

THE BIOTECHNOLOGY
EDUCATION COMPANY®

Edvo-Kit #

314

Edvo-Kit #314

In Search of the Cancer Gene

Experiment Objective:

In this experiment, students will gain an understanding of the effect of mutations in the p53 tumor suppressor gene and its role in familial cancers.

See page 3 for storage instructions.

Table of Contents

	Page
Experiment Components	3
Experiment Requirements	3
Background Information	4
Experiment Procedures	
Experiment Overview	10
Module I: Construction a Family Pedigree	11
Module II-A: RFLP Analysis of DNA Sample	13
Module II-B: Staining Agarose Gels Using FlashBlue™ (OPTIONAL)	17
Module III: Analysis of Chromatograms to Search for P53 Mutations	18
Study Questions	19
Instructor's Guidelines	20
Pre-Lab Preparations	21
Experiment Results and Analysis	23
Study Questions and Answers	26
Appendices	27

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Experiment Components

COMPONENTS

*Store samples A-E in the freezer immediately upon receipt.
All other components can be stored at room temperature.*

- A DNA Standard Marker
- B Control DNA
- C Patient Peripheral Blood DNA
- D Patient Colon Tumor DNA
- E Patient Normal Colon Tissue DNA

CHECK (✓)

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-

**Experiment #314 is
designed for 10 Groups.**

Store samples A-E in the
freezer immediately
upon receipt.

REAGENTS & SUPPLIES

- UltraSpec-Agarose™
- Electrophoresis Buffer (50x)
- Practice Loading Dye
- SYBR® Safe DNA Stain
- FlashBlue™ DNA Stain
- 1 ml pipet
- Microtipped Transfer Pipets
- Automated DNA Sequence Printouts

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All experiment components
are intended for educational
research only. They are not to
be used for diagnostic or drug
purposes, nor administered to
or consumed by humans or
animals.

Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets with tips
- Balance
- Microwave, hot plate or burner
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Small plastic trays or large weigh boats (for gel staining/destaining)
- UV Transilluminator or Blue Light visualization system (for visualizing SYBR® Safe stained gels)
- UV safety goggles (for visualizing SYBR® Safe stained gels using UV)
- White light visualization system (for visualizing FlashBlue™ stained gels)
- Distilled or deionized water
- Computer with Internet access
- Laboratory journal

Background Information

Chances are that you have a personal connection to cancer. Worldwide, cancer is the second leading cause of death, with about 1 in 6 deaths caused by cancer. Many more people have been treated for cancer. This means that the likelihood is high that a friend, family member, or colleague has been diagnosed with cancer at some point. But what is cancer, how does it affect the human body, and how can we test for it?

All cells divide in order to create more cells. In most cases, one parent cell will split into two identical daughter cells. This allows an organism to grow, to develop, and to replace old cells. In order for a cell to divide, it must increase in volume, duplicate its DNA and organelles, and then split all of the cellular components into two separate cells. This sequence of events, called the cell cycle, is tightly controlled so that cells only divide when and where they are supposed to. Cellular checkpoints block the passage of the cell from one phase to the next, preventing cells from dividing when they should not (Figure 1). Only when the cell has successfully completed one step is it allowed to clear the checkpoint and move to the next step of mitosis.

The cell cycle must be carefully regulated to ensure that the cell divides at the right time and in the right place. For example, cells in the epidermis divide frequently to replace the outer layers of skin, whereas mature neurons in the brain divide rarely if at all. Red blood cells, which have ejected their nuclear material, no longer divide; instead, when they are no longer useful, they are broken down and recycled into new cells. When mutations in a cell's DNA change the timing of signals that tell it when to grow and divide, it can multiply in a rapid, unregulated way.

This unregulated, abnormal growth of cells results in a group of diseases called cancer. Over time, the over-abundance of cells forms masses called tumors. Common cancers include skin, lung, breast, and colorectal cancer, each of which has similar characteristics. However, different tissues respond differently, and different gene mutations affect the progression of cancer. As the cells rapidly divide, proofreading during DNA replication becomes sloppy, creating additional mutations in genes. This can result in cancer cells leaving their primary site and invading surrounding tissues where they form satellite tumors in a process called metastasis.

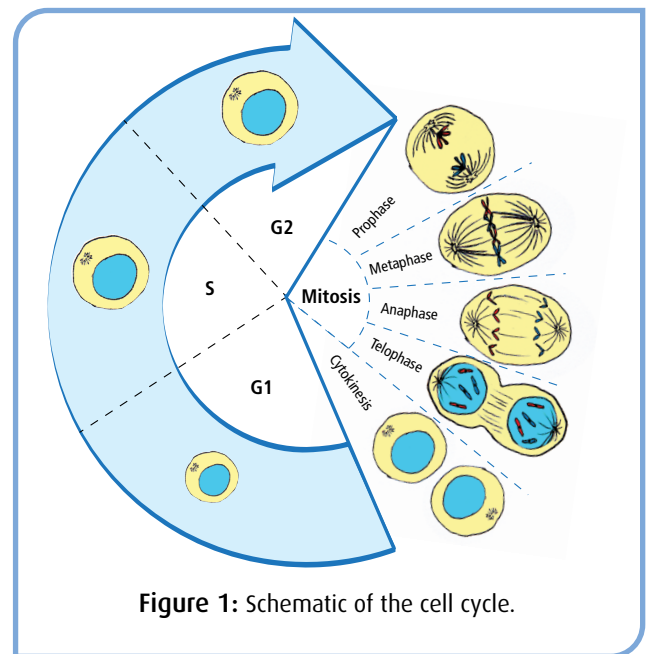


Figure 1: Schematic of the cell cycle.

TUMOR SUPPRESSOR PROTEINS AND ONCOGENES

The vast majority of cancers arise from mutations in genes controlling cellular growth. These mutations change the way proteins function and the way a cell behaves and divides, eventually leading to cancer. There are two main classes of genes that cause cancer:

- 1) **Oncogenes:** In healthy tissues, cells contain DNA that codes for proteins that promote normal cell growth and division. Mutations in the DNA sequence can cause these genes to become active at the wrong time or place. These “gain-of-function” mutations cause the genes to become more active, signaling for the cell to divide uncontrollably. Many of these mutations are dominant, meaning that only one mutant copy of the gene is necessary to cause unrestricted cell

growth. For example, in healthy tissues *MYC* genes code for proteins called transcription factors that turn on genes necessary for cell growth at specific times during development. When the genes are mutated, *MYC* is able to continuously turn on these pro-growth genes, resulting in uncontrolled cell proliferation.

- 2) **Tumor Suppressor Genes:** In healthy cells, tumor suppressor proteins inhibit cell growth and prevent tumor formation. Mutations in these genes remove the barriers preventing uncontrolled cell division. Many of these mutations are recessive, meaning that one mutant copy cannot cause uncontrolled cell growth. To promote cancer, both copies of the gene must be mutated. One example of a tumor suppressor protein is P53, encoded by the *TBP53* gene. P53 is an important protein in tumor suppression, regulating DNA damage repair, arresting cell growth, and initiating cell death in unhealthy cells.

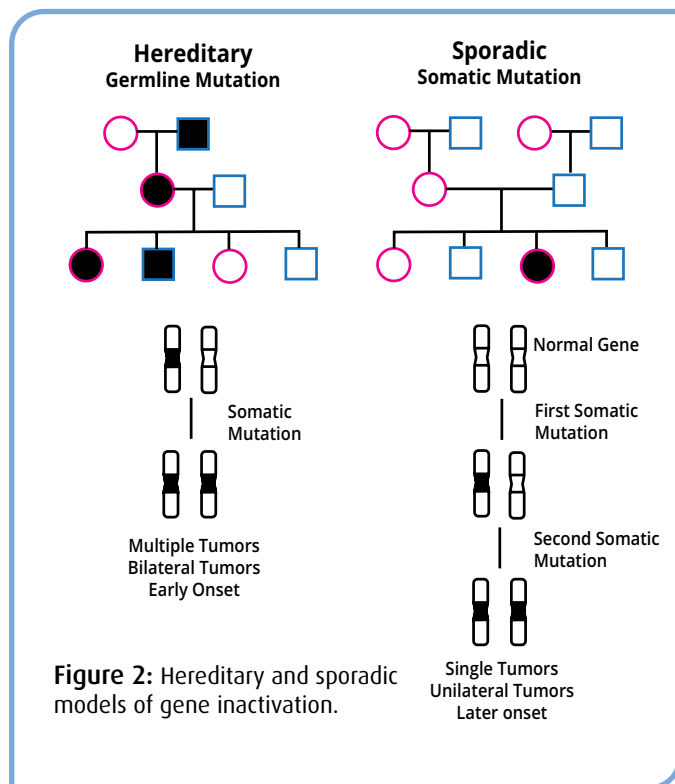
It is important to remember that cancer is not one disease. Even within a single type of cancer, like breast or colorectal cancer, different mutations can lead to the overgrowth of cells. Furthermore, it is rare for cancer cells to contain only a single mutation. In fact, initial mutations in pre-cancerous cells can destabilize the genome, leading to further mutations in that cell and its daughter cells. Cancer cells will often contain one or more oncogenes combined with the inactivation of an important tumor suppressor gene. Since cancer changes the nature of the cells, these mutations can be identified using modern molecular biology techniques.

