix structure is formed that resembles a spiral staircase. Each individual will have different sequences of A, T, G, and C in their DNA. There are highly similar and yet unique sequences of DNA that are used to identify humans by looking at the minute differences in their DNA. In this exercise, you will use several techniques to figure out if the DNA in any of the three samples matches up with the DNA of the unknown sample. In this exercise, you will use several techniques to figure out if the DNA in any of the three samples matches up with the unknown DNA sample.

We are very different from each other in many ways, but not as much as you might think on the genetic level. Our genome consists of over 3 billion base pairs, and yet the genetic makeup from one person to the next may differ by as little as 0.1%. Evidently, that 0.1% still makes a huge difference. Organisms need to be able to differentiate their species from that of closely related species; increasing genetic diversity is what makes that possible for them. Genetic diversity is the driving force behind speciation in any population of organisms. In the 1700's, Swedish scientist Carl Linnaeus devised his hierarchical classification system for naming organisms. The Linnaean system uses seven different tiers of classification in order to properly name every species because the genetic diversity on earth is so great. The differences between these genomes underlie the theory behind DNA profiling. There are a number of specific regions in our genomes that vary reliably between individuals. For this experiment, the focus will be on a "Variable Number Tandem Repeat (VNTR)" region. Throughout the genome there are segments that feature small repeating sequences of DNA. A repeated sequence is generally the same

between individuals, but the number of times it repeats can vary. By analyzing enough of the VNTR segments, a genetic “fingerprint” for an individual can be generated.

Genomes contain many random insertions by short repetitive interspersed elements (SINEs), and long repetitive interspersed elements (LINEs). Those elements have become randomly inserted within our genome, mostly in introns, over millions of years. VNTR and Alu elements are the most common.

Polymerase Chain Reaction (PCR) technology is a powerful tool to examine and compare genetic variations.

Full Protocol

Lab Setting

Materials are enough for 6 groups.

Preparation

1. Set up and program the thermal (see below).
2. Thaw 2x PCR Master Mix on ice.

Reagent Preparation

Refer to "Notes for Instructors – Preparation for PCR" on Page 2.

Pre-Experiment Observations

1. Describe the samples of DNA (physical properties: color, viscosity, etc.). Can you see the DNA?
2. Is there any observable difference between the samples of DNA?
3. Describe the appearance of the 2X PCR Master Mix? Can you see the enzymes?

PCR Reaction

Keep the 2X PCR Master Mix and all samples on ice when not in use.

1. *Wear gloves and handle solutions carefully.* Spin the "MM" tube for 10sec, vortex for 10sec, then spin for another 10sec.
2. **Using a NEW TIP for each sample**, pipette 10 µL of the 2X PCR Master Mix, (containing Taq DNA polymerase, nucleotides, primers, and PCR reaction buffer) into the sample tubes "A, B, C, and U," already containing 10 µL of each DNA and the "N" tube if your team received one.

PCR Reaction Mixtures			
DNA Samples		2X PCR Master Mix	Total Reaction Volume
Unknown DNA [U]	10 µL	10 µL	20 µL
Sample A [A]	10 µL	10 µL	20 µL
Sample B [B]	10 µL	10 µL	20 µL
Sample C [C]	10 µL	10 µL	20 µL
Negative control [N] (3 reactions)	10 µL	10 µL	20 µL

3. Pipette up and down carefully to mix well. Tightly cap each tube. Alternatively, mix the components by gently flicking the tubes with your finger. Arrange the tubes in a microcentrifuge and spin for 5 seconds to force all liquid to the bottom of the tubes. (Be sure the tubes are in a BALANCED arrangement in the rotor).

NOTE: If the teacher did not pre-aliquot samples, add the DNA samples THEN the master mix (enzyme) to your microcentrifuge tubes, changing tips each time.

4. Store the samples on ice until they are ready to be loaded into the thermal cycler.

PCR Parameters

Program the thermal cycler as follows:

1. 94ºC – 30 seconds
2. 94ºC denaturing – 20 seconds}
3. 58ºC annealing – 20 seconds} **repeat steps 2, 3, & 4 for 35 cycles**
4. 68ºC extension – 30 seconds}
5. 68ºC – 5 minutes
6. 4ºC – finished / hold

STOPPING POINT – For classes with shorter time periods, the PCR samples should be stored at 4ºC until the next lab period.

