

**The Influence of DNA Mismatch Repair on the Types of *p53* Mutations  
Found in *Msh2* Null and *Msh2/Atm* Double Null Transformants**

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Biochemistry 610  
Winter 2003**

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## **The Influence of DNA Mismatch Repair on the Types of p53 Mutations Found in Msh2 Null and Msh2/Atm Double Null Transformants**

### **ABSTRACT**

Bone marrow that was harvested from *Msh2* null and *Msh2/Atm* double null mice were previously infected by the oncogenic Abelson murine leukemia virus and grown until they became fully established cell lines in the Rosenberg Lab. By looking into exon 11 of the tumor suppressor gene, *p53*, I sought to determine if the absence of DNA Mismatch Repair (MMR) influences the types of *p53* mutations found in *Msh2* null and *Msh2/Atm* double null transformants. In this lab, I first used Polymerase Chain Reaction (PCR) to amplify samples isolated from 8 different cell lines. To be certain that the region had been amplified, I ran the PCR products on agarose gel and purified the samples. I then prepared two vectors: vBluescript SK+ and dTTP, which were cut with *EcoRV*. Next, I ligated the vector and PCR product, first with T4 DNA ligase and then Quick Ligase. In this study, the PCR cycling for all cell lines succeeded. Unfortunately, the T4 DNA ligase that I used was defective, thus my transformations failed to produce any colonies. I then switched to using Quick Ligase; however, due to the limited time in the fall, I was only able to do produce colonies for 18-63, 18-64, 22-60, and 22-90. In winter term, I then proceeded to grow the colonies in overnight cultures, isolate the DNA, and finally, do restriction analyses on them (first by using *EcoRI* and *HindIII*, then *PvuII*). Since I was unable to yield any inserts, I did new transformations for all cell lines (except 18-65); however, no colonies grew. I have decided not to continue working on the project for the remaining school year.

## INTRODUCTION

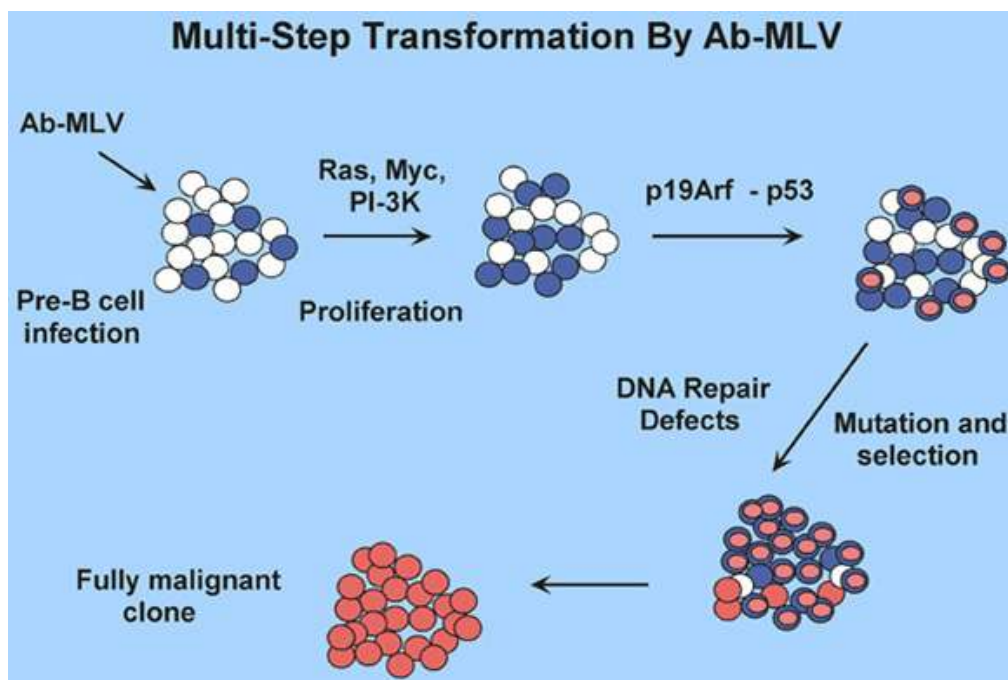
All cancers are multi-step in nature and require an accumulation of mutations that involve aberrant growth stimulation, inappropriate suppression of apoptosis and differentiation arrest (V). In about 50% of tumors, the *p53* pathway is disabled as the activated *p53* suppresses tumor formation by inducing cell cycle arrest and apoptosis, thereby eliminating potentially cancerous cells. The *p53* tumor suppressor gene is located in the short arm of the human chromosome 17. It encodes a protein of 393 amino acids, a transcription factor that controls the expression of many different genes involved in various cellular pathways (IV). Based on recent studies of a wide variety of human cancer, it is reported that mutations in the *p53* gene often lead to the synthesis of a stable, but inactive protein that accumulates in the nucleus of tumor cells (VIII). These mutations are often point mutations that inactivate the *p53* pathway, so that tumor cells are permitted to grow unregulated.

Before the DNA samples were sent to me, bone marrow was harvested from various mice, some of which were wildtype and some of which were *Msh2* nulls or *Msh2/Atm* double nulls. *Msh2* is a molecule that mediates DNA mismatch repair (MMR) in eukaryotic cells. MMR is a necessary defense mechanism for cells because it corrects base pair mismatches and other DNA polymerase errors. Besides *Msh2*, it is also orchestrated by 5 other proteins: Msh6 and Msh3 (along with *Msh2*) recognize mispaired nucleotides; Mlh2, Pms2 and Pms1 facilitate the assembly of factors required for DNA excision and resynthesis. Cells lacking MMR often show an increase in the frequency of *p53* mutations, thus resulting in a higher susceptibility to tumor formation. Like MMR-deficient cells, cells lacking *Msh2* exhibit dramatic instability in microsatellite sequences and show elevated rates of mutations in endogenous genes that are involved in control of cellular growth and apoptosis. In addition, *Msh2* itself also appears to play

a direct role in mediating apoptosis and regulating cell cycle since cells lacking *Msh2* activity fail to undergo cell cycle arrest in response to DNA damage (X).

*Atm* (ataxia-telangiectasia mutant) is a mouse homolog involved in cell cycle regulation, telomere length monitoring, meiotic recombination, and DNA repair (I). Like *Msh2* nulls, mice null for *Atm* exhibit dramatic acceleration of tumor formation. Also, mutations in *Atm* cause the human cancer –associated diseases ataxia-telangiectasia (XII). However, how the absence of MMR influences the types of mutations found in *Msh2* null and *Msh2/Atm* transformants is still not determined. The purpose of my lab is to answer the question above and see if some cell lines carry distinct mutations.

The primary pre-B-cell transformants (derived from *Msh2* *-/-* and *Msh2* *-/-* mice) used in this lab were previously infected by the highly oncogenic Abelson murine leukemia virus (Ab-MLV), a retrovirus that contains the v-abl oncogene and transforms pre-B-cells in vitro and in vivo (X). Primary transformants emerge as fully malignant cell lines after undergoing a prolonged apoptotic crisis in vitro. About 50% of those emerged from crisis contain mutated *p53* alleles while others have down-regulated the p19Arf protein, an important activator of *p53* (V):



After being infected with Ab-MLV, the bone marrow was grown for 10 days in soft agar, allowing isolation of clonal populations of infected cells. These cells were then grown until they became fully established cell lines. Since Ab-MLV-transformed pre-B cells expressing mutant forms of *p53* are resistant to apoptosis by ionizing radiation (IR) (V). The samples were treated with gamma irradiation to distinguish between wildtype and mutant *p53*. Observations showed that all cells except for 14-8 and 14-9 demonstrated a mutant phenotype. 14-8 and 14-9 appeared to be *p53* wildtype because they looked completely dead under the microscope after being irradiated, and therefore were chosen to be my controls for this lab.