MOLECULAR ANALYSIS OF INHERITED CANCER GENES

The vast majority of cancers result from acquired (somatic) mutations, which are changes in the DNA sequence that happen during a person's lifetime. These changes occur either through exposure to mutagens that change the DNA sequence or through errors in DNA replication. Now, not every change in a somatic cell's DNA (i.e., non-germ cells) may cause cancer; the changes may be in non-coding DNA, or they may be recessive to a functioning copy of the gene. But since these mutations occur in the body's cells, and not the germ cells, they cannot be passed to an individual's progeny so there is no direct genetic link between family members. In contrast, germline mutations are directly inherited through DNA changes that can be passed from one generation to the next as they are present in the gametes. As a result, they appear more frequently among family members. They can be tracked by creating a diagram that describes the relationships between family members, called a familial pedigree. Overall, familial cancers constitute a very small fraction of the total reported cancers. Pedigrees for both somatic and inherited cancers appear in Figure 2.

Some of the first cancer genes identified by researchers are responsible for inherited cancers. For example:

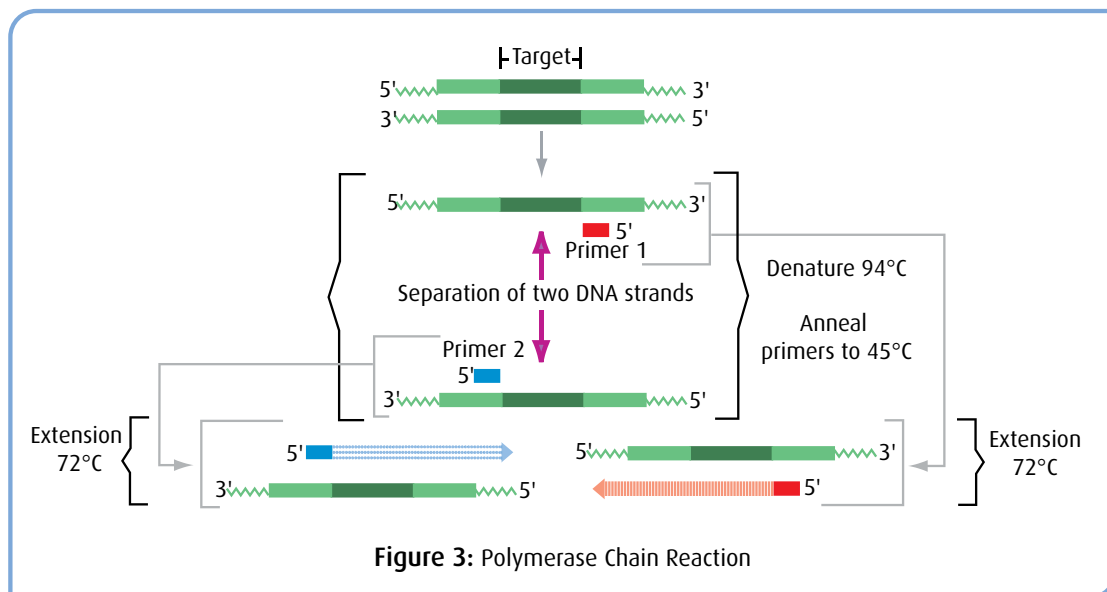
- **The retinoblastoma (*RB*) Gene:** a tumor suppressor gene that slows cell cycle progression. Mutations in this gene result in childhood cancer of the eye.
- **The Wilms' Tumor 1 Susceptibility (*WT1*) Gene:** a tumor suppressor gene that turns on genes necessary for development of the urogenital system. Mutations in this gene result in nephroblastoma, a cancer of the kidneys.



- **The Neurofibromatosis Type II (*Merlin*) Gene:** a tumor suppressor gene that codes for a protein that links the cytoskeleton to itself and to the cell membrane. Cells with mutations in this gene ignore contact inhibition when growing; that is, the signals that tell cells to stop dividing when they are touching other cells.
- **Li-Fraumeni Syndrome (*P53* gene):** a tumor suppressor gene that acts as a master regulator for the genes responsible for cell division and death. This gene is often called the “Guardian of the Genome” as it plays a major role in preventing changes to the genomic DNA. Just a single mutation in the gene greatly increases a person’s susceptibility to cancer. Not surprisingly, p53 is the most commonly mutated gene in cancer cells.

By studying inherited cancers, cancer biologists can search for genes that are critical for normal cell development and carcinogenesis. Using molecular biology techniques, researchers developed gene maps that describe the chromosomal locations of genes. These maps are used as tools for the identification of cancer-causing mutations for both inherited and non-inherited cancers. To create these maps, researchers isolate cancer cell DNA and analyze point mutations in hot spot areas in cancer-related genes, such as p53. Cancer genomes, or the whole DNA sequence from cancer cells, can be analyzed using techniques like Restriction Fragment Length Polymorphism (RFLP) analysis and DNA sequence analysis by chain termination.

For **RFLP analysis**, researchers first amplify DNA using the polymerase chain reaction (PCR), a biotechnology technique that quickly and accurately creates large amounts of DNA *in vitro* in a process similar to replication in a cell’s nucleus. The DNA samples are heated, which separates the two strands of the DNA duplex. Short, synthetic DNA primers, designed by researchers to target a specific sequence, bind to the DNA. This directs the DNA-building enzyme *Taq* DNA polymerase to build new DNA, starting at the primer location and continuing along the sequence. After the initial elongation, the sample is again heated to denature the newly formed DNA duplex, then cooled to allow primer binding and extension to happen again (Figure 3). Each time the sample cycles through the different temperatures, the amount of DNA doubles. This simple cycle – anneal, extend, denature – is the basis of PCR. By repeating this sequence of heating and cooling many times, billions of copies of a specific DNA sequence are produced in a matter of minutes. After amplification, the DNA is digested with restriction enzymes, which cut double-stranded DNA in a sequence-specific manner. The fragments are analyzed using agarose gel electrophoresis, a technique that separates DNA into discrete bands based on their molecular size.



DNA sequence analysis by chain termination (or Sanger sequencing) allows researchers to specifically target and sequence nucleotides from a specific location within a strand of DNA. As with PCR, genes are targeted using a short, synthetic DNA primer that base pairs with a specific DNA sequence. The sample to be sequenced is combined with the primer, the DNA-building enzyme DNA Polymerase I, and a blend of nucleotides. This mixture includes a high concentration of deoxynucleotides (dNTPs) and a low concentration of dideoxynucleotides (ddNTPs) (Figure 4A). During the sequencing reaction, DNA Pol I reads the DNA template and adds nucleotides to the primer to build a complementary strand of DNA. Most times, the polymerase will add a dNTP to the growing nucleotide chain. However, when DNA Pol I adds a ddNTP to the DNA strand, it is impossible for the polymerase to add another nucleotide to the end of the growing strand. This is because the ddNTPs lack the 3' hydroxyl group that DNA Pol I uses to link nucleotides to the growing DNA chain (Figure 4B). This stops the reaction, creating a series of DNA fragments of differing sizes. The site of the dideoxynucleotide incorporation allows one to determine the position of that base. The shortest fragments result from DNA strands that terminated near the primer, whereas longer fragments had more dNTPs linked to the growing DNA chain before the incorporation of the ddNTP (Figure 4B).