Agarose Gel Electrophoresis

General Procedure, detailed directions as given by instructor

1. Prepare 1% agarose.
2. Set up electrophoresis apparatus and pour in the 1% molten agarose with DNA dye for gelation.
3. For staining, use a DNA dye which is added directly to the molten agarose. For light sensitive dyes, keep the gel in the dark during gelation, either by performing in a dark room or placing a box over the gel.
4. Use at least 10 µL of PCR product to visualize results by electrophoresis on agarose gel. If gel well volume will accommodate more than 10 µL, a higher volume is preferred. Loading dye has already been added to the sample to ensure that the sample will sink to the bottom of the well and properly enter the agarose gel.
5. Run at ~100V for ~20 minutes and stop before loading dye has run off gel. Depending on the DNA dye used, caution may need to be taken to reduce exposure of gel to light.
6. Visualize under UV light exposure and record the results manually or by photography.

Suggested Gel Setup: Run 3 gels with 10 wells, 2 teams/gel.

Lane 1-4:	Team "1" samples A, B, C, and U up to 20 µL each.
Lane 5	10 µL DNA Ladder

Lane 6:	10 µL Negative Control
Lane 7-10:	Team "2" samples A, B, C, and U up to 20 µL each.

Results and Discussion

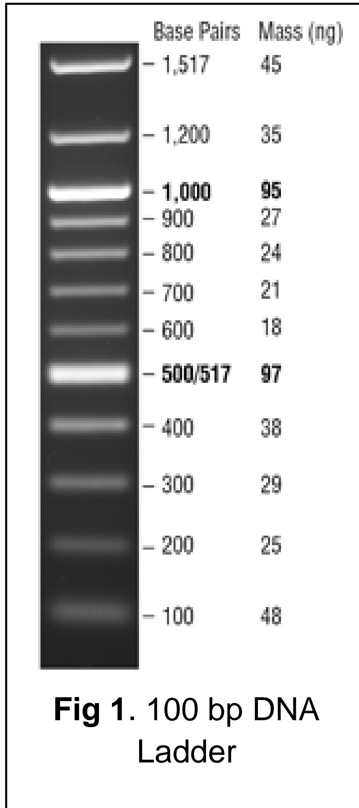

Fig 1. 100 bp DNA Ladder

Observe the bands visible in your samples on the gel. Recall which lanes contained the samples and which contained the unknown DNA sample. Do any of the samples match the unknown DNA sample on the gel?

Looking at the bands in relation to one another is quick and useful. What would be a more accurate way to infer band size and their distance traveled?

Compare the results from your gel with those of the other teams. Describe the similarities and differences.

Summarize the process of PCR using the correct terminology.

Describe a new experiment you could perform using PCR and DNA agarose gel electrophoresis.

Final Conclusions

1. Is there evidence that the stomach sample matches a known local food source?

2. Describe the evidence that supports your accusation.

3. Is it possible that the identified food source is not being eaten by the shark? Justify your explanation.

4. What further tests could be performed to support your claim?

5. What are some other ways marine biologists may use PCR in the identification of sharks?

6. What are some common shark food sources in your state or nearest coastal state? In the US? (Cite your sources).

GenoSensor Marine Biology: Shark Week Kit II: Background

Introduction to PCR

In 1983, during his time at Cetus Corporation, Kary Mullis developed a technique that significantly changed the field of genetics and that of all other biological sciences. This revolutionary process was termed “polymerase chain reaction,” or PCR. By 1993, he had earned the Nobel Prize in Chemistry for PCR. His new technique enabled researchers in numerous fields of biology, to easily and rapidly amplify DNA. Before that, amplification of DNA was extremely difficult and time consuming. Now, in the 21st century, it's not just research scientists who use this molecular biology technique. PCR has applications in a wide variety of areas including: gene detection and mapping, whole genome sequencing, analysis of gene expression, forensics, criminal justice, clinical diagnostics, pharmacogenomics, and dozens of others. Nearly every one of these applications were impossible prior to the implementation of PCR. Besides the initial investment in specialized machinery, the cost of performing PCR is relatively low, and the process is simple enough that nearly anyone can do it and get successful results every time!