The cells were then lysed; the DNA was purified from the extract and was sent to me. Two samples, isolated from cell lines 10-2 and 18-65, were sequenced previously and are known to have a single nucleotide deletion at a tract of 7 consecutive A's within exon 11 of the murine *p53* gene:

*Table.1: Msh2 null cell lines showing a deletion in the regions of the murine p53 gene consisting of repeated nucleotides. ( VI)*

<b>Cell Line</b>	<b>Mutation (on Exon 11)</b>	<b>Effect and <i>p53</i> phenotype</b>
<b>18-65</b>	...CAT <u>A</u> AAAAAACA...	No stop codon in the region amplified; abundant protein
<b>10-2</b>	...CAT <u>A</u> AAAAAACA...	No stop codon in the region amplified; no RNA; no protein

\* Deleted nucleotides are indicated by the underline.

The deletion causes a frameshift affecting the C-terminus of the protein and the loss of the normal stop codon (VI), which often results in reduced levels of mRNA and protein expression. However, while abundant *p53* protein was found in the transformant 18-65, there were none in 10-2. In the absence of MMR, insertions or deletions are common, particularly in

regions of the DNA containing repeated nucleotides, which implies a novel mechanism by which the *p53* gene is inactivated in Ab-MLV transformants. In this lab, I will do a sequence analysis of these two cell lines to see if they correspond to the results found previously by the Rosenberg Lab. By using PCR cycling, cloning, screening and sequencing techniques, I will find out what type of mutations my samples have and determine if they are distinct or not.

#### *A. Polymerase Chain Reaction and Gel Electrophoresis*

Prior to the sequence analysis, I first amplified the DNA samples with the PCR protocol that the Rosenberg Lab sent me. Polymerase Chain Reaction (PCR) is a method for making multiple copies of a specific segment of DNA. After amplifying the DNA, I purified and ran a small amount of each PCR product on agarose gel. If the PCR worked, I should be able to find a fragment of 161 bp on the gel, which is part of the exon 11 region of the *p53* gene.

#### *B. Cloning: preparation, ligation, transformation*

After the gel electrophoresis, I proceeded to clone my PCR DNA. However, since my primers were not incorporated with restriction endonuclease sites, I had to clone my PCR products as blunt-ended fragments. This method is usually not recommended, due to the template-independent terminal transferase activity of Taq DNA polymerase, which adds a single unpaired nucleotide at the 3' end of the fragment, leaving "ragged" ends on the amplified DNA (II). Therefore, to improve the efficiency of my ligations, I chose to clone my products using a method that employs a plasmid containing a single T overhang. In this experiment, part of my PCR products were cloned with Bluescript SK+ vector and the rest by preparing a T vector (dTTP).

The next step was to do ligations of the vectors and PCR products. I used two different ligases: T4 DNA ligase and Quick T4 DNA Ligase. The greatest difference between the two ligases is that Quick T4 DNA Ligase takes less time to ligate. However, both of them are more efficient in joining fragments with complementary sticky ends than blunt ends.

I spent the rest of the fall trimester doing transformations. Using the protocol provided by Life Technologies™, I transformed my ligation products into *Escherichia coli* DH5 $\alpha$  Competent Cells, so that my DNA can be amplified by the host cell DNA replication machinery of the competent cells into many copies per cell (III). I then plated the transformed cells into LB-amp plates at 37°C to obtain colonies. Due to the time limit, I kept all the plates in storage until winter term.

### *C. Screening: selective bacteria culture, DNA Isolation, restriction analysis*

In winter term, I picked out single transformed colonies produced the previous term, and placed them in LB-amp to grow overnight. This process increases the volume of cells, so as to maximize the amount of DNA available for isolation. If the cell contains the DNA plasmid, it will be resistant to the action of ampicillin and will grow. Since ampicillin is an antibiotic that disrupts the synthesis of the peptidoglycan layer that makes up the cell wall, it only kills cells that are actively creating cell walls and has no effect on cells that are not growing or actively dividing. Therefore, those that do not have the plasmid will be eliminated by the antibiotic. This method ensures that only cells surviving in the culture will have the plasmid (III).

After growing the cells in the overnight culture, I then isolated the DNA by doing miniprep. This involves isolating cells and resuspending them in a solution that encourages the cells to break open (III). By centrifuging the sample afterwards, the plasmid DNA is contained

in the supernatant, while the larger chromosomal DNA is found at the bottom, in the form of a pellet. The supernatant was then used for restriction analysis.

Restriction analysis involves the cutting of double-stranded DNA with restriction enzymes. These enzymes cut DNA at very specific recognition sites; the larger the DNA, the more sites present. The location of these sites can be mapped and used as an identifying characteristic of that particular DNA molecule. Restriction mapping is also the foundation of restriction fragment length polymorphism [RFLP] analysis techniques (III). In order to show that the segment of exon 11 is present in the vector, I used restriction enzymes to cut a fragment that should include *EcoRV*'s restriction site. This way, since I know the size of the bands I am looking for, by running the cut samples on an agarose gel, I will know if the exon 11 segment is there. If an insert is found, the sample should be sequenced and analyzed.

For this project, I first chose the two restriction enzymes, *EcoRI* and *HindIII* to digest my DNA. *EcoRI* cuts once at position 701 (G'AATT\_C) of pBluescript SK+, while *HindIII* cuts once at position 680 (A'AGCT\_T). The resulting fragment (12 bp) and the insert (161 bp) together should show a band of 173 bp on the agarose gel. However, since the fragment was too small to be seen on the gel, I switched to using *PvuII* instead, which cuts at positions 530 and 975 (CAG'CTG). The size of the band I was supposed to look for is 606 bp (161 bp + 445 bp).

To determine whether my enzymes were working or not, I cut fresh *pSK+* with *PvuII*. *pSK+* is a vector of 3204 bp. The *PvuII* restriction sites are located at position 2056 and 2438, creating two fragments of 382 bp and 2822 bp respectively.

If all goes well, those samples that showed an insert in the screening process should be sequenced. However, I will not know the final results of my project as I will not be continuing it next spring.

## MATERIALS AND METHODS

### Part I: Obtaining the Materials

I was sent from Tufts University a total of 8 different samples of DNA that were isolated from established cell lines, the names and the concentrations were as follow:

DNA samples (Isolated from cell lines)	Concentration (ng/ $\mu$ l)	Comments
14 – 8	508	Wildtype with wildtype <i>p53</i>
14 - 9	558	Wildtype with wildtype <i>p53</i>
18 – 63	100	Isolated from <i>Msh2</i> null mice
18 – 64	100	Isolated from <i>Msh2</i> null mice
18 – 65	184	Deletion of one of A's in region
10 – 2	1315	Deletion of one of A's in region
22 – 60	100	Isolated from <i>Msh2/Atm</i> double null mice
22 – 90	100	Isolated from <i>Msh2/Atm</i> double null mice

The two following primers were sent along with the samples and used for the PCR cycling:

*p53* L1 (20  $\mu$ M): 5' - GAAGACCAAGAAGGGCCAGT -3'

*p53* R1 (20  $\mu$ M): 5' - ACCGGGAGGATTGTGTCTC -3'

### Part II: Setting up the PCR

To prepare the DNA segment for PCR, I added *p53* left and right primers, which are complementary to the ends of the target DNA. rTAQ premix, which includes DNA polymerase and nucleotides, was also added to the mixture. The DNA was then heated to 94°C to separate the bonds that hold the double helix together. Next, the solution was cooled in order for the DNA polymerase to add nucleotides to the 3' ends of the primers, thereby doubling the targeted DNA sequence. The cycle is then repeated until the targeted sequence has been duplicated enough times. As a result, a large quantity of the targeted DNA was cloned.