To read the sequence, the DNA fragments are separated by capillary electrophoresis (Figures 4B, 4C). Automated machines separate DNA fragments through a polyacrylamide gel formed in a thin capillary tube. Importantly, each ddNTP is labeled with a different fluorescent marker, allowing the sequence of a particular DNA strand to be "read" by a laser that is focused on the capillary (Figure 4C). As the DNA fragments pass through the gel matrix, the laser excites the fluorophores on the DNA. The four different ddNTP fluorescence colors are then automatically detected and the fluorescence intensity translated into a data "peak" that represents the order of nucleotides in the

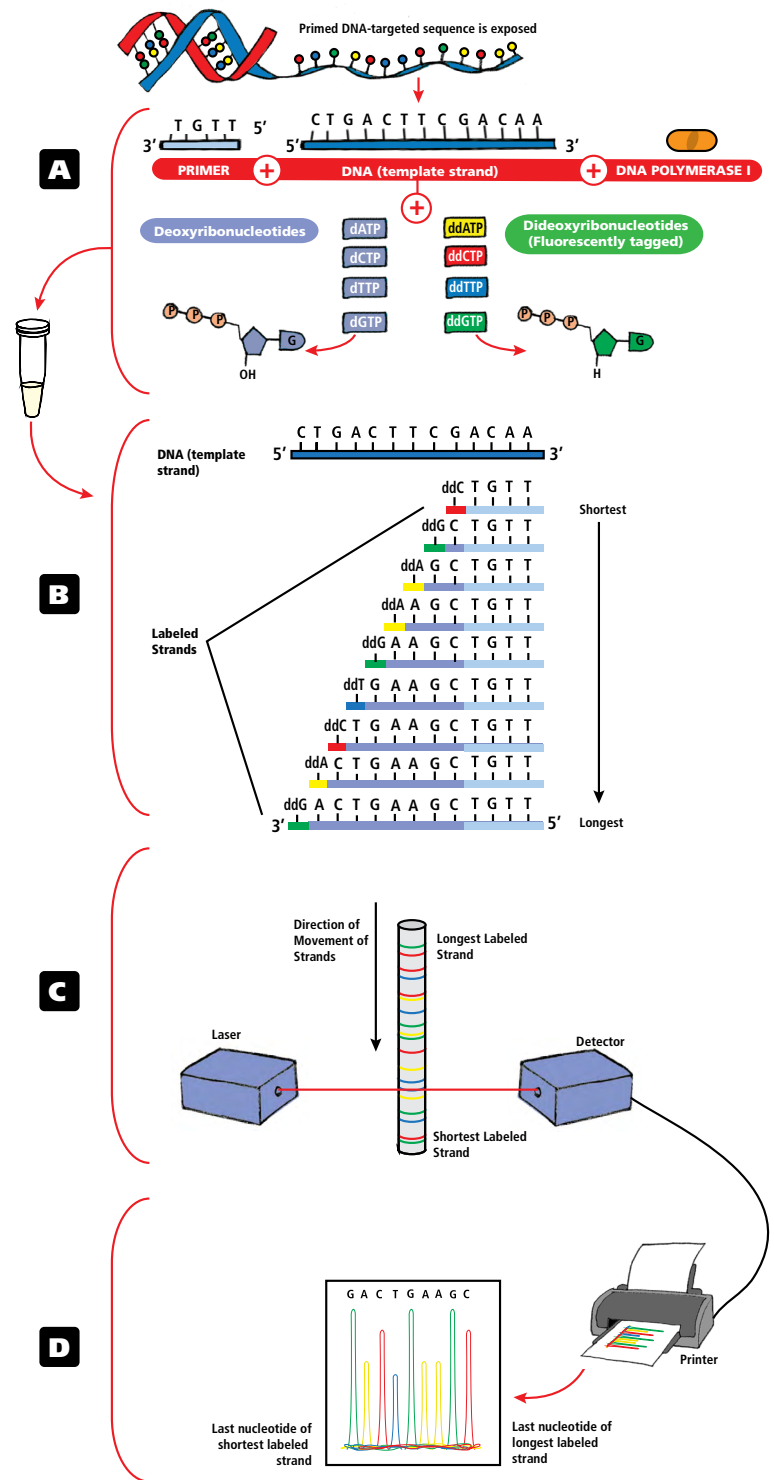


Figure 4: Sanger DNA Sequencing.

- Setting up the sequencing reaction.
- Incorporation of the ddNTPs create different size DNA fragments.
- The labeled mixture is sequenced using capillary gel electrophoresis. A laser detects the fluorescent label on each of the ddNTPs.
- The information is analyzed using a computer.

template DNA (Figure 4D). These high-throughput machines can provide fast, high-quality sequences for a fraction of the cost of traditional sequencing strategies.

LI-FRAUMENI SYNDROME (LFS) AND P53 MUTATIONS

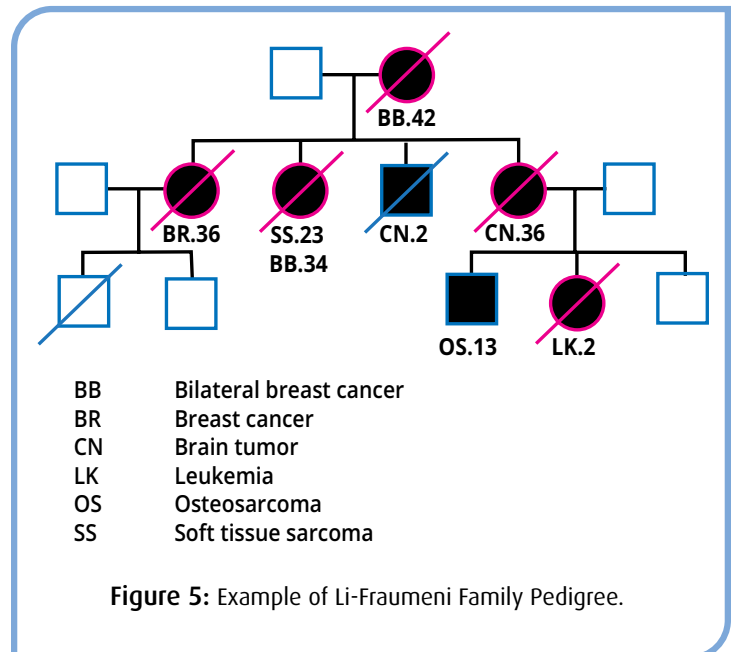
Two physicians, Frederick Pei Li and Joseph F. Fraumeni, Jr. first described a rare syndrome in 1969 after identifying 24 families with a history of childhood sarcomas, an uncommon bone and connective tissue cancer. Further analysis showed more than 50% of the family members were susceptible to cancers that include brain, colorectal, breast cancers and leukemias. To study the heredity of the syndrome, researchers took detailed patient histories and created family pedigrees tracing the occurrences of cancer back through several generations (Figure 5). After careful analysis of the family pedigrees, researchers concluded that this syndrome followed a dominant inheritance pattern. Using the data, Li and Fraumeni established specific guidelines for diagnosis of this familial cancer. All three of these guidelines must be met before LFS can be diagnosed.

- A tumor (sarcoma or other cancer common in Li-Fraumeni Syndrome patients) diagnosed before the patient is 45.
- One immediate relative (parents, siblings, or children) diagnosed with cancer before the age of 45.
- A second relative, either immediate or second-degree (grandparents/grandchildren, aunts/uncles, or nieces/nephews) diagnosed with cancer before the age of 45.