PCR: The Birth of Recombinant DNA Technology

PCR uses specific nucleotide sequences, named primers, to amplify segments of a genome from a very small amount of starting material, referred to as the template. DNA can be extracted and isolated from almost any cell type i.e., bacterium, blood cells, tissue cells, hair cells, cheek cells, etc. After proper treatment, with PCR, millions of copies of nearly any desired DNA sequence can be produced. The power of PCR is its specificity. PCR uses unique primers to target just the desired sequence of DNA out of the entire genome and amplifies only that segment with little error.

The basic components of PCR:

- Reaction Buffer
- DNA nucleotides (dNTP’s) of adenine, guanine, thymine, and cytosine
- DNA polymerase
- Forward and reverse DNA oligonucleotide primers
- Template DNA (starting material)

PCR Makes Use of Two Basic Processes in Molecular Genetics

1. **Complementary DNA strand hybridization**

For DNA to be amplified, one must have a known sequence that flanks the gene of interest both upstream and downstream. These sequences are used to create ‘oligonucleotide

primers,' meaning a short ~20 base pair nucleotide sequence which is used as a starting point for DNA replication. The primers are complementary to their target regions so they will anneal (attach) to those regions with great precision. Primers serve the same purpose that runways do for planes trying to lift off the ground. DNA polymerase cannot add nucleotides without a preexisting chain to start from. This process is referred to as primer extension. DNA polymerase recognizes the partially single stranded segment of DNA, attaches itself to the primer just as it normally would during the DNA replication phase of the cell cycle, and proceeds to add complementary nucleotides to fill in the gap.

Complementary strand hybridization occurs when two different oligonucleotide primers anneal to each of their respective complementary base pair sequences on the template. They are designed specifically to anneal at opposite ends of opposite strands of the specific sequence of DNA that is desired to be amplified.

2. DNA strand extension via DNA polymerase

In a PCR, a special type of DNA polymerase is used that is able to function properly and not become denatured during the temperature fluctuation cycles required for thermal cycling. Most mammalian DNA polymerases cannot tolerate the high temperatures and fluctuations from ~60ºC-94ºC. The breakthrough in PCR came with the isolation of DNA polymerase from a thermophilic bacterium known as *Thermus aquaticus*. This bacterial species lives in high temperature steam vents and its DNA polymerase has evolved to withstand the high temperatures of its environment.

During PCR, DNA is synthesized and its quantity doubles after each cycle making the reaction product grow at an exponential rate. In theory, after 30 cycles there will be 2^{30} -over a billion- copies of DNA. Yielding this much DNA allows it to be visualized after only a few simple procedures. One of the easiest and most popular methods of doing this is agarose gel electrophoresis.

Genes and DNA

The human genome contains 23 pairs of chromosomes that contain a total of thirty to fifty thousand protein coding genes. However, those genes only comprise about 5% of the genome, leaving 95% of it to be classified as non-coding DNA. This non-coding DNA is found not only between, but within genes, splitting them into segments. In eukaryotes, non-coding DNA sequences found within genes are known as **introns**. The sequences that do code for proteins are called **exons**. In eukaryotes, genomic DNA is transcribed into RNA molecules in its unmodified form containing both introns and exons from a particular gene. While the RNA is still in the nucleus (before being transported out of the nucleus), the introns (which **int**erfere with the gene product) must be removed from the RNA while the exons (excised from the original transcript)) are spliced together to form the complete messenger RNA sequence which will soon be translated into a protein. This process is called RNA splicing. Some genes may contain a few

introns, others may contain dozens. Interestingly, it is the non-coding ‘junk’ DNA that is useful to us when considering the DNA profile of an individual, rather than the protein coding DNA previously thought to be the most aspect of the genome.