Before the PCR, the following DNA samples needed to be diluted to a 100ng/ $\mu$ l concentration.

DNA sample	Original Conc.	Dilution	Final Amount & Conc.
14 – 8	508 ng/μl	0.2μl DNA; 0.8μl H <sub>2</sub> O	1μl of 100ng/μl
14 – 9	558 ng/μl	0.2μl DNA; 0.8μl H <sub>2</sub> O	1μl of 100ng/μl
18 – 65	184 ng/μl	0.5μl DNA; 0.5μl H <sub>2</sub> O	1μl of 100ng/μl
10 – 2	1315 ng/μl	0.1μl DNA; 0.9μl H <sub>2</sub> O	1μl of 100ng/μl

PCR cocktail and protocol:

- 25μl rTaq premix
- 0.5μl *p53R1* (20μM)
- 0.5μl *p53L1* (20μM)
- 24μl H<sub>2</sub>O
- 1μl DNA (100ng/μl)

Total: 51μl PCR cocktail

The solutions were mixed thoroughly and centrifuged briefly. The samples were amplified for 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1.5 min, followed by a 10 min extension at 72°C.

To be certain that the PCR cycling worked, I ran 10μl of each PCR reaction on 1.0% gel and looked for a fragment of 161 bp. A 2-log DNA ladder was also loaded into the first well.

### Part III: Purifying the PCR Products

To purify the PCR reactions, I used the QIAquick™ PCR Purification Kit. The protocol is designed to purify single or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge (protocol).

1. Add 5 volumes (200 μl) of Buffer PB to each 40μl PCR reaction and mix.
2. Place QIAquick spin column in a provided 2 mL collection tube.
3. To bind DNA, apply the sample to the QIA quick column and centrifuge for 30 – 60 seconds.
4. Discard flow-through, and place the QIAquick column back in the same tube.

5. To wash, add 0.75 mL of Buffer PE to the QIAquick column and centrifuge for 30 - 60 seconds.
6. Repeat step 4, and then centrifuged the column for an additional 1 minute at maximum speed.
7. Place QIAquick column in a clean 1.5 mL microfuge tube.
8. To elute DNA, add 50  $\mu$ l Buffer EB to the center of the QIAquick membrane and centrifuge the column for 1 minute.

#### **Part IV: Preparation of Vectors**

Two different vectors were made using the plasmid DNA Bluescript SK+. vBluescript SK+ refers to a vector that clones PCR products as blunt-ended fragments, while “dTTP” refers to a T vector. I prepared the T vector by digesting pBluescript SK+ with a restriction enzyme (*EcoRV*) that produces blunt ends and by adding a 3' terminal thymidine using deoxythymidine triphosphate (dTTP) to both ends (II).

The protocols for making the vectors are as follow:

##### vBluescript SK+

1. 5 $\mu$ l pBluescript SK+
2. 2 $\mu$ l Buffer 3
3. 13 $\mu$ l H<sub>2</sub>O
4. 1 $\mu$ l *EcoRV*
5. incubate digest in a 37°C waterbath for *at least* one hour
6. After incubation, remove digest from the water bath. If time permits, proceed to dephosphorylate digest by adding 1 $\mu$ l of CIP. Incubate at 37°C for one hour.

##### dTTP

1. Place 10 $\mu$ l of the digest into a 0.5 PCR tube
2. Add 2 $\mu$ l of 10x Taq Buffer
3. 1 $\mu$ l of 100mM dTTP
4. 7 $\mu$ l of H<sub>2</sub>O
5. 0.5 $\mu$ l of Taq DNA Polymerase

6. Place tube in the PCR machine. Conduct PCR cycling for 10 minutes at 72°C.

Before ligations, the vectors were purified by running them on 1.0% gel and extracting them with the aid of the QIAEX II<sup>®</sup> Gel Extraction Kit. Here is the protocol for the kit:

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel. Place gel in a 1.5 mL eppendorf tube.
2. Weigh the gel slice in the tube. Add 3 volumes of *Buffer QXI* to 1 volume of gel for DNA fragments 100bp – 4 kb.
3. Resuspend QIAEX II by vortexing for 30 sec. Add 10µl *QIAEX II* to  $\leq 2\mu\text{g}$  DNA.
4. Incubate at 50°C for 10 minutes to solubilize the agarose and bind the DNA. Mix by vortexing every 2 minutes to keep QIAEX II in suspension. Check that the color of the mixture is yellow.
5. Centrifuge the sample for 30 sec and carefully remove *supernatant* with a pipet.
6. Wash the pellet with 500µl of *Buffer QXI*. Resuspend by vortexing, centrifuge for 30 sec and remove all traces of supernatant with a pipet.
7. Wash the pellet *twice* with 500µl of *Buffer PE*. Resuspend by vortexing, centrifuge for 30 sec and remove all traces of *supernatant* with a pipet.
8. *Air-dry* the pellet for 10 – 15 min or until the pellet becomes white.
9. To elute DNA, add 20 µl of *Buffer EB* and resuspend the pellet by vortexing. Incubate at room temperature for 5 min.
10. Centrifuge for 30 sec. Carefully pipet the *supernatant* into a clean tube.
11. Store DNA in freezer.

## **Part V: Ligation**

For this lab, I used both T4 DNA ligase and Quick Ligase to ligate the purified PCR products and vectors. The general protocols are as follow:

### Using T4 DNA ligase

- 1µl vector (extracted from gel)
- 5µl PCR product

- 1µl 10x T4 ligase buffer
- 0.5µl T4 DNA ligase
- 2.5µl H<sub>2</sub>O
- leave sample at room temperature to ligate overnight

#### Using Quick T4 DNA Ligase

- 1µl vector (extracted from gel)
- 5µl PCR product
- 10µl 2x Quick Ligase buffer
- 1µl Quick T4 DNA Ligase
- 4µl H<sub>2</sub>O
- Flick mix, then centrifuge briefly
- Incubate sample at room temperature for one hour, then chill on ice.

#### **\* Controls:**

In order to determine the efficiency of the T4 DNA ligase and Quick T4 DNA ligase, several controls were done:

##### Control 1: with T4 DNA ligase

- make vBluescript SK+ (refer to Part IV: vBluescript SK+)
- make another digest with 5µl pBluescript SK+, 2µl Buffer 3, and 13µl H<sub>2</sub>O. (*N.B. do not add EcoRV*)
- Religate samples with T4 DNA ligase:
  - 8µl of each digest
  - 1µl of 10x T4 ligase buffer
  - 0.5µl T4 DNA ligase
  - Incubate overnight at room temperature

##### Control 2: with Quick ligase

- make vBluescript SK+ (refer to Part IV: vBluescript SK+)
- make another digest with 5µl pBluescript SK+, 2µl Buffer 3, and 13µl H<sub>2</sub>O. (*N.B. do not add EcoRV*)

- Religate samples with Quick T4 DNA ligase:
  - 4µl of each digest
  - 1µl of 2x Quick ligase buffer
  - 0.5µl of Quick T4 DNA ligase
  - Incubate for at least one hour at room temperature

\* Do transformations for all four controls (for protocol, refer to Part VI).

\* For comparison purposes, two more controls were made by transforming 1.3µl of vBluescript Sk+, and 1.3µl of a Bluescript SK+ digest that had not been cut with *EcoRV*.