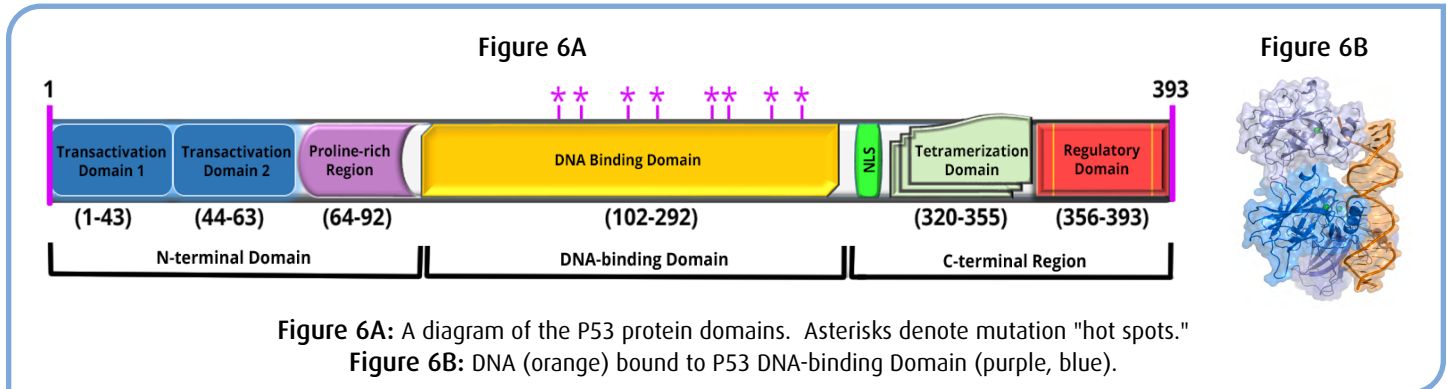
With the advent of DNA sequencing in the 1990's, researchers were able to link this condition, now called **Li-Fraumeni Syndrome** (or **LFS**) to the *P53* gene. The data showed that individuals with LFS have inherited one wildtype *p53* allele and one mutated allele. This means that a single somatic mutation within the gene will result in the inactivation of both alleles. In contrast, individuals with two wildtype copies of the *P53* gene require two sequential mutations to initiate tumors. This model is referred to as the "two-hit" hypothesis.

Cancer biologists have extensively studied and mapped the DNA sequence changes in *P53* that cause Li-Fraumeni syndrome and other cancers and found that over 50% of cancer genomes have mutations in this gene. Homozygous loss of *P53*, where mutations have arisen in both copies of the gene, is found in 65% of colon cancers, 30-50% of breast cancers, and 50% of lung cancers. Due to the prevalence of *P53* mutations, and the importance of the gene in driving cancer progression, it has become one of the most well studied cancer genes. Because of *P53*'s role as a master regulator in cell division and cell death, both the DNA and protein have become the focus of many cancer biology studies.

The gene for the *P53* protein is located on the short arm of chromosome 17. It was initially identified as a 53 kilodalton (kDa) protein by SDS-PAGE, hence the name *P53* (p for protein, 53 for the apparent molecular weight). After the sequence was determined, it was discovered that the protein's molecular weight was actually 43.7 kDa; the discrepancy in size came from the large number of prolines in the protein sequence which affected its migration through the gel. In the cell, *P53* regulates cell division and cell death through its function as a transcriptional regulator. Specific amino acids in the protein (called residues) allow it to bind to specific DNA sites. By binding the DNA, *P53* activates the transcriptions of genes necessary for cell division and cell death.



The P53 protein can be divided into three main domains (Figure 6A). The N-terminal region of the protein contains the transcriptional activation domain (TAD), which binds to and activates transcription factors. The central region of the protein is the DNA-binding domain (DBD), which binds to a specific promoter sequence to activate gene transcription. Sequences within this region are also responsible for binding to important co-factors. The C-terminal region of P53 is the most complex section of the protein. Oligomerization domains within this region allow P53 to bind to itself, and nuclear localization sequences (NLSs) allow the protein to be transported the cell's nucleus.



The majority of critical mutations are located within the central region of P53, which is where the protein binds DNA and transcriptional co-factors (Figure 6B). In human cancer genomes, mutations are detected in high frequency in these "hot spots"; in fact, nearly 95% of P53 mutations occur within this region. By changing the DNA sequence, the amino acid sequence changes, and P53 loses its ability to function. Examples of DNA hot spots that change the p53 protein sequence are found in the codons for residues 165 and 175 in exon 5, residues 196 and 213 in exon 6, residues 245 and 248 in exon 7, and residues 273 and 282 in exon 8. These mutations can change the conformation of the P53 protein, affect its ability to bind to DNA, and to increase the stability of the protein. Most mutations are recessive to the wild-type gene, meaning that for a tumor suppressor gene such as p53 to play a role in transformation in cancer, both alleles need to be altered. However, once both alleles have mutated, cells can grow uncontrolled.

Today, diagnosis of LFS includes sequencing to determine whether an individual has mutations in one or both copies of the *P53* gene, and where those mutations are located. Family pedigrees are constructed to identify other relatives who may have LFS. Under the guidance of their doctors, patients with LFS will be regularly screened for common cancers, including leukemia, brain, breast, and colorectal cancer. Furthermore, genetic counselors advise affected individuals about their disease and counsel them for any emotional distress that may arise from the diagnosis.

IMAGE CREDITS:

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Figure 6 (right) - RaihaT, CC BY-SA 3.0 <<https://creativecommons.org/licenses/by-sa/3.0/>>, via Wikimedia Commons.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will gain an understanding of the effect of mutations in the p53 tumor suppressor gene and its role in familial cancers.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. Exercise caution when using any electrical equipment in the laboratory.
4. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.



LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

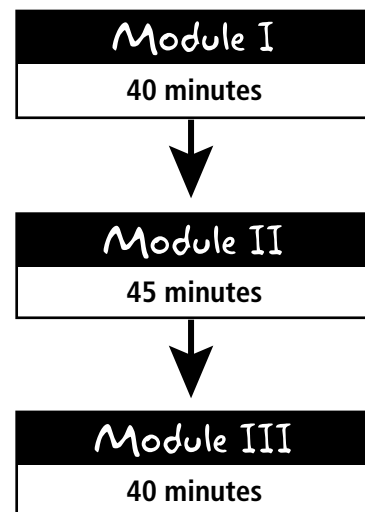
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.



Module I: Construction a Family Pedigree

The first part of the experiment is based on the information made available as part of a diagnosis by the family physician and the oncologist. The pedigree information that you will develop is for a young woman who is suspected to have the Li-Fraumeni syndrome. A first step in the search and assignment of Li-Fraumeni syndrome is to sequence the patient's DNA and establish their family pedigree.

DIAGNOSING COLON CANCER

Colorectal cancer is the second most deadly form of cancer (behind lung cancer), yet if detected early the survival rate is very high. Most colorectal cancers start as small, benign (non-cancerous) growths of cells on the inner lining (mucosa) of the large intestine, specifically the colon and the rectum. These clusters of cells, called polyps, are very common and are not necessarily pre-cancerous. Polyps can remain in the colon, unchanged and benign, for many years. In some cases, cancerous cells can form within a polyp, and the growths will begin to invade the surrounding tissue. At this point, doctors would remove and evaluate the polyps to determine the medical treatment. This could include surgery to remove polyps and regular colonoscopies, chemotherapy or radiation therapy, or even removal of the bowel and colon, depending on how advanced the cancer is.

Early symptoms of colorectal cancer tend to be mild, if noticeable. People may notice a change in bowel habits, a feeling of fullness in the bowel, or gastrointestinal bleeding which causes blood in the stool. Many times, patients will have weakness or fatigue from low red blood counts resulting from the undetected blood loss.

Risk factors for colorectal cancer include age, weight, level of activity, and diet. Furthermore, there is a genetic risk – it is estimated that 5-10% of colorectal cancers are inherited. When researchers analyzed genomic DNA from colorectal tumor cells, they found that mutations in the P53 gene were the second most common sequence changes. In colorectal cancers, high-frequency mutations occur in the DNA sequence that affect residues 245 and 248 in the P53 protein. Due to the mutations, individuals with Li-Fraumeni syndrome have an increased risk of developing colorectal cancer.

DIAGNOSING FAMILIAL CANCERS USING PEDIGREE ANALYSIS

At her first colonoscopy, doctors identified several polyps in Valerie Brown's colon (age 44). The polyps were biopsied, meaning that small samples of tissues were removed for closer examination by doctors. Upon closer examination, the polyps were shown to be neoplastic, meaning that they were precancerous.

Valerie was concerned because she knew that her mother had colon cancer when she was in her late thirties. She made an appointment with her physician, who referred her to a specialist at a local cancer center, where she was diagnosed as having colon cancer. As part of the medical work-up, the oncologist inquired about her family history of cancer.