As discussed, functional segments of genes (exons) code for proteins. Proteins are molecules that carry out most cellular functions. Exon sequences are therefore very similar among individuals. That is why when mutations in protein coding genes arise the result is very evident, and is sometime catastrophic. Many metabolic disorders and rare diseases are caused by mutated and nonfunctional proteins). Introns, however, often vary in size and number among individuals. Intron sequences are thought to be the result of the differential accumulation of mutations over time and through evolution are silently passed on to descendants after reproduction. It is differences in the presence and number of intron sequences that allow us to determine the diversity of human genetics. The recognition of these distinctive characteristics in DNA represents the molecular basis from which human identification and population genetics are made possible. Throughout evolution, intron sequences have been the target of random insertions by short repetitive interspersed elements (SINEs), or long repetitive interspersed elements (LINEs). Those elements have become randomly inserted within our introns over millions of years.

PCR Stages

The machinery required to perform PCR is known as a thermal cycler. The thermal cycler enables the steps of PCR to be automated. The reaction involves a repetition of cycles that promote template denaturation, primer annealing, and primer extension by the Taq DNA polymerase. A DNA sample is added to a mixture of the necessary reagents: oligonucleotide primers, thermostable DNA polymerase (Taq), the four nucleotides (A, T, G, C), and reaction buffer. These reagents are pre-mixed as a 2X PCR Master Mix in the GenoSensor Catch a Predator kit II. The tubes are placed into the thermal cycler which contains an aluminum block that holds the samples and can be rapidly heated and cooled by extreme temperature changes in a controlled environment. The rapid heating and cooling of this thermal block is called temperature cycling or thermal cycling.

The first step of the PCR temperature cycling procedure heats the sample to 94°C causing the template strands separate. This process is called **denaturation**.

The thermal cycler then rapidly cools to 60°C allowing the primers to anneal to the separated template strands. This is the **annealing** process. The two original template strands may re-anneal to each other or compete with the primers at the primers' complementary binding sites. However, the primers are added in excess so that the primers may out-compete the original DNA strands for their complementary binding sites.

Lastly, the thermal cycler heats the sample to 72°C (the usual environment temperature for *Thermus aquaticus)* so that Taq DNA polymerase can perform **primer extension** and produce complementary DNA strands of the target sequence. The two resulting new sets of

double-stranded DNA (dsDNA) will be used for the next cycle and proceeding strand synthesis. At this stage, a full temperature cycle (thermal cycle) will have been completed.

Each step takes 30 seconds to 1 minute, and will repeat for 30-40 cycles depending on how the user has programmed the thermal cycler. At the end of the pre-programmed number of cycles, the product is put on hold at 4°C until the user is ready to retrieve the PCR product and analyze its contents.