### **Part VI: Transforming DNA into DH5α Competent Cells**

1. Prepare required number of 1.5 mL eppendorf tubes and leave them on ice.
2. Obtain a tube of competent cells and gently mix them by pipetting the solution up and down.
3. Aliquot 50µl of DH5α competent cells into chilled eppendorf tubes.
4. To determine transformation efficiency, add 5µl of control pUC19 to one tube containing 50µl competent cells, moving the pipette through the cells while dispensing. Gently tap tube to mix.
5. Add 3µl of each DNA ligation directly into tube containing the competent cells. Move the pipette through the cells while dispensing and gently tap tube to mix.
6. Incubate cells on ice for 30 minutes.
7. Heat-shock cells for 20 seconds at 37°C.
8. Place on ice immediately afterwards for 2 minutes.
9. Add 0.95 mL of LB into each tube, and place in a 37°C waterbath as long as time permits.
10. Spin tubes down and resuspend solution by vortexing.
11. Plate 200µl of recovered cells onto LB-amp plates using *sterile* technique.
12. Incubate plates overnight at 37°C.

## Part VII: Growing Transformed Cells in an Overnight Selective Bacterial Culture

1. Mark 10 – 20 15mL snap cap tubes appropriately, and add 5mL of LB-amp into each tube using sterile technique.
2. Pick a single colony from each transformation selective plates with a sterile toothpick and swirl cell mass into the media.
3. Place the tube in the orbital shaker at 37°C. Shake at 300rpm and incubate overnight.

## Part VIII: DNA Isolation

1. Prepare the required number of 1.5 mL eppendorf tubes, and fill each with ~1.5 mL of the culture.
2. Spin for 30 seconds in microcentrifuge.
3. Remove the LB media from the pellet.
4. Resuspend the cell pellet in 50µl *lysis buffer* by pipetting up and down with the micropipettor or with the vortex.
5. Boil the suspension for 60 seconds and quickly chill on ice for 60 seconds.
6. Centrifuge for 15 minutes in a microcentrifuge at room temperature. <The cellular debris and chromosomal DNA is sompacted in the pellet, and the plasmid DNA is in solution in the supernatant.>
7. The *supernatant* will be used for the restriction analysis. Store remaining DNA in freezer.

## Part IX: Restriction Analysis

For my first trial, I attempted to perform a restriction analysis on cell-lines 18-64 and 22-90 using two restriction enzymes, *EcoRI* and *HindIII*.

1. Add 5µl of miniprep into a 1.5mL eppendorf tube.
2. 2µl of restriction buffer 2
3. 13µl of ddH<sub>2</sub>O
4. 0.5µl of *EcoRI*
5. 0.5µl of *HindIII*

6. Digest solution in a 37 °C water-bath for 30 min.
7. Store in freezer or run on gel if time permits.

For my next few trials, I performed the restriction analysis using *PVUII*.

1. Add 18µl of miniprep into a 1.5mL eppendorf tube.
2. 2µl of restriction buffer 2
3. 0.5µl of *PVUII*
4. Digest solution in a 37 °C water-bath for 30 - 60 min.
5. Store in freezer or run on gel if time permits.

In order to determine whether there was an insert or not, a *control* was done by cutting fresh *pSK+* with *PVUII*:

- Add 1µl of *pSK+*
- 1µl *PVUII*
- 2 µl restriction buffer 2
- 16µl H<sub>2</sub>O
- Digest for 30 – 60 minutes
- Store in freezer or run on gel if time permits.

## RESULTS

***8 DNA samples that have gone through PCR cycling contain exon 11 of the p53 tumor suppressor gene.*** From the PCR cycling, I expected to see a 161 bp fragment in my wildtype samples, 18-65, and 10-2. With the exception of cell line 18-65, all 8 other PCR products contained a fragment of 161bp, which represents exon 11 of the *p53* gene. When I received the samples from the university, the labels of two tubes, containing either 18-65 or 10-2 were rubbed off, thus I labeled them “1” and “2” (see fig. 1 and 2) for the time being, until I received new 18-65 and 10-2 in the mail.

*Fig. 1: This photo shows my first set of PCR on gel. All PCR products except for 14-8 and 22-90 contained a fragment of 161bp. As I was preparing the PCR cocktail for the reaction, I had forgotten to dilute 14-8 to a 100 ng $\mu$ l concentration, which could be the source of the problem.*

DNA	Lane
ladder	1
14-8	2
22-90	3
18-64	4
22-60	5
18-63	6
1	7
2	8



*Fig. 2: I redid all my PCR, the results of which are shown on this photo. Unlike my first trial, both 14-8 and 22-90 contained the targeted fragment while sample "1" did not. Note that on lanes 4 and 8, a lot of other fragments also showed up on the gel. The reason may be that some other DNA was accidentally mixed into it.*

DNA	Lane
ladder	1
14-8	2
18-64	3
22-60	4
22-90	5
18-63	6
1	7
2	8

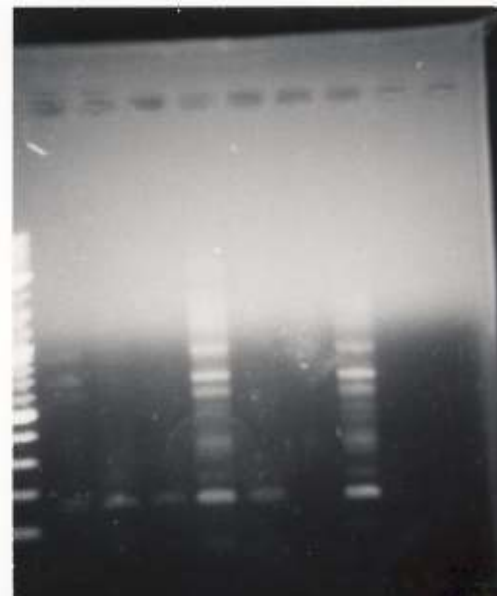


Fig. 3: I finally received new 14-9, 18-65 and 10-2 DNA, and therefore I did my PCR all over again. All of them worked except for 18-65, which is on the last lane. Since it is known definitely 18-65 should have the targeted fragment, there are two possible reasons that the PCR did not work – I diluted the DNA incorrectly, or that the DNA itself was defective.

DNA	Lane
	<b>1</b>
ladder	<b>2</b>
14-9	<b>3</b>
14-8	<b>4</b>
18-64	<b>5</b>
22-60	<b>6</b>
22-90	<b>7</b>
10-2	<b>8</b>
18-63	<b>9</b>
18-65	

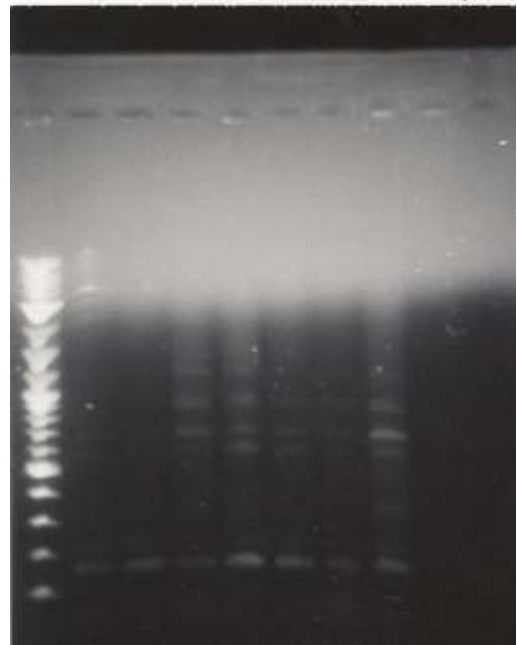


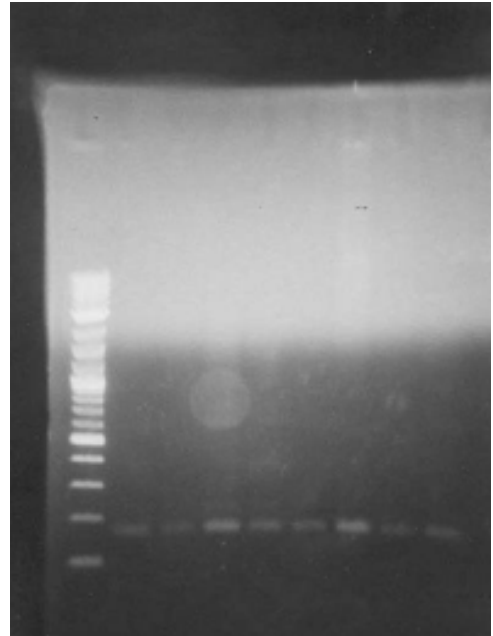
Fig. 4: I did another PCR for 18-65; however, it failed to work again. It was evident that something was wrong with the dilution or the DNA.