Upon consultation with her family, Valerie learned that her father and his family appeared to be free of cancer. However, in Valerie's mother's family, several family members had been diagnosed with different types of cancer. Given the high incidence of cancer in the family, the genetic counselor decided to chart the family's pedigree to determine whether there was a heredity component to the cancers.









Module I: Construction a Family Pedigree, continued

With the information given below, chart the family pedigree:

- Her mother, Diane, was diagnosed and treated for colon cancer at the age of 39.
- Valerie did not know that Diane had a sister, Mabel, who died at age 2 of a brain tumor.
- Diane's brother, James (age 40), underwent surgery, followed by chemotherapy for colon cancer.
- Her maternal grandmother, Elsie, died at age 42 from bilateral breast cancer.
- Her maternal grandfather, Elmer, was free of cancer and is 88 years old.
- Her maternal cousin, Patrick (son of James), died of brain cancer at 14.
- Her cousin (Patrick's sister), Jane, was diagnosed with childhood leukemia and subsequently died at age 2.
- Patrick's two other brothers, Richard (age 28) and Curtis (age 30), are in good health and free of cancer.
- Valerie's sister, Nancy (age 38), is free of cancer.
- Nancy's son, Michael, was diagnosed at age 3 as having sarcoma. Recently, at age 18, he was diagnosed as having osteosarcoma.
- Nancy's other son, John (age 16), and daughter, Jessica (age 8), are free of cancer.
- Valerie has five children, none have been diagnosed with cancer: Justin (male, age 16), Sheila (female, age 14), Robert (male, age 10), Angela (female, age 8), Anthony (male, age 6)

When drawing a family pedigree, the following are general guidelines to the symbols used and their representations:

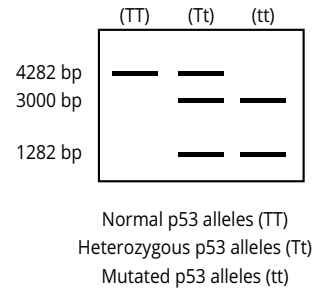
- A circle represents a female.
- A square represents a male.
- A shaded circle or square refers to a person having some form of cancer (affected).
- An open (non-shaded) square or circle represents a person who is free of cancer (unaffected).
- A circle or square (either shaded or open) with a diagonal slash through it represents a person who is deceased.
- A half shaded circle or square represents a carrier for the cancer gene who may get cancer in the future.

Female	Male	
		Unaffected
		Affected
		Deceased
		Carrier

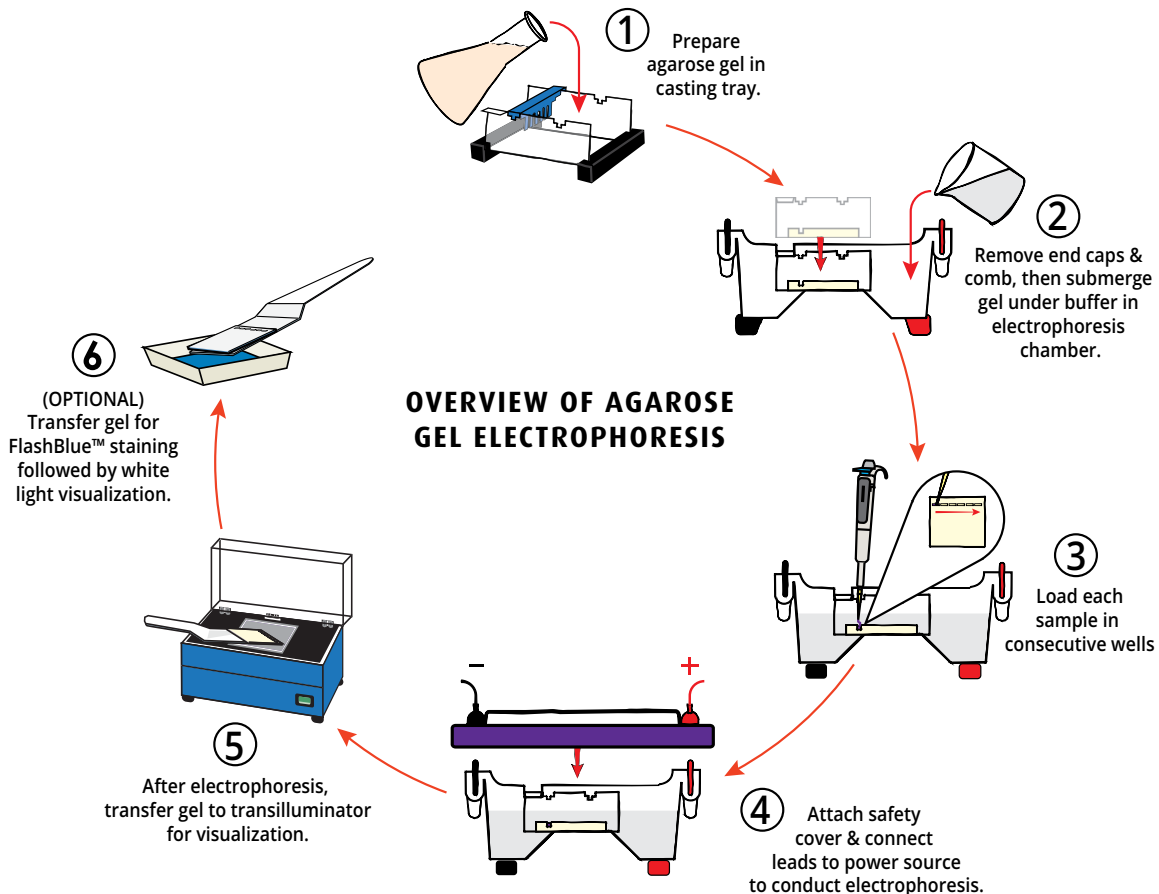
- 1) Using the family pedigree, do you believe that there is a genetic component to Valerie's cancer?
- 2) Does Valerie meet the required criteria for LFS (below)? Why or why not?
 - A tumor (sarcoma or other cancer common in LFS patients) diagnosed before the patient is 45.
 - One immediate relative (parents, siblings, or children) diagnosed with cancer before the age of 45.
 - A second relative, either immediate or second-degree (grandparents/grandchildren, aunts/uncles, or nieces/nephews) diagnosed with cancer before the age of 45.

Module II Overview

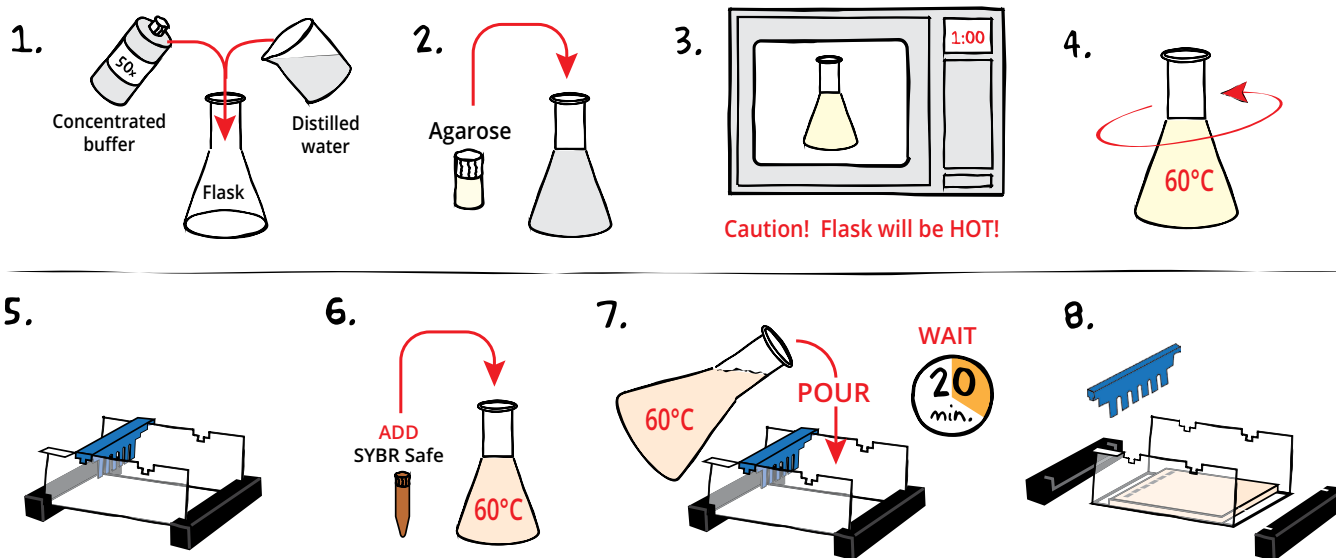
After building a family pedigree analyzing it using the LFS criteria, there is strong evidence that Valerie has Li-Fraumeni Syndrome. In such a case, a secondary diagnostic test is normally conducted in the form of DNA analysis. Doctors collect a blood sample, a tumor sample, and a sample from healthy tissue near the tumor for RFLP analysis of the P53 gene. DNA is extracted from the patient samples and a region the P53 gene is amplified using PCR. The PCR sample is then digested using a restriction enzyme that recognizes the mutant sequence that changes the hotspot codon at position 248 from Arg (CGG) to Trp (TGG), a high-frequency mutation found in colon cancer samples. The change in the DNA sequence allows the restriction enzyme *BsrI* to selectively digest the mutant sequence at the sequence ACTGGN, where N can be any base. The normal DNA sequence at this location cannot be cut by this enzyme because it reads ACCGGN, which is not recognized by the enzyme.