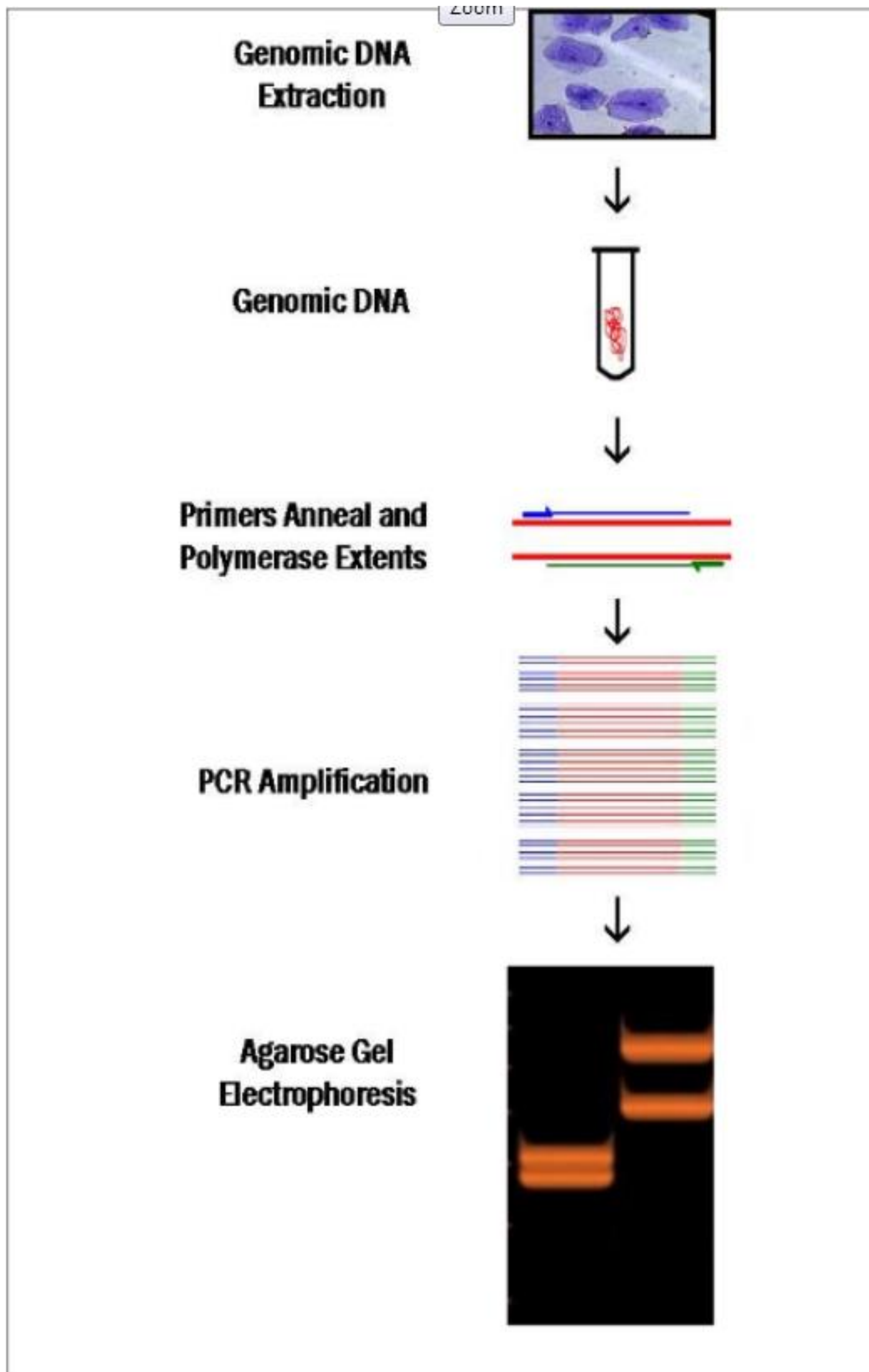

Figure 3. Experiment flowchart from start to finish

Troubleshooting

Symptom	Possible causes	Solutions
No amplification product	Questionable template quality	Analyze starting material
	Inhibitory Substance in reaction	Decrease sample volume
	Insufficient cycle #	Run additional cycles
	Incorrect thermal cycler program	Verify times and temperatures
	Errors in heat block incubation	Calibrate heating block, use sand or water to maximize contact with tube for proper heat transfer
	Contaminated tubes/solutions	Autoclave tubes and use filter tips
	Primer annealing temperature too high	Lower annealing temperature in 2ºC increments
Weak bands/faint signal	Low concentration of DNA template	Make sure enough DNA has been added; see recommended amount of DNA to load into PCR reaction.
	DNA Dye degradation during preparation	Light sensitive dyes should be kept in the dark during gel preparation. Prepare in dark room or place a box over the electrophoresis apparatus during gelation and electrophoresis.
	Expired, contaminated or degraded DNA dye	Verify that the DNA dye has not degraded in storage, been contaminated or expired.
Non-specific amplification product	Premature Taq-polymerase replication	Mix solutions on ice, place rxn directly into 94º thermal cycler
	Primer annealing temperature too low	Raise annealing temperature in 2ºC increments
	Insufficient mixing of reaction solution	Mix solutions thoroughly before beginning the reaction
	Exogenous DNA contamination	-Wear gloves -Use dedicated area for sample preparation -Use non-aerosol tips

Technical Service

For more information or technical assistance, please call, write, fax, or email.

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Limited Warranty

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