On the lane with the bright band (~3100bp) is the T vector dTTP. In order to get the vector purified, I cut the DNA out with a scalpel and used the QIAEX II<sup>®</sup> Gel Extraction Kit to extract the DNA from the gel.

DNA	Lane
	<b>1</b>
ladder	<b>2</b>
18-65	<b>3</b>
dTTP	



Fig. 5: This gel shows the last PCR that I did for this project. Surprisingly, all of them worked, including cell-line 18-65. This proves that I did not do the PCR properly in fall term (it might have been the dilutions).



DNA	Lane
ladder	1
22-90	2
22-60	3
18-63	4
18-64	5
18-65	6
10-2	7
14-8	8
14-9	9

**The T4 DNA ligase used was defective.** For my first three transformations (see table 3), I used T4 DNA ligase for my ligations; however, none of them yielded *E. coli* colonies. I suspected that something was wrong with the ligase itself; therefore I made 6 controls to test the efficiency of the ligase. For comparison, I first prepared cut and uncut Bluescript SK+ vectors and transformed them directly (i.e. no ligations). The vBluescript SK+ (cut vector) produced fewer colonies than that one that had not been cut. Then, I religated the leftover vectors with the T4 DNA ligase that I had been using for. The transformation of the religated vBluescript SK+ produced no colonies, while the uncut vector did (see Table 2). The 4 controls helped confirm that the T4 DNA ligase was not working.

I therefore switched to using Quick T4 DNA ligase for my transformations. To be sure that the ligase works, I made similar controls using the same vectors, but religating them with Quick Ligase instead. The one with *EcoRV* gave me colonies while the one without the enzyme did not. This corresponds nicely to my previous controls.

Table 2: Results of controls that test for the efficiency of T4 DNA ligase and Quick Ligase.

Control	No ligations done	religated with T4 DNA ligase	religated with Quick T4 DNA ligase
vBluescript SK+ (with <i>EcoRV</i> )	positive	negative	positive
Uncut vector (w/o <i>EcoRV</i> )	10x more colonies than vector with <i>EcoRV</i>	positive	negative

Luckily, once I started using the Quick Ligase, my transformation efficiency went up. I did a total of six transformations this trimester, three of them using the defective T4 DNA ligase, and Quick Ligase for the rest (see Table 3). The PCR products that used were all from my third set of PCR reactions (see Fig. 3). All but 18-65 of the PCR DNA have gone through at least one transformation.

My very first set of transformations yielded a lot of bacterial colonies, which made it obvious that my transformations failed.

My 2nd and 3rd sets did not produce any colonies. I had used vBluescript SK+ for the second one and dTTP for my third. Again, my transformations failed. Note that I used T4 DNA ligase for transformations 1, 2 and 3.

For my 4th set, I transformed only 2 cell lines - 14-8 and 14-9, because I wasn't sure if the Quick Ligase would work. I also made two of my controls – cut and uncut Bluescript SK+ religated with T4 DNA ligase. My controls worked out nicely, with colonies for religated uncut vector and none for religated vBluescript SK+. However, no colonies were produced by the 14-9 and 14-8 transformations.

A few colonies showed up for my fifth transformation! For this set, I did another two of my controls, this time religating the vectors with Quick Ligase. I used both dTTP (for 18-63 and 22-60) and vBluescript SK+ (for 18-64 and 22-90) this time. Though I didn't get any colonies for

22-60, 22-90, and 18-63, my 18-64 plate yielded 13 colonies. I therefore picked these colonies out and grew them in an overnight culture. My cells grew, but due to the limited time, I had to store them in the refrigerator to be mini-prepped after Winter Break.

The next day, I poured out 200  $\mu$ l of the excess LB from my remaining transformation mixtures (those used for transformation 5) to make them more concentrated. I resuspended the DNA at the bottom of the tubes and plated them on new LB-amp plates. My transformations were quite successful: 22-90 yielded 25 colonies; 18-64 yielded 172 and 18-63 yielded 13. There were however, too many colonies on 22-60 to be sure that they were colonies I wanted. The plates were stored in the refrigerator to be grown later on.

On the following day, I proceeded to do my ligations for the PCR products that haven't been processed yet: 10-2, 14-9, and 14-8. This time, I used dTTP for my 10-2 ligation, and vBluescript SK+ for 14-8 and 14-9 ligations. However, again due to the time limit, the transformations for the above cell-lines were postponed until winter term.

In winter term, due to my unsuccessful restriction analyses (see next section), I retransformed 22-60, 22-90, 18-63, and 18-64, using the same ligations I used for my 5<sup>th</sup> transformation. This time, however, I increased the concentration of the DNA by adding only 100  $\mu$ l LB before plating the entire sample. Unfortunately, I was unable to get any colonies.

Using the ligations for 14-8, 14-9, and 10-2 that were prepared in the fall, I transformed them, using again 100  $\mu$ l of LB only. There were no colonies found.

Finally, I decided to make new ligations with 18-63, 18-64 and 22-60, all with dTTP. I transformed the samples and yielded zero colonies.

Table 3.1: Summary of transformations done throughout the fall trimester.

	<b>Ligase used</b>	<b>vector used</b>	<b>DNA/Cell line</b>	<b>Results</b>	<b>Comments</b>
<b>1</b>	T4 DNA ligase	vBluescript SK+	All cell lines Except 18-65	No <i>E. coli</i> colonies Contaminated colonies	Bacterial colonies may be caused by unsterile spreader.
<b>2</b>	T4 DNA ligase	vBluescript SK+	All cell lines Except 18-65	All neg	Possibly due to T4 DNA ligase
<b>3</b>	T4 DNA ligase	dTTP	All cell lines Except 18-65	All neg	Possibly due to T4 DNA ligase
<b>4</b>	Quick ligase	dTTP	14-9, 14-8 Controls: T4 DNA ligase with <i>EcoRV</i> and without <i>EcoRV</i>	14-9, 14-8: neg Controls: with <i>EcoRV</i> : neg without <i>EcoRV</i> : pos	I forgot to heat shock samples during transformation process. Ligations were done for 5 minutes instead of 1 hour.
<b>5</b>	Quick ligase	a) dTTP b) vBluescript SK+	a) 18-63, 22-60 b) 18-64, 22-90 Controls: Quick ligase, with <i>EcoRV</i> and without <i>EcoRV</i>	18-64: 13 colonies 22-60, 22-90, 18-63: neg Controls: with <i>EcoRV</i> : pos without <i>EcoRV</i> : neg	DNA concentration might have been too low for 22-60, 22-90, 18-63
<b>6</b>	Quick ligase	a) dTTP b) vBluescript SK+	a) 18-63, 22-60 b) 18-64, 22-90 Controls: vBluescript SK+ Without <i>EcoRV</i> No ligations	22-60: too many colonies 22-90: 25 18-64: 172 18-63: 13 Controls: vBluescript SK+: neg without <i>EcoRV</i> : pos (approx. 10x more colonies than the cut vector)	Plated remaining DNA (200µl) from transformation 5, therefore DNA concentration is higher. Stored in fridge for next term.