In this module, you will perform agarose gel electrophoresis on Valerie's simulated samples. After amplification by PCR, the *BsrI* restriction enzyme was used to digest all three of Valerie's DNA samples, together with a wild-type control that does not have the DNA mutation. The samples will be analyzed using agarose gel electrophoresis, a biotechnology technique that uses electricity and a porous gel matrix to separate DNA fragments by size. If Valerie is positive for Li-Fraumeni syndrome, the DNA obtained from blood lymphocytes and the healthy tissue will show a pattern representing one normal allele and the second which is the mutant. If the mutation in the tumor cells occurred in the P53 gene, DNA analysis from the tumor tissue samples would only show the pattern representing the two mutant alleles.



Module II-A: RFLP Analysis of Tumor Samples



NOTE: If you are casting your own gels, review the following instructions. If you are using pre-cast gels, proceed to Step 8.

- DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
- MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
- DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
- COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
- While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch. **NOTE: This experiment requires 6 wells.**
- Before casting the gel, **ADD diluted SYBR® Safe** to the molten agarose and swirl to mix (see Table A).
- POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
- REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.



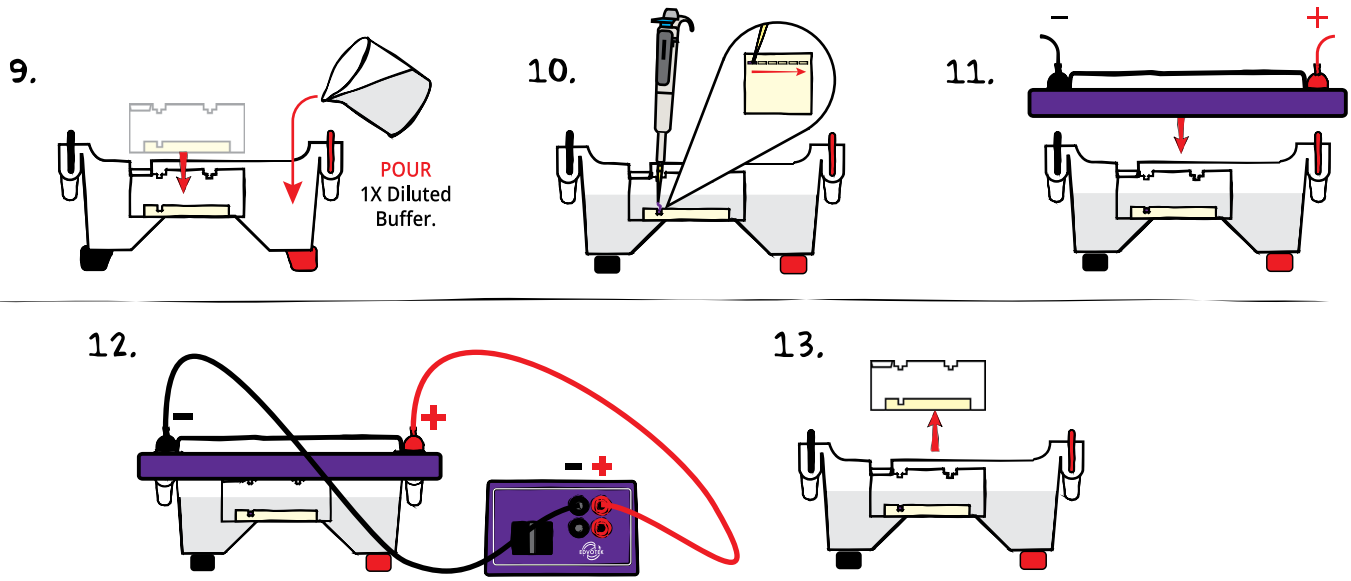
Reminder:
This experiment requires 0.8% agarose gels cast with 6 wells.

Size of Gel Casting tray	Concentrated Buffer (50x)	Distilled Water	Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.6 mL	29.4 mL	0.23 g	30 mL	30 µL
10 x 7 cm*	1.0 mL	49.0 mL	0.39 g	50 mL	50 µL
14 x 7 cm	1.2 mL	58.8 mL	0.46 g	60 mL	60 µL

* Recommended gel volume for the EDGE™ Integrated Electrophoresis System.



Module II-A: RFLP Analysis of Tumor Samples, continued



9. **PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **LOAD** 18-20 µL of each sample into the well in the order indicated by Table 1.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

Reminder:

Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

Table 1: Gel Loading

Tube	Lane	Sample
Tube A	Lane 1	DNA Standard Markers
Tube B	Lane 2	Control DNA
Tube C	Lane 3	Patient Peripheral Blood DNA
Tube D	Lane 4	Patient Colon Tumor DNA
Tube E	Lane 5	Patient Normal Colon Tissue DNA

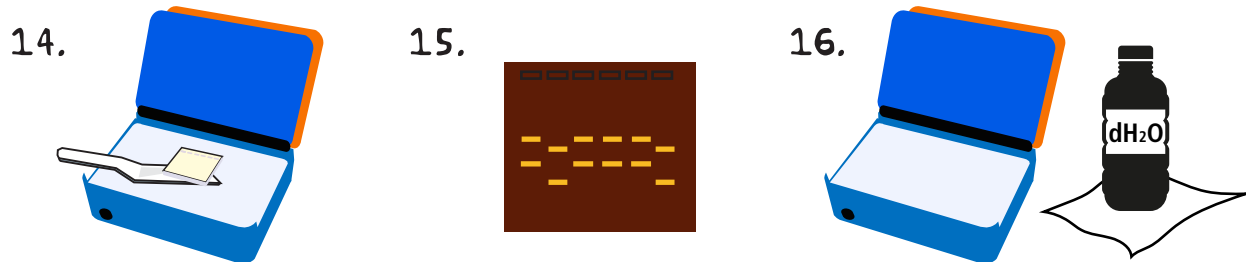
Table B
1x Electrophoresis Buffer (Chamber Buffer)

EDVOTEK Model #	Total Volume Required	Dilution	
		50x Conc. Buffer	+ Distilled Water
EDGE	300 mL	6 mL	294 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C
Time and Voltage Guidelines (0.8% Agarose Gel)

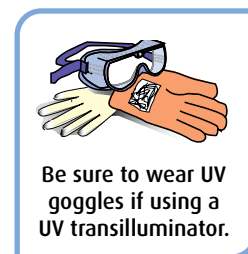
Volts	Electrophoresis Model	
	EDGE	M12 & M36
	Min. / Max.	Min. / Max.
150	10 / 15 min.	20 / 35 min.
125	N/A	30 / 45 min.
100	15 / 25 min.	40 / 60 min.