*Table 3.2: Summary of transformations done throughout the winter trimester.*

<b>7</b>	Quick ligase	a) dTTP b) vBluescript SK+	a) 18-63, 22-60 b) 18-64, 22-90	All neg	I retransformed the leftover ligations from the previous term. These ligations were also used in transformation 6. To increase DNA concentration, I added only 100µl of LB and plated all of it. DNA might have degenerated over Winter break.
<b>8</b>	Quick ligase	dTTP	10-2, 14-8, 14-9	All neg	These cell-lines were ligated the previous term and stored in the freezer. 100µl of LB was added. DNA might have degenerated over Winter break.
<b>9</b>	Quick ligase	dTTP	18-63, 18-64, 22-60	All neg	These cell-lines were newly ligated. 100µl of LB was added again. There might have been a problem with the vector itself.

***After being grown in an overnight culture, colonies from 22-60 and 18-63 did not grow.***

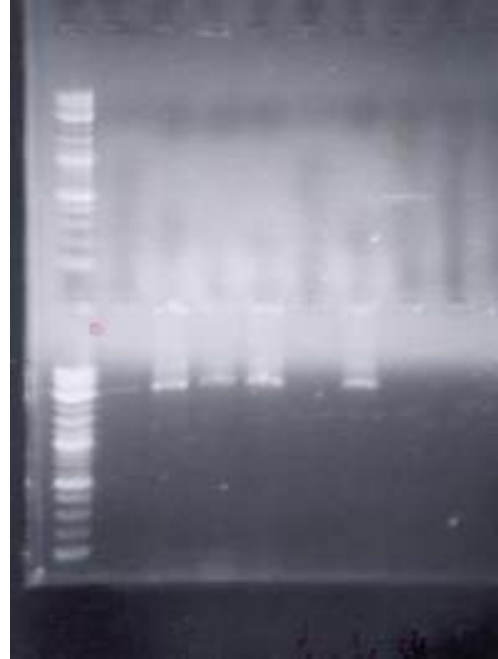
From transformations done in the fall, I obtained colonies for 18-63, 18-64, 22-60 and 22-90.

However, when I grew them in overnight cultures, there were no cells for 22-60 and 18-63. This proves that the colonies were not *E. Coli* cells. Cells grew for the other two samples, and therefore restriction analysis was done on them.

***No substantial results were obtained from restriction analysis.*** For my very first restriction analysis, I used *EcoRI* and *Hind III* to digest my DNA. However, since the resulting fragment was too small to be detected easily, *PvuII* was used in place of them. My results then seemed to get increasingly worse. The first analysis using *PvuII* was quite successful, since there were a bands of ~606 bp on the gel, despite the fact that they were very subtle. However, I am still not very sure why no inserts showed up on the other gels. The control also proved that the restriction enzyme was working.

Although cells did grow for 18-64 in the overnight culture, no bands showed up on the gels at all. There might have been a lack of DNA in the cells. Nevertheless, after getting nothing on the gel twice, I stopped trying to do restriction analysis on the sample.

Fig. 6: The photograph shows the results of my first restriction analysis using *EcoRI* and *Hind III*. The top row did not show any bands. There should be a band of 173 bp, but no inserts were found on either row (the fragment may be too small to detect). It is difficult to tell whether the DNA was cut or not, since the difference between 3000 bp and 2988 bp is too small.



Bottom Row (22-90)		Top Row (18-65)	
DNA	Lane	DNA	Lane
ladder	1	ladder	1
I	2	I	2
II	3	II	3
III	4	III	4
IV	5	IV	5
V	6	V	6
VI	7	VI	7
VII	8	VII	8

Fig. 7: *PvuII* was used instead. Again, there were no bands for 18-6). As for 22-90, bands (~600bp) were found on lanes 1-5 and 7. This may be the insert that I am looking for. Unfortunately, they were extremely subtle. The bands of ~2500 bp represent the other part of the fragment that doesn't include the insert. Nothing showed up on lane 6.

Bottom Row (22-90)		Top Row (18-65)	
DNA	Lane	DNA	Lane
ladder	1	ladder	1
I	2	I	2
II	3	II	3
III	4	III	4
IV	5	IV	5
V	6	V	6
VI	7	VI	7
VII	8	VII	8

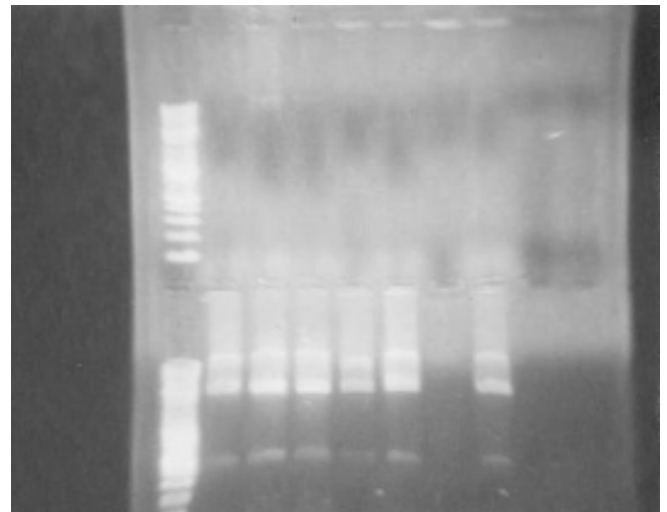
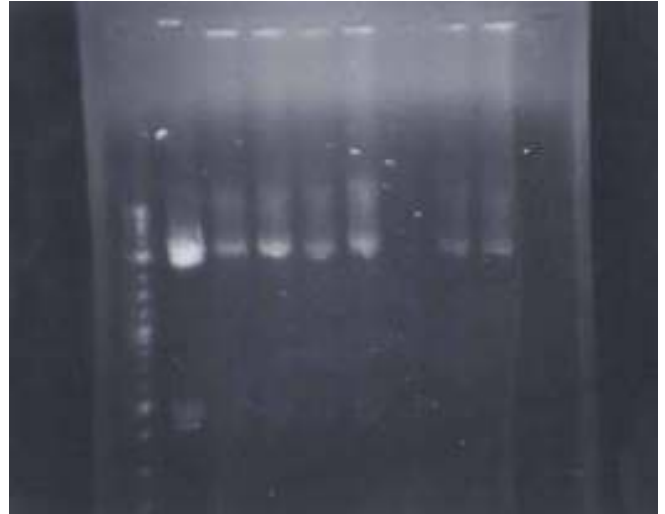
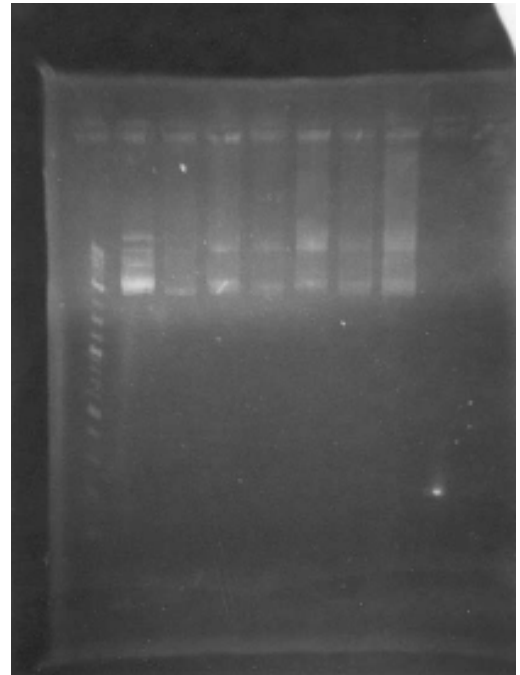


Fig. 8: The control pSK+ proved that the enzyme PVUII is working. Very subtle bands can be seen near the 500 bp mark, which could be the insert; however, I would not trust the results from this gel since the gel was dried up by the time I finished running it. In addition, nothing showed up on lane 7, meaning there wasn't any DNA in that particular miniprep.