Module II-A: RFLP Analysis of Tumor Samples, continued



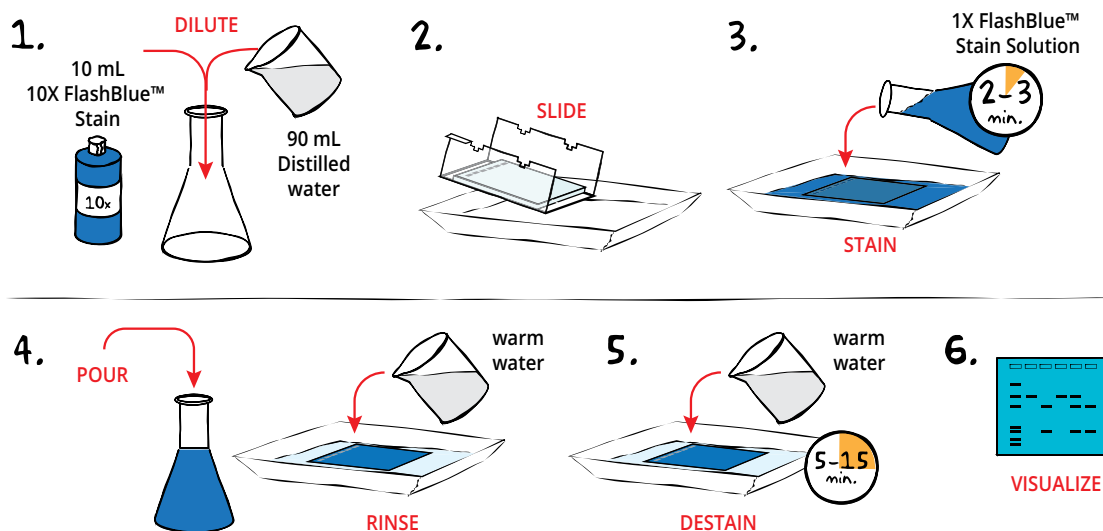
VISUALIZING THE SYBR® GEL

14. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.
15. **PHOTOGRAPH** results.
16. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.



Module II-B: Staining Agarose Gels Using FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. *IF staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.*



- DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
- REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
- COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
- POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45°C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
- COVER** the gel with clean, warm water (40-45°C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
- Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.



Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

- DILUTE** 1 mL of 10X FlashBlue™ stain with 149 mL distilled water.
- COVER** the gel with diluted FlashBlue™ stain.
- SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
- Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Module III: Analysis of Chromatograms to Search for P53 Mutations

Like RFLP analysis, DNA sequencing can be used to analyze the P53 gene. This technique "reads" every base in the targeted gene (for a refresher, see page 7), allowing us to identify mutations in DNA hot spots.

Valerie has five children. None of the children show signs of cancer at this time. Valerie has requested that DNA sequencing be performed for each of her children. This genetic test will allow Valerie's family to identify which children may also carry the mutant copy of the P53 gene, resulting in LFS. The results of this test will allow Valerie and her family, along with their medical team, to determine the correct course of action. This includes both preventative measures, genetic counselling, and regular cancer screenings.

1. Justin (male, age 16)
2. Sheila (female, age 14)
3. Robert (male, age 10)
4. Angela (female, age 8)
5. Anthony (male, age 6)

In this part of the experiment, clinicians have taken blood samples from each child and isolated the DNA. Using Sanger sequencing, they determined the nucleotide sequence of each child's P53 gene to determine whether or not there are mutations.

1. For each of Valerie's children, **READ** the DNA sequence information from the automated gel run sequence printout.
2. **COMPARE** each child's DNA sequence to the wild type sequence shown in the box below. **IDENTIFY** the location(s) of any mutant nucleotides. Which children have mutations in the P53 gene? What was the mutation?
3. Using the Expaty web server (<https://web.expasy.org/translate/>), **TRANSLATE** the DNA sequence into amino acids. Is the amino acid sequence different from the wild-type sequence? How could the DNA mutations affect the protein function?
4. Using the information, **UPDATE** Valerie's family pedigree.

Wild-type DNA and Protein Sequences:

DNA: gtt ggc tct gac tgt acc acc atc cac tac aac tac atg tgt aac agt tcc tgc atg ggc
 PRO: V G S D C T T I H Y N Y M C N S S C M G

DNA: ggc atg aac cgg agg ccc atc ctc acc atc atc aca ctg
 PRO: G M N R R P I L T I I T L

Study Questions

1. What is the difference between tumor suppressors and oncogenes? Describe how mutations affect each type of protein, and name specific examples.
2. What are P53 “hot spots”, and how do they affect p53 protein structure?
3. Why does Valerie's tumor DNA sample have fewer bands than the peripheral blood sample?
4. What is the purpose of the experimental controls?
5. Can a physician proceed with diagnosis of LFS based on the molecular data alone?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation for:	What to do:	When?	Time Required:
Module I: Creation of a Family Pedigree	Provide literature to students.	Any time before the experiment.	5 min.
Module II-A: RFLP Analysis of Tumor Samples	Prepare DNA samples	Up to one day before performing the experiment	45 min.
	Prepare diluted electrophoresis buffer		
	Prepare molten agarose and pour gels		
Module II-B: Staining Agarose Gels (OPTIONAL)	Prepare staining components	The class period or overnight after the class period	10 min.
Module III: Analysis of Chromatograms to Search for P53 Mutations	Provide literature to students, ensure access to internet database.	Any time before the experiment.	5 min.

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Pre-Lab Preparations: Module II-A

AGAROSE GEL ELECTROPHORESIS

This experiment requires a 0.8% agarose gel per student group. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Prepare SYBR® Safe Stain

1. Following the instructions in Appendix B, prepare 1x Electrophoresis Buffer by combining 20 µL of 50X Concentrated Buffer with 980 µL of distilled water.
2. Add 530 µL of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The diluted SYBR® Safe Stain is now ready to be used during agarose gel preparation.

Individual Gel Preparation

Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Part 1 in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water, agarose powder, and diluted SYBR® Safe Stain.

Batch Gel Preparation

To save time, a larger quantity of agarose solution can be prepared for sharing by the class. See Appendix B.

FOR MODULE II-A Each Group Will Need:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe Stain
- DNA Samples
- DNA Standard Marker

Preparing Gels in Advance

Gels may be prepared ahead and stored for later use. Solidified gels can be stored in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels.

Do not freeze gels at -20° C as freezing will destroy the gels.

Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Preparing the DNA Samples & DNA Standard Marker

The DNA samples and DNA Standard Marker for this experiment are provided in bulk in screw-top microcentrifuge tubes. Students can share the sample tubes, or they can be pre-aliquoted into individual tubes (not included).

Visualizing SYBR® Safe-Stained Gels

SYBR® Safe is a DNA stain that fluoresces when bound to double-stranded DNA, allowing us to visualize our samples. This DNA stain is compatible with both UV and blue-light transilluminators. For best results, we recommend the TruBlu™ 2 Blue Light Transilluminator (Cat #557).

Pre-Lab Preparations: Module II-B

STAINING WITH FLASHBLUE™ (OPTIONAL)

FlashBlue™ stain is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to “equilibrate” in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background. A white light box (Cat. #552) or the white light feature of the TruBlu™ 2 (Cat. #557) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.



FOR MODULE II-B

Each Group Will Need:

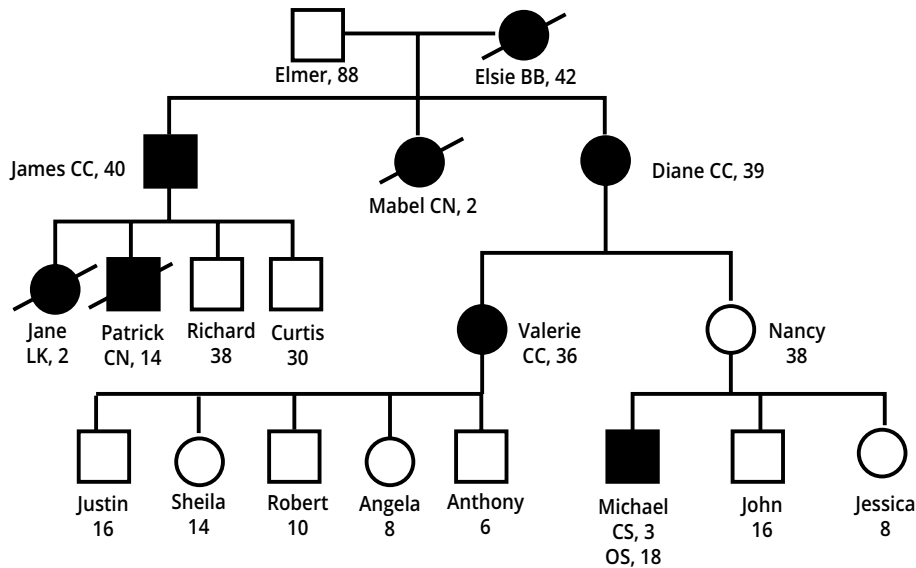
- 10 mL 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

PHOTODOCUMENTATION OF DNA (OPTIONAL)

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.

Experiment Results and Analysis

MODULE I - PEDIGREE



1. Using the family pedigree, do you believe that there is a genetic component to Valerie's cancer?

Yes, the pedigree identifies that there is a genetic component to Valerie's cancer because we can trace the frequent occurrence of different cancers among family members.

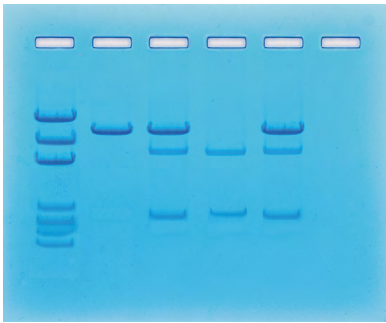
2. Does Valerie meet the criteria for LFS (below)? Why or why not?

Yes because of the following information:

- Valerie was diagnosed with a cancer that frequently occurs in individuals with LFS.
- Valerie's mother was diagnosed with cancer at age 39.
- Several of Valerie's second-degree relatives, including her grandmother and her aunt and uncle, were diagnosed with cancer before the age of 45.

Experiment Results and Analysis

MODULE II - RFLP ANALYSIS OF TUMOR SAMPLES



Lane	Tube	Sample	Molecular Weights (in bp)
1	A	DNA Standard Markers	6751, 3652, 2827, 1568, 1118, 825, 630
2	B	Control DNA	4282
3	C	Patient Peripheral Blood DNA	4282 3000 1282
4	D	Patient Colon Tumor DNA	3000 1282
5	E	Patient Normal Colon Tissue DNA	4282 3000 1282

Explanation of Gel Results

Lane 2: The normal (unmutated) p53 allele possesses no restriction sites for the enzyme.

Lane 3: Valerie's blood sample possesses one normal p53 allele and one mutated p53 allele that is point-mutated to acquire a restriction site for the enzyme. As shown in Figure 2 (the "two-hit" model, page 5), she inherits one "hit" in all her somatic cells (including peripheral blood). As she still possesses a normal p53 allele in this tissue, tumor formation remains suppressed.

Lane 4: Valerie's tumor biopsy shows only the mutated p53 pattern, present now on both alleles. She now has acquired a second "hit" in this tissue, which then becomes cancerous as she no longer possesses a normal p53 tumor suppressor.

Lane 5: Valerie's normal colon tissue DNA is the same as the peripheral blood DNA, with one mutated and one normal p53 allele. This tissue is not cancerous as one normal tumor suppressor is still present.

Experiment Results and Analysis

MODULE III - ANALYSIS OF CHROMATOGRAMS TO SEARCH FOR P53 MUTATIONS

Sheila (#2), Angela (#4), and Anthony (#5) have a normal DNA sequence for p53. Justin (#1) and Robert (#3) have a mutation in their DNA sequence, thus increasing their chance for developing a cancer at some point during their lifetimes.

In the parenthesis, say (sequence #1) or (sequence#2)

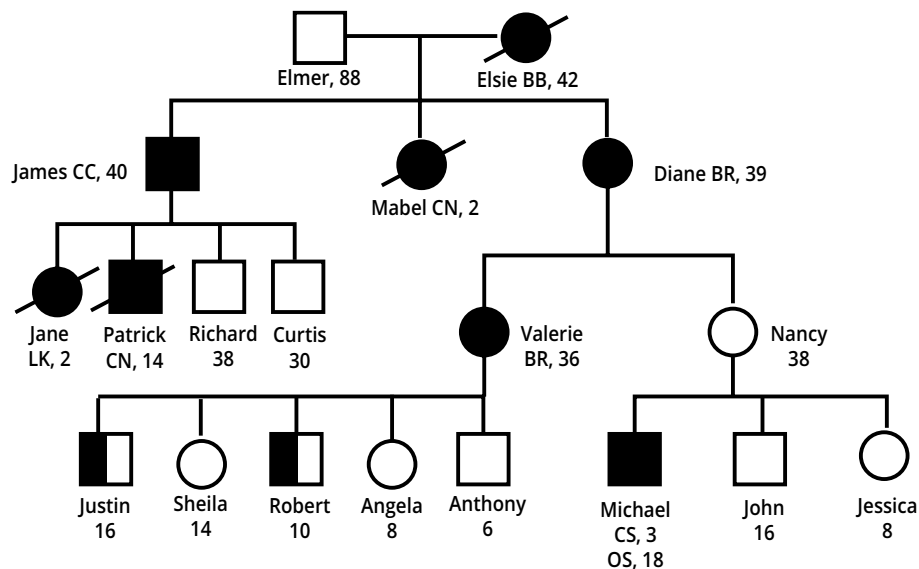
Justin and Robert's gene sequence (changed nucleotide in red):

GTTGGCTCTGACTGTACCACCATCCACTACAACACTACATGTGTAACAGTTCCTGCATGGGCGGCATGAAC**T**G-
GAGGCCCATCCTCACCATCATCACACTG

Mutant Protein Sequence: (changed AA in red):

VGSDCTTIHNYMNCNSSCMGGMN**W**RPILTIITL

The mutation changes the sequence of the codon at position 248 from CGG (Arg, basic) to TGG (Trp, non-polar). Because this amino acid is in the P53 DNA-binding domain, it affects the way the P53 protein binds DNA and therefore changes gene expression.



CHROMATOGRAM ANSWER KEY:

Chromatograms annotated with the correct DNA sequence are provided as a separate PDF file for reference. If you choose to print the file, please be sure to follow these guidelines for best results:

- Printing should be done on US Legal Sized paper (8.5 x 14").
- For best results, be sure that page sizing and handling is set to "Actual Size".
- The chromatograms must be printed in full color to allow for differentiation between nucleotides.

**Please refer to the kit
insert for the Answers to
Study Questions**

Appendix A

Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
There is only a small amount of SYBR® Safe in my tube.	SYBR® Safe is a concentrate that is diluted before use.	Centrifuge or tap the tube to move the contents to the bottom of the tube. Dilute the SybrSafe before use as outlined on page 21.
Bands are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained properly.	Ensure that diluted SYBR® Safe was added to the gel.
	The gel was not stained for a sufficient period of time with FlashBlue™.	Repeat staining protocol.
	The background of gel is too dark after staining with FlashBlue™.	Destain the gel for 5-10 minutes in distilled water.
DNA bands were not resolved.	Tracking dye should migrate at least 3.5 cm (if using a 7x7 cm tray), and at least 6 cm (if using a 14x7 cm tray) from the wells to ensure adequate separation.	Be sure to run the gel at least 6 cm before staining and visualizing the DNA (approximately one hour at 125 V).
DNA bands from FlashBlue stained gels fade when gels are kept at 4°C.	DNA stained with FlashBlue™ may fade with time	Re-stain the gel with FlashBlue™
There is no separation between DNA bands, even though the tracking dye ran the appropriate distance.	The wrong percent gel was used for electrophoretic separation.	Be sure to prepare the correct percent agarose gel. For reference, the Ready-to-Load™ DNA samples should be analyzed using a 0.8% agarose gel.

Appendix B

Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

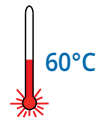
Table D Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2940 mL	3000 mL (3 L)

Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.

- Use a 1 L flask or bottle to prepare the diluted gel buffer.
- Pour 4.0 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
- With a marking pen, indicate the level of solution volume on the outside of the flask.
- Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
- Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.

Table E Batch Prep of 0.8% UltraSpec-Agarose™					
Amount of Agarose	+	Concentrated Buffer (50x)	+	Distilled Water	Total Volume
4.0 g		10 mL		490 mL	500 mL



- Add the entire tube of **diluted SYBR® Safe** stain (see page 21) to the cooled agarose and mix well.

- Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray, 50 mL for a 10 x 7 cm tray, and 60 mL for a 14 x 7 cm tray. **NOTE: For this experiment, 7 x 7 cm gels are recommended.**
- Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

NOTE:

The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.