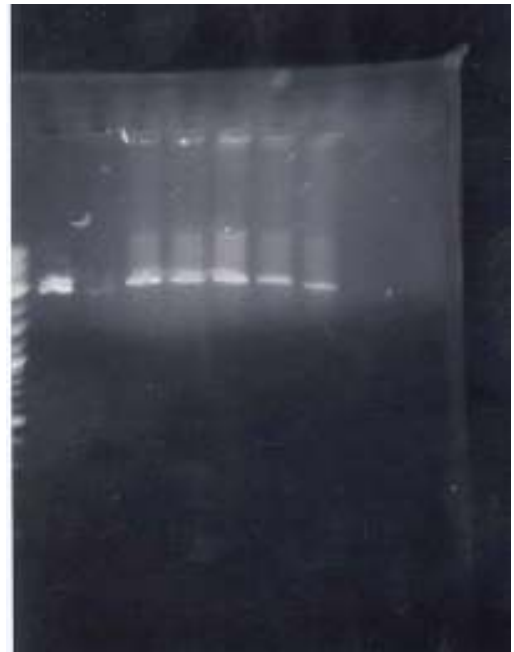
DNA	Lane
ladder	1
Control	2
I	3
II	4
III	5
IV	6
V	7
VI	8
VII	9

Fig. 9: Again, the control showed that the enzymes were working, and the DNA was cut. The smaller fragment is not seen, probably because there wasn't enough DNA. Bands are shown near the 2000 bp mark. However, there were no inserts shown on any of the lanes. One explanation is that the DNA was not completely digested.



DNA	Lane
ladder	1
Control	2
I	3
II	4
III	5
IV	6
VI	7
VII	8

*Fig. 10: On this gel, the control seems to show that the DNA did not cut properly, since it doesn't show one clear band (~2000-3000bp). Lanes 3, 4, and 5 also have two bands stuck together, while the last two show single clear ones. There are no inserts seen. It is possible that I left out a step as I was doing the analysis.*



Lane	1	2	3	4	5	6	7	8
DNA	ladder	Control	I	II	III	IV	VI	VII

## DISCUSSION

### *PCR results*

My gel results demonstrated that the success rate of my PCR excellent. The very first set I did, I had forgotten to dilute the DNA before the PCR; therefore overly high DNA concentration may have prevented it from working. As for 22-90, I cannot make any conclusions as to what might have happened the first time because it worked for the rest of my PCRs. For my second PCR, I'm not sure why sample "1" didn't work, especially when it worked in the first set. The third set was obviously the most successful PCR I did in fall term; however, 18-65 didn't show the fragment either time, therefore I stopped re-doing the PCR for that sample – until winter term. Strangely, when I did my very last PCR in the winter, all of them, including 18-65, worked. This time, the DNA was PCR cycled in their original

concentrations. Thus, I conclude that 18-65 did not work previously due to miscalculations with dilutions.

### ***Transformations***

Unlike my PCRs, I was rather unlucky with my transformations. For the first half of the term I used the T4 DNA ligase in the lab, which was later proved to be ineffective. That is the most likely reason I could come up with to explain the failure of my transformations. The first batch of transformations I did produced only contaminated cultures that did not grow in culture. The most likely reason to cause this is that I used 70% ethanol instead of 100% ethanol to sterilize my spreader, so that bacteria from the spreader got onto my plate.

I am almost positive that the reason for my unsuccessful 4<sup>th</sup> transformation was due to the fact that I forgot to heat-shock the cells before adding LB into it. What might have added to my disastrous results was that I ligated my samples with Quick Ligase for 5 minutes instead of the required one hour. However, the good news was that the results of my controls helped prove my hypothesis that the T4 DNA ligase wasn't working. This means that my first three transformations couldn't have worked properly, even if the human errors did not occur. Though the controls did in fact yield colonies, the overall number of them was still less than what was expected; this was probably because of the forgotten heat-shock step.

As for the low transformation efficiency on my 5<sup>th</sup> set, I think the main reason is that the transformation mixture was not concentrated enough, that is, not enough DNA was plated. I came up with this because comparing the results of the next set of plates, which contain the same transformation mixtures but with a higher DNA concentration, the transformation efficiency went up. Luckily, when I made the DNA more concentrated, colonies grew. As for the

overgrowth of colonies for 22-60, I am not sure what caused it, but after growing them in the winter, I proved that they were not *E. Coli* cells, since nothing grew in the overnight culture.

Returning from Christmas break, I grew the cells produced in transformation 6 in an overnight culture, isolated the DNA, and did a restriction analysis. Strangely, I was unable to get any inserts, which could mean that my plates have been contaminated by something. Or, another plausible reason is that the plates dried up when I was gone and my cells died. Therefore, I re-transformed the same ligations I used in transformation 5, in which I got colonies. This time I even made sure that the concentrations of the samples were higher by adding less LB media to it, but strange enough, no colonies grew. The most likely explanation is that the DNA had degenerated over time, or that the colonies I got previously were not *E. Coli* cells.

I left school last term after ligating 14-8, 14-9, and 10-2. Since I couldn't do the transformations then, I did it in the winter instead. Like all of my other plates, there were no colonies. Since none of my transformations are working, I've concluded that either my DNA degenerated over break (even though it is not likely to happen if DNA was stored in freezer), or the dTTP I've been using is defective.

Besides the known human errors, there are definitely other possible reasons for my unsuccessful transformations. It could be that the vBluescript SK+ wasn't working properly, since I incubated the sample at 42°C instead of 37°C as I was dephosphorylizing it. However, I doubt it made any difference because some of the successful transformations I did were done using that vector. Nevertheless, the best explanation for my unsuccessful first batch of transformations (those ligated with T4 DNA ligase) is the ligase itself, while those ligated with Quick Ligase didn't work initially because the DNA concentration was too low.

### ***Overnight Culture and Restriction Analysis***

The overnight cultures proved that the colonies yielded from the transformation of 22-60 and 18-63 were not *E. Coli* cells. I am not surprised that nothing grew for 22-60, since there was an overgrowth of colonies on the plate, which made it very unlikely to be *E. Coli* cells. As for 18-63, the plate might have been contaminated somehow, or dried up over winter break, which could have led to the death of the colonies.

My results with the restriction analyses were even worse than my transformations. First of all, I was unable to get any bands at all for 18-63. I suspect that there was a lack of DNA in my overnight cultures. It was difficult to understand why the experiments went wrong, since I know I've been very careful about putting all the necessary ingredients in. The only known human error occurred as I was running the gel Figure 8. I had put too little 1x TB to submerge the gel before I ran it, which caused the gel to dry up by the end of the gel electrophoresis. That might have been the cause for the subtleness of the bands.

Restriction Analysis using *EcoRI* and *HindIII*: The choice of enzymes was badly chosen, since the resulting fragment was quite small (~173 bp). That may have been the reason for not seeing any inserts. Also, I might have loaded too little DNA into the wells.

Restriction Analysis using *PvuII*: besides Figure 8, I am not sure why there were no inserts. The best explanation I could come up with is that the dTTP I've made is defective. It is possible that the dephosphorylization of the vBluescript SK+ was so efficient that the cells were unable to relegate afterwards. Another possible reason for the failure is that my DNA might have degenerated over winter break, though that rarely happens when the DNA is stored in the freezer. It couldn't have been the restriction enzymes' fault since my controls proved that the *pSK+* was cut.

### ***Controls***

My six controls made sense when I combined the results together. vBluescript SK+ should have fewer colonies than uncut Bluescript SK+, since restriction enzymes cut the vector into tiny fragments, making it more difficult to grow colonies. Also, if the T4 DNA ligase wasn't working, then vBluescript SK+ religated with the ligase should not produce any colonies because the vector cannot be religated. These 4 controls prove that the ligase was defective. The Quick ligase was proven to be working, since the transformation of the religated vBluescript SK+ gave me colonies.

### ***Efficiency of dTTP and "Normal" Bluescript SK+ vector***

As I have mentioned previously in the introduction, a T vector is supposedly more efficient in cloning blunt-end fragments. Unfortunately, none of my restriction analyses worked very well, and there is a great possibility it was because of a defective dTTP vector. Although I will not be continuing the project in the spring, I suggest using professionally-made dTTP to clone the samples instead. It will be costly, but worth a try.

## **CONCLUSIONS**

- I. My PCRs were successful for all DNA samples.
- II. The T4 DNA Ligase used was defective.
- III. Using Quick Ligase, I was able to obtain colonies from the cell lines 18-63, 18-64, 22-60 and 22-90; however, proved by the results from screening, the colonies for 18-63 and 22-60 were not *E. Coli* cells. The restriction analysis for the 18-64 and 22-90 did not work.
- IV. The dTTP vector may not be working properly. Professionally-made dTTP is recommended for usage.

## ACKNOWLEDGEMENTS

I am grateful to the Rosenberg Lab at Tufts Medical School for providing me with this project.

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XI. Vector map of pBluescript SK+.

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## APPENDIX A

### Sequence of Murine *p53* gene 3' region with exon 11 (VII)

1 ctacctgaag **accaagaagg gccagtctac** ttcccgccat aaaaaaaca tggtcaagaa  
61 agtggggcct gactca gact gactgcctct gcatcccgtc cccatcacca gcctccccct  
121 ctcttgctg tcttatgact tcagggtga **gacacaatcc tccgggtccc** tctgctgcc  
181 tttttacct ttagctagg gctcagcccc ctctctgagt agtgggtcct ggcccaagtt  
241 ggggaatagg ttgatagttg tcaggtctct gctggcccag cgaaattcta tccagccagt  
301 tgttgacce tggcacctac aatgaaatct caccctacce cacacctgt aagattctat  
361 cttgggcct cataggtcc atactctcca gggcctactt tccttcatt ctgcaaagcc  
421 tgtctgcatt taccacccc ccacctgct tccctctttt tttttttt acccttttt  
481 atatatcaat ttctatttt acaataaaat tttgtatca cttatatggt tttgagaggt  
541 tgatatcagc ataagctgct tg

\* In red: *p53* L1 primer: 5'- GAAGACCAAGAAGGGCCAGT -3'

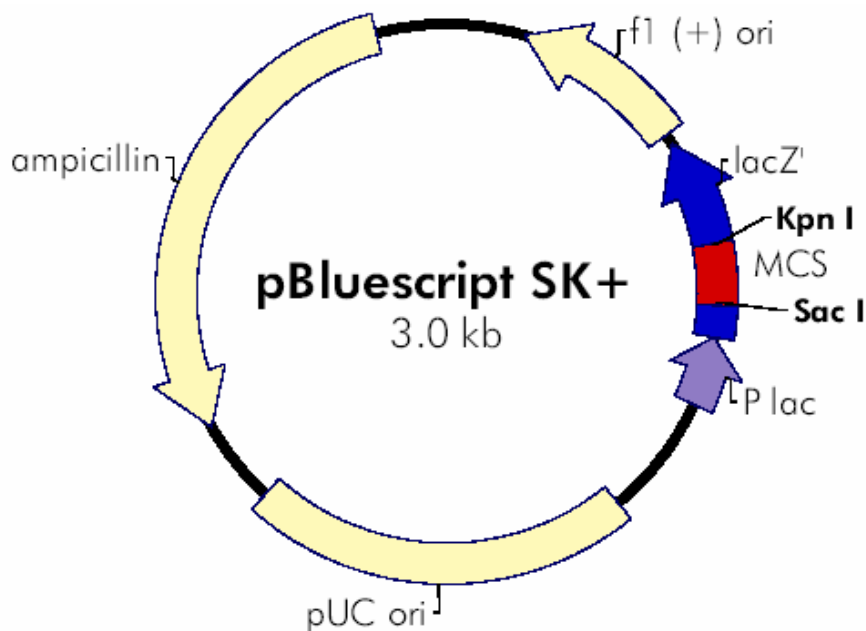
In green: *p53* R1 primer: 5'- ACCGGGAGGATTGTGTCTC -3'

In blue: target fragment (161bp)

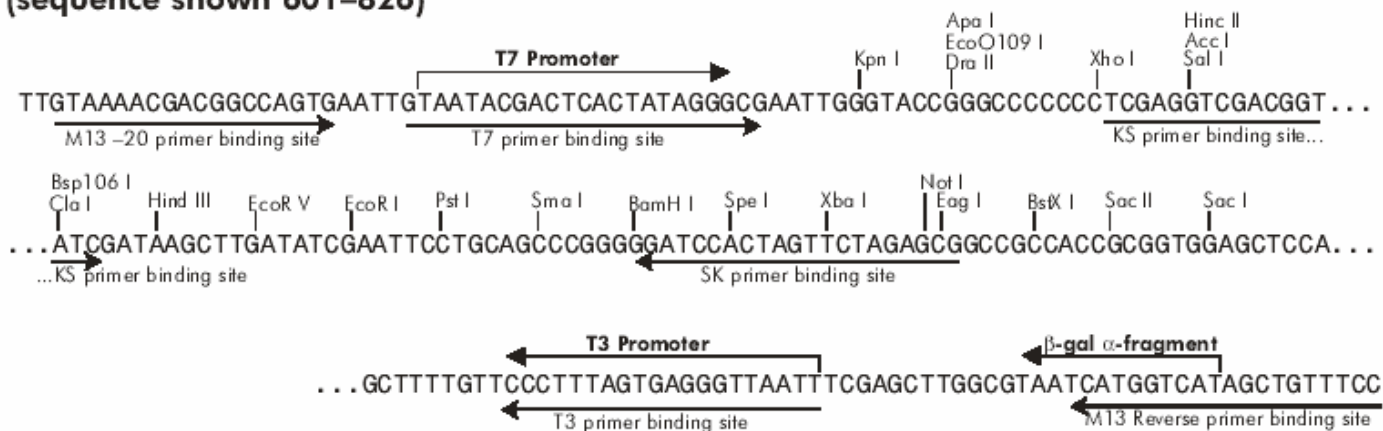
## APPENDIX B

### pBluescript SK+ vector Map (XI)

**f1 (+) origin 138–444**  
 **$\beta$ -galactosidase  $\alpha$ -fragment 463–816**  
**multiple cloning site 653–760**  
**lac promoter 817–938**  
**pUC origin 1158–1825**  
**ampicillin resistance (*bla*) ORF 1976–2833**



### pBluescript SK (+/-) Multiple Cloning Site Region (sequence shown 601–826)



# APPENDIX C

## pSK+ vector Map

